Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

1,4-dioxane

EC Number: 204-661-8
CAS Number: 123-91-1

CLH-O-0000001412-86-264/F

Adopted

15 March 2019

Annankatu 18, P.O. Box 400, FI-00121 Helsinki, Finland | Tel. +358 9 686180 | Fax +358 9 68618210 | echa.europa.eu

15 March 2019

CLH-O-0000001412-86-264/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: **1,4-dioxane**

EC Number: **204-661-8**

CAS Number: **123-91-1**

The proposal was submitted by the **Netherlands** and received by RAC on **13 February 2018.**

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

PROCESS FOR ADOPTION OF THE OPINION

The Netherlands 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 **9 April 2018**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **8 June 2018**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Christine Bjørge**

Co-Rapporteur, appointed by RAC: **Stine Husa**

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 **15 March 2019** by **consensus**.

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

	Index No	International Chemical Identification	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	603-024-00-5	1,4-dioxane	204-661-8	123-91-1	Flam. Liq. 2 Carc. 2 Eye Irrit. 2 STOT SE 3	H225 H351 H319 H335	GHS02 GHS08 GHS07 Dgr	H225 H351 H319 H335	EUH019 EUH066		Note D
Dossier submitters proposal	603-024-00-5	1,4-dioxane	204-661-8	123-91-1	**Retain** Flam. Liq. 2 Eye Irrit. 2 STOT SE 3 **Add** Muta. 2 **Modify** Carc. 1B	**Retain** H225 H319 H335 **Add** H341 **Modify** H350	**Retain** GHS02 GHS08 GHS07 Dgr	**Retain** H225 H319 H335 **Add** H341 **Modify** H350	**Retain** EUH019 EUH066		**Retain** Note D
RAC opinion	603-024-00-5	1,4-dioxane	204-661-8	123-91-1	**Retain** Flam. Liq. 2 Eye Irrit. 2 STOT SE 3 **Modify** Carc. 1B	**Retain** H225 H319 H335 **Modify** H350	**Retain** GHS02 GHS08 GHS07 Dgr	**Retain** H225 H319 H335 **Modify** H350	**Retain** EUH019 EUH066		**Retain** Note D
Resulting Annex VI entry if agreed by COM	603-024-00-5	1,4-dioxane	204-661-8	123-91-1	Flam. Liq. 2 Carc. 1B STOT SE 3 Eye Irrit. 2	H225 H350 H335 H319	GHS02 GHS08 GHS07 Dgr	H225 H350 H335 H319	EUH019 EUH066		Note D

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

Dossier submitters proposal

More information on the mutagenic and carcinogenic properties of 1,4-dioxane has become available in recent years, which warrants a review of the classification for carcinogenicity

The Health Council of the Netherlands published an evaluation of this substance in 2011 and concluded that 1,4-dioxane should be regarded as carcinogenic to humans (comparable with CLP category 1B) and considered the substance as a non-genotoxic carcinogen (HCN 1987, 2011).

In 2015, the Health Council of the Netherlands performed a re-evaluation of the mutagenic and carcinogenic properties of 1,4-dioxane, which included more recent studies. In this re-evaluation, additional studies where provided that confirmed the carcinogenic properties of 1,4-dioxane which forms the basis for this proposal for an update of the harmonised classification from Cat. 2 to Cat. 1B (H350) for carcinogenicity. In addition, an inclusion of a classification as Muta. 2 (H341) for germ cell mutagenicity is proposed by the DS.

It is noted that there has been a change in the classification criteria for carcinogenicity (CLP vs. DSD). Previously, under the DSD-regulation, a non-genotoxic chemical would in general not be classified as Carc. 2 (similar to the current 1B under the CLP-regulation). The current CLP-criteria do not exclude to consider non-genotoxic chemicals as presumed human carcinogens. Based on these considerations, a classification in category 1B (Carc. 1B; H350) is proposed.

<u>Toxicokinetics</u>

In four human healthy human volunteers, exposure to 50 ppm 1,4-dioxane showed rapid uptake and elimination of the parent compound and the metabolite hydroxyethoxyacetic acid (HEAA) (Yong *et al.*, 1977). A more recent study in 18 human volunteers exposed to 20 ppm 1,4-dioxane confirmed the rapid and almost complete metabolism of 1,4-dioxane to HEAA, reaching a steady state within 3-4h in the blood (Göen *et al.*, 2016). Overall the results reported by Göen *et al.* (2016) have shown to be in accordance with the study by Young *et al.* (1977). The elimination half-life of HEAA was found to be 3.4 hours, only slightly higher than the 2.7 hours found by Young *et al.* (1977). Further, Göen *et al.* (2016), noted that despite of the fast elimination kinetics, measurable amounts of HEAA were still detected in the urine 16h post-exposure. The levels were rather low compared to the maximum elimination levels of HEAA indicating nearly complete elimination and limited accumulation. These results were also in agreement with Young *et al.* (1977) where 99.3% of the absorbed dose was eliminated via the urine as HEAA, the remainder was unchanged 1,4-dioxane.

In mice 1,4-dioxane was rapidly and extensively absorbed following inhalation and oral exposure (Sweeney *et al.*, 2008). Dermal absorption occurred, but it was low, probably due to evaporation of the material (Marzulli, Anjo *et al.*, 1981).

In rats 1,4-dioxane is rapidly excreted in urine and the major metabolite is HEAA (Woo *et al.*, 1977, 1978). At low pH it was shown that this metabolite is rearranged (reversibly) to 1,4-dioxan-2-one.

1,4-dioxane was shown to be metabolised by cytochrome P450, possible of the 2A and 2D family, to the main metabolite HEAA (Sweeney *et al.*, 2008). Induction of cytochrome p-450 enzymes was shown to increase the rate of HEAA formation whereas inhibition decreased the HEAA formation (Woo *et al.*, 1977, 1978).

In rats a single oral dose of 10 mg/kg bw was rapidly metabolised and excreted (as HEAA) via the urine, while a single oral dose of 100 or 1000 mg/kg bw saturated the metabolism, resulting in a decreased proportion of urinary excretion of HEAA, and increased excretion of 1,4-dioxane in urine and the expired air (Dietz, Stott *et al.*, 1982; Reitz *et al.* 1990; Young, Braun, and Gehring, 1978). In mice a single oral dose of 20 mg/kg was shown to be rapidly metabolised. Saturation of metabolism was shown to occur above 200 mg/kg (Sweeney *et al.*, 2008).

It has been suggested by the Scientific Committee on Occupational Exposure Limits (SCOEL, 2004) that at high doses, another, presumably reactive metabolite of 1,4-dioxane, the HEA (β-hydroxyethoxyacetaldehyde) might be responsible for the (cyto)toxicity. This is based on the fact that in the toxicity studies morphological and biochemical changes were reported at saturated exposure levels and SCOEL postulated, without further evidence, that HEA may be assumed to be the reactive metabolite that is responsible for some of the toxicity observed following exposure to 1,4-dioxane such as carcinogenicity in experimental animals.

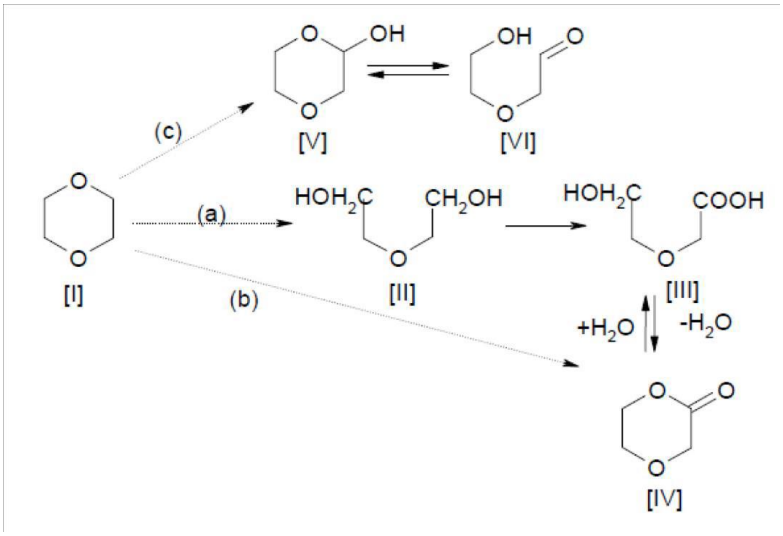


Figure from the Background document (BD): Suggested metabolic pathways of 1,4-dioxane in the rat (Woo *et al.* 1977). [I], 1,4-dioxane; [II], diethylene glycol; [III], -hydroxyethoxy acetic acid (HEAA); [IV], 1,4-dioxane-2-one; [V], 1,4-dioxane-2-ol; [VI] -hydroxyethoxy acetaldehyde (HEA). Note: Metabolite [V] is a likely intermediate in pathway b as well as pathway (c). The proposed pathways are based on the metabolites identified; the enzymes responsible for each reaction have not been determined. The proposed pathways do not account for metabolite degradation to the labelled carbon dioxide identified in expired air after labelled 1,4-dioxane exposure.

