To: European Chemicals Agency (ECHA) Telakkakatu 6, P.O. Box 400, 00121 Helsinki, Finland *Submitted via the [webform](#) of the [public consultation on the CLH report on dichloromethane](#)*	Chlorinated Solvents REACH Consortium ReachCentrum SA Cantersteen 47, 1000 Brussels, Belgium Company Number: BE 0882.099.291 Phone: +32 2 897 05 06 Email: chlorsolv@reachcentrum.eu Web: https://www.reachcentrum.eu/consortia/chlorinated-solvents-reach-consortium/ 15.11.2023

Response to the proposed harmonised classification of Dichloromethane (CAS no.: 75-09-2, EC no.: 200-838-9) as Carcinogen Category 1B and Mutagen Category 2

Dear Madame/Sir,

Please find herewith the comments to the public consultation on the proposal for harmonised classification and labelling (the CLH proposal) of dichloromethane (DCM), submitted by ReachCentrum on behalf of the members of the Chlorinated Solvents REACH Consortium and all co-registrants of the joint submission dossier of DCM.

The response is divided into 2 parts:

- Part 1: Response to the Carcinogen Category 1B classification proposal (p 2-14)
- Part 2: Response to the Mutagen Category 2 classification (p 15-22)

For any questions we remain to your disposal.

With kind regards,

[redacted]

General Manager
ReachCentrum SA

reachcentrum
an ERM Group company

Part 1

Response to the proposed harmonised classification of DCM as Carcinogen Category 1B

1. Background

On 21/07/2023 the Italian National Institute of Health on behalf of Ministry of Health (the "Dossier submitter" or "DS") submitted a proposal for harmonized classification and labelling (the "CLH proposal") for dichloromethane (DCM, also methylene chloride) to the ECHA RAC (see: https://www.echa.europa.eu/documents/10162/e58d1679-55f4-0851-1a4d-5385f5ce9059).

In this CLH proposal data on carcinogenicity of DCM are summarised in Table 15 for studies in experimental animals and in Table 16 for studies in humans. A summary and discussion of the overall relevance of the provided information is presented in Section 10.9.1 for both human and animal data. In the subsequent Section 10.9.2 short summaries of the human and animal data are compared with the CLP criteria for the various carcinogenicity categories. Section 10.9.3 provides the overall conclusion of the DS on classification and labelling for carcinogenicity, stating:

> "*Based on the results of the carcinogenicity studies available, there are limited evidence in human studies and sufficient evidence of DCM carcinogenicity in mice and rats. There is extensive evidence for genotoxicity, in association with metabolic pathways that are operative in humans, considering that the metabolic differences between species, organs, tissues and cells are quantitative but not qualitative. Overall, the available experimental evidence suggests that the mode of action of the carcinogenesis reported in animals is relevant for human.*
>
> *Based on the overall information the DS concludes that a classification as Carc 1B, H350 is warranted*."

As further demonstrated in detail below, however, this overall conclusion is not supported based on the available studies, information and analysis included in the CLH proposal. It does also not comply with the CLP requirements, which allow to classify a substance as carcinogen in category 1B.

2. Summary Consortium Assessment and Response

The DS proposes to classify DCM as carcinogen in category 1B based on
(i) limited evidence for carcinogenicity in human studies, and
(ii) sufficient evidence of DCM carcinogenicity in mice and rats, as observed in the available animal studies.
However, it fails to establish that these two types of evidence are indeed available in the present case. In particular:

1) According to Section 3.6.2.2.3(a), second indent of Annex I CLP limited evidence for carcinogenicity in human studies would exist when *"a positive association has been observed between exposure to the agent and cancer for which a causal interpretation is considered to be credible ..."*. This condition is however not met in the present case.

Firstly, the human epidemiological data relied upon by the DS does not support a credible positive association between DCM and cancer. In particular:

- Alleged positive association between DCM and cancer of the biliary tract is observed only in the first study by the group of Lanes et al. (1990). However, the authors concluded that the implications are "unclear". In a follow-up study, by the same group of investigators, that extended the follow up period to increase sensitivity, the association weakened making the presumed causal relationship implausible.
- Several other studies, with equal or greater number of subjects, do not report significant cancer effects (Gibbs et al., 1996; Hearne & Pifer, 1999; Tomenson et al., 2011)
- The German MAK Commission considered that the association between DCM and blood cancer is a "chance finding"

Secondly, the CLH proposal repeatedly states that it supports the evaluation of DCM based on IARC's Monograph 110. However, this evaluation took place in June 2014 and the alleged association between DCM and cancer was mainly based on the observations in a number of Japanese studies. Since then subsequent publications from the same group of investigators have shown that the observed carcinogenicity in the available human studies is due to exposure to 1,2 DCP (which is now classified as a known human carcinogen by IARC) rather than to exposure to DCM. Therefore, a positive association between DCM and cancer cannot be considered "credible" based on IARC 110. The human data cannot support the classification of DCM as carcinogen in category 1B.

2) Section 3.6.2.2.3(b), first indent of Annex I CLP also provides that the sufficient evidence of carcinogenicity in animal studies should be such to establish a *"causal relationship"* between DCM and the increased incidence of tumours development. The CLH proposal also fails to "establish" such a relationship. Instead, as explained in detail below, it is mostly based on incidental findings and confounding results observed only in mice in the condition of excessive toxicity at test doses. They have not been confirmed - even at high concentrations - in rats and hamsters. This cannot support the classification of DCM as carcinogen in category 1B.

3) CLP also provides in Section 3.6.1.1 of Annex I that the classification as a carcinogen can be based on animal studies *"unless there is strong evidence that the mechanism of tumour formation is not relevant for humans*". In this regard, the CLH proposal fails to observe that the available toxicokinetic data show that in the positive findings in studies with mice depend on a mode of action which - in practice - cannot be replicated in humans.

The substantial species differences in metabolism and kinetics between mice, rats and hamsters correlate well with the different observations of the carcinogenic potential of DCM, and explain the tumour formation in the liver and lung of mice exposed to very high concentrations of DCM, as well as the absence of DCM-induced formation in rats and hamsters. Considering that the glutathione-dependent pathway in humans is less active than even in the rat and the hamster, the carcinogenicity data from studies in the mouse are not relevant for the human situation as the relevant MoA is not relevant in humans under comparable conditions.

Furthermore, the CLP also provides that *"beyond the determination of strength of evidence for carcinogenicity, a number of other factors need to be considered that influence the overall*

likelihood that a substance poses a carcinogenic hazard in humans" (Section 3.6.2.2.4). These include, in particular, the *"possibility of a confounding effect of excessive toxicity at test doses"* (Section 3.6.2.2.6). The CLH proposal fails to take into account that the available animal studies only showed clear carcinogenicity at very high concentrations in mice but not in rats and hamsters, and that these differences can be fully explained by differences in enzymatic make-up and other aspects of the toxicokinetics. In addition, it was shown that the conditions under which carcinogenic responses are triggered in mice cannot be met in humans and that these results could therefore be considered confounding.

