Bundesanstalt für Arbeitsschutz
und Arbeitsmedizin
Federal Institute for Occupational
Safety and Health

SUBSTANCE EVALUATION CONCLUSION

as required by REACH Article 48

and

EVALUATION REPORT

for

(3E)-1,7,7-trimethyl-3-[(4-methylphenyl) methylene] bicyclo [2.2.1] heptan-2-one

List no. 701-394-3
(Formerly identified with EC no. 253-242-6)

CAS RN 1782069-81-1

Evaluating Member State(s): Germany

Dated: 27 February 2023

Evaluating Member State Competent Authority

BAuA

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Year of evaluation in CoRAP: 2020

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

(3E)-1,7,7-trimethyl-3-[(4-methylphenyl) methylene] bicyclo [2.2.1] heptan-2-one (referred to hereafter as "the Substance" or "4-MBC") was originally selected for substance evaluation to clarify concerns about potential endocrine disrupting (ED) properties for the environment. During the evaluation, no additional concerns were identified.

2. OVERVIEW OF OTHER PROCESSES / EU LEGISLATION

REACH

The evaluating Member State Competent Authority (eMSCA) of Germany conducted a Risk Management Option Analysis (RMOA) for 4-MBC in 2014, concluding in June 2015 that further risk management measures were necessary.[2] An Annex XV dossier was prepared by the eMSCA and submitted to ECHA in 2016 to identify 4-MBC as a substance of very high concern (SVHC) according to REACH article 57(f) due to its ED properties for the environment.[3] Following discussion at the 48th meeting of the Member State Committee (MSC), the dossier was withdrawn as the available information was considered to be insufficient to identify the Substance as an SVHC.[4]

The substance identifiers for 4-MBC were adapted by ECHA in 2021 (the previous EC number and CAS RN were 253-242-6 and 36861-47-9, respectively).

Based on an Annex XV dossier prepared by the Danish MSCA, 4-MBC was identified as an SVHC according to REACH article 57(f) due to its ED properties for human health by the MSC in November 2021.[5] Consequently, the Substance was added to the candidate list by ECHA on 17 January 2022.

Cosmetics Regulation

Entry 18 of Annex VI of the Cosmetics Regulation (EC) No 1223/2009 (UV filters allowed in cosmetic products) lists 4-MBC with a maximum concentration in ready for use preparation up to 4%. The Scientific Committee on Consumer Safety (SCCS)[6] re-evaluated the available data on 4-MBC and concluded that 4-MBC may act as an endocrine disruptor, affecting both the thyroid and oestrogen modalities. The SCCS also concluded that the provided information was insufficient to evaluate the potential genotoxicity of the Substance. The exposure assessment performed by the SCCS established a higher (i.e., fourfold) systemic exposure level than in previous assessments[7], indicating that the use of 4-MBC at a maximum concentration of 4% as a cosmetic ingredient would not be safe. The mandate of the re-evaluation by the SCCS did not extend to environmental aspects and, therefore, did not cover the safety of 4-MBC for the environment.

[2] RMOA conclusion for 4-MBC: https://echa.europa.eu/de/rmoa/-/dislist/details/0b0236e1809b80f7
[3] SVHC proposal for 4-MBC by the eMSCA: https://echa.europa.eu/de/registry-of-svhc-intentions/-/dislist/details/0b0236e180e47ae7
[4] Minutes of the MSC-48 meeting: https://echa.europa.eu/documents/10162/22837890/msc-48_meeting_minutes_en.pdf
[5] SVHC proposal for 4-MBC by the Danish MSCA: https://echa.europa.eu/de/registry-of-svhc-intentions/-/dislist/details/0b0236e186167ee3
[6] https://ec.europa.eu/health/document/download/bc7fc1c9-9a5e-4f7c-a67f-a03b4dea312b_en

[7] https://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_141.pdf

3. CONCLUSION OF SUBSTANCE EVALUATION

The evaluation of the available information on the Substance has led the eMSCA 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)	(x)*
Restrictions	
Other EU-wide measures	
No need for regulatory follow-up action at EU level	x

* In case the manufacture/import of the substance restarts, a new SEv needs to be considered to clarify the concern for environmental ED properties.

4. FOLLOW-UP AT EU LEVEL

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

4.1.1. **Harmonised Classification and Labelling**

N/A.

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

N/A.

4.1.3. **Restriction**

N/A.

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

N/A.

5. CURRENTLY NO FOLLOW-UP FORESEEN AT EU LEVEL

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

Table 2

REASON FOR REMOVED CONCERN The concern could be removed because	Tick box
Clarification of hazard properties/exposure	
Actions by the registrants to ensure safety, as reflected in the registration dossiers (cease of manufacture and/or import)	x

As detailed in Section 2, 4-MBC has already been identified as an SVHC due to its ED properties for human health. The substance evaluation of the MSCA aimed to clarify whether the Substance could also act as an ED in the environment. Further information would be necessary to clarify this concern. The eMSCA is of the opinion that, if manufacture in or import of the Substance to the EU were taken up again, this information on the environmental ED potential would need to be requested in another evaluation process from the respective registrants to clarify this concern, i.e., the SEv process may restart by including the Substance again in the CoRAP. Gathering the relevant information from the required vertebrates would potentially lead to the need to identify 4-MBC also as an ED for the environment.

On 6 April 2021, ECHA notified the registrant of a draft decision SEV-D-2114549796-30-01/D under Article 50(1) of REACH. On 10 November 2021, the sole registrant of 4-MBC ceased manufacture of the Substance in accordance with Article 50(3) of the REACH Regulation, before a decision with further information requirements was finalised to clarify the concern for environmental ED properties. Therefore, there is currently no formal addressee at EU level for further information requirements on the Substance. Consequently, the eMSCA expects that 4-MBC is no longer manufactured in the EU and might only be placed on the market in small quantities via import (i.e., in cosmetic products).

5.2. Other actions

N/A.

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

N/A.

Part B. Substance evaluation

7. EVALUATION REPORT

7.1. Overview of the substance evaluation performed

The Substance was originally selected for substance evaluation to clarify concerns about potential endocrine disrupting (ED) properties for the environment. During the evaluation, no additional concerns were identified.

Table 3

EVALUATED ENDPOINTS	
Endpoint evaluated	**Outcome/conclusion**
Endocrine disruption (for the environment)	**Concern unresolved** Further information is necessary to conclude on the ED properties for the environment, but it cannot be requested due to cease of manufacture and/or import.

7.2. Procedure

The formal substance evaluation of 4-MBC started in March 2020 with the publication of the 9th Community Rolling Action Plan (CoRAP). The evaluation focused on the clarification of the concern for endocrine disruption in the environment. Mammalian data and human health-related endpoints were checked by the eMSCA to support the overall assessment.

No additional concerns were identified throughout the evaluation. Besides data from the registration dossier, also information from the academic domain was used during the assessment.

Following conclusion of the initial evaluation period in March 2021, the eMSCA considered further information necessary to clarify the initial concern. Consequently, a draft decision was prepared by the eMSCA and sent to ECHA. The draft decision contained the request of a Fish Sexual Development Test following information request:

> *Fish Sexual Development Test (FSDT–according to OECD TG 234) using the Japanese medaka (Oryzias latipes) or zebrafish (Danio rerio), and with the Substance, with at least four test concentrations and clear identification and quantification of the isomer(s) tested. In addition, the following must be included:*
>
> > *The exposure must take place via testing water, and the use of a solvent must be avoided.*
> >
> > *If Oryzias latipes is chosen, the FSDT must include genetic sex determination as well as reporting of any change of the secondary sex characteristics.*
> >
> > *The histopathology of the gonads.*
> >
> > *The histopathology of liver and kidney.*

Further detail on the specifications of the test were included in the draft decision. The decision was sent to the registrant by ECHA who provided comments. The eMSCA took the comments into account and, following amendment of the draft decision, initiated the consultation of MSCAs for decision making. MSCAs and ECHA submitted proposals for

amendment to which the registrant again provided comments. Before the decision was finalised by the MSC, the sole registrant ceased manufacture of the Substance. As no addressee for the information requirement remained, no final decision was sent by ECHA.

As of July 2022, 4-MBC does not have any (remaining) active REACH registrants.

7.3. Identity of the substance

Table 4

SUBSTANCE IDENTITY	
Public name:	(3E)-1,7,7-trimethyl-3-[(4-methylphenyl) methylene] bicyclo [2.2.1] heptan-2-one
EC number:	-
CAS number:	1782069-81-1
Index number in Annex VI of the CLP Regulation:	-
Molecular formula:	$C_{18}H_{22}O$
Molecular weight range:	254.37 g/mol
Synonyms:	4-MBC. (3E)-1,7,7-Trimethyl-3-[(4-methylphenyl) methylene]-2-norbornanone. 3-(4-Methylbenzyliden) camphor (3E) (±)-1,7,7-trimethyl-3-[(4-methylphenyl) methylene] bicyclo [2.2.1] heptan-2-one

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

Structural formula:

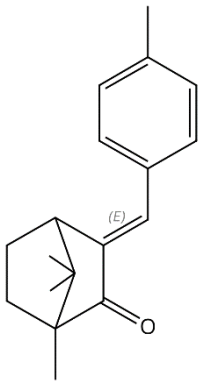

Table 5

Constituent			
Constituents	**Typical concentration**	**Concentration range**	**Remarks**
(1*R*,3*E*,4*S*)-1,7,7-trimethyl-3-[(4-methylphenyl) methylene] bicyclo [2.2.1] heptan-2-one	n.a.	n.a.	See annotation below the table.
(1*S*,3E,4*R*)-1,7,7-trimethyl-3-[(4-methylphenyl) methylene] bicyclo [2.2.1] heptan-2-one	n.a.	n.a.	

Annotation: The Substance is a multi-constituent substance due to its stereochemistry. It has an R/S isomerism whereas the double bond is present in the E configuration.

7.4. Physicochemical properties

Table 6

OVERVIEW OF PHYSICOCHEMICAL PROPERTIES	
Property	**Value**
Physical state at 20 °C and 101.3 kPa	*Pale white to white crystalline solid.*
Vapour pressure	*0.001 Pa at 25 °C, 0.029 Pa at 50 °C* *(Method: OECD 104 vapour pressure balance)*
Water solubility	*1.08 mg/L* *(Method: OECD 105, column elution method)*
Partition coefficient n-octanol/water (Log K_{ow})	*Log P_{OW} 5.1* *(Method: OECD 117 HPLC method)*
Granulometry	*Coarse fraction > 2000 µm amounts to 24%.* *Fine fraction:* *10% of the volume distribution is below / D10 = 6.5 µm.* *50% of the volume distribution is below / D50 = 43.8 µm.* *90% of the volume distribution is below / D 90 = 142.3 µm* *(Method: Laser diffraction, ISO 13320 (2009))*
Dissociation constant	*no dissociating properties*

7.5. Manufacture and uses

7.5.1. **Quantities**

As of May 2022, there is no longer an active REACH registration for 4-MBC. The sole registrant ceased manufacture in November 2022. The Substance was previously registered at an aggregated tonnage in the 10-100 tpa range.

7.5.2. **Overview of uses**

The Substance is used as an UV filter in cosmetics and personal care products. The following table lists the uses covered in the (no longer active) registration.

Table 7

USES	Use(s)
Formulation	Formulation of compounds/end-products: • Formulation of low viscosity liquids and solid cosmetics (small to large scale) • Formulation of high-viscosity body care products (small to large scale) • Formulation of Fine Fragrances – cleaning with water
Consumer Uses	Consumer (and professional) end-use of cosmetics • PC 39: Cosmetics, personal care products

7.6. Classification and Labelling

7.6.1. **Harmonised Classification (Annex VI of CLP)**

There is no harmonised classification for 4-MBC according to Annex VI of CLP regulation (Regulation (EC) 1272/2008).

