Committee for Risk Assessment

RAC

Annex 1

Background document

to the Opinion proposing harmonised classification and labelling at EU level of

trimethyl phosphate

EC Number: 208-144-8

CAS Number: 512-56-1

CLH-O-0000007318-70-01/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

8 June 2023

CLH report

Proposal for Harmonised Classification and Labelling

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

Chemical name:

Trimethyl phosphate

EC Number: 208-144-8

CAS Number: 512-56-1

Index Number: -

Contact details for dossier submitter:

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Version number: 02 **Date: May 2022**

CONTENTS

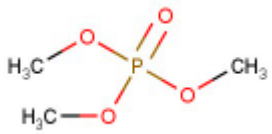
ABBREVIATIONS

ATE	Acute Toxicity Estimate
bw	body weight
CA	Chromosome Aberration
CAS	Chemical Abstract Service
d	day
DMP	Dimethyl phosphate
Drg	Danger
GLP	Good Laboratory Practice
ip	intraperitoneal
Kow	Partition coefficient octanol/water
LD50	Lethal dose, 50%
LC50	Lethal concentration, 50%
m/f	male/female
miSOD	Mitochondrial Superoxide
NMR	Nuclear Magnetic Resonance
MMS	Methyl Methane Sulfonate
MN	Micronucleus
MoA	Mode of Action
OECD	Organisation for Economic Co-operation and Development
PC	Product Category
PFRs	Phosphorus-containing flame retardants
ROS	Reactive Oxygen Species
SIDS	Screening Information Dataset
SSB	Single Strand Break
TMP	Trimethyl phosphate

1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

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)	trimethyl phosphate
Other names (usual name, trade name, abbreviation)	trimethylphosphate phosphoric acid, trimethyl ester TMP
ISO common name (if available and appropriate)	-
EC number (if available and appropriate)	208-144-8
EC name (if available and appropriate)	trimethyl phosphate
CAS number (if available)	512-56-1
Other identity code (if available)	-
Molecular formula	C3H9O4P
Structural formula	 (**source:** European Chemicals Agency, http://echa.europa.eu/)
SMILES notation (if available)	COP(=O)(OC)OC
Molecular weight or molecular weight range	140.07 g/mol
Information on optical activity and typical ratio of (stereo) isomers (if applicable and appropriate)	-
Description of the manufacturing process and identity of the source (for UVCB substances only)	-
Degree of purity (%) (if relevant for the entry in Annex VI)	-

1.2 Composition of the substance

Trimethyl phosphate (TMP) is a mono-constituent substance.

Table 2: Constituents (non-confidential information)

Constituent (Name and numerical identifier)	**Concentration range (% w/w minimum and maximum in multi-constituent substances)**	**Current CLH in Annex VI Table 3 (CLP)**	**Current self-classification and labelling (CLP)**
trimethyl phosphate EC 208-144-8	Conf.	-	Acute Tox. 4, H302 Skin Irrit. 2, H315 Eye Irrit. 2, H319 Muta. 1B, H340 Carc. 2, H351

Impurities registered are not relevant for classification.

Information on the test substances (if available) are given in the study descriptions.

2 PROPOSED HARMONISED CLASSIFICATION AND LABELLING

2.1 Proposed harmonised classification and labelling according to the CLP criteria

Table 3: For substance with no current entry in Annex VI of CLP

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATEs	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	No current Annex VI entry										
Dossier submitter's proposal	TBD	trimethyl phosphate	208-144-8	512-56-1	Carc. 1B Muta. 1B Repr 1B Acute Tox. 4 STOT RE 2	H350 H340 H360FD H302 H373 (nervous system)	GHS08 GHS07 Dgr	H350 H340 H360FD H302 H373 (nervous system)		oral: ATE = 1257 mg/kg bw	

Table 4: Reason for not proposing harmonised classification and status under consultation

Hazard class	Reason for no classification	Within the scope of 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	Acute Tox 4, H302	Yes
Acute toxicity via dermal route	*data conclusive but not sufficient for classification*	Yes
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	Muta. 1B, H340	Yes
Carcinogenicity	Carc. 1B, H350	Yes
Reproductive toxicity	Repr. 1B, H360FD	Yes
Specific target organ toxicity-single exposure	*hazard class not assessed in this dossier*	No
Specific target organ toxicity-repeated exposure	STOT RE 2, H373 (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	*hazard class not assessed in this dossier*	No

3 HISTORY OF THE PREVIOUS CLASSIFICATION AND LABELLING

Not relevant.

RAC general comment

Trimetyl phosphate (TMP) is used as a gasoline additive to prevent spark plug fouling and engine rumble. It is also used as a flame retardant for paints and polymers and it is a raw material for making insecticides. TMP is also used as a methylating agent.

TMP is used to manufacture fine and largescale chemicals. Formulation use is linked to re-packaging (laboratory chemicals). The uses are at industrial sites as intermediate and processing aid and by professional users as processing aid and laboratory use.

Studies assessed: According to the DS, there are only a few studies with TMP performed according to current OECD test guidelines (OECD TG). The DS mentioned specifically a combined repeated dose toxicity and reproductive screening study according to OECD TG 422 (Anonymous, 1994b), an *in vitro* cytogenicity / chromosome aberration study in mammalian cells according to Japanese Guidelines for Screening Mutagenicity Testing of Chemicals (Anonymous, 1994a) and a bacterial reverse mutation assay according to Japanese Guidelines for Screening Mutagenicity Testing of Chemicals (Anonymous, 1996). The studies have limitations, no original study reports were made available by the registrant(s), but for Anonymous (1994b) an English study summary (study in Japanese) and a tabular presentation of the results were provided. Most of the data referred to in the CLH report and this opinion are from the open literature. Some of these studies were considered by the DS to be similar to OECD TG studies. Some of the studies only had the abstract available, but they are included in the CLH report and this opinion since the results were considered to be supplementary to the other studies. In addition, a review by the US EPA on Toxicity values for TMP was also considered by the DS.

Toxicokinetics

Data from acute and repeated dose toxicity studies as well as toxicokinetic studies (Jackson and Jones, 1968) after oral administration indicate that TMP is bioavailable. The acute toxicity studies indicated that TMP is absorbed to a larger extent through the gastrointestinal tract than the skin. TMP is known as a methylating agent (Yamauchi *et al.,* 1976). ^{32}P-labellled TMP is metabolised to DMP in rats with intraperitoneal (i.p.) administration (1000 mg/kg) and in mice treated orally (100 mg/kg) and excreted primally in the urine (Jackson and Jones, 1968). Further investigation of exposure with ^{14}C-TMP demonstrates S-methyl cysteine in the urine. The DS also reported that S-methylcystein N-acetate was isolated as well as small amounts of S-methyl gluthatione. The metabolism of TMP was reported to be faster in mice compared to rats. Both mice and rats metabolise TMP quickly after oral administration and less quickly by i.p. administration. DMP was found urine and bladder 3h after oral administration in mice and in urine 16h after i.p. administration in rats. In rats and mice almost 90% of TMP is metabolised after 16h and almost everything is metabolised after 96h (Jackson and Jones, 1968; Jones, 1970).

The general pathway for TMP metabolism was reported by the DS as:

TMP → DMP and S-methylgluthione → S-methylcystein → S-methylcystein-N-acetate

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

There is no requirement for justification that action is needed at Community level.

TMP has to be harmonized classified for Mutagenicity, Carcinogenicity and Reproductive Toxicity. Harmonized classification for other endpoints (acute toxicity, STOT RE) is also proposed due to differences in self-classifications notified.

5 IDENTIFIED USES

Table 5: The following uses are indicated at ECHA dissemination site [accessed August, 2021]:

	Use(s)	Technical function
Manufacture	Manufacture of fine chemicals Manufacture of large scale chemicals	-
Formulation	Re-packing *(PC 21: Laboratory chemicals)*	-
Uses at industrial sites	Use as intermediate and processing aid *(PC 21: Laboratory chemicals; SU 8: Manufacture of bulk, large scale chemicals; SU 9: Manufacture of fine chemicals; SU 24: Scientific research and development)*	-
Uses by professional workers	Professional use as processing aid *(SU 9: Manufacture of fine chemicals)* Laboratory use *(PC 21: Laboratory chemicals; SU 9: Manufacture of fine chemicals; SU 24: Scientific research and development)*	-
Consumer Uses	-	-
Article service life	-	-

TMP is an antioxidant. It is used as a gasoline additive to prevent spark plug fouling and engine rumble. It is also used as a flame retardant for paints and polymers and it is a raw material for making insecticides[1]. TMP is also used as methylating agent[2].

6 DATA SOURCES

[1] Source PubChem (nih.gov)

[2] Römpp online lexicon; Jones et al. (1966): Jones FW, Osborne GO, Sutherland GJ, Topsom RD & Vaughan J (1966): J., Chem. Commun., 18 (1966).

ECHA dissemination site: Substance Information - ECHA (europa.eu)

The available data for TMP consist to a large part of studies from the open literature. Only few guideline studies were available, i.e. a combined repeated dose toxicity and reproductive screening study according to OECD 422 (Anonymous, 1994b), an *in vitro* cytogenicity / chromosome aberration study in mammalian cells according to Japanese Guidelines for Screening Mutagenicity Testing Of Chemicals (Anonymous, 1994a) and a bacterial reverse mutation assay according to Japanese Guidelines for Screening Mutagenicity Testing Of Chemicals (Anonymous, 1996). No original study reports were made available for these guideline studies by the registrant(s), but for Anonymous (1994b) an English study summary (study in Japanese) and a tabular presentation of the results were provided.

Also a review by the United States Environmental Protection Agency (US EPA, 2010) was considered, which is the most recent comprehensive assessment of toxicity data available for TMP.

7 PHYSICOCHEMICAL PROPERTIES

Table 6: Summary of physicochemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
Physical state at 20°C and 101,3 kPa	Liquid, colourless (20°C, 101.3 kPa)	ECHA dissemination site [Aug, 2021]	-
Melting/freezing point	-46°C	ECHA dissemination site [Aug, 2021]	Value taken from handbook
Boiling point	197°C (760 mmHg)	ECHA dissemination site [Aug, 2021]	Value taken from handbook
Relative density	1.197 g/cm^3 (20°C)	ECHA dissemination site [Aug, 2021]	Value taken from handbook
Vapour pressure	0.74.Pa (25°C)	ECHA dissemination site [Aug, 2021]	OECD 104
Surface tension	-	ECHA dissemination site [Aug, 2021]	waiving
Water solubility	500 g/L (25°C, pH ≥6 and ≤8)	ECHA dissemination site [Aug, 2021]	Value taken from handbook
Partition coefficient n-octanol/water	-0.46 (25°C)	ECHA dissemination site [Aug, 2021]	Value taken from handbook, OECD 107
Flash point	107°C (760 mmHg)	ECHA dissemination site [Aug, 2021]	Value taken from handbook
Flammability	-	ECHA dissemination site [Aug, 2021]	Waiving, substance is a liquid
Explosive properties	-	ECHA dissemination site [Aug, 2021]	Waiving; no chemical groups present in the molecule which are associated with explosive properties
Self-ignition temperature	380°C (1002.2 hPa)	ECHA dissemination site [Aug, 2021]	EU Method A.15
Oxidising properties	not oxidizing	ECHA dissemination site [Aug, 2021]	waiving

Property	Value	Reference	Comment (e.g. measured or estimated)
Granulometry	-	-	-
Stability in organic solvents and identity of relevant degradation products	-	-	-
Dissociation constant	-	-	-
Viscosity	-	-	-

8 EVALUATION OF PHYSICAL HAZARDS

Not assessed in this dossier.

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Table 7: Summary table of toxicokinetic studies

Method	Results	Remarks	Reference
^{32}P-TMP was administered intraperitoneally to mice and rats (no further details on number of animals or dosing regime).	Only one radioactive metabolite was formed, i.e. dimethyl phosphate (DMP), which was found in mouse urine and in the bladder after 3h. Also in the rat DMP was found in the urine, but after 16h and together with TMP. There is no evidence for further degradation to inorganic phosphate or even monomethyl phosphate in either species.	No guideline was followed	Jackson & Jones, 1968
In a preliminary *in vitro* study rat kidney, liver and intestinal tissue was used to investigate TMP metabolism (no further details were presented).	In the kidney tissue no conversion of TMP was observed, but liver and intestinal tissue converted TMP.	No guideline was followed	Jackson & Jones, 1968
^{14}C-TMP was administered to rats and mice (no further details on number of animals or route of exposure).	The formation of S-methyl cysteine was demonstrated in urine, this was seen after a view hours in mice, but was slower in rat.	No guideline was followed	Jackson & Jones, 1968
Investigation of the methylating properties of TMP.	See section on germ cell mutagenicity for detailed effects on the single bases of nucleic acids. Whereas other alkylating agents are little soluble in water, TMP is miscible freely with water and allowed alkylation reactions to be run in a homogenous aqueous phase.	No guideline was followed	Yamauchi et al., 1976

Method	Results	Remarks	Reference
	When reacting with the bases one methylgroup of TMP is used for the methylation reaction and dimethyl hydrogen phosphate remains, not exhibiting alkylating properties any longer.		

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

TMP is a colorless liquid, which is highly soluble in water (500g/l at 25 °C). The measured log Pow is -0.46 at 25 °C indicating a possible general absorption of TMP (see Table 6).

Absorption

Based on the observed acute toxicity after oral administration (see section on acute toxicity), bioavailability after single oral administration can be assumed. This is also demonstrated by oral toxicity observed in repeated dose toxicity studies (as outlined in this dossier) and oral toxicokinetic studies (Jackson & Jones, 1968).

In an acute dermal toxicity study with New Zealand White rabbits the LD_{50} was determined to be 3388 mg/kg TMP (Smyth et al., 1969). Due to the experimental acute oral and the lower dermal toxicity, it appears that TMP is absorbed to a larger extent through gastrointestinal tract than skin.

Metabolism

Rats treated orally at 100 mg/kg and mice treated i.p. at 1000 mg/kg with ^{32}P-labeled TMP excreted primarily dimethyl phosphate in the urine. Only traces of the parent compound were detected, and only in the rats at less than 6 h after treatment. S-methyl cysteine and S-methyl cysteine N-acetate were also isolated. Small amounts of S-methyl glutathione were detected, presumably the initial methylation product in this series of metabolites.

Metabolism of TMP was faster in the mouse than in the rat, but there was no evidence of further conversion to monomethyl phosphate in either species (Jackson & Jones, 1968). Both rat liver and rat intestinal tissue degrade TMP, but not the kidney.

TMP is degraded to dimethyl phosphate (DMP), not to monoalkyl phosphate or even to free phosphoric acid. The general pathway is:

TMP ----> DMP and S-Methylglutathion ----> S-Methylcystein ----> S-Methylcystein-N-acetat

TMP reacts almost quantitatively with glutathione *in vitro*. Both S-methylglutathione and dimethylphosphate are found on reaction with liver homogenate.

Mouse and rat metabolise TMP relatively quickly after oral, less quickly after intraperitoneal administration; in rat and mouse almost 90% is metabolised within 16 hours and almost everything is metabolised within 96 hours (Jackson & Jones, 1968; Jones, 1970).

Excretion

The metabolites are predominantly secreted with the urine (Jackson & Jones, 1968; Jones, 1970).

10 EVALUATION OF HEALTH HAZARDS

Acute toxicity

10.1 Acute toxicity - oral route

Table 8: Summary table of animal studies on acute oral toxicity

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance	Dose levels, duration of exposure	Value LD_{50}	Reference
Rabbit					
Predates guideline; 6 - 10 animals per group, sex not indicated; LD_{50} calculated by the maximum likelihood (Bliss et al., 1938)	Rabbit	TMP Oral (gavage)	742, 1125, 1436, 1676, 2514, 3830 & 5626 mg/kg bw (for more details see Table 9) Values converted from mL/kg bw to mg/kg bw based on the density of TMP of 1,197	1257 mg/kg bw	Deichmann & Witherup (1946)
Guinea pig					
Predates guideline; 2 animals per group, sex not indicated; An approximate lethal dose was determined as only 2 animals per group were used	Guinea pig	TMP Oral (gavage)	503, 742, 1125, 1676, 2514, 3830 & 5626 mg/kg bw (for more details see Table 9) Values converted from mL/kg bw to mg/kg bw based on the density of TMP of 1,197	1676 mg/kg bw	Deichmann & Witherup (1946)
Rat					
Predates guideline; 10 animals per group, sex not indicated; LD_{50} calculated by the maximum likelihood (Bliss et al., 1938)	Rat	TMP Oral (gavage)	1125, 1676, 2095, 2514, 3830 & 5626 mg/kg bw (for more details see Table 9) Values converted from mL/kg bw to mg/kg bw based on the density of TMP of 1,197	1975 mg/kg bw	Deichmann & Witherup (1946)
Predates guideline; The study tested a large amount of substances including TMP according to the method described by Smyth et al. (1962): 5m/group 14 day observation	Rat, male Carworth-Wistar 4-5 weeks of age	TMP Oral (intubation); It is stated that whenever possible undiluted test material was used - this seems to be the case for TMP.	Doses tested not indicated but stated that doses were arranged in a logarithmic series differing by a factor of two.	3388 mg/kg bw (converted from mL/kg bw to mg/kg bw based on the density of TMP of 1,197)	Smyth et al. (1969)

Method, guideline, deviations if any	**Species, strain, sex, no/group**	**Test substance**	**Dose levels, duration of exposure**	**Value LD_{50}**	**Reference**
period; The most probable LD_{50} value and its fiducial range are estimated according to Thompson et al. (1947) using the Tables of Weil (1952)					
Not indicated	Rat No information on strain, sex or number/group	TMP oral	No information	840 mg/kg bw	NIH national library [3] [cited in DFG, 1983, study could not be located]
Predates guideline; Animals were observed for mortality and toxic effects for 7 days; average lethal dose values were estimated non-statistically.	Albino rat, semi-adult 3-4 animals per sex per group	TMP (technical grade and purified) i.p. administration in glycol formal	Several doses were tested but not indicated.	Average lethal dose: <u>Technical grade:</u> Females: 1000 mg/kg bw Males: 500 – 1000 mg/kg bw <u>Purified:</u> Females: 1500 mg/kg bw Males 800 mg/kg bw	Sanderson et al., (1959)
Not indicated; No details presented.	Rat No information on strain, sex or number/group	TMP i.v., no vehicle	1800 & 2400 mg/kg bw	2400 mg/kg bw: lethal dose; 1800 mg/kg bw: sublethal dose, incoordination and pronounced weakness (at 6h), deep anaestesia and dyspnea (at 20 min), pronounced weakness and sleepiness (>24h) followed by	Vandekar (1957)

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance	Dose levels, duration of exposure	Value LD_{50}	Reference
				coma; No cholinergic symptoms	
Mouse					
Not indicated	Mouse No information on strain, sex or no/group	TMP oral	No information	1470 mg/kg bw	NIH national library [3] [cited in DFG, 1983, study could not be located]
Not indicated	Mouse No information on strain, sex or no/group	TMP oral	No information	3610 mg/kg bw	Newell et al. (1976) [cited in DFG, 1983, study could not be located]

10.1.1 Short summary and overall relevance of the provided information on acute oral toxicity

Several oral acute toxicity studies were located and relevant data are listed in Table 8. In most of these studies the test material was applied without dilution in a vehicle. These studies are of varying quality but none of the studies is conducted according to the most recent guidelines or GLP. Studies which lack essential information (e.g. on the route of exposure) were not included in the list. Some of the studies could not be located and reduced information on the applied procedure is available. Such studies are considered less relevant and are marked grey in the table. Studies using the i.v. or i.p. route were included in the table as in these studies also other effects than lethality were described. These studies are also marked grey in the table.

Oral LD_{50} values were derived for rat, mouse, rabbit and guinea pig and they ranged from 800 mg/kg bw to 3610 mg/kg bw. The majority of the studies with well described study protocol resulted in LD_{50} values <2000 mg/kg bw, with one exception being the rat study by Smyth et al (1969), which reported an LD_{50} value of 3388 mg/kg bw. The lowest of these values was the LD_{50} value of 840 mg/kg bw (NIH national library, cited by DFG, 1983) and although this LD_{50} was cited in many reports on TMP there are no details available on the applied protocol.

Deichmann & Witherup (1946) exposed rats, rabbits and guinea pigs to TMP via gavage. When absorbed in a lethal concentration from the gastrointestinal tract in rats, rabbits and guinea pigs a gradually decreasing rate and amplitude of respiratory movements (sometimes after a brief period of stimulation), general weakness, mild hyperirritability and fine tremors were observed. These signs were followed by marked dyspnea, collapse and death by respiratory failure. With increasing dose the time to death decreased. For further information on death rates and survival time see Table 9. The LD_{50} values in rats, rabbits and guinea pigs were in a comparable range.

[3] https://chem.nlm.nih.gov/chemidplus/rn/512-56-1; Progress Report for Contract No. NIH-NCI-E-C-72-3252, Submitted to the National Cancer Institute by Litton Bionetics, Inc. Vol. NCI-E-C-72-3252, Pg. 1973,

Table 9: Detailed results of the oral acute toxicity studies by Deichmann & Witherup (1946) in rat, rabbit and guinea pig

Number of animals used	Dose [mg/kg bw]	Percentage of death [%]	Survival time	LD_{50} [mg/kg bw]
Rats				1975
10	1185	0	-	
10	1676	50	2 to 8 days	
10	2095	30	30h to 5 days	
10	2514	100	24h to 8 days	
10	3830	100	20h to 3 days	
10	5626	100	15h to 3 days	
Rabbits				1257
6	742	0	-	
10	1125	20	5 and 7 days	
10	1436	80	30h to 5 days	
10	1676	100	30h to 48h	
6	2514	83	24h to 35h	
6	3830	100	24h to 36h	
6	5626	100	5h to 24h	
Guinea pig				1676
2	503	0	-	
2	742	0	-	
2	1125	50	1 died at 30h	
2	1676	100	24h and 30h	
2	2514	100	10h and 3 days	
2	3830	100	7h and 16h	
2	5626	100	7h and 8h	

10.1.2 Comparison with the CLP criteria

According to Table 3.1.1 of Regulation (EC) No. 1272/2008 a substance shall be classified as

- Acute Tox 4 (oral) if the LD_{50}/ATE values are > 300 and ≤ 2000 mg/kg bw.

- Acute Tox 3 (oral) if the LD_{50}/ATE values are > 50 and ≤ 300 mg/kg bw.

Overall the majority of reliable studies support classification in the oral acute toxicity category 4. Only one of the reliable studies resulted in an LD_{50} exceeding the upper limit for classification of 2000 mg/kg bw (i.e. 3388 mg/kg bw; Smyth et al. 1969).

Regarding the assignment of an ATE value the CLP guidance recommends to use the lowest most reliable LD_{50}. The lowest LD_{50} was 840 mg/kg bw from a rat study of low reliability (NIH national library, cited by DFG, 1983, protocol not well reported). The study by Deichmann & Witherup (1946) is rather old, but the

applied procedure is presented in detail. The study investigated rat, rabbits and guinea pigs. The lowest LD_{50} of 1257 mg/kg bw was derived for rabbits. There are no data available that would indicate that the rabbit is not relevant for humans and the derived LD_{50} values are in the same range for all investigated species.

10.1.3 Conclusion on classification and labelling for acute oral toxicity

In line with the criteria laid down in Regulation (EC) No. 1272/2008 a classification as Acute Tox 4, H302 and an ATE of 1257 mg/kg bw is proposed for TMP.

10.2 Acute toxicity - dermal route

Table 10: Summary table of animal studies on acute dermal toxicity

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance,	Dose levels duration of exposure	Value LD_{50}	Reference
Predates guideline; Refers to the method by Draize et al. (1944); 4 male rabbits per group; Fur was removed from the entire trunk by clipping and the dose is retained beneath an impervious plastic film (occlusive); Animals were immobilised during the 24 hours contact period, after which the film was removed and the rabbits caged for a 14 day observation period. The most probable LD_{50} value and its fiducial range are estimated according to Thompson et al. (1947) using the Tables of Weil (1952)	Rabbit, albino New Zealand Male	TMP Dermal, occlusive	Doses tested not indicated but stated that doses were arranged in a logarithmic series differing by a factor of two.	3388 mg/kg bw (converted from mL/kg bw to mg/kg bw based on the density of TMP of 1,197)	Smyth et al. (1969)

10.2.1 Short summary and overall relevance of the provided information on acute dermal toxicity

Only one dermal acute toxicity study could be located for TMP. Smyth (1969) is not conducted according to recent guidelines, but it is well reported and the procedure is comparable to current standards. Male albino New Zealand rabbits were exposed (occlusive) to TMP for 24h and observed for 14 days. An LD_{50} value of 3388 mg/kg bw was derived. No further details given.

10.2.2 Comparison with the CLP criteria

According to Table 3.1.1 of Regulation (EC) No. 1272/2008 a substance shall be classified as

- Acute Tox 4 (dermal) if the LD50/ATE values are > 1000 and ≤ 2000 mg/kg bw
- Acute Tox 3 (dermal) if the LD50/ATE values are > 200 ≤ 1000 mg/kg bw

The LD_{50} value of 3388 mg/kg bw exceeds the upper limit of 2000 mg/kg bw for classification.

10.2.3 Conclusion on classification and labelling for acute dermal toxicity

As the only available LD_{50} value for TMP exceeds the upper limit for classification no classification for dermal acute toxicity is proposed.

10.3 Acute toxicity - inhalation route

Not assessed in this dossier.

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

The DS proposed to classify TMP for Acute Tox. 4; H302 with an ATE of 1257 mg/kg bw based on results from oral (gavage) studies conducted in the rat, rabbit and guinea pig. The LD_{50} value from rabbit is the basis for the suggested ATE value.

The DS proposed no for classification for TMP via dermal route.

Acute toxicity via inhalation route was not assessed by the DS.

Acute oral toxicity

Five studies were assessed by the DS for acute toxicity in the CLH report (two of which are summarised in the table below). All the studies assessed either predated OECD TG or there was no indication whether the study was an OECD TG study. Two of the studies were considered sufficiently reliable for classification, although, both have deficiencies. Other studies were considered less reliable and only used to support the classification proposal, because the studies were not available (only referred to in reports) or had other deficiencies (reduced information on the applied procedure).

***Table**: Summary of the studies considered for the purpose of classification (adapted from Table 8 of the CLH report)*

Method, Guideline	Species, Strain, Sex, No/group	Test substance and type of administration	Value LD_{50}
Guideline: Predates guideline Deichmann & Witherup (1946)	Rabbit, guinea pig, rat. 6-8/group (rabbit), 2/group (guinea pig) and 10/group (rat). Sex not indicated.	TMP, Oral (gavage). Doses: 742-5626 mg/kg bw (rabbit), 503-5626 mg/kg bw (guinea pig), 1125-5626 mg/kg bw (rat)	LD_{50}: Rabbit: 1257 mg/kg bw, guinea pig: 1676 mg/kg bw, rat: 1975 mg/kg bw Effects: gradual decrease of rates and amplitude of respiratory movements, general weakness, mild hyperirritability and fine tremors. These signs were followed by marked dyspnea, collapse and death by respiratory failure Cause of death: Respiratory failure

Guideline: Predates guideline Smyth *et al.* (1969)	Rats (male Carworth-Wistar 4-5 weeks of age): 5/group. 14 days observation period	Large number of substances tested, TMP was one of them. Oral (intubation), TMP seems to have been undiluted. Dose levels: not reported but indicated that they were arranged in a logarithmic series differing by a factor of two.	LD_{50}: The most probable LD_{50} value and its fiducial range are estimated according to Thompson *et al.* (1947) using the Tables of Weil (1952) 3388 mg/kg bw (converted from mL/kg bw based on the density of TMP).

Deichmann & Witherup (1946) exposed rabbit, guinea pig and rats by oral (gavage). LD_{50} values were reported as 1257 mg/kg bw (rabbit), 1676 mg/kg bw (guinea pig) and 1975 mg/kg bw (rats). Cause of death were respiratory failure. Smyth *et al.* (1969) reported an LD_{50} value of 3388 mg/kg bw. The lowest LD_{50} value from the studies considered for classification was reported in rabbits (1257 mg/kg bw).

Other studies that were not considered sufficiently reliable for classification was an NIH national library report and Newell *et al.* (1976) both cited by Deutche Forschungsgemeinschaft - DFG (1983), Sanderson *et al.* (1959) and Vandekar (1957). The reported LD_{50} values or the lethal dose was in the range 840-3610 mg/kg bw in rats and mice. Most of the studies reported LD_{50} values in the same range as Deichmann & Witherup (1946). Based on the studies assessed a classification as Acute Tox 4, H302 and an ATE of 1257 mg/kg bw was proposed for TMP by the DS.

Acute dermal toxicity

One dermal acute toxicity study was provided for TMP (Smyth *et al.,* 1969). The study predated OECD TGs but was considered relevant for classification. Male albino New Zealand rabbits was exposed to TMP for 24 h and observed for 14 days. LD_{50} = 3388 mg/kg bw (converted from mL/kg bw based on the density of TMP of 1,197). The LD_{50} value exceeded the upper limit for classification and no classification for acute dermal toxicity was proposed by the DS.

Comments received during consultation

One comment was received from a member state competent authority (MSCA) supporting a classification as Acute Tox. 4; H302 following oral administration of TMP in rabbits with an ATE of 1257 mg/kg bw.

Assessment and comparison with the classification criteria

For acute oral toxicity, two studies are considered relevant for classification. In addition, RAC assessed one sperm motility assay (Cho & Park, 1994) reporting increased mortality after short time exposure. The study is assessed under STOT RE and germ cell mutagenicity. 20 animals/group were exposed by oral (gavage) for five days for up to five weeks. All animals in the 750 mg/kg bw/d dose group died within three days of exposure. There was no information on the exact timepoint the mortality occurred and whether the animals were exposed one or two times before they died. The CLP guidance states that mortalities that occur during the first 72 h after first treatment (in a repeated dose study) may also be considered for the assessment of acute toxicity, however since acute toxicity studies are

available RAC considers that the acute toxicity studies should be used as a basis for classification and ATE setting.

The preferred species for evaluation of acute toxicity by the oral route is the rat, but according to the CLP guidance on the application of the CLP criteria classification should be based on the lowest LD_{50} value in the most sensitive appropriate species. The study by Deichmann & Witherup (1946) was conducted in rats, rabbit and guinea pig. The LD_{50} values are all corresponding to classification as Acute Toxicity Category 4 (300 < ATE $\leqslant$ 2000 mg/kg bw). This is supported by other studies (not considered reliable for classification) showing LD_{50} values at similar ranges. The most sensitive species was rabbit with LD_{50} = 1257 mg/kg bw, RAC agrees with this LD_{50} value for ATE setting, but to round the ATE value to the closest two figures, which is 1300 mg/kg bw. There is no information available indicating that the effects in rabbits are not relevant for humans.

RAC agrees with the DS's proposal that a **classification as Acute Tox. 4, H302 is warranted**. Further, RAC agrees with the **ATE value of 1300 mg/kg bw**, as proposed by the DS, but rounded off.

For acute dermal toxicity, one study was considered relevant for classification. The LD_{50} value exceeded the upper limit for classification and RAC agrees with the DS's proposal that no classification is warranted.

10.4 Skin corrosion/irritation

Not assessed in this dossier.

10.5 Serious eye damage/eye irritation

Not assessed in this dossier.

10.6 Respiratory sensitisation

Not assessed in this dossier.

10.7 Skin sensitisation

Not assessed in this dossier.

10.8 Germ cell mutagenicity

Table 11: Summary table of mutagenicity/genotoxicity tests *in vitro*

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
Mechanistic studies				
In chemico experiment assessing the methylating	TMP	Reactions were carried out at 25, 37 and 60 °C; The bases were mixed with TMP	The applied procedure allowed the methylation reactions of nucleic acid-bases in homogenous aqueous phase owing to the water-soluble and stable	Yamauchi et al. (1976)

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
capacity of TMP – the reaction of TMP with nitrogen heterocycles of nucleic acids in an aqueous solution of pH 9-12 at 25-60°C.		in water at an appropriate pH: - uracil, thymine & adenine: pH 9-11; - cytosine & guanine: pH 11-12 Products were separated by a combination of extraction and column chromatography. Alkylation sites were determined by ultraviolet, NMR and mass spectra. Physical constants like Rf, melting point, elemental analysis, etc. were also employed for the identification of the products.	properties of TMP. TMP was found to alkylate the major heterocyclic moieties of nucleic acids and reactivity of heterocycles could be determined as follows based on the consumption of the starting materials: adenine > guanine > uracil ~ thymine > cytosine. Some of these reactions were shown to take place easily (e.g. the successive methylation of 1-methyluracil and 1-methylthymidine to 1,3-dimethyl derivatives). In addition to N-methylation of guanine, also O-6-methylation of guanine was demonstrated at a low yield. In each of these bases the first methylation occurs in the following order: adenine: N-9 ~ N-3 > N-7, N-1; guanine: N-1 > N-7 > N-3 > N-9, O-6; uracil: N-1 ~ N-3; thymine: N-1 ~ N-3; cytosine: N-1 > N-3.	
In vitro mode of action analysis of phosphorous containing flame retardants (alkyl-PFRs), including TMP	TMP (purity: ≥ 98%) vehicle: DMSO	A549 cells (widely used adenocarcinoma cell line, sensitive to divers stimuli; e.g. Yuan et al. 2019) Cells were grown at appropriate conditions in 96-well plates and exposed to TMP at 0, 1.02, 2.56, 6.4, 16, 40 & 100 µM for 96h. In the cytotoxicity assay doses up to 1024 µM were assessed. Commercially available test kits were applied according to the manufacturers recommendation to determine cytotoxicity, ROS & miSOD formation (miSOD, mitochondrial superoxide, is a by-product of oxidative phosphorylation), DNA content and mitochondrial impairment. Cell cycle and apoptosis assays were assessed using flow cytometry and p53 expression was detected using a p53 luciferase reporter gene analysis and quantitative real-time PCR was applied to analyse the expression of genes related to the p53-mediated pathway.	*Cytotoxicity:* TMP was the least cytotoxic with an LC_{50} of 311 µM. It was stated that chain length and log Kow would influence cytotoxicity of alkyl-PFRs (the lower the logKow and the shorter the chain length, the less cytotoxic). *ROS and miSOD formation:* In contrast to other alkyl-PFRs there was no strong increase in ROS or miSOD formation after TMP treatment. Also the induction of oxidative stress was associated with longer chain length of alkyl-PFRs. *Mitochondrial impairment:* TMP, as well as TEP (another short-chain alkyl-PFR) induced mitochondrial impairment. It was concluded that it was likely that the mitochondria-mediated pathway would be initiated by these substances, by a mode of action (MoA) other than cytotoxicity. *DNA damage:* DNA damage induced cell cycle arrest: TMP exposure increased the G1 phase distribution (cells in G1 phase) in the flow cytometric cell cycle analysis,	Yuan et al. (2020)

Method, guideline, deviations if any	**Test substance**	**Relevant information about the study including rationale for dose selection (as applicable)**	**Observations**	**Reference**
			indicating cell cycle arrest. TMP also increased the sub-G1 apoptosis peak, indicating apoptotic effects. The increase in apoptotic sub-G1 peak fits together with the increased expression of pro-apoptotic genes after TMP treatment (e.g. bax and the decreased expression of mdm2.	
Microbial *in vitro* test systems				
Bacterial reverse mutation assay; JAPAN: Guidelines for Screening Mutagenicity Testing Of Chemicals; GLP: no information available	TMP Purity: > 99%	0, 10, 50, 100, 500, 1000, 5000 µg/plate S. typhimurium TA 1535, TA 1537, TA 98 and TA 100; Target genes: His, Trp Without and with metabolic activation Liver S-9 fraction from Phenobarbital and 5,6-Benzoflavone pretreated male SD rats with NADPH-generating system Plate incorporation method; 3 plates per test; 2 replicates	The bacterial reverse mutation assay with TMP with and without metabolic activation gave negative results. Results were only presented for S. typhimurium TA 1535 und E. coli WP2 uvr A (although E. coli WP2 uvr A not among the strains indicated to be investigated). Positive and negative controls were reported to give valid results. For both strains 5000 µg/plate were identified as cytotoxic concentration with or without metabolic activation.	Anonymous (1996) (ECHA dissemination site; 12/10/2021)
Bacterial reverse mutation assay; Report on the test results of the capability of 311 chemicals to induce mutations in tester strains of *Salmonella typhimurium;* The tests were conducted within the National Toxicology Program (NTP) mutagenicity testing program.	TMP Vendour`s purity >99%	Initial tests were carried out with tester strains TA100 & TA98, for TMP no further strains were tested; Tests were carried out without S9 mix, with 30% rat S9 mix or with 30% hamster S9 mix (according to Haworth et al., 1983). The pre-incubation procedure according to Haworth et al. (1983) was applied with slight modifications. Initial testing was carried out in TA100 & TA98 without activation and with 30% rat and hamster S9 mix. If a positive response was obtained in one or both strains, only the positive test was repeated. If a negative response was obtained in these two strains the	The study authors concluded that the overall result was positive. TA 98 (+/-S9): no genotoxic effects TA 100 (+/-S9): dose dependent increase (see table in the line below).	Zeiger et al. (1992) NTP mutagenicity testing programme (Ames Conclusions | NTP Data Collections Guided Search (nih.gov))

<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance</th><th>Relevant information about the study including rationale for dose selection (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td></td><td></td><td>test was repeated in all four strains with and without S9 activation.

Chemicals were initially run in a toxicity assay to determine the appropriate dose range for the mutagenicity assay. Toxic concentrations were defined as those that produced a decrease in the number of his+ colonies, or a clearing in the density of the background lawn, or both.

At least five doses were tested in triplicates.</td><td></td><td></td></tr>
<tr><td></td><td></td><td colspan="2">TA100:
<table><tr><th>µg/Plate</th><th colspan="2">Without S9</th><th colspan="2">With 30% Rat S9</th><th colspan="2">With 30% Hamster S9</th></tr><tr><td>Neg. Co.[1]</td><td>130 ± 7.0</td><td>119 ± 9.2</td><td>159 ± 6.3</td><td>114 ± 8.7</td><td>143 ± 15.9</td><td>137 ± 8.9</td></tr><tr><td>333.0</td><td>127 ± 3.7</td><td></td><td>156 ± 9.0</td><td></td><td>170 ± 9.9</td><td></td></tr><tr><td>1000.0</td><td>135 ± 3.5</td><td>134 ± 4.0</td><td>159 ± 9.4</td><td>151 ± 9.6</td><td>189 ±3.2</td><td>174 ± 5.5</td></tr><tr><td>3333.0</td><td>156 ± 3.7</td><td>152 ± 8.5</td><td>193 ± 7.2</td><td>195 ± 14.8</td><td>184 ± 13.4</td><td>219 ± 3.8</td></tr><tr><td>6666.0</td><td>159 ±3.7</td><td>186 ± 13.9</td><td>198 ± 13.5</td><td>229 ± 5.0</td><td>231 ± 8.0</td><td>267 ± 5.2</td></tr><tr><td>10000.0</td><td>194 ± 3.8</td><td>227 ± 16.7</td><td>244 ± 14.1</td><td>265 ± 11.4</td><td>280 ± 15.7</td><td>315 ± 9.1</td></tr><tr><td>15000.0</td><td></td><td>233± 10.8</td><td></td><td>316 ± 5.2</td><td></td><td>353 ± 9.3</td></tr><tr><td>Pos. Co.</td><td>329 ± 9.5 [3]</td><td>422 ± 11.2 [3]</td><td>486 ± 38.8 [4]</td><td>535 ± 19.5 [4]</td><td>693 ± 49.0 [2]</td><td>489 ± 16.8 [2]</td></tr><tr><td>Trial Result</td><td>Equivocal</td><td>Positive</td><td>Weakly Pos.</td><td>Positive</td><td>Weakly Pos.</td><td>Positive</td></tr></table>
TA98:
<table><tr><th>µg/Plate</th><th colspan="2">Without S9</th><th colspan="2">With 30% Rat S9</th><th colspan="2">With 30% Hamster S9</th></tr><tr><td>Neg. Co.[1]</td><td>18 ± 2.5</td><td>-</td><td>44 ± 2.5</td><td>-</td><td>28 ± 1.9</td><td>-</td></tr><tr><td>333.0</td><td>16 ± 2.1</td><td>-</td><td>37 ± 2.6</td><td>-</td><td>26 ± 1.2</td><td>-</td></tr><tr><td>1000.0</td><td>17 ± 1.5</td><td>-</td><td>40 ± 2.6</td><td>-</td><td>24 ± 2.7</td><td>-</td></tr><tr><td>3333.0</td><td>17 ± 2.2</td><td>-</td><td>35 ± 4.0</td><td>-</td><td>30 ± 4.4</td><td>-</td></tr><tr><td>6666.0</td><td>15 ± 1.2</td><td>-</td><td>30 ± 0.3</td><td>-</td><td>25 ± 2.2</td><td>-</td></tr><tr><td>10000.0</td><td>17 ± 2.0</td><td>-</td><td>26 ± 5.8</td><td>-</td><td>29 ± 2.7</td><td>-</td></tr><tr><td>Pos. Co.</td><td>517 ± 24.0</td><td>-</td><td>161 ± 7.2</td><td>-</td><td>500 ± 28.4</td><td>-</td></tr><tr><td>Trial Result</td><td>Negative</td><td>-</td><td>Negative</td><td>-</td><td>Negative</td><td>-</td></tr></table>
1 … vehicle control: water; 2 … 1.0 µg/Plate 2-Aminoanthracene; 3 … 1.0 µg/Plate Sodium Azide; 4 … 2.5 µg/Plate 4-Nitro-O-Phenylenediamine</td></tr>
<tr><td>Bacterial reverse mutation assay;

Investigates the mutagenicity of tris (2,3-dibromopropyl) phosphate (Tris-BP) and its metabolites. TMP was tested as reference substance in the bacterial mutation assay.</td><td>TMP</td><td>Tester strain TA1535 & TA100 were applied.

The procedure of Ames et al. (1975) was followed.

The strains were tested with and without rat S9 mix.

Positive control: Tris-BP

Doses were tested in triplicates.</td><td>TMP increased the colony number in TA100, with and without S9 mix, but not in TA1535.

<table><tr><th>mmol/Plate</th><th colspan="2">TA1535</th><th colspan="2">TA100</th></tr><tr><td></td><td>- S9</td><td>+ S9</td><td>- S9</td><td>+ S9</td></tr><tr><td>Control</td><td>15 ±1</td><td>19 ± 5</td><td>115 ± 10</td><td>105 ± 10</td></tr><tr><td>1</td><td>11 ± 3</td><td>11 ± 3</td><td>134 ± 5</td><td>1338 ± 5</td></tr><tr><td>10</td><td>16 ± 7</td><td>13 ± 3</td><td>216 ± 8</td><td>222 ± 13</td></tr><tr><td>50</td><td>26 ± 2</td><td>18 ± 3</td><td>637 ± 23</td><td>678 ± 27</td></tr><tr><td>100</td><td>21 ± 1</td><td>17 ± 11</td><td>975 ± 50</td><td>1082 ± 2</td></tr><tr><td>250</td><td>-</td><td>-</td><td>1260 ± 28</td><td>1400 ± 57</td></tr><tr><td>Pos. Co.</td><td colspan="4">Conducted, but not presented</td></tr><tr><td>Trial Result</td><td>Negative</td><td>Negative</td><td>Positive</td><td>Positive</td></tr></table>
Mean his+ revertants per plate ± SD, 3 plates per dose.</td><td>Zeiger et al. (1982)</td></tr>
<tr><td>Bacterial reverse mutation assay;

120 organic chemicals including TMP</td><td>TMP</td><td>Tester strains: TA1535, TA1538, TA98, TA100 were applied.

Substances were tested once in batches of about ten substances, with the addition of S9 mix only. (S9 mix not further specified).</td><td>Positive results were obtained for tester strains TA1535 and TA100.

The study only presents the results at the dose at which the strongest effect was seen, which was 2500µg/plate for both positive strains. For TA1535 the increase</td><td>Purchase et al. (1978) and Appendix II, by Anderson & Styles</td></tr>
</table>

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
were analysed with 6 short-term assays. Only the TMP results of the bacterial mutation assay are presented here (TMP was negative in the remaining tests).		Positive and negative controls were included in each batch. Two plates per tested dose and three plates per positive and negative control were analysed. The procedure described by Ames (1973, 1975) was followed with slight modifications. Results were considered positive if: (a) there was a 2-fold increase over the negative control count for any strain (b) the negative-control cultures had counts within about 50% of the mean value (c) the positive-control cultures had counts greater than twice the negative control values (~ 10-fold) (d) the correct strains responded to the appropriate positive-control compounds (e) there was a background lawn indicating at least 10% survival	of revertants was 5-fold above controls and for TA100 the increase was 11-fold above controls. Tester strains TA1538 and TA98 gave negative results. The study authors concluded that the overall result was positive.	(1978)
Bacterial reverse mutation assay; 61 chemicals were investigated in the reverse mutation assay.	TMP	Tester strains TA1535, TA1537, TA 98 & TA 100 were obtained from Dr. B. Ames. All chemicals were tested at 0.05, 0.5, 5, 50 and 580 µg /plate, with and without S9 mix (rat). Results were considered positive if a 50% increase above the spontaneous frequency was observed	No detailed results were presented. The study authors concluded that the test was positive for TA100, with S9 and equivocal for the other strains.	Bruce & Heddle (1979)
Bacterial reverse mutation assay 29 chemicals were investigated in the reverse mutation assay using 4 different bacterial strains. Each chemical was assessed in two different	TMP Solvent: water	Tester strains *Salmonella typhimurium* TA102 & TA2638 (provided from Dr. B Ames) and *Escherichia coli* WP2/pKM101 & WP2*uvrA*/pKM101 were constructed by introducing the R-factor resistance plasmid pKM101 in strains WP2 & WP2*uvrA*, which were provided from Dr. T. Kada) Procedure: plate incorporation technique according to Maron & Ames (1983).	Number of revertants per plate see table below. All values are the average of three plates of the one experiment of each laboratory. Not indicated whether these results, presented in the table, are with or without metabolic activation. The study authors concluded that the test was positive in all 4 strains.	Watanabe et al. (1996)

<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance</th><th>Relevant information about the study including rationale for dose selection (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td>laboratories.</td><td></td><td>With and without addition of S9 mix (10% S) fraction).