3. Detailed Consortium Assessment and Response

As correctly noted on page 72-76 of the CLH proposal, the CLP Regulation allows classifying a substance as a carcinogen is based on the following criteria:

for Carcinogenicity Category 1A:

- *sufficient evidence of carcinogenicity: a causal relationship has been established between exposure to the agent and human cancer. That is, a positive relationship has been observed between the exposure and cancer in studies in which chance, bias and confounding could be ruled out with reasonable confidence;*
- *limited evidence of carcinogenicity: a positive association has been observed between exposure to the agent and cancer for which a causal interpretation is considered to be credible, but chance, bias or confounding could not be ruled out with reasonable confidence.*

for Carcinogenicity Category 1B:

- *sufficient evidence of carcinogenicity: a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single species in a well conducted study, ideally conducted under Good Laboratory Practices, can also provide sufficient evidence. A single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites;*
- *limited evidence of carcinogenicity: the data suggest a carcinogenic effect but are limited for making a definitive evaluation because, e.g. (a) the evidence of carcinogenicity is restricted to a single experiment; (b) there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the studies; (c) the agent increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential; or (d) the evidence of carcinogenicity is restricted to studies that demonstrate only promoting activity in a narrow range of tissues or organs.*
- *Some important factors which may be taken into consideration, when assessing the overall level of concern are:*
  (a) tumour type and background incidence;
  (b) multi-site responses;
  (c) progression of lesions to malignancy;
  (d) reduced tumour latency;
  (e) whether responses are in single or both sexes;

(f) whether responses are in a single species or several species;
(g) structural similarity to a substance(s) for which there is good evidence of carcinogenicity;
(h) routes of exposure;
(i) comparison of absorption, distribution, metabolism and excretion between test animals and humans;
(j) the possibility of a confounding effect of excessive toxicity at test doses;
(k) mode of action and its relevance for humans, such as cytotoxicity with growth stimulation, mitogenesis, immunosuppression, mutagenicity.
Mutagenicity: it is recognised that genetic events are central in the overall process of cancer development. Therefore evidence of mutagenic activity in vivo may indicate that a substance has a potential for carcinogenic effects.

With regard to the classification of DCM as carcinogen, the DS concluded that:

1. "*the assessment of the currently available pertinent epidemiological studies supports the evaluation of the IARC (IARC, 2017) that "there is limited evidence in humans for the carcinogenicity of dichloromethane". The DS conclusion is mainly based on the confirmed positive association between DCM exposure and cancer of biliary tract, and, at less extent, on evidence concerning non-Hodgkin lymphoma. Thus, a classification as Carc. 1A is not appropriate for DCM." and*
2. *"The classification in the category 1B is based on limited evidence in human studies and sufficient evidence in animal studies with a MoA relevant to humans. DS supports the evaluation of IARC (IARC, 2017) that "there is limited evidence in humans for the carcinogenicity of dichloromethane". These evidence are mainly based on two types of tumours: cancer of biliary tract, and, at less extent, on evidence concerning non-Hodgkin lymphoma."*

However, the CLH proposal fails to comply with the above requirements, because both conclusions are untenable considering all available data:

Conclusion 1

The 'confirmed positive' association between DCM exposure and cancer of the biliary tract was only reported by the group of Lanes and co-workers. In their initial study (Lanes et al., 1990), they found an SMR for biliary tract cancer of 5.75 (95% CI: 1.82-13.78) but concluded that the implications were unclear. In a follow-up study (Lanes et al., 1993) that extended the follow-up period of the previous study to increase sensitivity, the SMR dropped to 2.98 (95% CI: 0.81-7.63). Hence, in a more sensitive study the unclear association weakened and makes the presumed association less likely to be causal. The authors concluded that the wide confidence interval (that contains 1.0) indicate that the small excess of cancer observed is statistically unstable.

Importantly, in the classification proposal by the DS it is remarked for several other cohort studies with about an equal or even a larger number of subjects than the Lanes et al. studies - but which did not report significant cancer effects (Gibbs et al., 1996; Hearne & Pifer, 1999; Tomenson, 2011) - that the number of subjects was so low that it hampered proper analysis of exposure-response patterns. The DS failed to draw any conclusions from these findings that only further show that the initial study (Lanes et al., 1990) cannot be used to support the DS's conclusion.

In the classification proposal it is stated repeatedly that it supports the evaluation of DCM by IARC (IARC Monograph #110). However, this evaluation took place in June 2014 and covers only publications until early 2014. In the decade since this evaluation, numerous publications became available pointing out that co-exposure to 1,2-dichloropropane (1,2-DCP, which is classified as a Category 1 human Carcinogen by IARC and ECHA), is responsible for the observed carcinogenicity in studies investigating the carcinogenic potential of DCM in humans. The classification proposal puts a lot of emphasis on the various reports from a Japanese group of researchers investigating printing workers (Kumagai et al., 2013, 2014a, 2014b, 2016; Kubo et al., 2014, Yamada et al., 2014, 2015a, 2015b). However, virtually all subjects in these studies were co-exposed to 1,2-DCP. The few subjects that were not exposed to 1,2-DCP, were exposed to other halogenated solvents. In the initial reports by this research group, that were available in 2014, the suggestion was raised that both 1,2-DCP and DCM might be responsible for the observed cholangiocarcinomas. However, in subsequent reports, published after the IARC evaluation of DCM, the association with 1,2-DCP was established which led IARC decide to classify 1,2-DCP as a human carcinogen (Category 1). More importantly, in the last reports of the series, the authors not only conclude that cumulative exposure to 1,2-DCP is significantly associated with an increase of the relative risk of developing cholangiocarcinoma but also that DCM exposure is not significantly associated with the development of cholangiocarcinoma (Kumagai et al., 2016).

In addition, the same group of investigators showed that mice exposed to 1,2-DCP induced CYP450-dependent proliferation and apoptosis of cholangiocytes (Zhang et al., 2018), which is in line with the observed time- and dose-dependent genotoxicity of 1,2-DCP as demonstrated through the H2AX assay in cultured human hepatocytes and cholangiocytes (Toyooka et al., 2017) thus supporting a carcinogenic MoA of 1,2-DCP. In line with these findings, Suzuki and co-workers found that 1,2-DCP but not DCM showed a genotoxic response in the liver of mice exposed to DCM by inhalation (Suzuki et al., 2014).

In addition, as the German MAK commission already concluded, the association of DCM exposure with non-Hodgkin lymphoma (NHL) is most likely a chance finding (MAK, 2015). Finally, as also noted by the MAK commission, there is no concordance between the various study outcomes that suggest causality between carcinogenicity and exposure to DCM.

Hence, the overall conclusion must be that the available human data do not support a classification as Carcinogen Cat 1A, but also not as Carcinogen Cat 1B. Therefore, regarding the alleged limited evidence for carcinogenicity in humans, based on the studies available to date, the DS fails to demonstrate that the data allow establishment of a credible causal association between the exposure to DCM and cancer.

Conclusion 2

As explained above, the '*limited evidence in human studies*' is essentially non-existent, which leaves the alleged '*sufficient evidence in animal studies with a MoA relevant to humans'* as the main support for a possible classification as a Cat 1B carcinogen.

DCM is a very data-rich substance and not only are several studies available on the carcinogenicity of DCM in various species (mouse, rat, hamster) but also with a variety of exposure routes and concentration levels. In addition, detailed information is available on metabolism and kinetics in various species. The available information allows to compare the available studies and assess the implications of the applied concentrations and route of exposure on the operative MoA, covering (e), (f), (h), (i), (j) and (k) of the '*important factors which may be taken into consideration, when assessing the overall level of concern'* as indicated above for classification as Carcinogen Cat 1B based on animal data.

In a reliable two-year carcinogenicity study, Serota et al. (1986b) dosed male and female mice with DCM via drinking water with doses up to 238 mg/kg body weight/day and found no excess of cancer incidence as the incidence of tumours at the highest dose levels was in the range of the historical controls. Another study in mice with oral gavage of DCM (Maltoni et al., 1988) was of insufficient quality (Klimisch score of 3) to draw any reliable conclusions.

In a reliable two-year carcinogenicity study Serota et al. (1986a) also dosed male and female rats with DCM via drinking water with doses up to 269 mg/kg body weight/day and, again, found no dose-related excess of cancer incidence.

Studies with intra-peritoneal administration are nowadays considered to be less informative for hazard and risk assessment because the kinetics, MoA and bioavailability cannot easily be compared to those obtained by other, more relevant routes of exposure (Das & North, 2007). Nevertheless, it is worth noting that in a study in mice intraperitoneal administration of DCM at dose levels up to 800 mg/kg body weight, 3 times per week for 24 weeks, no tumours of the respiratory tract were found (Theiss et al., 1977).