It must be noted that approximately 660 notifiers self-classify the substance for aquatic toxicity, with old chemical identifiers in the C&L inventory (EC no. 253-242-6, CAS RN 36861-47-9). For the new chemical identifiers established by ECHA following adaptation, no notification in the inventory is available.

7.6.2. **Self-classification**

- In the registration (no longer active):

STOT RE 2	H373 (Thyroid) (oral and inhalation)
Aquatic Acute 1	H400 (M = 1)
Aquatic Chronic 1	H410 (M = 1)

- The following hazard classes are in addition notified among the aggregated self-classifications in the C&L Inventory with previous chemical identifiers*:*

Aquatic Acute 1	H400
Aquatic Chronic 1	H410
Repr. 2	H361
Skin Irrit. 2	H315
Eye Irrit. 2	H319

- The Substance with its new identifiers is not notified in the C&L inventory.

7.7. Environmental fate properties

The selected studies described in the following sections are reliable according to Klimisch categories 1 or 2. The same is considered for the models used to predict the environmental fate behaviour since the models are well described and documented in the literature.

7.7.1. **Degradation**

Abiotic degradation

Half-life in air due to degradation with hydroxyl radicals has been estimated with AOPwin v1.92 (US EPA, 2012) assuming a 12 hour-day and a hydroxyl concentration of 1.5×10^6 hydroxyl radicals/cm³.

The atmospheric half-life of 4-MBC was estimated to be 1.443 hours, the overall hydroxyl rate constant was estimated to be 8.897×10^{-11} cm³/molecules/sec.

It is expected that photodegradation in air is not a relevant pathway for removal from the environment since it is assumed that that the majority of 4-MBC will be emitted directly from the use in sunscreens and indirectly via sewage treatment systems as well as surface runoff into the aquatic compartment. Moreover, due to the very low vapour the Substance will not evaporate at ambient temperature. Therefore, photolytic degradation in air or aerosol binding is unlikely.

Photodegradation of 4-MBC is only expected to be a relevant degradation process in very shallow clear waters and in the first few centimetres' layer of the water column, decreasing rapidly in the lower layers of the water column. It is expected that environmental exposure of the Substance occurs in the whole water column.

Biodegradation

4-MBC is not readily biodegradable in the environment (Petersen et al. 2007).

Estimation of the biodegradation potential was carried out with BioWIN v4.10 (US EPA, 2012):

- Biowin2 (non-linear biodegradation probability) results in a value of 0.0193 indicating that the Substance does not rapidly biodegrade.
- Biowin6 (MITI non-linear biodegradation probability) results in a value of 0.1226 indicating that the Substance is not readily degradable.
- Biowin3 (Survey model – ultimate biodegradation) results in a value of 2.1155 indicating that ultimate biodegradation is expected after months.

7.7.2. **Environmental distribution**

The following values for the soil adsorption coefficient of 4-MBC have been estimated by using KOCWIN v2.00 (US EPA, 2012) resulting in K_{oc} values of 12290 L/kg (Log K_{oc}: 4.089) (MCI method) and 24840 L/kg (Log K_{oc}: 4.395) (Kow method), respectively.

It can be expected that the Substance will adsorb to a certain extent to sediment, soil, and organic matter.

Volatilisation

According to HENRYWIN v3.20 (US EPA, 2012) the Henry constant was determined to be 0.218 Pa×m³/mol indicating only little tendency for volatilisation.

Distribution modelling

According to Mackay Level III Fugacity Model (EpiSuite v.4.11) 4-MBC will be distributed as follows: 0.0317% to air, 11.9% to water, 77.3% to soil, and 10.8% to sediment. The results of this modelling indicate that most of the Substance will adsorb to sewage sludge, suspended organic matter or sediment, when considering that direct emission to soil is expected to be negligible regarding the uses of the Substance.

Field data

In screening sewage effluents from two Antarctic research stations (Scott Base and McMurdo Station) for personal care products (PCPs), Emnet et al. (2015) found bisphenol A (BPA), ethinyl-oestradiol (E2), estrone (E1), methyl triclosan, octylphenol (OP), triclosan (tric), and three UV filters: 2-hydroxy-4-methoxybenzophenone (BP-3) and 2,4-dihydroxybenzophenone (BP-1) and 4-MBC. The sewage treatment plants of the two stations produce approximately 17,000 L and 416,000 L of effluent per day over the summer season (October to February). Scott Base began to use ozone disinfection in the season the sample collection started (2009/10). Also, between August 2012 and February 2013 samples were taken and analysed using GC-MS. 2009/2010 the Scott Base wastewater treatment plant (WWTP) effluent contained 4-MBC as well as BP-3, BP-1, BPA, E1, OP, and tric. The McMundo station WWTP effluent only contained BP-3, BP-1, and BPA. All substances were detected in the seawater sampled nearby the research stations, at concentrations comparable to those reported for international coastal waters. Also, during the 2012/13 season 4-MBC, BP-1, BP-3, BPA, OP, tric, and methyl triclosan were detected in sewage effluents. The maximum concentrations of 4-MBC (2130 ng/L), methyl triclosan and OP in Scott Base WWTP effluent were close to the maximum concentrations observed internationally in WWTP effluents (2300 ng/L). Concentrations of the detected analytes did not correlate with the number of staff on base present at the time of sampling, or with the operating temperature of the WWTP (Pearson's Product–Momentum correlation test). Concentrations of 4-MBC demonstrated potential trends, increasing steadily throughout the research season from 321 ng/L in August to 2130 ng/L in January. In a seven-day study with daily measurement, maximum concentrations of 11,700 ng/L of 4-MBC were reported. In the seawater, the most detected analytes were 4-MBC, BP-3, BPA, methyl paraben, and OMC whose concentrations remained relatively constant throughout the area (Erebus Bay, Antarctica). Also, the sea ice was examined, and 4-MBC was also detected there. Biota was not examined for 4-MBC.

Tsui et al. (2014) reported the detection of 4-MBC (amongst other UV filters) in surface water samples from different cities from August 2012 to October 2013. In one city (Hong Kong) 4-MBC was found in a range of 173 to 379 ng/L (60 samples; detection frequency: 12). In the other cities (Tokyo, New York, Los Angeles, Shantou, Chaozhou, Bangkok, Arctic) the concentration of 4-MBC was below the limit of detection. 4-MBC is not permitted for use as a cosmetic ingredient in Japan and the United States, and thus was not detected in seawater there but in Hong Kong.

7.7.3. **Bioaccumulation**

The n-octanol/water partition coefficient of 4-MBC was determined according to OECD Guideline 117 (HPLC method). The log Pow was determined to be 5.1 at 23 °C. A maximum BCF value of 801 L/kg was reported based on the peak concentration of 4-MBC in mussels (*Mytilus galloprovincialis*) during the uptake period (information from registration dossier).

7.8. Environmental hazard assessment

7.8.1. Aquatic compartment (including sediment)

This chapter provides a short summary of test results for acute and chronic toxicity of 4-MBC in the aquatic compartment.

Aquatic invertebrates

Sieratowicz et al., 2011 reports a 48h-EC_{50} of 0.80 mg/L (nominal) for an OECD TG 202 acute toxicity test with *Daphnia magna*. The concentrations used were 0.4, 0.8, 1.6, 3.2, 6.4 mg/L. In the same study, the exposure of *Desmodesmus subspicatus* with 4-MBC resulted in a growth inhibition with a 72h-E_rC_{50} of 7.66 mg/L and a 72h-E_rC_{10} of 0.81 mg/L (both nominal) (Sieratowicz et al., 2011). The test was conducted according to OECD test guideline 201 with concentrations of 0.5, 1.0, 2.0, 4.0, 8.0 mg/L.

Schmitt et al., 2008 conducted additionally two sediment Yeast Oestrogen Screen (YES) assays with the freshwater invertebrates *Lumbriculus variegatus* and *Potamopyrgus antipodarum*. The 56-d sediment test with *P. antipodarum* started with the exposure of adult snails in a static system. Glass beakers measuring 10 cm in diameter were the test vessels, containing artificial sediment and reconstituted water. Test treatments contained 0.06, 0.26, 1.71, 7.65, and 32.9 mg/kg sediment dry weight (dw) 4-MBC as well as control and solvent control. All treatments were run in duplicate with 80 snails with a shell height of 4.0 ± 0.5 mm. After 14 days of exposure, 4-MBC increased the production of unshelled embryos significantly with a NOEC of 0.26 mg/kg sediment dw and an EC_{50} of 4.60 µM 4-MBC (= 1.17 mg/kg sediment dw), while increased mortality was only significant at the highest concentration. The study shows that 4-MBC affects the reproduction in the mud snail, which is an apical effect.

Fent et al., 2010 reported acute and chronic effects of 4-MBC on *Daphnia magna*. The chronic toxicity of 4-MBC was determined in a 21-d reproduction study performed according to OECD test guideline 211. At the highest concentration of 4-MBC (50 µg/L), reproduction and body length were reduced. No adverse effect on the sex of the offspring was observed. The study shows effects of 4-MBC on the reproduction of daphnia at the highest concentration tested. However, it cannot be ruled out that this is a toxic effect not related to endocrine disruption since the growth was also reduced and no effects were seen on the sex ratio of the offspring.

Ozáez et al. (2013) investigated the effects of 4-MBC on the midge *Chironomus riparius*. Different UV filters were tested in an OECD TG 218 spiked sediment test (OECD, 2004b). Fourth instar larvae (n = 90 at survival experiments, n = 30 at expression analysis) were exposed to a control, a solvent control (ethanol 0.02%), 0.1, 1, and 10 mg/L at 20 ± 1 °C under standard light-dark periods (16:8 in triplicate). After *in vivo* exposure where no significant effects on larval survival were found, expression levels of the genes coding for the ecdysone receptor (EcR), the ultraspiracle (USP, ortholog of the RXR) and the oestrogen-related receptor (ERR) were quantified by real-time PCR. The *EcR* gene was significantly upregulated by 4-MBC (and by octyl-p-methoxycinnamate (OMC) also called 2-ethylhexyl-4-methoxycinnamate (EHMC) and octyldimethyl-p-aminobenzoate (OD-PABA)), with a dose-related response following 24 h exposure. 4-MBC had the strongest effect, reaching over four-fold increases in mRNA *EcR* levels (at the lowest concentration). The transcription profiles of the *usp* and *ERR* genes were not significantly affected by any of the UV filters tested in the study.

Ozáez et al. (2016b) continued the work described in Ozáez et al. (2013) with *Chironomus riparius*. The test organisms were maintained under standard laboratory conditions according to OECD TG 218 (OECD, 2004b) and EPA 600/R-99/064 (EPA, 2000). Egg masses

younger than 12 h after oviposition were placed in 6-well culture plates. One half of the egg masses was used as UV-filter-free control, the other was used for treatment with different UV filters with a concentration of 1 mg/L. The egg masses were exposed for 24 h for expression studies and for developmental studies until hatching (3 days). Fourth instar larvae (n=90 for survival experiments and n = 30 for expression analysis) were maintained in glass vessels with culture medium. For the expression anaylsis, the larvae were exposed for 24 h with 1 mg/L UV filter. The survival experiments lasted 96 h and were conducted with the nominal concentrations of 0.1, 1, 10, and 100 mg/L. Ethanol (0.1% embryos and 0.02% larvae) was used as solvent. The test concentrations were analytically confirmed via HPLC-DAD. Similar to Ozáez et al. (2013), a real-time PCR was used to evaluate the expression profile of the *EcR* and *hsp70* genes. The expression levels of the genes docking for the ecdysone receptor (EcR) but also the heat shock protein HSP70 were investigated for different UV filters (4-MBC as well as OMC, EHMC, 4HB, OC, OD-PABA). A life-stage dependent sensitivity was found for some substances. The survival experiment resulted in a LC_{50} of 517.1 (24 h), 120.5 (48 h), and 10.9 (96 h) mg 4-MBC/L in fourth-instar larvae of *C. riparius*. Only 4-MBC and OMC/EHMC increased EcR and hsp70 mRNA levels in embryos and larvae. *EcR* and *hsp70* mRNA were found to be two to six times higher than in controls after 24 h exposure at 1 mg/L.