Positive controls:

S. typhimurium strains– C-Mitomycin without S9, at 0.05 µg/plate (TA102) & at 0.1 µg/plate (TA2638)

E. coli strains – 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide without S9, at 0,1 µg/plate (WP2/pKM101) & at 0.01 µg/plate (WP2 uvrA /pKM101)

All strains - 2-Aminoanthracene with S9, at 5 µg/plate (TA102), 10 µg/plate (TA2638 & WP2/pKM101) and 1 µg/plate (WP2 uvrA /pKM101)

Cytotoxic doses were identified by toxicity to the background lawn and reduction in the numbers of revertant colonies and were excluded from the analysis.</td><td></td><td></td></tr>
<tr><td colspan="3"><table>
<tr><td>Dose</td><td colspan="2">TA102</td><td colspan="2">TA2638</td><td colspan="2">WP2/pKM101</td><td colspan="2">WP2uvrA/pKM101</td></tr>
<tr><td>µg/plate</td><td>Lab. 1</td><td>Lab. 2</td><td>Lab. 1</td><td>Lab. 2</td><td>Lab. 1</td><td>Lab. 2</td><td>Lab. 1</td><td>Lab. 2</td></tr>
<tr><td>0</td><td>433</td><td>473</td><td>53</td><td>28</td><td>52</td><td>64</td><td>93</td><td>93</td></tr>
<tr><td>313</td><td>-</td><td>-</td><td>64</td><td>34</td><td>58</td><td>70</td><td>91</td><td>105</td></tr>
<tr><td>625</td><td>-</td><td>-</td><td>57</td><td>40</td><td>67</td><td>72</td><td>98</td><td>111</td></tr>
<tr><td>1000</td><td>463</td><td>497</td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td></tr>
<tr><td>1250</td><td>-</td><td>-</td><td>71</td><td>49</td><td>70</td><td>98</td><td>99</td><td>126</td></tr>
<tr><td>2000</td><td>516</td><td>494</td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td></tr>
<tr><td>2500</td><td>-</td><td>-</td><td>86</td><td>63</td><td>93</td><td>123</td><td>135</td><td>154</td></tr>
<tr><td>3000</td><td>513</td><td>528</td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td></tr>
<tr><td>4000</td><td>524</td><td>542</td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td></tr>
<tr><td>5000</td><td>552</td><td>550</td><td>128</td><td>93</td><td>126</td><td>187</td><td>172</td><td>211</td></tr>
</table>
- … not tested</td><td></td><td></td></tr>
<tr><td>Bacterial reverse mutation assay

106 chemicals were investigated in the reverse mutation assay using 5 different S. typhimurium strains.</td><td>TMP

Solvent: water</td><td>Tester strains: S. typhimurium TA1535, TA1537, TA1538, TA98, TA100

Procedure: according to Ames et al (1975).

Concentration range covered: 0.6 – 1.1 x 10^6 nmol/plate.

With and without S9 mix (rat).

Positive result: greater than 3-fold increase of induced versus spontaneous revertants.</td><td>Positive for TA100: 0.0003 revertants/nmol

S9 mix slightly enhanced the response.

All other strains were negative.</td><td>DeFlora et al. (1981, 1984)</td></tr>
<tr><td>Reverse Mutation

Non-guideline study</td><td>TMP

2% (v/v)</td><td>Klebsiella pneumonia.

No further information.</td><td>TMP at a concentration of 2% (v/v) resulted in an 11,8-fold increase in the mutation rate of Klebsiella pneumonia</td><td>Voogd et al., (1972)

[cited in Connor,</td></tr>
</table>

Method, guideline, deviations if any	**Test substance**	**Relevant information about the study including rationale for dose selection (as applicable)**	**Observations**	**Reference**
				1979[
Reverse Mutation Non-guideline study	TMP	*Neurospora crassa*. No further information.	First report of TMP induced mutagenicity in *Neurospora crassa*	Kölmark (1956) [cited in Connor, 1979[
Reverse mutation Non-guideline study	TMP	Tester strains: *Serratia marcesans* HY/α13 & HY/α21. Concentrations applied: 25, 50 & 100 mg/ml. No S9 mix was applied. The paper disc method was applied.	Dose-related effect in both strains. Significant effects at all doses in *S. marcesans* HY/α13 and at 50 & 100 mg/ml in HY/α21	Dean (1972) [cited in US EPA, 2010)]
DNA repair	TMP	Investigated endpoint: genotoxicity expressed by preferential killing of the DNA repair deficient as opposed to the proficient strain. Procedure: The test was carried out according to Mohn et al. (1984). Briefly, bacteria were incubated together with the substance, with or without S9 (rat), in liquid suspension, before they were spread on agar petri plates and the numbers of colonies of the two different strains were counted. Tester strains were provided by Prof. G. Mohn: DNA repair proficient *E. coli* 343/636 *uvrB*$^{+}$/*recA*$^{+}$/*lac*$^{-}$ and DNA repair deficient *E. coli* 343/591 *uvrB*$^{-}$/*recA*$^{-}$/*lac*$^{+}$ Positive control without S9: 4-Nitroquinoline-N-oxide. Positive control with S9 mix not conducted, as the activity of the S9 mix was demonstrated in a separate Ames test with 2-aminoanthracene, benzol[a]pyrene & cyclophosphamide.	The result with S9 mix was considered positive as the colonies of the deficient strain were significantly reduced at a lower concentration than that of the proficient strain.	Hellmer & Bolcsfoldi, (1992)

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
		<table><tr><th rowspan="2">S9 mix</th><th rowspan="2">Strain repair def. / prof. [a]</th><th colspan="3">Concentration (mmol/l)</th><th rowspan="2">Result</th></tr><tr><th>Highest tested concentration</th><th>Without surviving colonies</th><th>Significant reduction in # of colonies</th></tr><tr><td rowspan="2">-</td><td>def.</td><td>866</td><td>866</td><td>216</td><td rowspan="2">-</td></tr><tr><td>prof.</td><td>866</td><td>-</td><td>216</td></tr><tr><td rowspan="2">+</td><td>def.</td><td>866</td><td>-</td><td>108</td><td rowspan="2">+</td></tr><tr><td>prof.</td><td>866</td><td>-</td><td>433</td></tr></table> a … prof. : DNA repair proficient strain, def. : DNA repair deficient strain		
DNA repair	TMP	*trp- E. coli* tester strains were investigated: WP2 (repair-proficient), WP67 (uvrA polA) & CM871 (uvrA-recAlexA-); With and without S9 mix. Doses were not reported.	Positive in all strains. A weak potentiation of the mutagenic activity was seen with the addition of S9.	DeFlora et al. (1984)
DNA repair	TMP	Tester strains: *E. coli* P3110 (polA+) & *E. coli* P3478 (polA-); One test concentration: 25µl No S9 mix was used	Negative. The study authors concluded that the study in the applied form had limitations and questioned its usefulness as a pre-screening tool for chemical carcinogens.	Fluck et al. (1976) [cited in US EPA, 2010]
DNA damage – alkaline elution / rat hepatocyte assay to detect double strand breaks. 81 compounds were tested.	TMP Vehicle: 1% DMSO	Rat hepatocytes Doses applied: 0.03, 0.1, 0.3, 1, 3, 7 & 10 mM No S9 mix was used. Negative control: 1% DMSO. Harvest, culture and treatment of rat hepatocytes was comparable to the procedure by Williams et al. (1976, 1977) & Bonney et al. (1974). The DNA elution was carried out as described by (Bradley et al., 1982, Bradley & Sina, 1983). The following parameters assessing cell damage and cytotoxicity were evaluated; Trypan blue dye exclusion viability assay (TBDE) after 3h (end of cell treatment) and after 9h (6h post treatment), Sine et al. (1983), intracellular ATP-content (Armstrong et al. 1992, Elia et al., 1993), intracellular K^+ content, MTT assay, DNA double strand breaks were assessed with the pulsed field gel electrophoresis assay (Elia et al., 1993, 1994, Elia & Nichols,	A slight increase in the elution rate was seen at the two top doses (see table in the line below) No cytotoxicity or cell damage was observed (see line below): Storer et al. (1996) wrongly cited Sina et al. (1983), in that they stated that the result for TMP was negative, which is not correct (see next reference below).	Storer et al. (1996)

<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance</th><th>Relevant information about the study including rationale for dose selection (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td></td><td></td><td>1993).

Light-microscopic assessment of cell blebbing.</td><td></td><td></td></tr>
<tr><td></td><td></td><td colspan="2">A slight increase in the elution rate was seen at the two top doses:
<table>
<tr><th>Dose (mM)</th><th>Vehicle / elution slope (negative Control, concentration, slope)</th><th>Induced elution slope (treatment slope minus negative control slope)</th></tr>
<tr><td>0.03</td><td rowspan="4">1% DMSO, 0.004</td><td>0.003</td></tr>
<tr><td>0.1</td><td>0.005</td></tr>
<tr><td>0.3</td><td>0.003</td></tr>
<tr><td>1.0</td><td>0.001</td></tr>
<tr><td>3.0</td><td rowspan="3">1% DMSO, 0.018</td><td>0.002</td></tr>
<tr><td>7.0</td><td>0.012</td></tr>
<tr><td>10.0</td><td>0.030</td></tr>
</table>
No cytotoxicity or cell damage was observed:
<table>
<tr><th>Dose (mM)</th><th>DNA double-strand breaks</th><th>TBDE -0</th><th>TBDE -3</th><th>MTT</th><th>ATP</th><th>K+</th><th>Cell blebbing</th></tr>
<tr><td>0.03</td><td>-</td><td>102</td><td>104</td><td>99</td><td>107</td><td>92</td><td>-</td></tr>
<tr><td>0.1</td><td>-</td><td>102</td><td>97</td><td>115</td><td>137</td><td>92</td><td>-</td></tr>
<tr><td>0.3</td><td>-</td><td>103</td><td>103</td><td>98</td><td>93</td><td>93</td><td>-</td></tr>
<tr><td>1.0</td><td>-</td><td>101</td><td>97</td><td>100</td><td>103</td><td>95</td><td>-</td></tr>
<tr><td>3.0</td><td>-</td><td>97</td><td>105</td><td>88</td><td>107</td><td>95</td><td>-</td></tr>
<tr><td>7.0</td><td>-</td><td>102</td><td>102</td><td>82</td><td>106</td><td>106</td><td>-</td></tr>
<tr><td>10.0</td><td>-</td><td>100</td><td>102</td><td>78</td><td>112</td><td>98</td><td>-</td></tr>
</table></td><td></td></tr>
<tr><td>DNA damage – alkaline elution / rat hepatocyte assay to detect DNA single strand breaks (SSBs).

64 carcinogenic and 25 non-carcinogenic substances were tested.</td><td>TMP</td><td>Rat hepatocytes: freshly isolated by the collagenase perfusion technique of Williams et al. (1976, 1977) and Bonney et al. (1974).

Cells were exposed to the chemical for 3h.

Doses applied: 0.03, 0.3 & 3 mM.

No S9 mix was used.

Cells were harvested and subjected to the elution procedure (Bradley et al., 1982, Bradley & Sina, 1983).

Cytotoxicity was assessed by release of glutamate-oxaloacetate transaminase (GOT) to the medium at the end of the 3h treatment period.

Positive and negative controls were included in each experiment.</td><td>A 3-fold increase in elution rate compared with the concurrent control was considered a biologically significant increase in DNA SSBs, i.e. a positive result.

The study authors classified TMP as clearly positive in this assay.</td><td>Sina et al. (1983)</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance</th><th>Relevant information about the study including rationale for dose selection (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td></td><td></td><td>
<table>
<tr><th>Dose (mM)</th><th>Viability (% of Control): GOT [1]</th><th>Slope, control</th><th>Slope, treated</th><th>Extent DNA damage [2]</th></tr>
<tr><td>0.03</td><td>81</td><td>0.046</td><td>0.048</td><td>-</td></tr>
<tr><td>0.3</td><td>86</td><td>0.035</td><td>0.124</td><td>+</td></tr>
<tr><td>3</td><td>88</td><td>0.035</td><td>0.155</td><td>+</td></tr>
</table>
[1] … GOT: glutamate-oxaloacetate transaminase, [2] … -: elution rate < 3.0-fold control; +: elution rate 3.1 to 5.0-fold control
</td><td></td><td></td></tr>
<tr><td colspan="5">Mammalian in vitro test systems</td></tr>
<tr><td>In vitro cytogenicity / chromosome aberration study in mammalian cells;

JAPAN: Guidelines for Screening Mutagenicity Testing Of Chemicals,

comparable to OECD 473;

GLP: yes</td><td>TMP

Purity: 99.9%</td><td>Chinese Hamster Lung (CHL/IU) cells;

Solvent: acetone;

positive controls:

-S9: Mitomycin C

+S9: Cyclophosphamide;

Doses: 0, 0.4, 0.7, 1.4 mg/ml, either -S9 (continuous treatment), -S9 (short treatment) or +S9 (short treatment);

S9: Rat liver, induced with phenobarbital and 5,6-benzoflavone:

2 plates per test;</td><td>Cytogenetic effects were not seen under the conditions of this experiment.

Neither clastogenic nor aneugenic activity was seen with or without metabolic activation.

<table>
<tr><th></th><th colspan="3">clastogenicity</th><th colspan="3">polyploidy</th></tr>
<tr><th></th><th>+</th><th>?</th><th>-</th><th>+</th><th>?</th><th>-</th></tr>
<tr><td>without metabolic activation:</td><td>[ ]</td><td>[ ]</td><td>[*]</td><td>[ ]</td><td>[ ]</td><td>[*]</td></tr>
<tr><td>with metabolic activation:</td><td>[ ]</td><td>[ ]</td><td>[*]</td><td>[ ]</td><td>[ ]</td><td>[*]</td></tr>
</table>

The applied doses had no strong effect on cell growth. Cell growth was mostly close to 100% of control and did not go below 83% of the control.</td><td>Anonymous, (1994a)

[ECHA disseminatio n site; 10/2021 and from the Japanese study report. Tables are available in English]</td></tr>
<tr><td>Chromosome breaking effects in human lymphocytes;

Non-guideline study

GLP: no</td><td>TMP</td><td>Human lymphocytes were cultured according to Moorhead et al. (1960), with slight modifications.</td><td>Dose dependent increase of anaphase aberrations was observed.

<table>
<tr><th>Concentration of TMP (mM)</th><th>% anaphases with aberrations</th><th>Total cells scored</th></tr>
<tr><td>Control</td><td>8.0</td><td>50</td></tr>
<tr><td>1</td><td>7.9</td><td>38</td></tr>
<tr><td>10</td><td>12.2</td><td>41</td></tr>
<tr><td>50</td><td>14.3</td><td>28</td></tr>
<tr><td>75</td><td>15.2</td><td>46</td></tr>
<tr><td>100</td><td>17.4</td><td>46</td></tr>
</table>
Doses ≥ 250 mM were strongly toxic.

Metaphase chromosomes were studied in preparations from colcemid-treated cells.

The number of cells with breaks increased from 2% to 20% after 5h (see table below). After 24h the increase was even up to 65% cells with breaks, at the higher concentrations. Both the number of cells with breaks and the number of breaks per cell increased with increasing dose and exposure duration. The number of chromosome rearrangements also increased with exposure duration, which indicates that DNA repair takes place despite the presence of TMP.

The study authors concluded that the TMP concentrations needed to get chromosome breaks may seem high, but</td><td>Söderman (1972)</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance</th><th>Relevant information about the study including rationale for dose selection (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td></td><td></td><td></td><td>effects may arise from much weaker concentrations, if cells are submitted to chronic exposure. This is also indicated by the comparison of the results from the 5h and 24h experiments. The underlying DNA changes which cumulate to chromosome breakage are assumed to occur at lower doses already, with less impact on cell viability.</td><td></td></tr>
<tr><td></td><td></td><td colspan="2"><table>
<tr><th>Concentration of TMP (mM)</th><th colspan="2">% cells with break</th><th colspan="2">% cells with gaps</th><th colspan="2">Total cells scored</th></tr>
<tr><th></th><th>5h</th><th>24h</th><th>5h</th><th>24h</th><th>5h</th><th>24h</th></tr>
<tr><td>Control</td><td>< 1%</td><td>< 1%</td><td>3.0</td><td>4.0</td><td>250</td><td>350</td></tr>
<tr><td>0.01</td><td>-</td><td>2.7</td><td>-</td><td>8.0</td><td>-</td><td>75</td></tr>
<tr><td>0.1</td><td>0</td><td>10.0</td><td>4.0</td><td>8.0</td><td>50</td><td>50</td></tr>
<tr><td>1</td><td>2.0</td><td>16.0</td><td>10.0</td><td>8.0</td><td>50</td><td>50</td></tr>
<tr><td>2.5</td><td>-</td><td>12.0</td><td>-</td><td>6.0</td><td>-</td><td>50</td></tr>
<tr><td>5</td><td>-</td><td>14.0</td><td>-</td><td>9.0</td><td>-</td><td>100</td></tr>
<tr><td>10</td><td>6.0</td><td>20.0</td><td>7.0</td><td>10.0</td><td>100</td><td>100</td></tr>
<tr><td>25</td><td>6.7</td><td>37.0</td><td>10.0</td><td>7.0</td><td>30</td><td>100</td></tr>
<tr><td>50</td><td>9.0</td><td>65.5</td><td>5.0</td><td>7.2</td><td>100</td><td>55</td></tr>
<tr><td>75</td><td>12.0</td><td>-</td><td>4.0</td><td>-</td><td>100</td><td>-</td></tr>
<tr><td>100</td><td>20.0</td><td>-</td><td>15.0</td><td>-</td><td>40</td><td>-</td></tr>
</table>
5h experiment: ≥ 250 mM strongly toxic; 24h experiment: ≥ 75 mM strongly toxic</td><td></td></tr>
<tr><td>Micronucleus test in chinese hamster lung cells</td><td>TMP</td><td>Doses were not reported.
No S9 mix was applied.
No further information.</td><td>Positive result, no further information can be read from the English data table.</td><td>Ni et al. (1993; published in Chinese)
[cited in US EPA, 2010]</td></tr>
</table>

In a mechanistic study the alkylating properties of TMP on nucleic acid bases were investigated (Yamauchi et al., 1976). It is noted that the conditions in this study do not exactly mirror the situation in situ, i.e. the pH levels were different than in the cellular milieu, the temperatures are partly different and also the ratio of the amount of the specific base in relation to TMP was shown to influence the result. Nevertheless, TMP was found to methylate the major heterocyclic moieties of nucleic acids and some of these reactions were shown to take place easily (e.g. the successive methylation of 1-methyluracil and 1-methylthymidine to 1,3-dimethyl derivatives). In addition to N-methylation of guanine, also O-6-methylation of guanine was shown, which was considered relevant from the physiological point of view, despite its low yield, as such reactions build the basis of powerful mutagenic effects through atypical base pairing.

Yuan et al. (2020) also conducted a mechanistic *in vitro* study with TMP. They concluded that TMP produced hardly any cytotoxicity, which is in line with the results of the *in vitro* and *in vivo* studies presented later on. Yuan et al. (2020) also concluded that reactive oxygen species (ROS) formation was not involved in TMP toxicity, but they identified mitochondrial interference, but via different ways than ROS formation. In this *in vitro* assay also cell cycle arrest was induced by TMP as demonstrated by flow-cytometric cell cycle analysis.

TMP has been tested in many bacterial reverse mutation assays of varying quality and the results of these tests are described in varying detail. The vast majority of these studies was considered positive and the positive results achieved in *S. typhimurium* TA100 indicate that TMP induces base-pair mutations, but not frame-shift mutations (negative in in *S. typhimurium* strain TA98) (Connor, 1979). The positive results in TA 100 is reproducible, but conflicting results were obtained regarding the effect of metabolic activation on the outcome of the studies. There are positive results with and without metabolic activation and in other cases

only the addition of S9-mix lead to positive results, or increased the mutagenic response. Overall, it is concluded that TMP induces gene mutations in bacterial reverse mutation assays.

TMP was also tested in two assays in mammalian cell lines. Anonymous (1994a) conducted a chromosome aberration study (comparable to OECD 473) in Chinese hamster cells (CHL/IU) and produced negative results for clastogenicity and aneugenicity, whereas a scarcely described micronucleus test in Chinese hamster cells was positive (Ni et al., 1993, cited in US EPA, 2010).

Söderman (1972) investigated human lymphocytes and described a dose-dependent increase of anaphase aberrations upon TMP treatment. Also metaphase aberrations were investigated and a time dependent increase was observed. The presence of chromosome re-arrangements also increased with exposure duration, indicating that repair occurred despite the presence of TMP in the test system.

The conducted *in vitro* assays are not in compliance with currently accepted test guidelines and reporting is sometimes limited. However, overall the majority of the conducted *in vitro* studies, consisting of bacterial reverse mutation assays, DNA repair tests, *in vitro* mammalian micronucleus and chromosome aberration tests (in human lymphocytes) were positive, supporting a mutagenic potential of TMP in *in vitro* systems.

Table 12: Summary table of mutagenicity/genotoxicity tests in *Drosophila melanogaster*

Method, guideline, deviations if any	**Test substance,**	**Relevant information about the study including rationale for dose selection (as applicable)**	**Observations**	**Reference**
Drosophila melanogaster				
Drosophila melanogaster – induction of second chromosome recessive lethals. Comparable to OECD 477 (guideline was deleted on April 4th, 2014)	TMP feeding	Males and females from the Oregon-R stock of *Drosophila melanogaster* which had been rendered lethal-free by the Cy/NIL method, were mated and the females allowed to oviposit on standard maizemeal, yeast, agar media which had been prepared containing various concentrations of TMP. At 0.02 M survival was reduced to 20%, therefore no higher doses were applied. Concentrations tested: 0, 0.01, 0.015 & 0.02 M Emerging flies were collected and the males were mated individually to two Cy/BIL virgin females. Time until fertility was retained was recorded.	In most cases the males were sterile for varying periods of time. A clear and significant sterilising effect was seen down to the concentration of 0.002 M TMP (approx. 370ppm), when male larvae were fed on TMP containing agar. It appears that primarily the meiotic stages are affected at lower doses, whereas at higher doses, which produce sterility up to 12, 14 or more days also pre-meiotic stages are affected. No effects were seen on female fertility. At a concentration of 0.01 M no sperm was detected in mated females and no sperm bundles were seen in the males. Among treated males there is an increasing incidence of mutation with increasing dose. A significant increase in lethal mutations was seen at 0.01 M TMP. Positive result.	Dyer & Hanna, (1972)
Drosophila melanogaster– induction of second chromosome recessive	TMP feeding	Tested concentrations not indicated.	TMP was used as a positive control and induced a high level of accumulated mutations (83%) compared with negative control (9%). Positive result.	Hanna & Dyer (1975) [cited in US EPA 2010]

Method, guideline, deviations if any	Test substance,	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
leaks.				
Drosophila melanogaster -– induction of second chromosome recessive lethals.	TMP feeding	Concentrations tested: 0, 100, 300 & 1000 mg/kg bw	TMP was used as a positive control and gave negative results at 100 & 300 mg/kg bw, but was positive at 1000 mg/kg bw. Unclear whether doses were given as ppm or mg/kg bw. Positive result.	Valencia (1981) [cited in US EPA 2010]
Drosophila melanogaster MWh-flr^3 cross – somatic mutation (Wing-spot test)	TMP feeding	Tested concentrations: 0, 5, 10 & 20 mM for 48h	Dose dependent induction of all types of spots (small, large and twin); results inconclusive at 5 mM and positive ≥ 10 mM. Positive result.	Graf et al. (1989) [cited in US EPA, 2010]
Drosophila melanogaster – Eye mosaic assay	TMP feeding	Tested concentrations: 2 & 10 mM for 3 days and 10, 50, 100 & 200 mM on the surface of food given for 48h	Positive at both doses following the 3 day treatment. Positive at both doses following the 48 h treatment on food surface ≥ 50 mM.	Vogel & Nivard (1993) [cited in US EPA, 2010]

TMP was tested in 5 *Drosophila melanogaster* mutation assays, which gave all positive results. It was demonstrated that TMP fed to developing larvae produces males which are temporarily sterile, apparently by selectively killing pre-meiotic and meiotic germ cells, even at very low doses (Dyer & Hanna, 1972). Dyer & Hanna (1972) also concluded that TMP's action is different in rodents and *Drosophila*, but leaves no doubt that this compound induces mutations. In two of the studies TMP was used as positive control and gave appropriate results, with dose and time dependent increases in response. Also for somatic mutations a dose and time dependent increase was observed.

Table 13: Summary table of mutagenicity/genotoxicity tests in mammalian somatic or germ cells *in vivo*

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations	Reference
Mammalian Bone Marrow Chromosomal Aberration Test(screening test); Comparable to OECD 475	TMP (commercial grade) Single and repeated i.p. administration (TMP in water)	Male CD rats, 3-4 weeks old; Bone marrow samples were obtained and prepared according to Legator et al. (1969); Preparations were evaluated for structural chromatid aberrations such as gaps, chromatid breaks, isochromatid breaks & reunion figures.	Positive results. TMP induces chromosome aberrations. No acute toxic effects were observed at any dose. <u>Time dependence:</u> The incidence of chromatid aberration was maximal at 48h after a single i.p. injection of 2000 mg/kg bw TMP, although at 24h a similar value was approached. After 72 and 96 hrs there were no or less pronounced cytogenetic	Adler et al. (1971)

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations	Reference
		Each affected cell was classified in one of 4 categories: cells with gaps only, cells with chromatid breaks, cells with reunion figures & cells with more than 10 aberrations. *Dosing and sampling schemes:* Time response: after single i.p. dose of 2000 mg/kg bw bone marrow sample were gathered after 6, 12, 24, 48, 72 & 96h. The dose was shown to be subtoxic in a previous experiment. Dose range: Bone marrow samples were prepared 24h after single i.p. dosing with 500, 750, 1000, 1250, 1500 & 1750 mg/kg bw. The mitotic index was based on counting 500 – 600 cells per animal. Repeated dosing: i.p. injection of 500 mg/kg bw TMP was carried out on 4 consecutive days. Samples were prepared 6h and 24h after termination of treatment. No historical control data were presented. Number of animals per group did not always reach the recommended number of 5. The dosing scheme exceeded the requirements of OECD 475.	effects observed. The chromosome damaging effect at 2000 mg/kg bw TMP was markedly lower than at 10 mg/kg bw TEPA (which was also tested in the study, data not presented). Dose dependence: A dose related increase in incidence of chromatid aberrations and a concomitant dose-related decrease in the mitotic index was observed (single i.p. injection). The dose of 2000 mg/kg bw TMP induced comparable effects as TEPA at 2.5 – 5 mg/kg bw (i.p. injection, data not presented). Cumulative effect: The chromosome damaging effect was more pronounced after repeated exposure on 4 consecutive days than after single dosing. There was a substantial number of cells exhibiting more than 10 aberrations in the sample taken at 6h. TMP did not induce a significant decrease of the mitotic index, which was in contrast to TEPA (data not presented). Positive result.	
		Time dependence:		

<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance</th><th>Relevant information about the study (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td></td><td></td><td colspan="2">
2000 mg/kg bw TMP
<table>
<tr><th>Time (h)</th><th># of animals</th><th>Mitotic ind. (%) [a]</th><th>Total # of cells</th><th colspan="2">Cells with gaps only</th><th colspan="2">Cells with chromatid aberrations</th></tr>
<tr><th></th><th></th><th></th><th></th><th>Number</th><th>%</th><th>Number</th><th>% ± S.D.</th></tr>
<tr><td>6</td><td>5</td><td>7.1</td><td>360</td><td>11</td><td>3.1</td><td>7</td><td>1.9 ± 2.5</td></tr>
<tr><td>12</td><td>4</td><td>5.0</td><td>360</td><td>19</td><td>5.3</td><td>30</td><td>8.3 ± 5.0</td></tr>
<tr><td>24</td><td>5</td><td>5.9</td><td>360</td><td>15</td><td>4.2</td><td>73</td><td>20.3 ± 5.75</td></tr>
<tr><td>48</td><td>5</td><td>8.9</td><td>360</td><td>8</td><td>2.2</td><td>81</td><td>22.5 ± 9.45</td></tr>
<tr><td>72</td><td>3</td><td>8.1</td><td>200</td><td>2</td><td>1.0</td><td>12</td><td>6.0 ± 1.8</td></tr>
<tr><td>96</td><td>2</td><td>6.2</td><td>100</td><td>1</td><td>1.0</td><td>3</td><td>3.0 ± 0.0</td></tr>
<tr><td>control</td><td>17</td><td>11.7</td><td>1090</td><td>20</td><td>1.83</td><td>13</td><td>1.19</td></tr>
</table>
[a] … Based on 500-600 cells per animal.

<u>Dose dependence:</u>
<table>
<tr><th colspan="8">Samples were taken 24h after treatment</th></tr>
<tr><th>i.p. dose (mg/kg bw)</th><th># of animals</th><th>Mitotic ind. (%)</th><th>Total # of cells</th><th colspan="2">Cells with gaps only</th><th colspan="2">Cells with chromatid aberrations</th></tr>
<tr><th></th><th></th><th></th><th></th><th>Number</th><th>%</th><th>Number</th><th>% ± S.D.</th></tr>
<tr><td>500</td><td>5</td><td>8.4</td><td>240</td><td>4</td><td>1.7</td><td>1</td><td>0.4 ± 1.1</td></tr>
<tr><td>750</td><td>5</td><td>10.9</td><td>200</td><td>4</td><td>2.0</td><td>9</td><td>4.5 ± 3.7</td></tr>
<tr><td>1000</td><td>2</td><td>7.8</td><td>200</td><td>5</td><td>2.5</td><td>12</td><td>6.0 ± 1.8</td></tr>
<tr><td>1250</td><td>5</td><td>7.8</td><td>200</td><td>2</td><td>1.0</td><td>10</td><td>5.0 ± 3.05</td></tr>
<tr><td>1500</td><td>4</td><td>7.5</td><td>240</td><td>2</td><td>0.8</td><td>26</td><td>10.8 ± 4.3</td></tr>
<tr><td>1750</td><td>5</td><td>7.1</td><td>280</td><td>3</td><td>1.1</td><td>33</td><td>11.8 ± 7.0</td></tr>
<tr><td>2000</td><td>5</td><td>5.9</td><td>360</td><td>15</td><td>4.2</td><td>73</td><td>20.3 ± 5.75</td></tr>
<tr><td>Control</td><td>9</td><td>8.9</td><td>360</td><td>2</td><td>0.56</td><td>1</td><td>0.28</td></tr>
</table>
<u>Cumulative effect:</u>
<table>
<tr><th colspan="8">4 * 500 mg/kg bw</th></tr>
<tr><th>Sampling time</th><th># of animals</th><th>Mitotic ind. (%) [a]</th><th>Total # of cells</th><th colspan="2">Cells with gaps only</th><th colspan="2">Cells with chromatid aberrations</th></tr>
<tr><th></th><th></th><th></th><th></th><th>Number</th><th>%</th><th>Number</th><th>% ± S.D.</th></tr>
<tr><td>6h</td><td>2</td><td>5.7</td><td>200</td><td>4</td><td>2.0</td><td>14</td><td>7.0 ± 1.5</td></tr>
<tr><td>24h</td><td>2</td><td>6.5</td><td>200</td><td>3</td><td>1.5</td><td>9</td><td>4.5 ± 1.8</td></tr>
<tr><td>Control</td><td>4</td><td>6.7</td><td>200</td><td>5</td><td>2.5</td><td>0</td><td>0</td></tr>
</table>
a … Based on 500 cells per animal.
</td></tr>
<tr><td>Chromosome aberration test in bone marrow cells

Reproducibility of the study design was assessed in 4 different laboratories.</td><td>TMP

i.p. or gavage administration

vehicle: corn oil</td><td>Male Osborne rats.

Bone marrow samples were obtained and prepared according to Legator et al. (1969).

Dosing: single dose of 0 or 2000 mg/kg or at 0 or 1000 mg/kg bw/day on 5 consecutive days.

Rats were sacrificed 18, 24 or 48h after single application or 6h after the last of 5 applications.

To induce accumulation of metaphase figures animals received 4 mg/kg bw Colcemid (i.p.) 4-5h prior to sacrifice.

TMP was used as positive control.

Negative control: corn oil.

5 animals per dosed group, negative control consisted of 8 animals.</td><td>TMP was used as positive control, results for TMP:

Increases in the different types of aberrations were seen in all laboratories at all time points after single i.p. exposure as well as after exposure to 5 consecutive i.p. doses, but varied in degree. All laboratories reported a maximum increase of cells with more than 10 aberrations at 48h.

After oral application there was more variation between the laboratories but comparable trends were observed.

It can be concluded that TMP induced chromosomal aberrations after single and repeated oral and i.p. administration.

Positive result.</td><td>Legator et al. (1973)</td></tr>
<tr><td>Chromosome</td><td>TMP</td><td>Male Osborne rats.</td><td>TMP was used as positive control, results</td><td>Sheu et al.</td></tr>
</table>

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations	Reference
aberration in bone marrow cells	i.p. or gavage administration	Dosing: 0 (solvent control) or unspecified dose as a single dose or as 5 consecutive daily doses.	for TMP: Induced chromosomal aberrations by both routes and with both single and repeated exposure. Positive result.	(1979) [cited in US EPA 2010]
Chromosome aberration in bone marrow cells	TMP i.p. administration	Male Wistar rats. The study tested 6 chemicals, one of which was TMP. Dosing: Single dose of 0 or 3000 mg/kg bw & 0 or 1500 mg/kg bw 5 times in 1 day.	TMP induced chromosome aberrations including gaps, breaks, and fragments, and induced significantly greater numbers of abnormal cells following single and multiple doses. No further details were presented. Positive result.	Anderson & Richardson (1981)
Chromosome aberration in bone marrow cells	TMP gavage	Male & female Sprague-Dawley rats. 0 & 2000 mg/kg bw (single dose, 24h prior to sacrifice)	TMP was used as a positive control, results for TMP: Induced chromatid gaps (males only), breaks and exchanges, chromosome breaks in males, and severely damaged cells in both sexes; no effect on mitotic index. Positive result.	Sinha et al. (1983) [cited from US EPA, 2010]
Chromosome aberration in bone marrow cells	TMP route not indicated	Male mice (strain not specified). Dosing: 1250, 1500 & 1750 mg/kg bw	Only an abstract is available: No control is mentioned. Maximum number of chromosome aberrations observed after 48h. Maximum changes (breaks, gaps, and fragments) seen at highest dose, data not shown. Positive result.	Farrow, 1975 [cited in US EPA, 2010]
Chromosome aberration in bone marrow cells	TMP i.p. application Vehicle: buffered saline, pH 7.3	5 B6D2F1/J mice per group (sex not specified); Doses: 0, 500, 750, 1000 & 2000 mg/kg bw/day for 5 days. 50 metaphases were scored for each animal tested. The evaluation generally followed the protocol outlined by the *Ad Hoc* Committee on Chromosome Methodologies in Mutation Testing.	A dose related increase in chromatid breaks ≥ 500 mg/kg bw/day was observed. 2000 mg/kg bw/day was lethal. No other aberration than chromatid breaks was observed. The obtained results are in line with the induction of micronuclei investigated in the same study (see section on micronuclei below). No further information was presented. The conducting laboratory conducted 8 micronuclei tests with triethylenemelamine (TEM) over a period of 7 months and found consistent results, demonstrating proficiency in conducting	Weber et al. (1975)

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations	Reference
			the assay. Positive result.	
Chromosome aberration in bone marrow cells	TMP i.p. application	Male mice (Q strain). Doses not reported. Cells investigated 10 & 16 days after treatment.	TMP was used as a positive control, results for TMP: Induced chromosomal aberrations including breaks, exchanges and gaps. Positive result.	Moutschen-Dahmen et al. (1981)
In vivo bone marrow micronucleus test, Non-guideline, comparable to OECD 474	TMP i.p. application Vehicle: buffered saline, pH 7.3	5 B6D2F1/J mice per group (sex not specified); Doses: 0, 500, 750, 1000 & 2000 mg/kg bw/day for 5 days. Mice were sacrificed 4h after the last dose. The procedure by Matter & Schmid (1971) was followed, with the exception, that bone marrow was directly flushed into a minimal amount of fetal calf serum, slides were air dried for 1h & pH 6.0 phosphate buffer was used to dilute Wright and Giemsa stains. 1500 – 2000 nucleated bone marrow cells (including erythrocytes with micronuclei) per animal were analysed. (OECD 474 would require 4000 cells).	A dose-related increase in the frequency of micronuclei ≥ 500 mg/kg bw/day was observed.2000 mg/kg bw/day was lethal. The obtained results are in line with the induction of chromosomal aberrations investigated in the same study (see section on chromosomal aberrations above). No further information was presented. Positive result.	Weber et al. (1975)
In vivo bone marrow micronucleus test	TMP i.p. application	Female hybrid (C57BL/6 x C3H/He) mice, 11 – 14 weeks of age; Doses: 0 – 10000 mg/kg bw, doses were not explicitly reported but the range can be obtained from the figure plotting micronuclei; 8 animals per group; Treatment: 5 consecutive days, sacrifice 4h after the last dose 333 reticulocytes were counted for each of the 3 bone marrow preparations	An increase in micronuclei was observed at and above ~6000 mg/kg bw, as can be obtained from the figure plotting micronuclei. The study authors concluded that TMP was positive in this test.	Bruce & Heddle (1979)

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations	Reference
		per animal, resulting in ~1000 cells per treatment group. Results are presented as percent micronuclei after subtraction of the frequency found in the control group (which was treated simultaneously). Individual points considered: if the treated group exceeded that control group by 1% (10/1000) Agents were considered positive when results could be repeated and a dose-response curve was observed.		
In vivo bone marrow micronucleus test	TMP route not mentioned	Mice (sex and strain not reported); Dosing: 0, 1250, 1500 & 1750 mg/kg bw	Time and dose related increase in micronuclei, data not presented. Positive result.	Farrow et al. (1976) [cited in US EPA, 2010]
In vivo bone marrow micronucleus test	TMP i.p. application	Mice (sex and strain not reported). Dose not reported.	English data table; negative in vivo; no other details available in English. Negative result.	Ni et al. (1993) (published in Chinese) [cited in US EPA, 2010]
In vivo Comet assay, testicular cells, Non-guideline study but comparable to OECD 489	TMP Oral, gavage; Vehicle: water	Male CD1 mice, 5 weeks old; 5 animals per dose, positive and negative control group; Negative control: vehicle Positive control: Ethylmethanesulfonate (EMS), CAS: 62-50-0, in water, 300 mg/kg bw; Applied doses of TMP: 125, 250 & 500 mg/kg bw; administered twice – 24 hours apart; Two to four hours after the second dose animals were sacrificed. After macroscopic examination the testicles were excised and	TMP induced a significant positive effect at 500 mg/kg bw (see table in the line below) Histological examination of the left testis did not reveal any treatment related effects on testes. No signs of cytotoxicity were observed. No historical controls were presented in the publication, but from personal communication with the study authors, historical control data were received: Testes, water: 2.9 (2.3-3.5%). At 500 mg/kg bw/day TMP gives a mean median value of 8.2% for 5 mice. This clearly exceeds the historical controls. The values from the historical controls also confirm the adequacy of the concurrent negative controls. (Mean median values (95% CI) of % tail DNA were used because the distributions are skewed. Number of tests building the	Hansen et al. (2014)

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations	Reference
		weighed. After removing the capsule the right testicles were stored at -80°C until analysed. The DNA isolated from the testicular tissue origins from a mixture of different cell types. The alkaline version of the Comet assay was applied according to Tice et al., 2000, following the recommendations of Hartmann et al., 2003; with minor modifications according to the manufacturer of CometAssay ® Kit (Trevigen, Gaithersburg, Maryland); DNA damage was quantified as % tail DNA using a fully automatic scoring system; 2 gels per animal and 100 cells per gel were analysed. The left testicles were fixed in Bouin's fixative and routinely processed for paraffin fixation. One section (3µm) per testis were evaluated by an experienced pathologist. A detailed qualitative examination was made, taking into account the tubular stages of the spermatogenic cycle. Aim of the study: Develop a statistical methodology for the assessment of Comet assays in testicular cells, 11 test substances were tested, including TMP.	historical control: n=15-20 → no exact number can be given, because historical control data were also available for other organs and consisted of 15 to 20 experiments). The study predates the last up-date of the related OECD guidance 489, which was published in 2016, however its study design largely fulfils the requirements from this latest up-date. In the guidance document clear recommendations for conducting in vivo comet assays in rodent liver, jejunum or duodenum are included. However, it also states that any tissues can be used, if a positive control relevant for the respective tissue is included. The study is well conducted, positive and negative controls gave adequate results and a clear and statistically significant increase in % tail DNA was seen in the highest dose tested, with some increase in the mid dose. In order to distinguish genotoxic from cytotoxic DNA fragmentation, OECD 489 recommends to include one or more indicators of cytotoxicity in the protocol. The guideline further states that many measures of cytotoxicity have been proposed and of these histopathological changes are considered a relevant measure of tissue toxicity. In the present study it is stated that no treatment related effects were seen in testes upon histological examination. Overall the results are considered relevant and indicate genotoxic activity of TMP in mouse testicular cells in vivo. In line with OECD 489, Hansen et al. (2014) conclude that the testicular samples represent a mixture of different cell types including somatic cells as well as germ cells. Therefore positive results do not clearly demonstrate genotoxicity in germ cells, but they demonstrate that the substance reaches the gonads, where it interferes with the DNA. Personal communication with the study authors: TMP also gave positive results in the Comet assay in liver and kidney, but these data were not presented in the publication as the focus was on the development of a method to apply the Comet assay in testis.	

