Helsinki, 12 December 2023

Addressees
Registrants of 2-furaldehyde (EC no. 202-627-7) listed in the last Appendix of this decision.

Registered substance subject to this decision (the Substance)
Substance name: 2-furaldehyde
EC / List number: 202-627-7

Decision number: Please refer to the REACH-IT message which delivered this communication (in format SEV-D-XXXXXXXXXX-XX-XX/F)

DECISION ON SUBSTANCE EVALUATION

Under Article 46 of Regulation (EC) No 1907/2006 (REACH), you must submit the information listed below:

A. Information required to clarify the potential risk related to Mutagenicity

1. Transgenic rodent somatic and germ cell gene mutation assay (OECD TG 488) in transgenic mice, by the oral route (gavage), with the Substance, on the following tissues:
   - Liver, glandular stomach, duodenum, and urinary bladder.
   - Male germ cells from the seminiferous tubules must be harvested and stored for up to 5 years. Analysis of germ cells must be performed if the results in any of the somatic tissues (i.e., liver, glandular stomach, duodenum, or urinary bladder) are positive or inconclusive.

   Testing is further specified in Appendix A (section 2.1.b).

Deadlines

The information must be submitted by **19 March 2027**.

Conditions to comply with the information requested

To comply with this decision, you must submit the information in an updated registration dossier, by the deadline indicated above. The information must comply with the IUCLID robust study summary format. You must also attach the full study report for the corresponding study in the corresponding endpoint of IUCLID.

You must update the chemical safety report, where relevant, including any changes to classification and labelling, based on the newly generated information.

Justifications for the requests in this decision are provided within the Appendix entitled 'Reasons to request information to clarify the potential risk.'

Procedural steps followed to reach the adopted decision and some technical guidance are detailed in further Appendices.

Appeal

This decision may be appealed to the Board of Appeal of ECHA within three months of its notification to you. Please refer to http://echa.europa.eu/regulations/appeals for further information.

Failure to comply

If you do not comply with the information required by this decision by the deadline indicated above, ECHA will notify the enforcement authorities of your Member State.

Authorised[1] under the authority of Mike Rasenberg, Director of Hazard Assessment.

[1] As this is an electronic document, it is not physically signed. This communication has been approved according to ECHA's internal decision-approval process.

Basis for substance evaluation

The objective of substance evaluation under REACH is to allow for the generation of further information on substances suspected of posing a risk to human health and/or the environment ('potential risk').

ECHA has concluded that further information on the Substance is necessary to enable the evaluating Member State Competent Authority (MSCA) to clarify a potential risk and whether regulatory risk management is required to ensure the safe use of the Substance.

The ECHA decision requesting further information is based on the following:

(1) There is a potential risk to human health and/or the environment, based on a combination of hazard and exposure information.
(2) Information is necessary to clarify the potential risk identified; and
(3) There is a realistic possibility that the information requested would allow improved risk management measures to be taken.

Appendix A – Reasons to request information to clarify the potential risk related to Mutagenicity

1. Potential risk

1.1 Potential hazard of the Substance

Following its assessment of the available relevant information on the Substance, the evaluating MSCA has identified the following potential hazard which must be clarified.

Potential mutagenicity

The available information suggests that the Substance may have a mutagenic effect. However, the available and current information is not sufficient to clarify the identified concern.

Available *in vitro* and *in vivo* data were analysed using a weight of evidence approach. The *in vitro* data showed that the Substance can induce gene mutations in bacteria. Moreover, *in vitro* results demonstrated a clear ability for the Substance to induce chromosomal aberrations in mammalian cells. The available *in vivo* data reported in the chemical safety report is not sufficient to draw a firm conclusion on mutagenicity. Therefore, a concern for mutagenicity of the Substance cannot be excluded.

The original reports of the studies included in the registration dossier were provided by the Registrant(s). Additional published scientific data and opinions (SCCS 2012, EFSA 2011, EU 2008) were also considered in the evaluation of the Substance.

