SUBSTANCE EVALUATION CONCLUSION

as required by REACH Article 48

and

EVALUATION REPORT

for

Tert-butyl methyl ether

EC No 216-653-1

CAS RN 1634-04-4

Evaluating Member State: France

Dated: December 2021

Evaluating Member State Competent Authority

French Agency for Food, Environmental and Occupational Health Safety (ANSES)
on behalf French Ministry of Environment
14 rue Pierre et Marie Curie
94701 maisons-Alfort Cedex
(France)
Email: reach@anses.fr

Year of evaluation in CoRAP: 2014

Before concluding the substance evaluation a Decision to request further information was issued on: 7 February 2017.

Further information on registered substances here:

http://echa.europa.eu/web/guest/information-on-chemicals/registered-substances

DISCLAIMER

This document has been prepared by the evaluating Member State as a part of the substance evaluation process under the REACH Regulation (EC) No 1907/2006. The information and views set out in this document are those of the author and do not necessarily reflect the position or opinion of the European Chemicals Agency or other Member States. The Agency does not guarantee the accuracy of the information included in the document. Neither the Agency nor the evaluating Member State nor any person acting on either of their behalves may be held liable for the use which may be made of the information contained therein. Statements made or information contained in the document are without prejudice to any further regulatory work that the Agency or Member States may initiate at a later stage.

Foreword

Substance evaluation is an evaluation process under REACH Regulation (EC) No. 1907/2006. Under this process the Member States perform the evaluation and ECHA secretariat coordinates the work. The Community rolling action plan (CoRAP) of substances subject to evaluation, is updated and published annually on the ECHA web site[1].

Substance evaluation is a concern driven process, which aims to clarify whether a substance constitutes a risk to human health or the environment. Member States evaluate assigned substances in the CoRAP with the objective to clarify the potential concern and, if necessary, to request further information from the registrant(s) concerning the substance. If the evaluating Member State concludes that no further information needs to be requested, the substance evaluation is completed. If additional information is required, this is sought by the evaluating Member State. The evaluating Member State then draws conclusions on how to use the existing and obtained information for the safe use of the substance.

This Conclusion document, as required by Article 48 of the REACH Regulation, provides the final outcome of the Substance Evaluation carried out by the evaluating Member State. The document consists of two parts i.e. A) the conclusion and B) the evaluation report. In the conclusion part A, the evaluating Member State considers how the information on the substance can be used for the purposes of regulatory risk management such as identification of substances of very high concern (SVHC), restriction and/or classification and labelling. In the evaluation report part B the document provides explanation how the evaluating Member State assessed and drew the conclusions from the information available.

With this Conclusion document the substance evaluation process is finished and the Commission, the Registrant(s) of the substance and the Competent Authorities of the other Member States are informed of the considerations of the evaluating Member State. In case the evaluating Member State proposes further regulatory risk management measures, this document shall not be considered initiating those other measures or processes. Further analyses may need to be performed which may change the proposed regulatory measures in this document. Since this document only reflects the views of the evaluating Member State, it does not preclude other Member States or the European Commission from initiating regulatory risk management measures which they deem appropriate.

[1] http://echa.europa.eu/regulations/reach/evaluation/substance-evaluation/community-rolling-action-plan

Contents

Part A. Conclusion

1. CONCERN(S) SUBJECT TO EVALUATION

The Substance, Tert-butyl methyl ether (EC No 216-653-1, CAS RN 1634-04-4 or MTBE), was originally selected for substance evaluation in order to clarify concerns about:

- Human health/Potential endocrine disruptor;
- Exposure/Wide dispersive use,
- High (aggregated) tonnage.

During the evaluation, other concerns were identified. The additional concerns were:

- Mutagenicity
- Biodegradability and persistency in the environment,
- Risk for the environment.

2. OVERVIEW OF OTHER PROCESSES / EU LEGISLATION

Pursuant to Article 41(1) of REACH, ECHA performed a compliance check of the MTBE registration dossier. The compliance check was initiated on 28 September 2012 and was targeted on repeated dose toxicity and reproductive toxicity as well as at human exposure assessment.

The substance has the following harmonized classification: H225 (Flam. Liq. 2), Skin Irrit. 2 (H315).
Due to its flammability, MTBE is managed at industrial sites as a Seveso substance (categories P5a, P5b et P5c for Flammable liquids).

3. CONCLUSION OF SUBSTANCE EVALUATION

The evaluation of the available information on the substance has led the evaluating Member State to the following conclusions, as summarised in the table below.

Table 1

CONCLUSION OF SUBSTANCE EVALUATION Conclusions	Tick box
Need for follow-up regulatory action at EU level	X
Harmonised Classification and Labelling	
Identification as SVHC (authorisation)	
Restrictions	
Other EU-wide measures	
No need for regulatory follow-up action at EU level	

4. FOLLOW-UP AT EU LEVEL

4.1. Need for follow-up regulatory action at EU level

RMOA: An RMOA is needed to explore the possible need for restriction, SVHC identification or other EU-wide measures.

4.1.1. Harmonised Classification and Labelling

Not applicable.

4.1.2. Identification as a substance of very high concern, SVHC (first step towards authorisation)

Not applicable.

4.1.3. Restriction

Not applicable.

4.1.4. Other EU-wide regulatory risk management measures

Not applicable

5. CURRENTLY NO FOLLOW-UP FORESEEN AT EU LEVEL

5.1. No need for regulatory follow-up at EU level

Not applicable.

5.2. Other actions

An RMOA is needed before to conclude the need for a risk management measure.

6. TENTATIVE PLAN FOR FOLLOW-UP ACTIONS (IF NECESSARY)

Indication of a tentative plan is not a formal commitment by the evaluating Member State. A commitment to prepare a REACH Annex XV dossier (SVHC, restrictions) and/or CLP Annex VI dossier should be made via the Registry of Intentions.

Table 2

FOLLOW-UP		
Follow-up action	**Date for intention**	**Actor**
RMOA	2022	FR CA

Part B. Substance evaluation

7. EVALUATION REPORT

7.1. Overview of the substance evaluation performed

The Substance, Tert-butyl methyl ether (EC No 216-653-1, CAS RN 1634-04-4 or MTBE), was originally selected for substance evaluation in order to clarify concerns about:

- Human health/Potential endocrine disruptor;
- Exposure/Wide dispersive use,
- High (aggregated) tonnage.

During the evaluation, other concerns were identified. The additional concerns were:

- Mutagenicity
- Biodegradability and persistency in the environment,
- Risk for the environment.

Table 3

EVALUATED ENDPOINTS	
Endpoint evaluated	**Outcome/conclusion**
Potential endocrine disruptor	Concern refuted. For the Environment: potential estrogenic effect but without apical effect. Based on evaluated data, the current endocrine disruptor definition is not fulfilled for environment. Human health: ED related effects seen at high doses only. Not sufficient to request additional study. New data may require further consideration in an upcoming RMOA
Mutagenicity	Concern refuted. Based on the available database and weight-of-evidence, no classification is proposed for MTBE.
Biodegradability and persistency in the environment	Concern refuted. MTBE is not inherently biodegradable and not readily biodegradable. Therefore, MTBE is considered as potentially persistent.
Exposure/Wide dispersive use and aggregated tonnage	High production volume, with a possibility to decrease in future due to an increased use of ETBE.
Risk for the environment	Concern unresolved. Considering uncertainties about the degradation of MTBE in industrial and municipal wastewater treatment plants, no degradation in STP has been taken into account by the eMSCA when refining exposure scenarios, leading to unacceptable risks for the environment (see confidential annex). An RMOA is needed to clarify parameters to be used for risk characterisation and the need for risk management measures.

7.2. Procedure

On the basis of an opinion of the ECHA Member State Committee and due to initial grounds for concern related 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 and risks for the environment.

Regarding the endocrine disruptor (ED) potential, the concern was not sufficient to request further testing for the Human Health Part. However, the evaluating MSCA considered that further information was required to clarify the concerns of mutagenicity, ED properties for the environment, risk assessment for general population and environmental exposure.

ECHA sent a decision to the Registrants on 7 February 2017. The data were received in December 2018 and September 2019, and were evaluated in this conclusion document.

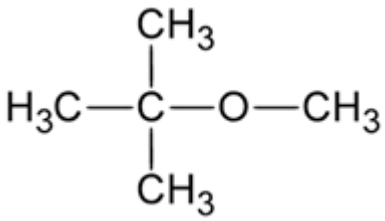
7.3. Identity of the substance

Table 4

SUBSTANCE IDENTITY	
Public name:	tert-butyl methyl ether (MTBE)
EC number:	216-653-1
CAS number:	1634-04-4
Index number in Annex VI of the CLP Regulation:	603-181-00-X
Molecular formula:	C5H12O
Molecular weight range:	88.17
Synonyms:	2-methoxy-2-methylpropane, MTBE

Type of substance ☑ Mono-constituent ☐ Multi-constituent ☐ UVCB

Structural formula:



7.4. Physico-chemical properties

Table 5

OVERVIEW OF PHYSICOCHEMICAL PROPERTIES	
Property	**Value**
Physical state at 20°C and 101.3 kPa	Colourless liquid with characteristic terpene-like odour
Melting point	-108 °C
Boiling point	55 °C
Vapour pressure	33 000 Pa at 20°C
Surface tension	72.5 mN/m (at 20 °C, 1.07 g/L)

Water solubility	41.8 g/L at 20°C and pH6.1
Partition coefficient n-octanol/water (Log Kow)	1.06 at 20°C
Flash point	-28 °C
Flammability	The substance is highly flammable (Flam. Liq. Cat. 2)
Self ignition temperature	460 °C
Explosive properties	Not explosive
Oxidising properties	Not oxidising
Granulometry	Not relevant
Stability in organic solvents and identity of relevant degradation products	Not relevant / Stable
Dissociation constant	Not relevant / None
Viscosity	0.464 mm^2/s at 20 °C 0.409 mm^2/s at 40 °C

7.5. Manufacture and uses

7.5.1. Quantities

Table 6

AGGREGATED TONNAGE (PER YEAR)				
☐ 1 – 10 t	☒ 10 – 100 t	☒ 100 – 1000 t	☐ 1000- 10,000 t	☒ 10,000-50,000 t
☒ 50,000 – 100,000 t	☒ 100,000 – 500,000 t	☐ 500,000 – 1000,000 t	☒ > 1000,000 t	☐ Confidential

The total tonnage band is 1 000 000 - 10 000 000 tonnes per annum as indicated in ECHA dissemination website.

7.5.2. Overview of uses

Table 7

USES	
	Use(s)
Uses as intermediate	-
Formulation	Gasoline/fuel blending with MTBE
Uses at industrial sites	Industrial distribution of MTBE and gasoline/fuel containing MTBE (transport of MTBE and distribution of gasoline containing MTBE, sampling and associated laboratory activities) Use at industrial site - Use as a process solvant and extraction agent
Uses by professional workers	Filling engines gasoline/fuel containing MTBE Other: in cleaning agents, coatings
Consumer Uses	Use of fuel containing MTBE

	Other: In cleaning agents, coatings
Article service life	-

7.6. Classification and Labelling

7.6.1. Harmonised Classification (Annex VI of CLP)

The harmonized classification of MTBE, as indicated in C&L inventory of ECHA dissemination website, is indicated below:

Table 8

HARMONISED CLASSIFICATION ACCORDING TO ANNEX VI OF CLP REGULATION (REGULATION (EC) 1272/2008)							
Index No	**International Chemical Identification**	**EC No**	**CAS No**	**Classification**		**Spec. Conc. Limits, M-factors**	**Notes**
				Hazard Class and Category Code(s)	**Hazard statement code(s)**		
603-181-00-X	tert-butyl methyl ether MTBE 2-methoxy-2-methylpropane	216-653-1	1634-04-4	Flam. Liq. 2 Skin Irrit. 2	H225 H315	-	-

7.6.2. Self-classification

There are no additional hazard classes notified among the aggregated self-classifications in the C&L Inventory.

However, considering the level of certain impurities identified in registration dossiers, there is a possibility for additional classifications that are listed hereunder:

- Material containing up to 12% alcanes C4-C6 and/or up to 20% alkenes C4-C7 may be classified as Flam. Liq. 1 (H224) instead of Flam Liq. 2 (H225), and may also be classified for aspiration hazard (H304) and Carc. 1A (H350).
- Material containing up to 0.2% benzene may be classified as Carc. 1A (H350) and Muta. 1B

7.7. Environmental fate properties

7.7.1. Degradation

7.7.1.1. Abiotic degradation

MTBE is stable to hydrolysis at the environmentally significant pH range.

The atmospheric half-life of MTBE is estimated at 5.65 days, based on degradation rate constant of 2.84E-12 cm^3/molecules/s and a radical concentration of 5E05 radicals/cm^3.

Aqueous photolysis can occur for substances which have UV/visible light absorption maxima in the range of 290 to 800 nm. MTBE does not adsorb light in the visible wavelength range (max t 289 nm); photo-transformation in water can be excluded.

Abiotic decomposition can be considered as a not significant degradation route for MTBE.

7.7.1.2. **Biodegradation**

7.7.1.2.1. Biodegradation in water

7.7.1.2.1.1. Screening tests

Table 9: Screening tests for biodegradation in water

Method	Results	Remarks	Reference
Test type: ready biodegradability activated sludge, domestic, non-adapted OECD TG 301 D (Ready Biodegradability: Closed Bottle Test)	% Degradation of test substance: 0 after 28 d (O_2 consumption)	1 (reliable without restriction) key study experimental result Test material (EC name): tert-butyl methyl ether	Unpublished study report , 1991a
Test type: ready biodegradability OECD TG 301 D (Ready Biodegradability: Closed Bottle Test)	% Degradation of test substance: 1.8 after 28 d (O_2 consumption)	2 (reliable with restrictions) key study experimental result Test material (EC name): tert-butyl methyl ether	Unpublished study report, 1996
Test type: ready biodegradability activated sludge, industrial (adaptation not specified) OECD TG 301 D (Ready Biodegradability: Closed Bottle Test)	% Degradation of test substance: 9.24 after 7 d (O2 consumption)	2 (reliable with restrictions) key study experimental result Test material (EC name): tert-butyl methyl ether	Unpublished study report, 2005

Discussion:

Three reliable studies testing the ready biodegradability of the MTBE according to the standard guideline OECD TG301D are available (table 9).

In an unpublished report (1991a) (Reliability index (RI)=1), a ready biodegradability test was performed on MTBE. The test substance (2 mg/L) was incubated during 28 days with a non-adapted inoculum sampled from a municipal treatment plant. Test substance degradation was estimated based on O_2 consumption. After 28 days, 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 was below the regulatory threshold (i.e. 60% of degradation based on O_2 consumption).

In an unpublished report (1996) a ready biodegradability test on MTBE was performed using a test substance concentration of 2 mg/L anda non-adapted inoculum and 28 days of incubation. Degradation was estimated based on O_2 consumption. After 28 days, 1.8% of the test substance was mineralised, which indicated that MTBE should be considered as not readily biodegradable, but not having inhibitory effects on the bacteria inoculum at the concentration tested (2 mg/L).

In addition, a biodegradability test on MTBE was performed according to the standard guideline OECD TG301D (non GLP-compliant, unpublished study report 2005, RI = 2). The test substance (2.5 mg/L) was 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 was estimated based on O_2 consumption. After 7 days of incubation, 9.24% of the test substance was mineralised. This percentage did not increased in the following

days of the test (i.e. up to day 28). 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.

As a conclusion, MTBE should be considered as not ready, nor inherently biodegradable.

7.7.1.2.1.2. Simulation tests (water and sediments)

Three studies on the degradation of MTBE in water/sediment system in aerobic and anaerobic conditions have been considered by the FR-MSCA. The studies did not follow any standard guideline.

