Ministry of Environment and Food of Denmark
Environmental Protection Agency

# SUBSTANCE EVALUATION CONCLUSION

## as required by REACH Article 48

## and

## EVALUATION REPORT

**for**

# 6,6'-di-tert-butyl-2,2'-methylenedi-p-cresol

**EC No 204-327-1**

**CAS No 119-47-1**

**Evaluating Member State(s):** Denmark

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# Evaluating Member State Competent Authority

**MSCA name**
Danish Environmental Protection Agency
Strandgade 29
DK- 1401 CopenhagenTel: +45 72544100
Email: mst@mst.dk
Contact person: Lea Stine Tobiassen
Tel: +45 72544173
e-mail: lesto@mst.dk

## Year of evaluation in CoRAP: 2016

Member State concluded the evaluation without any further need to ask more information from the registrants under Article 46(1) decision.

**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

6,6'-di-tert-butyl-2,2'-methylenedi-p-cresol (abbreviated DBMC), EC No 204-327-1, CAS No 119-47-1, was originally selected for substance evaluation in order to clarify concerns about:

- Toxicity to Reproduction
- Endocrine Disruption

During the evaluation no further concern was identified.

## 2. OVERVIEW OF OTHER PROCESSES / EU LEGISLATION

Registration: DBMC was registrered in 2010.

Testing proposal: A testing proposal for a 2-Generation Reproductive Toxicity Study (OECD 416) was filed by the registrant(s) in 2011. This proposal was included in the Commissions evaluation process of testing proposals in light of the amendment of REACH annexes including the substitution of the data requirement for this test with the Extended One Generation Reproductive Toxicity Study (EOGRTS) (OECD 443). The process of evaluation of the testing proposal is still ongoing at the time of preparation of this document. In the case of DBMC, the evaluating Member State (eMSCA) does not consider the performance of an EOGRTS as necessary in order to conclude on the classification as toxic for reproduction in category 1B, as the data available are evaluated by the eMSCA in the substance evaluation process to be sufficient to trigger that classification (see below). If classified Repr cat 1B, the adaptation possibilities under REACH, Annex X, 8.7., column 2 could be triggered, and the EOGRTS test may potentially become obsolete.

However, should the available data be evaluated to be insufficient for concluding a harmonised classification of DBMC as toxic for reproduction in category 1B, further testing for reproductive effects will be needed in order to fulfil the REACH data requirements and to clarify the concerns for reproductive toxicity. These studies could potentially be requested from the registrant(s) via the the pending examination of the testing proposal or via a compliance check.

Compliance Check: pursuant to the decision of a compliance check, the registrants have updated the registration in December 2016 to include two tests for terrestrial toxicity: an OECD 222 eathworm reproduction test and an OECD 208 terrestrial plant test which were requested in the Compliance Check from 18 June 2015.

CoRAP: DBMC was included in the CoRAP list 2016-18.

Substance Evaluation: Denmark initiated a substance evaluation on DBMC in March 2016.

# 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 | √ |
| Harmonised Classification and Labelling | √ |
| Identification as SVHC (authorisation) | |
| Restrictions | |
| Other EU-wide measures | |
| No need for regulatory follow-up action at EU level | |

**Background for CoRAP justification**

The justification for the initial grounds for concern leading to the CORAP nomination of DBMC was the following:

*"Human hazard*
In the reported repeated dose toxicity studies, effects on sperm in the cauda epididymis and histopathological changes in the testis, such as degeneration of step 19 spermatids and vacuolation of Sertoli cells, were observed in the 42.3 mg/kg and higher dose groups. In a reproductive/developmental toxicity screening study, the effects on reproductive parameters, such as decrease in number of corpora lutea, implantation scars and pups born, were observed in the 200 mg/kg/day and higher dose groups but not 50 mg/kg/day.
As for the developmental toxicity, low body weight gain of offspring and increased number of stillbirths were observed at 800 but not 200 mg/kg/day. No teratogenic effects were observed in a study with rats up to 375 mg/kg/day.
Due to effects on male resproductive organs, antiandrogenic and estrogenic effects are suspected. Due to the structure of the substance, effects on thyroid are suspected as well.

The potential for endocrine disrupting effects and toxic effects on reproduction and development seems relevant in the justification for the selection of 6,6'-di-tert-butyl-2,2'-methylenedi-p-cresol for CoRAP inclusion."

**Conclusions of the substance evaluation regarding reproductive toxicity**

The eMSCA has evaluated the available experimental studies in the registration dossier relevant for the end-points of reproductive toxicity. Original study reports and published articles on studies included in the registration dossier were provided by the registrant(s). A large number of repeated dose toxicity studies and a screening study for reproductive/developmental toxicity were available in the registration dossier. The eMSCA noted that the registration dossier does not include a higher tier reproductive toxicity study (a testing proposal has been submitted by the registrant(s)) or a pre-natal developmental toxicity study in a second species. The eMSCA conducted a literature search in SciFinder,

in PubMed and in ToxCast for additional available information on reproductive toxicity, not included in the registration dossier. However, no further information on reproductive toxicity was retrieved, but only *in vitro* studies investigating endocrine disrupting mechanisms and mode of action.

*Toxicity to fertility:*

The results from eight repeated dose toxicity studies (of variable quality) ranging from 28 days to 18 months exposure and one reproductive/developmental toxicity screening study in rats have consistently shown adverse effects on testes to be the critical effect of DBMC. Additionally, effects on sperm are reported in several studies. Both adverse effects on male fertility after DBMC exposure occur consistently in a dose-related manner at doses that only entail minor, non-significant effects on general toxicity (animal weight and liver weight changes). Detailed study descriptions of each of these studies is provided in part B of this document.

In conclusion, the observed effects in male rats consistently include markedly reduced testes weights, testis tubules atrophy and spermatogenic arrest at DBMC doses causing no or only small decreases in body weight (0-10%) and moderate increases in liver weight (20-30%). The exposure doses leading to these effects starts around 40 mg/kg bw/day, and seem to be independent of the duration of the individual studies and the number of animals used.

Adverse effects on female reproductive system were only observed at high doses (200-800 mg/kg) and generally accompanied by marked decreases in body weight and increased mortality. The effects on female reproductive organs are evaluated to be secondary to general toxicity.

A single 90-day study in Beagle dogs with limited power (n=2/sex) is available, showing no effects on the male or female reproductive system.

No *in vitro* data on endpoints relevant for toxicity to fertility are available in the registration dossier. However, the available *in vitro* data performed to study endocrine mechanisms revealed a strong cytotoxic effect of DBMC. It is unknown whether this effect is also present *in vivo* and may play a role in the observed adverse effects on male fertility. A possible mode of action suggested by Tagaki *et al*. (1994) in the observed testes-toxicity, is the molecular mechanism of uncoupling in mitochondria. Tagaki *et al*. (1994) showed that DBMC, and a structurally similar anti-oxidant (2,2'-methylenebis (4-ethyl-6-tert-butylphenol) (MBEBP) CAS no 88-24-2) exert an uncoupling action in isolated liver mitochondria. This could possibly inhibit the mitochondrial energy production in certain cells, resulting in a lack of ATP, which is necessary for cell division. Since testes are organs with a very high level of cell division and consequently a high energy consumption, this uncoupling in mitochondria if a dominant mode of action of DBMC *in vivo*, could possibly explain why adverse effects occur in the testes at lower doses of DBMC that any other organs. However, no experimental data are presently available to confirm this possible MoA.

*Conclusion of toxicity to fertility:*

The consistent findings of severe dose-related effects on the male rat testes and sperm across studies of different durations lead the eMSCA to conclude that DBMC is a reproductive toxicant that severely impairs male fertility in experimental animals, and that DBMC should be classified as repr.cat 1B, H360F: May impair fertility.

*Developmental toxicity:*

The two available studies with DBMC investigating developmental toxicity do not indicate any teratogenic effects of DBMC. The decreased number of live-born fetuses in the prenatal developmental toxicity study and the reduced number of pups in the high dose groups (800 mg/kg/day) in the reproductive screening study (TG 421), were most probably caused by maternal toxicity.

Overall, there are no indications of critical developmental effects of DBMC based on the available data. However, the eMSCA notes that no definitive conclusion can be drawn, as a prenatal developmental toxicity study in a second species is not available.

