Helsinki, 7 February 2017

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

DECISION ON SUBSTANCE EVALUATION PURSUANT TO ARTICLE 46(1) OF REGULATION (EC) NO 1907/2006

For tert-butyl methyl ether (MTBE), CAS No 1634-04-4 (EC No 216-653-1)

Addressees: Registrant(s)[1] of tert-butyl methyl ether (Registrant(s))

This decision is addressed to all Registrant(s) of the above substance with active registrations on the date on which the draft for the decision was first sent for comments, with the exception of the cases listed in the following paragraph. A list of all the relevant registration numbers subject to this decision is provided as an annex to this decision.

Registrant(s) holding active registrations on the day the draft decision was sent are *not* addressees of this decision if they are: i) Registrant(s) who had on that day registered the above substance exclusively as an on-site isolated intermediate under strictly controlled conditions and ii) Registrant(s) who have ceased manufacture/import of the above substance in accordance with Article 50(3) of Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH Regulation) before the decision is adopted by ECHA.

Based on an evaluation by tert-butyl methyl ether as the Competent Authority of France (evaluating MSCA), the European Chemicals Agency (ECHA) has taken the following decision in accordance with the procedure set out in Articles 50 and 52 of Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH Regulation).

This decision is based on the registration dossier on June 2014 while relevant elements of dossier updates submitted in 2015 and 2016 have been considered.

This decision does not imply that the information provided by the Registrant(s) in the registration(s) is in compliance with the REACH requirements. The decision neither prevents ECHA from initiating compliance checks on the dossier(s) of the Registrant(s) at a later stage, nor does it prevent a subsequent decision under the current substance evaluation or a new substance evaluation process once the present substance evaluation has been completed.

I. Procedure

Pursuant to Article 45(4) of the REACH Regulation the Competent Authority of France has initiated substance evaluation for tert-butyl methyl ether (MTBE), CAS No 1634-04-4 (EC No 216-653-1) based on registration(s) submitted by the Registrant(s) and other relevant and available information and prepared the present decision in accordance with Article 46(1) of

[1] The term Registrant(s) is used throughout the decision, irrespective of the number of registrants addressed by the" decision.

the REACH Regulation.

On the basis of an opinion of the ECHA Member State Committee and due to initial grounds for concern relating to Human health/Potential endocrine disruptor; Exposure/Wide dispersive use, aggregated tonnage, MTBE was included in the Community rolling action plan (CoRAP) for substance evaluation to be evaluated in 2014. The updated CoRAP was published on the ECHA website on 26 March 2014. The Competent Authority of France was appointed to carry out the evaluation.

In the course of the evaluation, the evaluating MSCA identified additional concerns regarding mutagenicity, biodegradability and persistency in the environment.

Regarding the endocrine disruptor (ED) potential, MTBE has a large database and ED related effects could only be seen at very high doses for the Human Health Part (effects at doses above the limit dose, 1000mg/kg bw/d). Therefore the concern was not sufficient to request further testing. However, for the Environmental Part, it was not possible to conclude on ED properties: even if an estrogenic effect is observed, there are no clear adverse effects *in vivo* (vitellogenin induction is not considered an adverse effect). Therefore further testing is needed.

The evaluating MSCA considered that further information was required to clarify the abovementioned concerns. Therefore, it prepared a draft decision pursuant to Article 46(1) of the REACH Regulation to request further information. It submitted the draft decision to ECHA on 26 March 2015.

On 7 May 2015 ECHA sent the draft decision to the Registrant(s) and invited them pursuant to Article 50(1) of the REACH Regulation to provide comments within 30 days of the receipt of the draft decision.

Registrant(s)' commenting phase

By 15 June 2015 ECHA received comments from the Registrant(s) of which it informed the evaluating MSCA without delay. The evaluating MSCA considered the comments received from the Registrant(s).

On basis of this information, Section II was amended. The Statement of Reasons (Section III) was changed accordingly.

Commenting by other MSCAs and ECHA

In accordance with Article 52(1) of the REACH Regulation, on 21 July 2016 the evaluating MSCA notified the Competent Authorities of the other Member States and ECHA of its draft decision and invited them pursuant to Articles 52(2) and 51(2) of the REACH Regulation to submit proposals to amend the draft decision within 30 days of the receipt of the notification.

Subsequently, two Competent Authority of the Member States and ECHA submitted proposals for amendment (PfAs) to the draft decision.

On 26 August 2016 ECHA notified the Registrant(s) of the proposals for amendment to the draft decision and invited them pursuant to Articles 52(2) and 51(5) of the REACH Regulation to provide comments on those proposals for amendment within 30 days of the receipt of the notification.

The evaluating MSCA reviewed the proposals for amendment received and amended the draft decision accordingly.

Referral to Member State Committee

On 5 September 2016 ECHA referred the draft decision to the Member State Committee.

By 26 September 2016, in accordance to Article 51(5), the Registrant(s) provided comments on the proposals for amendment. In addition, the Registrant(s) provided comments on the draft decision. The Member State Committee took the comments on the proposal(s) for amendment of the Registrant(s) into account. The Member State Committee did not take into account the Registrant(s)' comments on the draft decision that were not related to the proposal(s) for amendment made and are therefore considered outside the scope of Article 51(5).

After discussion in the Member State Committee meeting on 25 – 27 October 2016, a unanimous agreement of the Member State Committee on the draft decision as modified at the meeting was reached on 27 October 2016. ECHA took the decision pursuant to Article 52(2) and Article 51(6) of the REACH Regulation.

II. Information required

Pursuant to Article 46(1) of the REACH Regulation the Registrant(s) shall submit the following information using the indicated test methods (in accordance with Article 13(3) and (4) of the REACH Regulation) and the registered substance subject to the present decision:

1. Transgenic rodent somatic and germ cell gene mutation assays (TGR) (OECD TG 488). The TGR somatic test shall be conducted based on the guideline in male rodents treated for 28 days via inhalation route. Tissues (liver, kidney, nasal tissue, lymphatic tissue (lymph node or bone marrow)) and germs cells shall be harvested three days after cessation of treatment. Mutation frequency shall be assessed in liver, kidney, nasal tissue and lymphatic tissue (lymph node or bone marrow). In addition, spermatozoa from the vas deferens/cauda epididymis and developing germ cells from the seminiferous tubules shall be collected at the same time as the other tissues and stored at or below −70 °C. In case positive results are found in any somatic tissues, mutation frequency in the collected germ shall be assessed. The Registrant(s) may add an additional sampling time to accurately evaluate the effect of the treatment on cells that were spermatogonial stem cells during the exposure period.

2. Risk assessment of general population following indirect exposure of MTBE as specified in section III.

3. Clarification and detailed justification for each environmental exposure scenario as specified in section III.

4. Fish Sexual Development test (OECD TG 234) as specified in section III.

Deadline for submitting the required information

Pursuant to Article 46(2) of the REACH Regulation, the Registrant(s) shall submit to ECHA by **14 August 2018** an update of the registration(s) containing the information required by

this decision[2], including robust study summaries and, where relevant, an update of the Chemical Safety Report.

III. Statement of reasons

Based on the evaluation of all relevant information in the registration dossiers for MTBE, ECHA concludes that further information is required in order to enable the evaluating MSCA to complete the evaluation of whether the substance constitutes a risk to human health and environment.

1. TGR assay (OECD TG 488) in rodent inhalation route

Further information on mutagenicity, specifically gene mutation, is required in order to enable ECHA to conclude on whether the registered substance has the potential to cause gene mutations and to analyse the relevance of the carcinogenicity database. Indeed, both new mutagenicity data (since the Risk Assessment Report (RAR) carried out under Council Regulation (EEC) 793/93) together with remaining uncertainty on carcinogenesis after previous evaluation raises a concern.