Ozáez et al. (2016a) studied binary mixtures of UV filters by evaluating the larval mortality of *Chironomus riparius*. The test organisms were reared according to conditions described in OECD TG 233 (OECD, 2010). The same concentrations as in Ozáez et al. (2013) and (2016b) were used with 30 and 10 larvae for control and treatment. They used no sediment, cellulose tissue or food during the 24-h exposure period to prevent losses by adsorption during treatments. Fourth-instar larvae were exposed to different UV filters for up to 96 h in criystal vessels under semi-static conditions. Small amounts of either OMC or BP-3 counteracted the upregulatory effect of 4-MBC on *EcR* gene expression, as evidenced by mRNA levels similar to those of the control. Increasing concentrations of OMC or BP-3 with a small concentration of 4-MBC produce a reduction in mRNA levels at relatively lower doses than do single UV filter exposures seen in Ozáez et al. (2013). Ozáez et al. suggested from the data that 4-MBC may have antagonist effects on *EcR* gene transcription and a synergistic effect on hsp70 gene activation.

The ecdysone receptor (EcR) is a nuclear receptor that binds ecdysone, a steroid hormone of insects, and is considered the structural equivalent of the oestrogen receptor. It acts as a transcription factor that activates a cascade of hormonal effector genes. The effect of UV filter exposure on a key transcription factor for the ecdysone-genomic response in arthropods suggests the possibility of a broad and long-term effect on this endocrine pathway. Altered transcription rates or timing in larvae or embryos due to the presence of UV filters in the aquatic medium can also affect the timing of egg hatching or metamorphosis in insects and might be sufficient to alter developmental transitions. The upregulation of *hsp70* and other heat shock proteins in *C. riparius* and *SOD*, *GST*, *OGG1* and *P53* in *T. japonicus* indicates that 4-MBC also induces cellular stress response even at the level of the DNA.

Chen et al. (2018) performed an acute immobilisation toxicity test, as well as a multigenerational toxicity test with the marine copepod *Tigriopus japonicus*. The test organisms were kept under laboratory conditions at a temperature of 21 ± 1 °C and a 14:10 h light: dark cycle. For the 72-h immobilisation toxicity test, adult copepods were exposed to 10, 50, 150, 1000, and 5000 µg/L (0.039, 0.20, 0.59, 3.9, 20 µM) 4-MBC in seawater (nominal concentrations, measured concentrations were comparable). The mortality rate of *T. japonicus* exposed to 4-MBC developed in a dose-dependent way and in the two highest treatment groups mortality was 100%. The following effect values for 4-MBC were determined: LC_{50} = 92.9 µg/L, LOEC = 10 µg/L and NOEC < 10 µg/L. For the multigenerational toxicity test freshly hatched individuals of *T. japonicus* were exposed to 0, 0.5, 1, 5 and 10 µg/L (0, 2.0, 3.9, 20, 39 nM: all below LOEC) for 4 generations (F0-F3).

The test revealed a LC_{50} of the F0 generation of 8.5 µg/L (LOEC = 5 µg/L, NOEC = 1 µg/L). With the mortality occurring in the 5 first days of the test, the authors concluded that the nauplii stage of *T. japonicus* is more sensitive to toxic effects of 4-MBC than the copepodite and adult stages. Consequently, in the F1-F3 generation survival rates were > 90% for all treatment groups and no dose-dependent differences were observed. Additionally, the authors of the study described a significantly decreased developmental time of F2 and F3 individuals, a significantly decreased number of eggs in the F0 generation for the highest treatment group (but not in subsequent generations) and a reduction of the hatching rate of the F1 and F2 generation by approx. 70% after exposure to 5-10 µg/L 4-MBC. Chen and colleagues concluded that *T. japonicus* became more sensitive to 4-MBC after multigenerational exposure regarding reproduction and development but less sensitive regarding acute toxicity, maybe due to selective processes caused by exposure of the F0 generation. They surmised that 4-MBC might disrupt the endocrine system of *T. japonicus* and noted, that a decreased developmental time may not be of benefit for the organism, the population, or the ecosystem they live in. To investigate the mechanism of toxicity of 4-MBC in *T. japonicus*, Chen and colleagues additionally exposed adult individuals to 0, 1 and 10 µg/L 4-MBC for 7 and 14 days and investigated the expression of several genes with qPCR. They found the transcription of oxidative stress biomarker genes (SOD, GST) and DNA repair genes (OGG1, P53) to be increased in a dose-dependent way. The EcR gene was described as up-regulated as well, VTG was, however, not affected.

Martin-Fogar et al. (2017) investigated the response of different genes in *C. riparius*, coding for different groups of heat shock proteins, after exposure to 4-MBC. They exposed fourth instar larvae to 0.1 and 1 mg/L 4-MBC for 8 and 24 h and measured the gene expression levels through qPCR. Exposure was performed in crystal vessels (50 mL test solution) with three replicates per treatment and in the absence of light to prevent photodegradation of the test compound. A solvent control with 0.02% ethanol was prepared. For the investigation of the gene expression patterns, three larvae from each replicate were pooled together (n = 9). The study focussed primarily on the differences in the response patterns of the different heat shock protein types. In total, 12 genes coding for different heat shock proteins were assessed. They responded differently to exposure of *C. riparius* to 4-MBC. The study found all but four to be increased in their mRNA levels. Three genes were not expressed differently compared to the control and one was shown to be inhibited. Martin-Fogar and colleagues assumed that the differences in the responses could be related to endocrine disrupting effects of 4-MBC, as heat shock proteins are also involved in the development of the organisms.

Santonocito et al. (2020) exposed the marine clam *Ruditapes philippinarum* to 4-MBC in a semi static assay for 7 days, with a subsequent 3-day depuration period. Adult individuals of the clam were kept in groups of 50 in 50 L tanks and exposed to 4-MBC at 1, 10 and 100 µg/L (nominal concentrations), with three replicates per treatment. Chemical analysis revealed that the actual concentrations were about one order of magnitude lower. A water and solvent control (0.01% ethanol) were prepared. The authors of the study declared they also found low concentrations of 4-MBC in the water and solvent control in the range of 0.01 – 0.05 µg 4-MCB/l. The water in the tanks was changed daily. Assays were checked daily for mortality and dead individuals were removed. Mortality after 7 days of exposure was reported as 38.75% (nominal 10 µg/L 4-MBC) and 66.25 (nominal 100 µg/L 4-MBC) and increased to 92.50% and 100% respectively, during depuration. A LC_{50} value of 7.71 µg/L 4-MBC ± 95% confidence interval (6.29–9.73 µg/L 4-MBC) was derived. Additionally, Santonocito and colleagues performed a gene expression analysis with cDNA obtained from digestive glands of clams after 7 days of exposure and after the depuration period. Three clams per treatment group were taken for investigation of the expression of 14 different target genes. The authors described the gene encoding GST to be significantly up regulated in clams exposed to 1.34 and 10.79 µg/L 4-MBC (actual concentrations) after 7 days of exposure and a decrease in the expression after depuration. The genes coding for BCL-2, GADD45 and CAT showed a similar expression pattern, although for *CAT* differences

between treatment groups and control were not statistically significant. For *SOD* and *GPx* no significant changes in the expression were observed. The expression levels of the genes coding for EIF and GADPH were found significantly increased for the treatment groups 1.34 µg/L 4-MBC (*EIF*) and 10.79 µg/L 4-MBC (*EIF* and *GADPH*). Expression of *18s* was described as low and stable by Santonocito and colleagues, except for an increase in the 10.79 µg/L 4-MBC treatment. The genes encoding for THIO9, TPO53, and MT did not show any changes in their expression levels throughout the experiment. Concluding, the authors stated they observed significant mortality of *R. philippinarum* caused by 4-MBC at environmentally relevant concentrations (and referred to monitoring studies supporting this claim) and that they suspect 4-MBC to cause cellular stress in the clams at lower concentrations.

Other aquatic organisms

(Kunz et al., 2004) investigated whether 4-MBC may interfere with the thyroid and sex hormone system of the tadpoles of *Xenopus laevis* frogs during metamorphosis. At concentrations of 1, 5 and 50 µg/L (nominal), 4-MBC had no effect on the rate of metamorphosis and no obvious differences were observed in body length and tail length compared to controls. 4-MBC affected neither the sex ration of *X. laevis* tadpoles. The results indicate that 4-MBC does not negatively affect the thyroid system and sex ratio of amphibians at the tested concentrations. The tested concentrations were, however, lower than the LOEC determined for vitellogenin induction in male fish in Inui et al. (2003).

Quintaneiro et al. (2019) conducted a fish embryo toxicity assay (FET) with *Danio rerio* in accordance with OECD TG 236 (OECD, 2013). Exposure started at 3 hpf (128 cell stage) and lasted 96 hours. The fish embryos were exposed to 0.08, 0.1, 0.15, 0.26, 0.44 and 0.77 mg/L (measured concentrations at beginning of exposure, after 48 h concentrations were up to 51% lower) in 24-well plates with 2 ml test solution and one egg per well. A negative control (water from breeding system), solvent control (0,01% ethanol) and a positive control (4.0 mg/L 3,4-dichloroaniline) were prepared. Each plate contained an internal negative control. 20 replicates were prepared for each treatment, except for negative control (n = 56). Exposure solutions were renewed every 48 h. Tests were performed in an enviroment with stable temperature (27 ± 2 °C) with a light:dark period of 14:10 h. Temperature, pH, conductivity and dissolved oxygen of the test solutions were measured at the beginning and end of each test and conditions were stable throughout the whole exposure duration (mean values: dissolved oxygen of 7.8 ± 0.44 mg/L, pH of 7.6 ± 0.15, conductivity of 794.8 ± 112.54 mS/cm and temperature of 25.6 ± 0.77 °C) The assays were checked daily for number of dead, hatched embryos and malformations. Apical endpoints in the FET were assessed according to Kimmel et al. (1995) The EC_{50} for malformations in zebrafish embryos after exposure to 4-MBC was 0.28 ± 0.008 mg/L 4-MBC. The authors of the study mostly observed notochord curvature, delayed absorption of yolk sac and pericardial oedema. The overall survival rate at 0.77 mg/L 4-MBC was 65%. Furthermore, Quintaneiro and colleagues reported a significantly decreased heartbeat rate for embryos exposed to 0.77 mg/L. They suggested the decreased heartbeat rate might be related to the ability of 4-MBC to influence the Ca^{2+} influx (also described for human sperm cells by Schiffer et al. (Schiffer 2014)) as the cardiac rhythm partly depends on the function of Ca^{2+} ion channels. The study of Quintaneiro et al. (2019) also investigated effects of 4-MBC on Acetylcholinesterase (AChE) activity (using the method of Ellman et al. (1961) adapted by Guilhermino et al. (1996)), catalase (CAT) activity (method described by Clairborne (1985)) and glutathione-S-transferase (GST) activity (method by Habig and Jakoby (1981)). Therefore, embryos of *D. rerio* were exposed in similar conditions as described above (same test concentrations, exposure duration and environment) but without a positive control and in glass petri dishes with 8 ml test solution and 6 eggs. Five replicates per treatment were prepared. After exposure surviving embryos were pooled, frozen in liquid nitrogen and stored at −80 °C until the enzyme activity assays were performed. A significantly increased activity of AChE and GST in zebrafish embryos exposed

to 0.15, 0.26, 0.44 mg/L 4-MBC could be measured. No change in activity was observed for CAT in all treatments. Quintaneiro and colleagues concluded that the increased GST activity is a marker for an activated detoxification system of the exposed organisms. Furthermore, they suggested that the AChE induction might have affected the regular neurological function of the zebrafish embryos as they also observed loss of larvae equilibrium.

