Committee for Risk Assessment

RAC

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

Adopted

8 June 2023

8 June 2023

CLH-O-0000007318-70-01/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

Chemical name: **trimethyl phosphate**

EC Number: **208-144-8**

CAS Number: **512-56-1**

The proposal was submitted by **Austria** and received by RAC on **23 May 2022.**

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

PROCESS FOR ADOPTION OF THE OPINION

Austria has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **4 July 2022**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **2 September 2022**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Nina Tekpli**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **8 June 2023** by **consensus**.

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	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 submitters 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	
RAC opinion	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 = 1300 mg/kg bw	
Resulting Annex VI entry if agreed by COM	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 = 1300 mg/kg bw	

GROUNDS FOR ADOPTION OF THE OPINION

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

HUMAN HEALTH HAZARD EVALUATION

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.

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 animals. 9 weeks dietary study Non guideline study. Oishi *et al.* (1982) and US EPA (2010)	Dietary: 0-0.5% in diet. Concentration (calculated) 0 and 461 mg/kg day.	Effects observed at doses exceeding the guidance 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.	STOT RE 2 > 14 ≤ 140 mg/kg bw/day	323 mg/kg 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	**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	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	**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)

Study	Doses	Results	Corresponding guidance value (for STOR RE 2)**	Extrapolated effective dose to 90-day exposure* (study dose)
	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.	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: 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	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	
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 was followed by spasticity.	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)
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%.	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)

Study	Doses	Results	Corresponding guidance value (for STOR RE 2)**	Extrapolated effective dose to 90-day exposure* (study dose)
		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.		
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 3 times per week for 7 weeks	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)
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%).	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.

Study	Doses	Results	Corresponding guidance value (for STOR RE 2)**	Extrapolated effective dose to 90-day exposure* (study dose)
		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 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.		(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))
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 mutation test Epstein *et al.* (1970)	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 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.

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,	+ 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,	Adler *et al.* (1971) Comparable to OECD 475

Animal	Exposure	Results	Reference
	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.	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	
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)

Animal	Exposure	Results	Reference
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.

Animal	Exposure	Results	Reference
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 translocation test: 33-75 males/dose group	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. Heritable translocation test: i.p administration of 0, 1000 and 1500 mg/kg	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. Slight but significant reduction of young weaned at the highest concentration.	Tezuka *et al.* (1985)
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	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	Epstein *et al.* (1970)

Animal	Exposure	Results	Reference
	bw/day on 5 consecutive days.	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.	
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	i.p. administration. Doses: 100-1000	Increase in abnormal sperm at the two highest concentrations 1 week after	Wyborek *et al.* (1975)

Animal	Exposure	Results	Reference
genotype: (C57BL X C3H/Anf) F1 or (C57BL/6 X C3H/He)F1. 4/group	mg/kg bw. Mice were killed 1, 4 or 10 weeks after treatment.	exposure. No effects at the other weeks. Authors claim the results indicate that post-meiotic cells are affected.	
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 Sertoli cells was described.	Cho & Park (1994)
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	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.	Harbison *et al.* (1996)

Animal	Exposure	Results	Reference
	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.	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.	
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). No sign of mating with female virgin rats. Testes showed impaired spermatogenesis. Round spermatids showed vacuoles in round 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.	Hanna & Kerr (1981)

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.**

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. Study duration 30 months	Oral administration, mixed to drinking water weekly. 0, 1, 10 and 100 mg/kg bw/day. Highest concentration reduced to 50 mg/kg bw/day at month 24 (week 100) due to high mortality.	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)

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 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

[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.**

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)

Animal	Exposure	Results	Reference
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.

ANNEXES:

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).