1.1 Remaining uncertainty on carcinogenesis and mutagenicity after previous RAR evaluation

Regarding carcinogenesis, the RAR(2002)[3] concluded that "*there are indications of carcinogenicity in two species. However the treatment relation of the occurred tumours is equivocal in some studies (mouse adenoma) and the relevance of the mode of action is questionable in others (Leydig cell). Moreover, the tumours appear mostly at very high and systemically toxic doses, and MTBE is not genotoxic in vitro or in vivo. On the other hand, the human relevance of the testicular interstitial adenomas observed in rats on two separate rat strains cannot be neglected. In addition, certain uncertainty remains as to the significance of the lymphatic tumours found, in the light of the limitations of the study and inadequate reporting. The rapporteur considers MTBE as a borderline case between nonclassification and Carc. Cat. 3.*"

Indeed, hepatocellular adenomas were described in two inhalation mice studies (Moser, 1996[4] and Bushy Run[5], 1992) on females at the high dose (8000 ppm). Renal tubular cells tumours were observed in male mice following MTBE inhalation (Bushy Run, 1992), thought kidney toxicity was not limited to male and to tubular cells. However, when it evolves into nephro-carcinogenesis, it seems limited to high dose male giving credit to the alpha-2 microglobulin mode of action. Leydig cell adenomas were found in rats after inhalation and oral exposure to MTBE (respectively Bushy Run, 1992 and Belpoggi, 1995[6]). In the same oral study (Belpoggi, 1995), a dose-related increase in the incidence of lymphoimmunoblastic lymphoma in lungs and leukemias were described in female SD rats. Finally, astrocytomas were described in high dose treated rats within historical control range (Dodd, 2010[7]).

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[2] The deadline set by the decision already takes into account the time that registrants may require to agree on who is to perform any required tests and the time that ECHA would require to designate a registrant to carry out the test(s) in the absence of the aforementioned agreement by the registrants (Article 53(1) of the REACH Regulation).

3 Europen Union Risk Assessment Report, tert-butyl methyl ether, Volume 19, Finland, Final Report 2002.

[4] Moser GJ, Wong BA, Wolf DC, Fransson-Steen RL, Goldsworthy TL. Methyl tertiary butyl ether lacks tumor-promoting activity in N-nitrosodiethylamine-initiated B6C3F1 female mouse liver. Carcinogenesis. 1996 Dec;17(12):2753-61.

[5] Bird MG, Burleigh-Flayer HD, ChunJS, DouglasJF, Kneiss JJ and Andrews LS. Oncogenicity Studies of Inhaled Methyl Tertiary-butyl Ether (MTBE) in CD-1 Mice and F-344 Rats Journal of Applied Toxicology, Vol. 17(7), S45–S55 (1997)

[6] Belpoggi F, Soffritti M, Maltoni C. MTBE causes testicular and lymphohaematopoietic cancers in rats. Toxicology and industrial Health, Vol. 1 I , No. 2, 1995.

[7] Dodd D, Willson G, Parkinson H and Bermudez E. Two-year drinking water carcinogenicity study of methyl tertiary-butyl ether (MTBE) in Wistar rats. J. Appl. Toxicol. 2011

Regarding genotoxicity, RAR concluded that "*based on the available information, MTBE cannot be considered a mutagen*". Regarding the involvement of MTBE genotoxic metabolites in mutagenicity of MTBE, it was also concluded in the RAR that "*the study by Casanova et al. gives reassuring evidence that formaldehyde endogenously generated from MTBE does not have a significant genotoxic impact (Casanova et al. 1997[8]). Moreover, it is known that any generated formaldehyde rapidly reacts with glutathione, forming S-hydroxymethylglutathione, a substrate for formaldehyde dehydrogenase that swiftly catalyses the oxidation of the substrate to formylglutathione, which is subsequently hydrolysed to formate. This enzymatic event is known to take place in a number of tissues in a variety of species.*" In its comments the Registrant(s) emphasize this publication to conclude that there is no remaining concern. However, it should be noted that the positive control used was formaldehyde and not a DNA damaging agent that requires activation (e.g. Benzo[a]pyrene or other PAH); consequently the quality of metabolic activity of *ex vivo* hepatocytes was not tested in this study. The added value of this study to what would happen in humans exposed to MTBE is limited and does not clarify the remaining concern.

Moreover, one of the limitations of the analysis in the RAR is that most of the *in vivo* studies available while the RAR was agreed did not show any toxicity. This leads to uncertainty on the maximal dose tested together with the fact that MTBE reached the target organ. For example, it was specified in the micronucleus tests that PCE-NCE ratio did not show meaningful changes ([redacted]; 1993[9], Kado et al.; 1998[10]). In their comments to the draft decision, the Registrant(s) pointed out that "*based on the blood/air partition coefficient (17.7-20) and the olive oil/air partition coefficient (120-140) measured in vitro (cited in McGregor, 2006[11]) and its good water solubility it can be stated that MTBE is moderately soluble in blood and 7-10 times more soluble in fat tissue*". ECHA agrees with the Registrant(s) that based on the PBPK modelling, it is reasonably certain that these targets were in fact exposed to MTBE (and TBA) because they are highly perfused tissues. However, it should be noted that one of the possible explanations of the negativity of the test is the lack of a competent machinery for metabolising MTBE in bone marrow.

According to current knowledge, the enzyme catalysing MTBE biotransformation into formaldeyde and TBA in humans is mainly CYP2A6, which is found in significant quantities only in the liver. The rat is lacking this enzyme, so other CYP enzymes, notably 2B1 and 2E1 seem to be involved. In the rat, the olfactory and nasal epithelium CYP2A3 exhibited even higher metabolising activities than the previously mentioned liver enzymes. So MTBE would reach bone marrow (as well as other highly perfused tissues) but would not be transformed (or only in little quantities) to its reactive metabolites, as it has been shown that the level of CYP2E1 is much lower in this tissue (Bernauer et al 2000 [12]), than in liver. This is the reason why genotoxicity has to be tested on the organs were tumours have been found.

Another explanation would also be linked to the dose reaching the bone marrow together with the sensitivity of the test used. As an example, the diverging results regarding

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[8] Casanova, M., and H. d. A. Heck. 1997. Lack of Evidence for the Involvement of Formaldehyde in the Hepatocarcinogenicity of Methyl-t-Butyl Ether (MTBE). Chemico-Biological Interactions 105: 131-143.

[9] [redacted]. 1993. [redacted]

[10] Kado, N. Y., P. A. Kuzmicky, G. Loarca-Pina, and M. Molz-Mumtaz. 1998. Genotoxicity Testing of Methyl Tertiary-Butyl Ether (MTBE) in the Salmonella Micro Suspension Assay and Bone Marrow Micronucleus Test. Mutat. Res. 412: 131-138.

[11] McGregor D. Methyl tertiary-Butyl Ether: Studies for Potential Human Health Hazards. Crit Rev Toxicol. 2006;36:319-358.

[12] Bernauer U1, Vieth B, Ellrich R, Heinrich-Hirsch B, Jänig GR, Gundert-Remy U (2000). CYP2E1 expression in bone marrow and its intra- and interspecies variability: approaches for a more reliable extrapolation from one species to another in the risk assessment of chemicals. Arch. Toxicol., 73 (12), 618-624.

genotoxicity of Formaldehyde have been lengthily discussed[13] but did not prevent the RAC to finally decide that based on induction of mutagenic and genotoxic effects of formaldehyde on somatic cells at the site of contact, classification as a Category 2 mutagen was warranted (RAC Opinion proposing harmonised classification and labelling at EU level of Formaldehyde, adopted 30 November 2012). The discussion on formaldehyde pointed out that the sensitivity of the tests used could lead to contradictory results. Similarly, the lack of sensitivity of the tests used to detect genotoxicity of MTBE might explain the negative results considered in the RAR.

The genotoxicity of MTBE or its metabolites will be evaluated in any of the tissues where tumours are described.

1.2 New data on mutagenicity since RAR was published

Since the RAR was published in 2002 (with literature for genotoxicity taken into account up to 2001), new literature has been produced. Some show protective effects of fuel oxygenates to genotoxicity:

- In a paper reporting the development of yeast-based method for the detection of cyto- and genotoxicity and testing MTBE, it is stated that the presence of MTBE does not increase specific fluorescence emission, and thus genotoxic effect (data not shown) (Lichtenberg-Fraté H. et al., 2003[14]).