Genotoxicity *in vitro*

- There are six bacterial reverse mutation tests included in the Substance's registration dossier. In addition, the evaluating MSCA has identified three studies published in the open literature that were not included in the dossier. The available studies vary in reliability and in the results obtained as described below:
  - Two studies, both included in the dossier, reported that the Substance was positive in the tester strain *Salmonella typhimurium* TA100 where the strongest response was obtained without metabolic activation with S9-mix (Zdzienicka et al., 1978; unpublished report 1979). In another study by Loquet et al., (1981) (not included in dossier), the Substance was likewise positive in TA100 without S9, but here, toxicity was reported. In addition, the Substance was found to be weakly positive in the highest tested concentrations, in strain TA98 without S9 metabolic activation (7500 and 10000 µg/plate). However, at these concentrations, toxicity was also observed (unpublished report, 1979). Shane et al., (1988) (not included in the dossier), reported that the Substance was positive in TA104, but only when activated by S9-mix.
  - In a study by Mortelmans et al., (1986) (included in the dossier), the Substance was equivocal in the strain TA100 as it was found to be weakly positive in one laboratory but negative in another.
  - Three studies included in the registration dossier (Aeschbacher et al., 1989; Unpublished report 1982; Unpublished report 1999) and one identified in the open literature by the evaluating MSCA (Marnett et al., 1985) were negative in all tested bacterial strains. It should be noted that Aeschbacher et al., (1989) and Shane et al., (1988) studies only included three testing strains (TA98, TA100, and TA102, and TA100, TA102 and TA104 respectively) and the unpublished report (1982), lacks a cross-linking test strain (e.g. E.coli WP2 strains or S. typhimurium TA102). Only the unpublished report from 1999 included all the testing strains recommended in the current version of the OECD TG 471 (version 2020).

- The registration dossier also includes a pre-guideline L5178Y tk^+/tk^- Mouse Lymphoma Cell Forward Mutation Assay (McGregor et al., 1988). In this study, the Substance induced mutations without metabolic activation. Although this assay is generally used for identification of gene mutation potential in mammalian cells, mutant colonies were not characterized by colony sizing or growth. Therefore, it cannot be determined whether the observed mutagenicity of the Substance was caused by gene mutations or by chromosomal aberration mechanisms. The study is otherwise reliable and carried out in accordance with the OECD TG 490 with minor limitations in design and/or reporting.

- Gene mutation potential of the Substance was also tested in a Yeast cell assay in the strain *S. cerevisiae* JSC25-1 (Qi et al., 2019). This study, which is included in the dossier, showed that the frequency of point mutations was significantly elevated in the treated cells with the Substance. It should be noted however, that the reliability of this study is low due to among others, the use of an uncharacterized Yeast cell strain.

- The technical dossier contains several *in vitro* studies conducted in mammalian cells to address induction of chromosomal aberrations.
  - The most reliable and thoroughly described of these studies is a GLP compliant study performed according to the OECD TG 473 (Unpublished report, 1996). In this study, the Substance induced a significant increase in chromosomal aberrations and a positive dose-response trend in Chinese hamster ovary cells (CHO- K_1 cells) both with and without activation with S9.
  - Four additional studies included in the dossier support this finding by showing that the Substance induces chromosomal aberrations *in vitro* in CHO cells, V79 cells, Yeast cells (*S. cerevisiae* JSC25-1) and in human lymphocytes, although the reliability of these studies is low (Stich et al., 1981; Nishi et al., 1989; Qi et al., 2019; Gomez-Arroyo et al., 1985).

- Regarding DNA damage and repair induced by the Substance, positive, equivocal and negative results obtained in six available *in vitro* studies are presented in the technical dossier. It should be noticed that the reliability of these results is generally considered to be low by the evaluating MSCA. The studies are briefly summarised below:
  - In a DNA synthesis inhibition test, it was found that the Substance inhibited the DNA synthesis in immortalized human liver cells (HeLa S3 cells) (Heil and Reifferscheid, 1992). In studies by Uddin et al. (1993 and 1995) on DNA isolated from calf thymus, it was suggested that the Substance destabilized the secondary DNA structure (Uddin et al., 1993), and that the Substance induced DNA strand breaks in an exposure time dependent matter (Uddin et al., 1995). Induction of DNA strand breaks by the Substance was also observed by Hadi et al., (1989). They found that samples treated with the Substance showed a consistent increase in the number of breaks formed per unit of DNA as a function of increasing time of reaction. This degradation of double stranded DNA primarily occurred in AT sequences.
  - One study measuring DNA damage in an unscheduled DNA synthesis (UDS) assay conducted on tissue slides from human liver samples, was considered to be negative by the authors of the study. However, since the main outcome of the study (the net grain count) was significantly elevated in the highest doses of the 2-furaldehyde treated human liver tissue, the evaluating MSCA considers this study to be at least equivocal (Lake et al., 2001).
  - Finally, the Substance was reported to be negative in an UDS assay measuring induction of DNA repair (following DNA damage) in nasal epithelium (Wilmer et al., 1987).

In your comments to the draft decision, you did not consider the available *in vitro* studies individually. Instead, you commented on the data on a general level, which is considered by the evaluating MSCA in the conclusion of section 1.1.

Genotoxicity *in vivo*

- An *in vivo* chromosomal aberration study was performed by NTP and published in a risk assessment report in 1990 (Irwin, 1990). No guideline was mentioned, but the method is comparable to OECD TG 475.