Sediment organisms

Schmitt et al. (2008) conducted a 28-d sediment test with *L. variegatus* according to the draft OECD test guideline 218 with minor modifications. The sediment was spiked with concentrations 4-MBC 0.08, 0.4, 2, 10 and 50 mg/kg sediment dw dissolved in ethyl acetate. Besides, an un-spiked control also a solvent control was used. The measured concentrations in all tests conducted varied between 55.1 and 108%, in the lowest test concentration 4-MBC was below the limit of quantification. The observed factor of reproduction in the control group was with 3.98 (the draft guideline requests at least a factor of 1.8). The pH ranged between 7.4 and 8.6 and dissolved oxygen level was always above 60%. In contrast to the normal reproductive output in control and solvent control, the reproduction started to decrease already at 0.06 mg/Kg sediment dw to an average of 25 worms per test vessel. A significant decrease of reproduction was only found at a 4-MBC concentration of 6.18 mg/kg sediment dw. In contrast to the number of worms, their individual weight increased with increasing substance concentration. According to the authors, changes in the asexual reproduction of *L. variegatus* are more likely explained by general toxicity than by endocrine disruption. The fact that *L. variegatus* is affected by the two UV screens 3-BC and 4-MBC indicates that the worms are incorporating the substances. According to several studies, *L. variegatus* has a high potential for bioaccumulation of hydrophobic substances such as 17a-ethinylestradiol (Liebig et al. (2005) and the xenoestrogen 4-nonylphenol (Croce et al. (2005). Due to this, oligochaetes are assumed to act like a shuttle for certain substances within the food chain. This may have crucial implications for their predators and could be one of the reasons for the high concentrations of UV screens found in fish (Buser et al. (2006); Nagtegaal et al. (1997)).

7.8.2. **Terrestrial compartment**

Not assessed.

7.8.3. **Microbiological activity in sewage treatment systems**

Not assessed.

7.8.4. **PNEC derivation and other hazard conclusions**

Not assessed.

7.8.5. **Conclusions for classification and labelling**

The registrant classified the Substance as Aquatic Acute 1 (H400) and Aquatic Chronic 1 (H410) with acute and chronic M factors of 1 in the last version of the registration dossier prior to ceasing manufacture. Based on the assessment of the available information, the eMSCA supports this classification of 4-MBC and considers no change necessary.

7.9. Human Health hazard assessment

The assessment by the eMSCA was targeted on the potential endocrine disrupting properties of 4-MBC for the environment. Data relevant for human health hazard assessment was therefore only evaluated insofar as it was also considered relevant for the assessment of ED properties for the environment.

7.10. Assessment of endocrine disrupting (ED) properties

7.10.1. **Endocrine disruption – Environment**

In silico and in vitro tests

Table 8

NON-TEST INFORMATION CONCERNING 4-MBC					
Method	**Short Method description**	**Result**	**Description of results**	**References**	**Reliability**
In silico	Virtual screening of a 3d-structural database using pharmacophores of 17β-HSD3	- steroidogenesis	4-MBC was not recognised as an inhibitor of 17β-HSD3	Nashev et al. (2010)	2 – Acceptable, well-documented report
QSAR prediction tool VirtualToxLab	Virtual screening of 3D structures against binding affinity to various receptor proteins	+ antiandrogenic (+) oestrogenic	The prediction tool identified the AR as main target for 4-MBC binding ERβ was identified as the main target for binding of Benzylidene camphor	Vedani et al. (2009)	2 – Acceptable, well-documented report

Hypothalamus:Pituitary:Gonad (HPG) Axis

By virtually screening a 3D-structural database using pharmacophores of 17β-hydrosteroid dehydrogenase type 3 (17β-HSD3), Nashev et al. (2010) did not recognise 4-MBC as a potential inhibitor of 17β-HSD3, an enzyme that metabolises oestrogens and androgens. However, the same authors demonstrated *in vitro* using transfected HEK-293 cells that 4-MBC is an inhitor of 17β-HSD1, 2, and 3 (see Table 14).

***In vitro* assays providing data about selected endocrine mechanisms/ pathways:**

Several *in vitro* studies with 4-MBC have been performed. They are summarised in the following table. The description of the different studies is provided thereafter.

Table 9

In vitro assays with 4-MBC: Oestrogenic activity *(+ indicates a positive result; - indicates a negative result)*	**Result**	**Description of results**	**Results of positive control and/or antiestrogen treatments**	**References**	**Reliability**
Oestrogenicity:					
hERα and hERβ binding study (cell-free) Competitive ER binding assay (porcine uterus cytosol)	hERα: - oestrogenic hERβ: + oestrogenic	ERα: no binding observed up to 1mM ERβ: competitive binding (deliberation of radiolabeled E2) observed IC_{50} = 35.3 µM 4-MBC displaced E2 from ER (porcine uterus cytosol): IC_{50} = 112 µM	17β-Estradiol (E2): IC_{50}= 7.1 nM (hERα), and 2.1 nM (hERβ)	(Schlumpf et al., 2004a)	2 – Acceptable, well-documented report
E-screen (MCF-7) (pS2 gene expression)	+ oestrogenic	Increased expression of pS2 mRNA: EC_{50}= 1.90 µM	E2: EC_{50}= 4.80 pM	(Heneweer et al., 2005)	2 – Acceptable, well documented report
Competitive ER binding assay (immature rat uterus cytosol) Yeast ER transactivation assay (containing hERα) E-screen (MCF-7) (cell proliferation)	+ oestrogenic	4-MBC displaced E2 from ER; not more than 20% replacement achieved and no IC_{50} could be measured Response in the yeast ER transactivation assay: equivocal MCF-7 assay: stimulation of proliferation by 4-MBC (significant from 0.1 µM onwards)	E2	Tinwell et al. (2001)	2 – Acceptable, well-documented report

hERα and hERβ competitive binding study E-screen (MCF-7) (cell proliferation) CHOOSER assay (reporter gene assay based on CHO cells expressing hERα)	+ oestrogenic	hERα competitive binding: IC50 = 12 µM hERβ competitive binding: IC50 = 14 µM MCF-7 assay: REC_{10} (concentration showing 10% of the activity of E2) = 6.3 µM CHOOSER assay: REC_{10} = 4.8 µM	17β-Estradiol (E2) relative activity arbitrarily set to 10^6 Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Matsumoto et al., 2005)	2 or 4 – publication in Japanese with tables in English
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 4 µM 50% of E2 effect reached but no plateau was reached with highest test concentration	E2: EC_{50}= 1 nM	(Schlumpf et al., 2004a)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 3 µM 86% of E2 effect is reached	E2: EC_{50}= 1.2 pM Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Schlumpf et al., 2001)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 24.14 µM No antioestrogenic activity	E2	(Jimenez-Diaz et al., 2013)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation) Measurement of ERα, ERβ, pS2, and AhR mRNA in MCF-7 cells by RT-PCR	+ oestrogenic	Cell proliferation EC_{50}= 3.12 µM ERα, pS2, and AhR mRNA expression was dose-dependently up-regulated; no significant change in ERβ mRNA	E2 100 pM	Yazar and Oztas (2017)	2 – Acceptable, well-documented report
Yeast ER transactivation assay (containing hERα or rainbow trout (rt) ERα)	-	No oestrogenic activity at hERα or rtERα in this test system	E2: EC_{50}= 0.3 nM (hERα) or 18.1 nM (rtERα)	(Kunz et Fent, 2006)	2 – Acceptable, well-documented report
hERα activation in a Yeast Estrogen Screen (YES) with additional enzymatic degestion of the yeast cells	+ oestrogenic	Yeast hERα activation: EC_{50}= 44.3 µM (8% effect compared to E2)	E2: EC_{50}= 0.147 nM	(Schmitt et al., 2008)	2 – Acceptable, well-documented report with clear dose-response relationship

hERα and hERβ competitive binding study E-screen (MCF-7) (cell proliferation) CHOOSER assay (reporter gene assay based on CHO cells expressing hERα)	+ oestrogenic	hERα competitive binding: IC50 = 12 µM hERβ competitive binding: IC50 = 14 µM MCF-7 assay: REC_{10} (concentration showing 10% of the activity of E2) = 6.3 µM CHOOSER assay: REC_{10} = 4.8 µM	17β-Estradiol (E2) relative activity arbitrarily set to 10^6 Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Matsumoto et al., 2005)	2 or 4 – publication in Japanese with tables in English
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 4 µM 50% of E2 effect reached but no plateau was reached with highest test concentration	E2: EC_{50}= 1 nM	(Schlumpf et al., 2004a)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 3 µM 86% of E2 effect is reached	E2: EC_{50}= 1.2 pM Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Schlumpf et al., 2001)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 24.14 µM No antioestrogenic activity	E2	(Jimenez-Diaz et al., 2013)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation) Measurement of ERα, ERβ, pS2, and AhR mRNA in MCF-7 cells by RT-PCR	+ oestrogenic	Cell proliferation EC_{50}= 3.12 µM ERα, pS2, and AhR mRNA expression was dose-dependently up-regulated; no significant change in ERβ mRNA	E2 100 pM	Yazar and Oztas (2017)	2 – Acceptable, well-documented report
Transactivation assays (Luciferase expression in hERα and ERβ HELN reporter cell lines)	+ oestrogenic	Activates ERα at ≥ 3 µM Activity at ERβ unclear	E2	(Gomez et al., 2005)	2 – Acceptable, well-documented report
Transactivation assay (Luciferase expression in stable hERα and hERβ transfectants of HEK293 cells)	+ oestrogenic	Activation of transcription for hERα: IC_{50}= 6.2 µM Activation for hERβ: IC_{50}= 14 µM	E2l Activation of transcription for hERα: IC50= 0.0021 µM	(Schreurs et al., 2005)	2 – Acceptable, well-documented report

hERα and hERβ competitive binding study E-screen (MCF-7) (cell proliferation) CHOOSER assay (reporter gene assay based on CHO cells expressing hERα)	+ oestrogenic	hERα competitive binding: IC50 = 12 µM hERβ competitive binding: IC50 = 14 µM MCF-7 assay: REC_{10} (concentration showing 10% of the activity of E2) = 6.3 µM CHOOSER assay: REC_{10} = 4.8 µM	17β-Estradiol (E2) relative activity arbitrarily set to 10^6 Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Matsumoto et al., 2005)	2 or 4 – publication in Japanese with tables in English
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 4 µM 50% of E2 effect reached but no plateau was reached with highest test concentration	E2: EC_{50}= 1 nM	(Schlumpf et al., 2004a)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 3 µM 86% of E2 effect is reached	E2: EC_{50}= 1.2 pM Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Schlumpf et al., 2001)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 24.14 µM No antioestrogenic activity	E2	(Jimenez-Diaz et al., 2013)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation) Measurement of ERα, ERβ, pS2, and AhR mRNA in MCF-7 cells by RT-PCR	+ oestrogenic	Cell proliferation EC_{50}= 3.12 µM ERα, pS2, and AhR mRNA expression was dose-dependently up-regulated; no significant change in ERβ mRNA	E2 100 pM	Yazar and Oztas (2017)	2 – Acceptable, well-documented report
			Activation of transcription for hERβ: IC_{50}= 0.083 µM		
Transactivation assay (Luciferase expression in stable hERα and hERβ transfectants of HEK293 cells)	? oestrogenic	No induction of transcriptional activation		(Schreurs and van der Burg, 2002)	2 – Acceptable, well-documented report
Recombinant yeast systems carrying either a	- ? estrogen	Very high concentrations used (up to 1000 µM)	E2 or 4,5-dihydro-testosterone	(Kunz and Fent,	2- – Acceptable, well-