Several inhalation studies with DCM to investigate its carcinogenic potential were performed in mice, rats and hamsters. Both positive and negative results were reported in mice and rats, but only negative results in hamsters. Since studies in mice and rats were performed at various concentration levels the influence of kinetics and the MoA can be assessed.

In the only 2-year inhalation study in hamsters, the animals were exposed to 500, 1500 and 3500 ppm, 6 h/day, 5 days/week. These exposure concentrations correspond to daily doses of 640, 1920 and 4480 mg/kg body weight (assuming 60 g hamsters, a minute-volume inhalation of 61 ml/min, and 100% inhalation absorption), respectively. Despite the fact, that the highest two exposure levels exceeded the maximum dose level of 1000 mg/kg bw/day recommended by ECHA for oral repeated-dose studies, no significant carcinogenic response was observed. In fact, the positive results at the highest concentration in females (for both benign and malignant tumours), which are mentioned in the classification proposal, were not statistically significant when corrected for the survival according to the investigators (Burek et al., 1984).

In a 2-year inhalation study in rats, the animals were exposed to 50, 200 and 500 ppm of DCM for 6 h/day, 5 days/week, corresponding to 61, 242 and 605 mg/kg bw/day (assuming 250 g rats, a minute-volume inhalation of 250 ml/min, and 100% inhalation absorption), respectively. Apart from a slight deficit (!) in mammary gland tumours in the mid-concentration group of females, no effects on tumour incidence were found in either males or females (Nitschke et al., 1988).

In another 2-year inhalation study in rats, the animals were exposed to much higher exposure levels of 1000, 2000 and 4000 ppm, corresponding to 1210, 2420 and 4840 mg/kg bw/day (assuming 250 g rats, a minute-volume inhalation of 250 ml/min, and 100% inhalation absorption), respectively (Mennear et al., 1988). In this study an excess of mammary gland adenomas/fibroadenomas was observed in the highest concentration group in both male and female animals. In a similar 2-year inhalation study the rats were exposed to 500, 1500 and 3500 ppm of DCM for 6 h/day, 5 days/week, corresponding to 605, 1815 and 4235 mg/kg/day, respectively (Burek et al., 1984). In this study, no mammary gland tumours were observed in either male or female rats. However, in male rats of the highest dose group a significant increase in salivary gland carcinomas was observed.

In two 2-year inhalation studies in mice, the animals were exposed to concentrations of 2000 and 4000 ppm (Mennear et al., 1988) or to 1000, 2000 and 4000 ppm of DCM (Aiso et al., 2014) for 6 h/day, 5 days/week, corresponding to 1840, 3680 and 7360 mg/kg bw/day (assuming 25 g mice, a minute-volume inhalation of 1.46 ml/min, and 100% inhalation absorption),

respectively. In the study by Mennear and co-workers (1988), only the two highest exposure levels were applied, and an excess of broncheo-alveolar carcinomas and hepatocellular carcinomas was found in both males and females. In the study by Aiso and co-workers (2014) broncheo-alveolar carcinomas were found in males of the mid- and high-concentration groups and in females of the high-concentration group, whereas hepatocellular carcinomas were only found in males and females of the high-concentration group. In addition, a significantly higher incidence of haemangiomas was found in males of the highest concentration group. No excess of hepatocellular carcinomas was found in the 1000 ppm DCM concentration group (incidence equal or lower than controls) in both male and females.

The difference between the carcinogenic response following oral and inhalation exposure and between mice on the one hand and rats and hamsters on the other hand are striking and highly informative regarding the relevant MoA.

As described in the classification proposal, DCM is primarily metabolised by oxidative transformation catalysed by microsomal CYP2E1. However, this pathway gets saturated at higher concentrations at which a reductive pathway involving glutathione conjugation, catalysed by GSTT-1, becomes operative. This pathway leads to a reactive intermediate, S-(chloromethyl)glutathione which is genotoxic (MAK, 2015).

In the carcinogenicity studies with oral and intraperitoneal administration in mice and rats no significant increase in tumour incidences were found, which can be explained by the fact that these studies had maximum exposure levels of 800 mg/kg bw which is insufficient to trigger a carcinogenic response via the glutathione pathway.

In the available carcinogenicity studies with inhalation exposure, no tumours were seen in the hamster. In the rat an inconsistent pattern of tumours was seen, but only at exposure concentrations of 3500 ppm or higher. In mice no tumours were observed at exposure concentrations of 1000 ppm, but tumours were seen at higher exposure levels (2000 and 4000 ppm).

DCM is a data rich compound, and its metabolism has been well studied in several species. The observed differences in carcinogenic response between species and concentration/dose levels can be well explained with the available toxicokinetic and metabolic information, and be used to inform the actual carcinogenic risk for humans.

As described in the CLH proposal by the DS, there are two competitive pathways for the metabolism of DCM. The first pathway is via oxidation by cytochrome P450 (CYP2E1) yielding formylchloride which is either further metabolised to carbon monoxide or can conjugate with glutathione and subsequently further processed to carbon dioxide. The carbon monoxide may be further oxidised to carbon dioxide or react with hemoglobin, yielding COHb. In humans the formation of COHb is linear with exposures to DCM up to an order of magnitude over the current occupational exposure limits (DiVincenzo & Kaplan, 1981). As indicated above, the other pathway is conjugation of DCM itself with glutathione, catalysed by GSTT1. The reaction product, S-(chloromethyl)glutathione is highly reactive and will readily react with nucleophiles. In aqueous environments it is very short lived and forms S-(glutathionyl)methanol which is further processed, via formaldehyde and formic acid, to carbon dioxide but typically enters the C1-intermediate metabolism. Formaldehyde does not seem to play a role as genotoxic agent following exposure to DCM because, as already stated in the classification proposal, no cross-links or DNA damage were observed. However, apart from hydrolysis, the S-(chloromethyl-)glutathione may also react with cellular macromolecules and has therefore been considered the actual genotoxic metabolite responsible for the tumours observed at high concentrations of DCM.

Although the metabolism of DCM is qualitatively similar across species, there are substantial quantitative differences which are based on the species differences in CYP2E1 and GSTT.

As described in the classification proposal, the CYP2E1 pathway is predominant at lower concentrations since it is a high affinity/low capacity enzyme. Although the classification proposal states that it can be 'easily saturated', it has been shown that saturation only occurs at exposure levels greater than 500 ppm (Andersen et al., 1987), that is at least an order of magnitude higher than the current occupational exposure limits. In contrast, the GSTT-1 pathway is of low affinity/high capacity. Essentially, the balance between the CYP2E1 and GSTT-1 pathways is determined by the capacity and distribution of the CYP2E1 pathway in combination with the activity and distribution of the GSTT-1 pathway. This balance is distinctly different amongst species and, as a consequence, the relative contributions of both pathways in the metabolism of DCM are both highly dose- and species dependent (Andersen et al, 1987; 1991; Reitz et al., 1988; 1989; Pemble et al., 1994; Slikker et al., 2004, Dekant et al., 2021). Overall, and as also stated in the classification proposal by the DS, any genotoxicity of DCM would be associated with GSTT-based metabolism consistent with the formation of reactive metabolites through this pathway. There is, however, no evidence for any role of CYP450-dependent genotoxicity.