  The Substance was diluted in phosphate buffered saline (PBS) and was administrated by intraperitoneal injections using male B6C3F$_1$ mice. Doses were 0, 50, 100 and 200 mg/kg and eight mice were included in each dose group. The frequency of chromosomal aberration was analyzed at an early time point (17 hours after administration) and at a late time point (36 hours after administration). Induction of sister chromatid exchanges 23 hours after administration was also evaluated using the same dose groups but only five animals per dose group. The obtained results were negative under the condition of the study.

  The evaluating MSCA considers the reliability of the available NTP chromosomal aberration studies to be low due to significant limitations including the lack of information on the dose range-finding study, the missing individual data for each animal, and the low number of metaphases scored per animal (25-50 scored metaphases compared to the acceptance criteria in the latest version of the OECD TG 475 (2016) which is at least 200 per animal).

In your comments to the draft decision, you argue that the limitations of the available NTP study (Irwin, 1990) identified by the evaluating MSCA do not substantially question the reliability of the obtained negative results, and that the study is sufficient to confer a lack of clastogenic potential from the Substance.

Whilst the evaluating MSCA agrees to a certain extent to some of your observations when each limitation noted above is considered individually, the evaluating MSCA is of the opinion that when combined, the limitations are significant and jeopardize the reliability of the findings.

More specifically, regarding the absences of a dose range finding study, you mention that the NTP run dose range finding studies in-house but rarely present the data. In addition, you argue that the applied doses are in line with other data in the mouse, albeit via different routes of administration. The evaluating MSCA does not agree that the dose levels can be compared between different routes of exposure without further experimental or scientific support as ADME factors are known to vary significantly depending on administration route. Furthermore, as you consider in your comments, the evaluating MSCA is aware that individual data is rarely presented in the majority of peer reviewed studies published in the open literature. Still, presenting individual data is listed as a data presenting criterion in the OECD TG 475 and is important for the transparency and expert judgement of data reliability.

In your comments, you also argue that demonstration of target tissue exposure has been established since the Substance is known to be absorbed via the oral, inhalation and dermal routes, and to induce toxicological effects in various tissues. Upon further considerations, the evaluating MSCA agrees to this comment and the sentence "no evidence of exposure to target tissue" has been removed from the text above describing the limitations of the Irwin (1990) study.

Finally, you argue that the low number of metaphases scored is accounted for by a higher number of mice in each group (eight animals in each group compared to the minimum of five animals according the OECD TG 475). However, the OECD TG 475 does not offer the opportunity to include more animals to compensate for a low number of scored metaphases. The recommendations of scoring at least 200 metaphases is based on Adler et al., (1998). In this paper, it is stated that scoring of 100 cells is insufficient to detect at least one aberrant cell per animal. Therefore, scoring of 25-50 metaphases per animal is insufficient regardless of the group size.

In addition to the above described NTP study (Irwin, 1990), you mention in your comments to the draft decision that other regulatory bodies also refer to a chromosome aberration study where 4000 ppm furfural was administered to Swiss albino mice via feed for 5 days resulting in negative responses for chromosome aberrations (Subramanyam and Rathnaprabba, 1989). The evaluating MSCA is aware of this reference but has not succeeded in identifying either the abstract or a full-length article/study report describing these results. This is in accordance with the conclusion from the Risk assessment report carried out by the Netherlands (2008): "Evaluation of this result is not possible since this abstract only provided a very limited description, and no paper has been published since then in a peer reviewed journal." The Scientific Committee on Consumer Safety (SCCS), which also uses this reference, likewise only refers to the abstract in their opinion on the Substance from 2012 (SCCS, 2012).

- The Substance's potential to induce gene mutations in mice liver was examined in a GLP compliant pre-guideline *in vivo* λlacZ-transgenic mice assay (testing regime: 28+34/35 days) (Unpublished report, 2003). The Substance was dissolved in corn oil and administrated by oral gavage. Doses were 0, 37.5, 75, 150 and 300 mg/kg/day and 7-8 male mice were included in each dose group. The mutant frequency was not increased in response to exposure of the Substance.

  The method applied was similar to that described in the OECD TG 488, but only liver tissue was analysed, which is not considered to be sufficient to address the gene mutation concern of the Substance *in vivo*. Moreover, the study did not follow the recommended testing regime in OECD TG 488, i.e., 28+28 days to allow for mutagenic analysis of both fast and slowly dividing cell types. The study is otherwise considered to be reliable regarding the results obtained in the liver.

In your comments to the draft decision, you agree that the available OECD TG 488 study is reliable considering the results obtained in the liver. Your comments with regards to the testing regime are considered by the evaluating MSCA in section 2.1b.