**Conclusion of the substance evaluation regarding endocrine disruption**:

*Estrogenic/androgenic/antiandrogenic mode of action*

Several *in vivo* studies available in the registration dossier show adverse effects of DBMC on male fertility, but most of these do not investigate whether these testicular effects could be related to endocrine disrupting modes of action. No *in vivo* studies have been performed where endocrine sensitive endpoints like anogenital distance, nipple retention, timing of sexual maturation and estrous cyclicity have been investigated in animals that have been exposed to DBMC during development.

Only one study, Takahashi *et al.* (2006), investigated steroid hormone levels in DBMC exposed male rats having testicular atrophy and spermatogenic arrest. The authors did not see any significant effects on testosterone levels (in neither rats nor mice). However, these results do not rule out that the adverse fertility effects of DBMC could be hormonally mediated, since the group size in this study was rather limited (n=8) and only one dose (40-60 mg/kg/day) was tested.

*In vitro* studies and results from the ToxCast database. The studies from the open literature were retrieved by the eMSCA examined possible estrogenic/androgenic mode of action including affecting estrogen or androgen receptor (ER/AR) activity, evaluating the agonistic as well as the antagonistic effect of DBMC and its ERα- inhibitory activity and binding affinity. The studies were either negative or the effects were seen at cytotoxic levels.

QSAR predictions for *in vitro* ER binding from the Danish (Q)SAR database was positive, whilst the OECD QSAR Application Toolbox predicted no ER binding for DBMC. There was a negative prediction for ER agonism (Danish QSAR database).

QSAR predictions for *in vitro* human pregnane X receptor (PXR) binding and activation induction were positive (Rosenberg *et al*., 2017). Activation of PXR may lead to increased expression of hepatic enzymes involved in the metabolism of endogenous hormones, including estrogen, androgen and thyroid hormones, and can hereby potentially result in lower hormone plasma levels. However, the possible implications of these positive predictions with regard to adverse effects on human health for DBMC are presently unknown.

*Thyroid disruption mode of action*

*In vivo* thyroid gland weights and histopathology have been investigated in some of the available chronic and subchronic studies, but no adverse effects have been observed (Tagaki *et al.,* 1994, Unpublished study, 1965). In the study report from the unpublished subchronic toxicity study performed in 1965, all single animal data and group mean data are available, and no trends towards increased thyroid weights or incidence of histopatholgocal findings are evident. Unfortunately, no measurements of thyroid hormone levels have been performed in any of the *in vivo* studies.

*Ex vivo data in rats* on TPO inhibition from the US EPA ToxCast program indicate that the TPO enzyme is inhibited by DBMC (personal communication US EPA, 2016).

In a DTU Leadscope QSAR model for inhibition of the enzyme thyroid peroxidase (TPO), DBMC was included as part of a blinded external validation test set and was predicted positive (Rosenberg *et al*., manuscript in preparation).

It is possible that TPO inhibition also occurs *in vivo*, and that consequently circulating T4 levels are decreased in the animals, but that these effects on hormone levels do not lead to any measurable adverse effects on thyroid gland weight or histopathology.

*Conclusion on endocrine disruption*

With respect to androgenic/estrogenic mode of action the eMSCA concludes that, based on the available data, it cannot be ruled out that the adverse effects on male fertility effects are mediated by an endocrine mode of action. However, the mixed QSAR predictions, the overall ambiguous or negative *in vitro* data from published literature and from ToxCast, and the scarce *in vivo* data on endocrine endpoints do not give strong indications of DBMC having endocrine disrupting effects through an estrogen/androgen mode of action.

With respect to a possible thyroid endocrine mode of action, there is information from *in vitro and ex vivo* data showing TPO inhibition of DBMC. No investigation of thyroid hormone levels following exposure to DBMC *in vivo* has been conducted, However, the available *in vivo* data show no adverse effects on thyroid gland weight or histopathology. Based on the available data, a residual concern for possible thyroid inhibition subsists.

However, based on the overall level of evidence the eMSCA concludes this substance evaluation without requesting further information.

*Conclusion of the Substance Evaluation*

The eMSCA concludes from its evaluation that DBMC is a reproductive toxicant causing severe adverse effects on the testes and sperm in several animal studies. The eMSCA also concludes that the substance evaluation should be followed up by regulating DBMC through harmonised classification of the substance as reproductive toxicant in category 1B.

On the other end point of concern, endocrine disruption, the eMSCA evaluated that the available data point to a low concern that effects on reproductive endpoints are mediated through interference with the sex hormone system. A slight residual concern for a possible thyroid disrupting mechanism of action subsists based on the available evidence.

However, based on the overall level of evidence the eMSCA concludes this substance evaluation without requesting further information, but prioritises pursuing the concern for the endpoint of reproductive toxicity. The eMSCA has therefore filed a intention to submit a classification proposal for DBMC for a harmonised classification in category 1B for the effect on fertility.

# 4. FOLLOW-UP AT EU LEVEL

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

The data available in the registration dossier are deemed sufficient to warrant a classification as toxic for reproduction in category 1B. Yet, the self-classification notified, including that of the registrant, do not include that classification. Thus, the eMSCA finds it necessary that a harmonised classification of the substance is agreed upon.

#### 4.1.1. Harmonised Classification and Labelling

The substance is not at present included in Annex VI with a harmonised classification. Thus, the proposal will be a new entry in CLP-Annex VI.

As referred above under point 3. conclusion on substance evaluation, several studies of DBMC by repeated exposure and a screening reproductive toxicity study all demonstrate that DBMC is highly toxic to the testes and sperm development, and the data show that the substance affects fertility of experimental animals. The effects of DBMC on fertility occur at low doses without significant general toxicity and are consistently demonstrated in several valid experimental tests. The data are deemed sufficiently severe to warrant a classification as toxic for reproduction in category 1B.

A classification as toxic for reproduction in category 1B could lead to risk management through other REACH processes (e.g. REACH Annex XIV restriction of use in consumer products, nomination to candidate list or restriction proposal) and in other down-stream regulation and voluntary reduced marketing and use of the substance. These initiatives would increase protection of workers and consumers and of the environment from exposure to the substance.

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

No other actions than harmonised classification is considered at this point in time. Depending on the outcome of the process of harmonised classification for the end-point of reproduction toxicity is concluded, the need for inclusion on the REACH Candidate List may be considered.

#### 4.1.3. Restriction

No other actions than harmonised classification is considered at this point in time.

#### 4.1.4. Other EU-wide regulatory risk management measures

No other actions than harmonised classification is considered at this point in time.

# 5. CURRENTLY NO FOLLOW-UP FORESEEN AT EU LEVEL

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

As indicated above, this section is not relevant, as **there is** a regulatory follow-up action at EU level considered relevant, namely harmonised classification on the end-point of reproductive toxicity.

## 5.2. Other actions

No other actions than harmonised classification is considered at this point in time.

# 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 3**

| FOLLOW-UP | | |
|---|---|---|
| **Follow-up action** | **Date for intention** | **Actor** |
| CLP Annex VI dossier for harmonised classification (CLP) for reproductive toxicity | December 2017 | Member State: Denmark |

# Part B. Substance evaluation

## 7. EVALUATION REPORT

### 7.1. Overview of the substance evaluation performed

6,6'-di-tert-butyl-2,2'-methylenedi-p-cresol was originally selected for substance evaluation in order to clarify concerns about:

- Reprotoxic properties

- Potential endocrine disruptor

**Table 4**

| EVALUATED ENDPOINTS | |
|---|---|
| **Endpoint evaluated** | **Outcome/conclusion** |
| Toxicity to reproduction | Concern substantiated. Harmonised C&L process to be initiated. |
| Endocrine disruption | No further action. |

### 7.2. Procedure

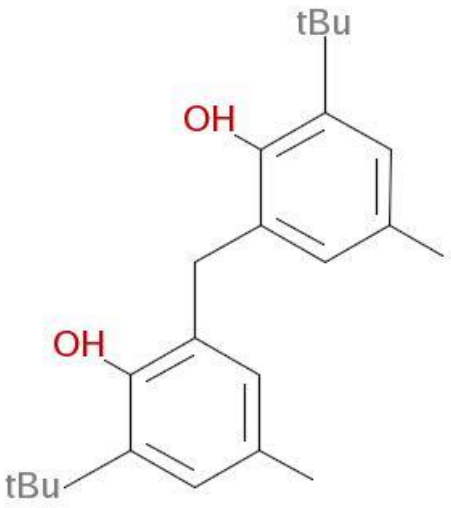
The eMSCA has evaluated the end-points of concern, reproduction toxicity and endocrine disruption based, on the registration dossier for which the applicant provided all original study reports and other literature upon request. Also, the eMSCA included articles from the open literature and conducted QSAR searches on specific end-points on endocrine disruption.