- In a paper published in 2005, MTBE was tested against S. typhimurium strains in two laboratories. The emphasis was placed on testing with S. typhimurium TA102 and the use of both dimethyl sulphoxide and water as vehicles. Dose levels up to 5000 microgram/plate were used and incubations were conducted in both the presence and absence of liver S9 prepared from male rats treated with either Arochlor 1254 or phenobarbital-beta-naphthoflavone. The experiments were replicated, but in none of them was a significant mutagenic response observed, indicating that MTBE is not mutagenic in bacteria (McGregor et al., 2005[15]).

- In a paper published in 2010, cytotoxic effects were investigated in murine fibroblasts (L929) using the neutral red uptake assay and mutagenicity using the bacterial reverse mutation assay The cells were treated with preparation coming from non-reformulated gasoline. This fuel was supplemented with 10%, 20%, 25% and 30% ETBE or 15% MTBE. The fuels were combusted in a gasoline engine at idling, part load and rated power. Condensates and particulate matter (PM) were collected and PM samples extracted with dichloromethane. PM-extracts showed mutagenicity with and without metabolic activation. Mutagenicity was reduced by the addition of MTBE and ETBE, 10% ETBE being most effective. The condensates produced no significant mutagenic response. The cytotoxicity of the condensates from ETBE- and MTBE-reformulated fuels was reduced as well. Reduction of mutagenicity in the PM-extracts is most probably caused by a lower content of polycyclic aromatic hydrocarbons (Westphal GA et al., 2010[16]).

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[13] As an example: Speit G, Schütz P, Weber I, Ma-Hock L, Kaufmann W, Gelbke HP, Durrer S.Mutat Res. 2011 Apr 3;721(2):127-35. Analysis of micronuclei, histopathological changes and cell proliferation in nasal epithelium cells of rats after exposure to formaldehyde by inhalation.

[14] Lichtenberg-Fraté H, Schmitt M, Gellert G, Ludwig J. A yeast-based method for the detection of cyto and genotoxicity. Toxicol In Vitro. 2003 Oct-Dec;17(5-6):709-16.

[15] McGregor DB, Cruzan G, Callander RD, May K, Banton M. The mutagenicity testing of tertiary-butyl alcohol, tertiary-butyl acetate and methyl tertiary-butyl ether in Salmonella typhimurium. Mutat Res. 2005 Jan 3;565(2):181-9.

[16] Westphal GA, Krahl J, Brüning T, Hallier E, Bünger J. Ether oxygenate additives in gasoline reduce toxicity of exhausts. Toxicology. 2010 Feb 9;268(3):198-203.

Some of this new literature also report positive results:

- In two papers in Chinese (only abstract available) from the same journal, positive results are reported.
  - *In vitro*, it was described that MTBE and its metabolites could have genotoxicity on human leukemia (HL-60) cells (Tang et al. 1997[17]). In their comments to the draft decision, the Registrant(s) identify limitations (such as the fact that this protocol is not validated, that following treatment of the cells with MTBE and two of its metabolites, the authors reported identical levels of DNA damage, that the cell line used in this study is not commonly used in the comet assays and is not known to be metabolically competent) leading them to conclude that "*the study methodology as well as its conclusions are of questionable reliability and thus should not alter the informed decision reached in the RAR that MTBE is not genotoxic*". However, ECHA believes that this paper emphasize the hypothesis of the evaluating MSCA. ECHA agrees that almost all tumour cell lines such as HL-60 are normally not known to be CYP450 active. However, other oxydases may play a role in drug activation that induce the formation of DNA adducts. Indeed, HL60 has been reported to activate for instance ellipticine derivatives by CYP3A4, CYP2E6 as well as peroxydases (Poljakova et al. 2006)[18]. In fact, despite a low level of activity specifically for CYP1A1 and others, HL60 cells express mainly peroxydases, cyclooxygenases, myeloperoxydases as well as reductases that participate to the activation of many compounds as tested by the formation of DNA adducts (for instance: Kizaki *et al.* 1996[19], Melendez-Colon *et al.*1999[20]). Moreover, the publication by Nagai et al. 2002[21] shows that they highly express CYP2E1 and CYP2A6 mRNA and would therefore be competent in transforming MTBE into its active metabolite. Despite the limitations given by the Registrant(s), there is no reason to discard this result.

  - Another study was performed both *in vitro* and on cells from animals exposed to MTBE by inhalation (Yang H. et al; 200522). In the *in vivo* comet assay, the lengths of DNA migration in hepatocytes of mice exposed by inhalation at 1440, 4968 mg/m(3) of MTBE, renal cells at all doses, and pneumocytes at 4968 mg/m(3) were greater than those in negative controls. There was a dose-effect relationship between the concentration of MTBE and hepatocytes DNA migration lengths in mice ($r = 0.997$, $P = 0.003$). MTBE in concentrations of 1440 and 4968 mg/m(3) contributed to a rise in MDA of renal homogenates in female mice ($P < 0.05$). MTBE above 0.050 mmol/L caused greater DNA migration in cultured rat type II pneumocytes and rat hepatocytes in vitro ($P < 0.05$), and also with dose-effect relationship (r(lung) = 0.967, r(liver) = 0.963, $P < 0.05$)). In an *in vitro* UDS assay, DNA synthesis of rat type II pneumocytes and rat hepatocytes were increased at the concentration of 5.0

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[17] Tang G, Wang J, Zhuang Z. [Cytotoxicity and genotoxicity of methyl tert-butyl ether and its metabolite to human leukemia cells]. Zhonghua Yu Fang Yi Xue Za Zhi. 1997 Nov;31(6):334-7. Chinese.

[18] Poljakova J *et al.* DNA adduct formation by the anticancer drug ellipticine in human leukemia HL-60 and CCRF-CEM cells. Cancer Letters 252 (2007) 270–279.

[19] Kizaki *et al.* Mechanisms of Retinoid Resistance in Leukemic Cells: Possible Role of Cytochrome P450 and P-Glycoprotein. 1996 87: 725-733.

[20] Meledenz-Colon et al. Comparison of Cytochrome P450- and Peroxidase-dependent Metabolic Activation of the Potent Carcinogen Dibenzo[a,l]pyrene in Human Cell Lines: Formation of Stable DNA Adducts and Absence of a Detectable Increase in Apurinic Sites. Cancer Res 1999;59:1412-1416.

[21] Nagai F1, Hiyoshi Y, Sugimachi K, Tamura HO. Cytochrome P450 (CYP) expression in human myeloblastic and lymphoid cell lines. Biol Pharm Bull. 2002 Mar;25(3):383-5.

[22] Yang H, Kong L, Zhao JS. [DNA damage induced by methyl tertiary-butyl ether in vivo and in vitro]. Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi. 2005 Oct;23(5):362-5. Chinese.

mmol/L and 10.0 mmol/L of MTBE (Yang H. et al; 2005).

In their comments to the draft decision, the Registrant(s) remind that "it is difficult to critically examine this paper either for the acceptability of the experimental methodology or for strength and reliability of the data" as only an abstract is available. ECHA never intended to use these data as definitive proof of MTBE's genotoxicity as the limitations identified by the Registrant(s) are correct. ECHA raises this publication as an additional alert on the genotoxicity.

- Genotoxicity of MTBE was assessed in human lymphocytes using *in vitro* comet assay. (Chen et al., 2008[23]) showed that MTBE could induce a variety type of DNA damage such as single-strand breaks (SSBs), double-strand breaks (DSBs), and oxidative base modification. Nevertheless, a few limitations were identified for this test: no image analysis technics was used. The induction of DNA strands break by MTBE was weak and not dose related. Cytotoxicity was not measured in this test.
    - In their comments on the draft decision, the Registrant(s) question the technical validity of this study in particular regarding the solubility of MTBE. Our understanding of the paper is different: MTBE is dissolved in DMSO (stock solution 20mMol that appears plausible), limiting the quantity of DMSO that can be used on cell culture. This experimental condition is no reason for disqualifying the study. ECHA believes that this experimental condition does not interfere with genotoxicity that appear at particularly low dose, explaining the weak response observed. The Registrant(s) conclude that "*DNA strand break response (reported in arbitrary units) to MTBE treatment was very weak with no apparent dose response*". ECHA would like to remind that in old Comet assays, the number of DNA break was quoted by cross. The report from Chen et al. 2008 was perfectly conducted showing no cell toxicity and using the classification after visual inspection by one person and encoded slides. The visual inspection is admitted in scientific papers and gives similar results as the semi or automatic determination of the percentage of DNA in the tail[24]. Its standardization is a long going process that led to its standardisation in 2014 for the *in vivo* part (TG489) while *in vitro* comet assay standardisation is still under discussion. ECHA agrees that cytotoxicity could lead to misinterpretation in the comet assay. However it is currently admitted that histopathological examination should be performed in the case of *in vivo* assay and that *in vitro* assay should be conducted with a cell survival rate at least equal to 90%.