- In contrast to the result obtained in the *in vivo* λlacZ-transgenic mice assay, the dossier contains a study published in the open literature (Reynolds et al., 1987) which gives some indications that the increased incidence in mouse liver tumors, observed following exposure to the substance as described by Irwin (1990), may be - at least in part - caused by the induction of weakly activating point mutations in ras oncogenes. The method applied in this study is however, only superficially described and it is not possible to assess the reliability of the obtained results.

- Effects in the liver were also the focus in a study examining unscheduled DNA synthesis (UDS) in hepatocytes from male and female B6C3F_1 mice and male F344 rats (Lake et al., 2001). No test guideline is mentioned, but the method is similar to the OECD TG 486, with some minor deviations. The Substance was diluted in corn oil, and animals were treated with single oral doses by oral gavage. The test was conducted 2 - 4 hours and 12 - 16 hours after exposure, and three mice were included

in each dose group, which is in line with the recommendations in OECD TG 486. The dose groups were 0, 50, 175 and 320 mg/kg. In the rat study, only two animals were included in each dose group, and doses were 0, 5, 16.7 and 50 mg/kg. Under the conditions of the test, the Substance did not induce DNA damage in mouse or rat liver cells.

In your comments to the draft decision, you argue that the negative result of the UDS study in hepatocytes supports that further gene mutation testing of the Substance is unnecessary. ECHA notes that the UDS study is an indicator test that detects some DNA repair mechanisms. However, it does not provide direct evidence of mutation such as the TGR assay. As reminded in the ECHA R.7A Guidance on information requirements and chemical safety assessment, Section R.7.7.6.3. (pages 571-572) (ECHA, 2017), the UDS test is sensitive to some (but not all) DNA repair mechanisms and not all gene mutagens are positive in the UDS test. Therefore, a negative result in a UDS assay alone is not a proof that a substance does not induce gene mutation (ECHA, 2017). In addition, the sensitivity of the UDS test has been questioned (Kirkland and Speit, 2008), and its lower predictive value towards rodent carcinogens and/or *in vivo* genotoxicants has been confirmed in comparison with the TGR assay (EFSA, 2017).

- In a publicly available pre-guideline comet assay, which is not included in the registration dossier, the genotoxicity of the Substance was tested at a concentration of 200 mg/kg in eight different tissues (Sasaki et al., 2000). The Substance was administrated once by oral gavage, four male mice were included in each group and organs were sampled 3, 8 and 24 hours after administration. In this study, the Substance was found to be positive in stomach, colon, liver, kidney, urinary bladder, lung, brain and bone marrow at the 8-hour and/or 24-hour time point. The strongest response was found in liver, stomach and urinary bladder.

In your comments to the draft decision, you argue that the available comet assay (Sasaki et al., 2000) has significant limitations and is therefore not sufficiently reliable to determine the potential mutagenicity of the Substance.

The evaluating MSCA agrees that there are limitations in the existing comet assay, namely: there is only one dose group included in the study preventing a dose-response evaluation; a low number of animals was tested in each group; only 50 nuclei were measured per slide per organ; and very sparse reporting of data. Moreover, as you indicate in your comments, there is no accompanying histopathology or assessment of apoptosis and no concurrent controls were used. Therefore, the evaluating MSCA agrees that a final conclusion on the mutagenicity of the Substance cannot be drawn based on the results obtained in this study which is, as you also mention in your comments, in line with the conclusion in the study review by the UK committee on toxicity of chemicals in food, consumer products and the environment (mutagenicity for dichlorvos, 2002). However, there is a substantial difference between drawing a conclusion on the endpoint of mutagenicity and raising a concern that, in combination with other available data, leads to the need for further testing. Hence, the evaluating MCSA disagrees that the positive results should be disregarded when considering the concern raised for mutagenicity. We also disagree that the UK committee report supports overlooking the data when considering a concern of a Substance and not the conclusion, as it is stated in the report that "*The Committee agreed that the positive results reported in the COMET assay using dichlorvos suggested that a full review of all the mutagenicity data was required*".

- In addition to the rodent studies, there are three Drosophila mutagenicity tests available in the registration dossier. A sex-linked recessive lethal (SLRL) test (Woodruff et al., 1985), a *mei-9*^{a} test in Drosophila sperm cells and a Wing Spot test. In the SLRL test, the Substance was administrated by injection or via feeding at

concentrations of 100 ppm and 1000 ppm respectively. In the *mei-9*[a] test and the Wing Spot test flies were exposed to the Substance by injection at concentrations of 0, 3750, 5000 or 7500 ppm. Positive results were obtained in all three tests, which can reflect induction of both gene mutations and/or chromosomal aberrations.

In your comments to the draft decision, you conclude that the positive results obtained for the Substance in Drosophila are not significant because the Substance induces sex-linked lethal mutations only when injected and not when administrated via feed. The evaluating MSCA agrees that these results are not sufficient to conclude on a mutagenic effect. However, the positive results obtained by injections cannot be neglected as supporting evidence of a mutagenic concern for the Substance, underpinning the need for further testing.