These studies indicated that under anaerobic conditions, degradation of MTBE in water/sediment system is not expected. The study in Bradley *et al.* (1999) shows that degradation of MTBE could occur at aerobic conditions with adapted inoculum. However, such result should be considered with caution for a regulatory purpose as MTBE is not readily biodegradable, and as only one study is available, with a low reliability and using adapted inoculum.

7.7.1.2.2. Biodegradation in soil

Table 10: Simulation tests for biodegradation in soil

Method	Results	Remarks	Reference
Biodegradation in static soil/water microcosms was evaluated under aerobic conditions.	% Degradation of test substance: 0 after 6 wk	2 (reliable with restrictions) supporting study experimental result Test material (EC name): tert-butyl methyl ether	Allard AS, Remberger M & Neilson AH (1996)
Test type: laboratory Aquifer material was collected from two locations (Cores X and Z) and used to prepare microcosms for evaluating the biodegradability of MTBE, MTBE plus BTEX and MTBE plus 100 mg/L NH4-N (aerobic Core X only) under aerobic, anaerobic-denitrifying, and low initial oxygen conditions. Microcosms were constructed in 225-mL serum bottles with 150 g wet soil, 140 mL mineral solution (medium from Zeyer et al. [1986] with 15 mg/L NO3-N and 10 mg/L PO4-P), and 45-mL headspace. They were sealed with Teflon© MiniInert© valves (Supelco. Inc.) and incubated at the ambient groundwater temperature (~14 ºC).	Evaporation of parent compound: no Volatile metabolites: no Residues: no Transformation products: TBA	2 (reliable with restrictions) supporting study experimental result Test material (EC name): tert-butyl methyl ether	Borden *et al*. (1997)

Discussion

Two studies which have tested the degradation of MTBE in soil at aerobic and anaerobic conditions have been considered by FR-MSCA. These studies did not follow any standard guideline (Table 10).

Yeh and Novak (1994; RI=2) studied the anaerobic biodegradation of MTBE in soils. Soils were collected from three sites and included unsaturated clay, sandy loam, and silty loam. Soils from the sites had not been previously exposed to fuels. Concentrations of MTBE were monitored for more than 250 days. Substantial numbers of anaerobic microorganisms were found at these sites. Three anaerobic metabolic processes were evaluated including 1) denitrification, 2) sulphate reduction and 3) anaerobic degradation. These processes were individually evaluated by adding nutrients that selectively encouraged their activity (i.e. nitrate, sulphate, and cysteine, respectively). **MTBE was not degraded in any of the unamended soil microcosms over 250 days of incubation time**. Under methanogenic conditions, degradation of MTBE occurred only in oligotrophic soils with low organic matter and at a pH between 5.0 and 6.0. The co-existence of ethanol and other easily-degraded organics inhibited MTBE degradation.

Borden *et al*. (1997; RI=2) collected contaminated gasoline aquifer material from two locations to be used to prepare microcosms for evaluating the biodegradability of MTBE, MTBE plus BTEX and MTBE plus 100 mg/L NH4-N under aerobic, anaerobic-denitrifying, and low initial oxygen conditions. MTBE and BTEX (when included) were added to the microcosms to achieve a final concentration of ~2 mg/L for MTBE, and BTEX. In all aerobic microcosms, MTBE biodegraded after a 20-day lag period. MTBE decreased from an initial concentration of 2.1 mg/L to between 1.0 and 1.5 mg/L by day 93. However after day 93, MTBE levels remained constant. Abiotic losses were minimal indicating the MTBE loss was due to biodegradation.

Laboratory microcosm studies confirmed **MTBE biodegradation under aerobic conditions. However, the extent of biodegradation was limited**.

These studies indicated that **in anaerobic conditions, degradation of MTBE in soil is not expected in most of type of soil and tested conditions**. However, such result should be considered with caution for a regulatory purpose as MTBE is not readily biodegradable, and because of the protocols applied for these tests.

7.7.1.3. **Summary and discussion on degradation**

The rate constant used in the assessment are:

Degradation for hydrolysis	0 d-1
Degradation for photolysis	0 d-1
Degradation in air	0.123 d-1
Degradation in STP	0 d-1
Biodegradation in soil	1E-03 d-1

7.7.2. Environmental distribution

7.7.2.1. **Adsorption/desorption**

The organic carbon-water partitioning coefficient (Koc) calculated from the octanol-water partition coefficient (log Kow = 1.06) using the equation from the TGD (predominantly hydrophobics) is 9.1 L/kg (log value = 0.93). This predicted value is used in the risk assessment.

7.7.2.2. **Volatilisation**

The Henry's Law constant (H) is calculated as 64.9 Pa m^3/mol, based on a vapour pressure of 33 kPa at 25°C and a water solubility of 4185 mg/L at 25°C (EUSES). The calculated H

value indicates that MTBE has the potential to volatilise rapidly from water to air. The value is used in the risk assessment.

7.7.3. Bioaccumulation

7.7.3.1. **Aquatic bioaccumulation**

With a logKow = 1.06, the potential for bioccumulation of MTBE is considered as low. This is supported by the study of Fujiwara *et al*. (1984; RI=3) that measured whole-body bioconcentration factors (BCF) of 1.4 and 1.5 for *Cyprinus caprio* exposed to 10 and 80 mg/l MTBE in a flow-through system at 25 °C. Fish exposed for 28 days and then transferred to clean water eliminated almost all MTBE residues within 3 days.

7.7.3.2. **Terrestrial bioaccumulation**

No relevant information available.

7.8. Environmental hazard assessment

7.8.1. Aquatic compartment (including sediment)

7.8.1.1. **Fish**

7.8.1.1.1. Short-term toxicity to fish

Table 11: Short-term effects on fish

Method	Results	Remarks	Reference
Poecilia reticulata freshwater semi-static OECD TG 203 (Fish, Acute Toxicity Test)	LC50 (96 h): > 893.1 mg/L TOC (estimated) based on: mortality	2 (reliable with restrictions) supporting study experimenwtal result Test material (EC name): tert-butyl methyl ether	Unpublished study report, 2005.
Danio rerio Freshwater static Method: no guideline followed	LC50 (48 h): 677 mg/L (measured (not specified)) based on: mortality	2 (reliable with restriction) supporting study experimental result Test material (EC name): tert-butyl methyl ether	Moreels *et al*. (2006).

Discussion

A non-GLP compliant acute toxicity test on fish (*Poecilia reticulata*) has been performed following the standard guideline OECD TG203. After 96h of exposure, the LC50(96h) was > 893.1 mg/L. No mortality was observed in any concentration tested (from 80 to 893.1 mg/L).

In addition, Moreels *et al.* (2006, RI=2) performed a well-documented series of toxicity tests on MTBE with the zebrafish (*Danio rerio*). The aim of the first experiment was to assess the acute toxicity of MTBE to thezebrafish. A 48h-exposure test was performed with adult zebrafish exposed to the concentration range 0 to 843 mg/L (mean measured concentrations). After a 48h-exposure, an LC50 of 677 mg/L was calculated.

Value used for the CSA

LC50 for freshwater fish: 677 mg/L.

7.8.1.1.2. Long-term toxicity to fish

Table 12: Long-term effects on fish

Method	Results	Remarks	Reference
Pimephales promelas freshwater early-life stage: reproduction, (sub)lethal effects flow-through ASTM E1241-92	NOEC (31 d): 299 mg/L test mat. (meas. (not specified)) based on: growth rate NOEC (31 d): 450 mg/L test mat. (meas. (not specified)) based on: mortality	1 (reliable without restriction) key study experimental result Test material (EC name): tert-butyl methyl ether	Unpublished study report (1999a)
Pimephales promelas freshwater early-life stage: reproduction, (sub)lethal effects flow-through OECD TG229 (Fish Short-Term Reproduction Assay) EPA OPPTS 890.1350 (Fish Short-Term Reproduction	NOEC (21 d): 62 mg/L test mat. (meas. (geom. mean)) based on: reproduction	1 (reliable without restriction) supporting study experimental result Test material (EC name): tert-butyl methyl ether Form: liquid (unspecified)	Unpublished study report 2013
Danio rerio freshwater early-life stage: reproduction, (sub)lethal effects flow-through OECD TG229 (Fish Short-Term Reproduction Assay) EPA OPPTS 890.1350 (Fish Short-Term Reproduction)	NOEC (21 d): 3.04 mg/L act. ingr. (meas. (geom. mean)) based on: reproduction	1 (reliable without restriction) supporting study experimental result Test material (EC name): tert-butyl methyl ether Form: liquid (unspecified)	Unpublished study report2012
Danio rerio freshwater flow-through Investigations on effects of short-term and long-term exposure of adult zebrafish (Danio rerio) to concentrations of MTBE in flow-through systems. Experiment 1 involved 48h exposures to effective concentrations of 400, 600, 652, 661, 730, 843 mg/l. Experiment 2 involved 3 week exposures to effective concentrations of 0.11, 2.7, 37 mg/l. Experiment 3 involved 8 week exposures to effective concentrations of 0.44, 2.2, 22,		2 (reliable with restrictions) supporting study experimental result Test material (EC name): tert-butyl methyl ether Form: liquid (unspecified)	Moreels *et al*. (2006)

Method	Results	Remarks	Reference
220 mg/l. Parameters observed included: survival, vitellogenin concentration, gonadosomatic index (GSI), fecundity, fertilization rate, hatch rate, and sperm motility.			

Discussion

Four reliable studies are available for assessing the chronic toxicity to fish of MTBE in accordance with a weight of evidence approach (table 12).

In an unpublished report (1999a), a GLP-compliant early-life stage toxicity test of MTBE was performed on the fathead minnow (*Pimephales* promelas) under flow-through conditions, according to the standard guideline ASTM E1241-92. 80 fathead minnow fertilized eggs (20 per replicate) were exposed during 31 days to the nominal MTBE-concentration of 0 - 214 - 342 - 547 - 875 - 1400 mg/L, correponding to the average measured concentration of 0 - 110 - 192 - 299 - 450 - 720 mg/L, respectively. Complete mortality was observed in the 720 mg/L treatment on test day 12. No significant mortality was observed for the duration of the test in the remaining treatments. After 31 days of exposure, based on mortality, a NOEC of 450 mg/L was then determined. After 31 days of exposure no deformities on survivals were observed in all treatment. A significant growth inhibition (based on fish lenght and weight) was measured in the 450 mg/L treament, corresponding to a NOEC of 299 mg/L.

A GLP-compliant Fish Short-Term Reproduction Assay was performed on MTBE with the Fathead Minnow (*Pimephales promelas*), according to the standard OECD TG 229 and US EPA OPPTS #890.1350 (Unpublished study report 2013, RI = 1). Breeding groups of fathead minnows (*Pimephales promelas*) were exposed to MTBE at mean measured concentrations of 0.60, 1.8, 6.2, 20 and 62 mg MTBE/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 reproduction (cumulative egg production, eggs per female reproductive day and fertilization success), secondary sex characteristics (including tubercle assessment), gonadosomatic index (GSI), vitellogenin (VTG) and gonad histopathology. In addition, survival, and growth (body length and wet weight) were measured as general indicators of toxicity. There were no apparent effects on survival, growth, reproduction, secondary sex characteristics, GSI, VTG or gonad histopathology in male or female fish exposed to MTBE for 21 days. Based on the endpoints evaluated, MTBE does not appear to interact with the estrogenic or androgenic hormone axes of fathead minnows at the tested concentrations.

A GLP-compliant Fish Short-Term Reproduction Assay was performed on MTBE with the zebrafish (Danio rerio), according to the standard guidelines OECD TGb229 and US EPA OPPTS #890.1350 (Unpublished study report 2012, RI = 1). 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. Exposure of fish to 0.122 and 3.04 mg/L MBTE has no effect on any of the endpoints measured except for a significant elevation in plasma VTG levels in male fish exposed to 3.04 mg/L. This elevation was low (3.4-fold compared to control) but significant. Exposure of fish to 147 mg/L MBTE 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 supported by Moreels *et al.* (2006; RI=2), who performed a well-documented series of toxicity tests on MTBE with the zebrafish (*Danio rerio*).

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 condition to MTBE at mean measured concentrations of 0.11 - 2.7 - 37 mg/L, corresponding to 0.01% - 0.39% - 5.5% of the LC50,48h respectively. The endpoints evaluated were VTG concentration in plasma for male and GSI for each sex. 21 days of exposure to MTBE at all doses had no significant effect on the female GSI 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 non-exposed male control group (1.76 vs 0.068 mg/mL). Exposure to the highest concentration of 37 mg/L again stimulated vitellogenin production in males (1.90 mg/mL) compared to the non-exposed male group.

This study demonstrates that MTBE can potentialy have an estrogenic activity at concentrations up to 0.11 mg/L, based on VTG induction in MTBE-exposed males compared to control. This result should be taken into account with caution considering that the tested concentration range (i.e. 3 tested concentrations) did not permit to define a clear dose-response relationship between MTBE concentration and VTG induction in males. No adverse effect on reproduction has been demonstrated, which could be explained by large experimental variations and low replication (i.e. low power to detect an effect).

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 - 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 non-exposed 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.

Based on the above-mentionned studies , the lowest chronical toxic effects 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 (Unpublished study report 2012 supported by Moreels *et al*. (2006)) and oocytes atresia in adult females (Unpublished study report 2012) exposed to MTBE at concentration up to 0.11 mg/L.

Value used for CSA

NOEC for freshwater fish: 3.04 mg/L.

7.8.1.2. **Aquatic invertebrates**

7.8.1.2.1. Short-term toxicity to aquatic invertebrates

Table 13: Short-term effects on aquatic invertebrates

Method	Results	Remarks	Reference
Daphnia magna freshwater flow-through EPA OPPTS 850.1010 (Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids)	EC50 (48 h): 472 mg/L (meas. (not specified)) based on: mobility (95% CL 357-712)	1 (reliable without restriction) key study experimental result Test material (EC name): tert-butyl methyl ether	Unpublished study report (1999b)
Daphnia magna freshwater semi-static OECD TG 202 (Daphnia sp. Acute Immobilisation Test)	EC50 (48 h): > 893.1 mg/L TOC (estimated) based on: mobility	2 (reliable with restrictions) supporting study experimental result	Unpublished study report (2005)

Method	Results	Remarks	Reference
		Test material (EC name): tert-butyl methyl ether	
Americamysis bahia saltwater flow-through EPA OPPTS 850.1035 (Mysid Acute Toxicity Test)	EC50 (96 h): 187 mg/L test mat. (meas. (not specified)) based on: erratic swimming (95% CI:149-231) LC50 (96 h): 200 mg/L test mat. (meas. (not specified)) based on: mortality (95% CI: 160-247)	1 (reliable without restriction) key study experimental result Test material (EC name): tert-butyl methyl ether	Rausina *et al*. (2002)
Americamysis bahia (reported as Mysidopsis bahia) saltwater static renewal conditions equivalent or similar to EPA OPPTS 850.1035 (Mysid Acute Toxicity Test)	LC50 (96 h): 44 mg/L test mat. (nominal) based on: mortality (95% CL = 36 - 53 mg/L)	2 (reliable with restrictions) key study experimental result Test material (EC name): tert-butyl methyl ether	Unpublished study report (1994)
Daphnia magna static Method: 84/449/EEC, C2	EC50 (48 h): 651.4 mg/L (meas. (not specified)) based on: mobility	2 (reliable with restrictions) supporting study experimental result Test material (EC name): tert-butyl methyl ether	Unpublished study report(1991b)
Callinectes sapidus flow-through Method: FIFRA, ASTM E729-88a	EC50 (96 h): 306 mg/L (meas. (not specified)) LC50 (96 h): 306 mg/L (meas. (not specified)) based on: mortality	1 (reliable without restriction) supporting study experimental result Test material (EC name): tert-butyl methyl ether	Unpublished study report (1999c)
Rhepoxynius abronius static renewal Method: ASTM E729 -96, E1367-96	EC50 (96 h): 294 mg/L (meas. (not specified))	1 (reliable without restriction) supporting study experimental result Test material (EC name): tert-butyl methyl ether	Unpublished study report (1999d)
Palaemonetes pugio flow-through Method: USEPA 850.1045	EC50 (96 h): 166 mg/L (meas. (not specified)) LC50 (96 h): 166 mg/L (meas. (not specified)) based on: mortality	1 (reliable without restriction) supporting study experimental result Test material (EC name): tert-butyl methyl ether	Unpublished study report (1999e)

Method	Results	Remarks	Reference
Mytilus galloprovincialis static Method: ASTM E729 -96	LC50 (96 h): 1950 mg/L (meas. (not specified)) based on: mortality	1 (reliable without restriction) supporting study experimental result Test material (EC name): tert-butyl methyl ether	Rausina *et al*. (2002).