hERα and hERβ competitive binding study E-screen (MCF-7) (cell proliferation) CHOOSER assay (reporter gene assay based on CHO cells expressing hERα)	+ oestrogenic	hERα competitive binding: IC50 = 12 µM hERβ competitive binding: IC50 = 14 µM MCF-7 assay: REC_{10} (concentration showing 10% of the activity of E2) = 6.3 µM CHOOSER assay: REC_{10} = 4.8 µM	17β-Estradiol (E2) relative activity arbitrarily set to 10^6 Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Matsumoto et al., 2005)	2 or 4 – publication in Japanese with tables in English
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 4 µM 50% of E2 effect reached but no plateau was reached with highest test concentration	E2: EC_{50}= 1 nM	(Schlumpf et al., 2004a)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 3 µM 86% of E2 effect is reached	E2: EC_{50}= 1.2 pM Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Schlumpf et al., 2001)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 24.14 µM No antioestrogenic activity	E2	(Jimenez-Diaz et al., 2013)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation) Measurement of ERα, ERβ, pS2, and AhR mRNA in MCF-7 cells by RT-PCR	+ oestrogenic	Cell proliferation EC_{50}= 3.12 µM ERα, pS2, and AhR mRNA expression was dose-dependently up-regulated; no significant change in ERβ mRNA	E2 100 pM	Yazar and Oztas (2017)	2 – Acceptable, well-documented report
human estrogen (hERα) or androgen receptor (hAR); YES and YAS assays	ic but anti-estrogenic	Estrogenic response in the yeast hERα assay as well as androgenic response in the yeast hAR assay: not detected Antiestrogenic responste in the yeast hERα assay: EC_{50}= 87.3 µM (181% effect compared to 4-hydroxy-tamoxifen)	(DHT)	2006b)	documented report

hERα and hERβ competitive binding study E-screen (MCF-7) (cell proliferation) CHOOSER assay (reporter gene assay based on CHO cells expressing hERα)	+ oestrogenic	hERα competitive binding: IC50 = 12 µM hERβ competitive binding: IC50 = 14 µM MCF-7 assay: REC_{10} (concentration showing 10% of the activity of E2) = 6.3 µM CHOOSER assay: REC_{10} = 4.8 µM	17β-Estradiol (E2) relative activity arbitrarily set to 10^6 Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Matsumoto et al., 2005)	2 or 4 – publication in Japanese with tables in English
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 4 µM 50% of E2 effect reached but no plateau was reached with highest test concentration	E2: EC_{50}= 1 nM	(Schlumpf et al., 2004a)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 3 µM 86% of E2 effect is reached	E2: EC_{50}= 1.2 pM Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Schlumpf et al., 2001)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 24.14 µM No antioestrogenic activity	E2	(Jimenez-Diaz et al., 2013)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation) Measurement of ERα, ERβ, pS2, and AhR mRNA in MCF-7 cells by RT-PCR	+ oestrogenic	Cell proliferation EC_{50}= 3.12 µM ERα, pS2, and AhR mRNA expression was dose-dependently up-regulated; no significant change in ERβ mRNA	E2 100 pM	Yazar and Oztas (2017)	2 – Acceptable, well-documented report
ER-transcription in primary cerebellar cell cultures from rats and modulatory role of intrinsic biological factors (E2, T3, Glia-cells)	+/- oestrogenic depending on intrinsic biological factors	Glia+: 2-fold increase in ER-mRNA levels (4-MBC alone, not significant), 3 fold increase (4 MBC + E2 + T3 treatment). Glia-: 2-fold decrease in ER-mRNA levels (4-MBC alone) and 7 fold decrease (4-MBC + E2 + T3)		(Jocsak et al., 2016)	2 – Acceptable, well-documented report
E-screen (MCF-7)	*E-*	*E-screen*: proliferation	E-screen:	(Klann et	2 –

hERα and hERβ competitive binding study E-screen (MCF-7) (cell proliferation) CHOOSER assay (reporter gene assay based on CHO cells expressing hERα)	+ oestrogenic	hERα competitive binding: IC50 = 12 µM hERβ competitive binding: IC50 = 14 µM MCF-7 assay: REC_{10} (concentration showing 10% of the activity of E2) = 6.3 µM CHOOSER assay: REC_{10} = 4.8 µM	17β-Estradiol (E2) relative activity arbitrarily set to 10^6 Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Matsumoto et al., 2005)	2 or 4 – publication in Japanese with tables in English
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 4 µM 50% of E2 effect reached but no plateau was reached with highest test concentration	E2: EC_{50}= 1 nM	(Schlumpf et al., 2004a)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 3 µM 86% of E2 effect is reached	E2: EC_{50}= 1.2 pM Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Schlumpf et al., 2001)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 24.14 µM No antioestrogenic activity	E2	(Jimenez-Diaz et al., 2013)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation) Measurement of ERα, ERβ, pS2, and AhR mRNA in MCF-7 cells by RT-PCR	+ oestrogenic	Cell proliferation EC_{50}= 3.12 µM ERα, pS2, and AhR mRNA expression was dose-dependently up-regulated; no significant change in ERβ mRNA	E2 100 pM	Yazar and Oztas (2017)	2 – Acceptable, well-documented report
(cell proliferation) ER binding assay in liver cells of *X. laevis* Gene induction assay with liver cells of *X. laevis*	*screen: proliferation index:* +oestrogenic *ER binding assay:* -estrogenic	index 22.27±3.8% *ER binding assay:* displacement of tracer was observed at concentrations higher than 10 µmol/L, tracer could not be entirely displaced by 4-MBC up to 1 mmol/L *Gene induction assay:* 4-MBC at 1 nmol/L ineffective in inducing the ER gene. At 0.1	proliferation index of E2: 21.07±1.1% Estrogen receptor binding assay: IC_{50} = 0.87 µmol unlabeled E2 (competitor), with [^{3}H]E2 as tracer	al., 2005)	Acceptable, well-documented report

hERα and hERβ competitive binding study E-screen (MCF-7) (cell proliferation) CHOOSER assay (reporter gene assay based on CHO cells expressing hERα)	+ oestrogenic	hERα competitive binding: IC50 = 12 µM hERβ competitive binding: IC50 = 14 µM MCF-7 assay: REC_{10} (concentration showing 10% of the activity of E2) = 6.3 µM CHOOSER assay: REC_{10} = 4.8 µM	17β-Estradiol (E2) relative activity arbitrarily set to 10^6 Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Matsumoto et al., 2005)	2 or 4 – publication in Japanese with tables in English
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 4 µM 50% of E2 effect reached but no plateau was reached with highest test concentration	E2: EC_{50}= 1 nM	(Schlumpf et al., 2004a)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 3 µM 86% of E2 effect is reached	E2: EC_{50}= 1.2 pM Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Schlumpf et al., 2001)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 24.14 µM No antioestrogenic activity	E2	(Jimenez-Diaz et al., 2013)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation) Measurement of ERα, ERβ, pS2, and AhR mRNA in MCF-7 cells by RT-PCR	+ oestrogenic	Cell proliferation EC_{50}= 3.12 µM ERα, pS2, and AhR mRNA expression was dose-dependently up-regulated; no significant change in ERβ mRNA	E2 100 pM	Yazar and Oztas (2017)	2 – Acceptable, well-documented report
	Gene induction assay: +oestrogenic	and 10 mmol/L induction of the ER gene almost as strongly as estradiol	Gene induction assay: E2 significantly induced the gene for the ER more than 1.5-fold (1 nmol/L–10 µmol/L)		
Alkaline phosphatase (AP) activity in Ishikawa cell line	*AP activity:* +/- oestrogenic	*AP activity:* induction at 0.1 nM, and 10 nM, but lacked any activity at doses up to 1 mM		(Mueller et al., 2003)	2 - Acceptable, well-documented

hERα and hERβ competitive binding study E-screen (MCF-7) (cell proliferation) CHOOSER assay (reporter gene assay based on CHO cells expressing hERα)	+ oestrogenic	hERα competitive binding: IC50 = 12 µM hERβ competitive binding: IC50 = 14 µM MCF-7 assay: REC_{10} (concentration showing 10% of the activity of E2) = 6.3 µM CHOOSER assay: REC_{10} = 4.8 µM	17β-Estradiol (E2) relative activity arbitrarily set to 10^6 Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Matsumoto et al., 2005)	2 or 4 – publication in Japanese with tables in English
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 4 µM 50% of E2 effect reached but no plateau was reached with highest test concentration	E2: EC_{50}= 1 nM	(Schlumpf et al., 2004a)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 3 µM 86% of E2 effect is reached	E2: EC_{50}= 1.2 pM Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Schlumpf et al., 2001)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 24.14 µM No antioestrogenic activity	E2	(Jimenez-Diaz et al., 2013)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation) Measurement of ERα, ERβ, pS2, and AhR mRNA in MCF-7 cells by RT-PCR	+ oestrogenic	Cell proliferation EC_{50}= 3.12 µM ERα, pS2, and AhR mRNA expression was dose-dependently up-regulated; no significant change in ERβ mRNA	E2 100 pM	Yazar and Oztas (2017)	2 – Acceptable, well-documented report
ER receptor binding/activation assay with Ishikawa cell line Metabolic activation in primary human or rat hepatocytes	depending on concentration *ER binding assay:* -oestrogenic *ER receptor activatio*	*ER receptor binding assay:* no significant binding affinity to ER up to the highest dose tested (IC50 > 8.2 mM) *ER receptor activation assay:* 4-MBC showed a higher potency to activate ERβ compared to ERα on the 3xERE (EC50 > 150 mM for ERα vs. 57 ± 9 mM for ERβ)	*ER receptor binding:* IC_{50} = 2,0 ±0,2 nM		report

hERα and hERβ competitive binding study E-screen (MCF-7) (cell proliferation) CHOOSER assay (reporter gene assay based on CHO cells expressing hERα)	+ oestrogenic	hERα competitive binding: IC50 = 12 µM hERβ competitive binding: IC50 = 14 µM MCF-7 assay: REC_{10} (concentration showing 10% of the activity of E2) = 6.3 µM CHOOSER assay: REC_{10} = 4.8 µM	17β-Estradiol (E2) relative activity arbitrarily set to 10^6 Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Matsumoto et al., 2005)	2 or 4 – publication in Japanese with tables in English
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 4 µM 50% of E2 effect reached but no plateau was reached with highest test concentration	E2: EC_{50}= 1 nM	(Schlumpf et al., 2004a)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 3 µM 86% of E2 effect is reached	E2: EC_{50}= 1.2 pM Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Schlumpf et al., 2001)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 24.14 µM No antioestrogenic activity	E2	(Jimenez-Diaz et al., 2013)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation) Measurement of ERα, ERβ, pS2, and AhR mRNA in MCF-7 cells by RT-PCR	+ oestrogenic	Cell proliferation EC_{50}= 3.12 µM ERα, pS2, and AhR mRNA expression was dose-dependently up-regulated; no significant change in ERβ mRNA	E2 100 pM	Yazar and Oztas (2017)	2 – Acceptable, well-documented report
	n assay: +oestrogenic *Metabolic activation:* +oestrogenic	*Metabolic activation:* no increased oestrogenic potency in rat or human hepatocytes compared to Ishikawa cells (EC_{50}>200 mM for ERα and 73±22 mM for ERβ)			
Transactivation assay (Luciferase expression in	+oestrogenic	ER-activated luciferase transcription: EC_{50}= $6*10^{-9}$ mol/L	E2 at 1 µmol/L	(Minh et al., 2008)	2 – Acceptable, well-