- In the study performed by Weng et al. (2013)[25] wild-type (WT) and Aldh2 knockout (KO) C57BL/6 mice were exposed to 0, 500, 1750, or 5000 ppm Ethyl tertiary butyl ether (ETBE) for 6h/day with 5 days per weeks for 13 weeks.
    - Responding to the Registrant(s)' comment, ECHA emphasizes that this study has been performed with a closely related substance (ethyl instead of methyl, same use - see table below for the comparison of their physicochemistry properties) to the one evaluated and is presented only as supportive alert.

[23] Chen CS, Hseu YC, Liang SH, Kuo JY, Chen SC. Assessment of genotoxicity of methyl-tert-butyl ether, benzene, toluene, ethylbenzene, and xylene to human lymphocytes using comet assay. J Hazard Mater. 2008 May 1;153(1-2):351-6.

[24] The comet assay: topical issues (2008) A.R. Collins et al, Mutagenesis 23(3):143-151

[25] Weng Z, Suda M, Ohtani K, Mei N, Kawamoto T, Nakajima T, Wang RS. Subchronic exposure to ethyl tertiary butyl ether resulting in genetic damage in Aldh2 knockout mice. Toxicology. 2013 Sep 15;311(3):107-14.

Phys-chem properties	ETBE	MTBE
CAS n°	637-92-3	1634-04-4
Molecular formula	C6H14O	C5H12O
Molecular weight (g / mol)	102,2	88,2
vapour pressure at 20 ° C	130	240
Solubility in water at 20 ° C g / L	23,7	42
Log Coefficient sharing octanol / water	1,28	1,06
Henry constant : Pa.m3/mole	140	43,8

After subchronic exposure to ETBE, there was significant increase in DNA damage in a dose-dependent manner in KO male mice, while only 5000 ppm exposure significantly increased DNA damage in male WT mice. Overall, there was a significant sex difference in genetic damage in both genetic types of mice. These results showed that ALDH2 is involved in the detoxification of ETBE and lack of enzyme activity may greatly increase the sensitivity to the genotoxic effects of ETBE, and male mice were more sensitive than females. This study demonstrates the potential interindividual variability to ETBE genotoxicity and intersexual difference of sensitivity.

- In their comments, the Registrant(s) question the design and reporting to undermine the results. On the other hand, it is important to note that this study has been performed using the hOGG1 human endonuclease well recognised as unstable but still increasing the signal. Therefore, it is also possible that the low signal reported would have been more pronounced while using the bacterial endonuclease FPG.

- In another study, the binding ability of MTBE to DNA have been measured by using doubly ^{14}C-labeled MTBE with an advanced, ultrasensitive technique: accelerator mass spectrometry (AMS). It was found that MTBE definitely formed adducts with DNA in mouse lung, liver, and kidney in a log/log linear dose-response relationship. The distribution sequence of DNA adducts in these tissues is: lung > liver > kidney. The level of MTBE-DNA adducts peaked at 12h post administration in the lung and peaked at 6h post administration in the liver. Then the adducts declined rapidly until 5 days post administration and thereafter declined much more slowly (Du HF et al., 2005)[26].

  - In their comments, the Registrant(s) have discussed the possibility that the radioactivity found might be due to metabolic incorporation of ^{14}C into DNA through cellular carbon pools fueled with ^{14}C originating from ^{14}C-MTBE metabolism instead of DNA adduct as the technique used requires DNA sample to be converted to elemental carbon or CO2 resulting in the loss of structural information for the adduct. Because liver, kidney and lung are tissues where replication is very limited, ECHA doubts that the ^{14}C incorporation is due to something other than adduct formation. Moreover, in order to respond to such comments, the authors have performed another

[26] Du HF, Xu LH, Wang HF, Liu YF, Tang XY, Liu KX, Peng SX. Formation of MTBE-DNA adducts in mice measured with accelerator mass spectrometry. Environ Toxicol. 2005 Aug;20(4):397-401.

study (Yuan et al., 2007) using a single labelling on the methyl group: it discard the possibility of formic acid in being reincorporated to metabolic incorporation of ^{14}C into DNA through cellular carbon pools fueled with ^{14}C originating from ^{14}C-MTBE metabolism (e.g. formation of ^{14}C-formate).

- In the study performed by Schreiner et al. (2014)[27], micronucleus and sister chromatid exchange (SCE) tests were performed for vapour condensate of baseline gasoline (BGVC), or gasoline with oxygenates, in which MTBE Sprague Dawley rats were exposed to 0, 2000, 10,000, or 20,000 mg/m(3) of each condensate (see Henley et al., 2014[28] for further details on the exposure), 6h/day, 5 days/week over 4 weeks. Positive controls (5/sex/test) were given cyclophosphamide IP, 24h prior to sacrifice at 5 mg/kg (SCE test) and 40 mg/kg (micronucleus test). Blood was collected from the abdominal aorta for the SCE test and femurs removed for the micronucleus test. Blood cell cultures were treated with 5 µg/ml bromodeoxyuridine (BrdU) for SCE evaluation. No significant increases in micronucleated immature erythrocytes were observed for any test material. Statistically significant increases in SCE were observed in rats given BGVC alone or in female rats given G/MTBE in a proportionally identical manner. The positive results of this publication are limited to one sex, and it is difficult to draw any conclusion from this article because of the cofounding effects of the vapour tested containing various chemicals. Moreover, the study design is complex. The review of the summary was not sufficient in the timeframe of the evaluation. A detailed evaluation of this study carried out by industry in their updated registration dossier would help distinguishing the effect of one compound or the other. Therefore, and as pointed out by the Registrant(s), this study is mentioned as an additional alert, having in mind that SCE indicates rather a chromosomal instability rather than genotoxicity.

Taken together, the data available point out for some coherent data regarding indirect mutagenicity of MTBE. *In vitro* genotoxicity tests performed with MTBE showed positive results in the MLA/TK only in the presence of S9 mix (see RAR), suggesting a bioactivation of MTBE in DNA reactive metabolites that induced mutations *in vitro*. Whereas no *in vivo* chromosomal damage was observed in bone marrow following inhalation or gavage with MTBE in rats, DNA adducts were observed in lung, liver and kidney in male mice following oral administration of MTBE, suggesting formation of DNA reactive metabolites in rodents as shown *in vitro*. Interestingly, two of these three organs showing DNA adducts formation were described for developing tumours after MTBE exposure via inhalation.

Further, the following aspects are to be considered:

- the positive results *in vitro* in MLA/TK with S9, that might come from the clastogenicity of formaldehyde generated extracellularly as stated by the Registrant(s) in their comments but which does therefore not preclude of what will happen *in vivo*, and DNA adducts formation in three organs in mice,
- all the *in vivo* and *in vitro* comet assays are positive pointing out for indirect mutagenicity when considered in comparison to all the other negative test (micronucleus, chromosomal aberration),
    - the sensitivity of this test rather *vivo* or *vitro* has been discussed in the Registrant(s)' comments by mentioning that "*tissue toxicity or cytotoxicity could be a confounder of comet results*". ECHA agrees that cytotoxicity, and more particularly heghogs cells, may bias the result of the comet assay. Later, the Registrant(s) state that "*there was no evidence for such a rigor in*

[27] Schreiner CA, Hoffman GM, Gudi R, Clark CR. Health assessment of gasoline and fuel oxygenate vapors: micronucleus and sister chromatid exchange evaluations. Regul Toxicol Pharmacol. 2014 Nov;70(2 Suppl):S29-34.
[28] Henley M, Letinski DJ, Carr J, Caro ML, Daughtrey W, White R. Health assessment of gasoline and fuel oxygenate vapors: generation and characterization of test materials. Regul Toxicol Pharmacol. 2014 Nov;70(2 Suppl):S13-7.

the conduct of the comet assays on MTBE". In that respect and as mentioned above, there is no objective data indicating that study from Chen et al. 2008 was not perfectly conducted. However it is considered that this study still needs to be taken into account although no definite conclusion can be drawn from this study alone.