**Table 5**

| SUBSTANCE IDENTITY | |
|---|---|
| **Public name:** | 6,6'-di-tert-butyl-2,2'-methylenedi-p-cresol |
| **EC number:** | 204-327-1 |
| **CAS number:** | 119-47-1 |
| **Index number in Annex VI of the CLP Regulation:** | - |
| **Molecular formula:** | C23H32O2 |
| **Molecular weight range:** | 340 |
| **Synonyms:** | - |

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

**Structural formula:**



## 7.3. Physico-chemical properties

**Table 7**

| OVERVIEW OF PHYSICOCHEMICAL PROPERTIES | |
|---|---|
| **Property** | **Value** |
| Physical state at 20°C and 101.3 kPa | Solid: a white solid powder with a faint odour |
| Vapour pressure | 0.00000033 |
| Water solubility | 0.007 mg/L at 20°C |
| Partition coefficient n-octanol/water (Log Kow) | Log Kow (Pow): 6.26 at 20°C |
| Flammability | End-point waived: REACH Guidance on information requirements and chemical safety assessment, Chapter R. 7.1.10, Table R.7.1-27, and Chapter R.7.1.10, Table R.7.1-27 does not apply to this substance, due to DBMC does not contain functional groups with pyrophoric properties and does not ignite when it comes into contact with water. |
| Explosive properties | End-point waived: The solid form of this substance does not have explosive properties and is for this reason not classified rearding its explosive properties |
| Oxidising properties | End-point waived**:** REACH Guidance on information requirements and chemical safety assessment, Chapter R.7.1.13.1, section 7.13 of Annex VII does not apply to this substance, due to DBMS does not contain chemical groups with oxidising properties. |
| Granulometry | 40%-75% < 10 µm<br>Mean particle size 6 µm-23 µm |
| Stability in organic solvents and identity of relevant degradation products | End-point waived: REACH Annex IX, Column 2, is not conducted, due to stability of the substance is not considered to be critical. |
| Dissociation constant | *pKa1: 11.3 at 20 °C* |

## 7.4. Manufacture and uses

### 7.4.1. **Quantities**

**Table 8**

| 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 |

### 7.4.2. **Overview of uses**

**Table 9**

| USES | Use(s) |
|---|---|
| **Formulation** | DBMC can be released to the environment through the formulation into solid matrix, into mixture and/or the synthesis of the substance.<br><br>DBMC is used both in closed, continuous systems, in systems where the substance or preparation is transferred at non-dedicated facilities and in industrial spraying ect. When used, oppoortunity for exposure may arise.<br><br>The use is for both preparations and articles. |
| **Uses at industrial sites** | DBMC can be released to the environment through the uses at industrial site. The use of DBMC at industrial site relates to inclusion into/onto articles, to non-reactive processing aid (no inclusion into/onto articles) and to the use as a functional fluid.<br><br>DBMC is used in mixing or blending batch processes for formulation of preparations and articles, and as a laboratory chemical. When used, opportunity for exposure may arise.<br><br>The uses of DBMC are fuels, adhesive, hydraulic fluids, lubricants, greases, metal working fluids, sealants, polymers preparations and compounds used in rubber and plastic products. |
| **Uses by professional workers** | DBMC can be released to the environment through the widespread use indoor and outdoor as a non-reactive processing aid and a functional fluid<br><br>When used, oppoortunity for exposure may arise.<br><br>The uses of DBMC are as a laboratory chemicals and, adhesive, sealants, fuel, hydraulic fluids, lubricants, greases, metal working fluids, polymer preparations and compounds.<br><br>The end use is in the manufacture of textile, leather, fur, wood, wood products, pulp, paper, paper products, manufacture of furniture, building and construction work, rubber and plastic products, general manufacturing, e.g. machinery, equipment. |
| **Consumer Uses** | DBMC can be released to the environment through the widespread use, both indoor and outdoor, as a non-reactive processing aid and a functional fluid<br><br>It is used in fuel, adhesive, sealants, hydraulic fluids, lubricants, greases and metal working fluids. |
| **Article service life** | DBMC can be released to the environment through the formulation and widespread use of articles, both indoor and ourdoor, e.g. vehicles, plastic and rubber articles. |

## 7.5. Classification and Labelling

### 7.5.1. Harmonised Classification (Annex VI of CLP)

No classification of the substance according to the entry in table 3.1 in Annex VI of CLP Regulation (Regulation (EC) 1272/2008) was available.

### 7.5.2. Self-classification

- In the registration(s)*:* Repr. 2 (Hazard Statement code(s): H361)
- The following hazard classes are in addition notified among the aggregated self-classifications in the C&L Inventory*:*

| | |
|---|---|
| Eye Irrit. 2 | (Hazard Stement Code(s): H319) |
| Skin Sens. 1 | (Hazard Stement Code(s): H317) |
| Aquatic Acute 1 | (Hazard Stement Code(s): H400) |
| Aquatic chronic 2 | (Hazard Stement Code(s): H411) |
| Aquatic chronic 3 | (Hazard Stement Code(s): H412) |
| Aquatic chronic 4 | (Hazard Stement Code(s): H413) |

## 7.6. and 7.7. Environmental fate properties and environmental hazard assessment

As the substance is used as an antioxidant in among others rubber and tyres, exposure of the genereal environment can be expected. As the initial concern of the eMSCA was reproductive toxicity and endocrine disruption, the eMSCA therefore also screened the information avaialable in the registration dossier on environmental fate properties and aquatic toxicity of the registered substance in order to assess the concern for endocrine disruption in the environment.

The screening concluded that no additional concern for endocrine disruption in the environment is warranted under this substance evaluation process. However, several aspects of the information provided in the registration dossier and new information gained in the process of evaluating the potential for endocrine disruption for human health led to the conclusion that if new information on the mode of action and/or the toxicity profile of the registered substance with relevance for aquatic organisms emerges, this could change the conclusion that no environmental concern for endocrine disruption is warranted, as discussed below.

The registered substance was evaluated by the PBT working group (ECB, 2009) which concluded that the substance is not considered PBT as it does not meet the B or T criteria. DBMC meets the screening criteria for P/vP.

However, the data on water solubility of the substance have changed considerably over the past years , from 0.92 mg/L in 1989 (OECD 105) to 0.02 mg/L in 1992 (MITI) to 0.007mg/L in 2010 (OECD 105). As the water solubility is an important parameter in environmental fate and toxicity testing, this change in water solubility has a significant impact on the reliability and conclusions of the available studies.

A ready biodegradation test with non-adapted activated sludge (equivalent to OECD 301C) showed 0% degradation with $O_2$ consumption as parameter and 1% primary degradation measured with HPLC (Chemicals Inspection & Testing Institute Japan 1992a, referenced in OECD (2000). No simulation degradation studies are available as the registrant states that the substance is insoluble in water.

However, in a bioaccumulation study (OECD 305 E, flow through + vehicle, uptake duration 60d) using *Cyprimus carpio* stabile water concentrations using vehicle under the water solubilty of 0,002 and 0,0002 mg/L were reported, yielding BCFs of 840 and 780, respectively (OECD, 2000). Lipid correction, growth dilution correction and depuration time and measurements were not reported in the study. The PBT working group reported that few details were included but the test was assumed to be carried out using the standard methods. Hence the PBT working group concluded that the substance did not meet the B criteria. As this study is conducted below the current water solubility of the substance the eMSCA is of the opinion that the substance has bioaccumulation potential which support the relevance of conducting long-term toxicity studies in the environment. As no new data is available, eMSCA has not been able to reevaluate the assessment of the PBT working group.

No vertebrate ecotoxicity studies with a duration over 96 hours are available and no amphibian or avian toxicity studies are available. Thus, no long-term environmental toxicity studies are available. All acute toxicity studies performed on fish, daphnia and algae have been performed with solubilizer and before the water solubility was determined at 0.007 mg/L. The PBT working group concluded that the substance is probably not T. Further, all tests were performed before the water solubility were determined to be 0.007 mg/L in 2010. Based on the available data it is the opinion of the eMSCA that it is not possible to derive a PNEC or adequately assess the validity of any of the reported ecotoxicological studies.

In conclusion, the environmental fate properties, the low quality of acute toxicity studies and the lack of chronic aquatic toxicity studies on vertebrates of the registered substance do not in themselves identify or dismiss a concern for endocrine distruption in the environment. However, if new knowledge about mode of action, toxicity and/or ecotoxicity becomes available from *in vitro, in vivo* or *in silico* sources, the environmental hazards, including endocrine disrupting properties, fate properties and risks, should be re-evaluated.