<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance</th><th>Relevant information about the study (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td></td><td></td><td colspan="2">The table below presents average % tail DNA with SD in parentheses. The values were calculated by first averaging the two summary statistics for each animal and from these values the average and SD were calculated. Data were analysed by means of a linear mixed-effects model as defined in model (1) with Dunnett's test to compare the dose groups to their corresponding control. Values marked grey indicate a significant difference: * p < 0.05, ** p < 0.01, *** p < 0.001.
<table>
<tr><th>Raw data</th><th>0 mg/kg bw/day</th><th>125 mg/kg bw/day</th><th>250 mg/kg bw/day</th><th>500 mg/kg bw/day</th><th>Positive control (EMS)</th></tr>
<tr><td>Mean</td><td>7.1 (1.9)</td><td>7.9 (3.7)</td><td>9.8 (2.2)</td><td>13.9 (3.6)***</td><td>12.4 (1.8)***</td></tr>
<tr><td>log(mean)</td><td>1.9 (0.3)</td><td>2.0 (0.5)</td><td>2.3 (0.2)</td><td>2.6 (0.3)**</td><td>2.5 (0.2)***</td></tr>
<tr><td>Median</td><td>2.5 (0.6)</td><td>2.1 (0.9)</td><td>4.4 (0.4)</td><td>8.2 (3.2)***</td><td>8.7 (2.6)***</td></tr>
<tr><td>65th perc.</td><td>4.7 (2.1)</td><td>5.4 (3.1)</td><td>8.0 (0.6)</td><td>12.4 (4.4)***</td><td>12.5 (2.6)***</td></tr>
<tr><td>75th perc.</td><td>9.5 (4.7)</td><td>10.8 (7.0)</td><td>11.7 (3.0)</td><td>17.9 (6.8)*</td><td>16.0 (2.4)**</td></tr>
<tr><td>85th perc.</td><td>14.9 (5.0)</td><td>16.6 (8.9)</td><td>21.6 (11.0)</td><td>28.8 (7.3)*</td><td>21.3 (3.4)*</td></tr>
<tr><td>95th perc.</td><td>25.4 (3.9)</td><td>32.7 (15.6)</td><td>38.7 (11.3)</td><td>46.6 (8.5)**</td><td>38.1 (3.9)***</td></tr>
</table>
<table>
<tr><th>log (data)</th><th>0 mg/kg bw/day</th><th>125 mg/kg bw/day</th><th>250 mg/kg bw/day</th><th>500 mg/kg bw/day</th><th>Positive control (EMS)</th></tr>
<tr><td>Mean</td><td>1.0 (0.2)</td><td>0.9 (0.4)</td><td>1.4 (0.1)</td><td>1.9 (0.4)***</td><td>1.9 (0.2)***</td></tr>
<tr><td>log(mean)</td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td></tr>
<tr><td>Median</td><td>0.8 (0.3)</td><td>0.6 (0.5)</td><td>1.5 (0.1)**</td><td>2.0 (0.4)***</td><td>2.1 (0.3)***</td></tr>
<tr><td>65th perc.</td><td>1.4 (0.4)</td><td>1.5 (0.6)</td><td>2.1 (0.1)*</td><td>2.5 (0.4)***</td><td>2.5 (0.2)***</td></tr>
<tr><td>75th perc.</td><td>2.1 (0.4)</td><td>2.2 (0.7)</td><td>2.4 (0.3)</td><td>2.8 (0.4)*</td><td>2.8 (0.2)**</td></tr>
<tr><td>85th perc.</td><td>2.6 (0.3)</td><td>2.7 (0.5)</td><td>3.0 (0.5)</td><td>3.3 (0.3)*</td><td>3.0 (0.2)**</td></tr>
<tr><td>95th perc.</td><td>3.2 (0.2)</td><td>3.4 (0.5)</td><td>3.6 (0.3)</td><td>3.8 (0.2)**</td><td>3.6 (0.1)***</td></tr>
</table></td><td></td></tr>
<tr><td>Chromosome aberration in spermatocytes</td><td>TMP
oral: gavage</td><td>Male Chinese Hamster
Dosing: 0 or 500 mg/kg bw/day for 2 days or
0 or 1000 mg/kg bw/day for 5 days.</td><td>At 500 mg/kg bw a significant increase in the number of aberrant metaphases was observed, when gaps were included – not significant (but still higher) when gaps were excluded. 3 translocations were observed.
At 1000 mg/kg bw: marked mitotic inhibition.</td><td>Machemer & Lorke (1975)
(abstract only)</td></tr>
<tr><td>Chromosome aberration in spermatocytes</td><td>TMP
i.p. application</td><td>Male mice (Q strain)
Doses not reported.
Cells investigated 10 & 16 days after treatment.</td><td>TMP was used as a positive control
Results for TMP: Positive
TMP induced chromosomal aberrations including breaks, exchanges and gaps.</td><td>Moutschen-Dahmen et al. (1981)</td></tr>
<tr><td>Chromosome aberration in spermatocytes</td><td>TMP
i.p. application</td><td>Male mice (Q strain).
20 animals per group, single i.p. dose.
14 organophosphorous substances were tested at the highest tolerated dose.
After a recovery period of 10 to 15 days, the cytogenetic effects were analysed in primary spermatocytes at diakinesis-metaphase I corresponding to the</td><td>TMP as well as the other 13 organosphosphorous compounds tested yielded negative results.
TMP did not induce chromosomal aberrations in this test.</td><td>Degraeve et al. (1984)</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance</th><th>Relevant information about the study (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td></td><td></td><td>treatment of A4-B type spermatogonia.
TMP:
0 or 1000 mg/kg bw
Positive control:
Methyl methane sulfonate (MMS): 60 mg/kg bw & Mitomycin C: 2 mg/kg bw.
Negative control: 20 untreated males.
Air-dried testes chromosome preparations were made according to the method of Evans et al. (1964)
500 well-spread spermatocytes were analysed per animal.</td><td></td><td></td></tr>
<tr><td></td><td></td><td colspan="2"><table>
<tr><th rowspan="2">Substance</th><th rowspan="2">Dose (mg/kg bw)</th><th rowspan="2">Recovery (days)</th><th rowspan="2"># of metaphases analysed</th><th colspan="4">Number of aberrations / 1000 cells</th></tr>
<tr><th>Breaks</th><th>Exchanges</th><th>Gaps</th><th>Total</th></tr>
<tr><td>Negative historical control</td><td>-</td><td>-</td><td>100000</td><td>2,8</td><td>0,12</td><td>0,31</td><td>3,3</td></tr>
<tr><td>Negative concurrent control</td><td>-</td><td>-</td><td>10000</td><td>3,4</td><td>0</td><td>0</td><td>3,4</td></tr>
<tr><td>Mitomycin C</td><td>2</td><td>10</td><td>1000</td><td>7</td><td>1</td><td>0</td><td>7</td></tr>
<tr><td></td><td></td><td>11</td><td>1000</td><td>157</td><td>6</td><td>7</td><td>170</td></tr>
<tr><td></td><td></td><td>12</td><td>80</td><td>112,5</td><td>0</td><td>12,5</td><td>125</td></tr>
<tr><td>MMS</td><td>60</td><td>10-11</td><td>2000</td><td>7</td><td>0</td><td>2,5</td><td>9,5</td></tr>
<tr><td></td><td></td><td>12-13</td><td>2000</td><td>8,5</td><td>0,5</td><td>2</td><td>11</td></tr>
<tr><td></td><td></td><td>14-15</td><td>2000</td><td>4</td><td>0</td><td>1</td><td>5</td></tr>
<tr><td>TMP</td><td>1000</td><td>10-11</td><td>2000</td><td>3,5</td><td>0</td><td>0,5</td><td>4</td></tr>
<tr><td></td><td></td><td>12-13</td><td>2000</td><td>4,5</td><td>0,5</td><td>0</td><td>5</td></tr>
<tr><td></td><td></td><td>14-15</td><td>2000</td><td>4</td><td>0</td><td>0,5</td><td>4,5</td></tr>
</table></td><td></td></tr>
<tr><td>Chromosome aberration in spermatocytes</td><td>TMP
i.p. application</td><td>Mice (sex and strain not reported).
3000 mg/kg bw TMP.
No mention of control.
Chromosome aberrations were scored at the paternal chromosome sets in the first cleavage metaphases after fertilization.</td><td>Positive result.
Chromosome aberrations were induced in the post-meiotic stages of the paternal male mice.
The most sensitive stage for the induction of chromosome aberrations was the late spermatid stage.
The structural chromosome aberrations induced were predominantly of the chromosome-type.
Data were not presented.</td><td>Katoh & Matsuda (1985)
(abstract only)</td></tr>
<tr><td>Sperm abnormality assay</td><td>TMP
i.p. application
sub-acute</td><td>25 chemical substances were tested, one of which was TMP.
Male hybrid mice of the genotype: (C57BL X</td><td>Percent abnormal sperm exceeded the background 90 percentile when mice were treated with the two top doses (≥ ~ 700 mg/kg bw) of TMP and sperm was analysed 1 week later. No increase in</td><td>Wyborek & Bruce (1975)</td></tr>
</table>

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations	Reference
		C3H/Anf) F1 or (C57BL/6 X C3H/He)F1. Age: 11 – 14 weeks. 4 mice per group. Mice were killed 1, 4 or 10 weeks after substance treatment with doses ranging from 100 to 1000 mg/kg bw TMP. After cervical dislocation the cauda epididymides was removed and 2 sperm suspensions were prepared, each from 4 cauda of two mice. For each suspension 1000 sperm were examined at 400-fold magnification ➔ resulting in a total of 2000 sperm / group.	abnormal sperm was seen after 4 or 10 weeks. The study authors concluded that these results indicate that post-meiotic cells are affected, but no effects are induced in pre-meiotic cells. They also concluded that these results are in line with the observations in the dominant lethal assays.	
Sperm abnormality assay	TMP i.p. application	Male hybrid (C57BL/6 x C3H/He) mice, 11 – 14 weeks of age; Doses: 0 – 10000 mg/kg bw, doses were not explicitly reported but the range can be obtained from the figure plotting abnormal sperms; 8 animals per group; Treatment: 5 consecutive days, sacrifice 35 days after the last dose	An increase in number of abnormal sperm was observed at and above ~7000 mg/kg bw, as can be obtained from the figure plotting number of abnormal sperm. The study authors concluded that TMP was positive in this test.	Bruce & Heddle (1979)
Sperm abnormality assay	TMP Oral: gavage Vehicle: distilled water	Computer-assisted sperm motion analysis (CASA) was applied to investigate 3 chemicals, one of which TMP, which were known to have adverse effects on male reproduction and sperm motility. Male Long-Evans hooded rats, ~15 weeks old; 20 / group Dosing: 5 consecutive days, 0, 100, 250 & 600 mg/kg bw/day. Animals were killed by CO_2 asphyxation and	TMP induced a significant animal weight loss at 100 & 250 mg/kg bw and at 600 mg/kg bw the weight loss was precipitous (-66g). No effects were seen on testis or whole epididymal weight at any dose, but cauda epididymal weight was significantly increased at 600 mg/kg bw. At 600 mg/kg bw marked neuro-muscular deficits were reported. Cauda epididymal sperm counts were reduced at 600 mg/kg bw. Also shape and movements of sperms were changed at the top dose, with some effects also seen at lower doses. The study authors considered the obtained results to be in line with previous	Toth et al. (1992)

<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance</th><th>Relevant information about the study (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td></td><td></td><td>testis and epididymis were excised.

Study does not indicate when the animals were sacrificed.

Epididymal sperm preparations were prepared according to Toth et al. (1991).</td><td>investigations of TMP, e.g. by Harbison et al. (1976).</td><td></td></tr>
<tr><td></td><td></td><td colspan="2">
<table>
<tr><th rowspan="2">Means (± SD) of male body weight, organ weight & cauda epididymal sperm counts</th><th colspan="8">Dose (mg/kg)
n = 20</th></tr>
<tr><th colspan="2">0</th><th colspan="2">100</th><th colspan="2">250</th><th colspan="2">600</th></tr>
<tr><td>Initial body weight (g)</td><td>409.6</td><td>(18.3)</td><td>407.2</td><td>(30.0)</td><td>411.9</td><td>(24.6)</td><td>412.0</td><td>(31.1)</td></tr>
<tr><td>Final body weight (g)</td><td>411.7</td><td>(21.5)</td><td>399.5</td><td>(31.3)</td><td>395.4 [a]</td><td>(25.5)</td><td>346.1 [e]</td><td>(31.7)</td></tr>
<tr><td>Weight difference (g)</td><td>2.2</td><td>(14.8)</td><td>-7.7 [c]</td><td>(16.5)</td><td>-16.5 [e]</td><td>(10.0)</td><td>-65.9 [e]</td><td>(11.6)</td></tr>
<tr><td>Testis weight (g)</td><td>1.387</td><td>(0.365)</td><td>1.493</td><td>(0.170)</td><td>1.459</td><td>(0.230)</td><td>1.331</td><td>(0.294)</td></tr>
<tr><td>C. epididymis weight (g)</td><td>0.164</td><td>(0.044)</td><td>0.170</td><td>(0.042)</td><td>0.173</td><td>(0.035)</td><td>0.206 [d]</td><td>(0.070)</td></tr>
<tr><td>Epdidymis weight (g)</td><td>0.469</td><td>(0.096)</td><td>0.481</td><td>(0.074)</td><td>0.474</td><td>(0,091)</td><td>0458</td><td>(0.104)</td></tr>
<tr><td>Sperm count (10^6 cells /g)*</td><td>897.2</td><td>(267.7)</td><td>907.6</td><td>(174.7)</td><td>842.9</td><td>(273.9)</td><td>275.3 [e]</td><td>(114.3)</td></tr>
</table>
* … Analysed with Kruskal-Wallis, Jonckheere's and Wolcoxon tests rather than ANOVA and regression.
Differes significantly from control (one-sided test): [a] … $0.05 < P \leq 0.10$, [b] … $0.03 < P \leq 0.5$, [c] … $0.01 < P \leq 0.03$
[d] … $0.001 < P \leq 0.01$, [e] … $P \leq 0.001$
</td><td></td></tr>
<tr><td>Sperm abnormality assay</td><td>TMP

(purity 99%)

Oral: gavage,

Vehicle: distilled water</td><td>Random-bred albino Sprague-Dawley descendants;

Dosing: 5 days / week, up to 5 weeks;

0, 400, 500, 750, 1000 & 1500 mg/kg bw/day;

5 rats in the control group (vehicle only), 20 rats per treated group.

4 rats per dose were sacrificed weekly and testes and adnexae were removed and stored for microscopic examination and evaluations of spermatogenic stages.

300 seminiferous tubules with an axial ratio of less than 2 in cross section were examined for maturation staging.</td><td>Mortality rates:

0 / 10 / 90 /100 / 100 / 100% in the control/ 400 / 500 /750 / 1000 / 1500 mg/kg bw/day groups, respectively.

Rats found dead were subjected to gross and microscopic examination. Almost all dead rats were anuric and anorexic prior to death. No remarkable finding, except severely distended bladders, with upon microscopic examination multifocal ulceration, loss of urothelial epithelium with marked thinning and atrophy of the muscle proper.

Spermatogenesis was affected immediately after dosing:

Aggregations of multinucleated giant cells were observed and their emergence peaked 1 week after dosing. These structures were described to be composed of late spermatids Also cytoplasmic vacuolation of Sertoli cells was described.

The study authors refer to publications that induced similar formations after direct irradiation or treatment with other chemical agents. Different theories of how such structures could emerge have been discussed, Cho & Park (1994) describe them as an aggregate of necrotizing stage-specific spermatids. It was postulated that giant cells result from injury of Sertoli cells, which would be in line with the observed vacuolation of Sertoli cells in the present study. Number</td><td>Cho & Park (1994)</td></tr>
</table>

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations	Reference
			of giant cells dropped after 2 weeks of TMP treatment. Maturation arrest at the spermatid level was most prominent at 3 weeks following treatment.	
Sperm motility	TMP (purity unknown); Oral: gavage	Male Sprague-Dawley rats, 10/dose; Dosing: 0 & 100 mg/kg bw/day, Once daily for 28 days. Rats were monitored for body weight and food consumption during the exposure period. 24 h after the final dose, animals were sacrificed and testes, epididymis, seminal vesicles and prostate were removed and weighed. Sperm samples were collected from the cauda epididymis and sperm number and viability was evaluated using flow cytometry and sperm motility and morphology was evaluated with the light microscope. Testes and epididymides were also examined microscopically.	No significant changes in body weights, food consumption or organ weights were observed in treated rats. No significant effect was seen on sperm numbers and viability, but sperm motility was reduced. Degenerative spermatogenic cells (1/10) and degenerative sperm (3/10) was observed in epididymal ducts. No histological changes were seen in testes, seminal vesicles or prostate.	Takizawa et al. (1998)
Sperm motility and count	TMP (no information on purity) oral	Male Wistar rats (number not indicated); Dosing: 250 & 500 mg/kg bw on 5 consecutive days, (not further specified). Sperm motility and numbers were investigated.	Decreased sperm motility and numbers at 500 mg/kg bw/day	Suzuki et al. (1996) [cited in US EPA, 2010]
Mechanistic study: effect of TMP on testosterone synthesis	TMP (purity unknown) oral: gavage	Male Wistar rats, 10-17 / group. Dosing: 0 & 100 mg/kg bw/day on 5 consecutive days. Organ weight and histology of the following	Decreased prostate weight. Decreased testosterone concentration in plasma and testes. Positive histochemical reaction for 3ß-hydroxysteroid dehydrogenase by the sperm tails. Increased number of immature Leydig	Carstensen (1971) [cited in US EPA, 2010]

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations	Reference
		organs was investigated: testes, prostate, seminiferous tubules, pituitary, adrenal glands, liver and kidney. Testosterone levels in plasma and testes tissue were determined. Histochemistry of testes was performed.	cells. Increased interstitial fluid in the testicular tissue.	
Dominant lethal mutation & heritable translocation assay	TMP i.p. application	Male & female C3H mice, 9 weeks old. Male mice were treated with i.p. injection of TMP. Dosing: 0, 1000 & 1500 mg/kg bw; single dose. Control animals were treated with saline. Positive control: Methyl methane sulfonate (MMS) at 50 mg/kg bw. Number of animals per group not indicated. 7 days after the injection males were mated with 2 untreated females each for 1 week. Females bearing offspring – whether alive or dead, or showing any sign of pregnancy, were considered to be fertile. Fertility of all F1 male offspring was determined at 9 - 18 weeks of age: After 9 weeks each male was caged with 2 virgin females for 1 week. Pregnant females were sacrificed 12 – 17 days after conception for scoring numbers of live and dead implants and the fertility status was determined by the method of Carter et al. (1955) ➔ fertile, inconclusive, semi-sterile or sterile. Fertile males were discarded and the	The aim was to investigate the induction of translocations by TMP in late spermatids. A significant decrease in the number of live young at birth in treated groups compared with the control was observed, indicating marked increase in the frequency of pre- and post-implantation losses. A slight but significant reduction in the number of young weaned was observed at 1500 mg/kg bw (decrease in viability) (see table in the line below). Results of the fertility analysis and the frequencies of translocation carriers of all F1 male progeny (see line below). A clear increase in semi-sterile and sterile F1 males was observed and the number of translocation carriers was increased. Both effects were dose dependent. The study authors concluded that TMP is capable of inducing chromosomal breakage in mouse post-meiotic germ cells (spermatids). The breakage induced heritable translocations. The incidence of translocations observed at 1500 mg/kg bw TMP was comparable to the positive control MMS. Both are methylating agents.	Tezuka et al. (1985)

<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance</th><th>Relevant information about the study (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td></td><td></td><td>remaining males were killed for cytological analysis. At least 50 cells per male were scored.</td><td></td><td></td></tr>
<tr><td></td><td></td><td colspan="2">A significant decrease in the number of live young at birth in treated groups compared with the control was observed, indicating marked increase in the frequency of pre- and post-implantation losses.
<table>
<tr><th>Chemical</th><th>Dose (mg/kg)</th><th># of males treated</th><th># of females fertile/ mated (%)</th><th># of life young at birth [a] Mean ± SD</th><th># of males weaned (%)</th></tr>
<tr><td>Control</td><td>0</td><td>33</td><td>62/66 (93.9)</td><td>7.2 ± 1.8</td><td>219 (96.9)</td></tr>
<tr><td>TMP</td><td>1000</td><td>50</td><td>86/100 (86.0)</td><td>6.3 ± 2.0 [c]</td><td>284 (96.3)</td></tr>
<tr><td>TMP</td><td>1500</td><td>75</td><td>130/150 (86.7)</td><td>3.5 ± 2.3 [b]</td><td>207 [d] (92.0)</td></tr>
<tr><td>MMS</td><td>50</td><td>53</td><td>83/106 [c] (78.3)</td><td>3.1 ± 2.3 [b]</td><td>121 (96.0)</td></tr>
</table>
[a] … Based on fertile matings. [b, c, d] … Significantly different from control at p < 0.001, p < 0.01 & p < 0.05, resp.

Results of the fertility analysis and the frequencies of translocation carriers of all F1 male progeny:
<table>
<tr><th rowspan="2">Chemical</th><th rowspan="2">Dose (mg/kg)</th><th rowspan="2"># of F1 males</th><th colspan="4">Fertility</th><th colspan="2">Translocation carrier mice</th></tr>
<tr><th>Fertile</th><th>Incon-clusive</th><th>Semi-sterile</th><th>Sterile</th><th># / total</th><th>%</th></tr>
<tr><td>Control</td><td>0</td><td>219</td><td>181</td><td>31 (0) [a]</td><td>6 (0) [a]</td><td>1 (0) [a]</td><td>0/129</td><td>0.0</td></tr>
<tr><td>TMP</td><td>1000</td><td>284</td><td>218</td><td>41 (2)</td><td>21 (9)</td><td>4 (4)</td><td>15/284 [c]</td><td>5.3</td></tr>
<tr><td>TMP</td><td>1500</td><td>203</td><td>141</td><td>29 (2)</td><td>24 (18)</td><td>9 (9)</td><td>29/203 [b]</td><td>14.3</td></tr>
<tr><td>MMS</td><td>50</td><td>118</td><td>89</td><td>14 (0)</td><td>11 (10)</td><td>4 (3)</td><td>13/118 [b]</td><td>11.0</td></tr>
</table>
[a] … The value in parentheses is the number of translocation carrier mice.
[b,c] … Significantly different from control at $p < 0.001$ and $p < 0.01$</td></tr>
<tr><td>Dominant lethal mutation test</td><td>TMP

single i.p. application</td><td>Fertile male and virgin female Swiss mice CF1 strain, 8 – 10 weeks old;

8 males per dose;

Application: single i.p. dose;

Applied doses: 1000 & 2000 mg/kg bw as a 400 mg/ml solution in distilled water.

Males were test mated for 8 weeks: the males were caged with 3 randomly selected females for 7 days, repeated weekly, mating was presumed to have occurred by the mid-week, thirteen days after the presumed mating the female mice were killed and the uterus removed for examination. Non-pregnant animals were noted, the numbers of early fetal deaths, live fetuses and late fetal</td><td><u>Toxicity:</u>
2000 mg/kg bw: 4 / 8 males died within 7 days
1000 mg/kg bw: 1 / 8 males died within 7 days

<u>Effect on pregnancies:</u>
At 2000 mg/kg bw there was a clear effect on percent pregnancies of mated females (see table in the line below)

<u>Effect on the total number of fetal implants:</u>
At 2000 mg/kg bw there was a clear effect on the number of fetal implants (see table in the line below).

<u>Effect on early fetal death:</u>

A considerable and statistically significant increase in early foetal deaths per pregnant female was seen at 1000 mg/kg bw in the first and second week of mating. At 2000 mg/kg bw there was also an increase in the first three weeks of mating, but due to the limited number of pregnant animals no statistical analysis was possible (see table in the line below).</td><td>Dean & Thorpe (1972)</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance</th><th>Relevant information about the study (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td></td><td></td><td>deaths were recorded for each pregnant female.

The weekly mean values for total foetal implants and early fetal deaths were analysed by standard analysis of variance techniques.</td><td></td><td></td></tr>
<tr><td></td><td></td><td colspan="2">
<u>Effect on pregnancies:</u>
At 2000 mg/kg bw there was a clear effect on percent pregnancies of mated females
<table>
<tr><th>Time of mating after dosing male (weeks)</th><th>Control (untreated)</th><th>1000 mg/kg bw (i.p.)</th><th>2000 mg/kg bw (i.p.)</th></tr>
<tr><td>1</td><td>58</td><td>90</td><td>17</td></tr>
<tr><td>2</td><td>88</td><td>95</td><td>0</td></tr>
<tr><td>3</td><td>77</td><td>81</td><td>33</td></tr>
<tr><td>4</td><td>85</td><td>71</td><td>58</td></tr>
<tr><td>5</td><td>73</td><td>81</td><td>75</td></tr>
<tr><td>6</td><td>81</td><td>81</td><td>66</td></tr>
<tr><td>7</td><td>81</td><td>76</td><td>67</td></tr>
<tr><td>8</td><td>77</td><td>- [c]</td><td>67</td></tr>
<tr><td>Mean</td><td>80.9</td><td>-</td><td>-</td></tr>
<tr><td>Total # of males mated / week</td><td>16</td><td>7 [a]</td><td>4 [b]</td></tr>
<tr><td>Total # of females mated per week</td><td>48</td><td>21</td><td>12</td></tr>
</table>
[a] … One male died after treatment; [b] … Four males died after treatment

<u>Effect on the total number of fetal implants:</u>
At 2000 mg/kg bw there was a clear effect on the number of fetal implants during weeks 1 - 3.
<table>
<tr><th>Time of mating after dosing male (weeks)</th><th>Control (untreated)</th><th>1000 mg/kg bw (i.p.)</th><th>2000 mg/kg bw (i.p.)</th></tr>
<tr><td>1</td><td>11.9 ± 0.60</td><td>13.8 ± 0.88</td><td>(3.0)</td></tr>
<tr><td>2</td><td>11.9 ± 0.61</td><td>11.3 ± 0.88</td><td>0</td></tr>
<tr><td>3</td><td>12.8 ± 0.54</td><td>14.1 ± 0.80</td><td>(6.3)</td></tr>
<tr><td>4</td><td>12.6 ± 0.49</td><td>11.6 ± 0.81</td><td>13.1 ± 0.43</td></tr>
<tr><td>5</td><td>13.0 ± 0.51</td><td>12.2 ± 0.73</td><td>11.1 ± 1.00</td></tr>
<tr><td>6</td><td>12.9 ± 0.55</td><td>12.3 ± 0.84</td><td>11.3 ± 1.23</td></tr>
<tr><td>7</td><td>12.4 ± 0.59</td><td>14.1 ± 0.91</td><td>11.5 ± 1.28</td></tr>
<tr><td>8</td><td>14.7 ± 0.42</td><td>- [c]</td><td>14.6 ± 0.90</td></tr>
</table>
[c] … males not mated

<u>Effect on early fetal death:</u>
</td><td></td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance</th><th>Relevant information about the study (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td></td><td></td><td colspan="2">
<table>
<tr><th>Time of mating after dosing male (weeks)</th><th>Control (untreated)</th><th>1000 mg/kg bw (i.p.)</th><th>2000 mg/kg bw (i.p.)</th></tr>
<tr><td>1</td><td>0.73 ± 0.16</td><td>1.21 ± 0.23</td><td>(3.0)</td></tr>
<tr><td>2</td><td>0.76 ± 0.16</td><td>2.45 ± 0.24 ***</td><td>0</td></tr>
<tr><td>3</td><td>0.86 ± 0.15</td><td>0.82 ± 0.22</td><td>(1.0)</td></tr>
<tr><td>4</td><td>0.71 ± 0.17</td><td>1.07 ± 0.28</td><td>0.86 ± 0 40</td></tr>
<tr><td>5</td><td>0.86 ± 0.20</td><td>1.29 ± 0.29</td><td>0.89 ± 0.40</td></tr>
<tr><td>6</td><td>0.64 ± 0.14</td><td>0.53 ± 0.22</td><td>0.75 ± 0.22</td></tr>
<tr><td>7</td><td>0.69 ± 0.18</td><td>1.19 ± 0.28</td><td>0.75 ± 0.40</td></tr>
<tr><td>8</td><td>1.35 ± 0.24</td><td>- [c]</td><td>1.50 ± 0.51</td></tr>
</table>
[c] … males not mated; *** … significant difference from control P < 0.001
</td><td></td></tr>
<tr><td>Dominant lethal mutation test</td><td>TMP

single i.p. administration
oral (gavage) on 5 consecutive days</td><td>Swiss (ICR/Ha) mice, males, 56 days old;
Single i.p. doses of TMP diluted in distilled water: 200, 500, 850, 1000, 1250, 1500 & 2000 mg/kg bw;
Volume: 0.1 ml
Gavage dosing on 5 consecutive days: 500 & 1000 mg/kg bw/day;
Males were test mated for 8 weeks: the males were caged with 3 randomly selected females for 7 days, repeated weekly (duration of the spermatogenic cycle).
Females were autopsied 13 days after the midweek of their caging and presumed mating (➔ untimed pregnancies – ranging from 9 to 15 days).
Each female was scored for pregnancy and numbers of implants, including living implants, early foetal deaths and occasional late foetal deaths (corpora lutea were not counted).
The percentage of pregnancies and mean numbers of total implants, including living foetuses</td><td>TMP was generally not toxic at the tested doses.
Pregnancy rates did not differ consistently, but the incidence of pregnancy was generally reduced at the highest dose tested (data not presented per week, but only as mean over all 8 weeks).
Weekly means of total implants per pregnant control ranged from 10.5 to 12.6. After i.p. administration of 200 and 1000 mg/kg bw, reductions of numbers of total implants were seen during the first 3 mating weeks. This effect was significant and dose related (see table inserted below).
This effect was however not repeated when TMP was tested over a wider range of i.p. dosages (500 – 2000), though lower numbers of implants were noted at the 2nd week of mating (see table inserted below).
After gavage exposure over 5 days to 500 or 1000 mg/kg bw reductions in numbers of implants in the first 3 weeks of mating was significant and related to dosage (see table & graphs inserted below). At 1000 mg/kg bw implants were also reduced at the 5th week.
Mean numbers of early fetal deaths per pregnant control ranged from 0 to 0.68 with a mean of 0.33.
A highly significant increase in early fetal deaths occurred during the first 3 weeks of mating for all experiments (see table in the line below). A dose dependent increase in early fetal deaths occurring in the second week of mating at all dose groups, except the top dose via gavage,</td><td>Epstein et al. (1970)</td></tr>
</table>

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations	Reference
		and early foetal deaths were determined weekly and analysis of variance of dose as a function of weeks after TMP administration was performed with matrix inversion techniques.	where effects were already seen in the first week of mating. (Absence of early foetal death in the latter dose group was probably due to reduced pregnancies and losses before implantation.) Numbers of total implants and early fetal deaths as mean values per pregnant female.	

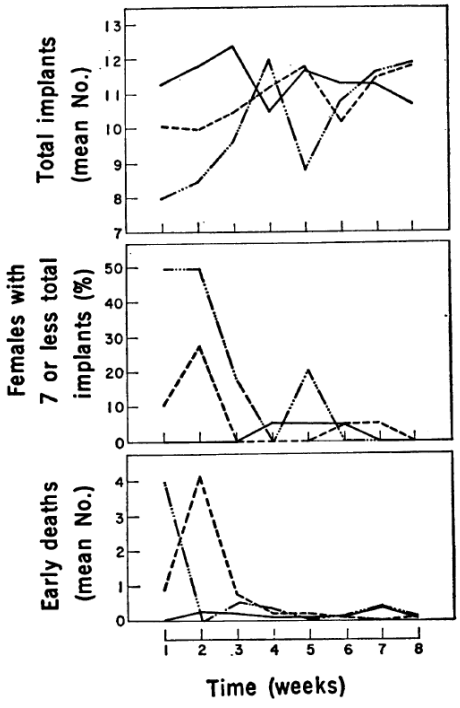
Effect on early fetal death:

Time (weeks)	Dose (mg/kg)	Route	Within subclass mean square (degrees of freedom)	F values (degrees of freedom)		
				Dose	Week	Interaction
Total implants per pregnancy						
1-3	200, 1000	I	4.69 (137)	5.33 * (2)	1.96 (2)	1.06 (4)
4-8	200, 1000	I	5.10 (227)	2.06 (2)	1.38 (4)	0.49 (8)
1-3	500-2000	I	4.22 (182)	0.29 (5)	4.79 * (2)	1.25 (10)
4-8	500-2000	I	4.21 (302)	0.19 (5)	2.30 (4)	1.82 # (20)
1-3	500, 1000	G	5.64 (95)	12.96 * (2)	1.69 (2)	0.24 (4)
4-8	500, 1000	G	3.91 (198)	0.22 (2)	1.29 (4)	3.01 * (8)
Early deaths per pregnancy [a]						
1-3	200, 1000	I	0.91 (137)	14.65 * (2)	0.73 (2)	3.40 # (4)
4-8	200, 1000	I	0.74 (227)	2.83 (2)	0.64 (4)	2.45 # (8)
1-3	500-2000	I	1.02 (182)	7.86 * (5)	5.64 (2)	1.19 (10)
4-8	500-2000	I	0.51 (302)	1.01 (5)	0.07 (4)	0.85 (20)
1-3	500, 1000	G	1.19 (95)	13.32 * (2)	7.03 * (2)	14.03 * (4)
4-8	500, 1000	G	0.35 (198)	0.03 (2)	0.07 (4)	0.87 (8)

[I=intraperitoneal; G=gavage (Table 2 of the publication)]

* … significant at P = 0.01; # … significant at P = 0.05
[a] … Data transformed by Freeman-Tukey Poisson transformation before analysis

Numbers of total implants and early fetal deaths as mean values per pregnant female.



<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance</th><th>Relevant information about the study (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td></td><td></td><td colspan="2">Time refers to week of mating after TMP treatment of males: (____) Control, (- - - - -) 500 mg/kg bw, (___ … ___) 1000 mg/kg bw.</td><td></td></tr>
<tr><td>Dominant lethal mutation test</td><td>TMP
i.p. application</td><td>Male & female C3H mice, 9 weeks old.
Male mice were treated with i.p. injection of TMP.
Control animals were treated with saline.
Positive control: Methyl methane sulfonate (MMS) at 50 mg/kg bw.
Dosing: 0, 1000, 1250 & 2500 mg/kg bw; single dose.
Number of animals per group not indicated.
After injection each male was mated with 2 virgin females for 1 week from day 7. Females were sacrificed 12 – 15 days after conception to analyse uterine content.
Based on own investigations and the reports from Dean & Thorpe (1972, Epstein et al. (1970) & Lorke & Machemer (1975) it was concluded that the spermatid was the most sensitive stage for TMP induction of dominant lethality.</td><td>Frequency of dominant lethal mutations estimated from the mean litter size at birth in each test group was 13% (1000 mg/kg bw TMP), 51.2% (15000 mg/kg bw TMP) and 57.6% (MMS).</td><td>Tezuka et al. (1985)</td></tr>
<tr><td colspan="3"><table>
<tr><th>TMP dose (mg/kg)</th><th># of fertile matings</th><th># of corpora lutea [a] Mean ± SD</th><th># of implants [a] Mean ± SD</th><th># of living embryos [a] Mean ± SD</th><th># of early deaths Mean ± SD (%)</th><th>Induced dominant lethal mutations (%)</th></tr>
<tr><td>0</td><td>23</td><td>11.0 ± 1.2</td><td>10.2 ± 0.9</td><td>8.6 ± 1.3</td><td>1.1 ± 1.0 (11.1)</td><td>-</td></tr>
<tr><td>1000</td><td>22</td><td>10.5 ± 1.1</td><td>8.9 ± 1.4 [b]</td><td>6.4 ± 1.5 [b]</td><td>2.0 ± 1.4 [c] (22.6)</td><td>26.1</td></tr>
<tr><td>1250</td><td>21</td><td>10.5 ± 0.9</td><td>9.3 ± 1.1 [c]</td><td>6.3 ± 1.9 [b]</td><td>2.8 ± 1.3 [b] (30.1)</td><td>26.5</td></tr>
<tr><td>2500</td><td>22</td><td>10.5 ± 2.2</td><td>8.8 ± 2.0 [c]</td><td>4.3 ± 2.8 [b]</td><td>4.3 ± 2.0 [b] (49.2)</td><td>49.8</td></tr>
</table>
[a] … All data are based on fertile matings, [b,c] … Significantly different from control at p < 0.001 & p < 0.01, resp.</td></tr>
<tr><td>Dominant lethal mutation test</td><td>TMP
oral (unspecif.)</td><td>NMRI mice
0 & 1000 mg/kg bw
Single dose.</td><td>TMP was used as a reference material.
No effect on pre-implantation loss was observed, but marked increase in post-implantation loss in the 2nd week of mating was reported.</td><td>Lorke & Machemer (1975)
[cited in US EPA, 2010]</td></tr>
<tr><td>Dominant</td><td>TMP</td><td>Mice (strain not specified)</td><td>Significant lethality occurred maximally</td><td>Farrow et al</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance</th><th>Relevant information about the study (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td>lethal mutation test</td><td>i.p. application or gavage</td><td>1250 mg/kg (i.p.) or
500 mg/kg bw/day for 5 days (gavage)
Controls not mentioned.</td><td>in the 2nd week of mating (i.p.) and in the 1st and 2nd week of mating after gavage administration of TMP.
Data not presented.</td><td>(1975)
[cited in US EPA, 2010]</td></tr>
<tr><td>Dominant lethal mutation test</td><td>TMP
oral (unspecif.)</td><td>Mice (strain not specified)
Dosing on 5 consecutive days.
Doses not reported.
No mention of controls.</td><td>Dominant lethal effects were seen for 2 weeks after 5 days treatment.</td><td>Newell et al (1976)
[cited in US EPA, 2010]</td></tr>
<tr><td>Dominant lethal mutation test</td><td>TMP
Route not specified</td><td>Mice (strain not specified)
1000 mg/kg bw.
No mention of control.</td><td>High mutagenicity particularly at post-meiotic stages.
Data not shown.</td><td>Degraeve et al. (1979)
[cited in US EPA, 2010]</td></tr>
<tr><td>Dominant lethal mutation test</td><td>TMP
i.p. application</td><td>Mice (Q strain).
0 & 1000 mg/kg bw.
TMP was used as positive control.</td><td>Significant increase in the frequency of pre-implantation and post-implantation losses 2 weeks after injection.</td><td>Moutschen-Dahmen et al. (1981)</td></tr>
<tr><td>Dominant lethal mutation test</td><td>TMP
i.p. & oral application</td><td>Rat & mice (strain not specified);
5 rats per group, 8 mice per group;
Rats received either 5 x 250 mg/kg bw or 5 x 100 mg/kg bw p.o.
Mice received either 5 x 1000 mg/kg bw p.o. or 5 x 1000 mg/kg bw i.p.
Treatment was on 5 consecutive days.</td><td>TMP affected fertility in rat and mouse, but the doses needed to induce these effects in mice was about 10-fold higher than in rats. From the study in rat it can be concluded that TMP was equally active when administered orally or by intraperitoneal injection (only oral data presented).
Total weekly offspring from male rats and mice treated with TMP (see line below).
Male rats were completely sterile 3 and 4 weeks after exposure to the lower TMP dose, while at the higher dose complete sterility was seen from the 2nd week up to the fifth week after exposure.
In mice full sterility was seen up to the second week after exposure.</td><td>Jackson & Jones (1968) / Jones & Jackson (1969)
(Both publications are listed together, as both include relevant information on the same experiments)</td></tr>
<tr><td></td><td></td><td colspan="2">Total weekly offspring from male rats and mice treated with TMP:
<table>
<tr><td></td><td colspan="12">Weeks from first dose</td></tr>
<tr><td></td><td>1</td><td>2</td><td>3</td><td>4</td><td>5</td><td>6</td><td>7</td><td>8</td><td>9</td><td>10</td><td>11</td><td>12</td></tr>
<tr><td>Rats:</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>5 x 250 mg/kg bw p.o.</td><td>24</td><td>0</td><td>0</td><td>0</td><td>0</td><td>20</td><td>14</td><td>15</td><td>20</td><td>16</td><td>10</td><td>23</td></tr>
<tr><td>5 x 100 mg/kg bw p.o.</td><td>-</td><td>3</td><td>0</td><td>0</td><td>5</td><td>30</td><td>32</td><td>30</td><td>19</td><td>5</td><td>41</td><td>27</td></tr>
<tr><td>Mice:</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>5 x 1000 mg/kg bw p.o.</td><td>0</td><td>0</td><td>5</td><td>32</td><td>46</td><td>64</td><td>49</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>5 x 1000 mg/kg bw i.p.</td><td>-</td><td>0</td><td>8</td><td>22</td><td>34</td><td>15</td><td>25</td><td></td><td></td><td></td><td></td><td></td></tr>
</table></td><td></td></tr>
<tr><td>Antifertility</td><td>TMP</td><td>Random-bred albino Sprague-Dawley</td><td>TMP induced reversible sterility in male mice, rats and rabbits. Induced sterility</td><td>Harbison et al.</td></tr>
</table>

Method, guideline, deviations if any	**Test substance**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
action	Route and vehicle used not indicated.	descendent rats; random bred albino Swiss-origin mice, New Zealand white rabbits. Human sperm samples. <u>Dosing schemes:</u> Mice: Subacute: 0, 750 & 1500 mg/kg bw on 5 consecutive days. Subchronic: 0 & 1500 mg/kg bw on 5 days / week for 1 month. Rats: Subchronic: 0, 100 & 600 mg/kg bw on 5 consecutive days. Chronic: 0 & 750 mg/kg bw once weekly for 12 (?) weeks. Rabbits: Chronic: 0, 200 & 325 mg/kg bw once weekly for 13 weeks. Control animals received the same volume of vehicle. <u>Fecundity:</u> Fecundity was measured by serial mating of males with corresponding females for 6 weeks. <u>Measurement of choline acetyltransferase in epididymal spermatozoa:</u> Spermatozoa were sampled from various segments of the epididymis: caput, proximal corpus, distal corpus, proximal cauda, distal cauda. Tissue was minced in Eagl's medium and total number of spermatozoa was determined.	was dependent on dosage and duration of treatment. <u>Mice:</u> *5 consecutive days at:* - 750 mg/kg bw: fecundity reduced to 13% in the first week - 1500 mg/kg bw: fecundity reduced to 0% (total sterility) in the first week, 29% in the second week At both doses fertility returned to normal. *5 days / week for 1 month:* - 1500 mg/kg bw: total sterility for 2 weeks, fertility gradually returned to normal after 6 weeks. <u>Rats:</u> *5 days / week for 1 month:* - 100 mg/kg bw: fecundity reduced to 29% in the first week. Fertility returned to normal during the second week. - 600 mg/kg bw: fecundity reduced to 0-5% for 4 weeks. Fertility returned to normal after 6 weeks. *Once weekly for 12 weeks:* - 750 mg/kg bw: fecundity was reduced to 50% during the first week and down to 0-6% by week 3 and through week 12. <u>Rabbits:</u> *Every 5 days for 13 weeks:* - 200 mg/kg bw: fecundity was reduced to 50% by the third week and to ~25% by the ninth week. - 325 mg/kg bw: fecundity was reduced to 37% by the second week and produced sterility from week 5 though week 13. Fertility returned to normal within one week of termination of treatment on week 13. *Single treatment:* - 750 mg/kg bw: fecundity was reduced to 34% during the first week. Fertility was normal in the second week.	(1976)

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations	Reference
		Fresh human sperm was obtained by ejaculation. After liquification it was diluted with Hanks' solution and centrifuged two times. Choline acetyltransferase activity was measured via the formation of ^{14}C-labeled acetylcholine from choline and ^{14}C-labeled acetyl-coenzyme A according to McCaman & Hunt (1965). Acetylcholine in sperm was assessed by gas chromatography and mass spectrometry. No details on how the in vitro studies were carried out were presented.	Mating behaviour was not affected. No observable changes in skeletal muscle activity of animal behaviour were reported. Testicular biopsies were taken at various times during and following treatment. No observable histological changes and spermatogenesis was normal. *Choline acetyltransferase activity in spermatozoa:* In all 3 species the amount of acetylcholine synthesised followed a developmental pattern (i.e. was in agreement with the expectation in that the lowest levels were measured in the caput and the highest levels in the distal cauda of the epididymis). TMP reduced the activity in a dose and time dependent fashion. After single treatment activity was reduced maximally at 72h and returned to control levels at 96 to 168h. When rats were treated with a single dose of 750 mg/kg bw the choline acetyltransferase activity was reduced to 25 to 50%. While brain choline acetyltransferase was only reduced to 65%. In both tissues enzyme reduction was significant after 24h. The study authors concluded that the rapid onset of enzyme activity depression corresponded with the rapid onset of infertility/sterility. Continued weekly treatment maintained depression of choline acetyltransferase activity. The effect on the enzyme activity was completely reversible, 1-2 weeks after termination of treatment enzyme activity returned to control levels at all segments of the epididymis. When comparing the enzyme activity in untreated spermatozoa of rat, rabbit and humans the following activity sequence was observed: rat >> rabbit ~ human. Spermatozoa of all 3 species were capable of producing and releasing acetylcholine in vitro.	

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations	Reference
			A concentration-dependent inhibition of spermatozoa mobility was seen when TMP was added to sperm suspensions in vitro. Conclusion: The study authors concluded that TMP induced time and dose-dependent sterility in mice, rats and rabbits, which was fully reversible. They also concluded that spermatogenesis was not affected, but that TMP interferes with the normal function of spermatozoa, probably by an action on sperm motility. The hypothesis is that choline acetyltransferase and acetylcholinesterase regulate the intracellular acetylcholine levels in spermatozoa, which plays a role in sperm mobility. Mammalian spermatozoa contain high levels of acetylcholinesterases, which is concentrated in the flagella. By inhibiting choline acetyltransferase TMP exposure reduces acetylcholine levels and interferes with sperm mobility.	
Antifertility action	TMP oral (in water)	Random-bred albino adult Sprague Dawley rats with proven fertility; Dosing: 250 mg/kg bw; 5 days / week for 30 days or 6 days / week for 60 days. Fertility was assessed after treatment by placing 1 male with 2 mature virgin females. Semen from the corpus was taken from 2 rats epididymis of the 30 day treatment group and presence of sperm and sperm morphology was assessed microscopically. Testes of 3 animals from each treatment group as well as from the control group were fixed by vascular perfusion and sections for microscopy were prepared.	TMP treatment with 250 mg/kg bw for 5 days/week for 30 days: Abnormal shape of epididymal spermatozoa, i.e. detached heads, abnormalities of head, middle piece and principal piece (not seen in controls). Virgin females mated with males from this group showed no signs of mating (i.e. no vaginal plugs), in contrast to control trials. Testes of males from this group showed impaired spermatogenesis due to abnormal spermiogenesis and depletion of the numbers of mature spermatids. Round spermatids showed vacuoles within their nuclei and extensive extracellular spaces were observed between the germ cells and Sertoli cells. These effects were not seen in controls. TMP treatment with 250 mg/kg bw for 6 days/week for 60 days: Germ cells were absent from the seminiferous tubules, which were collapsed and showed shrinkage – “Sertoli-cell-only” condition. The lumen of many seminiferous tubules was filled with processes of Sertoli cell cytoplasm.	Hanna & Kerr (1981)

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations	Reference
			The study authors concluded that prolonged dosing of TMP results in complete loss of germ cell activity.	

The *in vivo* mutagenic properties of TMP have been extensively investigated in the 1970-ties and 1980-ties and TMP has frequently been applied as positive control substance for the development and assessment of *in vivo* test methods for mutagenicity. The number of available studies is therefore rather high, however, not all of the studies are described in detail and in some of them, where TMP was used as positive control, only one dose was tested.

None of these *in vivo* studies was conducted according to accepted guidelines, but many of the applied procedures are considered equivalent or in some aspects even superior (e.g. more doses than required) to accepted test guidelines.

The range of the evaluated studies covers 8 cytogenetic (chromosomal aberration, CA) tests in bone marrow, 4 chromosomal aberration tests in spermatocytes, 4 bone marrow micronucleus tests, 1 *in vivo* Comet assay in testicular cells, 1 heritable translocation assay, 10 dominant lethal mutation tests, 2 studies with focus on TMP's antifertility action and 6 studies investigating sperm abnormality and/or motility. An additional mechanistic study (Carstensen, 1971) investigated hormonal involvement by measuring weight of male reproductive organs, testosterone levels in plasma and testis and testicular histochemistry.

In vivo studies in somatic cells:

All 8 available bone marrow CA tests gave positive results, both via the i.p. route as well as via the oral (gavage) route, in rat and mouse (Adler et al., 1971, Legator et al, 1973, Sheu et al., 1979, Anderson & Richards, 1981, Sinha et al., 1983, Farrow, 1975, Weber et al., 1975, Moutschen-Dahmen et al., 1981). Where several doses and time courses were investigated a dose- and time-dependent increase in CAs was observed. It was noted that when compared to other alkylating substances like TEPA (tris(2-methyladiridin-1-yl)phosphine oxide), CA-induction by TMP required relatively high doses, which did however not result in severe general toxicity.

From the 4 bone marrow micronucleus (MN) tests only one was negative (Ni et al., 1993), however, the study results were reported in Chinese and only an English abstract was available from which no details on the procedure and results could be derived. The three positive MN tests were conducted in mice and showed clear dose dependent increases in MN (Weber et al., 1975, Bruce & Heddle, 1979, Farrow, 1976).

In vivo studies in gonads and sperm cells:

The data base also includes a quite recent Comet assay conducted in testicular cells of male CD1 mice after gavage exposure (Hansen et al., 2014). TMP induced a significant positive effect at the top dose of 500 mg/kg bw, which clearly exceeded the historical controls. An increase was already seen at the mid dose, but not statistically significant. Histological examination of the other testis revealed no signs of cytotoxicity. In line with OECD 489, Hansen et al. (2014) conclude that the testicular samples represent a mixture of different cell types including somatic cells as well as germ cells. Therefore positive results do not clearly demonstrate genotoxicity in germ cells, but they demonstrate that the substance reaches the gonads, where it interferes with the DNA.

The induction of chromosomal aberrations (CA) was also investigated in spermatocytes of hamster and mice. Three studies are available for the i.p. route (mice) (Moutschen-Dahmen et al., 1981, Degraeve et al., 1984, Katoh & Matsuda, 1985) and in one study TMP was applied orally (gavage) to male Chinese hamster (Machemer & Lorke, 1975). The oral study in hamster tested two doses: 500 mg/kg bw on two consecutive days and 1000 mg/kg bw on 5 consecutive days and compared to respective negative controls. A significant increase in number of aberrant metaphases was observed at 500 mg/kg bw, when gaps were included. When gaps were not included the increase was not significant, but still higher than in the control. (According to OECD 483 the number of gaps should be reported, but not included in the CA counts.) At 1000 mg/kg bw

marked inhibition of mitosis was observed, indicating toxicity. As only an abstract was available no further information can be presented on this study.

In two of the spermatocyte CA i.p. studies TMP was used as positive control. While in the study by Moutschen-Dahmen et al. (1981) TMP gave the expected result (i.e. clear increase in CAs), it was negative in Degraeve et al. (1984), like all the other 13 organophsophorous compounds tested in this study. The fourth study investigating CAs in spermatocytes (Katoh & Matsuda, 1985) was again positive and identified the late spermatid as the most sensitive stage for the induction of CAs by TMP.