Discussion

The assessment of the acute toxicity to aquatic invertebrates of MTBE was based on 9 reliable data, including 2 freshwater species and 4 saltwater species (Table 13).

For freshwater species, the acute toxicity values (LC50 or EC50) ranged from 294 mg/L (with *Rhepoxynius abronius*) to > 893.1mg/L (with *Daphnia magna*).

For saltwater species, the acute toxicity values (LC50 or EC50) ranged from 44 mg/L (with *Americamysis bahia*) to 1950 mg/L (with *Mytitus galloprovincialis*).

Based on the ECHA Guidance R10, all data was pooled and the most sensitive endpoint was determined regardless of the medium, as the sensitivity difference between freshwater and saltwater species was less than a factor of 10.

Value used for CSA

LC50,96(*Americamysis bahia*) = 44 mg/L.

7.8.1.2.2. Long-term toxicity to aquatic invertebrates

Table 14: Long-term effects on aquatic invertebrates

Method	Results	Remarks	Reference
Daphnia magna freshwater flow-through EPA OPPTS 850.1300 (Daphnid Chronic Toxicity Test)	NOEC (21 d): 51 mg/L test mat. (meas. (geom. mean)) based on: reproduction, length, weight LOEC (21 d): 100 mg/L test mat. (meas. (geom. mean)) based on: reproduction, length, weight	1 (reliable without restriction) key study experimental result Test material (EC name): tert-butyl methyl ether	Unpublished study report (1999f)
Americamysis bahia (reported as Mysidopsis bahia) saltwater flow-through EPA OPPTS 850.1350 (Mysid Chronic Toxicity Test)	NOEC (28 d): 26 mg/L test mat. (meas. (not specified)) based on: reproduction LOEC (28 d): 50 mg/L test mat. (meas. (not specified)) based on: reproduction	1 (reliable without restriction) key study experimental result Test material (EC name): tert-butyl methyl ether	Unpublished study report (1999g)

Discussion

The assessment of the chronic toxicity of MTBE to aquatic invertebrates was based on 3 reliable data, including 2 freshwater species and 1 saltwater species (Table 14).

The chronic toxicity of MTBE to the saltwater mysid (Mysidopsis *bahia*; renamed *Americamysis bahia*) was evaluated over a 28-day exposure period under flow-through

conditions at measured concentrations of 0 - 16 - 26 - 50 - 103 - 207 mg/L. Mysids exposed to 16 and 26 mg/L showed no significant reductions in survival, reproduction and growth (length and dry weight). Mysids exposed to 103 and 207 mg/L showed significant reductions in survival prior to pairing on day 13 and from day 13 to test termination on day 28. The most sensitive biological endpoints measured during this study were reproduction, length and dry weight. Mysids exposed to 50 mg/L showed significant reductions in all of the above parameters. Consequently the NOEC and LOEC, based on reproduction, length and dry weight was 26 and 50 mg/L, respectively.

The chronic effects of MTBE on the survival, growth and reproduction of Daphnia *magna* were assessed with a GLP-compliant study , and according to the standard guideline EPA-OPPTS 850.1300 (1996). Daphnids (aged <24h at the start of the test) were exposed during 21 days to MTBE at the nominal concentration tested 0 - 25 - 50 - 100 - 200 - 400 mg/L in a flow through system. The mean measured concentration was defined as 0 - 26 - 51 - 100 - 195 and 405 mg/L. Reproduction (mean number of young per individually exposed adult), and growth (length and dry weight) were the most sensitive biological endpoints. A significant reduction in reproduction and growth was found in daphnids exposed to 100 mg/L. Survival was significantly reduced in the 195 and 405 mg/L treatment groups. For this study, the NOEC and the LOEC were 51 and 100 mg/L (mean measured concentration) based on reproduction and growth, respectively.

Based on the ECHA Guidance R10, all data were pooled and the most sensitive endpoint was determined regardless of the medium, as the sensitivity difference between freshwater and saltwater species was less than a factor of 10.

Value used for CSA

NOEC (*Americamysis bahia*) = 26 mg/L.

7.8.1.3. **Algae and aquatic plants**

Table 15: Effects on algae and aquatic plants

Method	Results	Remarks	Reference
Pseudokirchnerella subcapitata (reported as Selenastrum capricornutum) (algae) freshwater static ASTM E1218-90	IC50 (96 h): 491 mg/L test mat. (meas. (not specified)) (95% CI = 328 to 844 mg/l) IC20 (96 h): 103 mg/L test mat. (meas. (not specified)) (95% CI = 50 to 515 mg/l)	1 (reliable without restriction) key study experimental result Test material (EC name): tert-butyl methyl ether	Unpublished study report (1999h)
Desmodesmus subspicatus (algae) freshwater static OECD TG 201 (Alga, Growth Inhibition Test)	EC50 (72 h): > 908.7 mg/L TOC (estimated) based on: biomass EC50 (72 h): > 908.7 mg/L TOC (estimated) based on: growth rate NOEC (72 h): 489.3 mg/L TOC (estimated) based on: growth rate	2 (reliable with restrictions) supporting study experimental result Test material (EC name): tert-butyl methyl ether	Unpublished study report 2005
Skeletonema costatum (algae)	EC50(72 h) : 279 mg/L based on growth rate NOEC (72 h): 198 mg/L based on growth rate	1 (reliable without restriction) key study experimental result Test material (EC name): tert-butyl methyl ether	Rausina *et al*. (2002).

Discussion

The assessment of the toxicity to aquatic algae and cyanobacteria of MTBE was based on three reliable datas, including two freshwater species and one saltwater species (table 15).

Based on the ECHA Guidance R10, all data was pooled and the most sensitive endpoint was determined regardless of the medium, as the sensitivity difference between freshwater and saltwater species was less than a factor of 10.

Value used for CSA

ECr50,72h = 279 mg/L

NOEC,72h = 198 mg/L

7.8.1.4. Sediment organisms

No relevant information available.

7.8.1.5. Other aquatic organisms

No relevant information available.

7.8.2. Terrestrial compartment

Two studies for the acute toxicity on earthworms and one chronic toxicity study on springtail are available but are considered not reliable. Additional data from literature search are available for terrestrial plants however these data are also considered not reliable. The PNEC is calculated via EPM method.

7.8.3. Microbiological activity in sewage treatment systems

No data available.

7.8.4. PNEC derivation and other hazard conclusions

Table 16:

PNEC DERIVATION AND OTHER HAZARD CONCLUSIONS		
Hazard assessment conclusion for the environment compartment	**Hazard conclusion**	**Remarks/Justification**
Freshwater	$PNEC_{freshwater}$ = 304 µg/L	Assessment factor: 10
Marine water	$PNEC_{saltwater}$ = 30.4 µg/L	Assessment factor: 100
Sewage treatment plant	$PNEC_{stp}$ = 710 mg/L	Assessment factor: 1
Soil	$PNEC_{soil}$ = 142.8 µg/kg dw	Extrapolation method: EPM derivation

7.8.5. Conclusions for classification and labelling

There is no harmonized classification for the environment and based on the data available, MTBE is not classified.

7.9. Human Health hazard assessment

7.9.1. Toxicokinetics

MTBE is efficiently absorbed orally and via inhalation. Absorption through the skin is probably moderate under occlusive conditions, whereas in open contact rapid evaporation is expected to limit the uptake strongly. Apart from specific binding to male rat kidney protein, the extensive tissue distribution of MTBE appears to be determined by solubility:

concentrations in soft tissues are approximately the same as in blood with the exception of fat that may reach a ten-fold higher concentration.

MTBE is metabolised to formaldehyde and Tert-Butanol (TBA). Formaldehyde is believed to be metabolised extremely rapidly to formate (which is largely incorporated in the one-carbon pool) and to CO_2, and TBA is further metabolised to α-hydroxyisobutyric acid, 2-methyl-1,2-propanediol, TBA conjugates and acetone.

According to current knowledge, the enzyme catalysing MTBE biotransformation to formaldyde 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, CYP2A3 of the olfactory and nasal epithelium exhibited even higher metabolising activities than the previously mentioned liver enzymes.

In humans, CYP2E1 also appears to be active at lower MTBE concentrations. The capacity for MTBE metabolism is limited, although the rat has a higher capacity than humans. Saturation of metabolism was indicated in the rat after i.p. administration of 500 mg/kg, or during 6-h inhalation exposure to 8000 ppm MTBE.

In human inhalation exposures up to 75 ppm for four hours, no signs of saturation of metabolism were found. In most experimental conditions the major part of MTBE in the body was excreted as urinary metabolites, and less than a half was exhaled unchanged, however, if the uptake rate was high the opposite was true. The elimination half-time for MTBE in blood was about 0.5 hours in the rat and about ten times longer in humans.

After exposure to MTBE, TBA is found in the blood circulation for a longer period and at higher concentrations than MTBE. TBA is highly water-soluble and distributed in total body water. Apart from lower levels found in fat, soft tissues are expected to show approximately the same concentrations as the blood. The elimination half-time for TBA in blood was about 3 hours in the rat and about 10 hours in humans. The biotransformation capacity for TBA (by unidentified microsomal enzymes) appears to be markedly lower than that for MTBE in the rat, which explains its relatively low rate of elimination. The elimination half-times for the different urinary MTBE metabolites varied between 2.9 and 5 hours in rats and between 7.8 and 17 hours in humans. These data allow to conclude that MTBE or its metabolites will not accumulate in the human body significantly.

The generation of formaldehyde in MTBE metabolism is a point of major toxicological interest because this compound is reactive and mutagenic. The limited database available at the present time points to lacking, or greatly diminished, reactivity by formaldehyde when it is produced intracellularly from MTBE at rates, which are lower than those of its further metabolism.

7.9.2. Acute toxicity and Corrosion/Irritation

The registration dossier and the European Union Risk Assessment Report (EU RAR) assessed the same animal acute toxicity studies. The registrants and the EU RAR concluded the substance exhibits low acute toxicity in humans and animals whatever the exposure route and shall not be classified according to the CLP regulation (animal data: oral and dermal LD_{50} > 2000 mg/kg bw; inhalation $LC_{50} \geq 85$ mg/L). Based on the available information, FR-MSCA can support this conclusion.

Human male volunteers reported mild symptoms, mainly feelings in the head and feeling less cheerful (FIOH, 1997). The frequency of symptoms was related to the exposure level (0, 25, 75 ppm) and reached statistical significance at 75 ppm after 3 hours of exposure to MTBE. These effects were rated as slight. There was no indication found of an objective sign of CNS function impairment in terms of psychomotor performance, sustained attention, or standing steadiness. This study suggests a LOAEC of 75 ppm. It should be noted that although sensory symptoms may show greater sensitivity than objective indices, it is possible that symptoms are mediated by mechanisms other than CNS depression, especially as MTBE has a foul odour.

Another human volunteer study investigated whether CNS effects occurred after exposure up to 50 ppm MTBE for two hours while the persons were exercising at 50W on a bicycle ergometer (Nihlen *et al*. 1998). Effects were measured using a questionnaire. No

indications for the occurrence of CNS effects were reported. The study results suggested a NOAEC of 50 ppm.

FR-MSCA supports the assessment from the EU RAR for skin, eye and respiratory tract irritation. MTBE is a skin irritant but is not considered an eye and respiratory irritant.

Regarding corrosion, no specific data is available but results seen in skin irritation show that MTBE should not be considered as corrosive.

7.9.3. Sensitisation

FR-MSCA supports the assessment from the EU RAR for skin sensitisation. MTBE is not as skin sensitiser.

For respiratory sensitisation, there is no information indicating that MTBE is a respiratory sensitiser.

7.9.4. Repeated dose toxicity

The registration dossier and the EU RAR assessed the same repeated dose toxicity studies in animals. The registrants described three additional more recent studies by oral route (Table 17)

Table 17: Three additional studies on repeated dose toxicity after oral administration in the registration dossier

Method	Results	Remarks	Reference
90-day repeated dose oral toxicity study in rats Equivalent or similar to OECD TG 408 (Repeated Dose 90-Day Oral Toxicity in Rodents) Equivalent or similar to EPA OPPTS 870.3100 (90-Day Oral Toxicity in Rodents) GLP compliant Rat (Wistar) male/female Subchronic exposure by oral route (drinking water) for 13 weeks (maximum of 93 days), daily 0.5, 3, 7.5 and 15 mg/ml (nominal in water) Males: 37±10, 209±61, 514±142, 972±288 mg/kg bw/d (average daily dose calculated from water consumption) Females: 50±12, 272±51, 650±142, 1153±191 mg/kg bw/d (average daily dose calculated from water consumption) Vehicle: water Number of animals/sex/dose for 0, 0.5, 3 and 15 mg/ml: 20 in the core group, 20 in the clinical pathology subgroup, 30 in the cell replication subgroup For 7.5 mg/ml: 20 in the core group, 20 in the clinical pathology group, 0 in the cell replication group Clinical examination, body weight, food and water consumption, haematology, clinical chemistry, urinalysis, gross pathology Kidneys from male and female and testes from males were weighed and examined for histopathology and immunohistochemistry. Cardiovascular, respiratory, digestive, glandular, nervous, urogenital and skeletomuscular tissues were weighed and examined for histopathology in control and high dose group.	- Clinical examination, haematology, gross pathology: no treatment related effects. - Food consumption: unaffected in either sex. - Body weight: no significant effects in females; significant differences between control and the 15 mg/ml male group at weeks 12 and 13 and for the 7.5 mg/ml male group at week 13. - ↓Water consumption: 14, 23, 30, 35% less than controls in males and 22, 33, 40, 43% less than controls in females respectively at the four dose levels (likely due to poor palatability). - Clinical chemistry: ↑serum sodium levels in males and females at 15 mg/ml (likely due to reduced water consumption). - Urinalysis: ↑gravity and osmolality in urine collected from males. - Organ weights: ↑kidney weights in the 7.5 mg/ml male group compared to controls (absolute: +7.6%; relative: +21%) and in the 15 mg/ml male group (absolute: +5.7%; relative: +19%). ↑**relative kidney weights in the 7.5 mg/ml female group (+21%) and in the 15 mg/ml female group (absolute: +16%; relative: +30%).** - Histopathology: ↑cell replication in cortical epithelial cells at week 4 in the 15 mg/ml male group. Tubular cell regeneration in males at week 1 in cell replication subgroup males (2/5, 1/5 and 1/5 at 0.5, 3, 15 mg/ml) and at week 13 in core subgroup animals (2/10, 3/10, 3/10 and 6/10 at 0.5, 3, 7.5 and 15 mg/ml). ↑α2u-globulin levels in male kidneys, but not in females at weeks 1 and 4. NOAEL (male): 3000 mg/L drinking water (effects on body weights and kidney at 7500 and 15000 mg/L (↑weight, cell replication and α2u-globulin levels, tubular cell regeneration). Dose equivalent to 209 mg/kg/day. NOAEL (female): 3000 mg/L drinking water based on ↑relative combined kidney weight. Dose equivalent to 272 mg/kg/day.	key study experimental result **Test material (EC name): tert-butyl methyl ether**	Unpublished study report (2007) Bermudez *et al*. (2012)
1-year repeated dose oral toxicity study in rats Equivalent or similar to EPA OPPTS 870.4100 (Chronic Toxicity) GLP compliant Rat (Wistar) male/female	- Clinical examination, body weights: no effect. - ↓Water consumption: 24, 30, 35% less than controls in males and 20, 41, 48% less than controls in females respectively at the four dose levels. - Urinalysis: ↓ volume in all dose groups.	supporting study experimental result **Test material (EC name): tert-butyl methyl ether**	Bermudez *et al*. (2012)