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

For the assessment of germ cell mutagenicity, the Dossier Submitter (DS) included several *in vitro* studies in bacterial and mammalian cells, several *in vivo* studies in somatic and germ cells and one human study. A summary of the studies are included below:

In vitro: 1,4-dioxane was studied in six reverse mutation assays in bacterial cells, in two gene mutation assays, one micronucleus assay and two chromosome aberration tests in mammalian cells. These studies showed no mutagenic activity of 1,4-dioxane. Further, negative results were also reported in the unscheduled DNA synthesis assay and the sister chromatid exchange assay. In the Comet assay and in an alkaline elution assay in rat hepatocytes 1,4-dioxane induced DNA-damage, but only at cytotoxic concentrations (0.3 mM and higher where the following doses were tested: 0, 0.03, 0.3, 3, 10 and 30 mM).

In vivo (somatic cells): The genotoxicity of 1,4-dioxane was studied in somatic cells for 3 endpoints of genotoxicity: gene mutations, structural, and numerical chromosome aberrations. In most of the animal studies no data on cytotoxicity were reported, which limits the interpretation of the results. It should also be noted that in most studies the dose levels used exceeded the limit dose of 2000 mg/kg bw according to OECD TG 474, limiting the interpretations of the results. Furthermore, the different results reported among the studies could also be partially explained by the use of a small number of animals, different dose regimen and testing methods.

Due to these considerations, the *in vivo* genotoxicity studies showed contradictory results. Exposure to high doses of 1,4-dioxane, above the limit dose of 2000 mg/kg bw, resulted in an increase of cells with micronuclei indicating a relationship to cytotoxic rather than a genotoxic effect. However, in some studies positive results were also found in micronucleus tests with doses below the limit dose of 2000 mg/kg bw and not considered related to cytotoxicity. As these positive findings cannot be overlooked, 1,4-dioxane may have a genotoxic potential.

In genotoxicity tests in somatic cells studying aneuploidy, no positive results were reported. Further, the majority of the supportive *in vivo* genotoxicity tests confirmed both the positive and negative results reported regarding the induction of micronuclei in the *in vivo* studies.

In vivo (germ cells): No animal studies were performed according to acceptable test guidelines for the assessment of germ cell mutagenicity following *in vivo* exposure to 1,4-dioxane. The outcome of a sex-linked recessive lethal mutagenicity test using Drosophila melanogaster was negative (Yoon *et al.* 1985).

A dominant lethal study in male NMRI mouse (20/group) exposed to 2550 mg/kg bw 1,4-dioxane was negative (Klimisch 3; no positive control, no toxicity reported, and methodological deficiencies, ECHA, 2015).

Human data: Chromosomal aberrations (CA) was assessed in peripheral lymphocytes in 6 German workers exposed to unspecified levels of 1,4-dioxane for 6-15 years. No increase in CA was reported in the workers when compared to the control group (Thiess, Tress and Fleig, 1976).

In summary: The *in vitro* tests were negative but part of the *in vivo* tests were positive, predominantly at doses above the limit dose of 2000 mg/kg bw. The DS therefore concluded that 1,4-dioxane may be considered as a genotoxic substance, however, the positive results may be due to cytotoxicity and thus the induction of cell proliferation. The positive results found in the

tests measuring replicative DNA synthesis as a marker for cell proliferation confirm this mode of action. However, since positive results in the micronucleus tests were found at doses below the limit dose of 2000 mg/kg bw a genotoxic mechanism as a secondary mode of action cannot be excluded. Therefore, the DS considered 1,4-dioxane as mutagenic *in vivo* in mammalian cells.

The DS proposal is to classify 1,4-dioxane as Muta. 2 based on positive evidence from animal studies and/or from *in vitro* studies obtained from: somatic cell mutagenicity tests *in vivo*, or other *in vivo* somatic cell genotoxicity tests, which are supported by positive results from *in vitro* mutagenicity assays. 1,4-dioxane did not show genotoxicity *in vitro*. *In vivo* data showed an increase in micronuclei formation in several studies. Therefore, the DS recommended to classify 1,4-dioxane in category 2.

Comments received during public consultation

The classification of 1,4-dioxane as Muta. 2 was supported by 3 MSCAs based on the justification given by the DS. Comments were also submitted by three Industry or trade organisation, not supporting the proposed classification as Muta. 2, and were in favour of no classification for germ cell mutagenicity. The main arguments focused on the negative *in vitro* test showing that 1,4-dioxane has no direct DNA damaging potential. Further comments were related to the mixed results from the *in vivo* MN tests across labs and mice strains, and that these tests were not performed according to GLP or acceptable test guidelines. Many of the *in vivo* MN studies were performed with doses exceeding the maximum testing dose at 2000 mg/kg bw according to OECD TG 474. Therefore, they considered that the data should be interpreted as inconclusive rather than positive for mutagenicity and no classification for germ cell mutagenicity was proposed.

Assessment and comparison with the classification criteria

The DS's proposal is a classification as Muta. 2.

For the assessment of germ cell mutagenicity the DS included several *in vitro* studies in bacterial and mammalian cells, several *in vivo* studies in somatic and germ cells and one human study. It should be noted that none of the studies with positive results were performed according to GLP or to relevant OECD Test Guidelines. The studies are described below:

Human data: The induction of chromosomal aberrations (CA) was assessed in peripheral lymphocytes in 6 German workers exposed to unspecified levels of 1,4-dioxane for 6-15 years. No increase in CA was reported in the workers when compared to the control group (Thiess, Tress and Fleig, 1976).

In vitro studies: 1,4-dioxane was studied in six reverse mutation assays in bacterial cells (+/- S9-mix), in three gene mutation assays (two +/- S9-mix), one micronucleus assay (+/- S9-mix) and two chromosome aberration tests (+/- S9-mix) in mammalian cells. No mutagenic activity of 1,4-dioxane was reported in these studies indicating that 1,4-dioxane has no direct mutagenic potential. Further, negative results were also reported in the unscheduled DNA synthesis assay in rat hepatocytes and in the sister chromatid exchange assay (+/- S9-mix) in chinese hamster ovary (CHO) cells. 1,4-dioxane induced DNA-damage in a Comet assay and in an alkaline elution assay in rat hepatocytes, but only at cytotoxic concentrations (0.3 mM and higher, with the following doses tested: 0, 0.03, 0.3, 3, 10 and 30 mM).

In vivo studies (somatic cells): Several studies in mice have been performed to assess the mutagenic properties of 1,4-dioxane. The induction of micronuclei was mainly investigated in bone marrow cells (four studies), but also in peripheral blood cells (two studies) and in hepatocytes (two studies).

1,4-dioxane did not induce an increase in micronuclei in bone marrow cells in B6C3F1 male mice in two studies given intraperitoneal injection (ip) with doses up to 4000 mg/kg bw (Morita, 1994 and McFee *et al.*, 1994). In one of the negative ip studies a decreased ratio of polychromatic erythrocytes/normochromatic erytrhrocytes (PCE/NCE) was reported, indicating that 1,4-dioxane reached the bone marrow (McFee *et al.*, 1994). On the other hand, positive results were reported in two out of four studies in mice for the induction of micronuclei in bone marrow cells following oral exposure by gavage (Mirkova, 1994 and Roy *et al.*, 2005). In the study by Mirkova, 1994 a dose-related statistically significant increase in micronuclei was reported in C57BL6 male mice from 900 mg/kg bw and up to 3600 mg/kg bw, and in the study by Roy *et al.* (2005) a statistically significant dose-related increase from 1500 mg/kg bw (low dose) up to 3500 mg/kg bw (high dose) in male CD-mice, see table below. In the study by Mirkova (1994) 2000 PCE were assessed for micronucleae. The mean lethal dose was initially determined to be 4500 mg/kg bw in male C57BL6 mice. In the study by Roy *et al.* (2005) the increase in micronuclei was paralleled with a dose-related statistically significant decrease in the PCE/NCE ratio (16% reduction in PCE/NCE ratio at 1500 and 2500 mg/kg bw and 37% reduction at 3500 mg/kg bw), as a measure for cytotoxicity in bone marrow cells and thus bioavailability in bone marrow cells. Decreases in bone marrow cell proliferation were also reported. By using a CREST staining (Chen *et al.*,1994) it was shown that the majority of the induced micronuclei (90%) was CREST-negative indicating that they were caused by chromosomal breakage (0.4/2000 erythrocytes in control mice to 2.0, 2.2 and 4.2/2000 erythrocytes in mice treated with 1500, 2500 and 3500 mg/kg bw 1,4-dioxane). In contrast, with the spindle disrupting positive control vinblastine sulphate the majority (80%) of the induced micronuclei were induced by spindle disruption and consisted therefore of CREST-positive micronuclei, indicating that these micronuclei were performed from whole chromosomes.