## 7.8. Human Health hazard assessment

### 7.8.1. Toxicokinetics

Not assessed.

### 7.8.2. Acute toxicity and Corrosion/Irritation

Not assessed.

### 7.8.3. Sensitisation

Not assessed.

### 7.8.4. Repeated dose toxicity

The substance was included in CoRAP based i.a. on effects on the reproductive system in repeated dose toxicity studies. Several repeated dose toxicity studies in rats, mice and dogs have been performed with DBMC, primarily in rats. All the rat studies have shown that the compound adversely affects the male reproductive system at dose levels where no or only very minor signs of general toxicity are seen. The table below shows a summary of the performed repeated dose toxicity studies, where reproductive effects have been examined. The studies are presented in chronological order. More study details and discussion of the results are presented in the text below the table.

| Method | Results | Remarks | Reference |
|---|---|---|---|
| Rat, Sub-chronic toxicity study (90 days), n=25/sex, but part of the animals were sacrificed on day ?, and therefore n= 5-15/sex, for the endpoints investigated at necropsy.<br>Oral: feed<br>0, 330, 1000, 3000 ppm, corresponding to 0, 16,5, 50 and 150 mg/kg bw/day. | **NOAEL of 330 ppm (16.5 mg/kg)**<br>No adverse effects<br>**LOAEL of 1000 ppm (50 mg/kg)**<br>Increased liver weights in males and histopathological changes in the testes<br>**3000 ppm (150 mg/kg)**<br>Increased liver weights in males and histopathological changes in the testes, decreased food intake in both sexes, significant lower body weight at study termination in males, decreased kidney weight.<br>No adverse histological effects were seen in females. | 2, reliable with restrictions | 1965<br>Unpublished study report (english) available for the eMSCA. |
| Dog (Beagle) n=1 oral (feed)<br>90 days exposure<br>0, 330, 1000, 3000 ppm, corresponding to 0; 11; 33, 100 mg/kg bw/day | No adverse effects on organ weights or histopathology were observed | 3, not reliable. Due to the extremely limited group size, this study cannot be used to conclude that DBMC is not a reproductive toxicant in dogs | 1965<br>Unpublished study report (english) available for the eMSCA. |
| Rats and mice;<br>10 months exposure (0, 10 or 50 mg/kg/day)<br>n=not stated | **NOAEL of 10 mg/kg:**<br>No adverse effects were seen<br>**LOAEL of 50 mg/kg:**<br>Functional changes in the nervous system and the liver and morphological changes in the testes after 10 months but not after 4 | 3, not reliable. The number of animals per group is not stated and the study report is in Russian, meaning that documentation is not sufficient for assessment and not convincing for an expert judgement. However as effects seen here are similar to effects seen in other studies, this study is used as supporting evidence | Stasenkowa 1977.<br>Published article in Russian and study summary in German provided by the registrant both available for the eMSCA. |

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| Rat (Wistar) male/female, n=10/sex<br>Subchronic 13 week (oral: feed)<br>0,100, 330, 1000 or 3000 ppm in the diet (males: 0, 7.41, 24.91, 75.65, 281.64 mg/kg bw/d; females: 0, 9.66, 31.30, 113.16, 345.40 mg/kg bw/d) | 100 ppm (7.41 / 9.66 mg/kg): no adverse effects<br>**NOAEL of 330 ppm (24.91 / 31,3 mg/kg**): no adverse effects<br>**LOAEL of 1000 ppm (75.65 / 113.16 mg/kg)**<br>Male: Severe reduction in absolute (64%) and relative testes weights (66%), severe atrophy of the testes. Terminal BW was not significantly affected. A significant increase in relative liver weights (7%).<br>Female: decreased body weight gain during the study but no significant effect on terminal BW and no adverse effects on reproductive organs.<br>**3000 ppm** (281.6 / 345.4 mg/kg)<br>Male: almost 60% decrease in relative and absolute testes weight and severe atrophy of the testes. Terminal BW showed a non-significant 7 % decrease and a significant increase in relative liver weights (7 %).<br>Female: decrease in body weight gain during the study , absolute and relative liver weights were significantly increased (24 and 31 % respectively) and atrophy of both uterus horns was observed. | 2, reliable with restrictions. | (1982)<br><br>Unpublished study report in German, with tables and figures in English available to the eMSCA. |
| Rat (Wistar) male/female, n=10/sex, with half of the animals sacrificed after 4 weeks and the other half after 12 weeks,<br>subchronic, oral: feed<br>0, 1200, 6000, 30.000 ppm (corr. to 0, 88, 564, 3120 mg/kg bw/day for males and 0, 104, 618, 2610 mg/kg bw/d for females) (nominal in diet)<br>Exposure: 12 weeks (daily) | No NOAEL was found.<br>**LOAEL was 1200 ppm (88 / 104 mg/kg)**<br>Male: ~50% decrease in testes weight, decreased spermatogenesis, testicular tubule atrophy, appearance of giant cells, interstitial edema in the testis, sermatogenic arrest in testes and hypospermia in the epididymis. BW gain was not significantly affected and BW was appr. 4% decreased when measured at the 12 week necropsy. Relative liver weight was 24 % increased.<br>Female: BW was not significantly affected during the study. Relative liver weights were ~22% increased.<br>**6000 ppm (564 / 618 mg/kg)**<br>Male: increased mortality, decreased food intake, and severely reduced body weight (~ 44 % ). The general toxicity induced by this dose, makes the reproductive toxicity finding (decreased testis weight, decrease of spermatogenisis, testicular atrophy etc.) difficult to interpret.<br>Female: several signs of general toxicity, including decreased body weight and | 2, reliable with restrictions.<br>Since group size for the necropsies after four and twelve weeks of dosing was 5, and several of the mid and high dose animals died during the course of the study, n=1-2 for some of the groups at the 12 week necropsy | Takagi et al. (1994)<br><br>Peer reviewed publication |

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| | increased in liver weights. This makes reproductive effects (atrophy of the ovaries and uterus), difficult to interprete.<br>**30000 ppm (3120 / 2610 mg/kg)**<br>Male & female: highly increased mortality, decreased food intake and severely reduced body weight, makes all other adverse effects irrelevant for assessment of reproductive toxicity. | | |
| Rat (Wistar) male/female (n=30/sex),<br>Chronic feeding study<br>Exposure: 18 months (daily).<br>0, 100, 300, 1000 ppm (corresponding to 0, 4, 12.7, 42.3 mg/kg bw/day for males and 0, 5, 15.1, 54.2, mg/kg bw/d for females)<br><br>5 animals/dose/sex were sacrificed at 6 months and another 5 at 12 months for, histopathological, haematological and serum biochemical examinations. | 100 ppm (0.01 %) (4.23 / 5.1)<br>Male & Female: no adverse effects<br>**NOAEL of 300 ppm (0.03 %) (12.7 / 15.1 mg/kg)**<br>Male: Body weight was not significantly affected at any time point during the study. Relative liver weights showed a significant 9% increase at 18 months. Relative and absolute testes weights and testes and epididymis histopathology was not affected. Female: no adverse effects were noted.<br>**LOAEL of 1000 ppm (0.1 %) (42.3 / 54.2 mg/kg)**<br>Absolute and relative testes weights were significantly decreased throughout the study (by 58-75%). At all three time points, testis tubules atrophy, spermatogenic arrest and epididymis hypospermia was seen in all investigated animals. Survival rates were unaffected, small suppression of body weight gain from the 6$^{th}$ month but no significant body weigtht changes at 6 and 12 months. At 18 months a significant 9% decrease in body weight was observed. Increased absolute (15-20 %) and relative (22-27%) liver weight were seen. Female: suppression of body weight gain from 1$^{st}$ months, resulting in a 23-28% decreased BW at 12 and 18 months. Increased relative but not absolute liver weights were observed at 12 and 18 months (32-34%), whereas no significant changes were noted in the ovaries. | 2, reliable with restrictions.<br>Well conducted and valid but not GLP. A key study showing clear adverse testicular effects at a dose level that only induced slight systemic toxicity | Takagi, et al. (1994)<br><br>Peer reviewed publication |
| Rat (Crj: CD(SD)) male/female, n=6/sex<br>subacute 28-day toxicity study<br>oral: capsule (daily)<br>0, 50, 200, 800 mg/kg bw/day | No NOAEL was found.<br><br>**LOAEL was 50 mg/kg**<br>Male: Histological examination of testes showed an effect on degeneration of step 19 spermatids, but no other adverse effects on testes. Terminal body weight was not affected. A significant increase in | 2, reliable with restrictions | Ministry of Health and Welfare Japan (1996a)<br><br>Published report in Japanese, |

