EUROPEAN CHEMICALS AGENCY

Helsinki, 7 April 2017

Addressee
[redacted]

Decision number: CCH-D-2114356114-57-01/F
Substance name: Dinitrogen tetraoxide
EC number: 234-126-4
CAS number: 10544-72-6
Registration number: [redacted]
Submission number: [redacted]
Submission date: 18.12.2015
Registered tonnage band: 100-1000T

DECISION ON A COMPLIANCE CHECK

Based on Article 41 of Regulation (EC) No 1907/2006 (the 'REACH Regulation'), ECHA requests you to submit information on

1. **In vivo mammalian alkaline comet assay (Annex IX, Section 8.4., column 2; test method: OECD TG 489) in rats, inhalation route, on the following tissues: liver and lung with the registered substance or the analogue substance nitrogen dioxide;**

You may adapt the testing requested above according to the specific rules outlined in Annexes VI to X and/or according to the general rules contained in Annex XI of the REACH Regulation. In order to ensure compliance with the respective information requirement, any such adaptation will need to have a scientific justification, referring and conforming to the appropriate rules in the respective Annex, and an adequate and reliable documentation.

You are required to submit the requested information in an updated registration dossier by **16 April 2018**. You shall also update the chemical safety report, where relevant. The timeline has been set to allow for sequential testing.

The scope of this compliance check decision is limited to the standard information requirement(s) of Annex IX, Sections 8.4 and 8.7 of the REACH Regulation.

The reasons of this decision are set out in Appendix 1. The procedural history is described in Appendix 2. Advice and further observations are provided in Appendix 3.

Appeal

This decision can be appealed to the Board of Appeal of ECHA within three months of its notification. An appeal, together with the grounds thereof, shall be submitted to ECHA in writing. An appeal has suspensive effect and is subject to a fee. Further details are described under http://echa.europa.eu/regulations/appeals.

Authorised[1] by Claudio Carlon, Head of Unit, Evaluation E2

[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.

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Appendix 1: Reasons

1. In vivo mammalian alkaline comet assay (Annex IX, Section 8.4., column 2)

Pursuant to Articles 10(a) and 12(1) of the REACH Regulation, a technical dossier registered at 100 to 1000 tonnes per year shall contain as a minimum the information specified in Annexes VII to IX of the REACH Regulation.

"Mutagenicity" is an information requirement as laid down in Annex VIII, Section 8.4. of the REACH Regulation. Column 2 of Annex IX, Section 8.4. provides that "If there is a positive result in any of the *in vitro* genotoxicity studies in Annex VII or VIII and there are no results available from an *in vivo* study already, an appropriate *in vivo* somatic cell genotoxicity study shall be proposed by the Registrant."

ECHA notes that the genetic toxicity studies in the dossier are all conducted on a analogue substance, nitrogen dioxide. ECHA considers that the read across from nitrogen dioxide can be considered acceptable as there will always be a pressure and temperature dependent equilibrium between N_2O_4 and NO_2 irrespective which is the starting material. The technical dossier contains several non guideline *in vitro* studies performed with an analogous substance, i.e. the bacterial reverse mutation assay (Ames test) (Viktorin and Ståhlberg, 1988; Arroyo et al., 1992; Kosaka et al., 1986), the chromosome aberration assay in mammalian cells (Tsuda et al., 1981) and a DNA single strand breaks alkaline elution assay (Görsdorf et al., 1990) with the nitrogen dioxide that show positive results. The positive results indicate that the substance is inducing gene mutations and chromosomal aberrations under the conditions of the tests.