- The Registrant(s) submit that the "*comet assay data reported in relatively unknown journals and with only an abstract for review cannot and should not form a basis for requesting a transgenic rodent somatic and germ cell mutation assay*". ECHA points out that the journal in which Chen published has a 5-Year Impact Factor: 5.277. The "the elegant work by Casanova et al. (1997)" was published in a journal , which impact factor is of 2.57 (2014/2015)

- *in vivo* micronucleus tests and chromosomal aberration tests are not appropriate to detect gene mutations, and the possibility that unstable metabolites could have not reached the target organs (Dearfield et al., 2011[29]),
  - the Registrant(s) commented this point by arguing that "*based on FA metabolism and Chinese hamster V79 cells results, MTBE rather induces clastogenicity and that therefore, micronucleus and chromosomal aberration are appropriate tests*". ECHA cannot decide on the mode of genotoxicity so far and Mackerer et al. (1996) [30]corroborates with the hypothesis of the evaluating MSCA. At the time of the RAR, this study was disregarded based on Casanova's data. However, the new piece of data elight that this study should be considered in more detail. Indeed, it shows that the positive results obtained with S9 on MLA/TK decreased when formaldehyde dehydrogenase was added (Mackerer 1996[31]). Therefore, it was proposed that these results were at least partially due to formaldehyde than MTBE itself. As quoted by the Registrant(s) "*the report by Mackerer et al. (1996) does not give information on colony sizing of the mutants recovered in the mouse lymphoma assay which would have helped to decide whether the mutagenicity observed with MTBE was likely due to clastogenicity (primarily induction of small colony mutants) or due to point mutations (primarily large colony mutants)*".

    Moreover, the reports available from these tests do not ensure that the target tissue was reached. ECHA agrees that in principle, these tests are informative of the genotoxicity of a substance, would the organ tested be exposed to sufficient amount of the substance and later metabolised into its reactive metabolism. Moreover, Chinese hamster V79 cells should not be over-interpreted has it has been shown to be a test with small sensitivity compared to CHO for example (Erexson et al. 2001)[32].

- Formaldehyde is not the sole ultimate toxicant; oxidative stress reagents might also be acting. Mutagenicity could involve the formation of DNA adducts although other mechanisms may play a role (replicative stress, nucleotides imbalance etc.). ECHA agrees that the size of the colonies in the mouse lymphoma assay would have been helpful to quantify mutagenicity versus clastogenicity. However, the assays detecting

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[29] Dearfield KL, Thybaud V, Cimino MC, Custer L, Czich A, Harvey JS, Hester S, Kim JH, Kirkland D, Levy DD, Lorge E, Moore M, Ouédraogo-Arras G, Schuler M, Suter W, Sweder K, Tarlo K, van Benthem J, van Goethem F, Witt KL. Follow-up actions from positive results of in vitro genetic toxicity testing. Environ Mol Mutagen. 2011 Apr;52(3):177-204. doi: 10.1002/em.20617. Epub 2010 Oct 20. Review.

[30] Mackerer CR, Angelosanto FA, Blackburn GR, Schreiner CA. Identification of formaldehyde as the metabolite responsible for the mutagenicity of methyl tertiary-butyl ether in the activated mouse lymphoma assay. Proc Soc Exp Biol Med. 1996 Sep;212(4):338-41. PubMed PMID: 8751991.

[32] Erexson GL1, Periago MV, Spicer CS.Differential sensitivity of Chinese hamster V79 and Chinese hamster ovary (CHO) cells in the in vitro micronucleus screening assay. Mutat Res. 2001 Aug 22;495(1-2):75-80.

clastogenicity (CA, MN) did not give positive results either because of experimental conditions or due to mechanisms of carcinogenesis based on small genetic alteration. ECHA cannot say so far that Formaldehyde is the only responsible for MTBE potential mutagenicity. Therefore choosing TGR would allow to find small deletions and point mutations, in tissues including germ cells when Comet would have allowed to detect clastogenicity in a more limited number of tissues. The *in vivo* UDS test is not sensitive enough to detect genotoxic effects (Kirkland and Speit, 2008[33]),

- The Registrant(s) have commented this statement by stating that this UDS study was performed in liver, one of the target organs for tumours. In the cited paper and after an exhaustive comparison of the efficiency of UDS, MN, Comet and TGR to detect carcinogen compounds, Kirkland and Speit, 2008[34] concluded that *"the UDS test was disappointing and gave positive results with <20% of these carcinogens, some of which induced tumours in rat liver and produced DNA adducts in vivo."*. Although hepatocellular adenomas were reported with MTBE, but if MTBE is highly reactive, it is not sure that it would reach the liver after inhalation. As cited by the Registrant(s) in their comments from the guidance document: *"a negative result from the in vivo UDS assay is not a proof that the substance does not induce gene mutation"*. The Registrant(s) comment that collectively, the negative findings in the *in vivo* UDS assay and the *in vivo* hprt gene mutation assay provide compelling evidence for a lack of *in vivo* mutagenic potential for MTBE. Regarding the added value of the *in vivo* hprt gene mutation cited by the Registrant(s), in order to convince that if MTBE was mutagenic, that would be through clastogenic action, we have no data showing that the splenocytes were exposed to MTBE or in capability to metabolized it into its reactive metabolite as well as no experimental design since the reference corresponds to an abstract. Contrarily to what is stated by the Registrant(s), the available data do not allow to decide on mutagenicity of MTBE nor its mode of action.

As stated in section 1.1, the existing carcinogenic database raises uncertainty on organs susceptible of transforming MTBE into formaldehyde. Therefore, and based on Registrant(s)' comments on proposals for amendments (PfA), ECHA emphasises that TGR is requested to be conducted in exposed tissue (nasal tissue), on the tissues for which carcinogenicity (liver, lymphatic tissue (lymph node or bone marrow)) and/ or toxicity (kidney) has been described. While Leydig cells were initially considered to be necessary to be investigated as Leydig cells tumours have been described, following the Registrant(s)' comments ECHA agrees that there is no reason why this cell type would be more sensitive than others and at the same time a specific request would lead to an additional adaptative protocol not merited at this stage. Further, following the Registrant(s)' comments, the choice between lymph node and bone marrow is given for the investigation of lymphatic tissue.

If any of these tissues turns out to give positive results, mutation frequency shall be assessed on germ cells (spermatozoa from the vas deferens/cauda epididymis and developing germ cells from the seminiferous tubules). These cells will have been gathered at the same time as the other tissues. On a voluntary basis, in order to accurately evaluate the effect of the treatment on cells that were spermatogonial stem cells during the exposure period, the Registrant(s) may consider to have an additional sampling time at a minimum of 7 weeks (mice), or 10 weeks (rats), after the end of treatment.

Having taken into account all the remarks from the Registrant(s) as mentioned above, ECHA

[33] Kirkland D, Speit G. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens III. Appropriate follow-up testing in vivo. Mutat Res. 2008 Jul 31;654(2):114-32.
[34] Kirkland D, Speit G. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens III. Appropriate follow-up testing in vivo. Mutat Res. 2008 Jul 31;654(2):114-32.

requests performing additional *in vivo* genotoxicity tests to determine the mutagenicity of MTBE, mainly DNA strand breaks probably by an indirect effect based on metabolites as Formaldehyde as well as reactive oxygen species.

Because it is suspected that MTBE produces also DNA adducts (Du HF et al., 2005)[35], and because TGR is the only available test allowing to estimate mutagenesis in germ cells (there is no alternative available not using vertebrate test animals), ECHA requests to perform a TGR assay in rodents by inhalation route with MTBE (OECD 488) with the details of the protocol as mentioned in section 1.