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| A 14-day recovery group was also included (control and800 mg/kg animals) | relative liver weights (13%), and decrease in relative lung weight (8%) was seen, whereas other organ weights were not affected.<br>Female: No adverse effects.<br>**200 mg/kg**<br>Male: Significant effects on testes histopathology was seen in all examined males (sperm retention, vacuolation of Sertoli cells, degeneration of step 19 spermatids). Terminal body weight was not affected. Absolute and relative liver weights were moderately but significantly increased.<br>Female: significant increase absolute and relative liver weights, mild changes in liver histology and increased rel. adrenal weights (16%).<br>**800 mg/kg**<br>Male: Adverse effects on testes histopathology were seen in all males (sperm retention, vacuolation of Sertoli cells, degeneration of step 19 spermatids). Terminal body weight was not affected. But abs. and rel. liver weights were increased.<br>Female: increase in absolute and relative liver weights (30%), mild changes in liver histology<br>**800 mg/kg recovery**<br>Male: Histopathology of testes showed significant effects in all investigated parameters | | with abstract and result tables in English available to the eMSCA. |
| Male rats (F344/DuCrj (Fischer)), n=8, &<br>Male mice (Crj: CD(ICR)), n=8.<br>Exposure: 2 months (daily) in feed<br>rat: 0.06% (ca. 38.6-58.0 mg/kg bw/day)<br>mouse: 0.25 % in diet (ca. 371-447 mg/kg bw/day).<br>At study termination, repr. organ weights were excised and weighted, examined histologically and testosterone levels in | **LOAEL of 600 ppm, 40-60 mg/kg bw/day (Rat) (only one dose tested)**<br>Significant decrease in relative testicular (9%) and epididdymal (18 %) weights, histopathological changes, including vacuolisation of Sertoli cells disappearance of basement membrane and degeneration of spermatids . Moreover, the daily sperm production (DSP) was significantly decreased (~30 %). Terminal body weight was not significantly affected. Liver, kidney and spleen weights were not determined. Serum testosterone levels were not significant changed.<br>**2500 ppm, 371-447 mg/kg bw/day (mean of 414 mg/kg bw/day) (Mouse)**<br>Histopathological changes in testes, including giant cell formation, sloughing of seminiferous tubules and Leydig cell | 2 reliable with restrictions | Takahashi et al. (2006) peer reviewed publication |

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| serum were determined. | vacuolization. No significant changes in body weight gain, terminal body weight, absolute or relative weights of testes, sex accessory organ weights, liver and kidney. Serum testosterone levels were not significant changed.<br><br>No estrogenic activity in *vitro* in ERa-binding assay, up to $10^{-3}$ M, in in *vitro* ER alpha competitor screening assay. | | |

The chronic 18-months feeding study by Takagi *et al.* (1994), is by the eMSCA viewed as the key repeated dose toxicity study for evaluating the adverse effects of DBMC on male fertility. This study used an initial group size of 30/sex, and sacrificed 5 of these animals/dose/sex after both 6 and 12 months of dosing, and the remaing 20 after 18 months. The LOAEL dose of 1000 ppm (corresponding to 42.3 mg/kg in males), which was also the highest tested dose in this study, caused very severe effects on the testes, i.e. a 58%, 69% and 75% decrease in absolute testes weight, when measured after 6, 12 and 18 months exposure, respectively. In this dose group relative testes weights were also significantly decreased throughout the study (58-73%). Pathological findings at all three time points included testis tubules atrophy, spermatogenic arrest and epididymis hypospermia in all investigated animals. At this exposure dose survival rates were unaffected, and no significant body weight changes were seen after 6 and 12 months, but at 18 months a significant 9% decrease in body weight was observed. Increased absolute (15-20%) and relative (22-27%) liver weight were seen at all three time points at this dose level of 42.3 mg/kg bw/day. In this study, the NOAEL was the mid-dose level of 300 ppm (corresponding to 12.7 mg/kg in males). At this dose body weights and absolute liver weight were not affected, and neither were testes weights nor testes and epididymis histopathology. The adverse effects on male fertility at DBMC doses which cause no, or only minor signs of general toxicity in the Takagi *et al.* (1994) study, are corroborated by similar findings in a number of supporting studies.

In a 12 week subchronic oral toxicity study in rats (Takagi *et al*., 1994), a dose of 1200 ppm (corresponding to 88 mg/kg bw/day) adversely affected male fertility in a way that after 12 weeks of exposure, a ~50% decrease in testes weigh was seen. Furthermore, testicular tubule atrophy (5/5 animals), appearance of giant cells (3/5 animals), interstitial edema in the testis (4/5 animals), spermatogenic arrest in testes (5/5 animals) and hypospermia in the epididymis (5/5animals) was observed. Male BW gain was not significantly affected, and neither were absolute liver weights, but relative liver weight was significantly increased by 24%.

A similar picture was observed in an unpublished 13 week subchronic rat toxicity study from 1982. Here a dose of 1000 ppm (corresponding to 75.65 mg/kg bw/day) caused a marked reduction in absolute (64%) and relative (66%) testes weights, along with severe atrophy of the testes. At this dose terminal BW was not significantly affected, and only a small, though significant, increase in relative liver weights (7%) was seen.

In an even older sub-chronic 90-day toxicity study in rats (unpublished report, 1965), similarly a dose of 1000 ppm (corresponding to 50 mg/kg) caused histopathological changes in the testes without any adverse effects on body weight.

The only subchronic toxicity study not showing this adverse effect on male fertility was a 90-day study in Beagle dogs (n=2), also performed in 1965, receiving doses of 0, 330, 1000, 3000 ppm, corresponding to 0, 11, 33, 100 mg/kg bw/day. Whether the lack of any adverse effects in this study was because of the very limited power of the study due to the

low number of animals used, or whether male dogs are less sensitive than rats regarding the effects of DBMC, is presently unknown.

Also subacute repeated dose toxicity studies, where animals are exposed for a shorter period of time, show adverse effects on male fertility. In a 28-day toxicity study (n=6) (Ministry of Health and Welfare Japan 1996a) a dose of 50 mg/kg caused degeneration of step 19 spermatids (3/6 mild) and vacuolation of Sertoli cells in the testes. At this dose terminal body weight was not affected, whereas a significant 13% increase in relative liver weights was seen. At the next tested dose of 200 mg/kg all tested males showed sperm retention and degeneration of step 19 spermatids. Here terminal body weights were still not affected, but absolute and relative liver weights were significantly increased (by 25 and 19%, respectively), and mild changes in liver histology were seen.

Takahashi *et al*. (2006) investigated how a single dose of DBMC (600 ppm in rats and 2500 ppm in mice) would affect male fertility. In rats two months exposure to this dose, which corresponded to 40-60 mg/kg bw/day, resulted in significantly decreased relative testicular (9%) and epididdymal (18 %) weights, and histopathological changes in testes, including vacuolisation of Sertoli cells, disappearance of basement membrane and degeneration of spermatids. Moreover, the daily sperm production (DSP) was significantly decreased (~30 %). Terminal body weight was not significantly affected, and serum testosterone levels were not significant changed. In this study liver, kidney and spleen weights were not determined.

In male mice receiving a dose corresponding to 371-447 mg/kg bw/day, for two months, histopathological changes in testes, including giant cell formation, sloughing of seminiferous tubules and Leydig cell vacuolization was also seen. No significant changes in body weight gain, terminal body weight, absolute or relative weights of testes, sex accessory organ weights, liver and kidney were seen. Serum testosterone levels were not significant changed.

The results from the repeated dose toxicity studies presented above consistently show dose-related adverse effects on male fertility of rats and mice after DBMC exposure. These effects are seen after 1, 2, 3, 6, 12 and 18 months exposure in rats and in one mouse study, after 2 months of exposure, starting at doses of approximately 40 mg/kg bw/day and above. The effects were seen in all the available studies, including those using relatively few animals. Other adverse effects caused by DBMC at doses leading to testicular toxicity included moderate suppression of body weight gain and increased absolute and relative liver weights. At higher exposure doses increased mortality, severely reduced body weight and atrophy of the ovaries and uterus were seen.

The overall discussion and conclusions regarding the reproductive effects of DBMC are included in section 7.8.7, and also comprise the available studies on reproductive toxicity (a reproductive/developmental screening study, TG 421, and a prenatal developmental toxicity study, TG 414 ).