TMP was further tested in 10 dominant lethal mutation assays including one heritable translocation assay (Tezuka et al., 1985, Dean & Thorpe, 1972, Epstein et al., 1970, Lorke & Machemer, 1975, Farrow et al., 1975, Newell et al., 1976, Degraeve et al., 1979, Moutschen-Dahmen et al., 1981, Jackson & Jones, 1968 / Jones & Jackson, 1969) as well as in studies focussing on antifertility action (Harbison et al., 1976, Hanna & Kerr, 1981). These studies were conducted in rat, mice and rabbit, via oral (gavage) or i.p route. Effects were consistently seen during the 1st, 2nd and 3rd week after exposure (in two cases up to week 5 – Epstein et al., 1970 & Jackson & Jones, 1968, 1969) indicating that the spermatid was most sensitive (i.e. post-meiotic stages of spermatogenesis) for TMP induction of dominant lethality. The induced effects were a dose dependent reduction in fertility (reduced numbers of pregnancies) and increased mutation rates, reduced number of implants (pre-implantation loss) and increased early fetal death (post-implantation loss). Tezuka et al. (1985) also assessed F1 males and could observe a clear increase in semi-sterile and sterile F1 males (in mating experiments). Also the number of translocation carriers was increased. Both effects were dose dependent. Epstein et al. (1970) described such effects as the result of structural and/or numerical changes in the chromosomes of the germinal cells in sexually mature animals. The results clearly demonstrate that TMP acts mutagenic in male germ cells in rats and mice, which are transmitted to males of the F1 generation. No increase in late foetal death was observed and in Dean & Thorpe (1972) it was noted that late foetal death was unaffected, but such were considered of non-genetic origin.

Jackson & Jones, 1968, Jones & Jackson, 1969 concluded that the predominant effect of TMP was "functional" sterilising action involving spermatids from which intact motile but incompetent sperm continue to be produced. They also described that relatively high doses were required to induce this effect in the mouse (5 x 1000 mg/kg orally), whereas it was effective in the rat at one tenth of this dose. They further concluded that the antifertility action of TMP was probably related to methyl alkylation, comparable to MMS (methyl methane-sulfonate). Like MMS also TMP induces dominant lethal mutations at sub-sterilising doses.

Sperm numbers, number of abnormal sperm and/or sperm motility was investigated in further 6 studies in rats and mice after i.p. and oral route of exposure. In each of these studies the investigated parameters were affected, however, while Wyborek & Bruce (1975) detected only post-meiotic changes (effects were only seen 1 week after treatment, not after 4 or 10 weeks), Bruce & Heddle (1979) detected changes 5 weeks after exposure (earlier time points were not investigated). Clear effects on sperm were also seen in the study by Toth et al. (1992), including changed movement of sperm, but the study did not indicate the time of sacrifice after treatment, therefore, it cannot be derived which stage of spermatogenesis was impacted. Cho & Park (1994) found aggregations of multinucleated giant cells (composed of late spermatids) with a peak occurrence 1 week after end of treatment and maturation arrest at the spermatid level, which was most prominent at 3 weeks following treatment. They concluded that spermatogenesis was affected immediately after dosing.

Decreased sperm motility was also reported by Takizawa et al. (1998) and Suzuki et al. (1996, cited in US EPA, 2010).

Harbison et al. (1976) proposed a mechanism which might be related to reduced sperm motility, i.e. interference with choline acetyltransferase in spermatozoa. TMP was shown to suppress spermatozoan choline acetyltransferase activity, which correlated with TMP-induced sterility. Harbison et al. (1976) observed sterility in rats, mice and rabbits, after acute, sub-acute, sub-chronic and chronic exposure. Sterility was dose and exposure time dependent. They also could maintain sterility by continuous treatment. Harbison et al. (1976) concluded that TMP primarily affects epididymal spermatozoa, probably by action on sperm motility. This conclusion is somehow in contradiction to most of the other results which identified the late spermatid as the sensitive stage, but the different conclusion in contrast to studies which focused on

mutagenic / dominant lethal effects might be that Harbison et al. (1976) had their focus on inhibition of acetyl choline and sperm motility.

In most studies it appears that sterility was caused by effects on the post-meiotic stage of spermatogenesis and sterility was reversible (sterility returned to normal within 1 to 2 weeks after termination of treatment), but Hanna & Kerr (1981) observed that prolonged TMP exposure (up 30 & 60 days) resulted in complete and irreversible loss of germ cell activity.

It is noted that general toxicity induced by TMP in these studies was quite different in the single studies ranging from no observed effects at all to mortality, at comparable doses. The results from the investigations of testicular tissue are sometimes also diverging. For instance Takizawa et al. (1998) treated Sprague-Dawley rats orally (gavage) with 100 mg/kg bw TMP for 28 days and observed degeneration of spermatocytes (1/10 males) and sperm (3/10 males), as well as reduced sperm motility, but no effects on sperm viability and number and no effects on the weight of male reproductive organs (testes, epididymides, seminal vesicles or prostate). In contrast Carstensen (1971) administered doses of 100 mg/kg bw TMP to male Wistar rats on 5 consecutive days via gavage and observed decreased prostate weight, as well as some other changes. These studies did not follow any standardised procedure and also the strains used were different, indicating that slightly different treatment, differences in the assessment of the parameters or differences in the strains used could have caused diverging results. However, it is important to note, that the described mutagenic effects were seen across the different studies, despite the procedural differences and the use of different strains.

10.8.1 Short summary and overall relevance of the provided information on germ cell mutagenicity

TMP has been extensively investigated in *in vitro* and *in vivo* genotoxicity and mutagenicity assays. During the 1970-ties and 1980-ties it has been widely used as positive control for the development and assessment of methods to assess mutagenic properties of chemicals. The available data consist predominantly of studies from the open literature, with varying degree of details reported, ranging from comparable to test guideline requirements to very poor reporting. The only two studies conducted following accepted test-guidelines are Anonymous, 1996 (JAPAN: Guidelines for Screening Mutagenicity Testing of Chemicals: Bacterial Reverse Mutation assay) and Anonymous, 1994 (JAPAN: Guidelines for Screening Mutagenicity Testing of Chemicals: *In vitro* Mammalian Chromosomal Aberration test), but also for these two studies no detailed study report was available; the presented information was obtained from the ECHA dissemination website. All other studies are publications from the open literature with varying degree of quality and details reported. Some studies are reported in detail and the study design is comparable to accepted guidelines (e.g. Adler et al., 1971 or Hansen et al., 2014), others are scarcely reported or TMP was only used in a single dose, when only applied as positive control in the respective study. When used as positive control, the studies are sometimes only cited from review reports (i.e. Connor, 1979 and US EPA, 2010). All studies are listed in Table 11- Table 13.

Mechanistic studies (Yamauchi et al., 1976 and Yuan et al. 2020) demonstrated TMP's capacity to induce alkylation of nucleic acid base pairs, including modifications building the basis for powerful mutations through atypical base pairing (Yamauchi et al. 1976). Yuan et al. (2020) found that TMP does hardly induce cytotoxicity or ROS formation, but interferes with the integrity of mitochondria via another way than ROS formation.

Bacterial reverse mutation assays gave mainly positive results and the results for different strains indicate that TMP acts in these test systems via base-pair mutations rather than via frameshift mutations (Table 11). Only 3 studies were conducted in mammalian *in vitro* test systems, with a negative chromosome aberration test in Chinese hamster cells (Anonymous, 1994a), but a positive micronucleus test in Chinese hamster cells (Ni et al., 1993) and a positive chromosome aberration test in human cultured lymphocytes (Söderman, 1972).

Also the data from 5 *Drosophila melanogaster* mutation assays (Table 12) leave no doubt that TMP induces mutations, with the difference that in *D. melanogaster* not only the pre-meiotic, but also meiotic spermatids were affected, whereas in rodents only post-meiotic stages were affected.

TMP was clearly mutagenic in somatic *in vivo* studies in rodents investigating the formation of chromosome aberrations and micronuclei in bone marrow. These studies were carried out via the oral and i.p. routes in rats and mice, with the vast majority of respective studies giving positive results (Adler et al., 1971, Legator et al., 1093, Sheu et al., 1979, Anderson & Richardson, 1981, Sinha et al., 1983, Farrow, 1975, Weber et al., 1975, Moutschen-Dahmen et al., 1981, Bruce & Heddle, 1979, Farrow et al., 1976, Ni et al., 1993).

Several studies investigated TMPs potential to also induce mutations in gonads and germ cells: A positive Comet assay (Hansen et al., 2014) demonstrates that TMP exerts genotoxic activity in mouse testicular cells *in vivo*. The study included a histological examination of the testis where no signs of cytotoxicity were observed.

Several positive chromosome aberration studies in spermatocytes demonstrate that TMP can induce mutations in germ cells (Machemer & Lorke, 1975, Moutschen-Dahmen, 1981, Katho & Matsuda, 1985). This is further supported by positive results in dominant lethal mutation assays (Tezuka et al., 1985, Dean & Thorpe, 1972, Epstein et al, 1970, Lorke & Machemer, 1975, Farrow,1975, Newell et al. 1976, Degraeve et al., 1979, Degraeve et al., 1979, Moutschen-Dahmen et al., 1981, Jackson & Jones, 1968, Jones & Jackson, 1969) and a heritable transformation assay, which demonstrate that mutations are induced in germ cells and that these mutations are transmitted to F1 progeny (Tezuka et al., 1985).

Next to the demonstration that mutations are transmitted to F1 males, and that chromosomal aberrations are induced in spermatocytes, the observed pattern of increased pre- and post-implantation losses supports that the effects were caused by mutagenicity.

Despite the demonstration that TMP can induce mutations in male germ cells, also other effects on spermatids were observed. These include reduced sperm numbers, increased sperm abnormalities and decreased sperm motility. Next to possible involvement of TMP's mutagenic action in these effects as well, also other modes of action could be responsible or involved in addition. These include enzyme inhibition, as investigated by Harbison et al. 1976, who reported reduced choline acetyltransferase activity which correlated well with TMP induced sterility. Hormonal interference could play a role as Carstensen (1971) reported changes in the weight of reproductive organs and decreased testosterone levels in plasma and testis. Potentially the alkylating properties of TMP could also interfere with other biomolecules than DNA, thereby inducing the observed effects in sperm. However, the available information does not clearly demonstrate the relevance of one of these modes of action, despite the clear involvement of mutagenicity. The available data indicate that the relevance of cytotoxicity and the formation of ROS can be excluded as demonstrated by Yuan et al. (2020) and supported by lack of cytotoxicity in the available *in vivo* studies.

10.8.2 Comparison with the CLP criteria

Criteria according CLP regulation, Table 3.5.1

Categories	*Criteria*
CATEGORY 1	Substances known to induce heritable mutations or to be regarded as if they induce heritable mutations in the germ cells of humans. Substances known to induce heritable mutations in the germ cells of humans.
Category 1A	The classification in Category 1A is based on positive evidence from human epidemiological studies. Substances to be regarded as if they induce heritable mutations in the germ cells of humans.
Category 1B	The classification in Category 1B is based on:

	- positive result(s) from in vivo heritable germ cell mutagenicity tests in mammals; or - positive result(s) from in vivo somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations to germ cells. It is possible to derive this supporting evidence from mutagenicity/genotoxicity tests in germ cells in vivo, or by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells; or - positive results from tests showing mutagenic effects in the germ cells of humans, without demonstration of transmission to progeny; for example, an increase in the frequency of aneuploidy in sperm cells of exposed people.

CATEGORY 2	Substances which cause concern for humans owing to the possibility that they may induce heritable mutations in the germ cells of humans
	The classification in Category 2 is based on:
	- positive evidence obtained from experiments in mammals and/or in some cases from in vitro experiments, obtained from:
	  - somatic cell mutagenicity tests in vivo, in mammals; or
	  - other in vivo somatic cell genotoxicity tests which are supported by positive results from in vitro mutagenicity assays.
	Note: Substances which are positive in in vitro mammalian mutagenicity assays, and which also show chemical structure activity relationship to known germ cell mutagens, shall be considered for classification as Category 2 mutagens.

According to the CLP guidance classification in Category 1B may be based on positive results of at least one valid *in vivo* mammalian germ cell mutagenicity test. In case there are also negative or equivocal data, a weight of evidence approach using expert judgement has to be applied.

Annex I: 3.5.2.3.9 states: *The classification of individual substances shall be based on the total weight of evidence available, using expert judgement (See 1.1.1). In those instances where a single well-conducted test is used for classification, it shall provide clear and unambiguously positive results.*

There are no human data available for TMP that would allow to support a classification in Category 1A.

For TMP mutagenic effects have been clearly demonstrated in a long list of *in vivo* germ cell mutagenicity studies, dominant lethal assays in rodents and a heritable translocation assay. The vast majority of these studies was clearly positive and the observed results are very consistent (e.g. regarding the post-meiotic spermatid identified as the main target of TMP in all dominant lethal assays). The transmission of mutations to F1 offspring has been clearly demonstrated.

The available studies are of varying quality and none of the studies fully complies with an internationally accepted test guideline (often because they predate the according guideline and in many cases TMP was used as positive control in the course of the development of testing methods). Some studies are of lower quality or have limits in reporting, others were carried out according to a procedure comparable to nowadays test guidelines or in some aspects even superior to the relevant test guideline (e.g. more doses tested than required according to guideline). It is meaningful that many of the well conducted studies gave positive results and even more important, the vast majority of results across different protocols was positive. Also the types of effect induced were rather consistent.

The database of TMP also spans *in vitro* assays (bacterial and mammalian cell systems), mutagenicity tests in *D. melanogaster* (5/5 positive) as well as *in vivo* mutagenicity tests in somatic cells (bone marrow micronucleus (3/4 positive) and chromosome aberration tests (8/8 positive), with most of them giving positive results.

Some of these test methods like e.g. tests in *D. melanogaster* are not recommended for the assessment of mutagenic properties any longer, but as also in these studies positive results were obtained, they are considered as supportive evidence.

The available *in vivo* studies were carried out via intraperitoneal and oral route, demonstrating mutagenicity in somatic and germ cells via both routes. The oral route is considered more relevant from a physiological point of view.

Based on an overall weight-of-evidence analysis it can be concluded that this substance has clear mutagenic potential and induces heritable DNA damage.

10.8.3 Conclusion on classification and labelling for germ cell mutagenicity

Based on the available data a classification as Germ Cell Mutagen, Category 1B, H340 for trimethyl phosphate (TMP) is proposed.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The DS proposed a harmonised classification and labelling for germ cell mutagenicity in category 1B, H340.

For the assessment the DS included several *in vitro* studies in bacterial and mammalian cells as well as mechanistical studies, several studies in *Drosophila melanogaster* and several *in vivo* studies in somatic and germ cells.

In vitro (mechanistic): Several gene mutation, chromosomal aberration and mechanistic *in vitro* studies were available. None of the studies were guideline studies.

There were two mechanistic studies investigating TMP. Yamauchi *et al.* (1976) found that TMP alkylated the major heterocyclic moieties of nucleic acids in a reaction of TMP with nitrogen heterocycles of nucleic acids, however, the effects were seen at different conditions than in the cellular milieu. Yuan *et al.* (2020) found that TMP did not produce cytotoxicity or ROS but induced mitochondrial impairment in the adenocarcinoma cell line A549. Furthermore, TMP induced cell cycle arrest by increasing G1 phase distribution in the cells. A sub G1-phase peak was also found indicating apoptotic effects, this was supported by an increase in the expression of pro-apoptotic genes (bax and decreased expression of the p53 inhibitor mdm2).

In vitro (mutagenicity/genotoxicity): TMP was studied in seven bacterial reverse mutation assays (Anonymous, 1996; Zieger *et al.,* 1992; Zieger *et al.,* 1982; Purchase *et al.,* 1978; Bruce & Heddle, 1979 and DeFlora *et al.* 1981; 1984), three reverse mutation assays (Voogd *et al.,* 1972; Kölmark, 1956 and Dean 1972), one chromosomal aberration test (Anonymous, 1994a), one study on the chromosome breaking effects (Söderman, 1972), one micronucleus test (Ni *et al.* 1993), three studies on DNA repair (Hellmer & Bolcsfoldi, 1992; DeFlora *et al.,* 1984 and Fluck *et al.,* 1976) and two studies on DNA damage (alkaline elution) (Sina *et al.*, 1983 and Storer *et al.,* 1996). The studies assessed were not in compliance with current OECD TG. The bacterial reverse mutation assays had varying quality and none of the studies included all the five recommended bacterial strains according to the OECD TG 471. Most of the studies (4/5) that tested TMP in the bacterial strain S. typhimurium TA100 were positive, indicating that TMP induce base-pair mutations, while most of the assays with TA98 were negative, indicating that TMP does not cause frame-shift mutations. There were no clear effects on metabolic

activation with S9. For the S. typhimurium strains TA1535, 1537 and 1538 the results varied more (studies showing positive, negative and equivocal results). One study was positive for all the tested strains: S. typhimurium TA102, 2638 and E. coli WP2/pKM101 and WP2uvrA/pKM101. Three studies in mammalian cells investigated chromosomal aberration effects. One of the tests, considered comparable to the OECD TG 473 Chromosomal aberration test, was negative. The two other tests (Chromosomal breakage assay and MN-test) were positive. As a whole, the *in vitro* data were not conducted in compliance with current OECD TGs and there were variations in the outcome (positive, negative, equivocal) of the tests. Nevertheless, several of the bacterial reverse mutation tests, chromosomal aberration in human lymphocytes, MN-test as well as DNA repair tests indicate mutagenic potential for TMP.

Drosophila melanogaster: TMP was studied in five *Drosophila melanogaster* mutagenicity/genotoxicity tests. Three *D. melanogaster* induction of second chromosome recessive lethal test (one of them similar to the deleted OECD TG 477). Concentration range was 0-0.02M and significant increase of lethal mutations at 0.01 M (lowest concentration tested) for one of the studies and 0-1000 mg/kg bw with positive effects at the highest concentration (TMP was used as a positive control) for another study (Dyer & Hanna, 1972 and Valencia, 1981). The third study did not indicate the concentrations used, but TMP was used as a positive control (Hanna & Dyer, 1975). TMP was also studied in one MWh-flr3 cross – somatic mutation (Wing spot test) (Graf *et al.,* 1989) and in one eye mosaic assay (Vogel & Nivard, 1993). Concentrations used in the wing spot test was in the range of 0-20 mM for 48h with positive results at 5mM (lowest dose tested). Concentrations used for the eye mosaic assay was 2-10 mM for 3 days with positive effects at all doses and 10-200 mM for 48h with positive effects at concentrations over 50 mM. All the studies in *D. melanogaster* were positive indicating that TMP induce mutation in *D. melanogaster.*

In vivo: TMP was studied in 12 somatic mutagenicity tests and in 23 mutagenicity/genotoxicity studies or other relevant studies for assessment of TMP effects on germ cells. Most of the studies were from the open literature with varying quality ranging from comparable to OECD TG to poorly reported.

In vivo (somatic): Eight chromosomal aberrations in bone marrow studies in rats and mice and four micronucleus studies in bone marrow in mice were reported, for more details see the table below. All of the chromosomal aberration studies were positive. TMP was used as the positive control in four of them. None of the studies were OECD TG studies, but one of them was considered comparable to the OECD TG 475 (Adler *et al.,* 1971). Dose related effects were reported in dose range from 500 – 2000 mg/kg bw. Positive effects were reported after i.p. and oral administration. Two of the studies that used TMP as positive control did not report the doses that were applied. Of the four micronucleus studies three gave positive results and one was negative. One of the studies with positive results were considered comparable to the OECD TG 474 (Weber *et al.,* 1975). Doses were administrated i.p. in ranges from 500 – 2000 mg/kg bw and one study even used concentrations up to 10000 mg/kg bw. Dose related increase in micronuclei was reported from 500 mg/kg bw. One study was negative, but in this study the doses used were not indicated (Ni *et al.,* 1993).

***Table**: Effects of TMP in in vivo somatic tests (adapted from Table 13 of the CLH report)*

Animal	Exposure	Results	Reference
Effects of TMP in mammalian chromosomal aberration tests in bone marrow cells			
CD rats, male. Number of animals did not always reach 5.	i.p. administration. Vehicle: water Dose regime exceeds that of the OECD TG. Time response: 2000 mg/kg bw. Bone marrow gathered at 6, 12, 24, 48, 72 and 96h. (subtoxic dose at previous experiment). Dose range: Bone marrow samples prepared 24h after single exposure to 500, 750, 1000, 1250, 1500 and 1750 mg/kg bw. Repeated dosing: injection of 500 mg/kg bw TMP carried out on four consecutive days. Samples prepared 6h and 24h after termination of treatment.	+ Time response: 2000 mg/kg bw gave maximal increase of chromatid aberration after 48h, similar value also at 24h. Dose related effects: Dose dependent increase in incidence of chromatid aberrations (0.28, 0.4, 4.5, 6, 5, 10.8, 11.8, and 20.3 % for 0, 500, 750, 1000, 1250, 1500, 1750 and 2000 mg/kg bw, respectively). Dose related decrease in mitotic index. Repeated dosing: Chromosome damage more pronounced after repeated exposure compared to single exposure. The 6h sampling time had 14 (7%) cells with chromatid aberrations which was the highest number. Number of cells reduced to 9 (4.5%) after 24h. Controls had 0 cells with chromatid aberrations No acute toxicity observed	Adler *et al.* (1971) Comparable to OECD 475
Osborn male rats. 5/group. 8/control.	i.p. or oral (gavage) administration. Vehicle: corn oil. Exposed for single dose of 0 or 2000 mg/kg and sacrificed after 18, 24 or 48h. Or 0 or 1000 mg/kg bw/day on 5 consecutive days sacrificed 6h after last exposure.	+ both oral (gavage) and i.p. administration. TMP used as positive control. i.p. administration: Increase of all types of aberrations in four different laboratories at all time points (single and after exposure to 5 consecutive i.p. doses), but with variations in degree. Maximum increase was at 48h. Oral application: more variations, but comparable trends.	Legator *et al.* (1973)
Osborne male rats	i.p. or oral (gavage) administration. Dosing: 0 or unspecified dose or as 5 consecutive daily doses.	+ both oral (gavage) and i.p. administration from single and repeated exposure. TMP used as positive control.	Sheu *et al.* (1979) cited in US EPA (2010)
Wistar male rats	i.p. administration. Dosing: Single dose: 0 or 3000 mg/kg bw TMP. Repeated dose: 0 or 1500 mg/kg bw 5 times in 1 day.	+ at single and multiple doses. Chromosome aberrations included gaps, breaks, fragments and significant number of abnormal cells.	Anderson & Richardson (1981)
Sprague-Dawley male and female rats	Oral (gavage) administration. Dosing: single dose of 0 or 2000 mg/kg bw 24 h prior to sacrifice.	+ in males, but severely damaged cells for both sex. TMP used as a positive control. Male: Induced chromatid gaps, breaks and exchanges, chromosome breaks. No effect on mitotic index	Sinha *et al.* (1983) cited in US EPA (2010)

Mice, male (strain not specified)	Doses: 1250, 1500 and 1750 mg/kg bw.	+ Maximum number of chromosome aberrations after 48h. Maximum changes at 1750 mg/kg bw (breaks, gaps and fragments)	Farrow (1975) cited in US EPA (2010) Only abstract available
B6D2F1/J mice, no information on sex. 5/group.	i.p. administration. Vehicle: buffered saline, pH 7.3. Doses: 0, 500, 750, 1000 and 2000 mg/kg bw/day	+ dose related increase in chromatid breaks (over 500 mg/kg bw/day). The highest dose was lethal.	Weber *et al.* (1975)
Mice (Q strain), male.	i.p. administration. Doses not reported. Investigation of cells 10 and 16 days after treatment.	+ Induces chromosomal aberrations including breaks, exchanges and gaps. TMP used as a positive control	Moutschen-Dahmen *et al.* (1981)
Effects of TMP in mammalian bone marrow micronucleus tests			
B6DF1/J mice, 5/group. Sex not reported	i.p. administration. Vehicle: buffered saline pH 7.3. Doses: 0, 500, 750, 1000 and 2000 mg/kg bw/day for 5 days.	+ dose related increase in frequency of micronuclei over 500 mg/kg bw/day. Highest concentration was lethal.	Weber *et al.* (1975) Comparable to OECD TG 474
Female hybrid C57BL/6xC3H/He mice. 8/group	i.p. administration. Doses: 0-10000 mg/kg bw for 5 consecutive days and sacrified 4h after last treatment.	+ increase of micronucleus observed above ~6000 mg/kg bw.	Bruce & Heddle (1979)
Mice (sex and strain not reported)	Route not reported. Doses: 0, 1250, 1500 and 1750 mg/kg bw.	+ time and dose related increase in micronuclei. No data presented.	Darrow *et al.* (1979) cited in US EPA (2010)
Mice (sex and strain not reported)	i.p. administration. Doses not reported	- negative in data table. No other information available	Ni *et al.* (1993) cited in US EPA (2010) Study in Chinese

In vivo (germ cells): One *in vivo* Comet assay in testicular cells in mice, four chromosomal aberrations in spermatocytes studies in hamster and mice, four sperm abnormality tests in mice, rats and rabbit, two sperm motility tests in rats, ten dominant lethal tests in mice and rats, two antifertility action tests in rats and one mechanistic study in rats on the effect of TMP on testosterone synthesis were included by the DS. For more details see table below. TMP gave positive results in an *in vivo* comet assay in testicular cells (mix of germ cells and somatic cells) after oral (gavage) exposure. The study was considered comparable to the OECD TG 489 (Hansen *et al.,* 2014). Significant effects were seen at the highest concentration (500 mg/kg bw/day). Two of the studies on chromosomal aberration in spermatocytes after i.p. administration of TMP were positive although concentrations used were either not reported or high (3000 mg/kg bw) (Moutshen-Dahmen *et al.,* 1981 and Katoh & Matsuda, 1985). Effects were also seen on

aberrant metaphases in hamster at 500 mg/kg bw/day (Machemer and Lorge, 1975). 10 dominant lethal tests and four sperm abnormality tests showed effects during 1-3 weeks (and up to 5 for two of the studies) indicating that spermatids were most sensitive for TMP induced dominant lethality. Dose depended effects on fertility, increased mutation rates, reduced number of implants and increased early fetal death was reported. Exposure range was 0-2500 mg/kg bw. Dose related effects were reported from 200 mg/kg bw.

***Table**: Effects of TMP for evaluation of mutagenicity/genotoxicity in germ cells (adapted from Table 13 of the CLH report)*

Animal	Exposure	Results	Reference
Mammalian *In vivo* Comet assay in testicular cells			
CD1 mice, male. 5/group	Oral (gavage) administration. Vehicle: water. Doses 0, 125, 250 and 500 mg/kg bw administered twice 24 h apart. Animals sacrificed	+ significant positive results at 500 mg/kg bw. Testicular cells included a mix of somatic and germ cells. Authors reported to DS that there were positive results in kidney and liver as well. No other treatment effects were seen in histological examination of the testes.	Hansen *et al.* (2014) Comparable to OECD TG 489.
Chromosomal aberration in spermatocytes			
Chinese Hamster, male	Oral (gavage) administration. Doses: 0 or 500 mg/kg bw/day for 2 days. 0 or 1000 mg/kg bw/day for 5 days.	(+) significant increase in the number of aberrant metaphases at 500 mg/kg bw/day when gaps were included. At 1000 mg/kg bw/day marked mitotic inhibition was reported.	Machemer & Lorge (1975) Only abstract available
Mice (Q strain), male.	i.p. administration. Doses not reported	+ TMP used as positive control. Induction of breaks, exchanges and gaps	Moutshen-Dahmen *et al.* (1981)
Mice (Q strain), male. 20/group.	i.p. administration. 0 or 1000 mg/kg bw.	- TMP was used as positive control, all substances tested including TMP was negative in the test	Degraeve *et al.* (1984)
Mice (strain not reported)	i.p. administration. Doses 3000 mg/kg bw.	+	Katoh & Matsuda (1985) Only abstract available
Dominant lethal mutation tests			
CH3 mice, both sex Dominant lethal test:21-23 fertile mating/dose group. Heritable	Dominant lethal test: i.p. administration of male mice. Doses: 0, 1000, 1250 and 1500 mg/kg bw. It is unclear from the published article if the top dose was 1500 or 2500 mg/kg bw.	Dominant lethal test: Statistically significant decrease in number of implants and live young at birth indicating marked increase in the frequency of pre- and post-implantation losses. Heritable translocation test: Dose dependent increase in semi-sterile and sterile F1 males and increased number of translocation carriers.	Tezuka *et al.* (1985)

translocation test: 33-75 males/dose group	Heritable translocation test: i.p administration of 0, 1000 and 1500 mg/kg	Slight but significant reduction of young weaned at the highest concentration.	
Swiss mice CF1 strain, fertile male and virgin female. 8/group	i.p. administration. Doses: 1000 and 2000 mg/kg bw	4/8 and 1/8 males died within 7 days in the 2000 and 1000 mg/kg bw dose group, respectively. Statistically significant increase in early fetal death per pregnant female at 1000 mg/kg bw in the two first weeks of mating. For 2000 mg/kg bw the increase was seen the first 3 weeks, but no statistical analysis was performed due to limited number of pregnant animals. Clear effect on percent pregnancies and total number of fetal implants at the highest concentration.	Dean & Thorpe (1972)
Swiss (ICR/Ha) mice, male	i.p. and oral (gavage) administration. Vehicle i.p.: Water. Doses: i.p. 200, 500, 850, 1000, 1250, 1500 and 2000 mg/kg bw. Gavage 500 and 1000 mg/kg bw/day on 5 consecutive days.	TMP was generally not toxic at the tested doses. Reduced incidence of pregnancy at highest dose tested. Reduction of number of total implants the first three mating weeks after i.p. administration of 200 and 1000 mg/kg bw, effects were significant, and dose related. Effects not repeated upon testing of wide range of i.p. doses (500-2000), however lower number of implants were noted at the 2nd week of mating. Dose related reduction of numbers of implants were reported in the first three weeks after gavage administration. At 1000 mg/kg bw reduced number of implants were reported also at week 5. Significant increase in early fetal deaths occurred the first three weeks of mating for all experiments. Dose dependent increase in early fetal death occurred in the 2nd week of mating at all dose groups except top dose via gavage where the effects were seen at 1st week of mating.	Epstein *et al.* (1970)
NMRI mice	Oral (unspecified) administration. Doses: 0 and 1000 mg/kg bw	TMP was used as reference material. No effect on preimplantation loss but marked increase in post-implantation loss in the 2nd week of mating.	Lorke and Machemer (1975) cited in US EPA (2010)
Mice (no information on strain)	i.p. or gavage administration. Doses: 1250 mg/kg bw gavage or 500 mg/kg bw for five consecutive days	Significant increase in lethality occurred maximally in the 2nd week of mating (i.p.) and in the first two weeks of mating after gavage administration of TMP.	Farrow *et al.* (1975) cited in US EPA (2010)

Mice (no information on strain)	Oral (unspecified) administration. Controls not mentioned. Doses not specified.	Dominant lethal effects were seen for 2 weeks after 5 days treatment.	Newell *et al.* (1976) cited in US EPA (2010)
Mice (no information on strain)	Doses: 1000 mg/kg bw. Controls not mentioned.	High mutagenicity particularly at postmeiotic stages.	Degraeve *et al.* (1979) cited in US EPA (2010)
Mice (Q strain)	i.p. administration. Doses: 0 and 1000 mg/kg bw.	TMP used as positive control. Significant increase in the frequency of pre-implantation and post-implantation losses 2 weeks after injection.	Moutschen-Dahmen *et al.* (1985)
Rat and mice (strain not specified), 5 rats/group, 8 mice/group	i.p. and oral administration. Doses rats: 5x250 mg/kg bw or 5x100 mg/kg bw p.o. Doses mice: 5x 1000 mg/kg bw p.o. or i.p. Treatment for five consecutive days.	Fertility was affected in mice and rats. 10-fold higher effects in mice compared to rats. Male rats were completely sterile at week 3 and 4 after exposure to the lowest concentration and at week 2-5 for the highest concentration. Male mice were completely sterile at week 2 after exposure.	Jackson and Jones (1968) and (1969)
Sperm abnormality and motility assays			
Male hybrid mice of the genotype: (C57BL X C3H/Anf) F1 or (C57BL/6 X C3H/He)F1. 4/group	i.p. administration. Doses: 100-1000 mg/kg bw. Mice were killed 1, 4 or 10 weeks after treatment.	Increase in abnormal sperm at the two highest concentrations 1 week after exposure. No effects at the other weeks. Authors claim the results indicate that post-meiotic cells are affected.	Wyborek *et al.* (1975)
Male hybrid (C57BL/6 x C3H/He) mice. 8/group	i.p. administration. Doses 0-10000 mg/kg bw.for 5 consecutive days.	Increase in abnormal sperm above 7000 mg/kg bw.	Bruce & Heddle (1979)
Male long Evan Hooded rats. 20/group.	Oral administration. Vehicle distilled water. Doses: 0, 100, 250 and 600 mg/kg bw/day for 5 consecutive days.	Weight loss at all doses. Neural – muscular deficits also reported at the highest dose. No effect on testis or whole epididymal weight, but cauda epididymal weight was significantly increased at the highest dose and sperm counts reduced. Highest dose also showed changes in sperm shape and movement also slightly detected at lower doses.	Toth *et al.* (1992)
Random-bred albino Sprague-Dawley descendants. 20/exposed group, 5/ control group	Oral administration. Vehicle distilled water. Doses: 0, 400, 500, 750, 1000 and 1500 mg/kg bw/day for 5 days/week up to 5 weeks.	The study had high mortality rates: 0 / 10 / 90 /100 / 100 / 100% in the control/ 400 / 500 /750 / 1000 / 1500 mg/kg bw/day groups, respectively. Spermatogenesis affected immediately after dosing. Aggregations of multinucleated giant cells were observed, and their emergence peaked 1 week after dosing. These structures were described to be composed of late spermatids Also cytoplasmic vacuolation of	Cho & Park (1994)

		Sertoli cells was described.	
Male Sprague-Dawley rats. 10/group	Oral (gavage) administration. Dosing 0 and 100 mg/kg bw/day for 28 days.	Sperm motility was reduced. Degenerative spermatogenic cells (1/10) and degenerative sperm (3/10) was observed in epididymal ducts. No other effects were seen.	Takizawa *et al.* (1998)
Male Wistar rats.	Oral administration. Dosing: 250 and 500 mg/kg bw on 5 consecutive days.	Decreased sperm motility and count at 500 mg/kg bw/day.	Suzuki *et al.* (1996) cited in US EPA (2010)
Antifertility action			
Random bred albino Sprague-Dawley rats. Random bred albino swiss-origin mice. New Zealand white rabbits. Only males exposed for all species. Human sperm samples	Administration route not reported Dosing: Mice: Subacute: 0, 750 & 1500 mg/kg bw on 5 consecutive days. Subchronic: 0 & 1500 mg/kg bw on 5 days / week for 1 month. Rats: Subchronic: 0, 100 & 600 mg/kg bw on 5 consecutive days for 1 months. Chronic: 0 & 750 mg/kg bw once weekly for 12 weeks. Rabbits: Chronic: 0, 200 & 325 mg/kg bw once weekly for 13 weeks.	TMP induced reversible sterility in male mice, rats and rabbits. Induced sterility was dependent on dosage and duration of treatment. Mice: Subacute: Fecundity was reduced for both concentrations the first week (to 13% and 0% for 750 and 1500 mg/kg bw, respectively) and highest concentration the second week (29%). Subchronic: Exposure to 1500 mg/kg bw/day caused sterility the first 2 weeks. Fertility returned to normal for both subacute and chronic study. Rats: Subchronic: Reduced fecundity for both concentration the first week (29% and 0-5% for 100 and 600 mg/kg bw/day, respectively). For the highest dose the effects lasted for 4 weeks but turned normal again after 6 weeks. In the chronic study fecundity reduced to 50% the first week and 0-6% from week 3-12. Rabbit: Fecundity was reduced for both concentrations. For the lowest concentration 50% in week 3 and to 25% by week 9. The highest concentration resulted in 35% reduced fecundity in the 2nd week and sterility from week 5-13. After treatment fertility was normal within one week. A single dose of 750 mg/kg bw resulted in 34% reduction in fecundity. This was normalized the week after. Choline acetyltransferase activity in spermatozoa. TMP dose and time dependently reduced the activity in all species. The author concluded that rapid reduction in enzyme activity results in rapid infertility/sterility.	Harbison *et al.* (1996)
Random bred albino Sprague Dawley rats, males and females	Oral (in water) administration. Dosing: 250 mg/kg bw 5 days/week for 30 days or 6 days/week for 60	30 days treatment: Abnormal shape of epididymal spermatozoa, i.e. detached heads, abnormalities of head, middle piece and principal piece (not seen in controls). No sign of mating with female virgin rats. Testes showed impaired spermatogenesis. Round spermatids showed vacuoles in round	Hanna & Kerr (1981)

	days	spermatids and extensive extracellular spaces were observed between the germ cells and Sertoli cells. 60 days treatment: Germ cells were absent from the seminiferous tubules, which were collapsed and showed shrinkage – "Sertoli-cell-only" condition. The lumen of many seminiferous tubules was filled with processes of Sertoli cell cytoplasm. The study authors concluded that prolonged dosing of TMP results in complete loss of germ cell activity.	

Comments received during consultation

One comment received from a member state competent authority (MSCA) supported classification as Muta. 1B based on many studies that documented the mutagenic effects of TMP *in vitro* (bacterial and mammalian cell systems), mutation assays in Drosophila melanogaster, as well as *in vivo* - in mice, rats, and rabbits - inducing chromosomal aberrations in somatic cells and spermatocytes. The transmission of mutations to F1 offspring has been demonstrated.

Assessment and comparison with the classification criteria

The DS proposed to classify TMP as Muta 1B, H340.

For the assessment of germ cell mutagenicity, the DS included a large number of *in vitro* and *in vivo* (somatic and germ cell) studies. It is noted that most of the studies are from the open literature and are not conducted according to recent OECD TGs or GLP. TMP has been extensively studied and is used as a positive control for mutagenicity testing (both in somatic and germ cell tissue) in several of the reported studies. There are large variations on the quality of the studies assessed and DS highlighted that several have limitations but does not provide a reliability score for the different studies assessed. Some of the studies are similar to OECD TG standard, but many of them are poorly reported, only abstracts are available or there are quite large deficiencies in the reporting (such as lack of reporting the doses used, vehicle, species strain, cytotoxicity and more). The doses used are also in many of the studies high and similar or higher than the LD_{50} doses reported under the acute toxicity hazard assessment.

In vitro studies: TMP was studied in seven bacterial reverse mutation assays, three reverse mutation assays, one chromosomal aberration test, one study on the chromosome breaking effects, one micronucleus test, three studies on bacterial DNA repair and two studies on DNA damage (alkaline elution) in rat hepatocytes. For the seven bacterial reverse mutation assays doses used were in the range from 0-15000 µg/plate. The bacterial strain *S. typhimurium* TA100 and TA98 was studied in 5 and 4 studies, respectively. TA100 was positive in 4/5 studies, while most of the assays with TA98 was negative in 3/4 studies and equivocal in 1/4 (Anonymous, 1996; Zieger *et al.,* 1992; Zieger *et al.,* 1982; Purchase *et al.,* 1978; Bruce & Heddle, 1979 and DeFlora *et al.,* 1981; 1984). There were no clear effects on metabolic activation with S9. For the other strains the results varied more (studies showing positive, negative and equivocal results). One study was positive for all the tested strains (Watanabe *et al.,* 1996): *S.*

typhimurium TA102, 2638 and *E. coli* WP2/pKM101 and WP2uvrA/pKM101. Bacterial DNA repair effects in different *E. coli* strains were assessed in three studies. Two of the studies were positive (one with S9 activation and one positive both with and without S9) and one study was negative (Hellmer & Bolcsfoldi, 1992; DeFlora *et al.,* 1984 and Fluck *et al.,* 1976). Three studies in mammalian cells investigated chromosomal aberration effects. One of the tests, considered comparable to OECD TG 473 Chromosomal aberration test, was negative in the dose range 0-1.4 mg/mL (Anonymous, 1994a). The two other tests, Chromosomal breakage assay with concentration range 0-100 mM and MN-test (doses not reported), were positive (Söderman, 1972 and Ni *et al.,* 1993). Two studies on DNA damage by alkaline elution in rat hepatocytes also showed increase in elution rate compared to controls. Sina *et al.* (1983) reported increased DNA damage from 0.3 mM TMP while Storer *et al.* (1996) reported slight increase in elution rate at 7 and 10 mM.

In summary, none of the tests were performed according to the current OECD TGs and there were some variations in the outcome (positive, negative, equivocal) of the *in vitro* mutagenicity tests. However, several of the bacterial reverse mutation tests, chromosomal aberration in human lymphocytes, MN-test as well as DNA repair tests indicate mutagenic potential for TMP.

In vivo (somatic): All together 12 somatic mutagenicity studies were assessed for TMP in the CLH-dossier. All the tests investigated the potential for TMP to induce chromosomal aberration. Eight of the tests were chromosomal aberration in bone marrow tests, all of which gave positive results. The doses ranged from 500-3000 mg/kg bw. Adler *et al.* (1971) found dose dependent increase in chromosomal aberration after i.p. injection from 750 mg/kg bw TMP in CD-rats bone marrow cells. Mitotic index was also dose related decreased, which may indicate cytotoxicity. Upon repeated injection of 500 mg/kg bw on four consecutive days, increased number of chromatid aberrations were observed when investigated 6 and 24h after exposure with 14 (7%) and 9 (4.5%) of the cells with chromatid aberrations, respectively. No chromatid aberration was observed in the controls, and the mitotic index was not significantly affected. No acute toxicity was observed in the study. The study was well conducted and comparable to OECD TG 475. More doses than recommended in the OECD TG was included in the study, but less cells were counted (5-600 cells) for mitotic index compared to the OECD TG recommendation (1000 cells), there was no information on historical control data (HCD) and the dose groups sometimes had less than 5 animals/group. Altogether, it was concluded that TMP induced chromosome aberrations in the study. Oral (gavage) administration was performed in three of the studies in rats where TMP was used as a positive control. Doses used were 2000 mg/kg bw for single exposure and 1000 mg/kg bw for repeated exposure (Legator *et al.,* 1973 and Shina *et al.,* 1983) the third study did not specify the dose (Sheu *et al.*, 1979). All the studies were positive, although Legator *et al.* (1973) reported more variations but similar trends when compared to the results after i.p. injection. Shina *et al.* (1983) reported severely damaged cells after exposure to a single dose of 2000 mg/kg bw, induction of chromatid gaps, breaks and exchanges and chromosome breaks in the male rats, there was no effect on mitotic index. Four *in vivo* mammalian micronucleus studies in mice were assessed. Three of the studies were positive, the one study that was negative did not report the doses used in the study. Weber *et al.* (1975) exposed mice i.p. for 0, 500, 750, 1000 and 2000 mg/kg bw/day for 5 days. Dose related increase in micronucleus was observed at all doses (500 mg/kg bw/day and above). The highest concentration was lethal. The study was comparable with OECD TG 474 but with

some deviations (1500-2000 bone marrow cells were analysed compared to the 4000 that is required in the OECD TG). More information on the studies can be found in the table above. In summary, there were different quality in the studies performed, some were considered of good quality and some of the studies have severe limitations. Overall, the studies indicate that TMP induces chromosomal aberrations in somatic tissue in rats and mice after oral (gavage) and i.p. administration.

In vivo (germ cells): TMP has been studied in a large number of tests related to germ cell mutagenicity/genotoxicity, this includes chromosomal aberration in spermatocytes, comet assay in testicular cells, dominant lethal tests, sperm abnormality and mobility tests and anti-fertility tests (see table above for more information). Hansen *et al.* (2014) conducted an *in vivo* comet assay in testicular cells in mice. The study was well conducted and considered comparable to OECD TG 489. Testicular cells consisted of a mix of somatic cells and germ cells in different stages of spermatogenesis, so the data is an indication that TMP reaches the gonads. Significant increase in average % tail DNA was reported at 500 mg/kg bw administered on two consecutive days. The average % tail DNA was 7.1, 7.9, 9.8 and 13.9 when exposed to 0, 125, 250 and 500 mg/kg bw/day, respectively. In comparison the positive control Ethylmethanesulfonate had an average % tail DNA of 12.4. Four chromosomal aberrations in spermatocytes were reported, one in hamster and three in mice. There were limitations with most of these studies. Two of them only had abstract available, another did not report the doses used. In Chinese Hamster (gavage administration) there was an increase in aberrant metaphases only when gaps were included at 500 mg/kg bw/day on two consecutive days, when gaps were excluded, it was still an increase from control but not significant. At the highest concentration (1000 mg/kg bw/day) inhibition of mitosis was reduced, indicating toxicity (Machemer and Lorge 1975). Increase in chromosomal aberrations were also reported from two studies in mice (i.p. administration) at 3000 mg/kg bw (Katoh & Matsuda, 1985) and unknown concentration (Moutshen-Dahmen *et al.*, 1981). One study in mice using TMP as a positive control was negative, although no positive results were reported from this study (Degraeve *et al.,* 1984). Ten Dominant lethal mutations tests including one with heritable translocation assay (Tezuka *et al.,* 1985) and two anti-fertility assays were reported from mice, rats and rabbits after oral (gavage) or i.p. route. Dose concentration in the dominant lethal tests ranged from 200-2500 mg/kg bw of single dose or for 5 consecutive days. Dose concentrations for the anti-fertility assays were 100- 1500 mg/kg bw for 1-5 days during 4-13 weeks. Toxicity was reported in Dean and Thorpe (1972) were i.p. injection of 1000 and 2000 mg/kg bw resulted in 4/8 and 1/8 deaths, respectively. Most of the studies reported effects 1-3 weeks after exposure, in addition Epstein *et al.* (1970) and Jackson & Jackson (1968)/Jackson & Jackson (1969) reported effects up to week 5 after exposure. Effects reported was dose dependent reduction in fertility (reduced number of pregnancies), increased early fetal deaths (pre-implantation loss) and/or increased late fetal death (post-implantation loss), increase in sterile male rats and mice and increased mutation rates. Tezuka *et al.* (1985) also found a dose-dependent increase in semi-sterile and sterile F1 males and increased number of translocation carriers. Epstein *et al.* (1970) described such effects as the result of structural and/or numerical changes in the chromosomes of the germinal cells in sexually mature animals. Harbison *et al.* (1976) also found dose and time dependent reduction in the Choline acetyltransferase activity in spermatozoa, which may also indicate that other modes of action then mutagenicity could have an impact on the observed sterility. In summary, there are different quality in the studies performed, some

are considered of good quality and some of the studies have limitations. Overall, the studies indicate that TMP induces mutagenic/genotoxic effects in germ cells and germ cell tissue after oral (gavage) and i.p. administration.

According to the CLP criteria a substance is classified as Muta. 1 when positive evidence for *in vivo* heritable germ cell mutagenicity in humans (1A) or mammals (1B) has been reported. No acceptable data have been presented on human germ cell mutagenicity. Since no positive evidence for heritable germ cell mutagenicity of TMP in humans is shown, a classification as Muta. 1A is not justified.