hERα and hERβ competitive binding study E-screen (MCF-7) (cell proliferation) CHOOSER assay (reporter gene assay based on CHO cells expressing hERα)	+ oestrogenic	hERα competitive binding: IC50 = 12 µM hERβ competitive binding: IC50 = 14 µM MCF-7 assay: REC_{10} (concentration showing 10% of the activity of E2) = 6.3 µM CHOOSER assay: REC_{10} = 4.8 µM	17β-Estradiol (E2) relative activity arbitrarily set to 10^6 Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Matsumoto et al., 2005)	2 or 4 – publication in Japanese with tables in English
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 4 µM 50% of E2 effect reached but no plateau was reached with highest test concentration	E2: EC_{50}= 1 nM	(Schlumpf et al., 2004a)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 3 µM 86% of E2 effect is reached	E2: EC_{50}= 1.2 pM Oestrogen antagonist ICI 182,780: Inhibition of the proliferative effect of 4-MBC	(Schlumpf et al., 2001)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation)	+ oestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 24.14 µM No antioestrogenic activity	E2	(Jimenez-Diaz et al., 2013)	2 – Acceptable, well-documented report
E-screen (MCF-7) (cell proliferation) Measurement of ERα, ERβ, pS2, and AhR mRNA in MCF-7 cells by RT-PCR	+ oestrogenic	Cell proliferation EC_{50}= 3.12 µM ERα, pS2, and AhR mRNA expression was dose-dependently up-regulated; no significant change in ERβ mRNA	E2 100 pM	Yazar and Oztas (2017)	2 – Acceptable, well-documented report
stable hERα and hERβ transfectants of CHO-K1 cells)					documented report
ELISA-based oestrogen receptor competitive binding assay (ER-ELISA) Modified yeast two-hybrid oestrogen assay	*ER-ELISA:* -estrogenic *Yeast-assay:* -oestrogenic	No oestrogenic effects observed up to 37,5 µM(ER-ELISA) and 10 µM (Yeast-assay)	E2 at 300 pM (Yeast assay), DES at 300 nM (ER-ERLISA)	(Morohoshi et al., 2005)	2 – Acceptable, well-documented report

Regarding oestrogenic activity the following tests are available:

- 8 MCF-7 cell proliferation assays analysing cell proliferation due to hER activation
- 4 luciferase transactivation assays
- 4 transactivation assays (YES) with yeast cells transfected with hERalpha
- 3 competitive ER binding assays
- 2 transactivation assays with human HEK293 cells transfected with hERalpha and hERß receptors
- 1 CHOOSER assay
- 1 cell free binding study
- 1 ELISA based ER Binding assay
- 1 gene expression test with yeast cells transfected with rtERalpha
- 1 ER transcription assay in primary cerebellar cells from rats
- 1 ER binding assay in liver cells
- 1 gene induction assay in liver cells
- 1 alkaline phosphatase (AP) activity assay in Ishikawa cell line
- 1 ER receptor binding/activation assay with Ishikawa cell line
- 1 metabolic activation in primary human or rat hepatocytes

In summary, two studies reported a clear oestrogenic activity of 4-MBC for ERα and ERβ. 3 studies, performed with yeast, found oestrogenic effects of 4-MBC towards ERα (one only weak) but not ERβ. Two studies described oestrogenic effects for ERα but unclear, or no results for ERβ and one study, working with yeast and uterus cytosol, reported unclear results for both ER receptor types. Furthermore, all assays performed with MCF-7 cell lines were positive regarding the oestrogenic activity of 4-MBC. In conclusion, 4-MBC itself seems not to be able to bind to the E2 binding site of hERα and cause its activation but it may have antiestrogenic activity although at moderate concentrations. Whether it shows oestrogenic or antiestrogenic activity by binding to the E2 binding site of the hERβ remains unclear. 4-MBC showed oestrogenic activity mainly in mammalian cell lines with metabolic activity (compared to yeast assays without metabolism). Therefore, it is presumed that the observed oestrogenic activity of 4-MBC is caused by its two main metabolites.

Table 10

In vitro assays with 4-MBC: Androgenic activity (+ indicates a positive result; - indicates a negative result)					
	Result	**Description of results**	**Result positive control**	**References**	**Reliability**
hAR-mediated reporter-gene activiation assay in MDA-kb2 cells (endogenously expressing hAR and hGR)	- androgenic - antiandrogenic	No agonistic or antagonistic (in 0.5 or 0.1 nM DHT) action on AR (0.001 to 1 µM)	Androgen agonist: 4,5-dihydro-testosterone (DHT) EC_{50}=0.136 nM Antiandrogen: Flutamide in 0.5 nM DHT IC_{50}= 3.62 µM	(Ma et al., 2003)	2 – Acceptable, well-documented report
AR CALUX transactivation assay (U2-OS cells containing 3x3 ARE-TATA-Luc-reporter and a hAR expression plassmid)	+ antiandrogenic	Repression of hAR activation: IC_{50}= 7.1 µM	Flutamide IC_{50}= 0.5 µM	(Schreurs et al., 2005)	2 – Acceptable, well-documented report
hAR transactivation assay using PALM (PC-3-Androgen receptor-Luciferase-MMTV) cells	+ antiandrogenic	No androgenic activity Repression of hAR activation: IC_{50}= 9.1 µM	R1881	(Jimenez-Diaz et al., 2013)	2 – Acceptable, well-documented report
hAR transactivation assay using transfected HEK-293 cells	+ antiandrogenic	No androgenic activity Repression of hAR activation (84% of testosterone induced activity)	Testosterone	Nashev et al. (2010)	2 – Acceptable, well-documented report
Recombinant yeast systems carrying either a human oestrogen (hERα) or androgen receptor (hAR); YES and YAS assays	+antiandrogenic	Antiandrogenic response in the yeast hAR assay: EC_{50}= 11.8 µM (107% effect of flutamide)		(Kunz and Fent, 2006a)	2 – Acceptable, well-documented report

With regard to androgenic activity the following tests are available:

- three AR-mediated gene-reporter activation assays in cells
- one gene expression test with hAR (AR CALUX Bioassay)
- one assay with recombinant yeast cells

In summary, 4-MBC did not show any androgenic activity in the tests described above up to 10 µM. With regard to antiandrogenic activity, results were mostly positive. While it showed antiandrogenic activity in four studies (Schreurs et al. (2005) and Jimenez-Diaz (2013), Kunz and Fent (2006), Nashev et al. (2010)), it was not found antiandrogenic in only one study (Ma et al. (2003)).

Table 11

In vitro assays with 4-MBC: Gestagenic activity (+ indicates a positive result; - indicates a negative result)					
	Result	Description of results	Result positive control	References	Reliability
Progesterone receptor (PR) CALUX transactivation assay (U2-OS cells containing 3x3 PRE-TATA-Luc-reporter and a hPR expression plassmid)	+ antigestogenic	Repression of hPR activation: IC_{50}= 0.9 µM	ORG2058 (PR agonist): EC_{50}= 30 pM RU486 (PR antagonist): IC_{50}= 4.9 pM	(Schreurs et al., 2005)	2 – Acceptable, well-documented report
Human sperm: activation of the CatSper channel	+ gestogenic activity	4-MBC activates the CatSper Ca^{2+} channel and increased Ca^{2+} influx: EC_{50}= 6.8 µM (for comparison: 4-octylphenol (4-OP) EC_{50}= 5.93 ± 0.40 µM) 4-MBC competes with progesterone for CatSper activation 4-MBC changes sperm motility and stimulates acrosomal exocytosis similar to progesterone	Inhibition of 4-MBC mediated Ca^{2+} influx by MDL12330A (a CatSper inhibitor): 96.6% → 4-MBC increases Ca^{2+} influx via CatSper (For comparison: Inhibition of 4-OP mediated Ca^{2+} influx by MDL12330A: 62.5%)	(Schiffer et al., 2014)	2 – Acceptable, well-documented report
SULT1E1 mRNA expression in human endometrial epithelial adenocarcinoma Ishikawa cells	- antigestogenic	No antagonistic effect (in 0.1% DMSO) on progesterone (0.1 µM) induced SULT1E1 mRNA from 0.001 to 100 µM	Antigestogens showed inhibition of progestone (0.1 µM) induced SULT1E1 mRNA; RU486: EC_{50} = 1.4 nM; UPA: EC_{50} = 9.5 nM; ZK137316: EC_{50} = 6 nM	Yin et al. (2015)	2 – Acceptable, well-documented report

Human sperm: activation of the CatSper channel	+ gestogenic acitivity	4-MBC activates the CatSper Ca^{2+} channel and increased Ca^{2+} influx: EC_{50}= 0.52±0.32 µM	Inhibition of 4-MBC mediated Ca^{2+} influx by MDL12330A (not quantified)→ 4-MBC increases Ca^{2+} influx via CatSper	(Rehfeld et al., 2016)	2 – Acceptable, well-documented report
Human sperm: acrosome reaction, penetration, hyperactivation and viability	*acrosome reaction:* + gestogenic acitivity *penetration*: + gestogenic acitivity	*Acrosome reaction:* induction at 10 µM *Penetration:* induction of rise in [Ca^{2+}] I (Increment in cell density at 1 cm into a viscous medium) at 10 µM *Hyperactivation:* percentage of hyperactivated sperm cells not significantly changend at 10 µM *Viability:*no significant effect on viability at 10 µM	*Acrosome reaction:* 10 µM progesterone and 2 µM ionomycin *Penetration:* 5 µM progesterone *Hyperactivation:* 5 µM progesterone *Viability:* 0,5%Triton	(Rehfeld et al., 2018)	2 – Acceptable, well-documented report

With regard to gestagenic activity the following tests are available:

- 3 tests with human sperm cells
- 1 PR reporter gene assay with U2-OS cells containing a 3xPRE-TAT-Luc-reporter construct in combination with a hPR expression plasmid (PR CALUX)
- 1 gene expression test with human endometrial epithelial adenocarcinoma Ishikawa cells and SULT1E1 as target gene

All studies performed with human sperm cells reported positive results for the gestagenic activity of 4-MBC. On the other hand, a PR reporter gene assay (PR CALUX) demonstrated anti-gestogenic activity. One study with human endometrial epithelial adenocarcinoma Ishikawa cells was not able to demonstrate anti-gestogenic activity of 4-MBC on progesterone-induced upregulation of SULT1E1 mRNA-expression.