### 7.8.5. **Mutagenicity**

Not assessed.

### 7.8.6. **Carcinogenicity**

Not assessed.

### 7.8.7. **Toxicity to reproduction (effects on fertility and developmental toxicity)**

Concern for toxic effects to reproduction, i.e fertility and development, was one of the concerns for the inclusion of DBMC in CORAP. No reproductive toxicity study as required under point 8.7.3 of Annex IX and X of REACH is available for DBMC. A reproduction/developmental toxicity screening test (OECD TG 421) and a prenatal

developmental toxicity study (OECD TG 414) have been performed. The results of the two studies are summaries in the table below, and are hereafter discussed in further detail. Furthermore, several repeated dose toxicity studies (reviewed above in section 7.8.4) have reported reproductive toxicity effects of DBMC.

| Method | Results | Remarks | Reference |
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| rat (Wistar), n=24-20-20-22(20). oral: gavage<br>93,5; 187 or 375 mg/kg bw<br>Exp: 7th to 17th day of pregnancy (daily) with sacrifice on GD 20. Developmental toxicity study/teratogenicity | **93.5 mg/kg**<br>no adverse effects<br>**187 mg/kg**<br>Slight suppression of BW gain and food consumption. No effects on mean number of corpora lutea, implants and live foetuses, no effects on offspring body weights and no malformations.<br>**375 mg/kg**<br>Increased maternal mortality, diarrhoea, hair fluffing, decreased food consumption and suppression of BW gain No effects on mean number of corpora lutea, no. of implants and no. of live foetuses, but a non-significant decreased in number of live born fetuses. Fetal body weights were unaffected, and no effects on skeletal variations or malformations was seen. | 2, reliable with restrictions. Teratogenicity study, well conducted but not according to OECD test guideline, and not GLP | Tanaka et al (1990)<br><br>Published study report in Japanese, summary and result tables in English. Available to the eMSCA. |
| rat (Crj: CD(SD)) male/female n=12/sex<br>screening oral: gavage 0, 12.5, 50, 200, 800 mg/kg bw and day (nominal)<br>Exposure: male: 50-52 d, female: 40-48 d (from 14 days before mating to the day 3 of lactation) (daily)<br><br>OECD Guideline 421 (Reproduction / Developmental Toxicity Screening Test), | **12.5 mg/kg bw/day:** No adverse effects in males and females<br>**50 mg/kg bw/day:**<br>Male: Giant cell formation in the testes and significant adverse effects on semen quality, but no effect on body weight. Females: no adverse effects and no effects in offspring.<br>**200 mg/kg bw/day**:<br>Male: Reduction in absolute and relative testes and epididymides weights. Atrophy of testes and epididymis and adverse effects on sperm. No effect on body weight gain or food consumption and no significant effects on body weight.<br>Female: Lower food consumption during pre-mating, pregnancy and lactation. At necropsy on PND 4, body weight was significantly decreased. Offspring: A slight decrease in number of live pups born and live pups on day 4 of lactation (~12 %)<br>**800 mg/kg bw/day:** | 1, reliable without restrictions,<br>key study, performed according to OECD guideline 421, GLP | Ministry of Health and Welfare Japan (1999b)<br><br>Published report in Japanese with abstract and result tables in English Available to the eMSCA. |

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| | Male: Testes and epididymides weights severely decreased, atrophy of testes and epididymis, atrophy of seminal vesicles and of seminiferous tubules and no motile sperm. But no effect on terminal body weight.<br>Female: suppression of body weight gain during pre-mating, pregnancy, and lactation. At necropsy body weight was decreased and a small but significant decrease in number of pups born was seen. Offspring birth weights were unaffected but a ~10 % decreased in BW was seen in offspring of both sexes on PD 4. | | |

In the prenatal developmental toxicty study (Tanaka *et al.,* 1990) a dose of 93.5 mg/kg from GD7-17 caused no adverse effects in dams or offspring. Dams receiving a dose of 187 mg/kg bw/day showed slight signs of general toxicity (diarrhea, hair fluffing, suppression of BW gain (approximately 6% decrease in BW on GD20) and suppressed food consumption). No adverse effects on mean number of corpora lutea, implants and live foetuses, and no effects on offspring body weights and malformations were seen. At the highest tested dose of 375 mg/kg, two dams died during the study. Other dams were affected by diarrhoea, hair fluffing, decreased food consumption and suppression of BW gain (appr. 16% decrease in BW on GD20). This dose also did not affect the mean number of corpora lutea, no. of implants and no. of live foetuses and fetal body weights. However, a non-significant increase in dead implants (69 compared to 26 in controls) and in dam with only dead implants (5/20 compared to 1/24 in controls) was seen, and therefore offspring from only 15 litters could be examined for malformations. No foetuses showed skeletal malformations and no significant differences were seen in number of foetuses with variations. This study indicated that DBMC is not a teratogenic compound, as the decreased number of live-born foetuses in the high dose group was probably caused by maternal toxicity.

In the reproduction/developmental toxicity screening study (Ministry of Health and Welfare Japan 1999b), adult male rats and pregnant female rats were exposed to different doses of DBMC for approximately 50 days. The dose of 12.5 mg/kg bw/day caused no adverse effects in either males or females.

The LOAEL of the study was 50 mg/kg bw/day, as male fertility was adversely affected by this dose. At necropsy no effects on testes and epididymis weights were seen, but giant cell formation in the testes (2/12) was present. Furthermore, statistically significant adverse effects on semen quality were seen, including a 16% decrease in sperm motility ratio, a 10% decrease in sperm viability ratio, a 20% decrease in sperm survivability ratio, and a ~30% decrease in number of sperm in the epididymis cauda. Also a 5 times increase in the abnormal sperm ratio was observed. No effect on body weight or food consumption was seen in males from this dose group and in the females and offspring no adverse effects, including no decrease in body or ovary weight were noted.

At 200 mg/kg bw/day absolute and relative testes weights were reduced by ~ 16% and absolute and relative epididymides weights were reduced by ~ 12%. Atrophy of testes and epididymis, including atrophy of seminiferous tubules (6/12), and giant cell formation in the testes (2/12) was seen. The adverse effects on sperm were very severe and included an increase in abnormal sperm ratio (from 1.55% in control to 56.3% in this dose group), and a ~80% decrease in the sperm motility ratio, a 26% decrease in sperm viability ratio, ~50% decrease in sperm survivability ratio, and ~66% decrease in number of sperm in

the epididymis cauda. No effect on male body weight or food consumption was seen during the dosing period and at necropsy no significant effects on body weight were seen.

Females in this dose group showed a reduced body weight gain (non-significant) compared to controls. This effect became significant in the lactation period (day1-4). Additionally, lower food consumption was seen periodically during pre-mating, pregnancy and lactation. At necropsy on PND 4, body weight was significantly decrease (by 7%), whereas absolute and relative ovary weights were not significantly affected, and neither were the number of corpora lutea and implantation scars. A slight decrease in number of live pups born and live pups on day 4 of lactation (~12%) was seen, and both male and female offspring had higher birth weights than control pups.

The dose of 800 mg/kg bw/day caused absolute and relative testes weights to be decreased by ~ 50% and a ~25% decrease was seen in absolute and relative epididymis weights. Histological examination showed atrophy of testes and epididymis, atrophy of seminal vesicles and of seminiferous tubules (12/12). No motile sperm was evident in any of the 12 examined males, and the number of abnormal sperm was very high. Moreover, the total number of sperm in cauda epididymis was ~80% decreased, compared to control males. No effect on body weight was seen during the study, but a transient decrease in food consumption (one day in the beginning of the dosing period) was observed. At necropsy no effect on body weight was noted. In the females, suppression of body weight gain was seen during the pregnancy and lactation periods, and lower food consumption was seen periodically during pre-mating, pregnancy, and lactation. At necropsy body weight was 9% decreased, ovary weights were not affected. A 14% decrease in the number of corpora lutea and 8% decrease in number of implantation scars was seen. Offspring: A small but significant decrease in number of pups born. Furthermore 1 dam was unable to deliver pups, and 1 dam lost all pups during lactation. Offspring birth weights were unaffected but a ~10% decreased in BW was seen in offspring of both sexes on PD 4.