Method	Results	Remarks	Reference
Chronic exposure by oral route (drinking water) for 1 year. Interim sacrifice group of males at 6 months. 0.5 (males only), 3, 7.5, 15 (females only) mg/ml (nominal in water) 0.48 (males only), 2.9, 7.4, 14.8 (females only) mg/ml (analytically measured concentrations in drinking water) Males: 29, 166, 384 mg/kg/day (actual ingested) Females: 54, 258, 1119 mg/kg/day (actual ingested) 10 animals/per/dose; 15 used for cell replication study Vehicle: water Clinical examination, body weight, food and water consumption, urinalysis, histopathology on cardiovascular, respiratory, digestive, glandular, nervous, urogenital and skeletomuscular tissues in control and high dose group. Kidney histopathology and gross lesions in all dose groups.	- Organ weights: ↑relative left testis and kidney weights in mid and high dose male groups. ↑relative right kidney weight at all doses male groups. ↑**relative left kidney in the high dose female group.** - Histopathology: at 1 year, ↑incidence of minimal nephropathy in males. After 6 months, slightly higher but not significant mean chronic progressive nephropathy grade and mean number of foci in the 7.5 mg/ml male group. At 1 year, these effects were significantly different from control in the high dose male group. NOAEL (male): 480 mg/L drinking water (effect on kidney and testis weight at 2900 and 7400 mg/L). Dose equivalent to 29 mg/kg/day. NOAEL (female): 7400 mg/L drinking water (effect on kidney weight at 14800 mg/L). Dose equivalent to 258 mg/kg/day.		
Short-term repeated dose oral toxicity in rat Follows the basics of an OECD TG407 study but is principally designed to examine effects on blood and clinical chemistry and does not examine other endpoints in detail. GLP compliance: not specified Rat (Sprague-Dawley) male Subacute exposure by oral route (gavage) Group A: 2 weeks; Group B: 4 weeks (daily) 0, 400, 800, 1600 mg/kg (actual ingested) 10 animals/sex/dose Vehicle: peanut oil Clinical examination, body weight, food and water consumption, haematology, clinical chemistry, gross pathology and histopathology on brain, heart, liver, spleen , lung, kidneys, testes, epididymis, thymus and prostate.	Mortality: 30% at 400 mg/kg in group B. Clinical signs: transient signs of central nervous system effects including ataxia, hypoactivity, blepharospasm, lack of startling reflex (especially at 1600 mg/kg) Body weight: no significant effects. Haematology: Group A: significant ↑WBC, LYM, GRA, EOS counts compared to controls at 1600 mg/kg. Group B: significant ↓EOS at 800 mg/kg and ↑HGB at 1600 mg/kg. Clinical chemistry: Group A: significant ↓ALP at 800 and 1600 mg/kg, ↓UREA at 400 mg/kg, dose-dependent ↓CREA, significant ↑CHOL at 1600 mg/kg. Group B: significant ↓CREA at all dose groups but not between dose groups, significant ↑HDL and CHOL at 400 mg/kg, ↓LDL at 800 and 1600 mg/kg whereas significant ↑ALT and AST at 800 mg/kg. Organ weights: Group A: significant ↑relative heart and liver weights at 1600 mg/kg, significant ↓ relative testes weight in all dose groups, significant ↓ relative thymus weight at 800 and 1600 mg/kg. Group B: significant ↑relative liver and kidney weights at 800 mg/kg, significant ↓ relative thymus and prostate weights at 1600 mg/kg. No significant effect on testes.	supporting study experimental result **Test material (EC name): tert-butyl methyl ether**	Dong-mei *et al*. (2009)

Method	Results	Remarks	Reference
	LOAEL: < 400 mg/kg bw/day (actual dose received) (creatinine levels in blood reduced in all dose groups at 2 and 4 week observations) NOAEL: 400 mg/kg bw/day (actual dose received) (other observed effects on biochemistry)		

Summary and discussion on repeated dose toxicity

In the study from Dong-mei *et al.* (2009), a large number of variations in relative organ weights was observed. However, none of the findings appeared to have been reproduced between the experiments, even though conducted at the same exposures and experimental conditions. Moreover, there was no evidence of dose response in any of the organ weights. An unexplained high (30%) mortality in the low dose group gives further doubts on the reliability of this study. Therefore this study has been disregarded for risk assessment purposes.

The EU RAR and the registration dossier reported kidney impairment in males (Unpublished study report, 1992a, NOAEL of 90 mg/kg bw/d; Williams *et al.* 2000, LOAEL of 250 mg/kg bw; Bermudez *et al.* 2012, NOAEL of 480 mg/L drinking water (~29 mg/kg/d); Dong-mei *et al*. 2009, LOAEL < 400 mg/kg bw/d). However, FR-MSCA would like to point out that kidneys were also impaired in females as an increase in relative kidney weights was observed (Confidential 2007, NOAEL of 3000 mg/L drinking water (~272 mg/kg/d); Bermudez *et al*. 2012, NOAEL of 7400 mg/L drinking water (~258 mg/kg/d); Unpublished study report 1993a), NOAEC of 400 ppm). In female rats, renal toxicity cannot be ruled out since this increase in relative kidney weight was associated with nephropathy (defined by NTP as an increase in the regeneration of the tubular epithelium) from 850 mg/kg bw/d (corresponding to a LOAEL), thought we could not identify where this value come from.

In the publication of Bird *et al*. (1997), glomerulosclerosis was observed in rats at 3000 and 8000 ppm. Relative liver and kidney weights were increased in the two highest female dose groups, but the pathological changes, mainly chronic progressive nephropathy (CPN), were generally less severe in females than in males. In the 3000 and 8000 ppm dose groups, there were slightly more deaths due to CPN than in control females. This finding was confirmed histopathologically in males and to a lesser extent in females. In males, typical CPN signs, such as glomerulosclerosis, tubular proteinosis and interstitial nephritis and interstitial fibrosis, were observed at all treatment levels.

In the 90-day repeated dose oral toxicity study from Robinson *et al*. (1990), aldosterone level in female rats showed a 36% and 57% increase in medium and high dose groups compared to controls, although this change was not considered statistically significant. Aldosterone is a mineralocorticoid hormone secreted by the adrenal glands mainly in response to stimulation by angiotensin 2 or an elevation in serum potassium. It has a crucial role in maintaining plasma blood volume and blood pressure, and serum potassium, via its action on the kidney urinary sodium reabsorption and potassium secretion in the urine. It is also a major component of the renin-angiotensin-aldosterone system. This shows that contrarily to what is claimed by Robinson *et al*. (1990) that clinical chemistry of the females showed no adverse signs that would support kidney toxicity at that level, there is sign of modification of the hydric balance that could be explained by kidney impairement.

In oral exposure, male rats exhibit hyaline droplet formation in the proximal convoluted tubules at 440 mg/kg (Unpublished study report, 1992a), an effect that is male rat specific. In the 90-day Sprague-Dawley study by Robinson *et al*. (1990), there is an absence of significant findings at 100 mg/kg. At 300 mg/kg only the female rats had a statistically significant increase in kidney weight. However, this was not accompanied by degenerative microscopical findings, which only appeared in the males of the 1200 mg/kg group. However, these microscopical findings were described in female after longer studies. In addition, clinical chemistry of the females showed no adverse signs that would support kidney toxicity at that level (Robinson *et al.* 1990). Williams *et al.* (2000) reported seemingly different results. They found increased relative kidney weight and protein droplet nephropathy with significantly increased severity and incidence in the same male rat species (Sprague-Dawley) already at 250 mg/kg. However, in a recent study (Unpublished study report 2007 equivalent of an OECD TG408) in which MTBE was given in drinking water at 0.5; 3; 7.5 and 15 mg/mL (nominal in water) for 13 weeks, relative kidney weights were elevated in males and females at the higher tested dose levels (21% for males at 514 mg/kg bw/day and females at 650 mg/kg bw/day, 19% for males at 972 mg/kg bw/day and 30% for females at 1153 mg/kg bw/day) at the end of 13 weeks of

exposure. The effects seen in liver, namely weight increase, hypertrophy and slight morphological changes, are mostly seen at doses 500 mg/kg and higher. These effects may be adaptive responses, which is corroborated by the fact that there are few signs of remarkable liver toxicity even in the 2-year carcinogenicity studies (described in detail in the carcinogenicity section).

The results in females and the presence of glomerulosclerosis suggest that α2u-globulin is not the only mode of action to explain the observed effects of MTBE in the kidney. These effects lead to the progressive CKD, and later tumors in males. FR-MSCA proposes a NOAEL for this purpose based on the females of 272 mg/kg bw/d for oral route (Unpublished study report 2007) and a NOAEC of 400 ppm after inhalation (Unpublished study report 1993a).

Justification for classification or no classification

Considering the above NOAEL of 272 mg/kg bw/d for oral route and the NOAEC of 400 ppm after inhalation exposure in female rats, no classification is considered relevant.

7.9.5. Mutagenicity

The data taken into account for the evaluation were all the publications and study reports provided by the registrant for MTBE, and the EU RAR. A literature search has also been performed until March 2015.

MTBE has been extensively tested for genotoxicity in a variety of test systems both *in vitro* and *in vivo* (data not shown). Although not all results have been consistently negative (one positive result in an Ames test (TA102); (Williams-Hill *et al.* 1999) with S9 metabolic activation, one positive mouse lymphoma mutagenicity test with metabolic activation (Unpublished study report 1980), the conclusion in the registrantion dossier and the EU RAR was that MTBE is not a geno-toxicant.

However, in the course of the evaluation, FR-MSCA identified limitations of the analysis in the EU RAR and additional concerns regarding mutagenicity. Indeed, both new mutagenicity data (since the EU RAR) together with remaining uncertainty on carcinogenesis and mutagenicity after previous evaluation raised a concern that led FR-MSCA requesting a new study after evaluating the substance in 2014 (see below for further details).

Remaining uncertainty on carcinogenesis and mutagenicity after previous RAR evaluation

Regarding carcinogenesis, the EU RAR concluded that "*there are indications of carcinogenicity in two species. However the treatment relation of the occurred turnouts is equivocal in some studies (mouse adenorna) and the relevance of the mode of action is questionable in others (Leydig cell). Moreover, the turnouts 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 turnouts 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 *et al.* 1996 and Bird *et al*. 1997) on females at the high dose (8000 ppm). Renal tubular cells tumours were observed in male mice following MTBE inhalation (Bird *et al*. 1997), 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 Bird *et al*. 1997 and Belpoggi, 1995). 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 *et al.* 2013).

Regarding genotoxicity, the EU RAR concluded that "*based on the available information, MTBE cannot be considered a mutagen*". Regarding the involvement of formaldehyde, MTBE genotoxic metabolite, in mutagenicity of MTBE, the RAR reported 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). Moreover, it is known that any generated formaldehyde rapidly reacts with glutathione, forming S-hydroxymethyiglutathione, 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." However, FR-MSCA noted that the quality of metabolic activity of *ex vivo* hepatocytes was not tested in this study, as the positive control used was formaldehyde and not a DNA damaging agent that requires activation.

Moreover, one of the limitations of the analysis in the EU RAR is that most of the *in vivo* studies available while the EU RAR was agreed did not show any toxicity. This leads to uncertainty on the maximal dose tested. Besides, possible explanations of the negativity of some tests could be the lack of competent machinery for metabolizing MTBE in the target organ (bone marrow in the studies from Unpublished study report 1993b and Kado *et al.* 1998) or the lack of sensitivity of the test used to detect genotoxicity.

New data on mutagenicitiy of MTBE since the EU RAR was published are described in the following table.

Table 18: Summary of *in vitro* and *in vivo* mutagenicity studies with MTBE

Method	Concentration/cytotoxicity	Results	Observation and remarks	References
RAD54 activation test Detection of cyto- and genotoxicity by fluorescence *Saccharomyces cerevisiae.*	Without S9 3.1 to 4.4 µg/mL	**Negative**: No increase in specific fluorescence emission, and thus no genotoxic effect	Test material MTBE Purity: not specified	Lichtenberg-Fraté *et al.* (2003) (abstract only)
Ames test Similar to OECD TG471 *S. thyphimurium* TA 102	± rat S9 mix 0.1 to 5 mg/plate	**Negative**: No significant mutagenic response observed with and without rat S9-mix	Test material: MTBE Purity: 99.8% Solvent: either in deionised water or in dried DMSO	McGregor *et al.* (2005)
Ames test *S. thyphimurium* TA 98 and TA 100 The fuels (non-reformulated gasoline) 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.	±S9 mix Non-reformulated gasoline supplemented with 10%, 20%, 25% and 30% ETBE or 15% MTBE Cytotoxic effects investigated in murine fibroblasts (L929) using the neutral red uptake assay	PM-extracts: mutagenicity with and without S9-mix (most probably caused by a lower content of polycyclic aromatic hydrocarbons); ↓ **mutagenicity by the addition of MTBE** and ETBE, 10% ETBE being most effective Condensates: no significant mutagenic response; ↓ cytotoxicity from ETBE- and MTBE-reformulated fuels	Test material: Fuel supplemented with ETBE or MTBE	Westphal *et al.* (2010) (abstract only)
In vitro Comet assay Human leukemia (HL-60) cells	Without S9 mix 1 to 30 mmol/L	**Positive** at 1 mM	Test material: MTBE Purity: not specified Absence of cytotoxicity at genotoxic concentrations, but high concentrations	Tang *et al.* (1997)
In vitro Comet assay Cultured rat type II pneumocytes and rat hepatocytes	Use of S9 mix: not mentioned in the abstract Concentrations not mentioned in the abstract	**Positive** Greater DNA migration in the cultured cells with MTBE above 0.050 mmol/L (dose-effect relationship) ↗ DNA synthesis of rat type II pneumocytes and rat hepatocytes at 5.0 and 10 mmol/L of MTBE	Test material: MTBE	Yang *et al.* (2005) (abstract only)

UDS assay Performed in hepatocytes, renal cells and pneumocytes of mice administrated MTBE by inhalation for 20 consecutive days	Concentrations: 0, 108, 1440 and 4968 mg/m^3	**Positive** ↗ lengths of DNA migration in mice hepatocytes at 1440 and 4968 mg/m^3 (dose-effect relationship) ↗ lengths of DNA migration in mice renal cells at all doses ↗ lengths of DNA migration in mice pneumocytes at 4968 mg/m^3	Test material: MTBE	Yang *et al.* (2005) (abstract only)
In vitro Comet assay Human lymphocytes	Without S9 mix 50 to 200 µmol/L 1h of treatment	**Positive** from 50 µM Single-strand breaks and double-strand breaks, oxidative base modification	Test material: MTBE Purity: not specified Vehicle: DMSO Limitations: no image analytics used; weak and not dose-related induction of DNA strand breaks by MTBE; very short treatment time (1h); no cytotoxicity evaluation	Chen *et al.* (2008)
Subchronic study Wild-type (WT) and Aldh2 knockout (KO) C57BL/6 mice	0, 500, 1750, 5000 ppm Inhalation, 6h/d, 5d/w for 13 weeks	**Positive :** Dose-dependent and significant ↗ in DNA damage in KO male mice while ↗ in male WT mice only at 5000 ppm	Test material: ETBE	Weng *et al.* (2013)
DNA adducts measurement Kunning male mice (sacrifice at 2, 6, 12h and every 3 days until 21 days)	0.95; 1.09; 5.71; 6.18; 75.59 µg/kg	**Positive :** Dose-dependent DNA adducts formation in liver, kidney and lungs	Test material: MTBE-C^{14}	Du *et al.* (2005)
Sister chromatid exchange (SCE) and Micronucleus test Sprague Dawley rats (male and female) SCE: Blood collected from the abdominal aorta Blood cell cultures treated with 5 µg/mL bromodeoxyuridine (BrdU) Micronucleus test: femurs removed	2000, 10000, 20000 mg/m^3 of condensates Inhalation, 6h/d, 5d/w for 4 weeks	SCE : **Positive** Statistically significant ↗ in SCE in male rats given BGVC at 10000 mg/m^3 or in female rats given G/MTBE at all doses Micronucleus test: **negative**	Test material: vapour condensate of baseline gasoline (BGVC) or gasoline with oxygenated (G/MTBE) Purity: not specified	Schreiner *et al.* (2014)