Substances may be classified as Muta. 1B if there are "positive results from *in vivo* somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations to germ cells". The latter may be based on a) "supporting evidence from mutagenicity/genotoxicity tests in germ cells *in vivo*", or b) "by demonstrating the ability of the substance or its metabolites to interact with the genetic material of germ cells". For TMP a large number of studies demonstrated mutagenic effects *in vitro*, in *D. melanogaster* and *in vivo* mammalian somatic tissue and germ cells, TMP is also often used as a positive control in mutagenicity assays. The studies varied in quality from comparable to OECD TG studies to having several limitations. However, both the well-conducted studies and the studies with lower quality, showed similar mutagenic effects. Altogether, several *in vitro* mutagenic tests were positive. 5/5 mutagenicity tests in *D.melanogaster* was positive, even though these studies are not recommended for mutagenicity assessment they can still be considered supportive. Of the *in vivo* mutagenicity tests in somatic tissue 3/4 bone marrow micronucleus tests and 8/8 chromosomal aberration tests were positive. Mutagenic effects have also been demonstrated in several *in vivo* germ cell mutagenicity/genotoxicity studies, dominant lethal assays in rodents and a heritable translocation assay. Mutagenicity (somatic tissue and germ cells) was demonstrated after i.p. injection and oral (gavage) administration. **In conclusion**, using a weight-of-evidence analysis, RAC considers that TMP induces mutations in somatic tissue and germ cells and a **classification as Muta. 1B, H340 is warranted.**

10.9 Carcinogenicity

Table 14: Summary table of animal studies on carcinogenicity

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Chronic Toxicity/ Carcinogenicity Study **Fischer 344 rat** 20/sex in vehicle control	TMP Purity: 99% Oral (gavage), vehicle: distilled water; 3 times per week; 104 weeks	**Chronic study (104 wks) in rats :** Survival rates were high in males and females and considered adequate to allow the assessment of late appearing tumours - all rats lived beyond week 52 on study; Mean body weights of males and females were decreased in treated groups compared to control;	NTP (1978)

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
50/sex/treated group [a] Animal age at start: 42 days	0/50/100 mg/kg bw/day; Exposure duration: 104 weeks, rats were observed from an additional week following the exposure period. Observation: animals of the control group were observed 105 weeks Rats were observed twice daily for clinical signs and body weights were measured at regular intervals. At each weighing rats were palpated for masses. Pathological evaluation: Gross and microscopic examination of all major tissues, organs and gross lesions.	LOAEL: 100 mg/kg bw/day (43 mg/kg bw/d; duration adjustment) (reduced body weight) NOAEL: 50 mg/kg bw/day (21 mg/kg bw/d, duration adjustment) Increase in incidence of subcutaneous fibromas in males (stat. significant, dose-related). No evidence of carcinogenicity in females. Detailed results are presented in the text below as well as in Table 15 and Table 17. <u>Dose range study (7 weeks), 5/sex/group:</u> 0 / 100 / 147 / 215 / 316 / 464 / 681 / 1000 & 1470 mg/kg bw/d; 3 times per week All surviving animals were killed 1 week after dosing and necropsied. Distended bladder and gastrointestinal haemorrhage were observed in these rats. All males and females dosed with 681 mg/kg bw/d or greater died, 1 male dosed with 464 mg/kg bw/d also died. Body weight: in the animals of the 464 mg/kg bw/d group body weights were lowered (males: - 44%; females: - 32%) ➔ Doses for the chronic study: low: 50 mg/kg bw/d; high: 100 mg/kg bw/d.	
Chronic Toxicity/ Carcinogenicity Study **B6C3F1 mice** 20/sex in vehicle control 50/sex/treated group [b] Animal age at start: 42 days	TMP Purity: 99% Oral (gavage), vehicle: distilled water; 3 times per week; 103 weeks 0/250/500 mg/kg bw/d Exposure duration and observation: 103 weeks Mice were observed twice daily for clinical signs and body weights were measured at regular intervals. At each weighing mice were palpated for masses.	**Chronic study (103 wks) in mice:** Survival rates were high in males and females and considered adequate to allow the assessment of late appearing tumours. Mean body weights of females were decreased in treated groups compared to control; male body weight was unaffected. $NOAEL_{males}$: 500 mg/kg bw/d (241 mg/kg bw/d; dose adjustment) (males) $NOAEL_{females}$: 250 mg/kg bw/d (107 mg/kg bw/d; dose adjustment) $LOAEL_{females}$: 500 mg/kg bw/d (241 mg/kg bw/d; dose adjustment) (reduced bw) Increased adenocarcinoma of the uterus/endometrium in females (stat. significant, dose-dependent) Detailed results are presented in the text below as well	NTP (1978)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
	Pathological evaluation: Gross and microscopic examination of all major tissues, organs and gross lesions.	as in Table 16 and Table 17. <u>Dose range finding study (7 weeks), 5/sex/group:</u> 0 / 147 / 215 / 316 / 464 / 681 / 1000 / 1470 & 2150 mg/kg bw/d 3 times per week Subchronic studies - were conducted in order to estimate the maximum tolerated dose in the chronic study: All surviving animals were killed 1 week after dosing and necropsied (results not shown). All males and 1 females of the 2150 mg/kg bw/d group and 2 females of the 1470 mg/kg bw/d grouped died. Body weight: in males body weight was slightly lowered ≥ 681 mg/kg bw/d; hardly affected in females (data not shown) ➔ Doses for the chronic study: low: 250 mg/kg bw/d; high: 500 mg/kg bw/d.	
Chronic Toxicity / Carcinogenicity Study **Duration 30 months** **Wistar rat** 60/sex/group; 10/sex/group ➔ sacrificed at 12 months; 50/sex/group ➔ sacrificed after 30 months Animals were 5 – 6 weeks old at the start of the study	TMP Purity: 99% Oral, admixed to drinking water weekly; 0/1/10/100 mg/kg bw/day; 100 mg/kg bw/day: reduced to 50 mg/kg bw/day after 54 weeks. Due to poor general conditions and increased mortality, sacrificed at month 24 (week 100) due to the high mortality. Monitoring during the exposure period: Appearance & behavior daily. Body weights recorded weekly during the first 3 months and once every other week – the rest of	**100 / 50 mg/kg bw/day:** *Clinical signs and mortality:* Weakness of hind limbs beginning with week 46 (55 males, 26 females). Increased incidence of sunken flanks (especially in males), distended abdomen (especially in females), poor general condition. Dose reduction to 50 mg/kg bw/day at week 54 had no remarkably improving effect on these clinical signs. Mortality was increased starting within the period between week 39 and 52. Mortality increased further to 70% in week 100 (despite dose reduction). *Body weights, feed and water intake:* Body weight was reduced by up to 20% in males and up to 15% in females During the first 54 weeks a slightly reduced mean feed intake in males – but when adjusted to body weight a slightly higher feed intake was seen in males and females compared to controls. No effect on water consumption. *Clinical laboratory investigations:* Slight haematological changes, considered secondary	Bomhard et al. (1997)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
	the study. Food and water consumption were monitored weekly. Ophthalmological examination: In Weeks 98/99 and 128 on 10 rats/ sex/dose. Clinical laboratory investigations: 10 rats/sex/dose. Parameters: RBC, reticulocytes, leukocytes, differential leukocyte, platelet counts, Hgb, Hct, MCV, MCH, MCHC & thromboplastin time Urinalyses: volume, total protein, specific gravity, pH [Month 14 only], sediment [microscopically examined] & semi-quantitative measurements for blood, glucose, bilirubin, protein, ketone bodies, and pH [except Month 14]. Blood and urine samples were collected at 6, 12, 14, 18, 23/24, and 30 months. Clinical chemistry: ALP, lactate dehydrogenase, AST & ALT activity, total bilirubin, cholesterol, creatinine, albumin, total protein, urea nitrogen, triglycerides, inorganic phosphate, calcium, potassium, sodium & chloride - on serum samples collected at 6, 12, and 14 months. Necropsies: All rats found dead or	to the other toxic effects: reduced haemoglobin, haematocrit, erythrocyte counts, increased reticulocyte numbers and thrombocyte counts, shift in differential blood count. Slightly increased cholesterol levels in males and females. Shifts in serum protein electrophoresis. Slightly increased protein excretion (especially in males) and reduced urinary pH value (in females) *Gross pathology:* At 12 month interim termination signs of hind limb skeletal muscle wasting in males and females, slight increase in testis changes (small, contents fluid). Animals that died prematurely showed skeletal muscle wasting, changes in the lungs (e.g. mottled, reddish, pale), changes in the heart (e.g. Thick, hard, abnormal color), changes in the liver (e.g. thick, scarring), kidney (scarring), small seminal vesicle, changes in testes (small, soft), fluid contents in the thoracic cavity in males and skin edema in females. At final necropsy (month 24) animals showed a slight increase in small hindlimbs, scarring of the kidneys and small, soft testes. *Organ weights:* Absolute organ weights were hardly affected, but statistically significant increase in relative weights of heart (m: +29%, f: +17%), liver (m: +12%, f: 17%) and kidney (m: +23%, f: +23%) were seen in top dose males and females, in males also the relative weights of the lungs (m: +30%) were statistically significantly increased. The increased relative organ weights were considered to be caused by the decreased body weights and mainly attributable to the neurotoxicity and muscle wasting. *Histopathology - Non-neoplastic changes:* 12 months interim termination: Peripheral nerve and spinal cord degeneration was seen in males and females. Myopathy of skeletal muscles was seen in some animals, see table 15 below. Moderate to marked interstitial edema in the testes of two animals (only minimal in controls). Spermatozoa were absent in both epididymides of one animal. Three animals dying close to the interim sacrifice showed a moderate (1 animal) or marked (2 animals) tubular	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
	terminated in extremis. 10 rats/sex/group 12 months after study initiation. All high-dose rats at 24 months. All other surviving rats at 30 months. Organ weights - recorded at scheduled necropsies: adrenals, brain, heart, kidneys, liver, lungs, ovaries, spleen, and testes. All rats were subjected to complete gross and histopathological evaluations.	atrophy of the testes. <u>Main study groups (top dose: 24 months):</u> Microscopy revealed a statistically significantly ($p < 0.05$ by Fisher's exact test) increased incidence of degeneration and loss of spinal cord nerve fibres males and females. Fiber damage in the peripheral nerves in these animals was associated with reactive cell proliferation, resulting in hypercellularity, which was statistically significantly increased in top-dose males and females ($p < 0.05$ by Fisher's exact test), see table 15 below. **10 mg/kg bw/day:** *Clinical signs and mortality:* Hind limb weakness in a few animals, mostly starting around week 120, was attributed to old age. Mortality was slightly increased towards the end of the study. The study authors questioned this to be a substance related effect as it was within the historical controls and there were no indications for substance specific deaths reported. *Body weights, feed and water intake:* Body weight was reduced by up to 10%. *Histopathology - Non-neoplastic changes:* <u>12 months interim termination:</u> Peripheral nerve degeneration was seen in 1 female, see table Table 18 below. <u>Main study groups (top dose: 24 months):</u> Microscopy revealed degeneration of spinal cord nerve fibres in 1 male and peripheral nerve hypercellularity in 1 female, see Table 19 below. **1 mg/kg bw/day:** Hind limb weakness in a few animals, mostly starting around week 120, was attributed to old age. <u>12 months interim termination:</u> No effects in nerves. <u>Main study groups (top dose: 24 months):</u> Microscopy revealed degeneration of spinal cord nerve fibres in 2 males and peripheral nerve hypercellularity in 2 females, see table Table 19 below. **Control:**	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
		Hind limb weakness in a few animals, mostly starting around week 120, was attributed to old age. No ophthalmological effects were seen at any dose. No significant treatment-related differences in the incidence, time of occurrence, spectrum of types or localisations of tumours were observed among treated rats when compared with concurrent controls. A survival-adjusted statistical analysis did not reveal any significant increase in any tumours in the top dose group, but early termination for the high-dose group as well as the reduction of dose after week 54 limits the interpretation of these results. Mortality may have precluded the formation and detection of late tumours. The study authors derived a NOAEL of 1 mg/kg bw/day based on suppression of body-weight gain in males at 10 mg/kg bw/day.	

a … One animal in the high dose male group was found to be a female, one animal of the high dose female group was found to be a male. These animals were removed from the assessment.
b … One animal in the high dose female group was found to be a male. This animal were removed from the assessment.

Results of the chronic study in rats (cited from the NTP (1978) report and from the summary included in US EPA, 2010):

A slight dose dependent decrease in mean body weights of the low and high dose males and females was observed. Body weight data were presented as growth curves. From visual examination, it appears that terminal body weights of high dose males and females were decreased by slightly more than 10%. No treatment related clinical signs were reported. All rats on the study lived beyond 52 weeks. No positive dose-related trend in mortality was observed. In males 17/49 (35%) of the high dose group, 28/50 (56%) of the low dose group and 8/20 (40%) of the control group lived until the end of the study. In females, survival was 27/49 (55%) in the high dose, 36/50 (72%) in the low dose and 12/20 (60%) in the control group.

Histopathology revealed a variety of degenerative and inflammatory conditions related to aging, but no treatment related non-neoplastic lesions were observed. Based on reduced body weights in the top dose 100 mg/kg bw/d was identified as LOAEL and 50 mg/kg bw/d as NOAEL (duration-adjusted doses by multiplying with 3/7: NOAEL = 21 mg/kg bw/day, LOAEL = 43 mg/kg bw/day).

Table 15 gives an overview of the observed tumours. There was a statistically significant dose-related increase ($p < 0.01$ by Cochran-Armitage test) for the incidence of subcutaneous fibromas in males across all dose groups and the incidence of fibromas in high-dose males was statistically significantly increased compared to controls ($p < 0.05$ by Fisher's exact test). These benign tumours were characterised by layers of well-differentiated fibroplastic cells separated by dense bands of mature collagen. The fibromas ranged from 5 cm to 9 cm in diameter and were located along the axillary, thoracic, abdominal and inguinal regions. Fibromas are occasionally encountered in aged rats, but the observed dose related increase was considered unusual, though no historical control values were presented.

Apparent dose-related increases in the incidences of several other tumours were observed in male rats, but none of these were statistically significant in trend or pairwise tests. These include increased incidence in lung alveolar/bronchiolar tumours for adenoma alone (Control & low dose: 0, top dose 4/46 (9%)) and when adenoma and carcinoma are considered together (Control: 0; low dose: 2/49 (4%) & top dose: 5/46 (11%)). In addition, there was an increase in adenomatous hyperplasia in the lung of low and top dose male rats (Control: 0; low dose: 1/49 (2%); top dose: 4/46 (9%)). Also, for adrenal pheochromocytoma an increase in the dosed groups was observed, though not statistically significant (control: 1/20 (5%), low dose: 4/48 (15%) & top dose: 4/47 (15%)). Without any historical control data that would give an indication of the background incidence of these tumours it is difficult to interpret the relevance of this non-significant increase. While the lung alveolar/bronchiolar adenoma/carcinomas are malignant, adrenal pheochromocytomas are benign tumours, but at least in humans considered a rare lesion (Ni & Htet, 2012).

The authors noted low incidences of several "unusual" tumours in female rats, including glioblastoma multiforme in 1/48 high-dose females, myxosarcoma in 2/49 high-dose females and malignant reticulosis in 1/50 low-dose females. These tumours are all malignant and are considered rare lesions, though no historical control data were presented. No pre-neoplastic lesions were seen in the respective tissues, but as these tumours are considered rare events pre-neoplastic lesions are not necessarily expected. The increase was not significant as only single incidences of tumours were observed.

In female rats a significant dose-related trend in the negative direction (p = 0.043) was observed for the incidence of endometrial stromal polyps of the uterus (incidence in control group exceeded that of the dosed groups). The effect cannot be accounted for by differential survival.

Table 15: Statistical analysis of primary tumours which occurred in at least two animals in one group and with an incidence of at least 5% in one or more than one group (NTP, 1978).

Tumour type & site	Control	50 mg/kg bw	100 mg/kg bw
Male rats			
Subcutaneous tissue; fibroma	0/20 (0%)	2/50 (4%)	9/49 (18%)
P-values	P = 0.006	N.S.	P = 0.036
Lung; alveolar / bronchiolar adenoma or carcinoma	0/19 (0%)	2/49 (4%)	5/46 (11%)
P-values	N.S.	N.S.	N.S.
Hematopoetic system, leukemia or lymphoma	8/20 (40%)	20/50 (40%)	25/49 (51%)
P-values	N.S.	N.S.	N.S.
Pituitary; chromophobe adenoma	4/16 (25%)	13/44 (30%)	8/38 (21%)
P-values	N.S.	N.S.	N.S.
Adrenal, pheochromocytoma	1/20 (5%)	4/48 (8%)	7/47 (15%)
P-values	N.S.	N.S.	N.S.
Thyroid; C-cell adenoma or carcinoma	1/19 (5%)	3/45 (7%)	2/46 (4%)
P-values	N.S.	N.S.	N.S.
Testis; interstitial-cell tumour	11/16 (69%)	33/46 (72%)	25/46 (54%)
P-values	N.S.	N.S.	N.S.
Total animals with primary tumours	19/20 (95%)	48/50 (96%)	43/49 (87%)
Total primary tumours / mean # of primary tumours per tumour bearing animal	28 / ~1.5	91 / ~1.9	92 / ~2.1
Total animals with malignant tumours	8/20 (40%)	24/48 (50%) [a]	29/43 (67%) [a]
Total malignant tumours / mean # of malignant tumours per tumour bearing animal	9 / ~1.1	26 / ~1.1	34 / ~1.2
Female rats			
Lung; alveolar / bronchiolar adenoma or	0/20 (0%)	0/50 (0%)	3/45 (7%9

carcinoma			
P-values	N.S.	N.S.	N.S.
Hematopoetic system, leukemia or lymphoma	3/20 (15%)	14/50 (28%)	12/49 (24%)
P-values	N.S.	N.S.	N.S.
Pituitary; chromophobe adenoma	9/20 (45%)	21/48 (44%)	18/41 (44%)
P-values	N.S.	N.S.	N.S.
Thyroid; C-cell adenoma or carcinoma	2/19 (11%)	6/47 (0%)	5/43 (5%)
P-values	N.S.	N.S.	N.S.
Mammary Gland; fibroadenoma	2/20 (10%)	3/50 (6%)	5/49 (10%)
P-values	N.S.	N.S.	N.S.
Uterus; endometrial stromal polyp	2/20 (10%)	1/45 (2%)	0/44 (0%)
P-values	P = 0.043 (N)	N.S.	N.S.
Total animals with primary tumours	15/20 (75%)	40/50 (80%)	42/49 (86%)
Total primary tumours / mean # of primary tumours per tumour bearing animal	22 / ~1.5	51 / ~1.3	54 / ~1.3
Total animals with malignant tumours	8/20 (40%)	17/50 (34%)	19/48 (39.6%)
Total malignant tumours / mean # of malignant tumours per tumour bearing animal	8 / 1	17 / 1	21 / ~1.1

a … in total 3 tumours in 3 males could not be allocated to either benign or malign status in mid and top dose each
N.S. … not significant
P-values… Beneath the incidence in the control group is the probability level for the Cochran-Armitage test when $P < 0.05$ (otherwise N.S. is indicated). Beneath the incidence of each dosed group is the probability level for the Fisher exact test for the comparison of that dosed group with the control group when $P < 0.05$ (otherwise N.S. is indicated).
N … Negative trend: indicates a lower incidence in a dosed group than in the control group.

It is noted that a considerable number of animals was affected by pneumonia and / or parasitism of the gastrointestinal tract. In summary 28% to 43% of males and 36% to 50% of females were affected by pneumonia and 15% to 26% of males and 9% to 14% of females were affected by some kind of gastrointestinal parasitism. However, as effects on body weight were only slight, as no other indications of general toxicity were described and survival was considered adequate (all animals survived beyond week 52 and a sufficient number of animals survived to assess late occurring tumours) the study is considered acceptable.

The study authors concluded that under the conditions of this bioassay TMP induced benign tumours in male rats and that no evidence of carcinogenicity in female rats can be derived.

Results of the chronic study in mice (cited from the NTP (1978) report and from the summary included in US EPA, 2010):

A slight decrease in mean body weights of the low and high dose females was observed, while body weight of male mice was unaffected. No other treatment related clinical signs were reported and no positive dose-related trend in mortality was observed. In males 39/49 (80%) of the high dose group, 44/50 (88%) low dose group and 14/20 (70%) of the control group lived until the end of the study. In females survival was 29/49 (59%) in the high dose, 31/50 (62%) in the low dose and 18/20 (90%) in the control group. Statistical tests for a dose-related increase in mortality did not achieve significance in either sex ($p > 0.05$, Tarone's test). It can be concluded that sufficient numbers of each sex survived long enough to adequately assess the occurrence of late-appearing tumours.

A dose related decrease in mean body weights was observed among female mice, while mean body weights of male mice were comparable to controls throughout the study. Based on visual assessment of the presented growth curves it can be concluded that body weight of top dose female mice was reduced by at least 10% at the end of the study.

Most non-neoplastic lesions observed in treated mice were considered to be either spontaneous or common in mice in long-term studies.

500 mg/kg bw/d (214 mg/kg bw/d when multiplied by 3/7, to adjust for duration) was identified as NOAEL in male mice and LOAEL in female mice, based on reduced body weights. For females the NOAEL was 250 mg/kg bw/d (corresponding to 107 mg/kg bw/d when duration adjusted).

Table 16: Statistical analysis of primary tumours which occurred in at least two animals in one group and with an incidence of at least 5% in one or more than one group (NTP, 1978).

Tumour type & site	Control	250 mg/kg bw	500 mg/kg bw
Male mice			
Lung; alveolar / bronchiolar adenoma or carcinoma	3/20 (15%)	11/50 (22%)	9/49 (18%)
P-values	N.S.	N.S.	N.S.
Hematopoetic system, leukemia or lymphoma	3/20 (15%)	5/50 (10%)	9/49 (18%)
P-values	N.S.	N.S.	N.S.
Liver, hepatocellular carcinoma	4/20 (20%)	9/48 (19%)	8/49 (16%)
P-values	N.S.	N.S.	N.S.
Liver, hepatocellular adenoma or carcinoma	4/20 (20%)	10/48 (21%)	8/49 (16%)
P-values	N.S.	N.S.	N.S.
Total animals with primary tumours	11/20 (55%)	26/50 (52%)	26/49 (53%)
Total primary tumours / mean # of primary tumours per tumour bearing animal	14 / ~1.3	31 / ~1.2	32 / ~1.2
Total animals with malignant tumours	9/20 (45%)	20 / 50 (40%)	22/49 (45%)
Total malignant tumours / mean # of malignant tumours per tumour bearing animal	10 / ~1.1	23 / ~1.2	25 / ~1.1
Female mice			
Lung; alveolar / bronchiolar adenoma or carcinoma	3/20 (15%)	0/48 (0%)	6/45 (13%)
P-values	N.S.	P = 0.023 (N)	N.S.
Hematopoetic system, leukemia or lymphoma	5/20 (25%)	14/50 (28%)	11/47 (23%)
P-values	N.S.	N.S.	N.S.
Liver, hepatocellular adenoma or carcinoma	2/20 (10%)	4/50 (8%)	0/44 (0%)
P-values	N.S.	N.S.	N.S.
Uterus/endometrium; adenocarcinoma	0/16 (0%)	7/40 (18%)	13/37 (35%)
P-values	P = 0.003	N.S.	P = 0.004
Uterus; endometrial stromal polyp	0/16 (0%)	2/40 (5%)	1/37 (3%)
P-values	N.S.	N.S.	N.S.
Total animals with primary tumours	11/ 20 (55%)	28/50 (56%)	30/49 (61.2%)
Total primary tumours / mean # of primary tumours per tumour bearing animal	14 / ~1.3	33 / ~1.2	36 / ~1.2
Total animals with malignant tumours	8/20 (40%)	25/50 (50%)	26/49 (53%)
Total malignant tumours / mean # of malignant tumours per tumour bearing animal	9 / ~1.1	27 / ~1.1	27 / ~1.0

N.S. … not significant; N … Negative trend: indicates a lower incidence in a dosed group than in the control group

In females there was a high incidence of endometrial adenocarcinomas and a few other types of malignant uterine tumours. The incidence of endometrial adenocarcinomas was significant in comparison to controls in the high dose group and there was a significant dose related trend (Table 16).

Grossly, the uterine tumours were masses of 1 cm to 2 cm in diameter, which were usually limited to one horn. Microscopically, the majority of these tumours appeared to arise from the endometrium as irregular acinar structures with slit-like lumens that were composed of flat to low cuboidal hyperchromatic epithelial cells. The neoplastic glandular structures widely invaded the myometrium and often extended to the serosa. The remainder of the tumours appeared to arise from endometrial polyploid structures that contained columnar shaped cells with high nuclear/cytoplasmatic ratios and numerous mitoses. A few of these formed papillary structures had cystic areas. Overall, the uterine adenocarcinomas appeared to be highly malignant. There was vascular involvement and pulmonary metastases in one low dose and four high dose females. The tumours appeared to be more aggressive in the high dose animals, since metastasis frequently occurred in this group. In addition to the above described adenocarcinomas of the endometrium, there was also one case of squamous-cell carcinoma of the endometrium in one high dose female (1/37 (3%)) and one case of uterine leiomyosarcoma (1/40 (3%)) in a low dose female.

The study authors described spontaneous uterine adenocarcinomas as uncommon in mice and considered their high incidence as treatment related. No occurrence of endometrial adenocarcinoma was reported in the historical control data, consisting of 100 female B6C3F1 mice. No further details on the historical control data were presented.

Like in rats, several unusual tumours were seen at low incidence. These consisted of two interstitial-cell tumours of the testes in two low dose males (sheets of basophilic, round to polygonal cells that separated and displaced seminiferous tubules), one rhabdomyosarcoma in a low dose male, a gastric squamous-cell carcinoma in a high dose male, an adenocarcinoma of the lacrimal gland in a high dose male, an osteosarcoma that metastasized to the lung and kidney in a low dose female, and oligodendroglioma of the brain in a low dose female, an ameloblastoma of the mandible in a high dose female, and an arrhenoblastoma (Sertoli cell tumour) of the ovary in a high dose female.

Two non-neoplastic changes appeared to be related to the observed uterine tumours. One was hydronephrosis, which occurred in six animals, four of which had endometrial adenocarcinoma and one had uterine leiomyosarcoma. Involvement of the urinary tract was microscopically demonstrated in two of these cases, but it was described that it was likely that obstruction of the urinary tract by the tumour occurred also in the other cases at some point along the urinary tract. The other change was extensive thrombosis of the pulmonary arteries that occurred in three high dose females with pulmonary metastases of the endometrial adenocarcinomas.

No other remarkable non-neoplastic findings were reported in male and female mice.

Like in the rat carcinogenicity study, also in the mouse study animals were affected by pneumonia and/or gastrointestinal parasitism, though fewer mice than rats were affected. Like for the rat study also the mouse study is considered acceptable, as effects on body weight were only slight and only seen in females, no other general toxicity was described and study survival was sufficiently high in order to consider the study reliable.

The authors of the NTP (1978) study concluded that under the conditions of this bioassay TMP was carcinogenic in female B6C3F1 mice, inducing adenocarcinoma of the uterus/endometrium. TMP was associated with the induction of benign fibromas of the subcutaneous tissue in male Fischer 344 rats. The study authors concluded that there was no evidence for carcinogenicity in male mice.

Table 17: Summary of the most relevant tumours in male F344 rats and female B6C3F1 mice (NPT, 1978).

Male F344 rats			
Parameter	**Control**	**50 mg/kg bw/d**	**100 mg/kg bw/d**
Subcutaneous tissue, fibroma	0/20 (0%) [a]	2/50 (4%)	9/49 (18%) [b]

Alveolar/bronchiolar, adenoma or carcinoma	0/19 (0%)	2/49 (4%)	5/46 (11%)
Adrenal, pheochromocytoma	1/20 (5%)	4/48 (8%)	7/47 (15%)
Female B6C3F1 mice			
	Control	**250 mg/kg bw/d**	**500 mg/kg bw/d**
Uterus, adenocarcinoma	0/16 (0%)	7/40 (18%) [a]	13/37 (35%) [c]

a … Significant dose-related increase by Cochran-Armitage test at $p < 0.01$
b … Significant pairwise difference from control by Fisher's exact test at $p < 0.05$
c … Significant pairwise difference from control by Cochran-Armitage test at $p < 0.01$

The authors of the NTP (1978) study concluded that under the conditions of this bioassay TMP was carcinogenic in female B6C3F1 mice, inducing adenocarcinoma of the uterus/endometrium. TMP was associated with the induction of benign fibromas of the subcutaneous tissue in male Fischer 344 rats. The study authors concluded that there was no evidence for carcinogenicity in female rats and male mice.

In the **Chronic Toxicity / Carcinogenicity Study by Bomhard et al. (1997)** Wistar rats were exposed to 0, 1, 10 or 100 mg TMP/kg bw/d via drinking water for 30 months (60 rats/sex/group). 10/sex/group were sacrificed at 12 months and 50/sex/group were sacrificed after 30 months. The study design and most of the results are described in detail in Table 14. The results of the microscopic investigations of nerve fibres are presented in Table 18 and Table 19 below.

Table 18: Significant changes in Wistar rats treated with TMP via drinking water for up to 12 months (Bomhard et al, 1997).

Parameter	Control	1 mg/kg bw/day	10 mg/kg bw/day	100 mg/kg bw/day
Males				
Degeneration of peripheral nerve fiber	0/10 (0%)	0/10 (0%)	0/10 (0%)	8/10 (80%) [a]
Degeneration of spinal cord nerve fiber	0/10 (0%)	0/10 (0%)	0/10 (0%)	4/10 (40%)
Females				
Degeneration of peripheral nerve fiber	0/10 (0%)	0/10 (0%)	1/10 (10%)	9/10 (90%) [a]
Degeneration of spinal cord nerve fiber	0/10 (0%)	0/10 (0%)	0/10 (0%)	4/10 (40%)

a … Significantly different from control at $p < 0.05$ by Fisher's exact test performed (performed by US EPA, 2010)

Table 19: Significant changes in Wistar rats treated with TMP via drinking water for up to 24/30 months (Bomhard et al, 1997).

Parameter	Control	1 mg/kg bw/day	10 mg/kg bw/day	76 mg/kg bw/day [a]
Males				
Peripheral nerve hypercellularity	0/50 (0%)	0/49 (0%)	1/48 (2%)	11/47 (23.4%) [b]
Degeneration of spinal cord nerve fiber	0/50 (0%)	2/49 (4%)	1/48 (2%)	6/47 (12.8%) [b]
Loss of spinal cord nerve fiber	0/50 (0%)	0/49 (0%)	0/48 (0%)	15/47 (31.9%) [b]

Females				
Peripheral nerve hypercellularity	0/49 (0%)	2/49 (4%)	1/50 (2%)	6/50 (12%) [b]
Loss of spinal cord nerve fiber	0/49 (0%)	0/49 (0%)	0/50 (0%)	10/50 (20%) [b]

a … Time-weighted average (100 mg/kg bw/day for 54 weeks and 50 mg/kg bw/day for 50 weeks)
b … Significantly different from control at $p < 0.05$ by Fisher's exact test performed (performed by US EPA, 2010)

10.9.1 Short summary and overall relevance of the provided information on carcinogenicity

There are no human data available for assessment of the carcinogenic hazard of TMP.

As presented in Table 14, two adequate long-term studies on carcinogenicity with TMP administered by gavage are available in two species, rat and mouse (NTP, 1978). In addition, the carcinogenic potential was assessed in a third chronic toxicity/carcinogenicity study in rat (Bomhard et al., 1997) in which TMP was administered via drinking water.

The NTP (1978) study has some deficiencies in that there were no historical controls available for rat and for mice they are only scarcely reported and the test design with doses administered on only 3 days per week is not fully equivalent to life-long continuous exposure. A clear drawback of the study is also the low number of animals in the control groups (20 versus 50 in the treated groups) which might have hindered the detection of effects. But the study is well conducted, it assessed the relevant parameters needed in a carcinogenicity study, including detailed reporting on histopathology, survival was adequate and clear dose-related tumour increases were seen in male rat and female mice.

The study by Bomhard et al. (1997) also has deficiencies, mainly because considerable toxicity resulting in mortality was seen in the top dose of 100 mg/kg bw/day and the dose had to be reduced to 50 mg/kg bw/day after week 54, but no improvement of the animals condition resulted from this dose reduction. It is unclear why such high general toxicity was seen in top dose animals of this study, which is in contrast with the results from the NTP (1978) study and several other repeated dose toxicity studies. Due to the high mortality in the top dose group it might be that late occurring tumours have been missed. In addition, it is noted that the mid dose in this study was 10 mg/kg bw/day, a dose not included in the rat NTP (1978) carcinogenicity study: the lowest dose in NTP (1978) was 50 mg/kg bw/day in rat, a dose already revealing subcutaneous tissue fibroma.

Table 20: Compilation of factors to be taken into consideration in the hazard assessment.

Species and strain	Tumour type and background incidence	Multi-site responses	Progression of lesions to malignancy	Reduced tumour latency	Responses in single or both sexes	Confounding effect by excessive toxicity?	Route of exposure	MoA and relevance to humans
F344 rat	**Subcutaneous fibromas.** Not observed in concurrent controls. Occasionally encountered in aged rats, but the observed dose related increase was considered unusual.	No [a]	No	No interim sacrifice. No masses were reported.	Males only	No	Oral, gavage	Substance is mutagenic. Considered relevant for humans.

Species and strain	Tumour type and background incidence	Multi-site responses	Progression of lesions to malignancy	Reduced tumour latency	Responses in single or both sexes	Confounding effect by excessive toxicity?	Route of exposure	MoA and relevance to humans
	No historical control data available.							
B6C3F1 mice	**Adenocarcinoma of the uterus.** The tumour is considered uncommon in mice and the high incidence is considered treatment related. No such tumour was seen in the historical control data consisting of 100 female B6C3F1 mice.	No [a]	The tumour was described to be highly malignant. There was vascular involvement and pulmonary metastasis. The tumour was more aggressive in the top dose, than in the lower dose (frequency of metastasis).	No interim sacrifice. No masses were reported.	Females only [b]	No	Oral, gavage	Substance is mutagenic. Considered relevant for humans.

a … It should be noted that several "unusual" tumours were described in this study in rats which occurred at very low incidences. The relevance of these tumours cannot be fully assessed. b … Uterine tumours are female specific.

10.9.2 Comparison with the CLP criteria

Criteria according CLP regulation, Table 3.6.1

Categories	*Criteria*
CATEGORY 1	Known or presumed human carcinogen A substance is classified in Category 1 for carcinogenicity on the basis of epidemiological and/or animal data. A substance may be further distinguished as:
Category 1A	Category 1A, known to have carcinogenic potential for humans, classification is largely based on human evidence, or
Category 1B	Category 1B, presumed to have carcinogenic potential for humans, classification is largely based on animal evidence. The classification in Category 1A and 1B is based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived from: - human studies that establish a causal relationship between human exposure to a substance and the development of cancer (known human carcinogen); or - animal experiments for which there is sufficient (1) evidence to demonstrate animal carcinogenicity (presumed human carcinogen). In addition, on a case-by-case basis, scientific judgement may warrant a decision of presumed human carcinogenicity derived from studies showing limited evidence of carcinogenicity in humans together with limited evidence of carcinogenicity in experimental animals.
CATEGORY 2	Suspected human carcinogens

	The placing of a substance in Category 2 is done on the basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B, based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived either from limited (1) evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies.

The available experimental carcinogenicity data (NTP, 1978) demonstrate a causal relationship between TMP exposure and significant increase in tumour incidence in male rats and female mice. While the subcutaneous fibromas observed in male rats were of benign nature, the uterine adenocarcinomas in female mice were malign. The treatment related effect is substantiated by the fact that the increase in the incidence of tumours is dose-related. In addition, several unusual malign tumours were seen at low incidence (1 or 2 tumours in treated groups) in mice and rats.

No tumour increase was seen in a second carcinogenicity study in rat (Bomhard et al., 1997), but this study was affected by high mortality in the top dose, which had to be reduced after 54 weeks and the result can therefore not be directly compared with the first carcinogenicity study in rat. Also, the administration form (gavage 3 times a week vs application via drinking water) was different between these two carcinogenicity studies in rats and different strains of rat were used, i.e. the study by Bomhard et al. (1997) was conducted in Wistar rats, the NTP (1978) study used Sprague Dawley rats.

Furthermore, severe neurotoxic effects were observed in the study with Wistar rats, which seems to be the most prominent adverse effect in this study. No other carcinogenicity study is available.

The CLP guidance (ECHA, 2017) further states that additional aspects need to be considered in a weight-of-evidence analysis and lists several important factors that need to be considered for a decision:

Tumour type and background incidence	All observed tumours are considered to have a low background incidence, which is supported by historical control data for uterine adenocarcinoma (no single tumour was seen in 100 control B6C3F1 mice).
Multi-site response	Only for two tumour types (subcutaneous fibroma, uterine adenocarcinoma) a causal relationship between TMP exposure and tumour increase can be unequivocally demonstrated. For a number of unusual tumours, which occurred at very low incidence, this relation cannot be unequivocally demonstrated. The dose related increase in rat lung adenoma/carcinoma and adrenal pheochromocytoma was not significant and only seen in males. These tumours were not described as rare, but no historical control data were available. At least in humans adrenal pheochromocytoma is considered a rare lesion (Ni & Htet, 2012).
Progression of lesions to malignancy	The uterine adenocarcinomas showed a high degree of malignancy, indicated by vascular involvement and pulmonary metastasis. The tumour was more aggressive in the top dose compared with the low dose (higher frequency of metastases). Subcutaneous fibroma is a benign tumour type. Several of the unusual tumours observed at low incidence were malignant.
Reduced tumour latency	No information available.
Whether response are in single or both sexes	A clear increase in tumour incidence was seen in male rats and female mice. No tumour type was seen in both sexes. As the tumours in uterus are female specific the relevance of sex specificity

	is reduced.
Whether responses are in a single species or several species	Tumours were seen in rats and mice.
Structural similarity to a substance(s) for which there is good evidence of carcinogenicity	No data available.
Routes of exposure	Only the oral route was investigated.
Comparison of absorption, distribution, metabolism and excretion between test animals and humans	There is no information on species differences regarding ADME available. The observed tumours are considered relevant for humans.
The possibility of a confounding effect of excessive toxicity at test doses	The study in which the tumours were observed was not impaired by excessive toxicity, but animals were affected by pneumonia and parasites in the GI tract.
Mode of action and its relevance for humans, such as cytotoxicity with growth stimulation, mitogenesis, immunosuppression, mutagenicity	A huge data-base demonstrates that TMP acts mutagenic *in vitro* and *in vivo*, including somatic cells as well as germ cells (see Chapter 10.8). Other modes of action are not well investigated, but in the available studies it was noted that cytotoxicity and cell death was hardly induced by TMP, thereby increasing the relevance of its genotoxic effects.

The available experimental carcinogenicity data support that TMP is carcinogenic in female mice and that it induces benign tumours in male rats. The occurrence of several unusual tumours at low incidences in treated rats and mice is regarded as supportive evidence of TMPs carcinogenicity.

Several of the above evaluated factors relevant for the decision on classification increase the concern for TMP, most relevant is here the high degree of malignancy of uterine adenocarcinomas as well as the well documented mutagenicity of TMP in several organs and tissues, including germ cells. It is recognized that genetic events are central in the overall process of cancer development and the CLP Regulation states that mutagenic activity may indicate that a substance has a potential for carcinogenic effects. Though the relevance of the different types of rare tumours which occurred at low incidences cannot be fully assessed, they might mirror the action of a mutagen with moderate potency that is widely distributed through the mammalian body. This multisite carcinogenic activity is also supported by the dose related occurrence of tumours in the lung (lung alveolar/bronchiolar adenoma/carcinoma) and the adrenals (pheochromocytoma), but also their relevance cannot be fully estimated.

10.9.3 Conclusion on classification and labelling for carcinogenicity

Based on an overall weight-of-evidence assessment of all the available data, classification for carcinogenicity is warranted. A classification of TMP as Carcinogen, Category 1B, H350 is proposed.

RAC evaluation of carcinogenicity
Summary of the Dossier Submitter's proposal

For the evaluation of carcinogenicity, the DS included three chronic toxicity (carcinogenicity studies in mice and rats that were exposed by the oral route (gavage and by drinking water). The studies are summarized below.

***Table**: Carcinogenic effects after TMP exposure (adapted from Table 14 of the CLH report)*

Study	Doses	Results	Reference
F344 rats 20/sex in vehicle control. 50 animals/sex/treated group. Study duration 104 weeks	Oral (gavage) administration. 0, 50 and 100 mg/kg bw/day 3 times a week for 104 weeks. Control group observed for 105 weeks. Vehicle: distilled water	Increased incidence of subcutaneous fibromas (benign) in males (significant and dose related). 0, 4 and 18% when exposed to 0, 50 and 100 mg/kg bw/day, respectively. In addition, non-significant increase of alveolar/bronchiolar adenoma (or carcinoma 0, 4 and 11% at 0, 50 and 100 mg/kg bw/day, respectively) and adrenal pheochromocytoma (5, 8 and 15% at 0, 50 and 100 mg/kg bw/day, respectively) were reported. Other tumour types were also reported, but with less difference from the incidence in controls. No evidence of carcinogenicity in females. Significant decrease in uterus endometrial stromal polyp was reported (10, 2 and 0% at 0, 50 and 100 mg/kg bw/day, respectively). General: Survival rate considered adequate for assessment of late tumours. In males survival was 40%, 56% and 35% and in females 60%, 72% and 55% at 0, 50 and 100 mg/kg bw at the end of the study. All rats lived beyond 52 weeks. Mean body weight slightly reduced for male and female (LOAEL 100 mg/kg bw/day). Pneumonia and/or parasitism in GI tract were reported, 28-50% with pneumonia and 9-26% with GI parasitism, however bw were only slightly affected.	NTP (1978)
B6C3F1 mice 20/sex in vehicle control. 50/sex/treated group. Study duration 103 weeks	Oral (gavage) administration. 0, 250 and 500 mg/kg bw/day 3 times a week for 103 weeks. Control group observed for 105 weeks. Vehicle: distilled water	Increase in adenocarcinoma of the uterus/endometrium in females. 0, 18 and 35% when exposed to 0, 250 and 500 mg/kg bw/day, respectively (significant and dose related effects) General: Survival rate high in males and females and considered adequate for assessment of late tumours. In males survival was 70%, 88% and 80% and in females 90%, 62% and 59% at 0, 250 and 500 mg/kg bw at the end of the study. Mean body weight was unaffected for males and reduced for females (LOAEL 500 mg/kg bw/day).	NTP (1978)
Wistar rats 60/sex/group 10/sex/group sacrificed at 12 months 50/sex/group sacrificed after 30 months.	Oral administration, mixed to drinking water weekly. 0, 1, 10 and 100 mg/kg bw/day. Highest concentration reduced to 50	No tumour increases, mortality may have precluded the formation and detection of late tumours. General: Increased mortality between week 39 and 52. Highest concentration reduced to 50 mg/kg bw/day because of mortality, still mortality increased to 70% in week 100. Several other clinical signs were reported. Neurotoxicity highlighted (further assessed under STOT RE).	Bomhard *et al.* (1997)

Study duration 30 months	mg/kg bw/day at month 24 (week 100) due to high mortality.		

In the NTP (1978) study, F344 rats (50/sex/treated group) were exposed to 0, 50 and 100 mg/kg bw/day by oral (gavage) administration 3 times a week for 104 weeks. The control group only included 20 animals/sex. Statistically significant dose-related increase in subcutaneous fibromas in males were observed in males (trend test), the high-dose males also had a statistically significant increase compared to controls. Subcutaneous fibromas are benign tumours with a layer of well-defined fibroblastic cells separated by dense bands of mature collagen. The fibromas ranged from 5-9 cm in diameter and were located along the axillary, thoracic, abdominal and inguinal regions. Fibromas can be encountered in aged rats, but this type of dose related increased is unusual, however no HCD were presented. Increased incidences of several other tumours were also reported in male rats, although not statistically significant. This includes lung alveolar/bronchiolar tumours for adenoma alone and for adenoma and carcinoma together, which are malignant tumours. Increase was also observed for adrenal pheochromocytoma, which is a benign tumour. The data on tumour incidences in male rats can be seen in the table below (minor adjustment from table 15 in the CLH report). In female rats there were noted some incidences of rare malignant tumours including glioblastoma multiforme (1/48 high dose females), myxosarcoma (2/49 high dose females), malignant reticulosis (1/50 low dose females). No pre-neoplastic effects were detected in respective tissues. No HCD were provided.

A slight dose dependent decrease in mean body weights (around 10%) was observed for both sex. No other treatment related clinical signs were reported. Histopathology showed a variety of degenerative and inflammatory conditions related to aging, but treatment related non-neoplastic effects. Increase in number of animals affected by pneumonia and/or parasitism in the gastrointestinal tract was also detected, but there was no indication on general toxicity and survival was considered adequate.

***Table**: Summary of tumour incidence in male rats with incidence of at least 5% (NTP, 1978) (adapted from Table 15 of the CLH report)*

Tumour type & site	**Control**	**50 mg/kg bw**	**100 mg/kg bw**
Subcutaneous tissue; fibroma [a]	0/20 (0%)	2/50 (4%)	**9/49 (18%)***
Lung; alveolar/bronchiolar adenoma or carcinoma	0/19 (0%)	2/49 (4%)	5/46 (11%)
Hematopoetic system, leukemia or lymphoma	8/20 (40%)	20/50 (40%)	25/49 (51%)
Pituitary; chromophobe adenoma	4/16 (25%)	13/44 (30%)	8/38 (51%)
Adrenal, pheochromocytoma	1/20 (5%)	4/48 (8%)	7/47 (15%)
Thyroid; C-cell adenoma or carcinoma	1/19 (5%)	3/45 (7%)	2/46 (4%)
Testis; interstitial-cell tumour	11/16 (69%)	33/46 (72%)	25/46 (54%
Total animals with primary tumours	19/20 (95%)	48/50 (96%)	43/49 (87%)
Total primary tumours / mean # of primary tumours per tumour bearing animal	28/~1.5	91/~1.9	92/~2.1

Total animals with malignant tumours	8/20 (40%)	24/48 (50%)	29/43 (67%)
Total malignant tumours / mean # of malignant tumours per tumour bearing animal	9/~1.1	26/~1.1	34/~1.2

[a] statistically significant dose related trend P=0.006

In the NTP (1978) study, B6C3F1 mice (50/sex/treated group) were exposed to 0, 250 and 500 mg/kg bw/day by oral (gavage) administration 3 times a week for 103 weeks. The control group only included 20 animals/sex. A dose-related increase in incidences of endometrial adenocarcinoma were reported in females, the findings were also statistically significantly in the high dose group compared to controls. The data on tumour incidences in female mice can be seen in the table below (minor adjustment from table 16 in the CLH report). Uterine tumours were reported as masses of 1-2 cm in diameter usually limited to one horn. Most of the tumours appeared to arise from endometrium as irregular acinar structures with slit-like lumens that were composed of flat to low cuboidal hyperchromatic epithelial cells. The neoplastic glandular structures widely invaded the myometrium and often extended to the serosa. Remaining tumours appeared to arise from endometrial polyploid structures that contained columnar shaped cells with high nuclear/cytoplasmatic ratios and numerous mitoses. A few of these formed papillary structures with cystic areas. The uterine tumours seem to be highly malignant. Vascular involvement and pulmonary metastases were reported for one low dose and four high dose females. Tumours seems to be more aggressive in high dose animals. In addition to the adenocarcinoma tumours in uterus/endometrium, squamous-cell carcinoma of endometrium was detected in one high dose female (1/37 (3%)) and one case of uterine leiomyosarcoma (1/40 (2.5%) was observed in the low dose group). There was no occurrence of endometrial adenocarcinoma in the HCD.