Conclusion

In conclusion, the available *in vitro* data shows that the Substance induces chromosomal aberrations in mammalian cells. Although the obtained results for induction of gene mutations are more divergent, there are available studies showing that the Substance also induces gene mutations *in vitro* in the bacterial strain *Salmonella typhimurium* TA100 and potentially also in mouse lymphoma cells. Data from DNA damage and repair tests supports the finding that the Substance is genotoxic *in vitro*.

In your comments to the draft decision, you stated that: *"We do not disagree with ECHA that whilst there are a large number of predominantly negative in vitro genotoxicity data sets, some of these are not clearly negative and of questionable quality but do show adverse responses albeit at high toxicity, where toxicity has not been measured adequately or under conditions that are not biologically relevant."* You had no further comments to the available *in vitro* data.

In vitro assays are generally designed to only capture a well-defined part element of a full biological process. Therefore, discussing biological relevance of *in vitro* assays is not necessarily meaningful. Yet, the evaluating MSCA agrees that the reliability of the available *in vitro* studies varies significantly. However, there are available reliable positive studies for both gene mutations and chromosomal aberrations that demonstrate a concern for genotoxicity (Zdzienicka M et al., (1978) McGregor et al., (1988); Unpublished report (1996)).

Positive results of mutagenicity were obtained *in vivo*: in a pre-guideline mouse comet assay, showing positive results in several organs; and in three non-mammalian *Drosophila melanogaster* assays. Although these studies have significant limitation and are not sufficiently reliable to conclude on the mutagenicity of the Substance, the findings enhance the concern for mutagenicity identified *in vitro*, including germ cell mutagenicity.

In your comments to the draft decision, you conclude that you have "*adequately demonstrated the suitability of the NTP data for clastogenicity and the lac Z transgenic mutation data for point mutagenicity*" and that both studies are "*robust and adequately confer a lack of clastogenicity and point mutation*" for the Substance.

However, the evaluating MSCA does not consider the available negative *in vivo* data to be sufficient to adequately clarify the concern raised. More specifically, the evaluating MSCA does not agree that the '*current NTP studies are sufficient*' to conclude on the clastogenic potential of the Substance, since as explained above, the two negative chromosomal aberration studies published by NTP in 1990 (Irwin, 1990) are considered to be of low reliability. Additionally, regarding the available TGR, which is otherwise considered to be reliable, it only included measurements of gene mutations in the liver.

1.2 Potential exposure

According to the information you submitted in all registration dossiers, the aggregated tonnage of the Substance manufactured or imported in the EU is in the range of 10,000 – 100,000 tonnes per year.

Furthermore, you reported the use of the Substance among others:

- As a component in polymer production, in coating products (paints, thinners, paint removers), in refractory product production (bricks and other ceramic shapes), as manufacturing agent of abrasive wheels (brake linings and refractories), as extraction agent in the petroleum refining industry, as laboratory agent, and as intermediate in the production of furan derivates.

In the published literature, it is also reported that the Substance is used in the production of biocides, pesticides and fertilizers and found in various foods and beverages either added intentionally as a flavouring compound or because it is formed during preparation/heating (NTP 1990, EU 2008, EFSA 2011).

In addition, examination of chemical emission and/or migration from consumer products performed for the Danish EPA have shown that the Substance is released from products such as incense (while burning), toys (squishies, surface treated wooden toys) pine tar products and various Do-IT-Your-Self products such as paints, coatings, and oils (Danish EPA 2004, 2005, 2012, 2018a and 2018b).

Previously, the Substance was also frequently found in cosmetic products. However, the use of the Substance was regulated in the EU cosmetics regulation in 2019 and can no longer be used in cosmetic products in concentrations exceeding 0.001 % (Regulation No 1223/2009)[2].

Although some of the described uses and exposures of the Substance are not covered by the REACH regulation, they are still contributing to the combined exposure of the Substance. Taken together, the known uses and exposure sources of the Substance indicate that there is wide dispersive use of the Substance and exposure to consumers, workers, and the environment cannot be excluded.

1.3 Identification of the potential risk to be clarified

Based on all information available in the registration dossier and information from the published literature, there is sufficient evidence to justify that the Substance may cause gene mutation effects on somatic and/or germ cells.

The information you provided on manufacture and uses in addition to information from the published literature demonstrates a potential for exposure of consumers and workers.

Based on this hazard and exposure information, the Substance poses a potential risk to human health.

As explained in Section 1.1 above, the available information is not sufficient to conclude on the potential hazard. Consequently, further data is needed to clarify the potential risk related to mutagenicity.