Inhalation is the requested route as humans (workers, consumers and general population) will be exposed to MTBE via that route in the first place. Therefore, in order to carry on an appropriate evaluation, and as mentioned in REACH annexes, inhalation appears to be most appropriate route of administration, having regard to the likely route of human exposure.

The requested information is thus needed to determine whether the substance has the potential to cause gene mutations and to analyse the relevance of the carcinogenic effects. If the concern is confirmed this would potentially lead to classification as germ cell mutagen and/or carcinogen, which would also be a criterion for selection as a substance of very high concern (SVHC) under the REACH Regulation and trigger further risk reduction measures as defined in REACH and other downstream legislation.

2. Risk assessment of general population following indirect exposure of MTBE.

There is concern for the general population based on the potential contamination of drinking water with MTBE as this would affect the aesthetic quality of the water and expose the population to this substance that is suspected to potentially have mutagenic and carcinogenic properties.

Drinking water can be prepared from surface water or from groundwater. Groundwater can be contaminated by MTBE through leaching from the soil surface, surface water can be polluted through direct or indirect release. The physico-chemical properties of MTBE increase the possibility of serious contamination of groundwater (high water solubility, mobility through groundwater). Furthermore, assessment of indirect exposure is generally conducted if the tonnage is superior to 1,000 t/y (ECHA Guidance R16, 2016).[36]

MTBE enters surface water and groundwater mainly because of fuel leaks of underground storage tanks and spillage from overfilling the tanks. In urban areas, the rainwater contains low concentration of MTBE, which causes slightly elevated MTBE concentration in groundwater. When contaminated groundwater is used as drinking water, people are exposed to MTBE (RAR, 2002).

Based on monitoring data MTBE has been shown in some cases to be present in drinking water at concentrations exceeding taste and odour thresholds (RAR, 2002). While the number of pollution cases can be considered relatively restricted at the EU level the problem is more pronounced in some Member States. Therefore, Finland considered justified to conclude, in 2002, that MTBE is causing a risk for the aesthetic quality of drinking water (RAR 2002).

No risk assessment has been performed by the Registrant(s) and no argumentation was

[35] Du HF, Xu LH, Wang HF, Liu YF, Tang XY, Liu KX, Peng SX. Formation of MTBE-DNA adducts in mice measured with accelerator mass spectrometry. Environ Toxicol. 2005 Aug;20(4):397-401.
[36] (ECHA 2016) Guidance on information requirements and Chemical Safety Assessment. Chapter R.16: Environmental exposure assessment. Version 3.0, February 2016

provided that would enable ECHA to verify whether the concern regarding the aesthetic quality expressed in the RAR (2002) is still relevant nor to evaluate human health risks for potential carcinogenicity and germ cell mutagenicity due to consumption of drinking water containing MTBE once the hazard concerns are clarified. Therefore the Registrant(s) are required to submit a risk assessment based on realistic parameters considering actual uses of MTBE and what risk management measures are already implemented to avoid/reduce leaching to the ground water.

The Registrant(s) claim that this request *is contrary to ECHA guidance on the development of exposure scenarios that provide a worst case estimate of the health risks associated with the 'normal' use of a product (ECHA 2012). ECHA's guidance on the development exposure scenarios states that the reasonable worst-case should not include misuse, exposure which results from accidents or exposures in situations where workers do not follow the instructions or do not use the required RMM.*

Indeed, according to the Registrant(s), *the release of MTBE to groundwater is an accidental environmental release that does not normally occur at refueling stations: the request for an indirect consumer exposure scenario for the MTBE released under these circumstances goes beyond the intended scope of REACH.* Furthermore, the Registrant(s) argued that *the 2002 RAR for MTBE was created as required by Council Directive 793/93 on the regulation and control of existing substances, which did not distinguish between the risks associated with accidental and non-accidental exposures (Council Regulation (EEC) No 793/93 of 23 March 1993 on the evaluation and control of the risks of existing substances). As a result, the Registrant(s) believe that the request for an indirect consumer exposure scenario for the MTBE accidentally released into drinking water unfairly penalizes this chemical relative to others found in the marketplace.*

The question of accidental releases has been discussed in the RAR. In particular, in this assessment it is stated that "data which are known to originate from distinct large-scale accidents such as in transportation are omitted, while data which are related with subtle malfunctions and misconduct of common systems are largely included [...] This is felt to be in line with the overall aim of realistic conservative assumptions in the assessment." Continuous release from corroded tanks or releases at the petrol stations are not considered as accidental situations.

It has also been suggested that storm-water runoff and atmospheric transport are contributors to the low environmental and water concentrations of the MTBE (Delzer et al., 1996; Squillace et al., 1997 cited in the RAR, 2002). MTBE has been detected also in wells without no known leaks from the adjacent petrol stations. The contamination of groundwater by MTBE was not considered exceptional in the RAR (2002).

ECHA does not consider that REACH changes the objective of the former regulation on the evaluation and control of the risks of existing substances (No 793/93) in the way to consider exposure of humans indirectly exposed through the environment. ECHA R16 Guidance (version 3.0, February 2016) describes, in the notes of Appendix A.16-1: Environmental Release Categories, the release factors to soil and water from closed systems, such leakage rates for engine oil from cars. Based on an average leakage rate, annual number of kilometres travelled per vehicle and the amount of engine oil per vehicle the release factor can be calculated as follows: a leakage rate of 10 mg/km and a mileage of 20 000 km per year and four litres of engine oil per vehicle results in a release factor of about 5% per year. The average percentage of MTBE in gasoline may then be used to estimate regional and local predicted environmental concentrations (PECs). R16 Guidance refers also to releases to water that have also to be taken into account because of the possible spills to (waste) water during transfer or delivery procedures or leakage from equipment (such storage

tanks).

As a result, ECHA requests to submit information that allows to verify whether the conclusion in the RAR (2002) regarding the risk for affecting the aesthetic quality of water is properly addressed and to evaluate human health risks for potential carcinogenicity and germ cell mutagenicity due to consumption of drinking water containing MTBE. The following elements shall be taken into account in the exposure scenario:
- actual uses of MTBE in the European Union;
- risk management measures in particular for storage tanks in refuelling stations to avoid release to soil and groundwater;
- and recommendations of ECHA Guidance R16 (2016) for estimation of release factors.

In their comments to the PfAs, the Registrant(s) expressed their wish to analyse all existing MTBE groundwater databases rather than estimate the release factors according to R16 Guidance. Such analysis may have limitations e.g. due to analytical methods or representativeness of tested samples. ECHA however welcomes this initiative as a complement to the revision of exposure scenario for the discussion of the reliability of calculated concentrations.

3. Clarification and detailed justification for each environmental exposure scenario; Revision of environmental risk assessment in relation to the consideration of no-ready and no inherent biodegradation potential.

As mentioned in the justification document for the selection of MTBE for substance evaluation to be listed on the CoRAP, the inclusion of MTBE was motivated not only because of its potential endocrine disruptor properties, but also because of its wide dispersive use, and its high aggregated tonnage. Wide dispersive use and high aggregated tonnage implies that uses of MTBE will induce MTBE releases to the environment. Those concerns justify that the exposure and risk assessment for the environment should performed by ECHA during the REACH evaluation process, in order to check if all MTBE uses are safe for the environment. In case of identification by ECHA of unsafe uses for the environment, authorisation/restriction processes could be needed at the latest stage if no risk mitigation measures are clearly identified.

During the REACH evaluation process, an additional concern on biodegradability and persistence of MTBE was detected. According to the Registrant(s), data provided in the IUCLID dossier can support the hypothesis that MTBE should be considered as "inherently biodegradable, not fulfilling criteria" for non-adapted sewage sludge. For adapted sludge MTBE can be characterised as "ready biodegradable". ECHA considers that available data do not permit to support the Registrant(s)' conclusion.

Three reliable studies which had tested the ready biodegradability of the MTBE according to the standard guideline OECD301D are available.