All pharmacologically based kinetic models support a distinct species difference in the kinetics of DCM with a non-linear formation of glutathione conjugates in rodents with the CYP2E1 being dominant up to inhalation concentrations of 500 ppm. At higher concentrations, a clear difference is seen as in mice the production of glutathione conjugates, particularly in the lungs and the liver, increases sharply compared to rats (Andersen et al., 1987;1991). This is explained by the fact that mouse lung and liver show almost 4 and 7-fold higher GSTT1 activity than rat lung and liver, respectively. Considering that the distribution and activity of human GSTT1 is very different than found in rodents, with much lower activity than even rat in both lungs and liver, it is not expected that significant levels of the glutathione metabolite can be formed in humans (Sherratt et al., 1997; 2002). Moreover, unlike rodents, human erythrocytes have GSTT1 activity and glutathione conjugation of DCM metabolites in the blood will even further decrease the presence of these metabolites in lung and liver (Pemble et al., 1994). Although intra-individual differences may exist for CYP2E1 activity in humans, it was shown with actual data obtained from human volunteers exposed to DCM that the actual variability is limited (Sweeney et al., 2004).

4. Overall Conclusion on Classification for Carcinogenicity

Considering that, according to Section 3.6.2.2.3(a), second indent of Annex I of the CLP Regulation, limited evidence for carcinogenicity in human studies would exist when "a positive association has been observed between exposure to the agent and cancer for which a causal interpretation is considered to be credible ..." it must be concluded that this condition is not met in the present case.

In addition, according to Section 3.6.2.2.3(b), first indent of Annex I of the CLP Regulation, sufficient evidence of carcinogenicity in animal studies should be such that a "causal relationship" between DCM and the increased incidence of tumour development can be "established". However, the CLH proposal fails to establish such a relationship. Instead, it is mostly based on incidental findings and confounding results observed under conditions of excessive toxicity at test doses. As a consequence, the conditions, as laid out in the CLP Regulation, to support the classification of DCM as carcinogen in category 1B are not met in the present case.

In conclusion, the substantial species differences in metabolism and kinetics correlate well with the different observations of the carcinogenic potential of DCM and explain the tumour formation in the liver and lung of mice exposed to high concentrations of DCM as well as the absence of DCM-induced tumour formation, even at high concentrations, in rats and hamsters. Considering that the glutathione-dependent pathway in humans is less active than even in the rat and the hamster, the carcinogenicity data from studies in the mouse are not relevant for the human situation as the relevant MoA is not operational in humans. Hence, a classification as Carcinogen in Category 1B is not warranted based on studies in animals.

Moreover, as stated above, DCM is a data-rich substance, and many relevant studies are available that should be taken into account in a weight-of-evidence approach rather than simply focussing on the studies in mice exposed to very high concentrations of DCM by inhalation. A weight-of-evidence approach clearly shows that the difference between mice on the one hand and rats and hamsters on the other hand can fully be explained by differences in toxicokinetics, especially with regard to enzymatic make up, of these animals. The in vitro studies with human tissues strongly indicate that humans would be even less prone to develop tumours following exposure to DCM than rats and hamsters.

As a consequence, according to the CLP regulation, a classification as Carcinogen Category 2 would be applicable because some positive animal studies have been reported.

In fact, this conclusion is corroborated by both the opinion of the EU Scientific Committee of Consumer Safety (SCCS, 2012) and of the German MAK Committee (2015) that concluded that DCM should be classified in Group 5 considering that DCM might potentially be genotoxic but that its potency is so low that - provided that occupational exposure limits are respected - no significant risk of any contribution to human cancer risk is to be expected.

5. References

Aiso S, Take M, Kasai T, Senoh H, Umeda Y, Matsumoto M, et al. Inhalation carcinogenicity of dichloromethane in rats and mice. *Inhal Toxicol* 2014;**26:**435-451.

Andersen ME, Clewell HJ, Gargas ML, Smith FA, Reitz RH. Physiologically based pharmacokinetics and the risk assessment process for methylene chloride. *Toxicol Appl Pharmacol* 1987;**87:**185-205.

Andersen ME, Clewell HJ, Gargas ML, MacNaughton MG, Reitz RH, Nolan RJ, McKenna MJ. Physiologically based pharmacokinetic modeling with dichloromethane, its metabolite, carbon monoxide, and blood carboxyhemoglobin in rats and humans. *Toxicol Appl Pharmacol* 1991:**108;**14-27.

Arimoto A, Kawada N, Fujikawa M, Fujishima H, Sugawara Y, Tanaka S, Toyokawa H, Kuwae Y, Ohsawa M, Uehara S, Sato KK, Hayashi T, Endo G. Case series of 17 patients with cholangiocarcinoma among young adult workers of a printing company in Japan. *J Hepatobiliary Pancreat Sci* 2014;**21:**479-488.

Burek JD, Nitschke KD, Bell TJ, Wackerle DL, Childs RC, Beyer JE, et al. Methylene chloride: a two-year inhalation toxicity and oncogenicity study in rats and hamsters. *Fundam Appl Toxicol* 1984;**4:**30-47.

Das RG, North D. Implications of experimental technique for analysis and interpretation of data from animal experiments: outliers and increased variability resulting from failure of intraperitoneal injection procedures. *Laboratory Animals* 2007;**41:**312-320.

Dekant W, Jean P, Arts J. Evaluation of the carcinogenicity of dichloromethane in rats, mice, hamsters and humans. *Regul Toxicol Pharmacol* 2021;**120:**104858.

DiVincenzo GD, Kaplan CJ. Uptake, metabolism, and elimination of methylene chloride vapor by humans. *Toxicol Appl Pharmacol* 1981;**59:**130-140.

Green T. Species differences in carcinogenicity:the role of metabolism in human risk evaluation *Teratogenesis, carcinogenesis and mutagenesis* 1990;**10:**103-113.

Green T. Methylene chloride induced mouse liver and lung tumours: an overview of the role of mechanistic studies in human safety assessment. *Human Exp Toxicol* 1997;**16:**3-13.

Hearne FI, Grose F, Pifer JW, Friedlander BR, Raleigh RL. Methylene chloride mortality study: dose response characterization and animal comparison. *J Occup Med* 1987;**29:**217-28.

Hearne FI, Pifer JW, Grose F. Absence of adverse mortality effects in workers exposed to methylene chloride: an update. *J Occup Med* 1990;**32:**234-240.

Hearne FT, Pifer JW. Mortality study of two overlapping cohorts of photographic film base manufacturing employees exposed to methylene chloride. *J Occup Environ Med* 1999;**41:**1154-69.

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, No. 71. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon (FR): International Agency for Research on Cancer, 1999.

IARC monographs on the evaluation of carcinogenic risks to humans ; volume 110. Lyon (FR): International Agency for Research on Cancer. On the identification of carcinogenic hazards to humans: Some chemicals used as solvents and in polymer manufacture, 2016.

Kari FW, Foley JF, Seilkop SK, Maronpot RR, Anderson MW. Effect of varying exposure regimens on methylene chloride-induced lung and liver tumors in female B6C3F1 mice. *Carcinogenesis* 1993;**14:**819-26.

Kubo S, Nakanuma Y, Takemura S, Sakata C, Urata Y, Nozawa A, Nishioka T, Kinoshita,M, Hamano G, Terajima H, Tachiyama G, Matsumura Y, Yamada T, Tanaka H, Nakamori S, Kumagai S, Kurumatani N, Arimoto A, Ichihara G. Cholangiocarcinoma among offset colour proofprinting workers exposed to 1,2-dichloropropane and/or dichloromethane. *Occup Environ Med* 2013;**70:**508-510.

Kumagai S. Two offset printing workers with cholangiocarcinoma. *J Occup Health* 2014a;**56:**164-168.

Kumagai S, Kurumatani N, Arimoto A, Ichihara G. Time Course of Blood Parameters in Printing Workers with Cholangiocarcinoma. *J Occup Health* 2014b;**56:**279-284.

Kumagai S, Sobue T, Makiuchi T, Kubo S, Uehara S, Hayashi T, Sato KK, Endo G., Relationship between cumulative exposure to 1,2-dichloropropane and incidence risk of cholangiocarcinoma among offset printing workers. *Occup Environ Med* 2016;**73:**545-552.