There were no differences in tumour incidence for exposed males compared to controls.

A slight dose dependent decrease in mean body weights was observed for females (at least 10% at highest dose), but not for males. No other treatment related clinical signs were reported. Increase in number of animals affected by pneumonia and/or parasitism in the gastrointestinal tract was also detected, but less than for rats. There was no indication of general toxicity and survival was sufficiently high for the study to be considered reliable.

***Table**: Summary of tumour incidence in female mice with incidence of at least 5% (NTP, 1978) (adapted from Table 16 of the CLH report)*

Tumour type & site	**Control**	**250 mg/kg bw**	**500 mg/kg bw**
Lung; alveolar/bronchiolar adenoma or carcinoma	3/20 (15%)	0/48 (0%)	6/45 (13%)
Hematopoetic system, leukemia or lymphoma	5/20 (25%)	14/50 (28%)	11/47 (23%)
Liver, hepatocellular adenoma or carcinoma	2/20 (10%)	4/50 (8%)	0/44 (0%)
Uterus/endometrium; adenocarcinoma[a]	0/16 (0%)	7/40 (8%)	**13/37 (35%)***
Uterus/endometrium; stromal polyp	0/16 (0%)	2/40 (5%)	1/37 (3%)
Total animals with primary tumours	11/20 (55%)	28/50 (56%)	30/49 (61.2%)
Total primary tumours / mean # of primary	14/~1.3	33/~1.2	36/~1.2

tumours per tumour bearing animal			
Total animals with malignant tumours	8/20 (40%)	25/50 (50%)	26/49 (53%)
Total malignant tumours / mean # of malignant tumours per tumour bearing animal	9/~1.1	27/~1.1	27/~1.0

[a] statistically significant dose-related trend P=0.003

In the study by Bomhard *et al.* (1997) Wistar rats were exposed to 0, 1, 10 and 100 mg/kg bw/day via drinking water for 30 months (60/sex/group). 10 rats/sex/group were sacrificed after 12 months and the remaining animals after 30 months. High mortality was reported for the highest dose (100 mg/kg bw/day) and the highest concentration was reduced to 50 mg/kg bw/day after 54 weeks. Due to high mortality, it is difficult to compare the results in this study with the results from the NTP carcinogenicity study in rats, in addition administration route by drinking water differed from the NTP (gavage treatment) study as well as rat strain used in the studies (Wistar and F344 rats). The study reported no effect on carcinogenicity.

Comments received during consultation

One comment received from a member state competent authority (MSCA) supported classification as Carc. 1B based on the significantly dose-related increased incidences of subcutaneous fibromas in male rats, adenocarcinoma in female mice (uterus/endometrium), as well as occurrence of rare tumours and dose-related occurrence of tumours in the lung (lung alveolar/bronchiolar adenoma/carcinoma) and the adrenals (pheochromocytoma) indicate carcinogenic potential for humans.

Assessment and comparison with the classification criteria

The DS proposal was to classify TMP as Carc. 1B. According to the CLP criteria a classification as Carc. 1B is based on that a substance is presumed to have carcinogenic potential for humans and that classification is largely based on animal evidence.

- The classification in Category 1A and 1B is based on strength of evidence together with additional considerations. Such evidence may be derived from:
- human studies that establish a causal relationship between human exposure to a substance and the development of cancer (known human carcinogen); or
- animal experiments for which there is sufficient (1) evidence to demonstrate animal carcinogenicity (presumed human carcinogen).

The DS included three animal studies for assessment of carcinogenicity, two with oral (gavage) administration, one in mice and one in rats (NTP, 1978) and one in rats with administration through drinking water (Bomhard *et al.,* 1997).

The NTP (1978) study predates the test OECD TG and has some deficiencies. No HCD were available for rats and in mice and the studies were scarcely reported. The control animal group consisted of a low number of animals, only 20 animals (OECD TG recommends 50) compared to 50 in the exposed groups. This together with the lack of HCD may affect detection of some effects (such as further assessment of the low incidence of rare tumours). Exposure was also only for 3 days per week without

justification, which is not in line with the carcinogenicity TGs (OECD TG 451 and 453). Although, the dose-range finding study also exposed the animals for 3 times per week. Even though there were deficiencies in the study it was considered as well conducted, assessing relevant parameters, including detail reporting of histopathology and survival that was considered adequate.

In rats a causal relationship between TMP exposure and increased tumour incidence was reported with a significant dose related increase of fibroma in subcutaneous tissue. These tumours are benign. Fibromas are occasionally detected in aging rats, but the dose related effect observed was considered unusual. The tumours are also considered relevant for humans. In addition, there were non-significant increases in lung alveolar/bronchial adenoma or carcinoma (0, 4 and 11% at 0, 50 and 100 mg/kg bw, respectively) and adrenal pheochromocytoma (5, 8 and 15% at 0, 50 and 100 mg/kg bw, respectively) in male rats. However, according to the CLP guidance, section3.6.2.3.2, it should be noted that adrenal pheochromocytoma is a tumour which has a high spontaneous tumour incidence in F344 rats. There were also non-significant incidences of several rare malignant tumours in female rats. This includes glioblastoma multiforme (1/48 high dose), myxosarcoma (2/49 high dose) and malignant reticulosis (1/50 low dose).

In mice a causal relationship between TMP exposure and increased tumour incidence was reported with a significant dose related increase in uterine adenocarcinoma. The tumours were malign. Vascular involvement and metastases were also reported, the tumours were considered more aggressive in the high dose group. The tumours are also considered relevant for humans.

The third study by Bomhard *et al.* (1997) also had deficiencies. The study induced excessive toxicity in rats and resulted in increased mortality in the highest dose group (100 mg/kg bw/day). This resulted in that doses had to be reduces after week 54, but there was no improvement of the animals condition from this dose reduction. Excessive toxicity may have affected the carcinogenic responses in this study.

Weight of evidence assessment:

Tumour type and background incidence
Increased incidence of uterine adenocarcinoma in mice. Background incidence reported to be low from HCD (no single tumour was seen in 100 control B6C3F1 mice). Increase incidence of subcutaneous fibroma in rats. No HCD on rats were available for assessment of background incidences.
Multi-site response
Significant effects: Dose related increase in tumours were reported in uterus and subcutaneous tissue. Non-significant effects: Increases in tumours in male rats were reported in lung and adrenal gland. Several rare tumours that occurred in very low incidence was also reported in female rats.
Progression of lesions to malignancy
Uterine tumours had a high degree of malignancy. This was demonstrated by vascular involvement and pulmonary metastasis. The tumours in the animals exposed to the highest dose was more aggressive when compared to the lower dose (higher frequencies of metastasis). Subcutaneous fibroma is a benign tumour type. Non-significant effects: Several of the rare tumours reported in female rats were malignant.

Reduced tumour latency
No information available. No interim sacrifice.
Whether response are in single or both sexes
The tumours were detected in female mice and male rats. The uterine adenocarcinomas that were detected in mice are sex specific and did only occur in females, the relevance of sex specificity is therefore reduced.
Whether responses are in a single species or several species
Tumours in mice and rats.
Structural similarity to a substance(s) for which there is good evidence of carcinogenicity
No data available.
Route of exposure
Oral route.
Comparison of absorption, distribution, metabolism and excretion between test animals and humans
No information on ADME species differences. The tumours are considered relevant for humans.
The possibility of a confounding effect of excessive toxicity at test doses
The two NTP studies did not report excessive toxicity. Animals in both the studies were affected by pneumonia and parasites in the GI tract. The third study from Bomhard *et al.* (1997) was excluded from the carcinogenicity assessment based on excessive toxicity.
Mode of action and its relevance for humans, such as cytotoxicity with growth stimulation, mitogenesis, immunosuppression mutagenicity
TMP has been shown to be mutagenic in a large number of publications *in vitro* and *in vivo* in both somatic tissue and germ cells as assessed under germ cell mutagenicity, and a classification as Muta. 1B is proposed. It is also noted that TMP did not produce cytotoxicity and cell death which increase the relevance of its genotoxic effects.

In summary: A classification in category 1A is largely based on human evidence, while category 1B is largely based on animal evidence.

Such evidence may be derived from:

- human studies that establish a causal relationship between human exposure to a substance and the development of cancer (known human carcinogen); or
- animal experiments for which there is sufficient (1) evidence to demonstrate animal carcinogenicity (presumed human carcinogen).

Human data were not available for TMP and classification in category 1A is not justified.

TMP induced uterine adenocarcinoma in female mice and subcutaneous fibroma in male rats. The uterine tumours were malignant and vascular involvement and pulmonary metastasis were detected. The fibromas were benign. Both tumour types were considered relevant for humans. *In vivo* mutagenicity data clearly demonstrates a mutagenic mode of action for TMP (classification as Muta. 1B is proposed). The guidance on the application of the CLP criteria states that *a single positive carcinogenicity study in one species and*

sex in combination with positive in vivo mutagenicity data would be considered to provide sufficient evidence of carcinogenicity. According to this the uterine tumours could alone be considered sufficient for classification as Carc. 1B. The subcutaneous fibromas that are benign tumours are given less weight for classification as Carc. 1B and can be considered to be supportive. Several rare tumours occurred in very low incidence in female rats, the effects were not statistically significant. Increases in tumours in male rats were reported in lung (alveolar/bronchiolar adenoma/carcinoma) and adrenals (pheochromocytoma), however, spontaneous tumours incidences of adrenal pheochromocytoma may occur in F344 rats and the data were non-significant. Without HCD it is difficult to further assess the relevance of these tumours.

In conclusion, RAC agrees with the DS that a **classification as Carc. 1B, H350 is warranted.**

10.10 Reproductive toxicity

10.10.1 Adverse effects on sexual function and fertility

Table 21: Summary table of animal 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
Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test OECD 422 Sprague Dawley Rat (Crj:CD); 13/sex/group	TMP (purity: 99.9%); 0 (vehicle), 40, 100 & 250 mg/kg bw/day; Oral (gavage), Vehicle: distilled water; Males were exposed 2 weeks prior to mating, during the 2 weeks of mating and 2 weeks after mating (42 days). Females were dosed to maximum four weeks pre-mating, during mating period, during pregnancy and up until day 3 post-delivery (approximately 63 days).	**Repeated dose toxicity:** <u>250 mg/kg bw/day:</u> *Mortality:* 12 m and 1 f died (during weeks 4 to 6 of dosing) *Body weight gain:* significantly lower in m and f compared to control animals (f in the pre-mating period). In males body weight gain was affected from the 1st week of dosing, in females only in the 2nd week of dosing *Clinical signs:* Progressive paralytic gait and decreased motor activity in males and females, starting at the 2nd week of exposure, was seen in those animals that died later on. *Food consumption:* significantly reduced in m compared to control animals *Hematology and clinical chemistry:* similar alterations in the single surviving male as seen at 100 mg/kg bw/d *Histopathology:* see Table 22 <u>100 mg/kg bw/day:</u> *Body weight gain:* significantly lower in 2 pregnant f in mid and late pregnancy (only 2 females were pregnant)	Anonymous (1994b) (Study in Japanese, abstract available in English, data tables presented with English descriptions)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
		Hematology and clinical chemistry: in males significantly decreased erythrocyte counts, haemoglobin concentration, haematocrit and A/G (albumin/globulin) ratio; increased platelet count, percentage of segmented neutrophils, cholinesterase activity, total cholesterol and calcium levels. *Organ weights:* significant increase in kidney and thymus weight in males, significant decrease in epididymis weight *Histopathology:* see Table 22 <u>40 mg/kg bw/day:</u> *Body weight gain:* significantly lower in 12 pregnant f in mid and late pregnancy (but final body weight was comparable to controls) *Organ weights:* significant increase in kidney and thymus weight in males, significant decrease in epididymis weight; significant increase in thymus weight in females (not assessed in females of the mid and top dose, only 2 females pregnant in mid dose – no parturition, no females pregnant in top dose) *Histopathology:* see Table 22 **Sexual function and fertility:** <u>250 mg/kg bw/day:</u> 2/13 mated pairs showed copulation – copulation index was 15.4% vs 100% in all other groups Number of pregnant animals and fertility index was zero. Pairing days until copulation: 5.0 (SD = 4.2) Times of vaginal estrous: not affected Testis: all males had testis atrophy (7 moderate, 6 severe) Epididymal sperm number: reduced in all males (1 moderate, 12 severe) Ovaries: 6 of 13 females had an increase in atretic follicles (very slight to moderate) <u>100 mg/kg bw/day:</u> 13/13 mated pairs showed copulation – copulation index was 100 % Number of pregnant animals: 2, none of the 2 achieved parturiency	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
		Fertility index: 15.4% vs 100% in control Pairing days until copulation: 2.2 (SD = 1.2) Times of vaginal estrous: not affected Testis: One male had testis atrophy (moderate) Epididymal sperm number: decreased in one male (moderate) <u>40 mg/kg bw/day:</u> 13/13 mated pairs showed copulation – copulation index was 100 % Fertility index: 92.3% vs 100% in control Pairing days until copulation: 3.4 (SD = 2.3) Testis: atrophy in one male (very slight) Number of pregnant animals: 12 vs 13 in control; fraction of pregnant females delivering litters with live pups was lower than in controls (10/12 vs 13/13), and the average number of life pups was markedly reduced (-43%, $p < 0.01$) (increase in intrauterine mortality). No additional effect on pup viability between days 0 and 4 of lactation. Pup weights were statistically significantly higher than in control ($p < 0.01$) from birth to terminal necropsy at lactation day 4. Times of vaginal estrous: not affected Gestation length in days: 22.2 (SD: 0.6), n = 10	

In a combined repeated dose toxicity study with the reproduction/developmental toxicity screening test (Anonymous, 1994b) Sprague Dawley Rat (Crj:CD) were exposed (oral, gavage) to 0, 40, 100 or 250 mg TMP/kg bw/day. 13 rats/sex and dose were exposed for 42 (males) or 63 (females) days. For further details see Table 21 and below.

Twelve males and one female given 250 mg/kg died during the 4th to 6th week of the dosing period. These rats showed progressive development of a paralytic gait and decreased motor activity before death. Males were more sensitive than females as indicated by increased mortality, reduced food consumption, paralytic gait and reduced motor activity which was more pronounced in males than in females in top dose animals.

Histopathology:

The study report states that on histopathological examination, major lesions were noted in males and females given 100 mg/kg bw/day or more and included nephropathy characterized by tubular and papillary alterations such as increased eosinophilic droplets in tubular epithelium, increased regeneration of tubules and papillary necrosis, atrophy of the thymus, liver and testis, increased atretic follicles in the ovary (250 mg/kg bw/day females only), and degeneration of nerve fibres in the spinal cord or the peripheral nerves (e.g. sciatic nerve). The incidence and severity of these lesions increased with dose and were greater in males

than in females. The following contains a more detailed description which is based on the raw data and which is slightly diverging from the study summary.

In males the interpretation of the results from the top dose group needs to be assessed in relation to the high mortality observed in this group (12/13). 5 animals died during the fourth week, 4 during the fifth week and 3 during the sixth week.

12/13 top dose males showed **thymus** atrophy, which was judged severe in 10 of them. No other effects were seen in the thymus of top dose animals or animals of any other group. Top dose males were also affected by **hepatocyte atrophy**. Again 12/13 animals were affected and severity ranged from very slight to moderate. It might be the case that thymus and hepatocyte atrophy were related to the observed mortality. No relevant effects were seen in thymus or liver of females.

Kidney effects were seen in all dosed males. Eosinophilic droplets in the tubular epithelium were seen in all low (slight to moderate) and mid (moderate) dose males. Only 2 animals had this effect in the top dose (1 slight and 1 moderate). Eosinophilic droplets were seen in all animals of low and mid dose (mainly moderate), but only in one male of the top dose (moderate). Regenerated tubules were seen in males of all groups (except one animal of the top dose), but incidence and severity was higher in treated groups compared to controls.. Dilation of tubule was seen in 6 top dose males (mainly of slight severity) and slight neutrophil infiltration in 2 of the top dose males.

Some incidences of kidney effects were observed in females. These consisted of regenerated tubules of very slight nature in the mid dose and slight to moderate nature in the top dose. This was supported by the observation of cell debris in tubular lumen in 1 female in mid and top dose each (very slight). In top dose females aggregation of platelets in capillary of papilla (very slight to moderate) and debris in papillary interstitium (very slight to slight) were also noted.

All top dose males had atrophy of follicle in **spleen**, in nine of them this effect was severe, while in four it was of slight nature. One male of mid and top dose each showed slight spleen congestion and for deposited pigment an increase in severity was noted in the top dose (all animals of control, low and mid dose had slight pigment deposits, 4 of the top dose had moderate pigmentation). In contrast for extramedullary hematopoesis a decrease in incidence and severity was seen with dose.

Like in males also in females the severity of deposited pigment increased with dose, while the opposite was the case for extramedullary hematopoesis. Both effects were also seen in the control females.

In the mid and top dose males there were some incidences of degenerated **skeletal muscle nerve**, with only 4 animals affected very slightly in the mid dose, but 9 animals affected slightly and 1 moderately in the top dose group. In the top dose group 11 animals were also affected by atrophy of **skeletal myofiber**, 10 of them slightly (1 very slight). Mid and top dose males also showed degeneration of **sciatic nerve fiber**. In 9 animals of the mid dose this effect was of very slight nature, while all animals of the top dose group were affected (mainly slight, 1 moderate). In addition degeneration of nerve fibres in the **fasciculus gracilis of the cervical cord** were seen in mid and top dose males (mid dose: 1 very slight, 1 slight; top dose: 3 slight) and one male of the mid dose showed very light degeneration of **nerve fibres in the dorsal funicle of the lumbar cord**.

In top dose females there were some incidences of degenerated **skeletal muscle nerve**, one animal with slight atrophy of **myofibre in skeletal muscle** and several animals of the top dose showed degeneration of the **sciatic nerve** (very slight to slight). In addition two females of the top dose with degenerated nerve fibres in the **fasciculus gracilis of the cervical cord** (1 very slight, 1 slight) as well as two animals of the top dose with degenerated **nerve fibres in the dorsal funicle of the lumbar cord** (both very slight).

Testis atrophy was seen in one male of the low and mid dose each (low dose: very slight, mid dose: moderate) and in all males of the top dose (7 moderate, 6 severe) and **epididymal sperm number** was decreased in 1 male of the mid dose (moderate) and in all males of the top dose (12 severe, 1 moderate). No information on sperm motility was presented.

6 of 13 females of the top dose had an increase in atretic follicles in the **ovaries** ranging from very slight to moderate severity.

For number of animals affected see also Table 22.

Table 22: Overview of repeated dose and reproductive parameters investigated in Sprague Dawley rats treated with TMP via gavage (OECD 422) (Anonymous, 1994b).

Males				
Parameter	**Control**	**40 mg/kg bw/day**	**100 mg/kg bw/day**	**250 mg/kg bw/day**
Group size	13	13	13	13
Mortality [c]	0	0	0	12
Terminal body weight (g)	479 ± 84.7	480.7 ± 27.2	468.8 ± 28.8	244.8
Histopathology:				
Thymus: atrophy	0/13	0/13	0/13	12/13 *
Liver: hepatocellular atrophy	0/13	0/13	0/13	12/13 *
Kidney:				
- eosinophilic droplet in tubular epithelium	1/13	13/13 *	13/13 *	2/13
- regenerated tubule	6/13	13/13 *	13/13 *	12/13 *
- eosinophilic body	5/13	13/13 *	13/13 *	1/13
Adrenal: hypertrophy of cortical cell	0/13	0/13	0/13	8/13 *
Spleen: atrophy of follicle	0/13	0/13	0/13	13/13 *
Testes: atrophy	0/13	1/13	1/13	13/13 *
Epididymis: decreased number of sperm	0/13	0/13	1/13	13/13 *
Skeletal muscle: atrophy of myofiber	0/13	0/13	0/13	11/12 *
Skeletal muscle: degeneration of nerve fiber	1/13	0/13	4/13	10/12 *
Sciatic nerve: degeration of nerve fiber	0/13	0/13	9/13 *	12/12 *
Females				
Group size / number mated	13	13	13	13
Mortality [c]	0	0	0	1
Number copulated	13	13	13	2 *
Copulation index [a]	100%	100%	100%	15.4%
Number pregnant	13	12	2 *	0
Fertility index [b]	100%	92.3%	15.4%	0
Pairing days until copulation (Mean ± SD)	2.8 ± 1.2	3.4 ± 2.3	2.2 ± 1.2	5.0 ± 4.2
Times of vaginal estrus (Mean ± SD)	1.0 ± 0.0	1.2 ± 0.4	1.0 ± 0.0	1.0 ± 0.0
Mortality	0	0	0	1
Body weight on GD 20 (g)	404.2 ± 24.4	357 ± 26.9 *	319.5	-

Histopathology:				
Thymus: atrophy	13/13	2/13	0/13	7/13
Liver: hepatocellular atrophy	0/13	0/13	0/13	3/13
Kidney:				
- eosinophilic droplet in tubular epithelium	0/13	0/13	0/13	0/13
- regenerated tubule	1/13	1/13	7/13	5/13
- aggregation of platelets in capillary of papilla	0/13	0/13	0/13	8/13 *
- debris in papillary interstitium	0/13	0/13	0/13	6/13 *
Spleen:				
- atrophy of follicle	0/13	0/13	0/13	2/13
- deposit of pigment	13/13	13/13	13/13	13/13
- extramedullary haematopoiesis	13/13	13/13	13/13	12/13
Ovary: increase in atretic follicle	-	-	0	6/13
Cervical cord: degeneration of nerve fiber	0/13	0/13	0/13	2/13
Lumbar cord: degeneration of nerve fiber	0/12	0/12	0/13	2/13
Skeletal muscle: atrophy of myofiber	0/13	0/13	0/13	1/13
Skeletal muscle: degeneration of nerve fiber	0/13	0/13	0/13	9/13 *
Sciatic nerve: degeneration of nerve fiber	0/13	0/13	0/13	11/13 *

* … Significantly different from controls ($p < 0.01$); a … Copulation index = (Number of copulated pairs / Number of mated pairs) x 100 (%); b … fertility index = (Number of pregnant animals / Number of copulated pairs) x 100 (%); c … Animals died between weeks 4 and 6.

The following tables give details on organ weights and body weights from males and females on this study.

Table 23: Organ weight values F0 males (Anonymous, 1994b).

Weight		**0 mg/kg bw/day**	**40 mg/kg bw/day**	**100 mg/kg bw/day**	**250 [a] mg/kg bw/day**
Final body weight (g)		479.7	480.7	468.8	244.8
Liver	Abs. (g)	18.4	19.6	20.0	9.91
	Rel. (%)	3.8	4.1	4.3**	4.1
Kidneys	Abs. (g)	2.97	3.49**	3.46**	2.87
	Rel. (%)	0.62	0.73**	0.74**	1.17
Thymus	Abs. (g)	337.7	409.2	458.7**	222.6
	Rel. (%)	69.8	85.6	98.4**	90.9

Epididymis	Abs. (g)	1.15	1.08	0.89**	0.50
	Rel. (%)	0.24	0.22	0.19**	0.20

[a] … n = 1; ** … Significant difference from control, p < 0.01

Table 24: Organ weight values F0 females (Anonymous, 1994b).

Weight		0 mg/kg bw/day	40 mg/kg bw/day	100 [a] mg/kg bw/day	250 [b] mg/kg bw/day
FBW (g)		307.8	317.4	-	-
Liver	Abs. (g)	13.2	13.19	-	-
	Rel. (%)	4.3	4.2	-	-
Kidneys	Abs. (g)	2.0	2.1	-	-
	Rel. (%)	0.7	0.7	-	-
Thymus	Abs. (g)	138.1	261.9**	-	-
	Rel. (%)	44.7	82.5**	-	-

[a] … only 2 females pregnant, none showed parturition; [b] … no animal pregnant; ** … Significant difference from control, p < 0.01

Table 25: Body weight (g) in F0 males and females (Anonymous, 1994b)

Body weight (g)	0 mg/kg bw/day	40 mg/kg bw/day	100 [a] mg/kg bw/day	250 [b] mg/kg bw/day
Males				
Week 0 (init. weight)	304.8 (13)	303.5 (13)	303.8 (13)	304.4 (13)
Week 1 (day 7)	351.8 (13)	352.9 (13)	343.7 (13)	333.8** (13)
Week 2 (day 14)	386.6 (13)	386.1 (13)	378.2 (13)	324.8** (13)
Week 3 (day 21)	413.2 (13)	410.1 (13)	402.2 (13)	269.6** (13)
Week 4 (day 28)	439.3 (13)	437.7 (13)	431.7 (13)	234.0** (8)
Week 5 (day 35)	461.3 (13)	460.4 (13)	452.0 (13)	216.3** (4)
Week 6 (day 42)	479.7 (13)	480.7 (13)	468.8 (13)	244.8** (1)
Females				
Week 0 (init. weight)	212.4 (13)	212.7 (13)	213.0 (13)	213.8 (13)
Week 1	229.4 (13)	228.9 (13)	228.5 (13)	226.6 (13)
Week 2	247.0 (13)	244.4 (13)	246.7 (13)	237.4 (13) [c]
Week 3 (pregn. day 0)	256.3 (13)	253.0 (12) [d]	-	-
Week 4 (pregn. day 7)	290.4 (13)	286.3 (12)	-	-
Week 5 (pregn. day 14)	327.9 (13)	316.0 (12)	-	-
Week 6 (pregn. day 20)	404.2 (13)	357.2** (12)	-	-
Week 7 (lact. day 0)	277.2 (13)	295.4* (12)	-	-
Week 8 (lact. day 7)	307.8 (13)	317.4 (12)	-	-

[a] … only 2 females pregnant, none showed parturition; [b] … no female performed copulation or no pregnancy was attained; [c] … body weight gain was affected at this time point (-39%); [d] … one female was not pregnant; * … Significant difference from control, p = < 0.05; ** … Significant difference from control, p < 0.01, Number in parenthesis is the number of animals evaluated.

Reproductive performance was clearly affected at all doses and no viable offspring was obtained in mid and top doses. Also in the low dose the fraction of females delivering litters was lower than in controls and average number of live pups per litter was reduced (-43%, $p < 0.01$). These effects were caused by a significant increase in intrauterine mortality. No effects were seen on pups during lactation. The higher pup weight seen in that period might be related to the lower number of pups in that group compared to controls.

Animals were clearly affected by general and specific toxicity, with the males being more sensitive than the females. In the top dose 12 / 13 males and 1 / 13 females died, body weight gain (in females only relevant in the 2nd pre-mating week as no females got pregnant, in males throughout the study) and food consumption was affected and in animals that died later a progressive paralytic gait and decreased motor activity was noted after the 2nd week of dosing. Males of the mid and top dose also showed hematology and clinical chemistry alterations. In males of the low and mid dose only minor effects on body weight were observed. In females of the low dose group (mid and top dose not assessed, due to absence or low number of pregnancies) body weight was lower throughout pregnancy, reaching significance during the last part of pregnancy. However, as body weight was comparable or slightly higher than in controls after parturition, it can be concluded that the effect was caused by intrauterine mortality (average number of life pups was reduced to -43%) (see Table 27). A statistically significant increase in kidney and thymus weights and decrease in epididymis weights was seen in males of the low and mid dose group (top dose not assessed), in females only the low dose group was assessed and showed a statistically significant increase in thymus weight (see Table 23 and Table 24). In males the organ weight changes in the kidney were accompanied by eosinophilic droplet in tubular epithelium, regenerated tubule and eosinophilic body in all animals of the low and mid dose (animals of the top dose not representative as 12/13 died), but such observations (though with lower incidence) were also seen in the controls (see Table 22). For effects on nerves and muscles see section on STOT RE.

No NOAEL could be derived, the LOAEL for repeated dose toxicity and reproductive and developmental toxicity was 40 mg/kg bw/day. The LOAEL repeated toxicity is mainly based on kidney toxicity seen in males and thymus effects seen in females of the low dose group. The LOAEL reproductive toxicity is based on reduced numbers of females pregnant and pregnant females with pups alive. Also the number of pups born, delivery index, number of pups alive (day 0), birth index and number of pups alive (day 4) was significantly reduced at this dose.

Overall it can be concluded that the general condition of low and mid dose animals was not severely affected and the observed adverse effects on reproductive function and fertility appear to be independent of the observed effects in males (histological & weight changes in kidneys) or females (increased thymus weight).

Table 26: Fertility parameters (Anonymous, 1994b).

Dose [mg/kg bw/day]	**0**	**40**	**100**	**250**
Number of pregnant females	13	12	2	0
Number of pregnant females with pups alive	13	10	0	
Gestation index (A)	100.0	83.3	0.0	
Gestation length (days)	21.7 ± 0.5 (13)	22.2 ± 0.6 (10)	(a)	
Number of corpora lutea	21.2 ± 3.4 (13)	2002 ± 2.0 (12)	13.5 (2)	
Number of implantation sites	16.7 ± 3.6 (13)	16.6 ± 1.6 (12)	4.0 (2)	
Implantation index (B)	80.0 ± 18.7 (13)	83.2 ± 6.3 (12)	32.5 (2)	

Number in parenthesis indicates the number of litters evaluated. (A) … Gestation index = (Number of pregnant females with pups alive / Number of pregnant females) x 100 (%), (B) … Implantation index = (Number of implantation sites / Number of corpora lutea) x 100 (%), (a) … No animals showed parturition.

Table 27: Pup parameters (Anonymous, 1994b).

Dose [mg/kg bw/day]	**0**	**40**	**100**	**250**
Day 0 of lactation:				
Number of pups born	14.0 ± 3.6 (13)	6.4 ± 4.4 ** (12)	0.0 (2)	-
Delivery index (C)	85.6 ± 17.5 (13)	38.4 ±26.5 ** (12)	0.0 (2)	-
Number of pups alive	13.4 ± 3.7 (12)	7.6 ± 3.8 ** (10)	-	-
Birth index (D)	82.3 ± 19.8 (13)	45.5 ± 22.8 ** (10)	-	-
Live birth index (E)	95.0 ± 8.1 (13)	90.0 ± 31.6 (10)	-	-
Sex ratio (F)	46.2 ± 14.2 (13)	49.2 ± 14.8 (9)	-	-
Day 4 of lactation:				
Number of pups alive	12.8 ± 4.4 (13)	8.4 ± 2.9 ** (9)	-	-
Viability index (G)	92.3 ± 21.9 (13)	100.0 ± 0.0 (9)	-	-

Number in parenthesis indicates the number of litters evaluated. (C) … Delivery index = (Number of pups born / Number of implantation sites), (D) … Birth index = (Number of pups alive on Day 0 / Number if implantation sites) x 1000 (%), (E) … Live birth index = (Number of pups alive Day 0 / Number of pups born) x 100 (%), (F) … Sex ratio = (Number of male pups alive on Day 0 / Number of pups alive on day 0) x 100 (%), (G) … Viability index = (Number of pups alive on Day 4 / Number of pups alive on Day 0) x 100 (%).

TMP has also been investigated in 1 heritable translocation assay, 10 dominant lethal mutation tests, 2 studies with focus on TMP's antifertility actions and 6 studies investigating sperm abnormality and/or motility. These studies are described in detail in the section on germ cell mutagenicity (Chapter 10.8).

The results of these studies clearly demonstrate reduced to absent fertility and intrauterine mortality upon treatment of male animals only, indicating damage to the genetic material in the male germ cells. All 10 dominant lethal assays were positive. The results showed induction of pre- and post-implantation loss and partly / full sterility (depending on dose) and as the major effects were seen in the animals mated 1 to 3 weeks after exposure to TMP, it can be concluded that the late spermatid was the main target of toxicity. This pattern of effects matches with germ cell mutagenicity. Chromosomal aberrations were induced in spermatocytes after i.p. and oral TMP administration (Machemer & Lorke, 1975, Moutschen-Dahmen et al., 1981, Katoh & Matsuda, 1985). In the heritable translocation assay (Tezuka et al. 1985) a clear increase in semi-sterile and sterile F1 males was observed and the number of translocation carriers was increased.

It is notable that the results seen in the dominant lethal assays (i.e. pre- and post-implantation loss, semi-sterility/sterility), where only males were exposed, were comparable to the effects seen in the OECD 422 study, where both males and females were exposed. The observed effects were sterility/semi-sterility and intrauterine mortality. Therefore it might be concluded that the main effect was on male animals (male germ cells).

TMP induced antifertility was investigated in male rodents and rabbits and different dosing and time schemes also demonstrated that the most sensitive stage of spermatogenesis was the post-meiotic stage (Harbison et al. 1976; Hanna & Kerr, 1981).

Next to the clear support for the involvement of mutagenicity in the induction of the observed fertility effects the contribution of other modes of action needs to be considered. In several studies sperm motility and abnormalities were investigated, but only two studies (Harbison et al., 1976; Carstensen, 1971) investigated alternative modes of action other than mutagenicity as potentially contributing factor to the observed fertility effects.

One mode of action proposed was inhibition of choline acetyltransferase (Harbison et al. 1976), which was observed in sperm and correlated with TMP induced sterility in rats, mice and rabbits. Harbison et al. (1976) concluded that the main target of TMP toxicity were epididymal spermatozoa, where it interferes with choline acetyltransferase resulting in impaired sperm motility. In contrast the vast majority of other studies identified the late spermatid as the target. But the focus of Harbison et al. (1976) was on TMP's effect on choline acetyltransferase. However, based on the observations in this study it is not possible to conclude whether the effects on enzyme activity were the cause of the observed sperm effects or whether the decrease in choline acetyltransferase was a consequence of other effects on sperm (e.g. mutagenicity). One major deficiency of the study by Harbison et al. (1976) is that the route of exposure was not reported. A direct comparison with other studies is therefore not possible.

Reduced sperm motility as well as reduced sperm numbers were also reported upon 5 days oral treatment of male rats with 500 and 600 mg/kg bw/day by Toth et al. (1992) and Suzuki et al. (1996), respectively. Takizawa et al (1998) reported reduced sperm mobility (no effects on sperm numbers) upon 10 days oral treatment of male rats with 100 mg/kg bw/day, but they also observed degenerative spermatogenic cells in testis (1/10) and degenerative sperm in epididymal ducts (3/10).

In contrast to Harbison et al. (1976), Jackson & Jones / Jones & Jackson (1968, 1969) concluded that the predominant effect of TMP was "functional" sterilising action involving spermatids from which intact motile but incompetent sperm continue to be produced.

The information from the OECD 422 study by Anonymous (1994b) only includes sperm number, no information on sperm motility and shape is presented. Sperm numbers were not affected at the low dose of 40 mg/kg bw/day, but in one male of the mid dose (100 mg/kg bw/day) and in all top dose males (12/13 severe). Despite no effects on sperm numbers at 40 mg/kg bw/day there was still a clear decrease in fertility and increase in intrauterine death at this dose. It cannot be excluded that sperm motility was affected at this dose, which could be relevant for the reduced fertility (but not for the intrauterine mortality).

Another mode of action proposed was hormonal interference, indicated by decreased testosterone levels in plasma and testis as well as decreased prostate weight upon 5 days oral treatment with 100 mg/kg bw/day (Carstensen, 1971). Atrophy of testis (1/13 males at 40 mg/kgbw/day, slight; 1 /13 males of the 100 mg/kg bw/day, moderate; all males at 250 mg/kg bw/day had testis atrophy, 7 moderate, 6 severe) and reduced epididymidal weight (statistically significant at 100 mg/kg bw/day, abs. weight: -23%, rel. weight: -21%) was described by Anonymous (1994b). No further details on potential hormonal interference are available.

In order to clarify the potential role and contribution of these other modes of action to the observed effects on male germ cells in addition to the clear involvement of mutagenicity, it is indicated to compare the doses which induced the described fertility effects with the doses inducing mutagenic or different effects (reduced sperm motility, enzyme inhibition) in the germ cells.

The following table summarises the relevant studies (studies where insufficient information on dosing and route of dosing was presented are excluded).

Table 28: Studies relevant for the assessment whether effects on sexual function and fertility as well as development were seen at doses lower than doses applied for the demonstration of germ cell mutagenicity.

Test method	Test substance, dose levels, duration of exposure	Results	Reference
In vivo Comet assay, testicular cells, Non-guideline study but comparable to OECD 489	TMP oral, gavage; Vehicle: water Male CD1 mice; 5 animals per dose Doses tested: 0 / 125 /250 / 500 mg/kg bw/day	Statistically significant effect above historical controls at 500 mg /kg bw/day Some increase also at 250 mg/kg bw/day	Hansen et al. (2014)
Chromosome aberration in spermatocytes	TMP oral: gavage	At 500 mg/kg bw/d a significant increase in the number of aberrant metaphases was observed, when gaps	Machemer & Lorke (1975)

	Male Chinese Hamster Dosing: 0 or 500 mg/kg bw/day for 2 days or 0 or 1000 mg/kg bw/day for 5 days.	were included – not significant (but still higher) when gaps were excluded. 3 translocations were observed. At 1000 mg/kg bw/d: marked mitotic inhibition.	(abstract only)
Chromosome aberration in spermatocytes	TMP Single i.p. application: 0 or 1000 mg/kg bw Male mice (Q strain), 20 animals per group,	negative	Degraeve et al. (1984)
Chromosome aberration in spermatocytes	TMP i.p. application, 3000 mg/kg bw TMP Mice (sex and strain not reported).	An increase in chromosome aberrations was scored at the paternal chromosome sets in the first cleavage metaphases after fertilization. Data were not presented.	Katoh & Matsuda (1985) (abstract only)
Dominant lethal mutation & heritable translocation assay	TMP Single i.p. application; 0, 1000 & 1500 mg/kg bw Male & female C3H mice Male mice were treated	A significant dose dependent decrease in the number of live young at birth in treated groups compared with the control was observed, indicating marked increase in the frequency of pre- and post-implantation losses. A slight but significant reduction in the number of young weaned was observed at 1500 mg/kg bw (decrease in viability). A clear increase in semi-sterile and sterile F1 males was observed and the number of translocation carriers was increased. Both effects were dose dependent. The study authors concluded that TMP is capable of inducing chromosomal breakage in mouse post- meiotic germ cells (spermatids). The breakage induced heritable translocations. The incidence of translocations observed at 1500 mg/kg bw TMP was comparable to the positive control MMS. Both are methylating agents.	Tezuka et al. (1985)
Dominant lethal mutation test	TMP single i.p. application; 1000 & 2000 mg/kg bw (Vehicle: distilled water) 8 males per dose; Fertile male and virgin female Swiss mice CF1 strain	<u>Effect on pregnancies:</u> At 2000 mg/kg bw there was a clear effect on percent pregnancies of mated females <u>Effect on the total number of fetal implants:</u> At 2000 mg/kg bw there was a clear effect on the number of fetal implants <u>Effect on early fetal death:</u> A considerable and statistically significant increase in early foetal deaths per pregnant female was seen at 1000 mg/kg bw in the first and second	Dean & Thorpe (1972)

		week of mating. At 2000 mg/kg bw there was also an increase in the first three weeks of mating, but due to the limited number of pregnant animals no statistical analysis was possible.	
Dominant lethal mutation test	TMP Swiss (ICR/Ha) mice, males single i.p. administration: Single i.p. doses of TMP diluted in distilled water: 200, 500, 850, 1000, 1250, 1500 & 2000 mg/kg bw; oral (gavage) on 5 consecutive days: Gavage dosing on 5 consecutive days: 500 & 1000 mg/kg bw/day	Incidence of pregnancy was generally reduced at the highest dose tested After i.p. administration of 200 and 1000 mg/kg bw, reductions of numbers of total implants were seen during the first 3 mating weeks. This effect was significant and dose related. This effect was however not repeated when TMP was tested over a wider range of i.p. dosages (500 – 2000), though lower numbers of implants were noted at the 2nd week of mating. After gavage exposure over 5 days to 500 or 1000 mg/kg bw/d reductions in numbers of implants in the first 3 weeks of mating was significant and related to dosage. At 1000 mg/kg bw/d implants were also reduced at the 5th week. A highly significant increase in early fetal deaths occurred during the first 3 weeks of mating for all experiments. A dose dependent increase in early fetal deaths occurring in the second week of mating at all dose groups, except the top dose via gavage, where effects were already seen in the first week of mating. (Absence of early foetal death in the latter dose group was probably due to reduced pregnancies and losses before implantation.)	Epstein et al. (1970)
Dominant lethal mutation test	TMP Male & female C3H mice single i.p. application: 0, 1000, 1250 & 2500 mg/kg bw	Frequency of dominant lethal mutations estimated from the mean litter size at birth in each test group was 13% (1000 mg/kg bw TMP), 51.2% (15000 mg/kg bw TMP) and 57.6% (MMS).	Tezuka et al. (1985)
Dominant lethal mutation test	TMP NMRI mice Single, oral (unspecif.): 0 & 1000 mg/kg bw	No effect on pre-implantation loss was observed, but marked increase in post-implantation loss in the 2nd week of mating was reported.	Lorke & Machemer (1975) [cited in US EPA, 2010]
Dominant lethal mutation test	TMP Mice (strain not specified) i.p. application 1250 mg/kg oral (gavage): 500 mg/kg bw/day for 5 days	Significant lethality occurred maximally in the 2nd week of mating (i.p.) and in the 1st and 2nd week of mating after gavage administration of TMP. Data not presented.	Farrow et al (1975) [cited in US EPA, 2010]
Dominant lethal	TMP	Significant increase in the frequency of pre-implantation and post-implantation	Moutschen-Dahmen et al.

mutation test	Mice (Q strain) i.p. application: 0 & 1000 mg/kg bw	losses 2 weeks after injection.	(1981)
Dominant lethal mutation test	TMP Rat & mice (strain not specified); 5 rats per group, 8 mice per group i.p. & oral application Rats received either 5 x 250 mg/kg bw or 5 x 100 mg/kg bw p.o. Mice received either 5 x 1000 mg/kg bw p.o. or 5 x 1000 mg/kg bw i.p. Treatment was on 5 consecutive days.	TMP affected fertility in rat and mouse - doses needed to induce these effects in mice was about 10-fold higher than in rats. Rat: TMP was equally active when administered orally or by intraperitoneal injection (only oral data presented). Male rats were completely sterile 3 and 4 weeks after exposure to the lower TMP dose, while at the higher dose complete sterility was seen from the 2nd week up to the fifth week after exposure. Mice: In mice full sterility was seen up to the second week after exposure.	Jackson & Jones (1968), Jones & Jackson (1969)

The relevant studies mainly investigated the mouse, in addition there is also one study in the rat and another in the Chinese Hamster. Studies were conducted using the oral (gavage) and i.p. routes of administration. Compared to the OECD 422 study (Anonymous, 1994b) only rather high doses were used, but only single up to maximally 5 doses were applied, whereas in Anonymous (1994b) the rats were exposed for 12 to 15 days (pre-mating period until fertilisation, this can therefore be considered the relevant exposure duration in this study). Jackson & Jones, 1968 / Jones & Jackson, 1969 was the only study using rat. Upon comparison they concluded that rats were about 10 times more sensitive than mice. At an oral dose of 100 mg/kg bw/day for 5 consecutive days male rats were completely sterile 3 and 4 weeks after exposure. At 250 mg/kg bw/day for 5 days complete sterility was seen from the 2nd week up to the fifth week after exposure.

It is plausible that the observed fertility effects are resulting solely from the genetic damage of male germ cells (including the observations at the lowest dose tested in the OECD 422 study (Anonymous, 1994b), i.e. 40 mg/kg bw/day). However, the applied doses used in the *in vivo* studies demonstrating germ cell mutagenicity (see discussion above, Table 28 as well as section on germ cell mutagenicity) cannot be directly compared to the doses used in the OECD 422 study, as only higher doses were used, with mostly single or two applications 24 hours apart, or up to a maximum of 5 daily doses and only in one study the same species (rat) was used. In contrast, fertilisation in the OECD 422 study occurred after 12 to 15 days of exposure. Nevertheless, the effects observed in the dominant lethal studies (where only males were exposed) and the OECD 422 study (where both sexes are exposed) are the same, i.e. reduced numbers of life offspring due to intrauterine mortality. And no further effects are seen in the surviving pups after birth (from day 0 to 4 of lactation). Overall the results of the OECD 422 study (Anonymous, 1994b) and the studies listed above clearly demonstrate interference with male reproduction. A range of mechanistic studies demonstrates that this effect is induced via genetic damage of male germ cells, but a contribution of other modes of action (e.g. interference with choline acetyltransferase or hormonal interference or other) cannot be fully excluded. No effects on female reproductive organs were observed, except an increase in atretic follicles in the ovaries of some top dose animals. Female germ cells were not investigated in the studies assessing TMP's mutagenic potential.

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

The only available guideline study relevant for the assessment of sexual function and fertility (OECD 422, Anonymous, 1994b) clearly demonstrates adverse effects on sexual function and fertility, i.e. no viable offspring in mid and top dose, reduced number of females delivering litters also in the low dose group and

also the average number of life pups was reduced. Based on a huge database demonstrating mutagenicity of TMP and demonstrating TMP induced germ cell mutagenicity in males as well as transmission to F1 males (dose dependent increase in semi-sterile and sterile F1 males and number of translocation carriers) it is likely that the observed effects are caused by genetic damage to germ cells.