			Positive control: cyclophosphamide IP (SCE: 5 mg/kg; micronucleus test: 40 mg/kg) Limitations: no toxicity in the bone marrow, no evidence of tissue exposure	
<u>In vivo mammalian somatic and germ cell study</u> Equivalent or similar to OECD TG488 GLP compliant Fischer 344 Big Blue®homozygous transgenic rats (male) 10 males in the control and high exposure group 6 males in the low and mid exposure group 6h whole body exposures once daily for 28 consecutive days Collected tissues from the first 5 animals/group: nasal epithelium, liver, kidney, bone marrow, spermatozoa from the vas deferens/cauda epididymis and developing germ cells from the seminiferous tubules Mutant frequency evaluated in the first 5 animales/group	0, 400, 1000, 3000 ppm (target exposure concentrations) 0, 400, 992, 2977 ppm (mean analysed concentrations)	**Negative** MTBE exposure: no statistically elevated mutant frequencies at the cII gene in bone marrow, liver, kidneys and nasal epithelium of Big Blue® male Fischer 344 rats Positive control: statistically significant increase in the frequency of cII mutants for all tissues tested	Test material: MTBE Purity: not specified Non concurrent positive control: DNA from tissue samples from 5 animals treated with 20 mg/kg N-ethyl-N-nitrosourea (oral gavage) (tested as part of a separate study)	Unpublished study report, 2018

Summary and discussion of mutagenicity

From the studies described above and the EU RAR, it was concluded in the ECHA final decision of 7 February 2017 that there are some coherent data regarding possible 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 EU RAR, Unpublished study report 1980), suggesting a bioactivation of MTBE in DNA reactive metabolites that induced mutations *in vitro*. This result might come from the clastogenicity of formaldehyde generated extracellularly but it does therefore not preclude of what will happen *in vivo*. Mackerer *et al*. (1996) corroborated this hypothesis as the positive results obtained with S9 on MLA/TK decreased when formaldehyde deshydrogenase was added. Thus, these results were at least partially due to formaldehyde rather than MTBE itself.

Whereas no *in vivo* chromosomal damage was observed in bone marrow following inhalation or gavage with MTBE in rats (see RAR), DNA adducts were observed in lung, liver and kidney in male mice following oral administration of MTBE (Du *et al.* 2005), 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** (Moser *et al*. 1996 and Bird *et al*. 1997).

Moreover, all the *in vivo* and *in vitro* comet assays were positive (Tang *et al.* 1997, Lee *et al.* 1998 (see EU RAR), Yang *et al.* 2005, Chen *et al.* 2008) pointing out for **indirect mutagenicity when considered in comparison to all the other negative test** (micronucleus, chromosomal aberration).

The negative results obtained in *in vivo* micronucleus tests and chromosomal aberration tests could be explained by the fact these 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).

Actually, formaldehyde could not be the sole ultimate toxicant; oxidative stress reagents might also be acting. Mutagenicity could involve the formation of DNA adducts although other mechanisms might play a role (replicative stress, nucleotides imbalance etc.).

Based on these results that raise alert on mutagenicity and based on the remaining uncertainty regarding carcinogenicity, further investigations had been requested in order to clarify the potential mutagenicity of MTBE (ECHA decision of 26 March 2015). Thus, a Trangenic rodent somatic and germ cell gene mutation assay (TGR) (OECD TG 488) was requested to be conducted by the registrants according to the OECD in male rodents treated for 28 days via inhalation route. It was requested to measure mutation frequency in exposed tissue (nasal tissue), on the tissues for which carcinogenicity (liver, lymphatic tissue (lymph node or bone marrow)) and/or toxicity (kidney) have been described. While Leydig cells were initially considered to be necessary to be investigated as Leydig cells tumours have been described, it was later agreed that there was 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 necessary at this stage. Somatic tissues and germ cells (spermatozoa from the vas deferens/cauda epididymis and developing germ cells from the seminiferous tubules) were collected at the same time and stored. In case positive results are found in any somatic tissues, mutation frequency in the germ (spermatozoa from the vas deferens/cauda epididymis and developing germ cells from the seminiferous tubules) was planned.

In December 2018, the registrants updated their dossier with this requested TGR study. The study was performed in compliance with the ECHA decision requirements described above and no deviations were noticed compared to the OECD TG 488.

Big Blue® male Fischer 344 rats were exposed by inhalation to 0, 400, 1000 and 3000 ppm MTBE for 6h once daily for 28 consecutive days. The assay was negative as MTBE did not cause statistically elevated mutant frequencies at the cII gene in bone marrow, liver, kidneys or nasal epithelium of the rats. On the contrary, the positive control group with N-ethyl-N-nitrosourea produced statistically significant increases in mutant frequencies for

all tissues tested, demonstrating the utility of the test system to detect and quantify induced mutants following exposure to a known mutagen.

The tested concentrations were selected based on a 24-month inhalation study in male Fischer 344 rats from Bird *et al.* (1997), cited by McGregor (2006). In this study, rats were exposed to 0, 400, 3000 and 8000 ppm MTBE for 6h/day, 5 day/week. Significant increases in renal and testicular tumors were observed in the rats at 3000 ppm. The incidences of renal tubular cell tumors were 2, 0, 16 and 6% and that of interstitial adenomas of the testes were 64, 70, 82 and 94% at 0, 400, 3000, and 8000 ppm, respectively. Based on these results, the registrant justified the choice of the highest target concentration of 3000 ppm. It was also indicated that the intermediate concentration of 1000 ppm represented an inflection point where a change from linear to non-linear metabolism was predicted to occur (based on simulation; Unpublished study report 2015). Finally, the lowest concentration of 400 ppm was the no effect level (for tumors) in the inhalation 24-month carcinogenicity study.

However, according to FR-MSCA, the registrant's arguments for dose selection are questionable:

- First, based on a 2-year carcinogenicity study, the registrant cannot guarantee that the selected concentrations will be high enough to induce effects in the TGR assay where animals are exposed 28 days.
- Second, Bird *et al*. suggested that the maximum tolerated dose (MTD) was exceeded in the test as high mortality occured in the 8000 ppm-exposed group and all animals showed absolute body weight and body weight gain decreases (by 19% and 29% for male rats and by 13% and 22% for female rats) at this same exposure level. In the 3000 and 8000 females, absolute and relative liver weight increases (being 20% and 42% respectively) as well as relative kidney weight increases (being 18% and 29% respectively) were also observed. Based on this result, it could be assumed that the MTD was > 3000 ppm and < 8000 ppm in this study.

  Moreover, a 28-day MTBE vapor inhalation study was also performed on the same species and with the same exposure levels (Bird *et al.* 1997). No exposure-related mortality was observed. The same clinical signs were observed as occured in the 2-year oncogenicity study (i.e. blepharospasm, hypoactivity, ataxia and lack of startle reflex). Absolute body weight and body weight gain were generally decreased in male and female rats from the 8000 ppm group throughout the study. These results suggested that the MTD was ≥ 8000 ppm in this 28-day study.

Therefore, the criteria used by the registrant for the selection of highest target concentration (3000 ppm) are not consistent with those recommended in the guideline, i.e. the highest dose should be the MTD. The highest tested concentration in the TGR assay should have been > 8000 ppm.

Moreover, the reliability of the study from *Bird et al*. (1997) is questionable. IARC noted that mortality-adjusted analysis was not performed and that therefore the tumour incidence in the high dose group (8000 ppm) may have been underestimated. IARC also highlighted the unusually low incidence of interstitial adenomas of the testis in control rats compared with the historical control incidence in the laboratory.

No tissue exposure was experimentally confirmed in the TGR assay. Indeed, the registrant used the publication of Leavens and Borghoff, 2009 to claim that tissue exposure occured. This opportunity was given by the draft guideline (October 2018) that "ADME data, obtained in an independent study using the same route and same species can be used to demonstrate tissue exposure". In this study, MTBE has been shown to be systematically available following inhalation exposure of Fischer 344 rats. However, this publication cannot guaranty that exposure levels reaching the organs in the TGR assay newly performed were high enough to induce effects. It is difficult for eMSCA to understand why this parameter, that is not difficult to measure, has not been included in a newly conducted study.

Additionally, although the number of animals used in the study was the minimal number required by TG 488 (i.e. 5 animals/group), it should have been discussed. Some dispersal in the mutation frequencies measured for kidneys could be noted in control animals. Sequencing of mutants should have been performed.

Overall, the dose selection was the main limitation in the TGR assay. This limitation does not allow to conclude on the absence of mutagenicity of MTBE in this study. Nevertheless, there is no sufficient grounds to require an additional study.

Justification for classification or no classification

Based on the available studies, MTBE has a low capacity to induce heritable mutations. There are still uncertainties in the mutagenic potential of MTBE due to positive results *in vitro* in MLA/TK with S9, DNA adducts formation in three organs in mice (lung, kidney and liver) and positive results in all the *in vivo* and *in vitro* comet assays pointing out for indirect mutagenicity.

Nevertheless, MTBE is not mutagenic in more than 10 Ames tests with and without metabolic activation. An *in vitro* and an *in vivo* liver UDS (conducted with CD-1 mice with a maximum dose of 8000 ppm in the *in vivo* test) were negative. Another mammalian *in vitro* measure for mutation frequency, i.e., Chinese hamster V79 cells gave no signs of mutagenic activity. No significant increase in chromosomal aberration has been demonstrated *in vitro* or *in vivo*. The mouse micronucleus test also failed to show any positive effect in two independent studies conducted *via* inhalation or intraperitoneally.

In conclusion, based on the available database and weight-of-evidence, no classification is proposed for MTBE. The recent TGR performed did not show any increase in mutation frequency but some limitations described above hamper the added value of this study. However, these limitations are too minor to allow eMSCA to request an additional testing. Therefore, eMSCA concludes that this study lowers the concern regarding genotoxicity but does not clarifiy it entirely. As identified before, mutagenicity and further carcinogenicity of MTBE, if it exists, appears at high doses, higher than those tested in the most recent study.

7.9.6. Carcinogenicity

Since EU-RAR, some reviews have been published. However, only one new study was published (table below).

Table 19: Summary of one new study regarding carcinogenicity published since EU RAR

Method	Results	Remarks	Reference
Rat (Wistar) male/female for 743 days Males: 0.5 (0.04), 3 (0.29), 7.44 (0.45) mg/l in drinking water (SD in brackets). Females: 0.5 (0.04), 3 (0.29), 14.96 (0.79) mg/l in drinking water (SD in brackets) (analytical conc.) Males: 25 (11), 140 (63), 330 (139) mg/kg/day in drinking water (SD in brackets). Females: 49 (14), 232 (66), 1042 (280) mg/kg/day in drinking water (SD in brackets). (actual ingested) Equivalent or similar to OECD TG 451 (Carcinogenicity Studies)	NOAEL (carcinogenicity): 330 mg/kg bw/day (actual dose received) (male) based on findings deemed significant. No biologically significant findings seen at maximum tested dose. NOAEL (carcinogenicity): 1042 mg/kg bw/day (actual dose received) (female) (No significant findings seen at maximum tested dose.) Neoplastic effects: yes (Only statistically significant finding in brain)	1 (reliable without restriction) supporting study experimental result Test material (EC name): tert-butyl methyl ether	Unpublished study report (2010)

This study does not modify the outcome of the analysis in the EU-RAR.

Kidney tumours in Fisher-344 rat

Renal tubular cells tumors were observed in male rats following MTBE inhalation (Bird *et al*. 1997) and the association with a mode of action related to the alpha-2 microglobulin seems acceptable. To be more precise, it is clear that there is kidney toxicity that is not limited to male and to tubular cells. However, when it evolves into nephro-carcinogenesis, this mode of action (MoA) is probably involved and a cause of the tumors observed as only male show this evolution.

Liver tumours in CD-1 mice

Two mice studies (Moser *et al.* 1996 and Bird *et al.* 1997) describe an increase in the incidence of hepatocellular adenomas in females at the high dose (8000 ppm). Since MTBE is so far considered as genotoxic (and that it is not a direct genotoxicant), the postulated MoA for MTBE causing liver tumors in female mice is that MTBE interferes with the ability of estrogen to suppress liver tumor promotion. Unleaded gasoline has been proposed to cause liver tumors in female mice through this postulated MoA (Standeven and Goldsworthy, 1993; Standeven *et al.* 1994a,b). Estrogens suppress liver tumor promotion in mice (Vesselinovitch and Mihailovitch, 1967; Hanigan *et al.* 1993; Lee *et al.* 1989; Yamamoto *et al.* 1993). Therefore, MTBE does not affect the estrogen receptor, but it increases estrogen catabolism, increases liver P450 activity, increases hepatocyte cell proliferation, and induces changes in estrogen-sensitive tissues. Although not confirmed in an initiation- promotion assay, the data suggest that MTBE induces liver tumors in female mice through interference with the ability of estrogen to suppress liver tumor promotion.

Induction of liver tumors in mice by changes in estrogen hormone function is not relevant for human risk assessment because human liver tumors are not under estrogen control. (Cruzan *et al.* 2007). This last assertion is not so clear as recent publication implicates estrogen as a protective factor against hepatocellular carcinoma: indeed, female patients had a less invasive tumor phenotype and different prognostic factors from male patients. Estrogen may have a protective effect against early- but not late-stage HCC (Li *et al.* 2014). However, it should be noted that this finding is not found in rat neither after inhalation nor oral exposure to MTBE. Moreover, it appears in mice only at high doses (8000 ppm). Therefore, it is believed that this finding is linked to high dosage that is not relevant for humans.

Leydig cells tumours in Fisher-344 and Sprague-Dawley rat

Several alternatives have been listed as plausible mechanisms in the aetiology of Leydig cell tumours in rats (Cook *et al*. 1999). In the case of MTBE, some of the testicular paracrine factors have been under a scrutiny. Typically, there is a disturbance in hormonal homeostasis, e.g., reduced testosterone or estradiol level followed by a compensatory action which is typically seen as a high level of circulating luteinising hormone (LH). A weakening of a hormonal signal may also cause compensation by an antagonist or agonist action of the chemical.

An example of this kind of complex mechanism is agonist action on dopamine receptors, which causes a decrease in serum prolactin levels. Reduction in prolactin serum level causes LH-receptor down-regulation and a subsequent increase in LH. A number of other mechanisms have been reported. The interference that occurs in the hypothalamic-pituitary-testis axis, resulting in higher levels of luteinising hormone may be of relevance to humans due to the similarities of the regulatory pathways in humans and rats. Studies that investigate potential hormonal mode of actions of the hypothalamic-pituitary-testis axis are available.

The role of testosterone in Leydig cell tumorigenesis was investigated in an experiment, where Sprague-Dawley rats were given gavage MTBE doses ranging 40-800 mg/kg for 28 days (Day *et al.* 1998). The highest dose produced a significantly reduced plasma testosterone level and an increased level of corticosterone. The authors suggested that, at high dose, the evidence support MTBE involvement in alteration of male endocrine function in several organs, including testes. However, they saw no change in the LH-level. Allgaier and de Peyster conducted a similar test with equal dose but measured the plasma LH level

already after 2-5 h or 5 days (Allgaier and de Peyster 1999). Again, there was no increase in the circulating LH level. Both of the above studies were reported as abstracts.