Lanes SF, Cohen A, Rothman KJ, Dreyer NA, Soden KJ. Mortality of cellulose fiber production workers. *Scand J Work Environ Health* 1990;**16:**247-251.

Lanes SF, Rothman KJ, Dreyer NA, Soden KJ. Mortality update of cellulose fiber production workers. *Scand J Work Environ Health* 1993;**19:**426-428.

MAK, 2015, Deutsche Forschungsgemeinschaft (commission for the investigation of health hazards of chemical compounds in the work area): dichloromethane. In: MAK (Ed.), 2015. Supplement 2015; Series: MAK Value Documentation. The MAK Collection for Occupational Health and Safety.

Maltoni C, Cotti G, Perino G. Long-term carcinogenicity bioassays on methylene chloride administered by ingestion to Sprague-Dawley rats and Swiss mice and by inhalation to Sprague-Dawley rats. *Ann N Y Acad Sci* 1988;**534:**352-66.

NTP. NTP toxicology and carcinogenesis studies of dichloromethane (methylene chloride) (CAS No. 75-09-2) in F344/N rats and B6C3F1 mice (inhalation studies). *Natl Toxicol Program Tech Rep Ser* 1986;**306:**1-208.

Olvera-Bello AE Estrada-Muniz E Elizondo G Vega L. Susceptibility to the cytogenetic effects of dichloromethane is related to the glutathione S-transferase theta phenotype. *Toxicol Lett* 2010;**199:**218-224.

Reitz RH. Estimating the risk of human cancer associated with exposure to methylene chloride. *Ann Ist Super Sanita* 1991;**27:**609-614.

Reitz RH, Mendrala AL, Guengerich FP. In vitro metabolism of methylene chloride in human and animal tissues: use in physiologically based pharmacokinetic models. *Toxicol Appl Pharmacol* 1989;**97:**230-246.

Reitz RH, Mendrala AL, Park CN, Andersen ME, Guengerich FP. Incorporation of in vitro enzyme data into the physiologically-based pharmacokinetic (PB-PK) model for methylene chloride: implications for risk assessment. *Toxicol Lett* 1988;**43:**97-116.

SCCS. Scientific Committee on Consumer Safety: Opinion of Dichloromethane. European Commission, Health & Consumers, Directorate D: Health Systems and Products - Risk Assessment, Brussels, 2012.

Serota DG, Thakur AK, Ulland BM, Kirschman JC, Brown NM, Coots RH, et al. A two year drinking- water study of dichloromethane in rodents. I. Rats. *Food Chem Toxicol* 1986a;**24:**951-958.

Serota DG, Thakur AK, Ulland BM, Kirschman JC, Brown NM, Coots RH, et al. A two year drinking- water study of dichloromethane in rodents. II. Mice. *Food Chem Toxicol* 1986b;**24:**959-963.

Sherratt PJ, Pulford DJ, Harrison DJ, Green T, Hayes JD. Evidence that human class Theta glutathione S-transferase T1-1 can catalyse the activation of dichloromethane, a liver and lung carcinogen in the mouse. Comparison of the tissue distribution of GST T1-1 with that of classes Alpha, Mu and Pi GST in human. *Biochem J* 1997;**326:**837-846.

Sherratt PJ, Manson MM, Thomson AM, Hissink EAM, Neal GE, van Bladeren PJ, Green T, Hayes JD. Increased bioactivation of dihaloalkanes in rat liver due to induction of class theta GSTT-T1. *Biochem J* 1998;**335:**619-630.

Sherratt PJ, Williams S, Foster J, Kernohan N, Green T, Hayes JD. Direct comparison of the nature of mouse and human GST T1-1 and the implications on dichloromethane carcinogenicity. *Tox Appl Pharmacol* 2002;**179:**89-97.

Slikker W, Andersen ME, Bogdanffy MS, Bus JS, Cohen SD, Conolly RB, David RM, Doerrer NG, Dorman DC, Gaylor DW, Hattis D, Rogers JM, Setzer RW, Swenberg JA, Wallace K. Dose-dependent transitions in mechanisms of toxicity: case studies. *Toxicol Appl Pharmacol* 2004;**201:**226-294.

Suzuki T, Yanagiba Y, Suda M, Wang RS. Assessment of the genotoxicity of 1,2- dichloropropane and dichloromethane after individual and co-exposure by inhalation in mice. *J Occup Health* 2014;56:205-214.

Sweeney LM, Kirman CR, Morgott DA, Gargas ML. Estimation of interindividual variation in oxidative metabolism of dichloromethane in human volunteers. *Tox Lett* 2004;**154:**201-216.

Thier R, Wiebel FA, Hinkel A, Burger A, Brüning T, Morgenroth K, Senge T, Wilhelm M, Schulz TG. Species differences in the glutathione transferase GSTT1-1 activity towards the model substrates methyl chloride and dichloromethane in liver and kidney. *Arch Toxicol* 1998;**72:**622-629.

Tomenson JA, Bonner SM, Heijne CG, Farrar DG, Cummings TF (1997). Mortality of workers exposed to methylene chloride employed at a plant producing cellulose triacetate film base. *Occup Environ Med* 1997;**54:**470-476.

Tomensen JA. Update of a cohort mortality study of workers exposed to methylene chloride employed at a plant producing cellulose triacetate film base. *Int Arch Occ Env Health* 2011;**84:**889-897.

Toyooka T, Yanagiba Y, Suda M, Ibuki Y, Wang R-S. 1,2-Dichloropropane generates phosphorylated histone H2AX via cytochrome 2E1-mediated metabolism. *Tox Lett* 2017;**272:**60-67.

Yamada K, Kumagai S, Nagoya T, Endo G. Chemical exposure levels in printing workers with cholangiocarcinoma. Tokyo: Occupational Health Research and Development Center, Japan Industrial Safety and Health Association. 2014.

Yamada, K., Kumagai, S., Endo, G. Chemical exposure levels in printing workers with cholangiocarcinoma (2. report). *J Occup Health* 2015a;**57:**245-252.

Yamada, K., Kumagai, S., Kubo, S., Endo, G. Chemical exposure levels in printing and coating workers with cholangiocarcinoma (third report). *J Occup Health* 2015b;**57:**565-571.

Zhang X, Zong C, Zhang L, Garner E, Sugie S, Huang C, Wu W, Chang J, Sakurai T, Kato M, Ichihara S, Kumagai S, Ichihara G. Exposure of mice to 1,2-dichloropropane induces CYP450-dependent proliferation and apoptosis of cholangiocytes. *Tox Sci* 2018;**162:**559-569.

reachcentrum
an ERM Group company

Part 2

Response to the proposed harmonised classification of DCM as Mutagen in Category 2

1. Background

On 21/07/2023 the Italian National Institute of Health on behalf of Ministry of Health (the "Dossier submitter" or "DS") submitted a proposal for harmonized classification and labelling (the "CLH proposal") for dichloromethane (also methylene chloride or DCM) to the ECHA RAC (see https://www.echa.europa.eu/documents/10162/e58d1679-55f4-0851-1a4d-5385f5ce9059).

A summary of the in vitro and in vivo studies evaluated by the DS is presented in the Tables 9 and 10 in Section 10.8 (Germ Cell Mutagenicity) of the CLH proposal. A short summary and discussion of the overall relevance of the provided information and studies on the alleged germ cell mutagenicity of DCM is provided in Section 10.8.1, while Section 10.8.2 provides a comparison of the data with the classification criteria established by CLP. Section 10.8.3 provides the conclusion on classification and labelling for germ cell mutagenicity, the last two paragraphs of which are copied here:

"*The available data demonstrated a clear correlation between the observed genotoxicity in vitro and in vivo and the activity of GST pathway, but a role of P450 metabolic pathway in the induction of genotoxic effects cannot be ruled out.*

Altogether, the available data show evidence of genotoxicity both in vitro and in vivo. In particular, it is noted that the effects observed in vivo were in association with metabolic pathway operative also in humans. Then, a classification as mutagen category 2, H341 is warranted."