However, in the study by Tinwell and Ashby (1994) no induction of cells with micronuclei was reported following exposure to 1800 and 3600 mg/kg bw 1,4-dioxane (one dose below the limit dose of 2000 mg/kg bw according to the OECD TG), although in one of the three experiments included in the study a decreased ratio of PCE/NCE was reported.

Table: *Micronuclei formation in bone marrow cells following exposure to 1,4-dioxane by gavage.*

Animal	Exposure	Results	Reference
C57BL6 male mice, 10/group	0, 450, 900, 1800, 3600 mg/kg bw for 24 hr, 3600 mg/kg bw also for 48 h	+ (dose-related increase, st.sign, from 900 mg/kg bw) no data on cytotoxicity	Mirkova, 1994
C57BL6 male mice, 4/group	0, 900, 1800, 3600 mg/kg bw for 24 hr, 3600 mg/kg bw also for 48 h	+ (dose-related increase st.sign, from 900 mg/kg bw) no data on cytotoxicity	
C57BL6 male mice, 10/group	0 and 3600 mg/kg bw for 24 h	+ (no data on cytotoxicity)	
C57BL6 female mice, 5/group	0 and 5000 mg/kg bw for 24 h or 48 h	+ (no data on cytotoxicity)	
BALB/c male mice, 6/group	0 and 5000 mg/kg bw for 24 h	- (1/6 death occurred in 5000 mg/kg bw after 24 h); irrelevant exposure levels. No data on cytotoxicity	
CD-1 male mice, 5/group	1500, 2500 and 3500 mg/kg bw by gavage for 5 days with 24 h sampling time; CRESH and FISH staining used to demonstrate aneuploidy; implantation of BrdU releasing osmotic pumps used to demonstrate cell proliferation in liver and to increase sensitivity of the test.	+ (dose-related increase in MN frequency and decrease in PCE/NCE ratio; >90% micronuclei caused by chromosome breakage; induction of cell proliferation.	Roy *et al.*, 2005

CBA male mice, 4 animals	1800 mg/kg bw (oral, gavage, sacrifice 24 hours after dose); Giemsa staining*	- (decreased PCE/NCE ratio)	Tinwell and Ashby 1994. Follow-up of study Mirkova, 1994
CBA male mice, 8 animals	1800 mg/kg bw (oral, gavage, sacrifice 24 hours after dose); Acridine orange staining	- (no decrease in PCE/NCE; acridine orange staining*)	
C57BL6 mice, male bone marrow, 4 animals	3600 mg/kg bw (oral, gavage, sacrifice 24 hours after dose); Acridine orange staining	- (max. dose level; no decrease in PCE/NCE ratio methodological deficiencies; acridine orange staining*)	

* According to OECD guideline, the Giemsa stain is preferred for detection of micronuclei; the acridine orange stain is a DNA stain that can eliminate artefacts.

In male mice the formation of micronuclei was also studied in two studies in hepatocytes and one study in peripheral blood cells, see table below. In hepatocytes from male mice with partial hepatectomy a statistically significant dose-related increase in MN was reported from 2000 mg/kg bw (Morita and Hayashi, 1998). In this study the mean number of micronucleus were: 0.43±0.14, 0.49±0.19, 1.02±1.04* and 1.45±0.33* in the 0, 1000, 2000 and 3000 mg/kg bw exposed groups. In the positive control group exposed to Mitomycin C (1mg/kg bw) the incidence was 2.65±1.08. The study by Roy *et al.* (2005) was a follow up study from Morita and Hayashi (1998). However, to avoid partial hepatectomy young mice (28-days old) were used that were implanted with BrdU-releasing pumps to assess the frequencies of micronucleus in proliferating BrdU-labelled cells and in non-proliferating non-labelled cells. In the study by Roy *et al.* (2005) a statistically significant dose-related increase in micronucleus was reported from 2500 mg/kg bw in proliferating liver cells, however, no increase was seen in non-proliferating liver cells. It was shown that approximately 85% of the micronucleus was due to chromosome breakage shown by using a FISH technique. The frequency of FISH-negative micronucleus increased from 3.4/2000 labelled hepatocyte in control mice to 4.2, 9.2 and 12/2000 labelled hepatocytes in the 1500, 2500 and 3500 mg/kg bw dose groups. In contrast, with the spindle disrupting positive control vinblastine sulphate the majority (70%) of the induced micronucleus in hepatocytes were induced by spindle disruption and consisted therefore of FISH-positive micronuclei, indicating that these micronuclei were performed from chromosome loss.

In peripheral blood cells no induction of MN was reported in the study by Morita and Hayashi (1998). with doses up to 3000 mg/kg bw (0.05% to 0.16% compared to 0.15 in the control animals)

Table: *Micronuclei formation in hepatocytes and peripheral blood cells from male mice.*

Animal	**Exposure**	**Results**	**Reference**
CD-1 mice, hepatocytes; 5/group	1500, 2500 and 3500 mg/kg bw by gavage, 5 days, 24 h sampling time; CRESH and FISH staining used to demonstrate aneuploidy; implantation of BrdU releasing osmotic pumps used to demonstrate cell proliferation in liver and to increase sensitivity of the test.	From 2,500 mg/kg bw dose-related increase in MN in proliferating cells only; caused by chromosome breakage; induction of cell proliferation	Roy *et al.*, 2005
CD-1 mice, male peripheral blood and hepatocytes; 5/group	1000, 2000 and 3000 mg/kg bw by gavage; partial hepatectomy 24 h after dosing; peripheral blood obtained from tail vein 24 h after hepatectomy; hepatocytes analysed 5 days after hepatectomy.	- (in peripheral blood) + (in hepatocytes; from 2,000 mg/kg bw; dose-related increase).	Morita and Hayashi, 1998. Follow-up of study Mirkova, 1994, same dose levels.

In the majority of the *in vivo* studies, no information on cytotoxicity was reported, which makes it difficult to interpret the results. Further, in many studies the dose levels used exceeded the limit dose at 2000 mg/kg bw making them less relevant for the assessment for mutagenicity. The different results in the studies could also be partially related to the use of a small number of animals, different dose regimen and testing methods.

The DS also included other supporting *in vivo* genotoxicity studies. These included Unscheduled DNA Synthesis (UDS) in rat liver cells (6 studies) and in nasal epithelial cells (1 study), measurements of DNA single strand breaks by the Comet assay (1 study), the measurement of DNA alkylation in liver cells (1 study), and the measurement of cell proliferation by the replicative DNA synthesis assay (2 studies).

In the Comet Assay a dose-related increase in DNA single-strand breaks in rat liver cells was reported following oral exposure by gavage to 2500 and 5000 mg/kg bw 1,4-dioxane. At these relatively high dose levels no significant cytotoxicity were reported (Kitchin and Brown, 1990). In another study, 1,4-dioxane did not induce DNA-alkylation in liver cells from rats, which were given the substance by gavage at a concentration of 1000 mg/kg bw (Stott *et al.* 1981).

In the two cell proliferation assays in rats the measurement of replicative DNA synthesis in hepatocytes showed that cell proliferation was induced following exposure to 1,4-dioxane by gavage with doses up to 2000 mg/kg (Uno *et al.*, 1994 and Miyagawa *et al.* 1999). In the study by Uno *et al.* (1994) with doses of 0, 1000 and 2000 mg/kg cell proliferation was reported at 2000 mg/kg. Signs of cytotoxicity were observed at both doses. However this study had a Klimisch score of 3 due to non-validated test method. In the second study (Miyagawa *et al.,* 1999) cell proliferation were tested in a time-course experiment and in a dose-response experiment. In the time-course experiment a dose of 2000 mg/kg (gavage) was tested in two experiments, both with examination after 24, 39 and 48 hours. In the first experiment a statistically significant increase in replicative DNA synthesis (RDS) was reported after 24 hours, however in the second experiment it was only reported after 48 hours. In the dose-response experiment the RDS were measured 24 or 48 hours after gavage administration of 0, 1000, 1500, 2000 and 4000 mg/kg bw. After 24 hours a dose-related increase was reported from 1000 to 2000 mg/kg bw with no significant increase at 4000 mg/kg. After 48 hours no cell proliferation was reported at any concentrations tested. No hepatocytotoxicity was reported in this study. These two studies indicate that 1,4-dioxane may stimulate cell proliferation.

The *in vivo* UDS studies in rat liver cells and nasal epithelial cells were negative.

The supporting studies give some evidence that 1,4-dioxane may have genotoxic potential.

In conclusion: No acceptable data have been presented on human or animal germ cell mutagenicity. Since no positive evidence for heritable germ cell mutagenicity of 1,4-dioxane in humans is shown, a classification as Muta. 1A is not justified.