However, a dose-related decrease of serum LH was found by Williams *et al*. when they administered MTBE to the same rat species by gavage at 250-1500 mg/kg for 15 or 28 days (Williams *et al.* 2000). The change was significant only at the highest dose. The high dose rats also had a significantly decreased serum and interstitial fluid testosterone level at day 15. MTBE effect on luteinising hormone release hormone (LHRH) has been investigated in mice faeces by Billitti *et al*. They gave groups of five mice by gavage MTBE doses ranging from 400 to 1500 mg/kg for five days but found no difference in faecal LHRH (Billitti *et al*. 1999).

Based on the fact that there is numerous studies describing how high oral dosage of MTBE leads to decrease in serum testosterone, it could have been postulated that the mode of action for LCT would be a decrease of testosterone, leading to increases of LH levels in rats. However, as LH was either not modulated or increased: this MoA is not plausible.

Estrogen receptor (ER) expression has been reported by different authors in normal Leydig cells of adult testis (Pelletier *et al.* 2000; Taylor and Al-Azzawi, 2000; Saunders *et al.* 2001) indicating these cells as physiological targets for estrogens. Furthermore, cytochrome P450 aromatase, the enzyme catalyzing androgen aromatization into estrogens, has been detected in Leydig cells of normal human testis (Brodie *et al*. 2001), suggesting a paracrine action of estrogens, locally produced, in the steroidogenesis control (O'Donnell *et al.* 2001). However, estrogens are able to regulate cell growth and apoptosis (Blodbel and Orkin, 1996; Mabuchi *et al.* 2004); therefore, these hormones could also be involved in testicular neoplastic proliferation.

A possible involvement of estrogens in tumorigenesis in the human male gonad has been suggested by the increased incidence of testicular germ cell tumors after prenatal or occupational estrogen exposure (Weir *et al.* 2000 ; Dieckmann *et al.* 2001). Conversely, the link between estrogens and tumoral process is scarcely known in human testicular tumors originating from the gonadal stroma.

However, some studies on transgenic rodents suggested a role of estrogens in the development of Leydig cell hyperplasia and Leydig cell tumor (Fowler *et al.* 2000). The pattern of ER expression in neoplastic cells appears different from that of control Leydig cells exhibiting only ERb1 and ERb2 isoforms. The authors hypothesize how the high estrogen production could play a role in the neoplastic transformation of Leydig cells, while the exclusive presence of ERa in tumoral cells could amplify estradiol-17b signalling contributing to the tumor cell growth and progression (Carpino *et al.* 2007).

Available data do not show any impact of MTBE on estradiol serum concentration: Although MTBE increased the catabolism of estradiol in vitro, no decrease in serum estradiol levels occurred in mice exposed to 8000 ppm MTBE for 3 days to 8 months (Moser *et al.* 1998). Would anything happen, that would rather point to a decrease of oestrogens rather than the contrary. Moreover, it has been shown that MTBE did not compete with estradiol for binding to the estrogen receptor, nor did it activate human estrogen receptors transiently transfected in HepG2 cells. Exposure to MTBE also did not alter the location or intensity of the estrogen receptor in the uterus, cervix or vagina of MTBE-exposed mice (Moser *et al.* 1998).

The National Toxicology Program (NTP) in the United States has collected data that gives the incidence of interstitial tumours in control Fisher-344 rats since 1910. The average percentage of control animals with tumours is 89%, ranging from 64% to 98% (Haseman *et al.* 1990). An unpublished study report has reported an intra-laboratory Leydig cell tumour historical average incidence of 88% (Unpublished study report 1992b). A Leydig cell tumour (LCT) incidence higher than that was reached only at the highest MTBE dose. However, the incidence in high dose animals, euthanized earlier (82 weeks against 108 controls) remains higher (94%) even when compared with historical controls. A constant time, it seems likely that the impact of these lesions would be increased. This is also true for the 3000 ppm dose euthanized at 97 weeks with an incidence of 82%. In addition, the study was a lifetime study, where one can expect to have confounded results, because

LCTs are more frequent in the old rats. In other words, in this case, the longer surviving high dose rats would have been therefore more likely candidates to have a tumour. Moreover, a clear dose-response relationship was seen in this study. The dose-response of the Leydig cell tumours was not so evident in the results obtained by Belpoggi *et al.* who derived a dose-response curve from only two treatment groups. Moreover, a significantly lower mortality rate in the highest dose group than in the others skews the results setting restrictions to a valid interpretation of the data. There have also been doubts that the frequency of the neoplasm might be an overestimation, due to difficulties in distinguishing them morphologically from hyperplasia. Although there is no widely accepted criteria for distinguishing between the two lesions, principally, two arbitrary size based criteria have been used. Belpoggi *et al*. reported in their re-review that they followed the criteria established by NTP (Boorman, Chapin and Mitsumori, 1990).

In summary, there is evidence that MTBE causes an increase of LCT tumours in rats. In the current state of knowledge, only two mechanisms are known to be not relevant to humans: GnRH and dopamine agonists. Indeed, testicular GnRH receptors and prolactin are little or not expressed in humans. However, other mechanisms are considered relevant. However, despite that the data is still too limited to draw a conclusion on which mode of action induces the LCTs in the rats receiving MTBE.

Based on the available evidence, it seems that the typical mode of action, which involves elevated LH, is not the case for MTBE. The interpretation of these results is further complicated as it is unclear how the differences in physiology and anatomy between rat and human testis contribute to susceptibility to LCT tumours. Testicular cancer is a relatively uncommon cancer in humans. Most human testicular cancers originate either from germ or Sertoli cells. Tumours of the testes constitute about 1% of all human neoplasm; only 2-3% of all testicular tumours is of Leydig cell origin. Moreover, one cannot overlook the fact that the tumours appear in rats only at quite high doses and that MTBE lacks genotoxic properties.

In conclusion, it cannot be shown that these tumours are not relevant to man due to the lack of knowledge of the possible mode of action. It is therefore proposed to take into account these tumors for risk assessment from a dose of 3000 ppm and establish a NOAEL of 400 ppm for this effect (an increase is observed: 70% compared to the controls of the study but not compared to historical controls).

A weak tumorigenic response was reported for both MTBE and TBA in one tumor type (kidney) in male rats, for MTBE in one other tumor type (testicular) in male rats, for MTBE in one tumor type (liver) in female mice, and for TBA in one tumor type (thyroid) in female mice. The weight of evidence does not support a direct genotoxic MoA. More than the other MoAs suggested to explain the different tumors observed in animal models whose reliability to humans remains an opened question, the doses at which these effects appear are much higher in comparison with the conditions described in the exposure scenarios. Based on the data available on MTBE in the year of substance evaluation, it appears unnecessary to take into account the effects descibed for further risk assesment.

7.9.7. Toxicity to reproduction (effects on fertility and developmental toxicity)

Table 20: Studies on sexual function and fertility effects

Method	Results	Remarks	Reference
One-generation reproductive toxicity study No guideline followed GLP compliant: not specified Rat (Sprague-Dawley) male/female Target concentrations: 250, 1000 and 2500 ppm MTBE (900, 3600 and 9000 mg/m^3) Nomical concentrations: 300, 1300 and 3400 ppm MTBE Male exposure: by inhalation for 6h/d, 5d/w for 12 weeks Female exposure : same dosing scheme for 3 weeks; then 6h/d, 7d/w from gestation D1 to D20 and from lactation D5 to D20 P: Mortality, gross-toxicological signs, body weights, histopathological examination of testes, epididymis and ovaries F1a and F1b: bw, internal and external malformations, histopathological examination of gonads and abnormal tissue	P: non significant increased incidence of dilated renal pelvis at 250 and 2500 ppm; slightly but non significant lower pregnancy rate at 1000 ppm F1a: ↓* survival indices between lactation D0 and D4 at 250 and 1000 ppm but not at 2500 ppm F1b: ↓* pup viability indices at 1000 and 2500 ppm F1a and b: slight but non significant decrease in pup bw between D14 and 21	2 (reliable with restriction) Experimental study Supporting study Test material: MTBE Limitations: no GLP status, number of animals tested not specified	Biles *et al.* (1987) Cited by the EU-RAR
Two-generation reproductive toxicity study No guideline followed GLP compliant Rat (Sprague-Dawley) male/female 25 animals/sex/dose Target concentrations: 0, 400, 3000 and 8000 ppm MTBE Analytical concentrations: 0, 402, 3019 and 8007 ppm (SD between brackets) (P) Male exposure: by inhalation for 10 weeks before mating until delivery of the F1-litter (6h/d, 5d/w) Female exposure : by inhalation for 10 weeks before mating, during 21 day mating period, gestation and lactation from D5 until sacrificesame dosing scheme for 3 weeks; then 6h/d, 7d/w from gestation D1 to D20 and from lactation D5 to D20	**P:** hypoactivity and blepharospasms at 3000 and 8000 ppm; ataxia at 8000 ppm; ↓* bw and bw gain in the high dose male group at pre-mating period (PMP); ↑* bw gain in the high dose female group in PND21-28. **F1 litter:** ↑* number of dead pups on PND4 at 8000 ppm (likely not related to MTBE); ↓* male and female bw at PND 14-28 at 8000 ppm; ↓* female bw at PND 14 at 3000 ppm. **Parent F1:** hypoactivity and blepharospasms at 3000 and 8000 ppm; ataxia at 8000 ppm; ↓* bw and bw gain in the high dose male group at PMP; ↑* bw in the high dose female group at PND 14-21 and during lactation; ↓* food consumption in the high dose female group; ↑* relative liver weight in both sexes at 8000 ppm and in males at 3000 ppm; no histological change in any tissue.	2 (reliable with restriction) Experimental study Key study Test material: MTBE Purity: 99%	Bevan *et al*. (1997) Cited by the EU-RAR

(F1) new parents randomly selected and exposed on PND 28 (same exposure procedure) P: clinical examinations, bw, food consumtion, post mortem examination F1: bw, check for abnormalities, external examination (including cleft palate), post mortem examination P and F1 at control and 8000 ppm: histopathological examination of pituitary, testes, epididymis, prostate and seminal vesicles and vagina, uterus, ovaries, respiratory tract, gross lesions	**F2:** ↑* number of dead pups on PND4 at 8000 ppm; ↓* bw in male at 3000 (PND 14-28) and 8000 ppm (PND 7-28).		
28-day repeated dose toxicity study in rodent Equivalent to OECD TG 407 Rat (Sprague-Dawley) male Oral exposure: gavage 0, 400, 800, 1600 mg/kg/day for 2 or 4 weeks (group A and B resp.) Clinical signs, bw, food consumtion, blood collected, weight of liver, kidney, testis and epididymis, histopathological examinations of epididymis and testes	**Group A:** - Less compact cells in seminiferous tubules. - ↓* serum levels of testosterone at 800 and 1600 mg/kg/d, ↑* serum LH at 400, 800 and 1600 mg/kg/d, ↑ * serum FSH at 800 and 1600 mg/kg/d. - ↑* serum MDA at 1600 mg/kg/d, ↑* total antioxidant ability at 400, 800 and 1600 mg/kg/d. - ↓* mRNA level of ABP and OGG1, ↑* mRNA level of SOD(EX) at 1600 mg/kg/d. **Group B:** - Irregular and disordered arrangement of cells with the shedding of cellular material from the seminiferous epithelium at 800 and 1600 mg/kg/d. - ↑* % of abnormal sperm from 400 mg/kg/d (dose-dependent relationship). - ↑* serum levels of testosterone at 800 mg/kg/d. - ↑* serum MDA at 400 mg/kg/d, ↑* total antioxidant ability at 1600 mg/kg/d - ↓* mRNA level of ABP at 800 and 1600 mg/kg/d.	Test material: MTBE Vehicle: peanut oil	Li *et al.* (2008)
Non-guideline 5-day study on male mice CD-1 male mice Oral exposure: gavage on days 1, 3 and 5 with 0, 400, 1000 and 2000 mg/kg 5 animals/dose Faecal analysis used to determine T and LHRH levels, testis examination	- Little effect on T levels even at high doses - No difference in unstimulated or hCG stimulated fecal testosterone at any dose - No effect on mean bw and testes weights - No histopathological differences between control and high dose group in the % of tubules with SEV, MC, MNGC and sloughing - Greater number of tubules with gross disruption at 2000 mg/kg	Test material: MTBE Vehicle: canola oil Limitations: no GLP status, low number of animals tested	Billitti *et al.* (1999) (abstract only)

Non-guideline 7-day study on male mice CD-1 male mice Gavage on days 1, 3 and 5 with 0, 400, 1000 and 2000 mg/kg, injected i.p. with hCG (2.5 IU/g) on day 6, necropsied on day 7 6 animals/group Blood collected, organs weighed	- Some animals with ataxia and lethargy at 2000 mg/kg - No significant bw or organ weight differences - No effect in testis histology or testosterone levels - Considerable variability in testosterone values within groups - Control mouse with unilateral degeneration of the seminiferous tubules (only testicular abnormality in the pathology report) and highest testosterone value measured. Lowest testosterone value in another control mouse with no abnormal testicular histology.	Test material: MTBE Limitations: no GLP status, low number of animals tested	De Peyster *et al.* (2008)
Non-guideline 28-day study on adult male mice BALB/c adult male mice Exposure in drinking water to 80, 800 and 8000 µg/L for 28 days 6 animals/group Blood collected, tissue collection	- No significant treatment-related differences in mean bw or organ weight - No clear MTBE dose-related effect in mean serum testosterone, sperm permgcauda, or testis histology - Minimal or mild degeneration of seminiferous tubules in some control mice in addition to treated mice - All testicular lesions noted graded as minimal (grade 1) except for one tap water control mouse with unilateral mild degeneration (grade 2).	Test material: MTBE Limitations: no GLP status, low number of animals tested	De Peyster *et al.* (2008)
Non-guideline 51-day study on juvenile male mice BALB/c juvenile male mice Exposure in drinking water to 80, 800 and 8000 µg/L for 51 days 10 animals/dose Blood collected, organs weighed (testis, epididymis, liver)	- No effect on absolute mean bw, reproductive and other organ weights but ↑* relative seminal vesicle mean weight at 80 ppb and relative lung mean weight at 800 ppb - Variability in serum testosterone - No signs of hepatic oxidative stress	Test material: MTBE Limitations: no GLP status	De Peyster *et al.* (2008)
Non-guideline 28-day study on adult male mice BALB/c adult male mice Exposure in drinking water to 80, 800 and 8000 µg/L for 28 days 5 animals/dose	—↑* dose dependent mean combined testis weight at all dose; ↑ mean seminal vesicle weight at 800 and 8000 µg/L, ↑ mean seminiferous tubule diameter and incidence of abnormal tubules - ↓ serum testosterone at 800 and 8000 ppb	Test material: MTBE Limitations: no GLP status, low number of animals tested	Almeida *et al.* (2004) (abstract only)