The available *in vitro* data from the open literature and the ToxCast database on ER and AR agonist/antagonism (described under point 7.9 below) revealed a strong cytotoxic effect of DBMC. Whether this effect is also present *in vivo*, and whether it plays a role in the observed adverse effects on male fertility, is presently unknown. A possible mode of action suggested by Tagaki et al. (1994) in the observed testes-toxicity, is the molecular mechanism of uncoupling in mitochondria. Tagaki et al. (1994) showed that DBMC, and a structurally similar anti-oxidant (2,2'-methylenebis (4-ethyl-6-tert- butylphenol) (MBEBP, CAS no 88-24-2 exert an uncoupling action in isolated liver mitochondria. This could possibly inhibit the mitochondrial energy production in certain cells, resulting in a lack of ATP, which is necessary for cell division (Tagaki et al 1994). Since testes are organs with a very high level of cell division and consequently a high energy consumption, this uncoupling in mitochondria if a dominant mode of action of DBMC *in vivo*, could possibly explain why adverse effects occur in the testes at lower doses of DBMC that any other organs. However, no experimental data are presently available to confirm this possible MoA.

**Conclusions regarding reproductive toxicity**

The results from the repeated dose toxicity studies (presented in section 7.8.4 above) and the reproductive/developmental toxicity screening study have consistently shown dose-related adverse effects on male fertility after DBMC exposure, to be the critical effect of DBMC, in studies ranging from 28 day to 18 months exposure. The observed effects consistently include markedly reduced testes weights, testis tubules atrophy and spermatogenic arrest, at DBMC doses causing no or only small decreases in body weight (9%), and only moderate increases in liver weight (20-30%). In female rats, DBMC doses causing body weight decreases and liver weight increases are similar to those causing such effects in males, but at these exposure levels, no adverse effects were seen on the female reproductive system. In cases where adverse effects on female reproductive organs were present (200-800 mg/kg), these were generally accompanied by severe decreases in body weight and increased mortality.

Based on the results from the repeated dose toxicity studies, the reproduction/developmental toxicity screening study and the prenatal developmental toxicity study available in the registration dossier DBMC is concluded to be a reproductive toxicant for male fertility. DBMC consistently show dose-related adverse effects on male fertility after 1, 2, 3, 6, 12 and 18 months exposure, with effects starting at doses of 40 mg/kg bw/day, independent of duration of study at doses that do not cause significant general toxicity. The consistent findings of severe dose-related effects on the male testes across studies of different durations lead the eMSCA to conclude that DBMC should be classified as repr.cat.1B, H360F.

A prenatal developmental toxicity study in a second species is not available. However, the results of the available studies investigating developmental toxicity of DBMC do not indicate any teratogenic effects of DBMC. The decreased number of live-born fetuses in the prenatal developmental toxicity study in the first species and the reduced number of pups in the high dose groups (800 mg/kg/day) in the development/reproductive toxicity screening study were most probably caused by maternal toxicity. Overall, based on the available data, the eMSCA does not consider it plausible that developmental effects is a critical effect of DBMC.

#### 7.8.8. Hazard assessment of physico-chemical properties

Not assessed.

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

Not assessed.

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

The eMSCA concludes that DBMC, based on the consistent and serious effects reported in repeated dose toxicity studies and one screening reproductive/development toxicity study on male reproduction (sperm and testes) at low doses, fulfils the criteria for classification under CLP regulation as repr. cat. 1B; H360F.

### 7.9. Assessment of endocrine disrupting (ED) properties

DBMC was included on CoRAP due to concerns of a possible endocrine disrupting potential.

***Non-testing methods***

DBMC is predicted in the Danish (Q)SAR database to be positive (battery prediction on the basis of three models on the same training set in Leadscope, CASE Ultra and SciQSAR) for *in vitro* ER binding and negative for ER agonism. In the OECD QSAR Toolbox DBMC is predicted not to have ER binding due to impairment of the OH-group by substituents to both sides.

DBMC is predicted in the Danish (Q)SAR database to be positive (battery prediction) in a model for *in vitro* AR antagonism and the substance is included in the training set as a positive. The origin of the positive experimental result in the training set is Satoh *et al*. (2008). As discussed in more detail below (section 7.10.2.2) this result is, however, not reliable, as this publication showed no AR antagonistic effect at the lowest tested concentration of 1µM, and at the next concentration tested (10µM), where it showed an effect, cytotoxicity was oberserved.

DBMC is furthermore predicted in the Danish (Q)SAR database to be positive (battery prediction) for human *in vitro* Pregnane X Receptor (PXR) binding. DBMC is also predicted positive in Leadscope models for human *in vitro* PXR binding and activation, as well as CYP3A4 induction in new DTU models based on >1400 data points from US NIH and DTU-blindly external validations with >700 substances showing specificities above 85% (manuscript submitted). The possible implications of these positive predictions with regard to adverse effects on human health for DBMC are presently unknown. However, as PXR is a key regulator of many metabolic enzymes and transporters, activation of PXR may lead to perturbations of many different pathways Dybdahl *et al*. (2012). Activation of PXR may among other things lead to increased expression of hepatic enzymes involved in the metabolism of endogenous hormones and can hereby potentially result in lower hormone plasma levels (Murk *et al*., 2013). For example, a PXR-mediated and metabolism-based mechanism to reduce androgen activity was reported in Zhang *et al*. (2001). As another example on the AOP-Wiki site a suggested AOP draft entitled „Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals" identifies activation of PXR to be a molecular initiating event with strong evidence of being linked to the adverse outcome, but with weak quantitative understanding (AOPwiki, 2017).

In a new DTU Leadscope QSAR model for inhibition of the enzyme thyroperoxidase (TPO), which is essential in thyroid hormone synthesis, DBMC was predicted positive. According to a DTU-blinded external validation performed in collaboration with the US EPA a specificity of 91% (266x100%/(266 + 27)) was observed (manuscript under preparation). The substance was identified to be part of the external validation set and subsequently the detailed experimental test results were provided by the US EPA, see below under "ToxCast data".

***In vitro***

Some *in vitro* studies examining the mode of action of DBMC have been identified by the eMSCA in the open literature. Also the ToxCast database (US EPA) has been reviewed regarding *in vitro* studies investigating possible endocrine disrupting mode of action of DBMC. The two tables below summarize the *in vitro* data identified in the open literature.

**Estrogenic mode of action**

| Method | Results | Remarks | Reference |
|---|---|---|---|
| The estrogenic activity of DBMC was evaluated using the yeast two-hybrid assay with and without S9-mix (rat). | No estrogenic activity was found for DBMC neither as parent compound or its metabolite(s), tested after incubation with S9-mix | The *in vitro* test applied is not an OECD guideline test. However, the assay is a well-known *in vitro* test and there does not seem to be anything wrong with the way the tests were conducted**.** | *Ogawa et al. (2006)* |
| Estrogenic activity was tested using a recombinant yeast estrogen assay | No estrogenic activity was found for DBMC | Not a guideline test, but the test method used is well-known, so it should be reliable. | *Miller et al. (2001)* |
| ERα competitor screening assay | DBMC had no inhibitory activity against E2-ERα binding. | Not a guideline test, but seems reliable. | *Takahashi and Oishi (2006)* |

| | | | |
|---|---|---|---|
| In a reporter gene assay and a competitive binding assay for ER, DBMC was examined for effects via ER-mediated pathways. | No ER agonistic activity was found<br>No ER antagonistic effect was observed<br>DBMC had no ERα binding affinity | The applied ER reporter gene assay and ER binding assay is not the available OECD guideline tests, but they are very close to the guideline test. Thus they should be reliable | *Satoh et al. (2008)* |

## Androgenic/anti-androgenic mode of action

| Method | Results | Remarks | Reference |
|---|---|---|---|
| Using a (cell based) reporter gene assay as well as a (non-cell based) competitive binding assay for AR, DBMC was examined for effects via AR - mediated pathways. | No effect was found on AR agonist activity.<br>DBMC showed AR antagonistic effects, but only at concentrations where cytotoxicity was also observed (from 10µM -100µM).<br>In the non- cell based binding assay DBMC showed AR binding affinity starting at 10µM with an $IC_{50}$ of $1.8x10^{-5}$M (18µM) Overall, the results from Satoh et al. 2008 showed a strong cytotoxicity of DBMC, and any hormonal-like effects could not be clarified, although DBMC did show binding affinity for AR. | The applied AR reporter gene assay (the AR-Eco-Screen) is currently a draft OECD guideline test.<br>The AR binding assay is not a guideline test, but a well-described test | *Satoh et al. (2008)* |

ToxCast data

According to the ToxCast database DBMC is reported to be active on several endocrine disrupting endpoints/mechanisms of action including AR antagonism, AR agonism, ERα antagonism, ERα agonism, PPARγ activation/agonism and Aromatase inhibition.