As further demonstrated in detail below, however, this overall conclusion of the DS is not supported based on the available information and studies analysed in the CLH proposal. Also, this conclusion does not comply with the classification criteria established by the CLP Regulation, which allows classification of a substance as germ cell mutagen in category 2.

2. Summary Consortium Assessment and Response

The DS proposes to classify DCM as mutagen in category 2 based on (i) in vitro studies in bacteria, (ii) in vitro studies in somatic mammalian cells, and (iii) one in vivo genotoxicity study via inhalation in mice, which is however conducted under conditions which, as explained below, question its relevance for use in classification. Based on a comprehensive review of the available data and studies, the Consortium considers that the weight of evidence for in vitro mutagenicity or genotoxicity of DCM in mammalian somatic cells is equivocal, and that the weight of evidence for in vivo mutagenicity/genotoxicity of DCM in mammalian somatic cells is negative. This does not allow to draw the proposed DS's overall conclusion in compliance with the classification criteria established by the CLP regulation.

1) According to Section 3.5.2.1 of Annex I of CLP a classification in the hazard class mutagenicity applies to substances which *"may cause mutations in the germ cells of humans that can be transmitted to the progeny".* The CLP specifies that *"however,*

results from mutagenicity or genotoxicity tests in vitro and in mammalian somatic and germ cells in vivo are also considered in classifying substances" in this hazard class. This wording (the CLP says "however") clearly shows that the animal studies can only be used to support a classification if they allow to establish that a given substance "may" be mutagenic for humans. While the CLP does not require to establish that a substance "will" be mutagenic in humans, this should at least appear to be plausible based on the animal data at hand. The CLH proposal on DCM fails to meet this requirement.

In particular, the DS fails to observe that – based on the available data and studies on DCM – the mode of action of the substance could not be considered relevant for humans as effects would only be observed at concentrations/doses, which could not be achieved in humans, even under worst case conditions. This is because DCM metabolism in mice operates in a fundamentally different way than in rats, hamsters and a fortiori in humans. As further explained below, the metabolic pathway that occurs in humans does not result in the same level of metabolites that can cause mutations. The available evidence does therefore not demonstrate that DCM *"may cause mutations in the germ cells of humans [...]"* but only shows a possible concern for genotoxicity in mice.

2) The CLH proposal recognises on page 27 that "*No data are available for germ cell mutagenicity*" for DCM in humans and/or animals at all. It hence explicitly acknowledges that the proposed mutagenicity 2 classification is exclusively based on results in animals (mammalian somatic cells), *i.e.,* on the second part of the CLP provision cited above, which specifies: "*<u>however</u>, results from mutagenicity or genotoxicity tests in vitro and in mammalian somatic and germ cells in vivo are also considered in classifying substances*" (emphasis added). The CLH proposal fails to establish any plausible link between the observations in the animal data and the capacity of DCM to cause mutations in humans. The Consortium considers that the data and studies do simply not support such a link.

3) Section 3.5.2.2 and table 3.5.1 of Annex I to the CLP emphasizes that the classification of a substance as mutagen in category 2 should be based on *"<u>concern</u> for <u>humans</u> owing to the <u>possibility</u> that (a substance) may induce heritable mutations in the germ cells of <u>humans</u>"* (emphasis added). The CLP further specifies that animal studies can be used to demonstrate such a concern if they provide *"positive evidence obtained from <u>experiments in mammals</u> and/or in <u>some cases</u> from in vitro experiences"* (emphasis added). The wording of this provision is clear saying that the evidence from in vivo experiments in mammals should be leading, and that in vitro results can be used in *"some cases"* in support. This is also explicitly confirmed by the two examples provided by the legislation in second part of table 3.5.1 with relates to category 2, according to which the available evidence from "*in vivo somatic cell genotoxicity tests"* can be "*supported*" by positive results from in vitro mutagenicity assay. According to the CLP the classification cannot be based on in vitro results alone.

In the present case, the CLH proposal ignores the weight of evidence of in vivo animal genotoxicity data on DCM - which is negative in all studies with the exception of one single study via inhalation in mice, but which is of questionable relevance because of the conditions under which it was conducted, with very high inhalation exposure levels, while studies in mice

(and rats) via the oral route were negative. This cannot be justified given that, according to the CLP, the in vitro results can only be used to "support" relevant findings in vivo.

In particular, the CLH proposal fails to observe that:

- DCM produced mostly positive results when tested in bacterial mutation assays. This does not satisfy the CLP classification criteria for mutagenicity 2.
- The results of in vitro genotoxicity assays with mammalian cells are much more variable than those with bacteria/fungi. These results are inconsistent and provide only questionable evidence to support a concern for mutagenicity in humans.
- According to MAK the results of in vitro studies in mammalian cells were inconsistent viz. negative, positive and ambiguous results were reported.

The Consortium considers that the weight of evidence for *in vivo* mutagenicity/genotoxicity of DCM in mammalian somatic cells is negative. Only one study in the mice via inhalation is positive but is considered questionable for use in support of the present classification. All others *in vivo* results are negative, including a study via the oral route in the mouse. In the absence of positive *in vivo* assays, the *in vitro* results cannot support the proposed classification in line with the CLP regulation.

3. Detailed Consortium Assessment and Response

As correctly noted on page 31 of the CLH proposal, the criteria for the classification provided in the CLP regulation of a substance for germ cell mutagenicity category 2 is based on:

- *positive evidence obtained from experiments in mammals and/or in some cases from in vitro experiments, obtained from:*
- *somatic cell mutagenicity tests in vivo, in mammals; or*
- *other in vivo somatic cell genotoxicity tests which are supported by positive results from in vitro mutagenicity assays.*

However, the CLH proposal fails to comply with the above requirements, because:

1. <u>In vitro bacterial assays</u>

The DS mainly bases the classification proposal on positive in vitro bacterial assays. However, pursuant to Section 3.5.2.2 and table 3.5.1 of Annex I to the CLP such data cannot be used as main support to classify DCM as mutagenic in humans.

As noted by the DS itself, DCM produced mostly positive results when tested in bacterial mutation assays. As noted in IARC (1999; 2016) gene mutations were induced in *Salmonella typhimurium* strains TA100, TA1535 and TA98 exposed to dichloromethane vapour in a closed chamber with or without the addition of exogenous metabolic activation. However, glutathione-deficient strains NG 11 and NG 54 of TA100 were less responsive to the effects of dichloromethane than the parent strains. Studies using the liquid plate incorporation assay were negative, with the exception of one study which reported positive results in strain TA1535. DCM also induced mutations in *Escherichia coli* and gene conversion and mutation in *Saccharomyces cerevisiae*.

According to the CLP Regulation, however, positive results in bacterial mutation assays do not by themselves satisfy the classification criteria for Mutagenicity Cat 2.

2. *In vitro mammalian somatic cell assays*

The DS further supports the classification proposal based on inconclusive and inconsistent results *in in vitro* studies with mammalian somatic cells. Pursuant to Section 3.5.2.2 and table 3.5.1 of Annex I to the CLP, however, *in vitro* studies can be used only as supporting evidence in the classification for germ cell mutagenicity. In the absence of robust and consistent *in vivo* studies in mammals, such data cannot be used as the principal support to classify DCM as mutagenic in humans.

It is clear from the CLH proposal that the results of *in vitro* genotoxicity assays with mammalian cells were much more variable than those with bacteria/fungi, as noted in IARC (1999; 2016), ATSDR (2000) and MAK (Hartwig, 2016). They have been briefly summarized below.