For 1,4-dioxane no supporting evidence is available suggesting that the substance has potential to cause mutations in germ cells and a classification as Muta. 1B is not justified.

A substance may be classified as Muta. 2 if there is positive evidence from animal studies and/or from *in vitro* studies from somatic cell mutagenicity tests *in vivo*, or other *in vivo* somatic cell genotoxicity tests, which are supported by positive results from *in vitro* mutagenicity assays. 1,4-dioxane did not induce genotoxicity *in vitro* showing that it has no direct mutagenic potential. Results from *in vivo* studies showed an increase in MN formation in several studies in bone marrow cells and hepatocytes, but not in peripheral blood cells. However, the results from the studies were inconsistent. In the majority of the studies in bone marrow cells, an induction of MN was reported at levels above the limit dose of 2000 mg/kg bw, and in the hepatocytes an induction of MN was only reported at or above the limit dose of 2000 mg/kg. It should also be

mentioned that in most of the *in vivo* studies no data on cytotoxicity were reported, which limits the interpretation of the results.

In conclusion, RAC is of the opinion that a **classification of 1,4-dioxane for mutagenicity is not justified**.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

For the evaluation of carcinogenicity, the dossier submitter included several studies for different exposure routes (inhalation, oral, intraperitoneal injection and dermal) which have been summarised below.

Inhalation

In the study by Kasai *et al.* (2009) male F344/DuCrj rats (50/group) were exposed by inhalation (whole body) to 0, 180, 900 and 4500 mg 1,4-dioxane/m^3 (0, 50, 250, and 1250 ppm), for 6 hours a day, 5 days/week for 104 weeks. A statistically significant increase in hepatocellular adenomas and nasal squamous cell carcinoma were observed in the high dose group. Peritoneal mesothelioma was statistically significantly increased in the two highest dose groups. Pre-neoplastic lesions, such as squamous cell metaplasia were seen from 900 mg/m^3. Increased incidences of nuclear enlargement in the respiratory and olfactory epithelia, and atrophy and respiratory metaplasia in the olfactory epithelium, were noted in the nasal cavity of male rats from 180 mg/m^3.

In a study by Torkelson *et al.* (1974), 288 Wistar rats per sex were exposed to 111 ppm (400 mg/m^3) by inhalation for 7 hour/day for 5 days/week for two years. 192 Wistar rats/sex were used in the control group. The substance did not induce neoplastic lesions in this study, probably because the exposure level was too low. Moreover, the nasal cavity was not examined. The DS is therefore of the opinion that this study cannot be used to indicate a lack of carcinogenic potential of 1,4-dioxane.

Oral exposure

Several carcinogenicity studies with oral exposure (drinking water) of rats and mice are available. These are summarised in the table below.

***Table**: Summary table of animal studies on carcinogenicity studies (oral exposure)*

Study	Doses	Results	Reference
Rat F344/DuCrj 50 animals/sex/group; study duration 104 weeks; According to OECD TG 451 Klimisch-score: 2	0, 0.02, 0.1, 0.5% (w/w) in drinking water (*ad libitum*) Actual dose levels: m: 0, 11, 55, 274 mg/kg bw/d; f: 0, 18, 83, 429 mg/kg bw/d	Neoplastic lesions: + High dose group: Significant induction of nasal squamous cell carcinomas in females and hepatocellular adenomas and carcinomas in males and females, peritoneal mesotheliomas in males, and mammary gland adenomas in females. General: Significantly decreased survival rates at the high dose (m: 22/50; f: 24/50), retarded growth rates and decreased terminal body weights; relative liver weights significantly increased in mid and high dose group in males and high dose females; no effect on food or water consumption.	Kano *et al.*, 2009a

Study	Doses	Results	Reference
Mouse Crj:BDF1 50 animals/sex/group; study duration 104 weeks; According to OECD TG 451 Klimisch-score: 2	0, 0.05, 0.2, 0.8% w/w) in drinking water (*ad libitum*). Actual dose levels: m: 0, 49, 191, 677 mg/kg bw/d; f: 0, 66, 278, 964 mg/kg bw/d	Neoplastic lesions: + Significant induction of hepatocellular tumours in both sexes (in females from low-dose and in males from mid-dose). Two nasal tumours observed in the highest dose group. General: Significantly decreased survival rates at mid and high dose (29/50). Significantly retarded growth rates and terminal body weights in mid and high dose males and females. Relative liver weight significantly increased in mid and high dose males and high dose females; significantly decreased food and water consumption in high dose males and females.	Kano *et al.*, 2009b
Rat Sherman 60 animals/sex/ group; study duration 716 days; haematology, gross necropsy and histopathological examination Klimisch-score: 2	0, 0.01, 0.1, 1% in drinking water (*ad libitum*) Actual dose levels m: 0, 9.6, 94, 1,015 mg/kg bw/d f: 0, 19, 148, 1,599 mg/kg bw/d	Neoplastic lesions: + Treatment related hepatocellular carcinomas and nasal squamous cell carcinomas in high dose group. General: Body weights were significantly lower and water consumption slightly lower in the high dose group compared to controls. Severe reduction in survival rate on the high dose group during the first 4 months of study (66/120, $p < 0.05$); after 4 month the survival rate was the same for all groups; a significantly increased liver weight and liver/body weight ratio the high dose group; gross and histopathological examination revealed variable degrees of renal tubular epithelial and hepatocellular degeneration and necrosis, accompanied by regenerative activities in liver (hepatocellular hyperplastic nodule formation) and renal tubuli in rats in the mid and high dose. No exposure related effect observed on haematology	Kociba, *et al.*, 1974
Rat Osborne-Mendel 35 rats/sex/group; study duration 110 weeks; gross necropsy and histopathological examination Klimisch-score: 2	0, 0.5, 1% (v/v) in drinking water (*ad libitum*). Actual dose levels m: 0, 240, 530 mg/kg bw/d f: 0, 350, 640 mg/kg bw/d	Neoplastic lesions: + Significant induction of nasal squamous cell carcinomas in males in the high dose group and females in the mid and high dose group and hepatocellular adenomas in females in the high dose group. General: survival rate males: 33/35 high dose group, 26/35 low dose group, females: 29/35 high dose group, 30/35 low dose group; no clinical signs other than fluctuations in mean body weights of males probably due to mortality. Histopathology: Tubular degeneration in kidney. Liver: cytomegaly. Gastric ulceration of stomach in males (0/33, 5/28, 5/30). Pneumonia in males ((8/30, 15/31, 14/33) and females (6/30, 5/34, 25/32).	NCI, 1978
Mouse B6C3F1 50 mice/sex/group; study duration 90	0, 0.5, 1% (v/v) in drinking water (*ad libitum*).	Neoplastic lesions: + Significant induction of hepatocellular adenomas or carcinomas in the high dose	NCI, 1978

Study	Doses	Results	Reference
weeks; gross necropsy and histopathological examination Klimisch-score: 2	Actual dose levels m: 0, 720, 830 mg/kg bw/d f: 0, 380, 860 mg/kg bw/d	group females and in males from the mid dose group. General: survival rates males: 45/50 high dose group, 46/50 low dose group, females: 28/50 high dose group, 39/50 low dose group. Pneumonia in males (1/49, 9/50, 17/47) and in females (2/50, 33/47, 32/36). Rhinitis in males (0/49, 1/50, 1/49) and females (0/50, 7/48, 8/39).	
Rat SD 30 male/group; study duration 13 months; necropsy at 16 months; gross necropsy; histopathological examination only in nasal cavity with gross lesions Klimisch-score: 3	0, 0.75, 1.0, 1.4, 1.8% drinking water (*ad libitum*). Total dose/rat based on a daily fluid intake of 36 ml: 104, 142, 191, 198, 213 and 256 gram. Using a ref. body weight of 0,523 kg chronic exposure male CD: 0, 430, 574, 803, 1,032 mg/kg bw/d)	Neoplastic lesions: Nasal cavity: squamous cell carcinomas at the respective dose levels: 0/30, 1/30, 1/30, 2/30, 2/30.	Hoch-Ligeti *et al.*, 1970
Rat Wistar, 26 exposed males, 9 control males; study duration 63 wk; gross necropsy and histopathological examination Klimisch-score: 3	0, 1% in drinking water (*ad libitum*) (using a ref. body weight of 0,462 kg chronic exposure male Wistar: 640 mg/kg bw/d)	Neoplastic lesions in controls and dosed animals: lymphosarcoma (1/9, 0/26), liver tumours (0/9, 6/26) kidney cell carcinoma (0/9, 1/26). Histological changes in liver.	Argus *et al.*, 1965
Osborne rat and B6C3F1 mice, 35/sex/group; study duration 42 weeks. Control group 34 weeks Klimisch-score: 3	0, 0.5 and 1.0% in drinking water 0.5 and 1.0% in diet	Neoplastic lesions: - General: Survival rate male rats high dose: 24/35, low dose: 26/35, female rats high dose: 20/35, low dose: 32/35; Survival rate male mice high dose: 50/50, low dose: 49/50, female mice high dose: 49/50, low dose: 49/50; increased weight gain in male rat and mice; histopathological lesions of lung and liver in rats only.	King *et al.*, 1973

Dermal exposure and intraperitoneal injection

Two studies with dermal exposure and two studies with intraperitoneal injection described to be a pulmonary tumour assay were summarised by the DS. The studies were considered to be of low quality with low numbers of animals used in each dose group (between 16 and 35) and different exposure periods (from 16 to 78 weeks). The results of these studies were not included in the assessment of the carcinogenic properties by the DS due to the low quality of the studies.