In this respect CLP Annex I, section 3.7.1.1 needs to be considered, which states that: *for classification purposes, the known induction of genetically based heritable effects in the offspring is addressed in Germ Cell Mutagenicity, since in the present classification system it is considered more appropriate to address such effects under the separate hazard class of germ cell mutagenicity.*

However, a contribution of other modes of action than germ cell mutagenicity to the observed effects cannot be completely ruled out, as mutagenicity in germ cells was not investigated and therefore not demonstrated at the lowest dose at which effects on sexual function and fertility were seen in the OECD 422 study (Anonymous, 1994b). In this study exposure to 40 mg/kg bw/day resulted in a decrease in fertility (fertility index of 92.3% vs 100% in control), a reduction in females delivering litters with life pups: 83.3% vs 100% in controls; average number of live pups was markedly reduced (indicating intrauterine mortality: - 43%).

These effects did not occur secondary to general toxicity and demonstrate clear evidence for severe effects on sexual function and fertility.

10.10.3 Comparison with the CLP criteria

Categories	*Criteria*
CATEGORY 1	Known or presumed human reproductive toxicant Substances are classified in Category 1 for reproductive toxicity when they are known to have produced an adverse effect on sexual function and fertility in humans or when there is evidence from animal studies, possibly supplemented with other information, to provide a strong presumption that the substance has the capacity to interfere with reproduction in humans. The classification of a substance is further distinguished on the basis of whether the evidence for classification is primarily from human data (Category 1A) or from animal data (Category 1B).
Category 1A	Known human reproductive toxicant The classification of a substance in Category 1A is largely based on evidence from humans.
Category 1B	Presumed human reproductive toxicant The classification of a substance in Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on sexual function and 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.
CATEGORY 2	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 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.

No epidemiological data are available to demonstrate reproductive toxicity in humans. Therefore, classification in category 1A is not warranted.

There is only one screening study according to OECD 422 available, however, it fulfilled the study requirements and it clearly demonstrates severe effects on sexual function and fertility from the lowest dose

tested, with dose dependence. These effects are not considered to be a secondary non-specific consequence of other toxic effects.

Although there is clear evidence for TMP induced germ cell mutagenicity, a contribution of other modes of action than germ cell mutagenicity to the observed effects cannot be completely ruled out, as mutagenicity in germ cells was not investigated and therefore not demonstrated at the lowest dose at which effects on sexual function and fertility were seen in the OECD 422 study (Anonymous, 1994b).

A classification as Repro 1B, H360F is therefore proposed.

10.10.4 Adverse effects on development

There is only one guideline study available to assess developmental toxicity, a screening study according to OECD 422 by Anonymous (1994b). Although this combined study was designed to investigate reproductive capability in parental generation as well as development in F1 offspring, parameters to evaluate developmental toxicity were limited to only fetal body weights at day 0 and day 4 after birth, and autopsy findings at day 4 (further details see Chapter 10.10.1). Indications for developmental toxicity come nevertheless from increased intrauterine mortality observed in this study, however, as this effect was also seen in many studies from the open literature (see Chapter 10.8,Table 13) which observed the same effect after exposure of parental males only, it can be concluded that these effects are related to genetic damage of the parental male germ cells. In this respect also the findings from Tezuka et al. (1985) are of relevance as they found an increase in semi-sterile and sterile F1 males. Also the number of translocations in the germ cells of F1 males was increased. Both effects were dose dependent (see Chapter 10.8,Table 13).

No developmental toxicity study is available for TMP.

The mutagenic potential of TMP is well supported by a huge database consisting of numerous *in vitro* and *in vivo* studies covering somatic and germ cell mutagenic effects and a classification as Germ Cell Mutagen Category 1B is proposed. The positive results from dominant lethal assays and antifertility studies (see section on germ cell mutagenicity) are considered to be caused by damage of the genetic material of germ cells transmitted to the offspring and it is likely that also the intrauterine toxicity seen in the OECD 422 study by Anonymous (1994b) is caused by mutagenicity.

In this respect CLP Annex I, section 3.7.1.1 needs to be considered, which states that: *for classification purposes, the known induction of genetically based heritable effects in the offspring is addressed in Germ Cell Mutagenicity, since in the present classification system it is considered more appropriate to address such effects under the separate hazard class of germ cell mutagenicity.*

However, as already stated for the hazard class sexual function and fertility, mutagenicity in germ cells was not investigated and therefore not demonstrated at the lowest dose at which developmental effects (intrauterine mortality) were seen in the OECD 422 study (Anonymous, 1994b). For a detailed comparison of the doses used in the different studies see section on sexual function and fertility and table 28.

10.10.5 Comparison with the CLP criteria

Categories	*Criteria*
CATEGORY 1	Known or presumed human reproductive toxicant Substances are classified in Category 1 for reproductive toxicity when they are known to have produced an adverse effect on development in humans or when there is evidence from animal studies, possibly supplemented with other information, to provide a strong presumption that the substance has the capacity to interfere with reproduction in humans. The classification of a substance is further distinguished on the basis of whether the evidence for classification is primarily from human data (Category 1A) or from animal data (Category 1B).
Category 1A	Known human reproductive toxicant The classification of a substance in Category 1A is largely based on evidence from humans.

Category 1B	Presumed human reproductive toxicant The classification of a substance in Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect 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.
CATEGORY 2	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 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.

No epidemiological data are available to demonstrate reproductive toxicity in humans. Therefore, classification in category 1A is not warranted.

There is only one screening study according to OECD 422 available, however, it fulfilled the study requirements and it clearly demonstrated severe developmental effects, i.e. substantial increases in intrauterine mortality. These effects are not considered to be a secondary non-specific consequence of other toxic effects.

Although there is clear evidence for TMP induced germ cell mutagenicity, a contribution of other modes of action than germ cell mutagenicity to the observed effects cannot be completely ruled out, as mutagenicity in germ cells was not investigated and therefore not demonstrated at the lowest dose at which a strong increase in intrauterine mortality was seen in the OECD 422 study (Anonymous, 1994b).

A classification as Repro 1B, H360D is therefore proposed.

10.10.6 Adverse effects on or via lactation

No effects on lactation have been observed/described in the only available study (Anonymous, 1994b)

10.10.7 Conclusion on classification and labelling for reproductive toxicity

Interference of TMP with male reproduction and developmental toxicity (i.e. intrauterine mortality) is demonstrated in the available studies. Despite the clear involvement of germ cell mutagenicity in the observed effects, it is not possible to clearly rule out that also other mechanisms contribute to these effects and might be responsible for them at lower doses, where germ cell mutagenicity was not investigated. The observed effects were reported in a reliable OECD 422 study (Anonymous, 1994b) and demonstrate severe effects on sexual function and fertility as well as development. These effects are not considered to be a secondary non-specific consequence of other toxic effects.

A classification as Repro 1B, H360FD is proposed.

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

For the assessment of sexual function and fertility and developmental toxicity, one combined repeated dose toxicity study with reproductive/developmental toxicity screening test (OECD TG 422) was assessed (Anonymous, 1994b) by the DS. According to the DS the study was in Japanese, an English abstract and tables with English descriptions were available. In addition, the DS assessed sperm abnormality, sperm motility and antifertility studies as well as data from germ cell mutagenicity tests (Comet assay, chromosomal aberration, and dominant lethal tests) referred to in Table 13 and 28 in the CLH dossier.

Effects on sexual function and fertility

***Table**: Summary on the adverse effects on sexual function and fertility after TMP exposure in the Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test (OECD TG 422 (Anonymous, 1994b)) (adapted from Table 21 of the CLH report)*

Study	Doses	Results
Sprague Dawley Rat (Crj:CD). Males and femals 13/sex/group	Oral gavage administration 0, 40, 100 and 250 mg/kg bw/day Males: exposure 2 weeks prior, during and after mating (42 days) Females: max. 4 weeks prior to mating, during mating and pregnancy and until day 3 post-delivery (~63 days) Vehicle: distilled water	Copulation: Top dose showed reduced copulation with 2/13 mated pairs. Copulation index was 15.4% compared to 100% for all other groups. Number of pregnant animals were 13, 12, 2 and 0 with fertility index of 100, 92.3, 15.4 and 0% in the respective dose groups (0, 40, 100 and 250 mg/kg bw/day). None of the pregnancy in the mid dose group resulted in parturiency. In the lower dose group, the fraction of delivered litters (10/12) was lower than controls (13/13) and the average number of live pups were reduced (~43%, $p<0.01$). Pup weights were statistically significantly higher than in control ($p < 0.01$) from birth to terminal necropsy at lactation day 4. Pairing days until copulation was 3.4, 2.2 and 5.0 for the dose groups 40, 100 and 250 mg/kg bw/day Testis atrophy observed for all males in the top dose group (7 moderate and 6 severe) and one male in each of the lower (very slight) and mid (moderate) dose group. Epididymal sperm number was reduced in all males in the top dose group (1 moderate, 12 severe) and in one male in the mid dose group (moderate). Effects on repeated dose toxicity: 12 males and 1 female died in the top dose group (between week 4-6). Body weight gain was decreased significantly in both sex at the top dose group (female at exposure week 2 and males from week 1), in 2 pregnant females in the mid dose group (mid and late pregnancy) and in 12 pregnant females in the low dose group (mid and late pregnancy, final bw was

		comparable to controls). Kidney weight was increased in males at the low and mid dose groups and epididymis weight was decreased at the low and mid dose group. The top dose group only contained one male. Thymus weight was also increased in the low dose group in females (not assessed at top and mid dose). In the top and mid dose group decrease erythrocyte counts, haemoglobin concentration, haematocrit and A/G ratio was observed in males. Increased platelet count, percentage of segmented neutrophils, cholinesterase activity, and total cholesterol and calcium levels were increased in males in the mid and top dose groups. Histopathological examination reviled major lesions in both sex at mid dose and/or top dose groups (nephropathy, increased regeneration of tubules and papillary necrosis, atrophy of the thymus, liver and testis, increased atretic follicles in the ovary (at top dose), degeneration of nerve fibres in the spinal cord or the peripheral nerves).

The combined repeated dose toxicity study with reproduction/developmental toxicity screening (OECD TG 422, Anonymous 1994b) showed high lethality in males at the highest concentration (250 mg/kg bw/day). 12/13 males and 1/13 females died between week 4 and 6, these animals showed progressive development of paralytic gait and decreased motor activity before they died. Male animals being more sensitive than females at the top dose.

Histopathological analysis showed major lesions in males and females exposed to 100 mg/kg bw/day or higher. The effects included nephropathy, atrophy of thymus, liver and testis, increased atretic follicles in the ovary and degeneration of nerve fibres (spinal cord or peripheral nerves). Incidence and severity increased with dose and were greater in males. The histopathological effects should be viewed in relation to the high mortality in males at the top dose (12/13 males died at the top dose).

At the top dose in males it was reported thymus atrophy (12/13) which was severe in 10 males and hepatocyte atrophy (12/13) which was slight to moderate in severity. Effects in kidneys were seen at all doses in males. Eosinophilic droplets in tubular epithelium were reported in all low and mid dose males and in two top dose males with slight to moderate severity. Eosinophilic bodies were reported in males, 5/13 of the controls (severity not indicated), all low and mid dose males (mainly moderate) and one top dose male (moderate). Regenerated tubules were reported in 6, 13, 13 and 12 males exposed to 0, 40, 100 and 250 mg/kg bw/day, effects were more severe in exposed groups compared to controls. Some effects on kidneys were also observed in females, but effects were considered to be very slight to moderate in nature in the top dose. Atrophy of the follicle in spleen were reported for all top dose males, nine considered severe and four was slight in nature. Degeneration of the skeletal muscle nerve were reported for the mid and top dose, 4 males very slightly affected in the mid dose and 9 males slightly affected and one moderate at the top dose. Atrophy in the skeletal myofiber, degeneration of sciatic nerve fibres and nerve fibres in fasciculus gracilis of the cervical cord and nerve fibres in the dorsal funicle of the lumbar cord were also reported in the mid and top dose males, the effects were mostly slight or very slight in nature. Similar effects were also reported in females with degeneration of the sciatic nerve and skeletal muscles nerve at the top dose. Effects were very slight to slight in severity.

Testis atrophy was reported for all males at the top dose (7 moderate and 6 severe) and one male in the low (very slight) and mid dose (moderate). Epididymal sperm number was decreased in all males at the top dose (12 severe, 1 moderate) and one male at the mid dose (moderate).

For females increase in atretic follicles in the ovaries were reported in 6 females at top dose. The severity ranged from very slight to moderate.

***Table**: Fertility parameters (Anonymous 1994b) (adapted from Table 26 of the CLH report)*

Effect	**Fertility parameters at 0, 40, 100 and 250 mg/kg bw/day**
Number of pregnant females	13, 12, 2, 0
Number of pregnant females with pups alive	13, 10, 0, -
Gestation index (A)	100, 83.3, 0, -
Gestation length (days)	21.7±0.5 (13), 22.2±2.0 (10), no animal with parturition, -
Number of corpora lutea	21.2±3.4 (13), 20.2±2.0 (12), 13.5 (2), -
Number of implantation sites	16.7±3.6 (13), 16.6±1.6 (12), 4.0 (2), -
Implantation index (B)	80.0±18.7 (13), 83.2±6.3 (12), 32.5 (2), -

Number in parenthesis indicates the number of litters evaluated. (A) … Gestation index = (Number of pregnant females with pups alive / Number of pregnant females) x 100 (%), (B) … Implantation index = (Number of implantation sites / Number of corpora lutea) x 100 (%)

***Table**: Pup parameters (Anonymous 1994b) (adapted from Table 27 of the CLH report)*

Effect	**Pup parameters at 0, 40, 100 and 250 mg/kg bw/day**
Day 0 of lactation:	
Number of pups born	14.0±3.6 (13), 6.4±4.4** (12), 0 (2), -
Delivery index (C)	85.6±17.5 (13), 38.4±26.5** (12), 0 (2), -
Number of pups alive	13.4±3.7 (13), 7.6±3.8 **(10), -, -
Birth index (D)	82.3±19.8 (13), 45.5±22.8** (10), -, -
Live birth index (E)	95.0±8.1 (13), 90.0±31.6 (10), -, -
Sex ratio (F)	46.2±14.2 (13), 49.2±14.8 (9), -, -
Day 4 of lactation	
Number of pups alive	12.8±4.4 (13), 8.4±2.9** (9), -, -
Viability index (G)	92.3±21.9 (13), 100±0 (9), -, -

Number in parenthesis indicates the number of litters evaluated. (C) … Delivery index = (Number of pups born / Number of implantation sites), (D) … Birth index = (Number of pups alive on Day 0 / Number if implantation sites) x 1000 (%), (E) … Live birth index = (Number of pups alive Day 0 / Number of pups born) x 100 (%), (F) … Sex ratio = (Number of male pups alive on Day 0 / Number of pups alive on day 0) x 100 (%), (G) … Viability index = (Number of pups alive on Day 4 / Number of pups alive on Day 0) x 100 (%).

Reproductive performance was affected at all doses. There were no viable offspring at the mid and top dose group. At the lowest dose group, the average number of live pups per litter was also reduced with 43% and this effect was attributed to increased intrauterine mortality. Females in the low dose group also showed reduced bodyweight throughout the pregnancy, significant in the end of the pregnancy. After parturition bodyweight was slightly higher than controls which was concluded to be an effect of intrauterine mortality. In the study it was not possible to derive a NOAEL and the LOAEL for reproductive toxicity was based on reduced number of pregnant females and pregnant females with live pups of the low dose group (40 mg/kg bw/day). In addition, number of pups born, delivery index, number of pups alive (day 0), birth index and number of pups alive (day 4) was significantly reduced. There was an increase in pup weights between day 0 and 4 after birth, this may be related to the reduced litter sizes compared to controls. The effect on sexual function and fertility in the low- and mid-dose is considered to be a primary effect of TMP and not secondary to the general toxicity reported in males or females.

TMP has been studied in a large number of germ cell mutagenicity tests. One heritable translocation assay, 10 dominant lethal mutation tests, 2 studies focusing on antifertility action and 6 studies on sperm abnormality and/or motility. The studies are summarised in the assessment of germ cell mutagenicity (table on Effects of TMP for evaluation of mutagenicity/genotoxicity in germ cells). Briefly the studies showed reduced or absence of fertility and intrauterine mortality after treatment of male animals. All the dominant lethal tests were positive. Results showed pre- and post-implantation loss, partly of full sterility and the effects could be seen 1-3 weeks after exposure (indicating late spermatids as the target toxicity). Chromosomal aberrations were induced in spermatogonia (Machemer & Lorke, 1975; Moutschen-Dahmen *et al*., 1981; Katoh & Matsuda, 1985) and in the heritable translocation assay (Tezuka *et al.*, 1985) a clear increase in semi-sterile and sterile F1 males was observed and the number of translocation carriers was increased. Altogether the effects is in line with germ cell mutagenicity. The effects observed in the germ cell mutagenicity tests were similar as in the OECD TG 422 study (Anonymous, 1994b) inducing pre- and post-implantation loss and semi sterility/sterility and male animals seems to be affected, however, at lower doses than the germ cell mutagenicity tests.

The germ cell mutagenicity data demonstrate mutagenicity effects in germ cells as a mode of action for TMP, however, a few studies also investigated other modes of action. Harbison *et al.* (1976) studied effects of TMP exposure in rabbits, rats and mice and proposed that inhibition of Choline acetyltransferase correlated with the effects in sperm. Epididymal spermatozoa was suggested as target for TMP toxicity following interference with Choline acetyltransferase and impaired sperm motility. It should be noted that route of administration was oral or i.p., but no further information was reported in the study. Most of the other studies also identified spermatids as main target. Toth *et al.* (1992), Suzuki *et al.* (1996) and Takizawa *et al.* (1998) reported reduced sperm motility or mobility. Effects were reported after 5-10 days exposure to 100-600 mg/kg bw of TMP. Degenerative spermatogenic cells in testis (1/10) and degenerative sperm in epididymal ducts (3/10) was also reported (Takizawa *et al.,* 1998). Jackson & Jones (1968) and Jones & Jackson (1969) concluded that the predominant effect of TMP was "functional" sterilising action involving spermatids from which intact motile but incompetent sperm continue to be produced.

Carstensen (1971) suggested hormonal inference as mode of action with decreased

testosterone levels in plasma and testis and decreased prostate weight after oral treatment with 100 mg/kg bw for five days. Atrophy in the testis and reduced epididymal weight was reported by Anonymous (1994b), but no information on hormonal interference was available.

The OECD TG 422 (Anonymous, 1994b) showed no effects on sperm number at the lowest dose, one male had reduced numbers at the mid dose and 12 males had reduced numbers at the top dose, however, high mortality was reported in the top dose group. Even though sperm counts were not affected, reduced fertility and increase in intrauterine death was affected at the lowest dose. Sperm motility and mobility was not measured in the study.

Overall, most of the germ cell mutagenicity tests showed effect at higher concentrations and with single and up to 5 exposures compared to the OECD TG 422 (Anonymous, 1994b) where exposures were for a longer period of time. The OECD TG 422 was investigated in rats while most of the germ cell mutagenicity tests were investigated in mice. In the study by Jackson & Jones (1968) and Jones & Jackson (1969) both rats and mice were investigated, rats were 10 times more sensitive compared to mice. At an oral dose of 100 mg/kg bw/day for 5 consecutive days male rats were completely sterile 3 and 4 weeks after exposure. At 250 mg/kg bw/day for 5 days complete sterility was seen from the 2nd week up to the fifth week after exposure.

The results on fertility may be caused by mutagenicity in germ cells, however, the OECD TG 422 study reported fertility effects at lower concentrations with a longer exposure period than the mutagenicity tests. Similar effects were observed in the dominant lethal tests and the OECD TG 422. A lot of mechanistic data indicate effects induced by genetic damage. However other mechanisms cannot be ruled out (e.g., interference with Choline acetyltransferase or hormonal interference).

Comments received during consultation

One comment received from a member state competent authority (MSCA) supported classification as Repr. 1B FD based on the OECD TG 422 study demonstrating a clearly reduced fertility up to complete sterility at higher doses presumably caused by genotoxic effects on spermatocytes. The OECD TG 422 study also indicates developmental effects based on increased intrauterine mortality. Other modes of action than germ cell mutagenicity to the observed effects on fertility and development cannot be totally excluded.

Assessment and comparison with the classification criteria

Adverse effects on sexual function and fertility

No human data could be identified for the assessment of adverse effects on sexual function and fertility and a classification as Repr. 1A is therefore not justified. Only one reproductive toxicity study was available, a Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test, OECD TG 422 (Anonymous, 1994b). A limitation of this study was that the study was in Japanese, but it included an English summary and tables. The DS evaluated the study as adequate for classification purposes and RAC supports this assessment. In addition, the DS included studies assessing sperm abnormality, sperm motility, antifertility and germ cell mutagenicity.

Adverse effects on sexual function and fertility were reported in the OECD TG 422 study with oral (gavage) exposure to 0, 40, 100 and 250 mg/kg bw/day TMP. The study reported high mortality in males exposed to 250 mg/kg bw/day and 12 of the 13 males died between week 4 and 6. Less toxicity was reported in females, 1 female exposed to 250 mg/kg bw/day died. Repeated dose toxicity effects in females were also less severe compared to the effects in males. In the study there were no viable offspring at 100 and 250 mg/kg bw/day. There were reduced number of females delivering litters, 10/12 in the 40 mg/kg bw/day group and 2/13 in the 100 mg/kg bw/day group compared to 13/13 in the control group. The fertility index was 100%, 92.3% and 15.4% in the control, 40 mg/kg bw/day and 100 mg/kg bw/day dose groups, respectively. A significant reduction in the average number of live pups (~43%) at 40 mg/kg bw/day was reported. In summary, the effects observed in the low and mid dose group are considered to be a primary effect of TMP and not secondary to the general toxicity reported.

In several studies investigating effects on the male reproductive system in rats, rabbits and mice after oral administration at doses lower than the dominant lethal tests showed clear effects on fertility and sexual function (Toth *et al.,* 1992; Suzuki *et al.,* 1996; Takizawa *et al.,* 1998; Harbison *et al.,* 1976 and Hanna & Kerr, 1981). Effects in these studies included reduced sperm counts, altered shape and reduced movement, degenerative sperm cells, multinucleated giant germ cells, cytoplasmic vacuolisation of Sertoli cells, reversable reduced fertility and no signs of mating with virgin females. RAC notes that the copulation number was 2 and the copulation index 15.4% in the 250 mg/kg bw/day dose group in the OECD TG 422 study (Anonymous, 1994b), although a high mortality in males were also observed at this concentration (12/13 males died). However, similar effects on copulation were also observed in an antifertility study by Hanna & Kerr (1981) where there were no signs of mating after exposure to 250 mg/kg bw/day for 30 and 60 days. For further information see table below.

A number of mutagenicity studies demonstrated germ cell mutagenicity in males as well as transmission to F1 males and the effects are likely to be caused by genetic damage to the germ cells.

***Table**: Additional studies relevant for assessment of sexual function and fertility*

Animal	Exposure	Results	Reference
Sperm abnormality and motility assays			
Male long Evan Hooded rats. 20/group.	Oral administration. 0, 100, 250 and 600 mg/kg bw/day for 5 consecutive days.	Weight loss at all doses and reaching 16% at the highest dose. No effect on testis or whole epididymal weight, but cauda epididymal weight was significantly increased at the highest dose and sperm counts reduced. Highest dose also showed changes in sperm shape and movement also slightly detected at lower doses.	Toth *et al.* (1992)
Male Sprague-Dawley rats. 10/group	Oral (gavage) administration. Dosing 0 and 100 mg/kg bw/day for 28 days.	Sperm motility was reduced. Degenerative spermatogenic cells (1/10) and degenerative sperm (3/10) was observed in epididymal ducts. No other effects were seen.	Takizawa *et al.* (1998)
Male Wistar rats.	Oral administration. Dosing: 250 and 500 mg/kg bw on 5 consecutive days.	Decreased sperm motility and count at 500 mg/kg bw/day.	Suzuki *et al.* (1996) cited in US EPA (2010)

Antifertility action			
Random bred albino Sprague-Dawley rats. Random bred albino swiss-origin mice. New Zealand white rabbits. Only males exposed for all species. Human sperm samples	Administration route not reported Dosing: Mice: Subacute: 0, 750 & 1500 mg/kg bw on 5 consecutive days. Subchronic: 0 & 1500 mg/kg bw on 5 days / week for 1 month. Rats: Subchronic: 0, 100 & 600 mg/kg bw on 5 consecutive days for 1 months. Chronic: 0 & 750 mg/kg bw once weekly for 12 weeks. Rabbits: Chronic: 0, 200 & 325 mg/kg bw once weekly for 13 weeks. Choline acetyltransferase activity was measured by ^{14}C-labelled acetylcholine in sperm from the three species as well as fresh human sperm obtained by ejaculation	TMP induced reversible sterility in male mice, rats and rabbits. Induced sterility was dependent on dosage and duration of treatment. Mice: Subacute: Fecundity was reduced for both concentrations the first week (to 13% and 0% for 750 and 1500 mg/kg bw, respectively) and highest concentration the second week (29%). Subchronic: Exposure to 1500 mg/kg bw/day caused sterility the first 2 weeks. Fertility returned to normal for both subacute and chronic study. Rats: Subchronic: Reduced fecundity for both concentration the first week (29% and 0-5% for 100 and 600 mg/kg bw/day, respectively). For the highest dose the effects lasted for 4 weeks but turned normal again after 6 weeks. In the chronic study fecundity reduced to 50% the first week and 0-6% from week 3-12. Rabbit: Fecundity was reduced for both concentrations. For the lowest concentration 50% in week 3 and to 25% by week 9. The highest concentration resulted in 35% reduced fecundity in the 2nd week and sterility from week 5-13. After treatment fertility was normal within one week. A single dose of 750 mg/kg bw resulted in 34% reduction in fecundity. This was normalized the week after. *Choline acetyltransferase activity in spermatozoa.* TMP dose and time dependently reduced the Choline acetyltransferase activity in sperm from all three species. When comparing the enzyme activity in untreated spermatozoa of rat, rabbit and humans the following activity sequence was observed: rat >> rabbit ~ human. The author concluded that rapid reduction in enzyme activity interfered with sperm mobility and resulted in rapid infertility/sterility.	Harbison *et al.* (1996)
Random bred albino Sprague Dawley rats, males and females	Oral (in water) administration. Dosing: 250 mg/kg bw 5 days/week for 30 days or 6 days/week for 60 days	30 days treatment: Abnormal shape of epididymal spermatozoa, i.e. detached heads, abnormalities of head, middle piece and principal piece (not seen in controls). Testes showed impaired spermatogenesis. Round spermatids showed vacuoles and extensive extracellular spaces were observed between the germ cells and Sertoli cells. No sign of mating with female virgin rats, effects seen for both treatment groups (upon cessation of treatment each rat was placed with 2 virgin females to assess fertility). 60 days treatment: Germ cells were absent from the seminiferous tubules, which were collapsed and showed shrinkage – "Sertoli-cell-only" condition. The lumen of many seminiferous tubules was filled with processes of Sertoli cell cytoplasm. The study authors concluded that prolonged dosing of TMP results in complete loss of germ cell activity.	Hanna & Kerr (1981)

Mechanistic study on testosterone synthesis			
Male Wistar rats. 10-17/group	Oral (gavage). 0 and 100 mg/kg bw/day for 5 consecutive days	Decreased prostate weight. Decreased testosterone concentration in plasma and testes. Positive histochemical reaction for 3ß-hydroxysteroid dehydrogenase by the sperm tails. Increased number of immature Leydig cells. Increased interstitial fluid in the testicular tissue.	Carstensen (1971) cited in US EPA, 2010

Even though the genetic damage to the germ cells is the most likely mode of action resulting in reproductive toxicity, other mode of actions cannot be excluded. Harbison *et al.* (1976) reported that inhibition of choline acetyltransferase correlated with the effects observed in sperm. The hypothesis was that Choline acetyltransferase and Acetylcholinesterase regulate the intracellular acetylcholine levels in spermatozoa, which plays a role in sperm mobility. Mammalian spermatozoa contain high levels of Acetylcholinesterases, which is concentrated in the flagella. By inhibiting Choline acetyltransferase TMP exposure reduces acetylcholine levels and interferes with sperm mobility. Carstensen (1971) reported hormonal interference with decreased testosterone levels in plasma and testis and decreased prostate weight, effects that can affect fertility and sexual function. RAC consider that these modes of action also can be involved in the effects summarized in the table above as well as in the OECD TG 422 study (Anonymous *et al.*, 1994b).

The OECD TG 422 study reports similar effects as the effects seen in the germ cell mutagenicity studies, such as reduced pregnancy outcome, reduced number of fetal implants and intrauterine deaths. Although parameters assessing effects on sexual function and fertility was included in the OECD TG 422 study as in the dominant lethal assays, there were some differences in the protocols. Most of the germ cell mutagenicity tests are performed on mice, while the OECD TG 422 study was performed on rats. Only one study included rats and this study reports that rats are 10x more sensitive than mice (Jackson & Jones, 1968 and Jones & Jackson, 1969). Fertility effects were reported at the lowest dose tested in the OECD TG 422 (40 mg/kg bw/day) which is substantially lower than most of the germ cell mutagenicity tests (most of them around 1000 mg/kg bw, but ranging from 500-1000 mg/kg bw, oral; 200-2500 mg/kg bw, i.p.). Only a few studies with TMP indicate a mode of action other than genetic damage to the germ cells; however, these modes of actions can't be ruled out.

However, other studies provided for the assessment of reproductive toxicity indicate that other mode of actions can be involved in the observed effects on reproductive toxicity which are not considered to be covered by a germ cell mutagenicity classification, justifying a classification for reproductive toxicity.

Altogether RAC agrees with the DS that the effects in the OECD TG 422 at the lowest concentration tested, evident as a reduced fertility index, reduced number of litters born and reduced average number of live pups, demonstrate adverse effects on sexual function and fertility (Anonymous 1994b). Other fertility effects in rodents included no sign of mating with virgin females, effects on spermatogenesis, altered sperm shape and mobility, appearing at lower doses than the effective doses in the dominant lethal tests and after oral administration (Toth *et al*. 1992, Suzuki *et al*. 1996, Takizawa *et al*. 1998, Harbison *et al*. 1976 and Hanna & Kerr 1981). It cannot be excluded that modes of action other than germ cell mutagenicity may be involved in the reported effects on fertility and sexual function. In conclusion RAC agrees with the DS's proposal that a classification as

Repr. 1B, H360F is warranted.

Adverse effects on development

No human data was identified. No developmental toxicity study was available but an OECD TG 422 Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test was available (Anonymous, 1994b). Developmental effects were limited to assessment of pup weight at day 0 and 4 after birth and to autopsy findings at day 4. Developmental effects were reported as intrauterine mortality at 40 mg/kg bw/day. At this dose level, the number of females delivering litters was 10/12 compared to 13/13 in the control group. However, a significant reduction in the average number of live pups (~43% compared to controls) was reported. These effects were also reported in other studies (assessed under "Germ cell mutagenicity"), but at higher doses (around 1000 mg/kg bw, but ranging from 500-1000 mg/kg bw, oral; 200-2500 mg/kg bw, i.p.) and the contribution of other modes of action than germ cell mutagenicity to the observed effects cannot be completely ruled out.

In conclusion RAC agrees with the DS's proposal that a classification as Repr. 1B, H360D is warranted. RAC notes the clear involvement of germ cell mutagenicity, it is not possible to clearly rule out that also other mechanisms contribute to the increase intrauterine deaths at lower doses, where germ cell mutagenicity was not investigated.

Therefore, for TMP a **classification as Repr. 1B; H360FD is warranted.**

Adverse effects on lactation

No effects on lactation have been observed/described in the only available OECD TG 422 study (Anonymous 1994b). No classification for lactation is justified.

10.11 Specific target organ toxicity-single exposure

Not addressed in this dossier.

10.12 Specific target organ toxicity-repeated exposure

Repeated dose toxicity of TMP has been investigated in a wide range of studies covering exposure durations from 5 days to 30 months. These studies cover the oral route for mice, rats, rabbits and dogs. One study also investigated the dermal route in rabbits.

Several studies that are relevant for the assessment of the hazard class STOT RE, are described in detail in other sections of this dossier: The OECD 422 study by Anonymous (1994b) is reported in the section on reproductive toxicity (Chapter 10.10). The chronic NTP carcinogenicity study (NTP, 1978) in rat and mouse including a 7-week range findings study and a second rat carcinogenicity study by Bomhard et al. (1997) with a 30-month exposure are presented in the section on carcinogenicity (Chapter 10.9).

In addition there are several studies described in the section on germ cell mutagenicity with exposure durations ranging from 5 days to 13 weeks in rats, mice and rabbits. Only oral studies are considered, i.p. studies are excluded for this hazard class.

Additional repeated dose toxicity studies not reported in any other section of the dossier are presented in the table below and include a 7 week rat study (Oishi et al., 1982), a dog study with up to 4 months exposure

(Schaeppi et al., 1984, cited from US EPA, 2010) as well as two sub-acute rabbit studies (oral & dermal) (Deichman & Witherup, 1946).

Table 29: Summary table of repeated dose toxicity studies relevant for STOT RE (not reported previously)

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Non guideline study 9-week dietary study male JCL-Wistar rat 18 control & 6 treated animals.	TMP (purity unknown) dietary; 0 % 0.5% in diet Calculation based on a default body weight for male Wistar rats of 217g and a default food consumption rate of 0.02 kg/day (USEPA, 2010): approximate equivalent doses are 0 and 461 mg/kg-day.	<u>Investigated parameters:</u> Animals were weighed at study termination. Liver, kidneys, spleen and testes weights were determined. Prothrombin time & kaolin-activated partial thromboplastin time (kaolin-PTT) were determined. Leukocyte counts, erythrocyte counts, hemoglobin concentration, hematocrit and mean corpuscular volume were determined. Sera were analyzed for total protein, urea nitrogen, cholesterol, GOT activity, GPT activity and AIP activity and total bile acids, serum Na and K were measured. ChE activity was measured by the method of Garry and Routh. <u>Results - only parameters affected by TMP treatment are listed:</u> Body weights were significantly decreased. Absolute and relative kidney weights were significantly increased. Absolute testis weight was decreased. Erythrocyte counts and haemoglobin concentration were significantly reduced. Prothrombin time was significantly shorter and kaolin-PTT was significantly longer. GOT and GPT activities were significantly lower. LOAEL = 461 mg/kg bw/day **Effects are considered not supportive for a classification as STOT RE 2 and the single dose would be equivalent to 323 mg/kg bw/day when extrapolated to 90 days exposure.**	Oishi et al. (1982) & US EPA (2010)
Neurotoxicity study 1 – 4 months Beagle dogs: 5 adult animals: 2 males, 3 females: 1ml daily; 1 female: 2ml daily.	TMP No control group - but electro-physiological control values were available from pretest examination of the treated dogs and from previous	<u>Investigated parameters:</u> *Behaviour* *Neurological tests:* weekly examination of tonic neck reflexes, righting response, standing on a straight line, pain reflex, cornea reflex, pupil light response. *Electrodiagnostic test:* biweekly measurement of maximum nerve conduction velocity (MNCV). *Neuropathology* <u>Results:</u>	Schaeppi et al. (1984) [cited from US EPA, 2010]

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
	studies on untreated control dogs. 1ml daily (capsule), 1 females 2 ml daily (capsule) Body weight and duration adjusted dosing (USEPA, 2010): **88 & 121 mg/kg bw/day** for males exposed for 29 and 50 days; **105, 89 &** **106 mg/kg mg/day** for females exposed for 71, 101, or 121 days. 1 female received a 2ml capsule 5 days/week, for 150 days → daily exposure to **~181 mg/kg bw** (based on a ~body weight of 9.45 kg)	**At 88 - 121 mg/kg bw/day (2 males & 3 females):** All animals developed signs of neurotoxicity - impaired gait, hopping, tactile placing and landing, persistence in abnormal posture & decreased muscle tone. Severity of these effects increased progressively with dose and duration of exposure. Dogs receiving ≥ 50 treatments had prolonged distal latency for neuromuscular impulse transmission compared with pre-test values. Sensory MNCV was decreased in the dog receiving 121 doses. Peripheral MNCV was not affected in any dog receiving 1ml/day when compared with pre-treatment control values or untreated dogs (from previous studies). Neuropathology – no changes in dogs treated ≤ 71 days; dogs treated 101 & 121 days (2 females): degenerative changes in nerve fibers and demyelination of axons. **At 181 mg/kg bw/day (1 female):** Notable <u>weight loss</u> after 85 days (exceeds the upper guidance value of 100 mg/kg bw/day for a 90 day exposure for STOT RE2) <u>Inactivity</u> after 88 days (exceeds the upper guidance value of 100 mg/kg bw/day for a 90 day exposure for STOT RE2) Treatment was discontinued on days 93 – 112, resumed during days 113 – 149 and terminated following day 149 due to severe morbidity. Sacrifice on day 151 in poor general condition. <u>Neurotoxicity</u> increased with exposure duration: The following effects were observed at the indicated days and the dose of 181 mg/kg bw/day was extrapolated to a 90 day exposure (to allow comparison with the upper guidance value of 100 mg/kg bw/day for classification as STOT RE 2): - enhanced patellar reflex (day 18) → 36 mg/kg bw/day - attenuated extensor postural thrust (day 25) → 50 mg/kg bw/day - atactic gait (day 39) → 78 mg/kg bw/day - decreased muscle force and persistent abnormal posture (day 46) → 92 mg/kg bw/day - decreased muscle tonus and impaired hopping & landing (day 53) → 107 mg/kg bw/day <u>Neurophysiological testing:</u> attenuated MNCV and progressive decrease of central motor MNCV to as low as 50% of the pre-treatment value (day 150) <u>Neuropathology:</u> advanced distal degeneration of the long spinal tracts and the peripheral nerve fibers and demyelination of nerve fibers. Based on the differences between the treatment schemes (treatment was different for every single dog) and the fact that no control was included, no verified conclusion can be drawn, however, as comparable effects were seen in all dogs, the study gives an indication of the type of	

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
		neurotoxic changes that occur upon oral TMP treatment. **Neurotoxicity was seen in all treated dogs – starting from treatment with 88 mg/kg bw/day for 29 days. When extrapolated to 90 days exposure this equals to a dose of 28 mg/kg bw/day, indicating that the effects are relevant for classification as STOT RE 2.** **Most of the observed neurotoxic effects were seen under exposure conditions relevant for classification as STOT RE 2.**	
6 days oral (gavage). 3 rabbits in total; No control mentioned.	TMP Dosing: 0.3 ml TMP / kg bw/day = 359 mg/kg bw/day	*Body weight:* One animal gained 303g, while the other two animals lost 135g and 418g, respectively. All animals developed fine tremors, unsteadiness and weakness of the extremities after the second or third dose. All developed flaccid paralysis two days later (day 5). A view days after the last dose the initial flaccid paralysis was replaced by a state of spasticity.	Deichmann & Witherup (1946)
20 days dermal. First experiment: 6 rabbits; Second experiment: 3 further rabbits; No control mentioned.	TMP <u>First experiment:</u> Dosing: 2ml TMP / kg bw/day = 2394 mg/kg bw/day, 2h per day, on 20 days over a total period of 28 days. <u>Second experiment:</u> Exposure duration increased to 3h per day, up to 14 days. For the rest like the first experiment	<u>First experiment:</u> No local irritation. All animals survived. *Body weight:* Half of the animals lost weight (degree not indicated) *Other effects:* 1 rabbit developed flaccid paralysis after the last application. 2 days later this rabbit showed a hunch-backed position, fore-legs and hindlegs from knees to toes were rigidly extended, hind joint was flexed. <u>Second experiment:</u> *Mortality:* 1 rabbit died after 5 applications, another after 14 applications. *Body weight:* The two animals that had died had lost 546g and 1213g, respectively. The 3rd rabbit only lost 13g. <u>Other effects:</u> The two animals that had died showed fine tremors and unsteadiness, weakness and un-coordination of the lower extremities. After 3 to 4 applications the 3rd rabbit who had lost only 13 g, was lying with its legs extended. When touched it would assume normal sitting position or hop about, then exhibiting fine tremors and unsteadiness. Paralysis of the muscles of the extremities developed after the seventh application and was followed by spasticity.	Deichmann & Witherup (1946)

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

In a 9-week dietary study (Oishi, 1982) male JCL Wistar rats were exposed to 0 or approximately 461 mg TMP/kg bw/d. Detailed results are presented in the table below.

No treatment related histological changes were reported. The only dose tested of 461 mg/kg bw/day is the LOAEL, based on reduction in body weight and statistically significant ($p < 0.05$) hematological and biochemical changes as described above. Schaeppi et al (1984) conducted a neurotoxicity study with Beagle dogs, which were exposed 1 – 4 months to TMP (1ml or 2ml daily dose). Neurotoxicity was seen in all treated dogs – starting from treatment with 88 mg/kg bw/day for 29 days (see Table 29). When extrapolated to 90 days exposure this equals to a dose of 28 mg/kg bw/day, indicating that the effects are relevant for classification as STOT RE 2.

Table 30: Significant effects in JCL Wistar rats after treatment with TMP for 9 weeks (Oishi, 1982) (cited from US EPA, 2010).

Parameter	Control	461 mg/kg day
No of animals examined	18	6
Terminal body weight [g]	446.2 ± 10.7[a]	392.5 ± 3.9[b]
Hematology		
RBC (×106/mm3)	6.94 ± 0.072	6.63 ± 0.076[b]
Hgb (g/100 mL)	13.3 ± 0.12	12.7 ± 0.13[b]
Prothrombin time (second)	20.1 ± 0.54	17.6 ± 0.4[b]
Kaolin-PTT (second)	37.4 ± 1.2 (17)	43.2 ± 1.0[b]
Clinical chemistry		
AST (Karmen units)	79 ± 4.9 (16)	59 ± 2.9[b]
ALT (Karmen units)	32 ± 1.8 (15)	25 ± 1.4[b]
Absolute organ weights		
Kidneys (g)	3.36 ± 0.11	3.73 ± 0.051[b]
Testes (g)	3.69 ± 0.051	3.01 ± 0.19[b]
Relative organ weights		
Kidneys (g/100 g bw)	0.75 ± 0.017	0.95 ± 0.017[b]

[a] … Mean ± standard error (n, if different from group size).
[b] … Significantly different from control at $p < 0.05$.

Deichmann & Witherup (1946) investigated the effects of TMP after oral and dermal subacute exposure in rabbits. Animals developed neurotoxic effects like tremors, unsteadiness, weakness or flaccid paralysis. For further details see Table 29. The study authors concluded that these effects are comparable to those induced by other phosphoric and phosphorous acid esters. The observed deaths were considered to be a result of respiratory failure.

The table below compares the relevant effects seen in the available studies with the respective guidance value for classification as STOT RE after extrapolation to 90 days exposure.

Table 31: Extrapolation of equivalent effective dose for toxicity studies of greater or lesser duration than 90 days.

Study reference	Effective dose (mg/kg/d) & type of effect(s) observed ; Length of exposure	Extrapolated effective dose when extrapolated to 90-day exposure	Classification supported by the study
Bomhard et al. (1997) 30 month, Wistar rat, carcinogenicity study, Oral in drinking water. 0 / 1 / 10 / 100 mg/kg bw/day Top dose reduced to 50 mg/kg bw/day after week 54	100 mg/kg bw/day, reduced to 50 mg/kg bw after 54 weeks due to excessive toxicity → adjusted dose: Time-weighted average (100 mg/kg-day for 54 weeks and 50 mg/kg-day for 50 weeks): 76 mg/kg bw/day (USEPA, 2010). **100 mg/kg bw/d, week 46:** *Clinical signs:* hind limb weakness (55 males, 26 females), sunken flanks (especially in males), distended abdomen (especially in females) & poor general condition. *Increased mortality:* starting with week 39, increased to 70% after week 100, despite dose reduction. *Body weight:* lowered from the beginning, final body weight: Males: -20% Females: -15% **100 mg/kg bw/d, week 52 (12 months):** *Degeneration of peripheral nerve fiber:* Males: 8/10 vs 0/10 in control Females: 9/10 (1/10 at 10 mg/kg bw/day) vas 0/10 in control *Degeneration of spinal cord fiber:* Males: 4/10 vs 0/10 in controls Females: 4/10 vs 0/10 in controls **76 mg/kg bw/day, 24 months:** (top dose terminated earlier, other groups after 30 months) *Peripheral nerve hypercellularity:* Males: 11/47 (1/48 at 10 mg/kg bw/day) vs 0/50 in controls Females: 6/50 (2/49 at 1 mg/kg bw/day, 1/50 at 10 mg/kg bw/day) vs 0/49 in controls. *Degeneration of spinal cord fiber:* Males: 6/47 (2/49 at 1 mg/kg bw/day, 1/48 at 10 mg/kg bw/day) Females: - *Loss of spinal cord nerve fiber:*	**358 mg/kg bw/day** **404 mg/kg bw/day** **650 mg/kg bw/day**	No Clear neurotoxicity was observed, but the doses inducing the effects exceed the upper guidance value for STOT RE 2

Study reference	**Effective dose (mg/kg/d) & type of effect(s) observed ;** **Length of exposure**	**Extrapolated effective dose when extrapolated to 90-day exposure**	**Classification supported by the study**
	Males: 15/47 vs 0/50 in controls Females: 10/50 vs 0/49 in controls		
NTP (1978) Oral (gavage), 3 times per week, Fischer 344 rat, 0 / 100 / 147 / 215 / 316 / 464 / 681 / 1000 & 1470 mg/kg bw/day	**7 week exposure:** *Mortality:* 1 rat died at 464 mg/kg bw/day (All animals ≥ 681 mg/kg bw/ day died) (Distended bladder and gastrointestinal haemorrhage was seen in these rats). *Body weight:* Males gained 44% less weight, females gained 32% less weight than controls.	**253 mg/kg bw/day**	No Doses inducing the effects exceed the upper guidance value for STOT RE 2.
NTP (1978) Oral (gavage), 3 times per week, Fischer 344 rat, 0 / 50 / 100 mg/kg bw/day	**Chronic exposure (104 weeks):** *Mortality:* Survival rates were high in males and females, no death prior to week 52 on study. *Body weight:* Body weight in top dose males and females reduced by slightly more than 10%.		No relevant effect.
NTP (1978) Oral (gavage), 3 times per week, B6C3F1 mice, 0 / 147 / 215 / 316 / 464 / 681 / 1000 & 1470 & 2150 mg/kg bw/day	**7 week exposure:** *Mortality:* All males and 1/5 female mice died at 2150 mg/kg bw/day. 2 females died at 1470 mg/kg bw/day. *Body weight:* Slight depression in males ≥ 681 mg/kg bw/day. Females body weights not greatly affected.	**1170 mg/kg bw/day** **800 mg/kg bw/day** **370 mg/kg bw/day**	No Doses inducing the effects exceed the upper guidance value for STOT RE 2
NTP (1978) Oral (gavage), 3 times per week, B6C3F1 mice, 0 / 250 / 500 mg/kg bw/day	**Chronic exposure (104 weeks):** *Mortality:* Survival was high in males and females. *Body weight:* Slight decrease in female body weight (~10%), male body weight unaffected.		No relevant effect.
Anonymous (1994b) OECD 422, Oral (gavage), Rat, 0 / 40 / 100 / 250 mg/kg bw/day	**Exposure was 42 days in males and 63 days in females:** **At 250 mg/kg bw/day:** *Mortality:* 12/13 males died, 1/13 female died at 250 mg/kg bw/day; these animals showed progressive paralytic gait decreased motor	**Males: 117 mg/kg bw/day; Females: 175 mg/kg bw/day**	No. Dose inducing the effects exceeds the upper guidance value for STOT RE 2.