DCM induced DNA–protein cross-links *in vitro* in hepatocytes of male B6C3F1 mice but not in hepatocytes of Fischer 344 rats, Syrian hamsters or in human hepatocytes with functional GSTT1 genes. DNA–protein cross-links were also induced in Chinese hamster ovary CHO cells exposed to DCM with or without exogenous metabolic activation. DNA damage was greater in the presence of metabolic activation.

DCM induced single-strand DNA breaks in AP rat primary hepatocytes and B6C3F1 mouse hepatocytes and Clara cells, but not in Syrian hamster hepatocytes *in vitro*. DNA damage was reduced in Clara cells co-treated with a glutathione-depleting agent (buthionine sulfoximine). In another study, DNA single-strand breaks were increased in CHO cells cultured with DCM only in the presence of an exogenous metabolic activation system but was negative in its absence.

DCM did not induce unscheduled DNA synthesis or hprt locus gene mutations in Chinese hamster lung V79 cells in the absence of exogenous metabolic activation, but did induce a slight increase in sister chromatid exchange frequencies.

DCM was mutagenic in CHO cells at the hprt locus in one study, in the presence of exogenous metabolic activation, but produced equivocal results in the mouse lymphoma tk+/− assay in another study. DNA sequence analysis of the hprt mutants of CHO cells treated with DCM indicated that most were GC→AT transitions (4/8), with two GC→CG transversions and two AT→TA transversions.

DCM induced chromosomal aberrations in CHO cells in the presence and absence of an exogenous metabolic system in one of two studies but did not increase sister chromatid exchange frequencies.

Virus-infected Fischer rat and Syrian hamster embryo cells were transformed after treatment with DCM *in vitro*.

Neither DNA single-strand breaks nor unscheduled DNA synthesis were induced in human primary hepatocytes or AH fibroblasts, respectively, following DCM treatment.

Sister chromatid exchanges were induced in human peripheral blood lymphocyte cultures but only in those from donors lacking GST activity towards methyl bromide.

In human MCL-5 cells that stably express cDNA encoding human CYP1A2, CYP2A6, CYP3A4, CYP2E1 and in h2E1 cells which contain a cDNA for CYP2E1, DCM induced kinetochore-staining micronuclei indicative of aneuploidy and kinetochore-negative micronuclei. However, AHH-1 cells expressing CYP1A1 showed neither an increase in total micronucleus frequencies nor kinetochore-staining micronuclei after DCM exposure.

In summary, the results of *in vitro* mammalian somatic cell genotoxicity assays with DCM are inconclusive and inconsistent. It is worth highlighting that DCM exposure:

- did not induce DNA–protein cross-links in hepatocytes of Fischer 344 rats, Syrian hamsters or in human hepatocytes with functional GSTT1 genes
- did not increase unscheduled DNA synthesis or hprt locus gene mutations in Chinese hamster lung V79 cells in the absence of exogenous metabolic activation
- did not induce DNA single-strand breaks nor unscheduled DNA synthesis in human primary hepatocytes or AH fibroblasts
- did not increase total micronucleus frequencies nor kinetochore-staining micronuclei in AHH-1 cells expressing CYP1A1

As noted by MAK (Hartwig, 2016), the results of *in vitro* studies in mammalian cells were partly negative (no unscheduled DNA synthesis in cultured human cells, no DNA damage or gene mutations in rodent cell systems); partly positive (transformation of rat and hamster embryo cells, chromosomal aberrations in rodent cells) and partly ambiguous (in a test for sister chromatid exchange in cultured Chinese hamster cells).

The Consortium considers that the weight of evidence for *in vitro* mutagenicity or genotoxicity of DCM in mammalian somatic cells is equivocal.

3. <u>In vivo mammalian genotoxicity assays</u>

The DS fails to observe that pursuant to Section 3.5.2.2 and table 3.5.1 of Annex I to the CLP the in vivo studies should be the main driver of the classification in the mutagenicity hazard class. This condition was not met in the present case for DCM.

The majority of the in vivo genotoxicity studies with DCM provide evidence that DCM is not mutagenic or genotoxic in vivo, as noted in IARC (1999; 2016), ATSDR (2000) and MAK (Hartwig, 2016) and briefly summarized below.

The principal study cited in the CLH proposal by the DS to support in vivo genotoxicity of DCM is a US EPA study by Allen et al. (1990), in which chromosome damage was studied in female B6C3F1 mice exposed to DCM by subcutaneous or inhalation exposure. No increase in the frequency of either sister chromatid exchanges (SCEs) or chromosome aberrations (CA) in bone marrow cells was observed after a single subcutaneous injection of 2,500 or 5,000 mg/kg bw DCM. Inhalation exposure to DCM for 10 days at concentrations of 4,000 or 8,000 ppm resulted in significant increases in frequencies of SCEs in lung cells and peripheral blood lymphocytes, CAs in lung and bone marrow cells, and micronuclei (MN) in peripheral blood erythrocytes. Lung cell CAs and blood erythrocyte MN reached frequencies of approximately two times control levels. Following a 3-month inhalation exposure to 2,000 ppm DCM, mice showed a small but significant increase in lung cell SCEs and peripheral blood erythrocyte MN. The authors noted that under the experimental conditions used, inhalation exposure at a high concentration and short duration proved to be more effective in causing chromosome changes than low-concentration exposure for a longer period of time.

In another paper published by the same US EPA laboratory (Westbrook-Collins *et al*. 1990) further investigations of *in vivo* clastogenic potential of DCM was undertaken in 3- to 5-month-old male C57B1/6J mice. SCEs and CAs in mouse bone marrow cells were studied following intraperitoneal exposures of 100 - 2000 mg/kg bw DCM. In this study, DCM failed to increase the frequencies of either SCEs or CAs. The authors concluded that the negative results obtained in this study "*add to an increasing body of data that suggests that DCM exposure in vivo does not result in genetic damage. Taken together, the available studies provide no*

evidence of induced micronuclei, CAs or SCEs in marrow cells or UDS in liver cells of mice or rats exposed by i.p., gavage or inhalation routes to DCM".

In another *in vivo* micronucleus study with male and female C57BL/6J/Alpk mice (Sheldon *et al*., 1987) DCM was administered by gavage at 1250, 2500 and 4000 mg/kg bw in corn oil. The highest dose level selected for this study was the maximum tolerated dose. Micronucleated polychromatic erythrocytes (MPEs) in the bone marrow of treated mice were evaluated 24, 36, 48 and 72 hrs after dosing. No significant increases in the incidence of MPEs versus controls were observed for any of the test groups, and <u>it was concluded that DCM was not clastogenic in this assay.</u>

DCM did not cause micronucleus formation in male B6C3F1 mice exposed at 400, 800 or 1600 ppm by inhalation for 6 weeks (6 hours per day, 5 days per week) (IARC, 2016; Suzuki et al., 2014). The negative Suzuki et al., 2014 study, conducted at lower exposure level, should be considered much more relevant than the Allen et al. (1990) study.

Furthermore, rats exposed by inhalation to DCM concentrations up to 3,500 ppm for 6 months showed no evidence of CA in bone marrow cells (ATSDR, 2000; Burek et al. 1984). The difference in responses observed may be partly due to species differences or test methodology. ATSDR (2000) noted that the relevance of these two *in vivo* genotoxicity studies in the mouse and rat to clastogenic mechanisms in humans is uncertain.

The ability of DCM to induce unscheduled DNA synthesis (UDS) in the liver of male mice and rats was investigated *in vivo* (Trueman and Ashby, 1987). In the first study, Alpk:AP rats were dosed via oral gavage with 100, 500 or 1000 mg/kg bw DCM and hepatocytes were evaluated for UDS 4 and 12 hours after dosing. In a second study, Fischer F344 rats and B6C3F1 mice were exposed via inhalation to 2000 or 4000 ppm DCM for 2 or 6 hrs with UDS evaluated in hepatocytes immediately after exposure. DCM did not induce UDS in male mice or rats following oral or inhalation exposure.