[redacted] (1991; RI[37]=1)[38] performed a ready biodegradability test on MTBE. The test substance (2 mg/L) were incubated during 28 days with a non-adapted inoculum sampled from a municipal treatment plant. Test substance degradation were estimated based on O_2 consumption. After 28 days of incubation, 0% of the test substance was mineralised, which indicated that MTBE should be considered as not readily biodegradable, as the percentage of degradation after 28 days is below the regulatory threshold (i.e. 60% of degradation based

[37] RI : reliability index
[38] [redacted] (1991).

on O_2 consumption).

[redacted] (1996; RI=2)[39] performed a ready biodegradability test on MTBE. The test substance (2 mg/L) were incubated during 28 days with a non-adapted inoculum. Test substance degradation were estimated based on O_2 consumption. After 28 days of incubation, 1.8% of the test substance was mineralised, which indicated that MTBE should be considered as not readily biodegradable, but not have inhibitory effects on the bacteria inoculum at the concentration tested (2 mg/L).

In addition, [redacted] (2005; RI=2)[40] performed a biodegradability test on MTBE, according to the standard guideline OECD301D (non GLP-compliant). The test substance (2.5 mg/L) were incubated during 28 days with an inoculum (5 mL/L) originated from an industrial wastewater treatment plant that received effluent from manufacture of MTBE. No data is available about adaptation of the inoculum to the MTBE. However, the inoculum should be considered as adapted to the MTBE.

Test substance degradation were estimated based on O_2 consumption. After 7 days of incubation, 9.24% of the test substance was mineralised. The biodegradation result achieved after day 7 was not increased in the following days of the test (i.e. 28 days). As a consequence MTBE should be considered as not inherently biodegradable by adapted inoculum originated from industrial wastewater treatment plant that received effluent from manufacture of MTBE.

Moreover, a last study [redacted] (1981; RI=3)[41] performed a ready biodegradability test on MTBE. The test substance (4 ppm) were incubated during 5 days with a non-adapted inoculum. Test substance degradation were estimated based on O_2 consumption. After 5 days of incubation, 3% of the test substance was mineralised, which indicated that MTBE should be considered as not readily biodegradable.

In addition to data already mentioned above, other data was provided by the Registrant(s) and was considered by ECHA not reliable for a regulatory purposes. ECHA is of the opinion that the reports on biodegradation of MTBE in laboratory activated sludge reactors with adapted sludge, or microbial populations from waste water from the petrochemical industry, or laboratory selected micro-organisms for their ability to degrade MTBE, cited by the Registrant(s), do not provide data on MTBE degradation in environmental realistic conditions equivalent to the results that would be obtained by standard testing. The test conditions and the composition of the selected inoculum cannot be considered as environmentally relevant.

As a conclusion, MTBE should be considered as neither ready, nor inherently biodegradable. This conclusion implies that the environmental exposure assessment must be performed with the assumption of no degradation of the MTBE in the environment compartments, in accordance with the ECHA Guidance R16 (2016) for any substance considered as "inherently biodegradable, not fulfilling specific criteria" or as "not biodegradable".

Hence, as a starting point, unless they have new data to the contrary, the Registrant(s) should use for the environmental exposure assessment the modelling assumptions of no

39 [redacted] (1996).

40 [redacted] (2005).

41 [redacted] (1981).

degradation of the MTBE for the STP, aquatic compartment including sediment, and for the soil compartment.

In addition, for all environmental exposure scenarios described in the Registrant(s)' dossier, description and justifications are generally missing. Those are needed for the understanding of the uses, and the acceptance of the adequacy between a given use and its corresponding exposure scenario, in order to give a high confidence on environmental risk assessment conclusions. As examples:

- For all emission scenarios, the estimated annual tonnage for the corresponding use, the regional fraction, and the fraction of main source are missing. These inputs must be provided with justifications.

- When SPERC is applied, the adequacy between the described use and the applied SPERC should be explicitly justified. Additional justification should be provided if SPERC default value is not used.

- About on-site treatment mentioned in industrial use scenarios, a removal efficiency of > 90% is assumed without any justification.

To conclude, ECHA considers that the descriptions of emission scenarios are insufficient for allowing ECHA to perform an exposure and risk assessment for the environment. All emission scenarios should be reviewed by bringing more details on their descriptions and justifications, in accordance with the ECHA guidances R12 (2015)[42], R13 (2012)[43], R16 (2016), and the SPERC Factsheet Guidance Document (2016).

During the commenting period, the Registrant(s) agreed with ECHA's request on Clarification and detailed justification for each environmental exposure scenario, and informed about their willingness to update their dossiers, in order to provide clarification and detailed justification for each environmental exposure scenario.

In their comments on PfAs, the Registrant(s) indicated that a CSR update was submitted to ECHA on July 9, 2015, which aims to bring more clarification and detailed justification for each environmental exposure scenario. There was an additional update in 2016. ECHA considers that the adequacy between the described use and the applied SPERC are still missing. Therefore, ECHA cannot perform an exposure and risk assessment for the environment even when considering the last version of the registration dossier. As a consequence, clarification and justification for each environmental exposure scenario are still needed.

Therefore, pursuant to Article 46(1) of the REACH Regulation, the Registrant(s) are required to give clarifications and detailed justifications for each environmental exposure scenario, and to perform accordingly an environmental risk assessment in which the Registrant(s) should assume as the starting point no degradation of the substance in the environment compartment.

[42] ECHA (2015) Guidance on information requirements and Chemical Safety Assessment. Chapter R.12: Use description. Version 3.0, December 2015

[43] ECHA (2012). Guidance on information requirements and Chemical Safety Assessment. Chapter R.13: Risk management measures and operational conditions. Version 1.2, October 2012.

Environment concern on potential endocrine disrupting properties of MTBE

4. Fish Sexual Development test (OECD TG 234)

MTBE was included in the CoRAP in particular because of its potential endocrine disrupting properties. Data was provided in the Registrant(s)' dossier on these potential ED properties for the environment, including published and non-published data.

Three reliable studies are available for assessing the potential endocrine disrupting properties of MTBE on fish.

[redacted] (2012; RI=1)[44] performed in GLP-compliance a Fish Short-Term Reproduction Assay on MTBE with the zebrafish (*Danio rerio*), according to the standard guidelines OECD 229 and US EPA OPPTS #890.1350. Breeding groups of zebrafish were exposed to MTBE at mean measured concentrations of 0.122, 3.04 and 147 mg/L for 21 days. The endpoints evaluated, to determine if the test substance might interact with the estrogenic or androgenic hormones axes of fish, were fecundity, fertility, plasma VTG levels, and gonad histopathology. In addition, survival, body length, and wet weight were measured as general indicators of toxicity. A significant elevation in plasma vitellogenin (VTG) levels in male fish exposed to 3.04 mg/L was demonstrated. Exposure of fish to 0.122 and 3.04 mg/L MTBE has no effect on any of the other endpoints measured. Exposure of fish to 147 mg/L MTBE significantly reduced the total number of eggs produced and the number of eggs produced per female per reproductive day. This reduction in fecundity was accompanied by a significant increase in the incidence of oocyte atresia along with a significant increase in the accumulation of oocyte debris in the oviduct, which can be linked to an estrogenic activity of the MTBE at the tested concentration.

The estrogenic activity of MTBE is also supported by Moreels *et al.* (2006; RI=2)[45], who performed a well-documented series of toxicity tests on MTBE with the zebrafish (*Danio rerio*).
A first experiment was perform to assess the acute toxicity of MTBE to the zebrafish with a 48h-exposure test with adult zebrafish exposed to the concentration range 0 - 400 - 600 - 652 - 661 - 730 - 843 mg/L (mean measured concentration). After a 48h-exposure, an LC50 of 677 mg/L was calculated.

The chronic toxicity of MTBE was assessed with two experiments. In the first chronic exposure experiment, breeding groups of zebrafish were exposed for 21 days in flow-through conditions to MTBE at mean measured concentrations of 0.11 - 2.7 - 37 mg/L, corresponding to 0.01% - 0.39% - 5.5% of the LC_{50},48h respectively. The endpoints evaluated were VTG concentration in plasma for male and gonadosomatic index (GSI) for each sex. Exposure to MTBE at all doses during 21 days had no significant effect on the female GSI and on the male GSI. The lowest MTBE concentration of 0.11 mg/L induced a 26-fold and highly significant ($p = 0.001$) increase in vitellogenin concentration in males compared to the nonexposed male control group (1.76 vs 0.068 mg/mL). Exposure to the highest concentration of 37 mg/L also stimulated vitellogenin production in males (1.90 mg/mL) compared to the nonexposed male group.