Table 12

In vitro assays with 4-MBC: Thyroidal activity (+ indicates a positive result; - indicates a negative result)					
	Result	**Description of results**	**Result positive control**	**References**	**Reliability**
TH-responsive luciferase-based reporter gene assay HepG2 cells transfected with p(DR4)2-SV40-luc and pRS-rTRα1	+ thyroidal	4-MBC activates TRα (LOEC = 1 µM), and acts as an antagonist in the presence of T3 (LOEC = 10 µM) 4-MBC (10 µM) stimulates endogenous expression of DIO1 but does not suppress T3-induced DIO expression	T3, GC-1 (synthetic agonist), NH-3 (synthetic antagonist)	(Hofmann et al., 2009)	2 – Acceptable, well-documented report
TPO activity FTC-133 thyroid carcinoma cell stably transfected with human TPO	- thyroidal	No inhibition of TPO activity		(Schmutzler et al., 2004)	2 – Acceptable, well-documented report
Iodide uptake into FTRL-5 cells	+ thyroidal	4-MBC inhibited iodide uptake into FTRL-5 cells at 0.1 and 1.0 µM		(Schmutzler et al., 2007)	4, not assignable For the original description of methods and results, this review paper refers to a poster abstract not available to the eMSCA
Gene expression of marker genes for deiodinase activity in SH-SY5Y cells	+ thyroidal	4-MBC changed the expression potential marker genes for diodinase activity		(Song et al., 2013)	2 – Acceptable, well-documented report

With regard to thyroidal activity the following tests are available:

- 1 luciferase-based TRα-reporter gene assay using HepG2 cells
- 1 TPO activity assay with FTC-133 thyroid carcinoma cells stably transfected with human TPO
- 1 iodide uptake assay performed with FRTL-5 cells
- 1 expressional study on potential marker genes for deiodinase activity in SY5Y human neuroblastoma cells

With regard to thyroidal activity of 4-MBC, there is only a limited number of in vitro mechanistic studies. The available data indicate that 4-MBC weakly interacts with TRα and might modulate deiodinase expression and possibly activity. Furthermore, while TPO activity seems not to be inhibited, a review paper mentions that 4-MBC might interfere with iodide uptake in FRTL-5 cells. However, regarding the iodide uptake assay, no original data are available.

Table 13

In vitro assays with 4-MBC: Steroidogenesis activity (+ indicates a positive result; - indicates a negative result)					
	Result	**Description of results**	**Result positive control**	**References**	**Reliability**
Enzyme activity (17β-HSD1, 2, 3, 5) in transfected HEK-293 cells	+ steroidogenesis	4-MBC inhibits the activity of 17β-HSD1 (IC_{50} = 70 µM), 17β-HSD2 (IC_{50} = 5.9 µM), and 17β-HSD3 (IC_{50} = 10.7 µM); No inhibition of 17β-HSD5	Substrates: Radiolabelled estrone (17β-HSD1), E2 (17β-HSD2), or androstenedione (17β-HSD3, 5)	Nashev et al. (2010)	2 – Acceptable, well-documented report

With regard to steroidogenesis, one study showed that 4-MBC inhibits 17β-hydroxysteroid dehydrogenase (17β-HSD) 1, 2, and 3.

High-throughput tests

The ToxCast database lists 250 high throughput assays in which 4-MBC was screened. 45 of those assays are reported as active (ToxCast entries for 4-MBC, last checked 10 July 2020). Some of the assays include endpoints relevant for the identification of ED effects (e.g., ER-receptor) but, except for three, all the assays reported positive results for concentrations close to or above the cytotoxicity threshold. One of the assays, with active results below the cytotoxicity threshold, reported antagonism of the PR.

The ComTox database lists 25 assays with 4-MBC and 7 of those report an active result (1 Erα, 2 AR, 4 assays with thyroidal endpoints) (CompTox entries for 4-MBC, last checked 10 July 2020). However, herein also most of the active results were obtained at concentrations above the cytotoxicity threshold.

Summary of *in vitro* data:

The *in vitro* studies assessed during this evaluation show that 4-MBC displays endocrine activity related to the EATS modalities.

Five studies reported an oestrogenic activity of 4-MBC (mostly for ERα). Furthermore, all assays performed with MCF-7 cell lines were positive regarding the oestrogenic activity of 4-MBC. The Substance itself seems not to be able to bind to the E2 binding site of hERα and cause its activation, but it may have antiestrogenic activity although at moderate concentrations. Whether it shows oestrogenic or antiestrogenic activity by binding to the E2 binding site of the hERβ remains unclear. 4-MBC showed oestrogenic activity mainly in mammalian cell lines with metabolic activity (compared to yeast assays without metabolism). Therefore, it is presumed, that the observed oestrogenic activity of 4-MBC is caused by its two main metabolites. 4-MBC did not show any androgenic activity, but with regard to anti-androgenic activity, results were mostly positive.

All studies performed with human sperm cells reported gestagenic activity of 4-MBC, mimicking the effects of progesterone. On the other hand, a reporter gene assay (PR CALUX) demonstrated antigestogenic activity. However, another study with human

Ishikawa cells was not able to confirm anti-gestogenic activity on progesterone-induced marker gene expression.

Additionally, one study investigating the effects of 4-MBC on steroidogenesis demonstrated that 4-MBC inhibits the activity of 17β-HSD1, 2, and 3, enzymes important for steroid hormone metabolism.

With regard to thyroidal activity, the limited data available demonstrate interaction of 4-MBC with TRα, as well modulation of deiodinase expression and possibly activity. Furthermore, whereas 4-MBC seems not to be a TPO inhibitor, the substance is reported to interfer with iodide uptake in FRTL-5 cells.

The data from high throughput assays from the US EPA ToxCast ond ToxComp databases provides some evidence for endocrine disrupting properties of 4-MBC (e.g. effects on ER, AR, PR). But most assays report activity of 4-MBC only for concentrations above the cytotoxicity threshold, so information from these assays is very limited.

In vivo tests

Approach used for assessing the endocrine acitivity in fish:

This section focuses on the effects of 4-MBC observed in fish. Additionally, available supporting studies with mammals potentially indicating endocrine disrupting properties of 4-MBC are described.

The assessment of whether 4-MBC is actually an endocrine disruptor in fish was mainly based on the OECD guidance document No. 150 on standardised test guidelines for evaluating chemicals for endocrine disruption (OECD, 2018). Although this document focuses on validated OECD test guidelines, some general information on how to assess endocrine disrupting properties is provided. The guidance provided in this document has been supplemented with information from other guidance documents (e.g. OECD guidance document on the diagnosis of endocrine related histopathology in fish gonads (OECD, 2010)) and information from literature (e.g. (IPCS (2002); Kendall et al. (1998); Knacker et al. (2010); OECD (2004)). In general, two different types of effects are considered and analysed separately:

- Indicators of an endocrine mode of action via EATS modalities and
- Effects on apical endpoints that are sensitive to, but not diagnostic of, EATS parameters are considered to provide evidence that a substance exerts adverse effects.

Indicators of endocrine mode of action:

Indicators of an endocrine mode of action may be provided by biomarkers that are known to indicate a specific mode of action as well as by histological changes that are likely to be a direct response to an oestrogen mode of action.

One of the most common biomarkers indicating an oestrogenic or androgenic endocrine mode of action is vitellogenin (VTG). Vitellogenin is naturally produced by female fish as a precursor of yolk proteins which are incorporated in eggs (IPCS, 2002). Induction of VTG in female and (more pronounced) in male fish is a known indicator of an oestrogen receptor agonist mode of action (IPCS (2002); Kendall et al. (1998); Knacker et al. (2010); OECD (2004)).

With respect to histological changes, according to the OECD test guideline 229 for the fish short term reproduction assay (OECD, 2009b) and the guidance document on the diagnosis of endocrine related histopathology in fish gonads (OECD, 2010), the following endpoints are diagnostic for endocrine activity:

- Male: increased proportion of spermatogonia (early sperm cells), presence of testis-ova, increased testicular degeneration, interstitial (Leydig) cell hyperplasia/hypertrophy
- Female: increased oocyte atresia, perifollicular cell hyperplasia/hypertrophy, decreased yolk formation (aromatase inhibition), changes in gonadal staging.

Other effects such as decreased proportion of spermatogonia, altered proportions of spermatozoa (mature sperm cells) and gonadal staging in males are of secondary diagnostic interest as they are sensitive to, but not diagnostic of, EATS parameters.

Changes in the gonadosomatic index (GSI) may provide additional information about the gonad maturation and spawning readiness (OECD, 2004a). It describes changes in the relation of gonad to whole body mass and thus may be an indicator of the reproductive effort of organisms (Helfman et al. (1997)). Although GSI might be influenced by other modes of action too, reduction of GSI in male fish is regarded as a sensitive parameter in reproductive studies with oestrogenic substances (OECD, 2004a). However, care must be taken as the GSI is highly dependent on the individual fish (frequent spawners) or seasonal gonadal stage (seasonal breeders).[8]

In addition, the following apical endpoints are considered to be indicators of an oestrogen receptor agonist mode of action according to the OECD guidance document No. 150 (OECD, 2018).

- Depression of male secondary sex characteristics in fathead minnow or medaka
- Female biased phenotypic sex-ratio during sexual development

A decrease in *secondary sex characteristics* in males may indicate an oestrogenic or anti-androgenic mode of action but should be interpreted with caution and based on weight of evidence according to (OECD, 2009b). Induction of female secondary sex characteristics in males such as urogenital papillae in male zebrafish was shown to be significant after exposure to oestrogenic substances (Kendall et al. (1998); OECD (2004a)).

Change of sex-ratio towards females is a known result of oestrogen or antiandrogen exposure during sexual development (IPCS (2002); Kendall et al. (1998); OECD (2004)). In aquaculture, this phenomenon is frequently used to generate all female or partial female populations by exposing fishes to exogenous oestrogen active substances (Baroiller et al. (1999); Piferrer (2001)).

Whether or not endocrine mediated effects are observable highly depends on the life stage tested. For example testis-ova might be induced in adult males as at least in some species gonads remain bipotent, but sensitivity is usually highest during sexual development (e.g. Nakamura et al. (1998)). Differences in development of fish species must be considered. *O. latipes* for example is a differentiated gonochoric species that naturally develops either male or female gonads and sex is naturally not changed after gonadal development.

[8] The size of the sexual gonads (testes and ovaries) increases when gonads mature prior to spawning. Depending on the spawning strategy of fish species (total spawners, spawning only once in a breeding season or lifetime versus repeated, batch or serial spawners) the gonadal size and thus the GSI may substantially increase during a spawning season, reaching maxima just before spawning (Helfman et al., 1997). In repeated spawners, this process recurs and, as their spawning is usually not synchronized, individual gonadal growth differs in time.

Hormonal influence (especially of female hormones) in this species starts very early during pre-hatch development (OECD, 2004a) and thus life stages under exposure need to be considered carefully while analysing test results. If effects on gonadal staging are analysed the reproductive cycle of a species should be considered. Especially for total spawners having only one breeding season such as *O. mykiss* effects may be observed only during the process of maturing prior to spawning and may be missed at other times of the year.

Indicators that adverse effects are endocrine mediated

Alteration of the endocrine system may cause adverse effects that are endocrine specific but may also influence endpoints that are not endocrine specific (Kendall et al., 1998; Knacker et al., 2010; OECD, 2004a).

Secondary sex characteristics and sex-ratio are apical endpoints that are oestrogen specific.

Other endpoints such as growth, sexual maturity, reproduction, and behaviour are known to be sensitive to oestrogens or antiandrogens (IPCS, 2002; OECD, 2004a; OECD, 2011). Fertility rate, growth, time to first spawn, sex-ratio shift toward females (medaka and fathead minnow) and delay of male sexual development (zebrafish) are the most sensitive endpoints for oestrogen receptor agonists in fish full life cycle tests (Knacker et al. (2010)).

Thus, in combination with indicators of endocrine activity, they provide evidence of oestrogen mediated effects but alone (apart from sex-ratio) they are not diagnostic for this mode of action as they might also be influenced by other modes of action.

Error! Reference source not found. Below table summarises endpoints that are considered indicators of oestrogenic activity and may be affected because of this activity *in vivo*.