Dose range finding studies

In addition, the DS included two dose range finding studies (90 days) in rats and one in mice as supportive evidence for the long term carcinogenicity studies. These are summarised in the table below.

***Table**: Dose range finding studies supportive for the carcinogenicity studies*

Study	Doses	Results	Reference
Rats, F344/DuCrj 10 rats/sex/dose 1,4-dioxane, purity >99% Study duration: 90 days Exposure by inhalation for 6 h/day, 5 days/week OECD TG 413	0, 100, 200, 400, 800, 1600, 3200 and 6400 ppm Calculated as 0, 360, 720, 1440, 2880, 5760, 11520 and 23040 mg/m^3 by inhalation (vapour)	All males in the 6400 ppm dose group died due to renal failure during the first week, all animals in other dose groups survived until week 13 (no abnormal clinical signs). **Terminal body weights** significantly decreased in the 200 ppm and 3200 ppm dose groups for males and the 200 ppm and above 800 ppm dose groups for females. **Relative liver weight** increased from 800 ppm for both males and females. **Relative kidney weight** was increased from 800 ppm for females and from 3200 ppm for males. **Relative lung weights** were increased in the 200 ppm dose group and from 1600 ppm for males and from 200 ppm for females. **Liver enzymes**: ALT was increased in 3200 ppm (males/females) and AST was increased in the 200 ppm and 3200 ppm dose groups for females. Glucose and triglyceride levels were decreased in the 3200 ppm dose groups for males. **Histopathology** showed increased incidences of nuclear enlargement in respiratory (at >100 ppm), olfactory (at >200 ppm) and Trachea epithelia (at ≥1600 ppm) for both males and females. Nuclear enlargement was also reported at ≥1600 ppm (males) and ≥3200 ppm (females) in Bronchial epithelium. Single cell necrosis of hepatocytes was found in males at 3200 ppm. Centrilobular swelling of hepatocytes was found in males and females at 3200 ppm. GST-P positive liver foci were observed in 3/10 males and 2/10 females at 3200 ppm and in 4/10 females at 1600 ppm. Hydropic changes in renal proximal tubule were observed at 3200 ppm in females.	Kasai *et al.,* 2008
Rats F344/Du Crj 10 rats/sex/dose 1,4-dioxane, purity >99% Exposure by drinking water Study duration: 90 days OECD TG 408	0, 640, 1600, 4000, 10000 and 25000 ppm Actual doses: 0, 52, 126, 274, 657 and 1554 mg/kg bw/d (males) and 0, 83, 185, 427, 756 and 1614 mg/kg bw/d (females)	One female died in the highest dose group during the second week due to renal failure. **Food consumption** was decreased at 25000 ppm for males and from 10000 ppm for females. **Water consumption** was decreased from 4000 ppm for both males and females. **Terminal body weights** were reduced from 10000 ppm males and from 4000 ppm in females. **Relative kidney weight** increased from 4000 ppm in males and 1600 ppm in females. Absolute kidney weight only increased at the highest dose (m/f). **Relative lung weight** increased at 25000 ppm for males and from 10000 ppm in females. **Hematology:** RBC, haemoglobin, HTC, AST and ALT were increased in the highest dose group in males. Decrease in blood glucose was seen in ≥10000 ppm	Kano *et al.,* 2008

		(m/f). AST increased in females at 25000 ppm. Urinary pH decreased in ≥4000 ppm (m) and ≥10000 ppm (f). **Histopathology** showed nuclear enlargement in nasal respiratory epithelium at ≥1600 ppm (m/f) followed by enlarged nuclei of epithelial cells in olfactory epithelium and tracheal and bronchial epithelium. Centrilobular swelling occurred at ≥1600 ppm (m) and single cell necrosis and inflammatory cell infiltration increased in 4000 and 25000 ppm (m) and 25000 ppm (f). GST-P positive foci were found in all animals (m/f) of the highest dose group. Nuclear enlargement of renal proximal tubule epithelial cells at ≥10000 ppm (m/f) and hydropic change in the proximal tubules was seen at 25000 ppm (m/f). Vacuolic changes in the cerebrum were noted at 25000 ppm (m/f) in the corpus callosum, hippocampus and dentate gyrus.	
Mouse, Crj:BDF1 10 mice/sex/dose 1,4-dioxane, purity >99% Exposure by drinking water Study duration: 90 days OECD TG 408	0, 640, 1600, 4000, 10000 and 25000 ppm Actual doses levels were 0, 86, 231, 585, 882 and 1570 mg/kg bw/d (males) and 0, 170, 387, 898, 1620 and 2669 mg/kg bw/d (females)	One male died at 25000 ppm during the second week however the cause was unknown. **Food consumption** decreased at 25000 ppm (m) and **water consumption** decreased ≥10000 ppm (m/f). **Terminal body weights** only reduced at 25000 ppm (m). **Relative kidney and lung weight** was increased in 25000 ppm (m/f). **Hematology**: RBC, Hb and HTC increased in high dose males. The liver enzymes AST was increased at 25000 ppm (m/f) and ALT was increased at ≥10000 ppm (f). Glucose levels decreased at 10000 ppm (f) and 25000 ppm (m/f). Urinary pH was decreased at ≥10000 ppm (m/f). **Histopathology** showed nuclear enlargement in bronchial epithelium at ≥1600 ppm (f) and at ≥4000 ppm (m). Nuclear enlargement in olfactory epithelium at ≥4000 ppm (m/f), and respiratory epithelium at 25000 ppm (f). Single cell necrosis was increased at ≥4000 ppm (m/f). Centrilobular swelling occurred at ≥4000 ppm (m/f) and vacuolic change in the olfactory nerve (lamina propria) was observed at 25000 ppm (m/f).	Kano *et al.,* 2008

Other relevant information

Method/Cell type	Concentration	Results/remarks	Reference
Initiation/promotion studies			
Mice, SENCAR 25-40 females/dose; early papilloma development as potential predictor of carcinoma yields Klimisch: 2	1,000 mg/kg bw oral, subcutaneous, or dermal for 2 weeks, followed by 1 µg TPA dermal 3x/week for 20 weeks. A single dose of 1000 mg/kg bw in a satellite group followed by acetone dermal served as negative control. TPA is a tumour promotor	-	Bull *et al.*, 1986
Rat SD	Partial hepatectomy of rat was followed by 30 mg intraperitoneal treatment with	+ Increase in number and total volume of foci	Lundberg *et al.,* 1987

8-9 male/group GGT-enzyme altered foci of hepatocytes determined 10 days after last treatment sacrifice and staining liver sections for GGT Klimisch: 2	diethynitrosamine DENA/kg bw (initiator). Thereafter treatment with 0, 100 and 1000 mg 1,4-dioxane/kg bw (gavage 1/d, 5 times/week for 7 weeks. Controls with and without DENA initiation included	only at toxic doses of 1000 mg/kg bw/d	
Mice, Swiss-Webster 30/sex/group; study duration 78 weeks. Gross necropsy and histopathology Klimisch: 3	50 µg DMBA (dimethylbenzanthracene) for 1 week, as initiator, followed by 3 applications/week of 0.2 mM 1,4-dioxane solution on shaved back for 78 weeks. Acetone was the negative control and croton oil the positive control	+ Neoplastic lesions of skin, lung and kidney in survivors: 4 papillomas (2m, 2f); 6 suspected carcinomas (3m, 3f); 2(m) subcutaneous tumours. Skin tumours increased sharply after 10 weeks. No skin tumours observed after dermal application in absence of DMBA initiation (Table 11 of the BD). General: mortality up to 25/36 after 60 weeks	King *et al.*, 1973

Human data

Three epidemiological studies of low quality are summarised by the DS. These studies did not show any indications of carcinogenicity; however, all the studies had limited power and are insufficient for concluding on carcinogenicity.

In a cross-sectional study, 74 workers exposed for 3-41 years showed no evidence of liver or kidney cancer. There was no increase in cancer deaths compared to population at large. Two retired workers were diagnosed with cancer (squamous epithelial carcinoma and myelofibrosis leukemia) and died (Thiess *et al.*, 1976).