In the second chronic exposure experiment, breeding groups of zebrafish were exposed for 8 weeks in flow-through condition to MTBE at mean measured concentrations of 0.44 - 2.2 -

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44 [redacted] (2012). [redacted]

45 Moreels D, Van Cauwenberghe K, Debaere B, Rurangwa E, Vromant N, Bastiaens L, Diels L, Springael D, Merckx R & Ollevier F (2006). Long-term exposure to environmentally relevant doses of methyl-tert-butyl ether causes significant reproductive dysfunction in the zebrafish (Danio rerio). Environmental Toxicology and Chemistry, 25 (9), 2388–2393.

22 - 220 mg/L, corresponding to 0.06% - 0.32% - 3.25% - 32.5% of the LC50,48h respectively. The endpoints evaluated were the fecundity (number of eggs produced between four and eight weeks), the fertility and the hatchability of eggs. No significant difference in fecundity, fertility, and hatchability were observed between the nonexposed control and the MTBE-exposed groups. According to the authors, these results (*i.e.* no significant effect of MTBE treatments) could be explained by large experimental variations and low replication.
This study demonstrates that MTBE can potentially have an estrogenic activity at concentration up to 0.11 mg/L, based on VTG induction in MTBE-exposed males compare to control.

[redacted] (2013; RI=1)[46] performed in GLP-compliance a Fish Short-Term Reproduction Assay on MTBE with the Fathead Minnow (*Pimephales promelas*), according to the standard guidelines OECD TG 229 and US EPA OPPTS #890.1350. Breeding groups of zebrafish were exposed to MTBE at mean measured concentrations of 0.60, 1.8, 6.2, 20 and 62 mg/L for 21 days. Based on the endpoints evaluated (*i.e.* the same than wildlife Internationale (2012)), MTBE does not appear to interact with the estrogenic or androgenic hormone axes of fathead minnows at the tested concentrations.

Based on studies mentioned above the lowest chronic toxic effect were measured with the zebrafish with a NOEC of 3.04 mg/L, based on reproduction endpoint. This toxic effect is linked to a significant estrogenic activity of MTBE, *i.e.* VTG induction in adult males (Wildlife International (2012) supported by Moreels *et al.* (2006)) and oocytes atresia in adult females (Wildlife Internationale, 2012) exposed to MTBE at concentration up to 0.12 mg/L. Nevertheless, more robust data are needed to confirm this hypothesis of an estrogenic activity in fish.

The studies mentioned above should be considered at level 3 of the conceptual framework (*i.e. in vivo* assays providing data about selected endocrine mechanism(s) / pathway(s) according to the OECD Guidance Document on standardized test guidelines for Evaluating Chemicals for Endocrine Disruption (OECD, 2012). Their results (*i.e.* mainly the significant vitellogenin induction in fish male adults) indicate possibilities for adverse effects which can be highlighted in reproductive and developmental studies of levels 4 and 5 of the conceptual framework of OECD (2012). According to the OECD guideline, studies of level 4 and 5 that highlight adverse effects linked to the mode of action are needed to identify endocrine disruptive substance.

The Fish sexual Development Test (FSDT; OECD TG 234) is a partial lifecycle assay that can be used to show several types of *in vivo* endocrine disruption activities in fish, including estrogenic activity, and also to provide apical information relevant for the environmental risk assessment. This test is recommended by OECD (2012) as a conceptual framework level 4 test that covers a sensitive fish life stage responsive to both estrogen and androgen-like chemicals. Performing this test should allow confirming if MTBE has an estrogenic activity on fish, and if this estrogenic activity induces adverse effect on fish sexual development.

During the commenting period, the Registrant(s) provided comments on the FSDT (OECD TG 234) request, without any additional data. As a summary, the Registrant(s) argued that the FSDT (OECD TG 234) request is not scientifically justified considering that available data on fish concludes that there is no biologically relevant effect of MTBE on VTG in male or female fish under the test conditions.

46 [redacted] (2013).

As previously mentioned, two studies performed on adult zebrafish (*Danio rerio*) sustained the hypothesis that MTBE has an estrogenic activity on fish, mainly based on the significant induction of VTG in zebrafish male adults exposed to MTBE compared to control. In accordance with the OECD TG 229, "*vitellogenin measurements should be considered positive if there is a statistically significant increase in VTG in males*". As a consequence, this result should be considered in the OECD conceptual framework as a positive outcome *(i.e.* a statistically significant change(s) in an ED-specific endpoint) of Level 3 assays which indicates a possibility for adverse effects in the reproductive and developmental studies at Levels 4 and 5.

According to OECD guidance, studies of level 4 and 5 that highlight adverse effects linked to the mode of action are needed to identify endocrine disruptive substance. As a consequence, the FSDT (OECD TG 234) request is considered scientifically justified by ECHA. If the test outcome confirms the concern for environmental endocrine disruption MTBE might be considered as substance of equivalent concern under Article 57 of the REACH Regulation and considered a candidate for identification as substance of very high concern (SVHC).

Therefore, pursuant to Article 46(1) of the REACH Regulation, the Registrant(s) are required to perform a Fish sexual Development Test (FSDT test method: OECD TG 234) with Japanese medaka *Oryzias latipes* or Zebrafish *Danio rerio*. The genetic sex determination and secondary sex characteristics shall also be included if the determination of the parameters is possible for the selected test species.

Five test concentrations must be tested in a range between 0.1 and 10 mg/L expressed in measured concentration, in order to cover the highest tested concentration recommended by the OECD 234 standard guideline and the concentration for which significant effects were demonstrated in the studies mentioned above.

For a better assessment of the reliability of this study, in addition to the robust study summary, the full study report must be provided to ECHA, in order to have access to raw data.

In their comments on the PfAs, the Registrant(s) indicated their agreement to not proposed stickleback as test species for performing the FSDT.

IV. Adequate identification of the composition of the tested material

In relation to the required experimental stud(y/ies), the sample of the substance to be used shall have a composition that is within the specifications of the substance composition that are given by all Registrant(s). It is the responsibility of all the Registrant(s) to agree on the tested material to be subjected to the test(s) subject to this decision and to document the necessary information on composition of the test material. The substance identity information of the registered substance and of the sample tested must enable the evaluating MSCA and ECHA to confirm the relevance of the testing for the substance subject to substance evaluation. Finally, the test(s) must be shared by the Registrant(s).

V. Avoidance of unnecessary testing by data- and cost-sharing

In relation to the experimental stud(y/ies) the legal text foresees the sharing of information and costs between Registrant(s) (Article 53 of the REACH Regulation). Registrant(s) are therefore required to make every effort to reach an agreement regarding each experimental study for every endpoint as to who is to carry out the study on behalf of the other Registrant(s) and to inform ECHA accordingly within 90 days from the date of this decision

under Article 53(1) of the REACH Regulation. This information should be submitted to ECHA using the following form stating the decision number above at: https://comments.echa.europa.eu/comments_cms/SEDraftDecisionComments.aspx

Further advice can be found at http://echa.europa.eu/regulations/reach/registration/data-sharing.

If ECHA is not informed of such agreement within 90 days, it will designate one of the Registrant(s) to perform the stud(y/ies) on behalf of all of them.

VI. Information on right to appeal

An appeal may be brought against this decision to the Board of Appeal of ECHA under Articles 52(2) and 51(8) of the REACH Regulation. Such an appeal shall be lodged within three months of receiving notification of this decision. Further information on the appeal procedure can be found on the ECHA's internet page at http://www.echa.europa.eu/regulations/appeals. The notice of appeal will be deemed to be filed only when the appeal fee has been paid.

Authorised[1] by Leena Ylä-Mononen, Director of Evaluation

Annex: List of registration numbers for the addressees of this decision. This annex is confidential and not included in the public version of this decision.

[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.