In addition, the technical dossier contains several non guideline *in vivo* studies performed with an analogous substance, i.e. an alkaline elution assay examining alveolar macrophages, with DNA strand breaks caused by exposure to ozone and nitrogen dioxide (Bermudez et al., 1999), a chromosomal aberration study examining mouse leukocytes and spermatocytes following nitrogen dioxide inhalation (Gooch et al., 1977), a mouse bone marrow micronucleus assay with NO_2 (Victorin et al., 1990), a somatic mutation and recombination test in Drosophila (wing spot test) with genotoxic activity of 1,3-butadiene and nitrogen dioxide and their photochemical reaction products (Victorin et al., 1990). All showed negative results. There is also an *in vivo* chromosomal aberration study (Isomura et al., 1992, reliability 4) where lung cells from rats exposed to NO_2 showed a significant dose-related increase in aberrations (chromatid type) and chromatid breaks were increased 2.5- and 12-fold over the control at 8 and 27 ppm, respectively demonstrating that NO_2 and NO can induce mutations and that NO_2 also has the ability to induce chromosome aberrations in lung cells of rats *in vivo*.
The studies used exposure concentrations varying form 0.1 ppm to 27 ppm and the exposure periods varied from a single exposure of hours to exposures of three days. According to the endpoint summary for genetic toxicity, the Isomura et al, 1984 "*study design has severe limitations which makes the interpretation of the results difficult (very low cell survival, no validation of test conditions, lack of positive controls, frequency of chromosomal aberrations determined at very late timepoint*". Consequently, currently there is not an *in vivo* study available in the registration dossier that would be of sufficient quality. Hence, ECHA concludes that the tests provided are not appropriate to follow-up a concern for gene mutations and chromosomal aberrations. Moreover, the results of several repeated dose toxicity studies show that the lung is the primary target and that the substance causes, e.g., hyperplasia in the lungs.

In your comment to the draft decision, you propose that a paper from Han et al (2013) may provide appropriate data to fulfil the information requirement of a comet assay, but you have not provided the paper in your comment. Since the information had not been provided in the registration dossier in the form of a robust study summary, or in the comment, ECHA was unable to evaluate, for the purpose of current decision making process, whether this information would satisfy the information requirement.
In your comment, you indicate that you wish to perform classification based on the results of the in vivo mutagenicity study. Based on the information provided by you in the comment, i.e., a finding of a positive response in a comet assay in several tissues and in the micronucleus assay, you should self-classify the substance accordingly, and should provide a justification for your classification.

In your comment to the MSCAs' proposals for amendments you provided a copy of the study of Han *et al.* (2013) that includes results of the comet assay in liver, lung, brain, spleen, heart and kidney of rats following inhalation of NO2 (this article also described results on the micronucleus and DNA–protein crosslinks assays). The study has been conducted on nitrogen dioxide, which is the representative read-across substance for the registered substance, for the reasons expressed in your comments to the draft decision. You consider that these data are sufficient to fulfill the mutagenicity endpoint as requested in the current decision ("*In vivo* mammalian alkaline comet assay in rats, inhalation route, on the following tissues: liver, lung"). Moreover you expressed your intention to self-classify the registered substance N2O4 as Muta Cat 1B.

ECHA notes that the Han *et al.* study is published in a peer-reviewed journal, details are provided and basic scientific principles are met. ECHA also notes some deviations from the OECD test guideline for the in vivo comet assay (TG 489, 2014): Olive tail moment was used to evaluate DNA damage while % tail DNA is the parameter recommended in the TG 489; 50 cells per animal were analysed vs. 150/animal in TG 489; no positive control group was mentioned in the study report. ECHA however considers that, because the study shows a clear dose-related increase in the DNA damage in all five tissues analysed, these deviations do not affect the scientific acceptability of the study and thus do not invalidate the study results. ECHA also considers the read across from nitrogen dioxide as acceptable. ECHA thus concludes that, provided that the data contained in Han et al study is inserted as a robust study summary in an updated dossier, this data could be considered as fulfilling the information requirement for mutagenicity, removing the need to generate currently requested data.

Regarding the self classification, such action is the responsibility of the registrant.

However, an appropriate *in vivo* genotoxicity study to follow up the concern on gene mutations or chromosomal aberrations is not currently available in the dossier. Consequently there is an information gap and it is necessary to provide information on the registered substance, or the analogue substance nitrogen dioxide, for this endpoint, in the dossier.

According to the ECHA *Guidance on information requirements and chemical safety assessment* (version 4.1, October 2015) Chapter R.7a, section R.7.7.6.3, the *in vivo* mammalian alkaline comet assay ("Comet Assay", OECD TG 489) is suitable to follow up positive *in vitro* result for gene mutation and chromosomal aberrations. Hence, ECHA considers this test to be most appropriate for the substance subject to the decision.