A mortality follow up study from a chemical plant in the US evaluated 165 workers exposed to 1,4-dioxane (<25ppm or 90 mg/m^3) for 28-89 months. In the manufacturing department there were 7 deaths with 2 from cancer whereas the expected would be 4.9 and 0.9. Similarly, in the processing department there were 5 deaths with 1 from cancer whereas the expected would be 4.9 and 0.8 (Buffler *et al.*, 1978).

A retrospective study with 80 men exposed to 0.18-184 mg/m^3 for some years showed no exposure related health effects (NIOSH, 1977).

Based on the studies presented the DS proposed to classify 1,4-dioxane as Carc. 1B. They considered that data from the human studies showed no evidence of carcinogenicity and that a classification as Carc. 1A could not be justified.

As regards a classification as Carc. 1B, the DS considered that the studies by Kasai *et al.* (2009) and Kano *et al.* (2009a, 2009b) showed consistent carcinogenic effects (hepatocellular adenoma, squamous cell carcinoma in the nasal cavity and peritoneal mesothelioma) in rodents after exposure by inhalation and via drinking water, respectively. Histopathological effects were observed in the liver and the nasal epithelium in the repeated dose toxicity tests and in the chronic tests. No non-tumour histopathological effects were reported for the rat peritoneum.

Notably, this tissue is not normally assessed in toxicological studies. The available data suggest a contribution of cell proliferation which may be secondary to local necrosis. However, the available data is limited especially for organs other than liver. Therefore, it must be assumed that the observed increase in tumours is relevant for humans.

The DS calculated T25 for 1,4-dioxane and concluded that the substance could be of medium potency (T25 between 1 - 100 mg/kg bw/d). No SCL was therefore proposed by the DS.

Comments received during public consultation

The classification with category 1B for carcinogenicity as proposed by the DS was supported by two commenting MSCA. The induction of mesothelioma in the peritoneum in male F344 rats not only after oral exposure but also after inhalation exposure was considered as especially noteworthy. The MSCA considered the "new data", that was not available when 1,4-dioxane was classified in August 2001, justify a change in the classification of 1,4-dioxane for carcinogenicity.

Three Industry/Trade associations however disagreed with the proposed classification and considered that the current classification with category 2 for carcinogenicity should be retained. They questioned the lack of consideration for the relevance of metabolic saturation of 1,4-dioxane as the initiating event in the mode of action (MoA) for liver tumour induction, and that 1,4-dioxane seems to increase tumour incidences only at doses with saturated metabolism where the processes of detoxification and elimination is limited. The postulated MoA presented by Industry/Trade associations included therefore metabolic saturation of 1,4-dioxane followed by cytotoxicity occuring above saturation levels with regeneration and unregulated growth of liver cells (regenerative hyperplasia) leading to tumour formation (Dourson *et al.*, 2017) with a MoA for which the study results indicate the presence of a non-linear threshold.

Assessment and comparison with the classification criteria

The DS' proposal was to change the current classification as Carc. 2 to Carc. 1B. According to the CLP criteria, a classification as Carc. 1B is appropriate when a substance is presumed to have carcinogenic potential for humans and the classification is largely based on animal evidence.

For the assessment of carcinogenicity, the DS included several animal studies which are described below:

Inhalation

In the study by Kasai *et al.* (2009) male F344/DuCrj rats (50/group) were whole-body exposed to 0, 180, 900 and 4500 mg 1,4-dioxane/m^3 (0, 50, 250, and 1250 ppm), for 6 hours a day, 5 days/week for 104 weeks. The terminal survival rates of the control, 50, 250, and 1250 ppm exposed groups were 37/50, 37/50, 29/50, and 25/50, respectively. At 1250 ppm terminal body weights decreased, relative liver weight increased and plasma ALT, AST and gamma-GTP enzyme activities increased. As regards non-neoplastic lesions increased incidences of nuclear enlargement in respiratory and olfactory epithelia were seen in all exposed animals. In addition, increased incidences of nuclear enlargement were observed in liver of 1250 ppm and in kidneys of 250 and 1250 ppm exposed groups.

At 1250 ppm necrosis of hepatocytes and hydropic changes in renal proximal tubules were observed as well as squamous cell hyperplasia in nasal cavity and altered cell foci in liver.

1,4-dioxane induced a statistically significant increase in hepatocellular adenomas and in nasal squamous cell carcinoma in the nasal cavity in the high dose group. Peritoneal mesothelioma was statistically significantly increased in the two highest dose groups. Pre-neoplastic lesions, such as squamous cell metaplasia were seen from 250 ppm. Increased incidences of nuclear

enlargement in the respiratory and olfactory epithelia, and atrophy and respiratory metaplasia in the olfactory epithelium were noted in the nasal cavity of male rats from 50 ppm. See table below.

***Table**: Tumour incidence in male rats (50 per group) (Kasai et al. 2009)*

Exposure level (ppm, inhalation)	**0**	**50**	**250**	**1250**
Nose cavity, squamous cell carcinoma	0	0	1	6*
Liver: hepatocellular adenoma	1	2	3	21**
Liver: hepatocellular carcinoma	0	0	1	2
Kidney: renal cell carcinoma	0	0	0	4
Preitoneum: mesothelioma	2	4	14**	41**
Mammary gland: fibroadenoma	1	2	3	5
Mammary gland: adenoma	0	0	0	1
Zymbal gland: adenoma	0	0	0	4
Subcutis: fibroma	1	4	9**	5

Fisher exact test: *p≤0.05, **p≤0.01

In the study by Torkelson *et al.* (1974), 288 Wistar rats per sex were exposed to 111 ppm (400 mg/m^3) by inhalation for 7 hour/day for 5 days/week for two years. 192 Wistar rats/sex were used in the control group. The substance did not induce neoplastic lesions in this study, probably because the exposure level was too low. Moreover, the nasal cavity was not examined. RAC agrees with the DS opinion that this study cannot be used to indicate a lack of carcinogenic potential of 1,4-dioxane.

Oral

In the study by Kano *et al.* (2009a) F344/DuCrj rats (50/sex/dose level) were exposed to 1,4-dioxane (>99%) in the drinking water at levels of 0, 200, 1000, or 5000 ppm for 2 years. The doses were equivalent to approximately 0, 11, 55, or 274 mg/kg bw/d (males) and 0, 18, 83, or 429 mg/kg bw/d (females). General toxicity: significantly decreased survival rates at 274/429 mg/kg bw/d (m: 22/50; f: 24/50), retarded growth rates and decreased terminal body weights; relative liver weights significantly increased at 55 mg/kg bw/d for males and 274/429 mg/kg bw/d males and females; no effect on food or water consumption. Neoplastic lesions: Significant induction in high dose group of nasal squamous cell carcinomas in females. Additionally, in the nose cavity esthesioneuroepithelioma, rhabdomyosarcoma and sarcoma (not otherwise specified) were observed in the high dose group. Further, in the high dose group hepatocellular adenomas and carcinomas in males and females, peritoneal mesotheliomas in males, and mammary gland adenomas in females were observed. Details on tumour incidences for rats in the study by Kano *et al.* (2009a) are shown in the tables below.

***Table**: Tumour incidences in **rats** (Kano et al., 2009a)*

Doses (mg/kg bw/d) (m/f)	**0/0**	**11/18**	**55/83**	**274/429**
Nose cavity: squamous cell carcinoma (m/f)	0/0	0/0	0/0	3/7**
Nose cavity: esthesioneuroepithelioma (m/f)	0/0	0/0	0/0	1/1
Nose cavity: rhabdomyosarcoma (m/f)	0/0	0/0	0/0	1/0
Nose cavity: sarcoma (not otherwise specified) (m/f)	0/0	0/0	0/0	2/0
Liver: hepatocellular adenoma	3/3	4/1	7/6	32**/48**
Liver: hepatocellular carcinoma	0/0	0/0	0/0	14**/10**
Liver: combined hepatocellular adenoma and carcinoma	3/3	4/1	7/6	39**/48**

Peritoneum: mesothelioma	2/1	2/0	5/0	28**/0
Mammary gland: fibroadenoma or adenoma	1/8	2/8	2/11	6/18*
Subcutis: fibroma	5/0	3/2	5/1	12/0

Fischer exact test: * p≤0.05, ** p≤0.01

In addition, Crj:BDF1 mice (50/sex/dose level) were similarly exposed in the drinking water to 0, 500, 2000, or 8000 ppm of 1,4-dioxane (Kano *et al.,* 2009b). The doses were equivalent to approximately 0, 49, 191, or 677 mg/kg bw/d (males) and 0, 66, 278, or 964 mg/kg bw/d (females). General toxicity: significantly decreased survival rates at 191/278 mg/kg bw/d and 677/964 mg/kg bw/d (29/50). Significantly retarded growth rates and terminal body weights at 191/278 and 667/964 mg/kg bw/d in males and females. Relative liver weight significantly increased at 667/964 mg/kg bw/d in males and females and at 191 mg/kg bw/d in males; significantly decreased food and water consumption at 667/964 mg/kg bw/d in males and females. Neoplastic lesions: significant induction of hepatocellular adenomas and carcinomas in both sexes. Two nasal tumours were observed in the highest dose group, one adenocarcinoma in females and one esthesioneuroepithelioma in males.