[2] https://eur-lex.europa.eu/legal-content/DA/ALL/?uri=celex:32009R1223

1.4 Further risk management measures

If the gene mutation effect of the Substance is confirmed based on Request A.1, the evaluating MSCA will analyse the options to manage the risk.

New regulatory risk management measures could be a harmonisation of the classification for germ cell mutagenicity, as defined in the CLP Regulation, and an assessment of whether the Substance should be proposed for identification as a substance of very high concern under Article 57 of REACH. Eventually, inclusion on annex XIV of REACH (authorisation) or a proposal for restriction of the use of the Substance may be considered. This would result in stricter risk management measures, such as improved measures at manufacturing sites, better waste management and revised instructions on safe use as appropriate.

Moreover, considering the current classification of the Substance as Carc. Cat 2, a potential classification as germ cell mutagen in category 1B would further improve risk management measures currently in place and would also have consequences for the classification of mixtures containing the Substance, due to this classification's generic/specific concentration limits for products. If classified as germ cell mutagen, revised instructions on safe use could be applied, if appropriate.

2. How to clarify the potential risk

2.1 Request A.1:

a) Aim of the study

The TGR assay (OECD TG 488) in glandular stomach, duodenum, liver, urinary bladder, and male germ cells as further specified below, will clarify the concern for gene mutations of the Substance *in vivo*.

b) Specification of the requested study

To address the concern identified above, the OECD TG 488 *in vivo* transgenic rodent (TGR) somatic and germ cell gene mutation assay will allow to identify gene mutation potential of the Substance in both somatic tissues and germ cells, which are required to conclude on the mutagenic properties and the potential risk posed by the Substance in this regard.

Species, route of exposure and solvent

The Substance must be tested in transgenic mice, administrated by oral gavage and dissolved in olive oil, corresponding to the rodent species, administration route and vehicle used in the available positive pre-guideline comet assay (Sasaki et al., 2000).

Sampling time

Based on OECD TG 488, the selected tissues for mutant analysis must be collected 28 days after the final treatment (28+28d testing regime); this permits the testing of mutations in somatic tissues and as well as in tubule germ cells from the same animals.

Selection of tissues for mutant analysis

Mutant analysis must be performed in the following somatic tissues: liver, glandular stomach, duodenum and urinary bladder. Male germ cells from the seminiferous tubules must be collected and analysed if any of the selected somatic tissues are positive or inconclusive. Otherwise, germ cells must be stored at or below -70° C for up to 5 years.

Selecting several relevant tissues for mutant analyses from the same animals is considered to be in accordance with the three R's of responsible animal testing by maximizing the information obtained per animal and thus potentially limiting or avoiding the subsequent use of additional animals without compromising animal welfare[3].

- *Liver and gastro-intestinal tract (glandular stomach and duodenum)*

According to the test method OECD TG 488, the test must be performed by analysing liver tissue, as slowly proliferating tissue and primary site of xenobiotic metabolism, and from glandular stomach and duodenum, as rapidly proliferating tissue and site of direct contact.

Liver:

Summarizing previously obtained *in vivo* results for the liver, the Substance was positive in liver tissue in the comet assay (Sasaki et al., 2000), while it was negative in a TGR assay (Unpublished report, 2003). As explained above, due to its limitations, the existing comet assay does not however adequately clarify the mutagenicity concern for liver.

In the available negative pre-guideline TGR assay, tissue for mutant frequency analysis was collected on day 28+34/35, which is longer than the 28+28 testing regime recommended in the OECD TG 488. While the available literature suggests that longer sampling time may not affect the result for strong mutagens, sampling times greater than 28 days may produce false-negative results for mutagens that produce less strong increases in mutant frequencies in the TGR assay (Marchetti et al., 2021; Heddle et al., 2003).

In your comments to the draft decision you:

1) Argue that gene mutation testing in only the liver tissue is adequate for the evaluation of mutational ability of the Substance. You base this argumentation, among others, on the results obtained in the available comet assay (Sasaki, 2000). The comet assay shows the highest response in the liver at the 24-hour time point, and you therefore argue that the liver could be the most sensitive tissue. Therefore, since the liver was negative in the available TGR assay, you conclude that no further testing is needed.

2) Acknowledge that there are TGR assays that showed a decrease in sensitivity (lower mutant frequencies) over extended expression periods (Marchetti et al., 2021; Heddle et al., 2003). However, you further argue that the shortest time point showing a potential decrease in sensitivity is 42 days post-exposure (Marchetti et al., 2021), and that in the existing lac Z transgenic study, the sampling time is 34 days i.e., closer to 28 days than 42. You conclude that the 35-day sampling time is unlikely to be dramatically less sensitive after such a short elongation of the sampling time compared to current OECD guidance.