However, looking at the concentration-response curves for each of the assays effects on all the listed endpoints are only seen at concentrations above (and often far above) the cytotoxic limit listed. Thus, the results from the ToxCast database do not suggest that DBMC affects any of the classical (ER, AR etc.) endocrine disrupting endpoints, but the data supports the conclusion from Satoh *et al*. (2008), that the major effect of DBMC is a strong cytotoxic effect.

In a collaboration with the US EPA to externally validate a QSAR model on TPO inhibition DBMC was identified to be part of the ToxCast validation set (E1K EDSP set) and detailed results from the experimental *in vitro* assay on TPO inhibition were received from the US EPA. The results for TPO inhibition are positive with the following conclusion from the US EPA: "$AC_{50}$ is below cytotoxicity activity. $AC_{50}$ is below generic interference activity. Efficacy is well above noise threshold. Data supports TPO inhibition using our selectivity screening process". The TPO inhibition $AC_{50}$ is 0.79 µM, the selectivity score is 1.18 (min. 0.95 and max. 1.39) and the AUC is 149.1. (See Paul *et al*., 2014 and Friedman *et al*., 2016 for information on the experimental tests and the selectivity screening process).

***In vivo***

*In vivo* studies examining endocrine disrupting effects of DBMC are scarce. Several *in vivo* studies showing adverse effects of DBMC on male fertility exist, but most of these do not investigate, or even discuss, whether these testicular effects could be related to endocrine disrupting modes of action. No *in vivo* studies have been performed where endocrine sensitive endpoints like anogenital distance, nipple retention, timing of sexual maturation and estrous cyclicity have been investigated in animals that have been exposed to DBMC during development. In one study, Takahashi *et al*. (2006) investigated steroid hormone levels in DBMC exposed male rats having testicular atrophy and spermatogenic arrest. The authors did not see any significant effects on testosterone levels (in either rats or mice), indicating that the adverse effects on testes did not seem to be mediated by lowered circulating testosterone levels. However, these results do not rule out that the adverse fertility effects of DBMC could be hormonally mediated, since the group size in this study was rather limited (n=8) and only one dose was tested. No other *in vivo* studies have measured steroid hormone levels after DBMC exposure.

*In vivo* some thyroid endpoints have been investigated, but no adverse effects have been observed. In two subchronic 90 day studies in rats (Tagaki *et al.,* 1994; unpublished report, 1965) and in a chronic study in rats (Tagaki *et al.,* 1994) both thyroid gland weights and thyroid histopathology have been examined, and no significant adverse effects have been reported. In the Takagi *et al.* (1994) chronic feeding study in rats, doses up to 42.3 mg/kg bw/day for 18 months did not adversely affect the thyroid histopathology or weight. Higher doses of ~100-150 mg/kg bw/day given to rats for 90 days, caused slight signs of systemic toxicity in the animals, but did not adversely affect thyroid histopathology or weight (Unpublished report 1965, Takagi *et al.,* 1994). In the Takagi *et al.* (1994) study the two highest doses of ~600 and ~3000 mg/kg bw/day lead to markedly decreased body weights and severely increased mortality, but no adverse effects on the thyroid glands were reported. A subchronic study in dogs also examined thyroid weight and histopathololgy at doses up to 100 mg/kg bw/day (Unpublished report, 1965). This study did not show any adverse effects on the thyroid gland weight of histopathology, but it included so few animals that these results are not deemed reliable and they are therefore not further included in the performed weight of evidence analysis.

Results from the thyroid weight and histopathology assessments were not shown in the publication by Takagi *et al.* (1994), as the authors only showed endpoints which were significantly affected. It is therefore not possible to assess whether there is a non-significant trend towards increased thyroid weights or more histopathological findings with increasing doses of DBMC. In the study report from the unpublished subchronic study performed in 1965, all single animal data and group mean data are available, and here no such trends are evident.

None of the performed *in vivo* studies measured thyroid hormone levels in the animals.

**Discussion of possible estrogenic and antiandrogenic mode of action**

Based on QSAR predictions, the published *in vitro* data, ToxCast data and *in vivo* results, it cannot be ruled out that the adverse effects on male fertility effects are mediated by an endocrine mode of action, but there are no experimental data to support this concern, as there are no strong indications of DBMC having endocrine disrupting effects on any of the tested mechanisms of action, i.e. DBMC did not show ER or AR agonist and antagonist activity *in vitro* at non-cytotoxic concentrations.

The available *in vitro* data (from the open literature and the ToxCast database) revealed that the most pronounced effect of DBMC is a strong cytotoxic effect. Whether this effect is also present *in vivo*, and whether it plays a role in the observed adverse effects on male fertility is presently unknown. A mode of action, suggested by Tagaki *et al.* (1994) to play a role in the observed testes-toxicity, is uncoupling action in the mitochondria. In the test the authors showed that DBMC, and a structurally similar anti-oxidant, exert an uncoupling action in isolated liver mitochondria. This could possibly inhibit the mitochondrial energy

production in certain cells, resulting in a lack of ATP, which is necessary for cell division. Since testes are organs with a very high level of cell division and consequently a high energy consumption, and if this is a dominant mode of action of DBMC *in vivo*, this could possibly help explain why adverse effects occur in the testes at lower doses of DBMC that any other organs. This possible MoA is however, at present, only speculative.

**Discussion of thyroid disruption**

In a DTU Leadscope QSAR model for inhibition of the enzyme thyroid peroxidase (TPO), DBMC was predicted positive. In addition *in vitro* data on TPO inhibition from US EPA (described in section 7.10.2.2) indicate that the TPO enzyme is inhibited by DBMC *in vitro*.

*In vivo* thyroid gland histopatholgoly and weight have been investigated in chronic and subchronic studies, but no adverse effects have been observed (Tagaki *et al.,* 1994, unpublished report, 1965). In the study report from the subchronic study performed in 1965, all single animal data and group mean data are available, and no trends towards increased thyroid weight or incidence of histopatholgocal findings are evident.

Unfortunately, no measurements of thyroid hormone levels have been performed in any of the *in vivo* studies. It is possible that TPO inhibition also occurs *in vivo*, and that consequently circulating T4 levels are decreased in the animals, but that these effects on hormone levels do not seem to lead to any measurable adverse effects on thyroid gland weight or histopathology.

### 7.9.1. Conclusion on endocrine disrupting properties

Based on mixed QSAR predictions, the overall ambiguous or negative *in vitro* data from published literature and from ToxCast, where DBMC did not show ER or AR agonist and antagonist activity at non-cytotoxic concentrations and the scarce *in vivo* results or data on endocrine endpoints, it cannot be ruled out that the adverse effects on male fertility effects are mediated by an endocrine mode of action. However, the available data do no give strong indications of DBMC having endocrine disrupting effects.

Given the serious and clear effects on the male reproductive system which are described above, the eMSCA has concluded that the end-point of toxicity to reproduction, especially to fertility, should be pursued by further regulatory action in priority of further clarification of the concern on a possible effect on steroid hormones at this point in time.

*In vivo* thyroid gland weights and histopathology have been investigated in some of the available chronic and subchronic studies, but no adverse effects have been observed (Tagaki *et al*., 1994; unpublished study, 1965). In the study report from the subchronic toxicity study performed in 1965, all single animal data and group mean data are available, and no trends towards increased thyroid weights or incidence of histopatholgocal findings are evident.

Unfortunately, no measurements of thyroid hormone levels have been performed in any of the *in vivo* studies. It is possible that TPO inhibition also occurs *in vivo*, and that consequently circulating T4 levels are decreased in the animals, but that these effects on hormone levels do not lead to any measurable adverse effects on thyroid gland weight or histopathology.

In conclusion, even though *in vitro* data show TPO inhibition and a concern for thyroid inhibition remains, this mechanism is not supported by the findings in the available *in vivo* studies, as no adverse effects on thyroid gland weight or histopathology have been reported. Further investigations on possible thyroid disrupting effects of DBMC could address this concern. However, based on the overall level of evidence the eMSCA prioritises pursuing the concern for the endpoint of reproductive toxicity and conclude this substance evaluation without requesting further information.

## 7.10. PBT and VPVB assessment

PBT properties were not assessed during this substance evaluation. However, the eMSCA have included some observations of relevance for the PBT/vPvB assessment in section 7.6.

## 7.11. Exposure assessment

Not assessed.

## 7.12. Risk characterisation

Not assessed.

## 7.13. References

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