Study reference	Effective dose (mg/kg/d) & type of effect(s) observed ; Length of exposure	Extrapolated effective dose when extrapolated to 90-day exposure	Classification supported by the study
	activity *Body weight:* Males: Considerable decrease at the end of treatment (~-50%) Females: No effect. *Significant organ weight change:*		
	Males, relative weights of: Liver at 100 mg/kg bw/day +13% Kidney at 40 mg/kg bw/day +18% Thymus at 100 mg/kg bw/day +41%	**47 mg/kg bw/day** **19 mg/kg bw/day** **47 mg/kg bw/day**	Only kidney weight changes accompanied by histopathological changes.
	Females, relative weights of: Thymus at 40 mg/kg bw/day +85%	**28 mg/kg bw/day**	
	Relevant histological changes: Males: Kidney: eosinophilic droplets & regenerated tubule (very slight to moderate) ≥ 40 mg/kg bw/day;	**19 mg/kg bw/day**	Effects in male kidneys considered relevant
	Dilation of tubules (6/13) & slight neutrophil infiltration (2/13) at 250 mg/kg bw/day	**117 mg/kg bw/day**	Severity is borderline at doses relevant for classification as STOT RE 2.
	Degeneration of skeletal muscle nerve ≥ 100 mg/kg bw/day (very slight 4/13 at that dose)	**47 mg/kg bw/day**	Effects in the mid dose considered severe and supportive for STOT RE 2.
	Atrophy of skeletal myofiber, 11/13 at 250 mg/kg bw/day (1 very slight, 10 slight)	**117 mg/kg bw/day**	
	Degeneration of sciatic nerve fiber ≥ 100 mg/kg bw/day, (9/13, very slight)	**47 mg/kg bw/day**	
	Degeneration of nerve fibers in the fasciculus gracilis of the cervical cord ≥ 100 mg/kg bw/day (2/13, 1 very slight, 1 slight)	**47 mg/kg bw/day**	
	Females: Kidney: regenerated tubules (very slight) & cell debris in tubular lumen, (very slight, 1/13) % ≥ 100 mg/kg bw/day	**70 mg/kg bw/day**	Effects in female kidneys considered borderline relevant.
	Aggregation of platelets in capillary of papilla (very slight ot moderate) at 250 mg/kg bw/day	**175 mg/kg bw/day**	
	Several females of the top dose had degenerative changes in skeletal muscle nerve, myofiber in skeletal muscle, sciatic nerve, cervical or lumbar cord at 250 mg/kg bw/day.	**175 mg/kg bw/day**	No, dose inducing the effects exceeds the upper guidance value for STOT RE 2.

Study reference	**Effective dose (mg/kg/d) & type of effect(s) observed ;** **Length of exposure**	**Extrapolated effective dose when extrapolated to 90-day exposure**	**Classification supported by the study**
Oishi et al. (1982) 9 week dietary study Male JCL-Wistar rat	-	-	Results not relevant for classification as STOT RE. See description in Table .
Schaeppi et al. (1984) Beagle dog Each dog treated according to different scheme	Neurotoxicity was seen in all treated dogs – starting from treatment with 88 mg/kg bw/day for 29 days. When extrapolated to 90 days exposure this equals to a dose of 28 mg/kg bw/day, indicating that the effects are relevant for classification as STOT RE 2. Most of the observed neurotoxic effects were seen under exposure conditions relevant for classification as STOT RE 2.	28 mg/kg bw/day	Result considered supportive for classification as STOT RE 2 based on neurotoxic effects, study has drawbacks, see Table 29.
Deichmann & Witherup (1946) 6 days oral (gavage) 3 rabbits No control	At 356 mg/kg bw/day all 3 rabbits developed fine tremor, unsteadiness and weakness of extremities after 2 to 3 days. All animals developed flaccid paralysis after 5 days. A view days after the last dose the initial flaccid paralysis was replaced by a state of spacticity.	**36 mg/kg bw/day**	Clear neurotoxicity was observed. First signs of this effect were seen after 2 to 3 days, and could therefore be regarded as acute toxicity, not relevant for STOT RE. But flaccid paralysis and spasticity developed upon further dosing, which could be a repeated dose effect.
Toth et al. (1992) Sperm abnormality assay Male Long-Evans hooded rats (20 / group) Oral (gavage) 0 / 100 / 250 & 600 mg/kg bw/day for 5 days	At 100 & 250 mg/kg bw/day – significant weight loss. At 600 mg/kg bw/day – extreme weight loss (-66g) At 600 mg/kg bw/day – marked neuro-muscular deficits (no further details).	**6 & 14 mg/kg bw/day** **33 mg/kg bw/day**	Relevant effects for classification as STOT RE 1 (low dose) & 2 (mid and top dose), but missing details for neuro-muscular effects. The severe effect on body weight is considered supportive for STOT RE classification.
Cho & Park (1994) Sperm abnormality assay Random breed albino Sprague-Dawley descendents Oral (gavage) 0 / 400 / 500 / 750 / 1000 & 1500 mg/kg bw/day for 5 days	Mortality rates: 0% / 10% / 90% / 100% / 100% / 100% at 0 / 400 / 500 / 750 / 1000 & 1500 mg/kg bw/day. Rats that died were anuric and anorexic prior to death. No remarkable finding except severely distended bladder with multifocal ulceration, loss of urothelium and marked thinning and atrophy of the muscle proper.	**Considerable effects from the lowest dose tested:** **22 mg/kg bw/day**	Effects supportive for classification as STOT RE 2.

Study reference	**Effective dose (mg/kg/d) & type of effect(s) observed ;** **Length of exposure**	**Extrapolated effective dose when extrapolated to 90-day exposure**	**Classification supported by the study**
Takizawa et al. (1998) Oral (gavage) Male Sprague-Dawley rats, 0 / 100 mg/kg bw/day for 28 days.	No significant changes on body weight, food consumption or organ weights.	**31 mg/kg bw/day**	No effects were seen. Not supportive for classification as STOT RE.
Epstein et al. (1970) Oral (gavage) 0 / 500 & 1000 mg/kg bw/day for 5 days.	TMP was not toxic at the tested doses.	**55 mg/kg bw/day**	No effects were seen. Not supportive for classification as STOT RE.
Deichmann & Witherup (1946) dermal First experiment: 2h for 20 days, 6 rabbits Second experiment: 3h for 14 days; 3 rabbits; No control	<u>First experiment:</u> 6 animals were treated with 2394 mg/kg bw/day, all animals survived, half of the animals lost weight (degree not indicated); 1/6 rabbits developed flaccid paralysis after the last treatment, 2 days later this rabbit showed a hunch-backed position, fore-legs and hindlegs from knees to toes were rigidly extended, hip joint was flexed. <u>Second experiment:</u> 3 animals were treated with 2394 mg/kg bw/day, 2/3 died, 1 after 5 days, the other after 14 days. Body weight was severely reduced in the 2 animals that died, only slight in the third animal. The 2 animals that died showed fine tremors and unsteadiness, weakness and incoordination. Also the 3rd rabbit showed increasing severity of neurotoxic effects from the 3rd to the 4th application onwards. The observed deaths were described to be the result of respiratory depression.	**532 mg/kg bw/day** **130 mg/kg bw/day** (1st death after 5 days, GV for STOT RE 2 dermal is 200 mg/kg bw/day) **365 mg/kg bw/day** (2nd death occurred after 14 days, also the 3rd animal was treated for 14 days)	Effects seen above the relevant guidance value for STOT RE 2 (dermal, 200 mg/kg bw/day). This single death occurred at a dose relevant for classification – alone not supportive for classification, but it fits together with the observations in the other studies. Effects in the other animals above the guidance value.

The most prominent effects observed across several studies and species upon repeated exposure were neurotoxicity, kidney toxicity and mortality. Neurotoxicity and mortality was also seen in the single study in rabbits with dermal application (Deichmann & Witherup, 1946).

<u>Neurotoxicity:</u>

Neurotoxicity has been observed in rats, rabbits and dogs and consisted of behavioural effects, clinical signs, electrophysiological changes and histopathological changes (NTP, 1978, Bomhard et al., 1997, Anonymous, 1994b, Schaeppi et al., 1984, Deichman & Witherup, 1946, Toth et al., 1992).

Most information is available for the rat. In the 30 months carcinogenicity study (Bomhard, 1997), degenerative effects on peripheral nerve fiber and spinal cord fiber were seen in males and females of the top dose of 100 mg/kg bw/day after one year and this was accompanied by hind limb weakness in week 46 (55 males and 26 females). The dose was reduced to 50 mg/kg bw/day in the second year. These effects were

clearly adverse, however, as they were seen at doses exceeding the upper guidance value for STOT RE 2 they do not support classification. No such effects were seen in the other chronic carcinogenicity study in rats (NTP, 1978), which used much higher doses, but in contrast to Bomhard et al. (1997) who administered TMP via drinking water, NTP (1978) applied the doses via gavage.

Also in the OECD 422 study (Anonymous, 1994b) similar observations were made as by Bomhard et al (1997), though doses were applied via gavage. In the top dose progressive paralytic gait was observed in males and females and in mid and top dose degenerative effects were described in skeletal muscle nerve, skeletal myofiber, sciatic nerve and the cervical cord. These effects were seen in males and females, in males they occurred at doses relevant for the classification as STOT RE 2 (see Table 29).

No neurotoxicity was seen in a 9-week study in rat applying dietary exposure to 461 mg/kg bw/day (Oishi et al., 1982), but Toth et al. (1992) described marked neuromuscular deficits upon 5 days gavage treatment of male rats with 600 mg/kg bw/day (no further details were presented). When extrapolated to 90 day exposure the dose would be relevant for classification as STOT RE 2.

Schaeppi et al. (1984) investigated the effects of orally (capsule) applied TMP in dogs (see Table 29). Although the study investigated only 6 dogs (2 males, 4 females) and the treatment schemes (dose and duration) were different for every single dog, a detailed investigation of neurotoxic effects was carried out. The severity of effects increased with dose and exposure duration and as similar effects were seen in all 6 dogs this study is considered useful for the assessment of TMP's neurotoxicity. All animals developed signs of neurotoxicity including impaired gait, hopping, tactile placing and landing, persistent abnormal posture & decreased muscle tone. Neuropathological changes were seen in all dogs treated with ≥ 71 mg/kg bw/day at the end of treatment. Prolonged distal latency for neuromuscular impulse transmission compared with pre-test values was increased in 4 of the 6 dogs, in 3 of them at doses relevant for STOT RE 2 (i.e. male dosed with 121 mg/kg bw/day for 50 days, female dosed with 105 mg/kg bw/day for 71 days and female dosed with 98 mg/kg bw/day for 101 days). In one female treated with 181 mg/kg bw/day the following effects are considered supportive for a classification as STOT RE 2 (see Table): enhanced patellar reflex (day 18), attenuated extensor postural thrust (day 25), atactic gait (day 39), decreased muscle force and persistent abnormal posture (day 46), decreased muscle tonus and impaired hopping & landing (day 53). Overall, this is just a single study in dogs, which only used 6 animals of both sexes. However, the similarity of effects seen across the dogs and the detailed analysis of these effects, which showed a dose and time dependent pattern including at doses relevant for STOT RE 2 is considered supportive for a classification.

In rabbits neurotoxicity was seen upon oral as well as dermal treatment (Deichmann & Witherup, 1946). Again rather low numbers of animals were included and no control group was part of these studies. In the oral study a dose of 359 mg/kg bw/day was applied on 6 consecutive days. All 3 rabbits developed fine tremors, unsteadiness and weakness of the extremities after the second or third dose and all animals developed flaccid paralysis after 5 days. A few days after the last dose (on day 6) the initial flaccid paralysis was replaced by a state of spasticity. As the onset of effects was already after 2 to 3 days it can be discussed whether the observed neurotoxicity can be regarded as a repeated dose effect or as acute effect. Similar effects were seen in rabbits after single oral dose (included in the same study of Deichmann & Witherup, 1946, see also section on acute toxicity). General weakness, mild hyperirritability and fine tremors as well as gradually decreasing rate and amplitude of respiratory movements were observed. However, in the present repeated dose study, different effects, i.e. flaccid paralysis and spasticity developed upon further dosing. These effects were not described in the acute toxicity study, which also applied clearly higher doses and the LD_{50} was 1257 mg/kg bw/day.

The dermal study (Deichmann & Witherup, 1946) consisted of 2 parts. In the first experiment, 6 rabbits were dermally exposed to 2394 mg/kg bw/day for 2h per day for 20 days. One animal developed flaccid paralysis after the last application. Two days later this rabbit showed a hunch-backed position, fore-legs and hind-legs from knees to toes were rigidly extended and hip-joints were flexed. The finding is considered relevant, but occurred at a dose exceeding the cut-off for classification as STOT RE. In a second experiment in 3 rabbits exposure duration was increased to 3h per day. Two animals died after 5 and after 14 doses and neurotoxicity preceded death. In these two animals fine tremor and unsteadiness were observed as well as weakness and un-coordination of the lower extremities. After 3 to 4 applications the 3rd rabbit showed unusual posture (legs extended) and showed fine tremors and unsteadiness. Paralysis of the muscles of the extremities developed

after the seventh application and was followed by spasticity. These effects are considered to be of chronic nature, but were induced at a dose exceeding the cut-off for classification as STOT RE 2, except for the one rabbit that died after 5 days. The observed death could be a consequence of the observed neurotoxicity.

No neurotoxicity was seen in mice.

Mode of action considerations:

Conflicting results are available regarding TMP's potential to inhibit acetylcholine esterase (AChE) activity. Some older studies concluded that TMP did not have such activity (e.g. Jackson & Jones, 1968, Vandekar, 1957) and Oishi et al. (1982) did not report changes in cholinesterase activity. In contrast, the study summary from the OECD 422 study (Anonymous, 1994b) describes that AChE was reduced, the measured value for this parameter is, however, missing from the tabular presentation of the study results. Witherup & Deichmann (1946) concluded that the observations upon repeated TMP exposure resembled those made with other phosphoric and phosphorous acid esters.

Interference with TMP was demonstrated for a different enzyme, i.e. choline acetyltransferase, in sperm (Harbison et al., 1976)

TMP belongs to the chemical group of organophosphorous substances, members of which are known to inhibit AChE activity and related neurotoxicity is well described for this group of compounds. However, this mode of action is insufficiently investigated for TMP and no conclusion on whether TMP inhibits AChE, or not, can be drawn.

Kidney toxicity:

Kidney toxicity was seen in the OECD 422 study (Anonymous, 1994b) and consisted of an increase in relative organ weight in males by about 20% in low and mid dose (only 1 surviving male in the top dose) accompanied by histopathological changes, i.e. eosinophilic droplets and regenerated tubules (very slight to moderate) from the low dose and dilation of tubules (6/13) and slight neutrophil infiltration (2/13) in the top dose. In the females no change in kidney weight was registered, but histological changes were reported, which were, however, very slight at the mid dose, which would be relevant for classification when compared with the guidance value and were not seen at the low dose (see Table 29).

In the 9 week study in male rats by Oishi et al. (1982) increased relative and absolute kidney weight was reported, also in the carcinogenicity study by Bomhard et al. (1997) relative kidney weight was increased by 23% in top dose males and females. No other kidney changes were seen or reported in these studies. Bomhard et al. (1997) judged the increase in relative kidney weight a consequence of the decreased body weight at that dose. In two further rat studies, the 7-week range findings study (NTP, 1978) and the 5 day rat study (Cho & Park, 1994) it was observed that in animals that had died destended bladder was described. Cho & Park (1994) described that these animals were anuric before death and that the severely distended bladders had multifocal ulcerations, loss of urothelium and atrophy of the muscle proper.

Mortality:

In several repeated dose toxicity studies considerable increase in mortality was observed at moderate doses that could be relevant for classification as STOT RE.

In the rat chronic carcinogenicity study by Bomhard et al. (1997) mortality was significantly increased in the top dose after one year, so they reduced the dose from 100 to 50 mg/kg bw/day in the second year; but the dose exceeds the cut-off for classification as STOT RE 2. Increased mortality was also observed in the 7 weeks and chronic studies of NTP (1978), but also at doses too high for STOT RE classification. The same is the case for the OECD 422 study by Anonymous (1994b).

Significant weight loss was seen in the rat study by Toth et al. (1992) reaching -16% in the top dose of 600 mg/kg bw/day dosed for 6 days. This dose is supportive for STOT RE 2 when extrapolated to 90 day exposure. Also at the two lower doses body weight was reduced at -3% and -4% at low and mid dose respectively (significant at the mid dose) No death was observed in this study, but in the top dose considerable neurotoxicity was observed.

Cho & Park (1994) also in rats dosed orally for 5 days and considerable mortality was observed at all doses: at 400 / 500 / 750 / 1000 & 1500 mortality of 10% / 90% / 100% / 100% & 100% respectively was reported.

The animals of the 3 top doses all died within 3 days upon dosing. At 500 mg/kg bw/day all animals that died were dead within 7 days, while the two animals from the low dose died within 5 days. It is assumed that at the two high doses death is related to acute toxicity. Also the derived range of oral LD_{50} values is close to the doses applied in this study. In a poorly reported oral acute toxicity study an LD_{50} values of 840 mg/kg bw was obtained (NIH national library, cited by DFG, 1983), which is also close to the dose of 750 mg/kg bw. The low dose can be regarded as an LD_{10} and the observed deaths might also be related to acute toxicity of TMP.

10.12.2 Comparison with the CLP criteria

Categories	Criteria
Category 1	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; 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 dose/concentration values are provided below (see 3.9.2.9), to be used as part of a weight-of- evidence evaluation.
Category 2	Substances that, on the basis of evidence from studies in experimental animals can be presumed to have the potential to be harmful to human health following repeated exposure. Substances are classified in category 2 for target organ toxicity (repeat 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 below (see 3.9.2.9) in order to help in classification. In exceptional cases human evidence can also be used to place a substance in Category 2 (see 3.9.2.6).

Neurotoxicity:

The available data clearly describe neurotoxicity that occurred with dose and time dependence in rats, rabbits and dogs. The observed effects occurred in many cases at doses relevant for classification as STOT RE 2 and were of severe nature, including neuronal dysfunction resulting in severe impairment of the animals (paralysis, tremor, hunched-posture, un-coordination), supported by histopathological and electrophysiological evidence. It is not clear why the results in the different rat studies differed considerably. While in some studies effects were induced at doses relevant for STOT RE 2, in other studies no effects were induced at comparable or higher doses. Overall, the results clearly indicate that TMP can induce similar neurotoxicity in 3 species via the oral and dermal route, thereby supporting a classification as STOT RE 2, H373 targeting the nervous system.

Kidney toxicity:

Overall, it can be concluded that clear kidney toxicity was only seen in a single study and only in males at sufficient severity at a dose relevant for classification. The finding of severely distended bladder, accompanied by some histological changes, was seen in animals that died during the study, but is of minor relevance for the classification for nephrotoxicity. In conclusion, no classification as STOT RE for kidney effects is considered warranted.

Mortality:

Overall it can be concluded that the increased mortality seen in some studies at doses relevant for STOT RE are all studies of rather short duration (5 days). Though extrapolation to 90 day exposure results in values

that might be relevant for classification as STOT RE 2 it can be questioned whether it is appropriate to apply the Haber's Law in these cases. It is assumed that the observed deaths seen after only a few days and at rather high doses are related to acute toxicity and are covered under the classification for acute toxicity via the oral route.

10.12.3 Conclusion on classification and labelling for STOT RE

Based on the data presented above and the CLP criteria, a classification as STOT RE 2; H373 (nervous system) is proposed.

Route of exposure:

The majority of the studies available are for the oral route. The available toxicokinetic data do not allow to exclude the relevance of other routes of exposure. The acute toxicity studies via the dermal route resulted in mortality (though at doses exceeding the cut-off values for classification for acute dermal toxicity), supporting the relevance of the dermal exposure route. In addition, a single study investigated the effects of TMP in rabbits after repeated dermal application. Clear effects on body weight and neurotoxicity were observed, which were comparable to the effects seen after oral exposure. No inhalation toxicity studies are available, and this route does not appear to be of relevance for this substance (Registration Dossier - ECHA (europa.eu)), but inhalation toxicity upon repeated exposure cannot be excluded either. No route can therefore be excluded. Specification of a single route is therefore not justified.

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

Summary of the Dossier Submitter's proposal

The DS proposed a harmonised classification and labelling for STOT RE 2; H373 (nervous system).

Many studies on repeated dose toxicity were assessed by the DS (summarised below). Several species were investigated (mice, rats, rabbits and dogs) with an exposure duration ranging from 5 days to 30 months. Most of the studies had oral administration except one study that investigated the dermal route. Most of the studies were also assessed under other hazard classes than STOT RE and for these studies only the findings relevant for STOT RE classification will be reported here. Studies with i.p. administration were not considered.

***Table**: Studies assessed for effects after repeated exposure to TMP (adapted from Table 29 of the CLH report)*

Study	Doses	Results	Corresponding guidance value (for STOR RE 2)**	Extrapolated effective dose to 90-day exposure* (study dose)
JCL-wistar rats, male, 6 treated and 18 control	Dietary: 0-0.5% in diet.	Effects observed at doses exceeding the guidance	STOT RE 2 > 14 ≤ 140 mg/kg	323 mg/kg bw/day

animals. 9 weeks dietary study Non guideline study. Oishi *et al.* (1982) and US EPA (2010)	Concentration (calculated) 0 and 461 mg/kg day.	value for STOT RE. Body weight significantly decreased. Kidney weight significantly decreased (absolute and relative). Erythrocyte counts and haemoglobin concentration significantly reduced. Prothrombin time significantly shorter and kaolin-PTT longer. GOT and GPT activities were lower.	bw/day	(461 mg/kg bw/day)
Beagle dogs, 6 animals exposed, no controls but electrophysiological control values available from pretest examination of treated dogs and previous studies on untreated control dogs. Oral administration (1-2 mL capsules) daily. Duration 1-4 months. Neurotoxicity study Schaeppi *et al.* (1984), cited in US EPA (2010)	2 males and 3 females, oral exposure to 1 mL capsules daily. 1 female exposed to 2 mL daily. Duration was from 29-121 days (and up to 150 days). Concentrations (calculated): 1 mL capsule: 88 and 121 mg/kg bw/day for males exposed for 29 and 50 days. 105, 89 and 106 mg/kg bw/day for females exposed for 71, 101 or 121 days. 2 mL capsule: daily exposure estimated as ~181 mg/kg bw/day. Exposure was 5 days a week for 150 days.	**Neurotoxicity** observed in all dogs from lowest concentration. 88-121 mg/kg bw/day (2 males and 3 females): impaired gait, hopping, tactile placing and landing, persistence in abnormal posture and decreased muscle tone. Severity increased with dose and duration. Dogs receiving 50 or more treatments had prolonged distal latency for neuromuscular impulse transmission compared with pre-test values. Sensory maximum nerve conduction velocity (MNCV) decreased in one dog receiving 121 mg/kg bw/day. Dogs treated for 89 mg/kg bw/day for 101 and 106 mg/kg bw/day for 121 days (2 females), degenerative changes in the nerve fibers and demyelination of axons were observed. Effects on weight loss, inactivity and neurotoxicity was reported in the dog exposed to ~181 mg/kg bw/day. The concentration exceeds the upper guidance value for STOT RE 2. Study considerations:	STOT RE 2: 88 mg/kg bw/day for 29 days = GV > 3 ≤ 310 mg/kg bw/day 121 mg/kg bw/day for 50 days= GV > 18 ≤ 180 mg/kg bw/day 105 mg/kg bw/day for 71 days= GV >13 ≤ 130 89 mg/kg bw/day for 101 days= GV > 9 ≤ 90 mg/kg bw/day 106 mg/kg bw/day for 121 days= GV > 8 ≤ 75 mg/kg bw/day 181 mg/kg bw/day for 150 days= GV >6 ≤ 60 mg/kg bw/day	**28 – 67 mg/kg bw/day** (88 – 121 mg/kg bw/day) Note limitations in the study. **100** – 143 mg/kg bw/day (89-106 mg/kg bw/day)

		treatment different for every dog and no control animals in the study. Similar effects were observed in the dogs and the study give an indication of neurotoxic effects upon oral exposure to TMP		
Rabbits, 3-6 treated animals. No description of controls. Oral (gavage) exposure. 3 treated animals. Duration 6 days. Dermal exposure. 3-6 treated animals (2 experiments). Duration: 2-3 hrs a day for 20 days in a period of 28 days. Deichmann & Witherup (1946)	Oral: 0.3 mL TMP/kg bw/day (=356 mg/kg bw/day) Dermal: Experiment 1: 2 mL/kg bw/day (=2394 mg/kg bw/day) 2 hrs a day for 20 days during period of 28 days. Experiment 2: same condition as exp. 1 but with 3 hrs exposure for 14 of the days.	Oral: One animal gained 303 g, two animals lost 135 g and 418 g. **Neurotoxicity**: Fine tremors, unsteadiness and weakness of the extremities was observed in all animals after the second or third dose. Flaccid paralysis observed two days later (day 5) in all animals, this was replaced by a state of spasticity after a few days. Dermal: Experiment 1 (6 animals): 3 animals lost weight. Flaccid paralysis in one rabbit after last application. Hunch-backed position, fore-legs and hindlegs from knees to toes were rigidly extended and flexed hind joint was observed 2 days later in the same animal. Experiment 2 (3 animals): 2 rabbits died after 5 and 14 applications. The two rabbits that died had reduced body weight with 546 g and 1213 g, respectively. The last rabbit lost 13 g. **Neurotoxicity**: The animals that died had fine tremors and unsteadiness, weakness and un-coordination of the lower extremities. The last rabbit was lying with its legs extended after 3-4 applications. Upon touch the rabbit assumed normal position or hop, then exhibiting fine tremors and unsteadiness. Paralysis of the muscles of extremities after 7th application, this	Oral: STOT RE 2 > 100 ≤ 1000 mg/kg bw/day (duration less than 9 days)*** Dermal: STOT RE 2 > 90 ≤ 900 mg/kg bw/day for 20 days STOT RE 2 > 130 ≤ 1300 mg/kg bw/day for 14 days	Oral: **24 mg/kg bw/day** **(356 mg/kg bw/day)** Dermal: experiment 1; 532 mg/kg bw/day (2394 mg/kg bw/day) Experiment 2: 372 mg/kg bw /day. Mortality and neurotoxicity: One rabbit died after 5 days treatment, the estimated concentration was **130 mg/kg bw/day** (2394 mg/kg bw/day)

		was followed by spasticity.		
Wistar rats. Oral exposure in drinking water. 60/sex/group Duration 30 months Bomhard *et al.* (1997) Carcinogenicity study	0, 1, 10, 100 mg/kg bw/day 100 mg/kg bw/day for 54 weeks, reduced to 50 mg/kg bw/day for 50 weeks.	**Neurotoxicity** observed, but the doses exceed the guidance value for STOT RE 2. **Mortality**: Increase in mortality starting between week 39 and 52. Doses were reduced from 100 mg/kg bw/day at week 54 but mortality increased further to 70%. Kidney: Increase in relative kidney weight were reported for the highest dose (+23% in males and +23% in females). **Neurotoxicity**: 100 mg/kg bw/day: In week 46 hind limb weakness (55 males, 26 females). In week 52 degeneration of peripheral nerve fiber were observed in 8 males and 9 females and degeneration of spinal cord fiber in 4 males and 4 females. No degeneration in controls. 76 mg/kg bw/day: at 24 months peripheral nerve hypercellularity was observed in 11 males and 6 females and degeneration of spinal cord fiber in 6 males. Loss of spinal cord nerve fiber was observed in 15 males and 10 females. No effects in controls.	STOT RE 2 > 1 ≤ 11 mg/kg bw/day	358-650 mg/kg bw/day (100 mg/kg bw/day for 46 weeks – 76 mg/kg bw/day for 24 months)
F344 rats and B6C3F1 mice. Oral (gavage) (5/sex/group) Duration 7 weeks NTP (1978)	F344 rats: 0, 100, 147, 215, 316, 464, 681, 1000 and 1470 mg/kg bw/day 3 times per week for 7 weeks B6C3F1 mice: 0, 147, 215, 316, 464, 681, 1000, 1470 and 2150 mg/kg bw/day	F344 rats and B6CF1 mice: Effects observed above the upper guidance value for STOT RE. Effects were reduction in body weight (most pronounced in rats) and increased **mortality** (one rat died at 464 mg/kg bw/day and all ≥681 mg/kg bw/day. All male mice and one female died at 2150 mg/kg bw/day and 2 females at 1470 mg/kg bw/day).	STOT RE 2 > 18 ≤ 180 mg/kg bw/day	F344 rats: 253 mg/kg bw/day (464 mg/kg bw/day) B6C3F1 mice: 800- 1170 mg/kg bw/day (1470-2150 mg/kg bw/day)

	3 times per week for 7 weeks			
F344 rats and B6C3F1 mice. Oral (gavage) Duration 104 weeks Chronic exposure/carcinogenicity study NTP (1978)	F344 rats: 0, 50 and 100 mg/kg bw/day 3 times per week B6C3F1 mice: 0, 250 and 500 mg/kg bw/day 3 times per week	No relevant effects for STOT RE classification.	STOT RE 2 > 1 ≤ 12 mg/kg bw/day	-
Sprague Dawley Rat (Crj:CD). Oral (gavage). Duration: 42 days in males and 63 days in females. OECD TG 422 Anonymous (1994b)	0, 40, 100 and 250 mg/kg bw/day	**Mortality**: All males and 1 female exposed to 250 mg/kg bw/day died. The animals that died showed progressive paralytic gait and decreased motor activity. Males had reduced bodyweight at the end of treatment (~-50%) at 250 mg/kg bw/day. Males had increased relative organ weights of liver at 100 mg/kg bw/day (+13%), **kidney** at 40 mg/kg bw day (+18%) and thymus at 100 mg/kg bw/day (+41%). Females had increased relative thymus weight at 40 mg/kg bw/day (+85%). Histological changes: Effects in **kidneys** in males and females. Males had eosinophilic droplets and regenerated tubule (very slight to moderate in severity) at 40 mg/kg bw/day and higher. Dilation of tubules in 6 males and slight neutrophil infiltration in two males were also reported at 250 mg/kg bw/day. In one female regenerated tubules and cell debris in tubular lumen (very slight in severity) at 100 mg/kg bw/day. **Neurotoxicity**: From 100 mg/kg bw/day 4 males had degeneration of skeletal muscle nerve (very slight	STOT RE 2 (male) > 21 ≤ 210 mg/kg bw/day STOT RE 2 (female) > 14 ≤ 140 mg/kg bw/day	Mortality: male: 117 mg/kg bw/day. Female: 175 mg/kg bw day (250 mg/kg bw/day) Kidney: effects from **19 mg/kg bw/day** in males and **70 mg/kg bw/day** in females. (40 mg/kg bw/day (males), 100 mg/kg bw/day (females)) Neurotoxicity: From **47 mg/kg bw/day** in males and 175 mg/kg bw/day in females (100 mg/kg bw/day (males), 250 mg/kg bw/day (females))

		in severity), 9 males had degeneration of sciatic nerve fiber (very slight in severity) and 2 males had degeneration of nerve fibers in the fasciculus gracilis of the cervical cord (very slight-slight in severity). Atrophy of skeletal myofiber was observed in 11 males at 250 mg/kg bw/day (10 slight in severity, 1 very slight). For several females it was reported degenerative changes in skeletal muscle nerve, myofiber in skeletal muscle, sciatic nerve, cervical or lumbar cord at 250 mg/kg bw/day.		
Long-Evans hooded rats. 20/group. Oral (gavage) Duration: 5 days Toth *et al.* (1992)	0, 100, 250 and 600 mg/kg bw/day for 5 days.	Significant weight loss at 100 and 250 mg/kg bw/day, weight loss was (-66g, corresponding to 16%) at 600 mg/kg bw/day. At 600 mg/kg bw/day **marked neuromuscular deficits** were reported.	STOT RE 2 > 100 ≤ 1000 mg/kg bw/day (duration less than 9 days)***	**33 mg/kg bw/day** (600 mg/kg bw/day)
Random breed albino Sprague-Dawley. 20/group. Oral (gavage) Duration 5 days, up to 5 weeks. 60 out of 80 rats died within 5 days.of treatment. Mortality rate was 75% Sperm abnormality assay Cho & Park (1994)	0, 400, 500, 750, 1000 and 1500 mg/kg bw/day	**Mortality**: High mortality. All animals died after exposure to 750 mg/kg bw/day or higher. 10 % died at 400 mg/kg bw/day and 90% at 500 mg/kg bw/day. No mortality in the controls. The rats were anuric and anorexic prior to death. No remarkable finding except severely distended bladder with multifocal ulceration, loss of urothelium and marked thinning and atrophy of the muscle proper.	STOT RE 2 > 100 ≤ 1000 mg/kg bw/day (duration less than 9 days)***	**22 mg/kg bw/day** and higher (400 mg/kg bw/day for 5 days)
Sprague-Dawley rats, male. Oral (gavage). 10/dose. Oral (gavage). Duration: 28 days. Takizawa *et al*. (1998)	0 and 100 mg/kg bw/day	No relevant effects for STOT RE classification	STOT RE 2 > 30 ≤ 300 mg/kg bw/day	
Swiss (ICR/Ha) mice, male. Oral (gavage). Duration: 5 days Dominant lethal	0, 500 and 1000 mg/kg bw/day	No relevant effects for STOT RE classification	STOT RE 2 > 100 ≤ 1000 mg/kg bw/day (duration less	

mutation test Epstein *et al.* (1970)			than 9 days)***	

*The DS extrapolated to the effective dose compared to 90-day exposure to compare with the 90-day guidance value to STOT RE using Haber's rule. The extrapolated doses were in most cases calculated for the dose and the timepoint where the effect occurred. Effects on the nervous system, kidneys and mortality were considered most relevant for a classification as STOT RE. A value between 10 and 100 mg/kg bw/day is an effect relevant for classification as STOT RE 2, these values are marked in bold in the table above.
** In line with section 3.9.2.9.5 of annex I in the CLP regulation, the corresponding GV for STOT RE for the exposure duration of the different assays were calculated and concentrations used in the different studies can then be directly compared to the corresponding GV to evaluate if the effects are relevant for classification.
*** GV are set at 1000 mg/kg bw/day for the studies with duration less than 9 days. CLP guidance notes that it is problematic to adjust the GV for very short study durations and this can result in a higher GV than the limit for acute toxicity (2000 mg/kg bw). For studies with exposure durations shorter than 9 days the guidance value used should be no greater than 10 times the default guidance value. Oral route studies of 9 days or less should therefore be compared with a guidance value of 1000 mg/kg bw/day for STOT-RE Category 2.

Nervous system: Neurotoxic effects were observed in rats, rabbits and dogs. The effects consisted of behavioural effects, clinical signs, electrophysiological changes and histopathological changes (Bomhard *et al.,* 1997; Anonymous, 1994b; Schaeppi *et al.,* 1984; Deichman & Witherup, 1946; Toth *et al.,* 1992).

Bomhard *et al.* (1997) reported neurotoxic effects in Wistar rats in a 30-month carcinogenicity study at the top dose (100 mg/kg bw/day, the dose was reduced to 50 mg/kg bw/day at week 50). Degenerative effects on peripheral nerve fibre and spinal cord were reported in both sex after one-year exposure and these effects were accompanied by hind limb weakness. The effects in the study by Bomhard *et al.* (1997) were adverse, but the exposure exceeds the upper guidance value for STOT RE classification. The chronic toxicity/carcinogenicity study by NTP (1978) did not report similar effects. The OECD TG 422 study (Anonymous 1994b) showed similar effects as reported by Bomhard *et al.* (1997). Sprague-Dawley rats exposed to 250 mg/kg bw/day showed progressive paralytic gait in both sexes. At concentrations ≥ 100 mg/kg bw/day degenerative effects were reported in skeletal muscle nerve, skeletal myofiber, sciatic nerve and cervical cord. Degenerative effects were reported in almost all the animals, but the severity was reported to be very slight to slight. When extrapolating the doses where effects were observed they occur in doses relevant for classification as STOT RE 2.

The study by Schaeppi *et al.* (1984) investigated neurotoxicity in 6 dogs. There were limitations with the study, there were no controls, and it was a different treatment scheme for each dog. Neurotoxicity started from exposure to 88 mg/kg bw/day for 29 days, and the severity of the effects increased with dose and duration and similar effects were reported in all the dogs. Effects included impaired gait, hopping, tactile placing and landing, persistent abnormal posture and decreased muscle tone as well as neuropathological changes. Increased prolonged distal latency for neuromuscular impulse transmission compared with pre-test values was observed in 4 dogs (effect concentration was below the guidance value for STOT RE 2 for 3 of the dogs). In the female dog exposed to 181 mg/kg bw/day enhanced patellar reflex (day 18), attenuated extensor postural thrust (day 25), atactic gait (day 39), decreased muscle force and persistent abnormal posture (day 46), decreased muscle tonus and impaired hopping and landing (day 53) were reported at doses that are supportive of STOT RE 2 classification.

Deichmann & Witherup (1946) reported neurotoxic effects in rabbits exposed by oral (gavage) or dermal administration. The effects in 3 rabbits exposed by oral administration to 359 mg/kg bw/day for 6 days was fine tremors, unsteadiness and weakness of the extremities after second or third dose and flaccid paralysis (5 days) which was replaced by a state of spasticity. Effects started at day 2-3 and it can be discussed if the effects should be evaluated under repeated exposure or as acute effects, nevertheless, the doses are below the guidance value for STOT RE 2 classification. Similar effects were also observed upon single exposure in the same study however the effects on flaccid paralysis and spasticity developed after further dosing. In the rabbits exposed by dermal route, two exposure conditions were used. In the first experiment 6 rabbits were exposed for 2394 mg/kg bw/day for 2 hrs a day for 20 days. In the second experiment 3 rabbits received increased exposure duration to 3 hrs for 14 days. Neurotoxic effects were flaccid paralysis followed by hunch-backed position, fore-legs and hind-legs from knees to tows were rigidly extended and hip-joints flexed in experiment 1. The effects occurred at concentrations exceeding the upper guidance value for STOT RE 2 classification. In the second experiment fine tremor and unsteadiness as well as weakness and un-coordination of the lower extremities were observed in two of the animals that died after 5 and 14 applications. Unusual posture (legs extended) and fine tremors and unsteadiness was reported for the 3rd rabbit after 3-4 applications. After seven applications paralysis of the muscles of the extremities was developed and followed by spasticity. Only the neurotoxic effects observed in the rabbit that died after 5 applications were below the guidance value for STOT RE 2 classification in the dermal exposure study.

Mode of action: Impaired acetylcholine esterase (AChE) activity was suggested as a possible MoA, however, there are conflicting results available regarding TMP's potential to inhibit AChE. The OECD TG 422 study (Anonymous, 1994b) described that AChE was reduced (although the data was not presented) while Jackson & Jones (1968), Vandekar (1957) and Oishi *et al.* (1982) did not find changes in cholinesterase activity. Deichmann & Witherup (1946) concluded that the effects were similar as other phosphoric and phosphorous acid esters which are substances known to inhibit AChE activity and related neurotoxicity, but the MoA is not sufficiently investigated for TMP. Harbison *et al.* (1976) described interference with choline acetyltransferase in sperm.

Kidney: The OECD TG 422 (Anonymous, 1994b) reported effects in kidney that were more prominent in males with an increase in relative kidney weight at 40 mg/kg bw/day (+18%). There were also histopathological changes in the kidneys, eosinophilic droplets, and regenerated tubules (very slight to moderate) from 40 mg/kg bw/day, and dilation of tubules (6/13) and slight neutrophil infiltration (2/13) at 250 mg/kg bw/day. In females histological changes in kidneys were reported but they were very slight in severity at the doses relevant for STOT RE 2 classification. Oishi *et al.* (1982) reported increased relative and absolute kidney weight, and Bomhard *et al.* (1997) reported increased relative kidney weight in males and females at 100 mg/kg bw/day (+23%). The increased kidney weight was not accompanied with other kidney changes. Distended bladder was observed in rats that died after oral exposure to TMP in a 7-week range finding study (NTP, 1978) and in a study where rats were exposed for 5 days/week (Cho & Park, 1994). In the latter study the severely distended bladders had multifocal ulcerations, loss of urothelium and atrophy of the muscle proper.

Mortality: Bomhard *et al.* (1997) reported significant increase in mortality at 100 mg/kg

bw/day after one-year exposure and the doses were reduced to 50 mg/kg bw/day for the rest of the study. The 7-week dose range finding study (NTP, 1978) also reported increased mortality. However, the doses in both studies exceeded the upper guidance value for STOT RE 2 classification. In the study by Cho & Park (1994) there were significant mortality observed in rats after 5 days exposure. All rats exposed to ≥ 750 mg/kg bw/day died within 3 days of dosing, which is assumed to be related to acute toxicity. 90% of the rats exposed to 500 mg/kg bw/day died within 7 days and 10% of the rats exposed to 400 mg/kg bw/day died within 5 days. /day.

Comments received during consultation

One comment received from a member state competent authority (MSCA) supported classification as STOT RE 2, H373 (nervous system) based on neurotoxic effects in several repeated-dose toxicity studies in rats, rabbits, and dogs, with effective doses > 10 mg/kg bw/day and < 100 mg/kg bw/day.

Assessment and comparison with the classification criteria

Kidney toxicity: Only one study described clear kidney toxicity in males where increases in kidney weight were followed histological changes that were very slight to moderate in severity (Anonymous 1994b). Two other studies reported severely distended bladder accompanied by some histological findings in the animals that died during the study, these two studies were considered of minor relevance for classification (NTP, 1978 and Cho and Park, 1994). **In conclusion**, RAC agree with the DS that no classification for STOT RE for kidney effects is warranted.

Mortality: Some studies showed effects on mortality at relevant concentrations for a STOT RE 2 classification. The mortality was observed after short exposure duration (5 days). Given the short exposure duration the effects could be considered more of an acute effect rather than repeated toxicity. The doses are also in the similar range as the acute toxicity LD_{50} values after oral exposure. In addition, according to the CLP guidance caution should be given when adjusting the guidance value for very short study durations since this can lead to very high guidance values which are not appropriate for classification of STOT RE. **In conclusion**, RAC agree with the DS that no classification for STOT RE for mortality is warranted.

Neurotoxicity: Neurotoxic effects were reported in rats, rabbits and dogs. Although there were deficiencies in some of the studies, dose and time dependent effects on neurotoxicity were observed, and sever effects occurred at concentrations relevant for classification as STOT RE 2. Effects observed included neuronal dysfunction causing tremor, lack of coordination and paralysis. Histopathological investigations reported neuronal degeneration and electrophysiological evidence. Several studies were performed in rats and the effects on neurotoxicity varied. Some of the studies showed marked neuromuscular deficits, progressive paralytic gait, decreased motor activity and neuronal degeneration (Toth *et al*. 1994 and Anonymous 1994b) at concentrations below the STOT RE2 guidance value. Other studies in rats reported similar effects at concentrations exceeding the STOT RE2 guidance value (Bomhard *et al.* 1997) and five studies did not show neurotoxicity effects (Takizawa *et al.,* 1998; Cho & Park, 1994; NTP, 1978 and Oishi *et al.,* 1982). It is not clear why there are different results in the rat studies. The

data indicate neurotoxic effects in 3 species mainly exposed by the oral route, but effects were also reported in rabbits exposed via the dermal route (although only one animal had effects below the guidance value after dermal exposure) (Deichmann and Witherup 1946). Two of the studies (Toth *et al.,* 1992 and Deichmann and Witherup, 1946) showed neurotoxic effects after short exposure duration (5-6 days) and it could be argued whether the neurotoxicity reported were more related to an acute toxicity or STOT SE classification. However, neurotoxicity was also reported in studies after repeated exposure in rats and dogs for longer durations (42 days in rats and up to approximately 100 days in dogs) (Anonymous, 1994b and Schaeppi *et al.,* 1984) below the GV for STOT RE 2 and with lower effective doses when compared to the studies of shorter durations. In addition, one chronic exposure study (30 months) in rats also showed neurotoxic effects after exposure for 46 weeks, 12 months and 24 months, however at concentrations exceeding the guidance value for STOT RE 2 classification (Bomhard *et al.,* 1997). The CLP guidance states that "Where a number of studies are available these should be assessed using a weight of evidence approach to determine the most appropriate classification". Several studies showed adverse effects on the nervous system (Deichmann and Witherup, 1946; Toth *et al.,* 1992; Anonymous, 1994b; Schaeppi *et al.,* 1984 and Bomhard *et al.,* 1997) in rats, rabbits and dogs, the studies are of varying duration and quality. In most of the studies the neurotoxic effects are observed below the GV for STOT RE 2 classification (Deichmann and Witherup, 1946; Toth *et al.,* 1992; Anonymous, 1994b and Schaeppi *et al.,* 1984). In a weight of evidence assessment, the neurotoxic effects are considered relevant for repeated exposure and STOT RE classification.

In conclusion, RAC agrees with the DS that a classification as **STOT RE 2; H373 (nervous system)** is warranted.

Relevant exposure route: Most of the studies available were with exposure by the oral route, however, there were also effects after dermal exposure in rabbits. Acute toxicity studies after dermal exposure showed increased mortality in rabbits, although at concentrations that exceeded the cut-off values for classification. There were no inhalation studies available, and this exposure route does not seem to be relevant, but possible effects by inhalation cannot be excluded. Based on this it is not possible to specify a specific route of exposure for TMP.

10.13 Aspiration hazard

No assessed in this dossier.

11 EVALUATION OF ENVIRONMENTAL HAZARDS

No assessed in this dossier.

12 EVALUATION OF ADDITIONAL HAZARDS

No assessed in this dossier.

13 ADDITIONAL LABELLING

Not relevant.

14 REFERENCES

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