However, the evaluating MSCA does not agree with the above, because:

1) The available comet assay does not provide enough evidence to determine that the liver is the most sensitive tissue. Whereas the positive results obtained in the comet assay in combination with positive *in vitro* studies and non-mammalian *Drosophila melanogaster* studies support the need for further testing, the available comet assay has significant limitations as explained above. Therefore, a conclusion on

[3] https://nc3rs.org.uk/who-we-are/3rs

mutagenicity of the Substance and/or tissue sensitivity based on these results cannot be drawn.

2) You do not provide any further scientific evidence that the potential mutagenicity of the Substance in the TGR assay is not affected by a longer than 28-days sampling time. Hence, based on the available data, it is not possible to conclude that the sampling time of 34 days did not affect the results obtained in the available TGR.

Therefore, based on the above, the liver must be included in the analyses of the requested TGR assay (28+28 testing regime). Furthermore, collecting liver tissue for analysis in addition to the other selected tissues will increase the information obtained per animal without increasing the number of animals used or compromising animal welfare, which is in line with the 3R principle of responsible animal testing.

Gastro-intestinal tract:

Both glandular stomach and duodenum must be analysed as there are several expected or possible variables between these two tissues (e.g., different tissue structure and function, different pH conditions, variable physico-chemical properties and fate of the Substance, and probable different local absorption rates of the Substance and its possible breakdown product(s)). Considering these expected or possible variables, you must analyse both tissues to ensure a sufficient evaluation of the potential for mutagenicity at the site of contact in the gastro-intestinal tract.

In your comments to the draft decision, you argue that the request for collection of multiple sites of contact tissues in the digestive tract is "unusual" when considering the similar level at the 24-hour time point between glandular stomach and duodenum obtained in the available comet assay (Sasaki et al., 2000).

Several tissues were selected for analyses in the publicly available comet assay, but the duodenum was not included (Sasaki et al., 2000). Hence, it is not possible for the evaluating MSCA to comment on your comparison of the obtained results between the glandular stomach and duodenum. However, independent of this comparison, at least one rapidly dividing tissue must be selected for analysis according to the OECD TG 488, and because of the expected or possible variables between the two tissues as described above, the duodenum and the liver are standard tissues to be requested in combination and regarded as minimum requirements for the TGR assay according to OECD TG 488.

- *Urinary bladder*

In the available comet assay (Sasaki et al., 2000), the strongest positive responses were found in the stomach, liver and urinary bladder. As explained above, due to its limitations, the existing comet assay does not however adequately clarify the mutagenicity concern for urinary bladder. However, the study result still provides a strong concern for genotoxicity in the urinary bladder. In addition, the primary site of excretion of 2-furaldehyde derived radioactivity is through urine and only very little is excreted via faeces or exhalation. Hence, a high exposure of the urinary bladder to 2-furaldehyde metabolites is expected. Consequently, the urinary bladder must also be included in the analyses of the requested TGR.

In your comments to the draft decision, you express the opinion that the available comet assay (Sasaki et al., 2000) is not reliable enough to increase the range of tissues that should be tested in any follow up assays.

While the evaluating MSCA acknowledges the limitations of the study, the OECD TG 488 (2022) para. 52 states that "…the selection of tissues to be collected should be based upon the reason for conducting the study and any existing genotoxicity, carcinogenicity or toxicity data for the test chemical under investigation". Hence, the results of the available comet assay (Sasaki et al., 2000) should not be neglected when selecting additional tissues for the TGR assay. Furthermore, collecting the urinary bladder, in addition to the other, more regularly selected tissues for analyses, will increase the information obtained per animal without increasing the number of animals used or compromising animal welfare.

- *Male germ cells*

Male germ cells must be harvested from the seminiferous tubules and stored at or below -70° for at least 5 years. In case of a positive or inconclusive result in any of the analysed somatic tissues, the male germ cells must be analysed, as this information is needed for the overall assessment of germ cell mutagenicity.

Request for the full study report

You must submit the full study report, which includes:
- a complete rationale of test design and
- interpretation of the results
- access to all information available in the full study report, such as implemented method, raw data collected, interpretations and calculations, consideration of uncertainties, argumentation, etc.

This will enable the evaluating MSCA to fully and independently assess all the information provided, including the statistical analysis, and to efficiently clarify the potential hazard for the Mutagenicity of the Substance.

c) Alternative approaches and how the request is appropriate to meet its objective

The request is:
- Appropriate, because it will provide information which will clarify the germ cell mutagenicity of the Substance enabling the evaluating MSCA to conclude on whether a classification for germ cell mutagenicity is warranted.
- The least onerous measure because there is no equally suitable alternative method available that would clarify the potential hazard of germ cell mutagenicity without the need of running separate tests for mutagenicity on somatic tissue and germ cells.