*significant

Table 21: Studies on developmental toxicity

Method	Results	Remarks	Reference
According to EPA OTS 798.4350 (Inhalation Developmental Toxicity Screen) GLP compliant New Zealand White rabbit 15 females/group 0, 1000, 4000, 8000 ppm Exposure by inhalation from day 6 to 15 of gestation (6h/d)	Maternal toxicity: 8000 ppm: 15% increased liver weight and >70% reduction in food consumption during GDs 6-10 Developmental toxicity: No effect NOAEC (maternal toxicity): 4000 ppm NOAEC (fetotoxicity): 8000 ppm	1 (reliable without restriction) Key study Experimental study Test material (EC name): MTBE Purity: at least 99%	Bevan *et al.* (1997)
According to EPA OTS 798.4350 (Inhalation Developmental Toxicity Screen) GLP compliant CD-1 mice 30 females/group 0, 1000, 4000, 8000 ppm Exposure by inhalation from day 6 to 15 of gestation (6h/d)	Maternal toxicity: 4000 and 8000 ppm: ataxia, hypoactivity, prostration, laboured respiration and lacrimation 8000 ppm: reducted bw at GD 12, 15 and 18 and reduced bw gain throughout the treatment and post-treatment periods; uterine weight 50% lower* than controls Developmental toxicity: 8000 ppm: increased post implantation loss due to ↑* incidence of late resorptions and dead foetuses; ↓* live and male foetuses/litter; ↓* foetal bw; malformations (cleft palate*); skeletal variations (reduced ossification in various sites*) 4000 ppm: ↓* foetal bw; malformation (only one cleft palate); skeletal variations (reduced ossification) NOAEC (maternal toxicity): 1000 ppm NOAEC (fetotoxicity): 1000 ppm	1 (reliable without restriction) Key study Experimental study Test material (EC name): MTBE Purity: at least 99%	Bevan *et al.* (1997)
Equivalent or similar to OECD TG 414 (Prenatal Developmental Toxicity Study) GLP compliant Spraque-Dawley rat 25 females/group 0, 250, 1000, 2500 ppm Exposure by inhalation from day 6 to 15 of gestation (6h/d)	Maternal toxicity: ↓* food consumption at all doses on GD 9-12 Developmental effects: A preponderance of male pups over females at 1000 ppm* (biological variability) NOAEC (maternal toxicity): 2500 ppm NOAEC (fetotoxicity): 2500 ppm	1 (reliable without restriction) Key study Experimental study Test material (EC name): MTBE Purity: 95-98.9%	Conaway *et al.* (1985)

Equivalent or similar to OECD TG 414 (Prenatal Developmental Toxicity Study) GLP compliant CD-1 mice 30 females/group 0, 250, 1000, 2500 ppm Exposure by inhalation from day 6 to 15 of gestation (6h/d)	Maternal toxicity: Increased lacrimation and a slight, non-significant dose-related decrease in food consumption on days 12-15 in the treated groups and in water consumption during days 9-12 in the treated groups (no change in bw) Developmental effects: - A slight increase in the number of resorption sites and their proportion to implants at 250 and 2500 ppm - A slight increase in skeletal abnormalities at 2500 ppm - A slight increase of sternebrae malformations in all treated groups (0.6% (low), 1.2% (mid) and 2.1% (high)). (Investigators stated that historically seen with low incidence in control animals with 0.16% incidence. They concluded this was not treatment related since there were no increase of vertebral or rib effects usually associated with this malformation). NOAEC (maternal toxicity): 2500 ppm NOAEC (fetotoxicity): 2500 ppm	1 (reliable without restriction) Key study Experimental study Test material (EC name): MTBE Purity: 95-98.9%	Conaway *et al.* (1985)
Fischer 344 rat 500-1500 mg/kg bw/d Exposure: gavage from day 6 of organogenesis to 10 days post parturition (daily) Limitations :no GLP or guideline status, number of animals not specified	Developmental effects: No organ toxicity or histological changes to pup vasculature NOAEL (developmental toxicity): > 1500 mg/kg bw/d	2 (reliable with restriction) Supporting study Experimental study Test material (EC name): MTBE Purity: not specified	Unpublished study report 2011
Harlan Sprague-Dawley rat 5 females/group 0, 500, 1000, 1200, 1500 mg/kg/d Exposure: gavage from day 6 to 10 of gestation (once daily) At birth, sacrifice, necropsy and gross observations for one-half of the animals of each sex from each litter Histopathological examination of brain, lung, liver, kidney, heart and stomach Remaining animals from each litter: exposed by gavage to MTBE in corn oil once daily from birth until post-partum day 10 at the same level of the dams; then sacrified on post-partum day 11 and examined as described above	Maternal toxicity: Narcotic effects Developmental effects in the treated pups: - Narcotic effects at 1200 and 1500 mg/kg/d - No significant changes on pup bw gain, organ weights, no changes in vascular development following either gross or histological examination	Test material: MTBE Purity: not specified Vehicle: corn oil	Unpublished study report 2011

Statistical analysis: Dunnett's test Limitations: no GLP or guideline status			
According to EPA OPPTS 870.3700 (Prenatal Developmental Toxicity Study) GLP compliant Sprague-Dawley rat 24-25 females/dose 0, 2000, 10000, 20000 mg/m^3 Exposure: by inhalation from day 5 to 10 of gestation (6h/d)	Maternal toxicity: - Reduced bw gain* and food consumption* on GD 8-11 at 20000 mg/m^3 G/MTBE - No mortality or significant clinical effects, no effect on pregnancy rates - Mild stress related to exposure conditions at higher test concentrations Developmental effects: No evidence of treatment-related foetotoxicity, no treatment-related increases in external foetal abnormalities/variations, or in visceral malformations or variations, no increased frequency of skeletal malformations NOAEL (BGVC, maternal toxicity): 20000 mg/m^3 NOAEL (BGVC, developmental toxicity): 20000 mg/m^3 NOAEL (G/MTBE, maternal toxicity): 10000 mg/m^3 NOAEL (G/MTBE, developmental toxicity): 20000 mg/m^3	2 (reliable with restriction) Supporting study Experimental study Test material (EC name): baseline gasoline vapor condensate (BGVC) alone or vapors of gasoline blended with MTBE (G/MTBE)	Roberts *et al.* (2014a)
According to OECD TG 414 (Prenatal Developmental Toxicity Study) and US EPA OPPTS 870.3700 GLP compliant CD-1 mice Exp 1: exposure to 0, 2000, 10000, 20000 mg/m^3 BGVC or G/MTBE by inhalation from day 5 to 17 of gestation (6h/d) 25 females/group Exp 2: exposure to 0, 2000, 10000, 20000, 30000 mg/m^3 G/MTBE by inhalation from day 5 to 16 of gestation (6h/d) or exposure from GD 5-10 plus sham (air) exposures on GD 11-16 23 females/group	Maternal toxicity: - BGVC at 20000 mg/m^3: bw ↓ over GD 8-11 (1 dam), ↓* mean bw for this group from GD 11 - G/MTBE: disturbed breathing in 1 dam at 20000 mg/m^3 (GD 9 only) and in 1 dam at 30000 mg/m^3 (GD 10 only) - Exp 2: ↑* relative liver weight (dose dependent relationship) Developmental effects: - BGVC: ↓* mean foetal bw at 10000 and 20000 mg/m^3, ↓ * live foetuses/litter at 20000 mg/m^3 (related to a lower number of corpora lutea and an increase in resorptions - Exp 1, G/MTBE: 1 dead fetus with ectopia cordi at 2000 mg/m^3 and in 2 viable foetuses at 10000 mg/m^3. Effects not reproduced in Exp 2. 1 fetus with gastrochisis at 10000 mg/m^3 - Exp 2, G/MTBE: 1 foetus with gastrochisis at 30000 mg/m^3, associated with very low bw and cleft palate (low	2 (reliable with restriction) Supporting study Experimental study Test material (EC name): baseline gasoline vapor condensate (BGVC) alone or vapors of gasoline blended with MTBE (G/MTBE)	Roberts *et al.* (2014b)

	maternal bw at 30000 mg/m^3); increased incidence of cleft palate at 30000 mg/m^3; no ectopia cordis NOAEL (BGVC, maternal toxicity): 10000 mg/m^3 NOAEL (BGVC, developmental toxicity): 2000 mg/m^3 NOAEL (G/MTBE, maternal toxicity): 20000 mg/m^3, 10000 mg/m^3 (exp 1, exp 2 resp.) NOAEL (G/MTBE, developmental toxicity): 20000 mg/m^3 (exp 1 and 2)		

a) Effects on sexual function and fertility

The EU-RAR reported the effects of MTBE on fertility in one and two-generation studies in Sprague-Dawley rats (Biles *et al.* 1987, Bevan *et al.* 1997 respectively). Based on these studies, the EU-RAR concluded that MTBE does not cause significant reproductive toxicity in Sprague-Dawley rats as the reproductive function was not adversely affected and no pathological changes were observed in the gonad microscopy (see described results in Table 20). In addition, there were no adverse changes observed in the gonads in any of the sub-chronic or long-term toxicity studies.

In the one-generation study, the pup viability was significantly lower at 1000 and 2500 ppm for the F1b litter but there was no such change seen in the F1a litter. Furthermore, the control group of the F1b litter viability percentage was 99.0% while the F1a litter control viability was 97.6%. This may have skewed the significance seen. Moreover, no adverse effect on the survival of the pups was seen in any dose group of the second litter. There was also a significant reduction in pup survival index between lactation day 0 and day 4 at 250 ppm and 1000 ppm. Although this may be of toxicological importance, it should be noted that there was no difference in the highest dose or in the parallel F1a-litter. In addition, there was no significant change in the survival indices during the lactation period (days 4-21).

In the two-generation study, there were general toxicity signs at 3000 and 8000 ppm in both generations of parental animals (significant effect on body weights). No significant changes could be seen in the reproduction parameters even at the highest dose level in the Sprague-Dawley rat. There was a statistically significant increase of dead pups in both F1 and F2 generation litters with no change in survival indices. The authors did not consider the deaths in the F1 generation to be related to MTBE because the increase was due to a death of an entire litter of 16 animals.

Since the EU-RAR has been published, new sub-acute or sub-chronic studies investigating the effects of MTBE on male rats or mice reproductive system became available (see description in Table 20).

Studies from Billitti *et al*. (1999) (5-day study on CD-1 mice exposed to 400-2000 mg/kg/d) and De Peyster *et al.* (2008) (7-day study on CD-1 mice exposed to 400-2000 mg/kg/d and 28-day study on BALB/c mice exposed to 80-8000 µg/L) did not show any adverse effect on the reproductive system. There was no effect on testosterone level, testes weight or histology.It should be noted in the study from De Peyster *et al.* that there was considerable variability in testosterone levels within groups. Moreover, results obtained in control animals raised question on the reliability of the study as one mouse showed unilateral degeneration of the seminiferous tubules (the only testicular abnormality in the pathology report) and the highest testosterone value measured.

The only effect observed in the 51-day study on BALB/c juvenile mice from De Peyster was a significant increase in the relative semical vesicle mean weight at 80 µg/mL and the relative lung mean weight at 800 µg/mL.

The study of Almeida *et al*. (2004) reported a statistically significant increase in testes weight (both combined) at all dose levels and increased seminal vesicle weight at 800 and 8000 ug/L in BALB/c mice. Seminiferous tubule diameter increased with drinking water concentration of MTBE but was apparently not statistically greater. Serum testosterone appeared to be greatly decreased at 800 and 8000 ppb although this may not have been statistically different from the controls.

Finally, the 28-day repeated dose toxicity study in Spraque-Dawley rats from Li *et al.* (2008) was the only subacute study showing an adverse effect on the reproductive system. Male rats were exposed to 0, 400, 800 and 1600 mg/kg/day MTBE by gavage for 28 days. Secretions of T, LH and FSH as well as MDA content, total antioxidant ability, mRNA level of ABP, OGG1 and SOD(EX) were significantly disturbed at 800 and/or 1600 mg/kg/d. Moreover, a dose-dependant increase in the percentage of abnormal spermatozoids was observed and lead to establish a LOAEL of 400 mg/kg/d. Irregular and disordered arrangement of cells with the shedding of cellular material from the seminiferous

epithelium was also observed from 800 mg/kg/d. The authors concluded that relatively high doses of MTBE could exert reproductive system toxicity of male rats and disturb the secretions of T, LH and FSH, possibly due to oxidative stress induced by MTBE.

Conclusion on fertility

Inhalated MTBE does not cause significant toxicity to fertility in Sprague-Dawley rats. Orally, after subacute exposure, it affects spermatozoids and seminiferous tubules from 400 and 800 mg/kg bw/d respectively.

As MTBE becomes systemically available after oral and inhalation exposure, the discrepancies of the results depending on the route of exposure cannot be explained. For the effect on fertility, a LOAEL of 400 mg/kg bw/d is set up whatever the route of exposure.

b) Developmental toxicity

In the study from Bevan *et al.* (1997), malformations were observed at 8000 ppm in CD-1 mice but they occurred at a dose level of marked maternal toxicity. In the study from Conaway *et al.* (1985), the sternebrae malformations seen in CD-1 mice at 250-2500 ppm were not considered treatment related (see description in Table 21). Based on these results, the EU-RAR concluded that MTBE is not toxic for the development.

Since the EU-RAR has been published, new studies were available (see description in Table 21). Kozlosky *et al.* 2011 and Confidential, 2011 studied developmental toxicity of MTBE in rat after oral exposure. No developmental effects were observed. Roberts *et al.* (2014a and b) investigated developmental toxicity of MTBE in rats and mice after inhalation exposure to 2000, 10000 and 20000 mg/m^3 baseline gasoline vapor condensate (GBVC) alone or to vapors of gasoline blended with MTBE (G/MTBE) (additional concentration of 30000 mg/m^3). These studies are interesting because they mimick real life exposure. In CD-1 mice, developmental effects are reported. Two uncommon ventral wall closure defects occurred: gastroschisis (1 fetus at 10000 mg/m^3 (~3000 ppm)) and ectopia cordis (1 fetus at 2000 mg/m^3 (~600 ppm); 2 fetuses/1 litter at 10000 mg/m^3) but were not reproduced in a second study (G/MTBE-2). In this second study, an increased incidence of cleft palate was observed at 30000 mg/m^3 G/MTBE (~8000 ppm) but was associated with maternal toxicity. No ectopia cordis occurred in the replicate study, but a single observation of gastroschisis was observed at 300 00 mg/m^3.

Conclusion on developmental toxicity

Based on the EU RAR and the new findings, developmental effects were observed only in association with maternal toxicity. Thus, no classification for developmental toxicity is proposed.

7.9.8. Hazard assessment of physico-chemical properties

MTBE is not explosive nor pyrophoric and does not have oxidising properties.

When its purity is 97.9% w/w or over, its flash point is below 23 °C and initial boiling point is over 35 °C, therefore it should be considered as highly flammable (Flammable liq. Cat. 2, H225).

The presence of significant amount of impurities such as hydrocarbons in the technical material may lead to an over-classification, as flammable liquid cat. 1 (H224) instead of flammable liquid cat. 2 (H225). Moreover, considering the dynamic viscosity of the pure substance, the additional classification as Asp. Tox. Cat. 1 (H304) applies when the technical substance contains 10% or more of impurities classified for aspiration hazard.

Furthermore, considering the level of certain impurities indentified in registration dossiers, there are a possibility for additional classifications (e.g. if <4.5% methanol, <0.2% benzene, <12% alcanes C4-C6, <20% alcènes C4-C7).

7.9.9. Selection of the critical DNEL(s)/DMEL(s) and/or qualitative/semi-quantitative descriptors for critical health effects

Not assessed.

7.9.10. Conclusions of the human health hazard assessment and related classification and labelling

Regarding the effects on human health, the numerous studies do not allow identifying effects warranting a classification. Despite limitations (although conducted according to the guidelines, the mutagenicity studies lack information regarding exposure and they could have been conducted with higher doses), the data obtained show that MTBE does not meet the criteria of the CLP Regulation to be classified as mutagen. Likewise, the tumors observed in some models on the studies evaluated at the time of substance evaluation are observed after high doses of MTBE treatments and according to modes of action deemed irrelevant for humans. Regarding fertility, the effets observed do not justifiy a proposal of classification Regarding developmental effects, MTBE does not appear to induce developmental effects, except as a result of maternal toxicity. No classification for developmental toxicity is thus proposed.

7.10. Assessment of endocrine disrupting (ED) properties

7.10.1. Endocrine disruption – Environment

MTBE was included in the CoRAP in particular because of its potential endocrine disrupting properties. Data was provided in the registration 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 in fish (Table 22)Table 22. Reliable studies on the potential endocrine disrupting properties of MTBE in fish.