Details on tumour incidences for mice in the study by Kano *et al.* (2009b) are shown in the tables below.

***Table**: Tumour incidences in **mice** (Kano et al., 2009b)*

Doses (mg/kg bw/d) (m/f)	**0/0**	**49/66**	**191/278**	**677/964**
Nose cavity: adenocarcinoma (m/f)	0/0	0/0	0/0	0/1
Nose cavity: esthesioneuroepithelioma (m/f)	0/-	0/-	0/-	1/-
Liver: hepatocellular adenoma (m/f)	9/5	17/31**	23**/20**	11/3
Liver: hepatocellular carcinoma (m/f)	15/0	20/6*	23/30**	36**/45**
Liver: combined hepatocellular adenoma and carcinoma (m/f)	23/5	31/35**	37**/41**	40**/46**

Fischer exact test: * p≤0.05, ** p≤0.01

In the study by Kociba *et al.*, (1974) rats (Sherman) were exposed to 0, 10/19, 94/148, 1015/1599 mg/kg bw/d (m/f) in drinking water for 716 days. Body weights were significantly lower in high dose animals compared to control animals. A severe reduction in survival rate were seen in the high dose animals during the first 4 months (66/120, p <0.05). After 4 months the survival rate was the same for all groups. The liver weight and liver/body weight ratio were significantly increased in the high dose group. Gross and histopathological examination revealed variable degrees of renal tubular epithelial and hepatocellular degeneration and necrosis, accompanied by regenerative activities in liver (hepatocellular hyperplastic nodule formation) and renal tubuli in rats in the mid and high dose groups. Treatment related hepatocellular carcinomas and nasal squamous cell carcinomas in high dose group was reported, see table below.

***Table**: Tumour incidences in rats (males and females combined) (Kociba et al., 1974)*

Doses (mg/kg bw/d) (m/f)	0/0	10/19	94/148	1015/1599
Nose cavity: squamous cell carcinoma	0	0	0	3***
Liver: hepatocellular carcinoma	1	0	1	10**
Liver: hepatic tumours, all types	2	0	1	12*

Fisher exact probability test: *p=0.00022, **p=0.00033, ***p=0.05491

In the study by NCI (1978) rats (Osborne-Mendel) and mice (B6C3F1) were exposed to 1,4-dioxane for 110 and 90 weeks respectively. As regards rats, 35 animals per dose group were exposed to 0, 240/350, 530/640 mg/kg bw/d for males/females. The survival ratio was 33/35

for males in the high dose group and 26/35 in the low dose group. For females the survival ratio was 29/35 in the high dose group and 30/35 in the low dose group. Histopathology revealed vacuolar degeneration and/or tubular epithelial regeneration on the proximal cortical tubules and occasional hyaline casts in the kidneys. An increased incidence of hepatocytomegaly were observed in females. Gastric ulcers were seen in treated males. The incidence of pneumonia was increased in the high dose females compared to the controls. A statistically significant increase in nasal squamous cell carcinomas were observed in males in the high dose group and in both dose groups for females. Hepatocellular adenomas were statistically significantly increased in females in the high dose group (see table below).

***Table**: Tumour incidences in rats, 35 animals/dose group (NCI, 1978)*

Doses (mg/kg bw/d) (m/f)	**0/0**	**240/350**	**530/640**
Nose cavity: adenocarcinoma (m/f)	0/0	1/0	3/1
Nose cavity: squamous cell carcinoma (m/f)	0/0	12/10***	16***/8****
Nose cavity: rhabdomyoma (m/f)	0/-	1/-	0/-
Liver: hepatocellular adenoma (m/f)	2/0	2/10	1/11**
Liver: hepatocellular carcinoma (m/f)	0/-	1/-	0/-
Testis/epididymis: mesothelioma (m/f)	2/-	4/-	5/-

Fisher exact test: *p≤0.05, **p≤0.01, ***p≤0,001, ****p=0.003

As regards mice, 50 B6C3F1 mice per dose group were exposed to 1,4-dioxane at dose levels of 0, 720/380, 830/860 mg/kg bw/d (males/females). The survival rates were for males: 45/50 in the high dose group and 46/50 in the low dose group, while for females: 28/50 in the high dose group and 39/50 in the low dose group. Non-neoplastic lesions that were significantly increased included pneumonia in males/females (2%/2%, 18%/70%, 36%/89% in control, low dose group and high dose group) and rhinitis in females (0%, 14%, 21% in control, low dose group and high dose group). A statistically significant induction of hepatocellular adenomas or carcinomas were observed in the low dose group as well as the high dose groups for both males and females (see table below).

Table: Tumour incidence in mice (males/females) 50 animals/dose group (NCI, 1978)

Doses (mg/kg bw/d)	**0/0**	**720/380**	**830/860**
Nose cavity: adenocarcinoma	0/0	0/1	1/0
Liver: hepatocellular carcinoma	2/0	18***/12***	24***/29***
Liver: hepatocellular adenoma or carcinoma	8/0	19****/21***	28***/35***

Fisher exact test: *p≤0.05, **p≤0.01, ***p≤0,001, ****p=0.014

Three studies of lower quality were also evaluated by the DS. A 13-month study with 30 male/dose SD rats at doses of approximately 0, 430, 574, 803 and 1032 mg/kg bw/d showed a slight increase in squamous cell carcinomas in the nasal cavity (incidence with increasing dose; 0/30, 1/30, 1/30, 2/30 and 2/30) (Hoch-Ligeti *et al.*, 1970). In a 63 week study by Argus *et al.*, (1965) 26 male Wistar rats were exposed via drinking water to approximately 640 mg/kg bw/d. An increase in liver tumours were observed (0/9 in control and 6/26 exposed). In the third study, Osbourne rats and B6C3F1 mice were exposed in drinking water (0.5 and 1%) and diet (1%) for 42 weeks. In this study no neoplastic lesions were observed (King *et al.*, 1973).

Dose range finding studies

Two dose range finding studies with 1,4-dioxane of relevance for the classification for carcinogenicity were included in the CLH report. In a study by Kasai *et al.* (2008) rats

(F344/DuCrj) were exposed to 1,4-dioxane by inhalation at doses of 0, 100, 200, 400, 800, 1600 and 3200 ppm (corresponding to 0, 360, 720, 1440, 2880, 5760, 11520, 23040 mg/m^3) for 13 weeks. The study was performed according to OECD TG 413. In the study by Kano *et al.* (2008) rats (F344/DuCrj) and mice (Crj:BDF1) were exposed to 1,4-dioxane in drinking water at doses of 0, 52/83, 126/185, 274/427, 657/756, 1554/1614 mg/kg bw/d for male/female rats and 0, 86/170, 231/387, 585/898, 882/1620, 1570/2669 mg/kg bw/d for male/female mice. In both studies nuclear enlargement was observed in several epithelial tissues along the respiratory tract (olfactory, respiratory, tracheal and bronchial). However, these studies reported a difference in the location of the 1,4-dioxane induced enlarged nuclei of the nasal epithelial cells between oral exposure and inhalation (Kasai *et al.* 2008). After inhalation the enlarged nuclei in the respiratory epithelia expanded from the anterior region to cover also the posterior region. Oral administration in drinking water resulted in nuclear enlargement over the entire region of the respiratory epithelium without any anterior–posterior gradient. Centrilobular swelling of hepatocytes was observed at lower estimated body doses via the oral exposure (≥126 mg/kg bw/d) compared inhalation (3200 ppm corresponding to an estimated 2336 mg/kg bw/d). Other histopathological findings in the liver included single cell necrosis and GST-P positive liver foci at predominantly high doses. The histopathological changes seen in liver and nasal cavity in these two studies are in line with the observed carcinomas in the 2-year carcinogenicity studies by Kano *et al.* (2009a and 2009b) and Kasai *et al*. (2009).

Human data

The three epidemiological studies included in the CLH report are all of limited power and show no indications of carcinogenicity. Hence they do not support classification in category 1A.

Weight of evidence assessment

Tumour type and background incidence

Increased incidences of peritoneal mesothelioma, hepatocellular adenoma/carcinoma, squamous cell carcinoma (nasal cavity) was reported following exposure to 1,4-dioxane. However, no appropriate historical control data are available for the assessment of background incidences. These tumour types are considered relevant for humans. However, the mouse strain used in Kano *et al.* (2009b) is considered to be a sensitive specie for induction of liver adenomas/carcinomas.

Multi-site responses

Yes, tumors were reported in liver, nasal cavity and peritoneum.