According to the test method OECD TG 489, the test is routinely performed in rats. Having considered the anticipated routes of human exposure and adequate exposure of the target tissues performance of the test by the inhalation route is appropriate because the substance is a gas.

According to the test method OECD TG 489, the test can be performed by analysing tissues from liver as primary site of xenobiotic metabolism and lung as sites of contact.

Therefore, pursuant to Article 41(1) and (3) of the REACH Regulation, you are requested to submit the following information derived with the registered substance subject to the present decision or the analogue substance nitrogen dioxide:

In vivo mammalian alkaline comet assay (test method: OECD TG 489) in rats, inhalation route, on the following tissues: liver and lung.

Notes for your consideration

You are reminded that according to Annex IX, Section 8.4., column 2 of the REACH Regulation, if positive results from an *in vivo* somatic cell study are available, "the potential for germ cell mutagenicity should be considered on the basis of all available data, including toxicokinetic evidence. If no clear conclusions about germ cell mutagenicity can be made, additional investigations shall be considered".

You may consider examining gonadal cells, as it would optimise the use of animals. ECHA notes that a positive result in whole gonads is not necessarily reflective of germ cell damage since gonads contain a mixture of somatic and germ cells. However, such positive result would indicate that the substance and/or its metabolite(s) have reached the gonads and caused genotoxic effects. This type of evidence may be relevant for the overall assessment of possible germ cell mutagenicity including classification and labelling according to the CLP Regulation.

Deadline to submit the requested Information

In the draft decision communicated to you the time indicated to provide the requested information was 36 months from the date of adoption of the decision. This period of time took into account the fact that the draft decision also requested a pre-natal developmental toxicity study (Annex IX, 8.7.2.) and an extended one-generation reproductive toxicity study (Annex IX, 8.7.3.). As these studies are not requested by the present decision, ECHA considers that a reasonable time period for providing the required information in the form of an updated registration is 12 months from the date of the adoption of the decision. The decision was therefore modified accordingly.

EUROPEAN CHEMICALS AGENCY

Appendix 2: Procedural history

For the purpose of the decision-making, this decision does not take into account any updates of your registration after the date when the draft decision was notified to you under Article 50(1) of the REACH Regulation.

The compliance check was initiated on 13 April 2016.

The decision making followed the procedure of Articles 50 and 51 of the REACH Regulation, as described below:

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

ECHA took into account your comments and did not amend the requests.

ECHA notified the draft decision to the competent authorities of the Member States for proposals for amendment.

ECHA received proposals for amendment and modified the draft decision

ECHA invited you to comment on the proposed amendments.

ECHA referred the draft decision to the Member State Committee.

Your comments on the proposed amendment(s) were taken into account by the Member State Committee.

The Member State Committee reached a unanimous agreement on the draft decision during its MSC-52 meeting and ECHA took the decision according to Article 51(6) of the REACH Regulation.

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Appendix 3: Further information, observations and technical guidance

1. The substance subject to the present decision is provisionally listed in the Community rolling action plan (CoRAP) for start of substance evaluation in 2018.

2. This compliance check decision does not prevent ECHA from initiating further compliance checks on the present registration at a later stage.

3. Failure to comply with the request(s) in this decision, or to fulfil otherwise the information requirement(s) with a valid and documented adaptation, will result in a notification to the enforcement authorities of your Member State.

4. In relation to the information required by the present decision, the sample of the substance used for the new test(s) must be suitable for use by all the joint registrants. Hence, the sample should have a composition that is suitable to fulfil the information requirement for the range of substance compositions manufactured or imported by the joint registrants. It is the responsibility of all joint registrants who manufacture or import the same substance to agree on the appropriate composition of the test material and to document the necessary information on their substance composition. In addition, it is important to ensure that the particular sample of the substance tested in the new test(s) is appropriate to assess the properties of the registered substance, taking into account any variation in the composition of the technical grade of the substance as actually manufactured or imported by each registrant. If the registration of the substance by any registrant covers different grades, the sample used for the new test(s) must be suitable to assess these grades. Finally there must be adequate information on substance identity for the sample tested and the grade(s) registered to enable the relevance of the test(s) to be assessed.