OECD Level	Substance Data type	Study type	Guideline/GLP	Reliability	Endpoint	Type of dose-response relationship	Result	ED hypothesis checked	Conclusion based on data	Reference
3	MTBE Publication 3 tests	Fish *Danio rerio* *In vivo*	No standard guideline followed Not GLP-compliant 48h of exposure Flow-through condition Tested concentrations: 0 – 400 – 600 – 652 – 661 – 730 – 843 mg/L [mea] 2 replicate per treatment and control 10 adults per replicate	2 (well documented publication)	Mortality	Monotonic increase	LC50,48h = 677 mg/L [mea]	Estrogenic activity Androgenic activity	[+] [-]	Moreels *et al.* (2006)
			No standard guideline followed Not GLP-compliant 21d of exposure Flow-through condition Tested concentrations:		VTG concentration in plasma for males	No significant treatment-related effect	The lowest MTBE concentration of 0.11 mg/L induced a 26-fold and highly significant ($p = 0.001$) increase of vitellogenin concentration in males compared to the non exposed male control group			

			0 – 0.11 – 2.7 – 37 mg/L [mea], corresponding to 0 - 0.01% - 0.39% - 5.5% of the LC50, 48h respectively. 3 replicates per treatment and control 22 adults per replicate				(1.76 vs 0.068 mg/mL). No result mentioned in the publication for the 2.7 mg/L treatment 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.			
					GSI	No significant treatment -related effect	NOEC,21d ≥ 37 mg/L [mea]			
			No standard guideline followed Not GLP-compliant 8 weeks of exposure Flow-through condition Tested concentrations: 0 – 0.44 – 2.2 – 22 – 220 mg/L [mea],		Fecundity (number of eggs produced between four and eight weeks)	No significant treatment -related effect	NOEC,21d ≥ 220 mg/L [mea]			
					Fertility of produced eggs	No significant treatment -related effect	NOEC,21d ≥ 220 mg/L [mea]			

			corresponding to 0.06% - 0.32% - 3.25% - 32.5% of the LC50, 48h respectively. 3 replicates per treatment and control 30 adults per replicate							
3	MTBE Study report	Fish *Danio rerio* *In vivo* Short-term reproduction assay	OECD229 US-EPA OPPTS #890.1350 GLP-compliant 21d of exposure Flow-through condition Tested concentrations: 0 – 0.1 – 5 – 250 mg/L [nom] 0 – 0.122 – 3.04 – 147 mg/L [mea] 4 replicates per treatment and control 5 males and 5 females per replicate	2 (GLP; standard guideline followed with deviations)	Survival	No significant treatment -related effect	NOEC,21d ≥ 147 mg/L [mea]	Estrogenic activity Androgenic activity	[+] [-]	Unpublished study report 2012
					Length and weight of males and females	No significant treatment -related effect	NOEC,21d ≥ 147 mg/L [mea]			
					Fecundity : - Cumulative egg produced - Eggs per female reproductive day	Significant reduction at the highest tested concentration	NOEC,21d = 3.04 mg/L [mea] The mean cumulative number of eggs produced in the negative control group and the 0.122, 3.04 and 147 mg/L treatment groups was 6107, 5889, 6417 and 1620 eggs, respectively. The mean number of eggs per female reproductive day in the negative control group and			

							the 0.122, 3.04 and 147 mg/L treatment groups was 58.2, 50.7, 61.1 and 15.5 eggs per day, respectively.			
					Fertilization success	No significant treatment -related effect	NOEC,21d ≥ 147 mg/L [mea]			
					VTG concentration in plasma of male	No significant treatment -related effect	VTG levels in the plasma of male fish exposed to test substance at 3.04 and 147 mg/L were 3.4- and 2-fold higher compared to controls, respectively. These slight elevations were significant ($p \leq 0.05$, Jonckheere-Terpstra trend test). Mean VTG for the 3.04 mg/L treatment group was also significantly different from the control mean according to Dunnett's test ($p \leq 0.05$).			
					VTG concentrati	No significant	NOEC,21d ≥ 147 mg/L [mea]			

					on in plasma of female	treatment -related effect				
					Histopathology of gonads in males	Significant effect at the highest tested concentration	NOEC,21d = 3.04 mg/L [mea] Statistically significant differences between control and treated fish were observed only for the highest treatment group (147 mg/L) with respect to gonadal stage ($p \leq 0.05$).			
					Histopathology of gonads in females		NOEC,21d = 3.04 mg/L [mea] Exposure of female fish to the test material at 147 mg/L significantly ($p \leq 0.05$) increased the incidence of oocyte atresia and accumulation of oocyte debris in the oviduct as compared to controls.			
3	MTBE Study report	Fish *Pimephales promelas* *In vivo*	OECD229 US-EPA OPPTS #890.1350 GLP-compliant	1 (GLP; standard guideline followed)	Survival	No significant treatment -related effect	NOEC,21d ≥ 62 mg/L [mea]	Estrogenic activity Androgenic activity	**[-]** **[-]**	Unpublished study report 2013

		Short-term reproduction assay	21d of exposure Flow-through condition Tested concentrations: 0 – 1 – 3 – 10 – 30 – 100 mg/L [nom] 0 – 0.6 – 1.8 – 6.2 – 20 – 62 mg/L [mea] 4 replicates per treatment 6 replicates for control 2 males and 4 females per replicate		Fecundity : - Cumulative egg produced - Eggs per female reproductive day	No significant treatment-related effect	NOEC,21d ≥ 62 mg/L [mea]			
					Fertilization success of produced eggs	No significant treatment-related effect	NOEC,21d ≥ 62 mg/L [mea]			
					Secondary sex characteristics: - Tubercles - fatpads	No significant treatment-related effect	NOEC,21d ≥ 62 mg/L [mea]			
					GSI	No significant treatment-related effect	NOEC,21d ≥ 62 mg/L [mea]			
					Histopathology of gonads	No significant treatment-related effect	NOEC,21d ≥ 62 mg/L [mea]			
					VTG concentration in plasma of males and females	No significant treatment-related effect	NOEC,21d ≥ 62 mg/L [mea]			

					Length and weight of males and females	No significant treatment -related effect	NOEC,21d ≥ 62 mg/L [mea]			
4	MTBE study report	Fish Danio rerio In vivo Sexual development	OECD TG234 GLP-compliant 66d of exposure Flow-through conditions Tested (nominal) concentration: 1.6 – 3.1 – 6.3 – 13 and 25 mg/L 4 replicates per treatment 6 replicates for control 160 embryos per treatment 240 embryos in the control	1	Hatching success	No significant treatment -related effect	NOEC = 22 mg/L	Estrogenic activity	[-]	Unpublished study report, 2019
					Larval survival	No significant treatment -related effect	NOEC = 22 mg/L			
					Sex ratio	No significant treatment -related effect	NOEC = 22 mg/L			
					Growth and weight	No significant treatment -related effect	NOEC = 22 mg/L			
					VTG	No significant treatment -related effect	Male: statictically significant increase in VTG concentratiol for males fish at 2,4 and 11 mg/L following a parametric test. There is alo a slightly statistically significant increase at the highest dose (22 mg/L) with			

							non-parametric test Female: no statistically significant difference in VTG concentration using parametric and non-parametric test			

[nom]: nominal concentration
[mea]: measured concentration
GSI: Gonado-Somatic Index

A GLP-compliant Fish Short-Term Reproduction Assay was performed on MTBE with the zebrafish (Danio rerio), according to the standard guidelines OECD TG 229 and US EPA OPPTS #890.1350 (Unpublished study report 2012, RI = 1). 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 MBTE has no effect on any of the other endpoints measured. **Exposure of fish to 147 mg/L MBTE 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), who performed a well-documented serie of toxicity tests on MTBE with 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 other experiments. In the first chronic exposure experiment performed by Moreels *et al.* (2006), 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 LC50,48h 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 gr**oup (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.

This study demonstrates that MTBE can potentially have an estrogenic activity at concentrations up to 0.11 mg/L, based on VTG induction in MTBE-exposed males compared to control.

In the second chronic exposure experiment performed by Moreels *et al.* (2006), breeding groups of zebrafish were exposed for 8 weeks in flow-through condition to MTBE at mean measured concentrations of 0.44 - 2.2 - 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, 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.

A GLP-compliant Fish Short-Term Reproduction Assay was performed on MTBE with Fathead Minnow (*Pimephales promelas*), according to the standard guidelines OECD TG229 and US EPA OPPTS #890.1350 (Unpublished study report 2013, RI = 1). 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 the Unpublished study report 2012), MTBE does not appear to interact with the estrogenic or androgenic hormone axes of fathead minnows at the tested concentrations as no significant treatment-related effects were obersved.

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 (GD) on standardized test guidelines for Evaluating Chemicals for Endocrine Disruption (OECD, 2018). Their results (i.e. mainly the **significant vitellogenin induction in male adult fish**) indicate possibilities for adverse effects which can be highlighted in reproductive and developmental studies of levels 4 and 5 of the OECD conceptual framework. According to the OECD GD 150, studies of level 4 and 5 that highlight adverse effects linked to the mode of action are needed to identify endocrine disruptive substances.

Therefore, pursuant to Article 46(1) of REACH, a Fish Sexual Development Test (FSDT) was requested in a Substance Evaluation Decision with the following specifications :

- Five test concentrations must be tested in a range between 0.1 and 10 mg/L expressed in measured concentrations, in order to cover the highest tested concentration recommended by the OECD TG 234 standard guideline and the concentration for which significant effects were demonstrated in the studies of Moreels *et al.* (2006) and Unpublished study report (2012).
- The fish species medaka (*Oryzias latipes*) must be chosen in order to add the specific endpoints proposed by the standard guideline OECD TG 234 for this specie (*i.e.* genetic sex, secondary sexual characteristics) that improve the power of the test.

The FSDT 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 (2018) as a conceptual framework level 4 test that covers a sensitive fish life stage responsive to both estrogens and androgen-like chemicals.

The FSDT was provided by the registrant to determine the effect of MTBE on the sexual development of zebrafish (*Danio rerio*). Hatching success, vitellogenin concentration, phenotypic sex via gonadal histology, and sex ratio were evaluated. Zebrafish were exposed to five test concentrations (1.3 – 2.4 – 5.4 – 11 and 22 mg/L) and a negative control (dilution water) under flow-through conditions.

No effects were observed for the Hatching success, larval survival, sex ratio and growth. Concerning the vitellogenin in mal fish **there is a statictically significant increase in VTG concentration for males fish at 2,4 and 11 mg/L following a parametric test. There is also a slightly statistically significant increase at the highest dose (22 mg/L) with non-parametric test**. For female fish, there is no statistically significant difference in VTG concentration using parametric and non-parametric test. **In the absence of adverse effects, endocrine disruption cannot be demonstrated for fish.**

7.10.2. Endocrine disruption - Human health

ED properties of MTBE for human health have been examined in 2014-2015 in the context of SEv.

Numerous data indicates effects of MTBE on circulating hormones, in particular testosterone, corticosterone and LH. However, a consistent mode of action that could be associated with these changes has not been identified.

A decrease in serum testosterone is observed in many studies following oral exposure to high doses of MTBE but it has not been studied by inhalation. The hypothesis that MTBE can have a direct high-dose solvent effect, which would destroy testosterone by dissolution is not considered relevant as increase in serum corticosterone are observed.

Based on this data, it has not been considered to request additional data.

ED properties of MTBE for human health may require further consideration based on new data and will be reassessed by the endocrine disruptors working group of ANSES and discussed in an upcoming RMOA.

7.10.3. Conclusion on endocrine disrupting properties (combined/separate)

For the environmental part, even if an estrogenic activity was observed in male fish (vitellogenin induction), there were no adverse effects observed *in vivo*. In the absence of adverse effects, endocrine disruption cannot be demonstrated for fish.

For the Human Health Part, based on the data evaluated during SEv, ED related effects could only be seen at very high doses (effects at doses above the limit dose, 1000 mg/kg bw/d) and without a consistent mode of action.

The concern was not sufficient to request further testing.

ED properties of MTBE for human health may require further consideration based on new data and will be re-assessed by the endocrine disruptors working group of ANSES and discussed in an upcoming RMOA.

7.11. PBT and VPVB assessment

1) Persistence

MTBE is neither readily biodegradable nor inherently biodegradable (please refer the section 7.7.1.2.1.1). Therefore, MTBE is considered as potentially persistent.

2) Bioaccumulation

Based on the LogKow of 1.06 (Fujiwara *et al*. (1984; RI=3)) the potential of bioaccumulation of MTBE is considered as low. Therefore, MTBE is not considered as B.

3) Toxicity

According to Annex XIII of REACh, a substance fulfills the toxicity criterion (T) if the NOEC or EC10 < 0.01 mg/L for marine or freshwater organisms, or the substance is classified as carcinogenic (category 1A or 1B), germ cell mutagenic (category 1A or 1B), or toxic for reproduction (category 1A or 1B). MTBE does not fulfill any of these criteria, therefore MTBE is not considered as T.

4) Overall conclusion

Based on the available data, and screening criteria, MTBE is not considered as PBT/vPvB.

7.12. Exposure assessment

MTBE has a high production volume (total tonnage band: 1 000 000 - 10 000 000 tonnes per annum) and is mainly used in fuel production to enhance the octane index (fuel oxygenate). MTBE is also used as an intermediate (to produce isobutylene) and as a process solvent or extraction agent (for laboratory chemical analyses, research and development purposes…).

7.12.1. Human health

7.12.1.1. Worker

Based on exposure scenarios, inhalation is considered the most important exposure route for workers. Indeed, exposure to MTBE is mainly due to its high vapour pressure and easy evaporation. For skin contact, Lead registrant assumed that gloves will be worn in all contributing scenarios except completely closed systems.

Some measurements of MTBE are available in the COLCHIC database (Vincent, 2001), for the fuel distribution by professionals. COLCHIC is a French database collecting exposure measurements by inhalation carried out by the chemical laboratory services from CARSAT and INRS. It is important to note that these measures are not undertaken for the purpose of enforcement but for the purpose of prevention: they are at the initiative of health and safety services, usually for situations of concern. They can also be requested by the

company works doctor. The measured concentrations thus cannot be generalized to all situations found in the workplace.

MTBE monitoring data have been extracted from the COLCHIC database for the period 2000 to 2010 during distribution operation or tanker loading (wagon truck). There are 2 sorts of measurements: atmospheric and individual:

- The atmospheric samples correspond to stationary sampling, used to characterize ambient air pollution.

- The individual samples correspond to samples in the breathing area of workers.

The measurements were carried out by sampling MTBE vapors on activated carbon by active or passive sampling. The samples were analyzed by gas chromatography coupled with a flame ionization detector (GC-FID). Long-term sampling was between 120 and 480 minutes and most of the measures were carried out in 2009.

The table below give results and statistics for long-term measurements:

Table 23: Long-term measurements of MTBE (atmospheric and individual samples)

	Atmospheric samples ($mg.m^{-3}$ / ppm)	**Individual samples ($mg.m^{-3}$ / ppm)**
Number of samples	6	37
Minimum	0.2 / 0.06	0.3 / 0.08
Arithmetic mean	0.95 / 0.26	4 / 1.11
Geometric mean	0.48 / 0.13	1.7 / 0.47
Median	0.25 / 0.07	1.5 / 0.42
Percentile 90	3.2 / 0.89	11 / 3.06
Maximum	3.4 / 0.94	34 / 9.44

Even if the number of samples is limited (especially for atmospheric samples), measurements are lower than 8-hour estimates used in Lead registrant's exposure scenarios.

7.12.1.2. **Consumer/general population**

Exposure can take place during car refuelling or indirectly to contamination of soils or groundwater. Indeed, MTBE can enter surface water and groundwater because of fuel leaks and spills mostly at the service stations. 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. High exposures are caused by occasional or accidental leaks and spills.

7.12.2. Environment

See confidential annex.

7.13. References

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