Progression of lesions to malignancy

Yes, it was evident from the data in mice and rats that liver adenomas were progressing to carcinomas (Kano *et al.*, 2009b). The peritoneal mesothelioma and squamous cell carcinoma (nasal cavity) are considered as malignant tumours.

Reduced tumour latency

No information is available on tumour latency.

Whether responses are in single or both sexes

Hepatocellular adenomas/carcinomas and squamous cell carcinoma in the nasal cavity were observed in both males and females. Peritoneal mesothelioma was only observed in male rats.

Whether responses are in a single species or several species

Hepatocellular adenomas/carcinomas were observed in rats and mice.

Structural similarity to a substance(s) for which there is good evidence of carcinogenicity

No information available.

Routes of exposure

Routes of exposure of relevance for humans are used in the animal studies (inhalation and oral exposure).

Comparison of absorption, distribution, metabolism and excretion between test animals and humans

Limited information on the comparison of absorption, distribution, metabolism and excretion between test animals and humans are available. However, two studies showed that 1,4-dioxane was rapidly and extensively absorbed, metabolised and excreted after inhalation exposure to 4 and 18 healthy human volunteers (Young *et al.*, 1977, Göen *et al.*, 2016). This was also shown in mice following inhalation and oral exposure (Sweeney *et al*. 2008). Dermal absorption occurs, but it is low, probably due to evaporation of the material (ECETOC 1983).

The possibility of a confounding effect of excessive toxicity at test doses

In rats a single oral dose of 100 or 1000 mg/kg bw saturated the metabolism, while in mice saturation of metabolism seems to occur above 200 mg/kg bw. The saturation of metabolism in rats and mice have been questioned in relation to the relevance of liver carcinogenicity.

Mode of action and its relevance for humans, such as cytotoxicity with growth stimulation, mitogenesis, immunosuppression mutagenicity

Genotoxicity

Based on the mutagenicity studies and their assessment , RAC concluded the substance is not mutagenicity. Therefore, it is unlikely that a genotoxic mode of action (MoA) plays a role in the observed tumours.

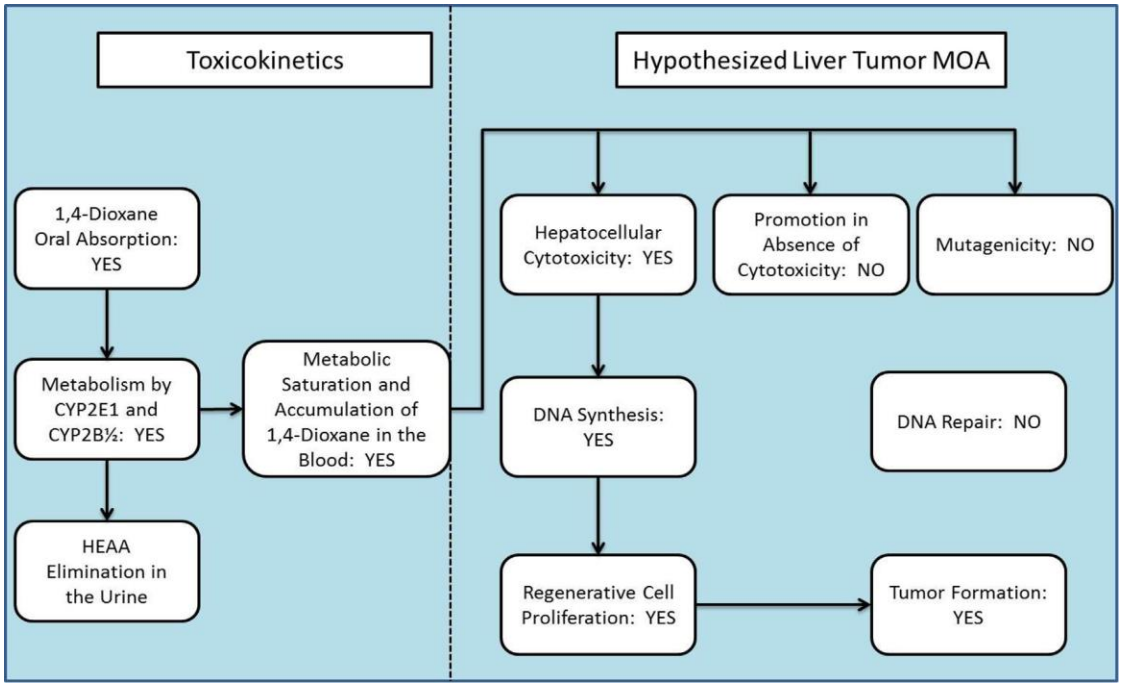
Toxicokinetics

A practical threshold based on saturated metabolism would require knowledge on which entity, 1,4-dioxane or a metabolite, causes the carcinogenic effects. It could be anticipated that 1,4-dioxane is responsible since most of the effects are observed at first pass organs and because of the difference in nasal tumour distribution throughout the respiratory tract in animals exposed via inhalation or via drinking water. However, it has also been reported that one of the metabolites of 1,4 dioxane, 1,4-dioxane-2-one is more toxic (ATSDR 2012) compared to 1,4-dioxane.

Liver tumour mode of action; non-genotoxic regenerative hyperplasia

In the study by Dourson *et al.* (2017) submitted during public consultation, a non-genotoxic regenerative hyperplasia MoA for the induction of liver tumours was discussed. In the figure below this MoA is presented along with its 4 key events;

- (KE1) metabolic saturation of 1,4-dioxane leading to accumulation of 1,4-dioxane in blood,
- (KE2) liver hypertrophy,
- (KE3) hepatocellular cytotoxicity with
- (KE4) regenerative cell proliferation leading to liver tumour formation:



This MoA may be plausible for the induction of liver tumours, but also a non-genotoxic MoA for the induction of tumours is considered relevant for classification. For the induction of the other tumour types reported following exposure to 1,4-dioxane, peritoneal mesothelioma and nasal cavity squamous cell carcinoma, no clear MoA has been postulated. There is also information showing that 1,4-dioxane could be considered as a genotoxic substance at higher dose levels, and toxicity of metabolites cannot be excluded. Therefore, it is considered that no definite conclusions can be made about the MoA for the induction of tumours following exposure to 1,4-dioxane.

In summary

RAC considers that the human data available for 1,4-dioxane do not justify a classification in category 1A.

RAC considers that a classification for 1,4-dioxane as Carc. 1B is warranted based on evidence of carcinogenicity in different tissues observed in two species at reasonable dose levels.

In conclusion: RAC supports the DS proposal that a **classification as Carc. 1B; H350 is justified for 1,4-dioxane.**

Specific concentration limit for carcinogenicity

The carcinogenic potency of 1,4-dioxane has been calculated by the DS according to the T25 concept (EC, 1999).

In the inhalation study by Kasai *et al.* (2009) peritoneal mesothelioma were considered the most sensitive endpoint, and a T25 of 46.6 mg/kg bw/d were calculated.

In the oral study by Kano *et al.* (2009a and 2009b) hepatocellular adenoma and carcinoma were considered as the most sensitive endpoint for rats as well as mice. As regards rats a T25 of 114.3 mg/kg bw/d (females) and 89.4 mg/kg bw/d (males) were calculated. As regards mice a T25 of 18.4 mg/kg bw/d (females) and 92.2 mg/kg bw/d (males).

A carcinogenic substance is considered to be of high potency if the substance has a T25 of less than 1 mg/kg bw/d, medium potency if the substance has a T25 between 1 - 100 mg/kg bw/d, and of low potency if the T25 is above 100 mg/kg bw/d.

1,4-dioxane is considered to be of medium potency based on the calculated T25 values and the selection of the lowest value relevant for human (18.4 mg/kg bw/d). It should be noted that the T25 calculation method is based on assumptions (e.g. linear relationship) and that the calculated value is preliminary. Additional elements may be taken into account to modify the preliminary potency, e.g. to compensate for non-correct assumptions. However, the T25 calculated from the inhalation study were in the same potency range as the one calculated based on the oral study, and both are far from the threshold values for the medium potency. Therefore, the influence of the 'modifier elements', such as a possible non-linear relationship between dose and effect, is expected to be low and not to influence the allocation of 1,4-dioxane in the medium potency category. It should also be noted that the tumour incidence in mice (Kano *et al.*, 2009b) is based on a tumour incidence in the high dose group which were higher than 25% (51-96%) and may therefore be less accurate.

Based on these T25 calculations, **no Specific Concentration Limit is proposed for 1,4-dioxane**.

Additional references

Dourson *et al.* (2017). Update: Mode of action (MOA) for liver tumors induced by oral exposure to 1,4-dioxane. Regulatory Toxicology and Pharmacology, 88: 45-55.

EC (1999) Guidelines for setting specific concentration limits for carcinogens in Annex I of Directive 67/548/EEC

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)..