Considering the existing harmonized classification of the Substance, as Carc. 2, the OECD TG 488 gene mutation assay is the only study that can be requested to potentially improve the risk management measures of the Substance based on the potential risk for mutagenicity. The OECD TG 488 is the only validated genotoxicity test that enables the measurement of mutagenicity in both somatic tissues and germ cells, simultaneously using the same animals for both measurements.

Gene mutation mode of action is pursued in this substance evaluation. A subsequent follow up on the chromosomal aberration mode of action may still be relevant, if the requested TGR/OECD TG 488 is negative or inconclusive. In that case, an updated thorough weight-of-evidence analysis will be performed taking all available information into account.

2.2 References relevant to the requests (which are not included in the registration dossier)

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The Danish (Q)SAR database: (http://qsar.food.dtu.dk/)

Appendix B: Procedure

This decision does not imply that the information you submitted in your registration dossier(s) is in compliance with the REACH requirements. ECHA may still initiate a compliance check on your dossiers.

12-month evaluation

Due to initial grounds of concern for mutagenicity, carcinogenicity and human exposure the Member State Committee agreed to include the Substance in the Community rolling action plan (CoRAP) 2022-2024 for evaluation in 2022. The Danish Environmental Protection Agency was appointed as the competent authority ('the evaluating MSCA') to carry out the evaluation.

In accordance with Article 45(4) of REACH, the evaluating MSCA carried out its evaluation based on the information in the registration dossier(s) you submitted on the Substance and on other relevant and available information.

The evaluating MSCA completed its evaluation considering that further information is required to clarify the following concern: mutagenicity. Therefore, it submitted a draft decision (Article 46(1) of REACH) to ECHA.

Decision-making

ECHA notified you of the draft decision and invited you to provide comments.

(i) Registrant(s) commenting phase

ECHA received your comments and forwarded them to the evaluating MSCA. The evaluating MSCA took your comments into account and the request was not amended while the deadline was amended.

In your comments on the draft decision, you requested an extension of the deadline. You reason that it is unlikely that 24 months from the date of adoption of the decision would be adequate to "*commission, run and report*" the required study. You have not provided documentary evidence to substantiate your claim.

Nevertheless, ECHA has exceptionally extended the standard deadline by 12 months to consider currently longer lead times in contract research organisations. On this basis, ECHA has extended the deadline to 36 months.

(ii) Notification to MSCAs

The evaluating MSCA notified the draft decision to the competent authorities of the other Member States and ECHA for proposal(s) for amendment.

As no amendments were proposed, ECHA took the decision according to Articles 52(2) and 51(3) of REACH.

(iii) Follow-up evaluation

After the deadline set in this decision has passed, the evaluating MSCA will review the information you will have submitted and will evaluate whether further information is still needed to clarify the potential risk, according to Article 46(3) of REACH. Therefore, a subsequent evaluation of the Substance may still be initiated after the present substance evaluation is concluded.

Appendix C: Technical Guidance to follow when conducting new tests for REACH purposes

Test methods, GLP requirements and reporting

Under Article 13(3) of REACH, all new data generated as a result of this decision must be conducted according to the test methods laid down in a European Commission Regulation or to international test methods recognised by the Commission or ECHA as being appropriate.

Under Article 13(4) of REACH, ecotoxicological and toxicological tests and analyses must be carried out according to the GLP principles (Directive 2004/10/EC) or other international standards recognised by the Commission or ECHA.

Under Article 10(a)(vi) and (vii) of REACH, all new data generated as a result of this decision must be reported as study summaries, or as robust study summaries, if required under Annex I of REACH. See ECHA Practical Guide on How to report robust study summaries[4].

Test material

Before generating new data, you must agree within the joint submission on the chemical composition of the material to be tested (Test Material) which must be relevant for all the registrants of the Substance.

1. Selection of the Test material(s)

The Test Material used to generate the new data must be selected taking into account the following:

- the variation in compositions reported by all members of the joint submission,
- the boundary composition(s) of the Substance,
- the impact of each constituent/ impurity on the test results for the endpoint to be assessed. For example, if a constituent/ impurity of the Substance is known to have an impact on (eco)toxicity, the selected Test Material must contain that constituent/ impurity.

2. Information on the Test Material needed in the updated dossier

a) You must report the composition of the Test Material selected for each study, under the 'Test material information' section, for each respective endpoint study record in IUCLID.

b) The reported composition must include all constituents of each Test Material and their concentration values.

This information is needed to assess whether the Test Material is relevant for the Substance and whether it is suitable for use by all members of the joint submission.

Technical instructions on how to report the above is available in the manual "How to prepare registration and PPORD dossiers"[5].

[4] https://echa.europa.eu/practical-guides
[5] https://echa.europa.eu/manuals