Other *in vivo* genotoxicity studies have attempted to elucidate mechanism(s) of genotoxic action of DCM. In one study, male B6C3F1 mice and Syrian Golden hamsters were pre-exposed for 2 days (6 hours/day) to 4,000 ppm of methylene chloride (ATSDR, 2000; Casanova et al.,1992). On the third day, animals were exposed for 6 hours to a decaying concentration of [14C] methylene chloride and then examined for the presence of DNA-protein crosslinks. DNA-protein crosslinks were detected in mouse liver, but not in mouse lung, hamster liver, or hamster lung. Similar results were observed in a second experiment (ATSDR, 2000; Casanova et al., 1996). B6C3F1 mice, exposed to DCM for 6 hours/day for 3 days at concentrations ranging from approximately 500 to 4,000 ppm formed DNA-protein crosslinks in the liver. The formation of DNA-protein crosslinks was a nonlinear function of airborne concentrations of DCM. In addition, mice exposed for 6 hours/day for 3 days to concentrations ranging from approximately 1,500 to 4,000 ppm showed an increased rate of DNA synthesis in the lung, indicating cell proliferation, but increased cell turnover was not detected in mouse lung at exposure concentrations of 150 or 500 ppm. <u>Hamsters showed no evidence of cell proliferation in the lung at any concentration, nor did cell proliferation occur in the livers of either species.</u>

In another study (ATSDR, 2000; Devereux et al., 1993) the presence of activated ras proto-oncogenes in liver and lung tumours induced in female B6C3F1 female mice by inhalation of 2,000 ppm of methylene chloride for 6 hours/day, 5 days/week exposure for up to 104 weeks was analysed. In methylene chloride-induced liver tumours, mutations (mainly transversions or transitions in base 1 or base 2) were detected; these were similar to those observed for the H-ras gene in spontaneous liver tumours. Mutations were also identified in the lung. The K-ras activation profiles in the methylene chloride-induced tumours were not significantly different

from those in spontaneously occurring tumours. No other transforming genes were found in the nude mouse tumorigenicity assay. The authors were unable to identify any transforming genes other than ras genes in either mouse liver or lung tumours.

Based on its review of the available studies, the Consortium considers that the weight of evidence for *in vivo* mutagenicity/genotoxicity of DCM in mammalian somatic cells is negative.

4. Overall Conclusion on Classification for Mutagenicity

The Consortium is in general agreement with the summary published by MAK (Hartwig, 2016), indicating that DCM is genotoxic in in vitro test systems in which the GSH-dependent metabolic pathway is active. However, positive results in in vivo genotoxicity studies were only observed in somatic sells of mice at high inhalation exposure concentrations which make it questionable for use in support of the proposed classification. It is also noted that GSTT1 isoenzyme activity in mice is significantly higher compared with humans and that the RNA-adduct formation and the mRNA expression of GSTT1 in the prostate, ovaries and placenta is also higher in mice than in humans. Consequently, due to the lower sensitivity of humans compared with mice under relevant conditions, DCM is not expected to be mutagenic in human germ cells.

The Consortium does not agree with the statement in Table 10.8.2 of the CLH document (highlight added): "*Moreover, it is important to note that, as reported in a study (Crebelli, 1999), the halogenated hydrocarbons (**such as DCM**) are not very effective in inducing micronucleus formation in mouse bone marrow, **therefore a negative bone marrow micronucleus assay is not sufficient to rule out the concern raised by the consistently positive in vitro results***". First, the Crebelli (1999) paper did not include DCM among the 10 halogenated hydrocarbons that were investigated in the study. Furthermore, the position suggested by the DS, that negative results from *in vivo* genotoxicity studies may be disregarded when not in agreement with results from *in vitro* mammalian cell assays, is not only doubtful from a scientific point of view, it is also inconsistent with the classification criteria established by the CLP Regulation. The Consortium therefore considers this statement to be highly subjective and inconsistent with the applicable legal CLP criteria for the classification of substances as mutagenic in category 2.

It should be noted that DCM produced a positive response in one *in vivo* genotoxicty study in mice, but only at very high exposure concentrations in excess of 4000 ppm. Other studies in the mouse via inhalation, ip injection or oral gavage were negative, as were studies in the rat via the oral and inhalation routes.

Based on its review of the available studies, the Consortium considers that the weight of evidence for:

- ***in vitro* mutagenicity/genotoxicity of DCM in mammalian somatic cells is equivocal**
- ***in vivo* mutagenicity/genotoxicity of DCM in mammalian somatic cells is negative**

5. References

Allen J. et al. (1990) Cytogenetic Analyses of Mice Exposed to Dichloromethane. *Environmental and Molecular Mutagenesis* **15**:221-228.

ATSDR (2000) Toxicological Profile for Methylene Chloride, US Dept of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry, https://www.atsdr.cdc.gov/ToxProfiles/tp14.pdf

Burek JD, Nitschke KD, Bell TJ, et al. (1984) Methylene chloride: A two-year inhalation toxicity and oncogenicity study in rats and hamsters. *Fund Appl Toxicol* **4**:30-47.

Casanova M, Deyo DF, Heck HA. (1992) Dichloromethane (Methylene chloride): Metabolism to formaldehyde and formation of DNA-protein cross-links in B6C3F1 mice and Syrian golden hamsters. *Toxicol Appl Pharmacol* **114**:162-165.

Casanova M. (1996) Are you a man or a mouse? *Regul Toxicol Pharmacol* **24**:106.

Crebelli R. et al. (1999) Evaluation of 10 aliphatic halogenated hydrocarbons in the mouse bone marrow micronucleus test. *Mutagenesis* **14**(2): 207–215.

Devereux TR, Foley JF, Maronpot RR, et al (1993) Rat proto-oncogene activation in liver and lung tumors from B6C3F1 mice exposed chronically to methylene chloride. Carcinogenesis **14**(5): 795-801.

Hartwig, A. (2016) Dichloromethane [MAK Value Documentation, 2015]. In The MAK-Collection for Occupational Health and Safety. https://doi.org/10.1002/3527600418.mb7509e5916

IARC (1999) IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, No. 71. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon (FR): International Agency for Research on Cancer. https://www.ncbi.nlm.nih.gov/books/NBK499382/#p251_s4

IARC (2016) IARC monographs on the evaluation of carcinogenic risks to humans; volume 110. Lyon (FR): International Agency for Research on Cancer, https://publications.iarc.fr/Book-And-Report-Series/Iarc-Monographs-On-The-Identification-Of-Carcinogenic-Hazards-To-Humans/Some-Chemicals-Used-As-Solvents-And-In-Polymer-Manufacture-2016

Sheldon T., Richardson C, and M. Elliott (1987) Inactivity of methylene chloride in the mouse bone marrow micronucleus assay. *Mutagenesis* **2**:57-59.

Suzuki T, Yanagiba Y, Suda M, Wang RS (2014). Assessment of the genotoxicity of 1,2-dichloropropane and dichloromethane after individual and co-exposure by inhalation in mice. *J Occup Health* **56**(3):205–14. doi:10.1539/joh.13-0236-OA, PMID:24739373, https://pubmed.ncbi.nlm.nih.gov/24739373/

Trueman R. and J. Ashby (1987). Lack of UDS activity in the livers of mice and rats exposed to dichloromethane. *Environ. Molec. Mutagen*. **10**:189-195.

Westbrook-Collins B. et al. (1990) Further Evidence that Dichloromethane does not Induce Chromosome Damage. *J. Appl. Toxicology* **10**(2):79-81.