Table 14

Summary of endpoints that are considered during analysis of fish data	
Endpoints indicating an oestrogen receptor agonist mode of action	**Endpoint considered to be sensitive to an oestrogenic mode of action *in vivo***
• Vitellogenin induction in males • increased proportion of spermatogonia (early sperm cells), presence of testis-ova, increased testicular degeneration, interstitial (Leydig) cell hyperplasia/hypertrophy in males • increased oocyte atresia, perifollicular cell hyperplasia/hypertrophy, decreased yolk formation (aromatase inhibition), changes in gonadal staging in females • Depression of male secondary sex characteristics in fathead minnow or medaka and induction of female secondary sex characteristics such as urogenital papillae in zebrafish • Female biased phenotypic sex-ratio during sexual development.	• Female biased phenotypic sex ratio during sexual development especially in medaka • Reproduction (fecundity, fertility, number of males or females with reproductive success) • Spawning behaviour • Growth of offspring

The following table summarises available *in vivo* assays providing data about endocrine activity. Test conditions and results are briefly described in the subsequent section followed by a summary with regard to endocrine acivity.

Table 15

***In vivo* assays providing data about selected endocrine mechanisms/pathways and adverse effects of 4-MBC**						
Species **Life stage/ duration**	**Concentration/ test condition/ tested substance/ solvent**	**Vitellogenin**	**Others**	**Result positive control**	**References**	**Reliability**
Oryzias latipes Adult male (body length: 2.5-3.5 cm)/ duration: 7 days	39, 390, 3900 µM 4-MBC or E2 (3.7, 37, 185 nM), solvent: 0.1% ethanol ELISA of plasmaVTG – real-time RT-PCR	Dose-dependent increase of plasma VTG: at 390 µM 4-MBC same level as 0.037 µM E2 Increase of plasma VTG + mRNA expression levels of VTG-1 and -2: LOEC = 39 µM (= 9.9 mg/l) Effect is comparable to the effect observed after E2 incubation.		positive control: 17β-Estradiol (E2)	(Inui et al., 2003)	2 – Acceptable, well-documented report
Pimephales promelas Mixed-sex juvenile (2-3 months, 19 to 27 mm body length)/ duration: 14 d	Solvent control (0.1 mL ethanol/l), 10, 100, 500 and 1000 µg 4-MBC/L (nominal) or 9, 435, 953 µg 4-MBC/L (real) 25 ± 1 °C; photoperiod 16 h light per day; 10 L stainless steal tanks; semi-static	No VTG induction	753 µg 4-MBC/L: 20% mortality	positive control 17β-Estradiol (E2): no difference in wet weight and mean length in SC and E2 Benzophenone-1 and -2: VTG induction at 4919 and 8783 µg/L	(Kunz et al., 2006)	2 – Acceptable, well-documented report

Danio rerio Embryos (3 hpf, 128 cell stage) /duration: 96 h	0,165 mg 4-MBC/l (measured, corresponds EC_{10} of FET performed in the same study (see 0)), solvent 0,01% ethanol, renewal of test solution after 48 h27 ± 2 °C, 14:10 h light:dark period 25 eggs with 8 ml test solution in glass petri dishes, 3 replicates Negative control and solvent control (0,01% ethanol)	Not assessed	Cyp19a2 expression significantly decreased: 1.54 ± 0.107 fold compared to solvent control	No positive control	Quintaneiro et al. (2019)	2 – Acceptable, well-documented report

Oryzias latipes Adult (3-4 months), mixed sex (ratio 1:1)/duration: 28 d	5, 50, 500 µg 4-MBC/l (nominal, measured concentrations > 80% of nominal) 1 l glass beaker (fecundity and fertility endpoints), 18 l glass aquarium (other endpoints), test solution renewed daily pH 7.2-7.6; 25 ± 1 °C, 16 h photoperiod water control/solvent control (0,01% ethanol)	Significantly increaesd plasma VTG in females in 5 and 50 µg 4-MBC/l treatments + increased *vtg* expression at 5 and 50 µg 4-MBC/l No significant changes for plasma VTG in males, but increased *vtg* expression in all treatment groups	Brain: *ara*, *era, erβ, cyp19b*, *fshb*, and *lhb* were significantly increased in females for all treatments Levels of *ara*, *era* and *erβ* in males significantly increased for all treatment groups increases in the levels of *fshb* and *cyp19b* obtained in males for 5 µg/l 4-MBC	No positive control	Liang et al. (2020)	2 – Acceptable, well-documented report

	ELISA of plasmaVTG, RT-PCR, histopathological endpoints, hatch-rate, time to hatch of F1 generation		liver: *araα* and *star* inhibited in females and males exposed to 500 µg 4-MBC/l, *eraα* and *erβ* found increased in females at 50 µg 4-MBC/l and in males at 50 and 500 µg4-MBC/l testes/ovaries: *eraα*, *erβ*, *cyp17a*, *hsd3b*, *star*, *fshr* and *lhr* increased in males for all treatment groups. *Star* and *lhr* increased in females for all treatment groups, *araα*, *erβ* and *cyp17a* increased in females exposed to 500 µg 4-MBC/l. *fshr* decreased in females after exposure to 5 µg 4-MBC/l			

Supporting information from mammalian toxicity tests:

Detailed study summaries of the *in vivo* studies are provided below:

Table 16

Supporting in vivo studies with mammals					
Short Method description	**Results**	**Description of results**	**Results of positive control**	**References**	**Reliability**
Mechanistic *in vivo* studies (OECD level 3)					
Uterotrophic assay OECD TG 440 Immature SD rats 0, 10, 100, 1000 mg/kg bw/d subcutanously	- oestrogenic	No effects on uterus weight		Orlando (2001) Not in IUCLID	2 – Acceptable, well-documented report
Uterotrophic assay Similar to OECD 440 21-d old, immature female LE rats 66, 119, 211, 337, 402, and 1980 mg/kg bw/d in feed duration: 4 days Positive control: EE2 (0.085, 0.34, 0.78, 0.86, 1.65, and 8.6 µg/kg bw/d)	+ oestrogenic	Dose-dependently increased (wet) uterus weight (2-fold compared to control), significant at ≥ 119 mg/kg bw/d ED_{50}= 309 mg/kg/d Maximum increase as percent of EE2: 35.5% No effects of 4-MBC on body weight	Ethinylestradiol-17α (EE): Increased (wet) uterine weight, significant at ≥ 0.34 µg/kg bw/d ED_{50}= 0.082 µg/kg bw/d No effects of EE2 on body weight	(Schlumpf et al., 2001) In IUCLID	2 – Acceptable, well-documented report
Uterotrophic assay 21-d old, immature rats (Nu (hairless) strain (Ico: OFA hr/hr)) 0, 2.5%, 5.0%, and 7.5% in	+ oestrogenic	Increased (wet) uterus weight; significant at 5% and 7.5% 4-MBC Estimated uptake in the 5% group assuming 0.6% penetration through the skin: 37 mg/kg bw/d		(Schlumpf et al., 2001)	2 – Acceptable, well-documented report

olive oil (vehicle) Dermal application twice daily (3-4 h interval) by whole body immersion followed by additional brushing of test soliution on the back		No effects of 4-MBC on body weight			
Uterotrophic assay 19-20 d old, immature female Alpk:APfSD rats 500 or 800 mg/kg bw/d in feed; duration: 3 days OR 500 or 1000 mg/kg bw/d) subcutanous (n = 12 nimals per group) Positive control: diethylstilbestrol (DES)	+ oestrogenic	Increased (wet and dry) uterus weight after dietary or subcutaneous exposure at ≥ 500 mg/kg/d Lower body weight at 500 and 800 mg/kg bw/d after dietary exposure	DES (5 µg/kg): increased (blotted and dry) uterus weight No effects on body weight	(Tinwell et al., 2002)	2 – Acceptable, well-documented report
Uterotrophic assay immature female Alpk:APfSD rats Experiment 1: 19-20-d old animals (Tinwell et al., 2002), Experiments 2, 3: 19-20d old animals; 1000 mg/kg bw/d alone or in combination with 300 mg/kg bw/d Antarelix (ANT; GnRH-receptor antagonist) Experiments 4, 5: Animals were either 19 - 20 d, 20 – 21 d, 21 – 22 d, or 22 - 23 d old; 1000 mg/kg bw/d	+ oestrogenic (weak; probably no direct effect of the uterus)	4-MBC increased uterus weight (blotted and dry) only when controls values are at the low end of their normal range. ANT abolished the effect of 4-MBC on uterus weight. The authors therefore suggest that 4-MBC-induced uterus growth is centrally mediated (possibly by centrally mediated release of endogenous estradiol from the ovariese or adrenals) No effects of 4-MBC on body weight were recorded.	DES (5 µg/kg): increased (blotted and dry) uterus weight Cotreatment with ANT did not prevent the DES-induced increase fo uterus weight No effect of DES on body weight	(Ashby et al., 2004)	2 – Acceptable, well-documented report

Positive control: DES (5 µg/kg bw/d) alone or in combination with ANT (experiments 2 and 3)					
Ovariectomised (ovx) Sprague Dawley rats n =12 per group Oral (diet) for 12 weeks 0, 60, or 285 mg/d Positive control: E2 (0.45 mg/d)	+EATS-mediated parameters	Reduced weight gain (both doses) and fat depots (low dose) Slightly increased uterus weight (both doses) Increased LH levels (both doses) Increased TSH (both doses) and decreased T4 (both doses), trend for increased T3 Reduced leptin levels (both doses) Trend for reduced triglycerides; increased HDL (low dose), trend for increased LDL; trend for increased cholesterol	E2: Reduced weight gain and fat depots Increased uterus weight Decreased LH levels Trend for increased TSH and T4 Reduced cholesterol, HDL, and LDL levels Reduced leptin levels	(Seidlova-Wuttke et al., 2006a) In IUCLID	2 (reliable with restrictions)
Ovariectomised (ovx) Sprague Dawley rats n = 12 per group Oral (diet) for 12 weeks 0, 57.5, or 250 mg/d After six weeks the high	+EAS-mediated parameters	Slightly increased uterus weight (significant only at high dose) and epithelial thickness of the endometrium Slight stimulation of vaginal epithelium (both doses) No effects on mRNA expression of ERα, ERβ, PR, IGF-1 in uterus and vagina	E2: Reduced body weight Increased uterus weight and epithelial thickness of the endometrium Proliferative effect	(Seidlova-Wuttke et al., 2006b) In IUCLID	2 (reliable with restrictions)

dose animals were supplied with the low dose due to reduced food intake Immediately put on test diet after ovarectomy Positive control: E2 (0.6 mg/d)		Prevention of bone mineral density in tibia after ovarectomy Increased osteocalcin levels (both doses) and decreased levels of C-terminal breakdown products of bone–specific collagen-1α1 (marker for osteoclast activity)	on myometrium Stimulation of vaginal epithelium Increased mRNA expression of PR (uterus and vagina) and IGF-1 (uterus), decreased mRNA of ERβ (uterus and vagina) Reduced serum osteocalcin		
Ovariectomised (ovx) Sprague Dawley rats n = 8-11 per group Ovarectoamy at 14 weeks of age Oral (diet) for 12 weeks 0, 66, or 310 mg/d (soyfree (sf) diet) OR 0, or 307 mg/d (soy containing (sc) diet) Positive controls: E2 (0.64 mg/d and 0.58 mg/d for soy-free and soy-containing food, respectively); 5α-androstane-3β,17β-diol (Adiol; 2.8 mg/d for soy-	+ thyroid	Increased TSH (sf and sc diet), decreased T4 (sf diet), and increased T3 (sf diet) No effect on hepatic 5'deiodinase (5'DI) activity Slight increased malic enzyme activity (as a readout for thyroid hormone action) in liver (sc diet).	E2: Increased TSH, increased T3 and T4 (sc diet) Decreased hepatic 5'DI activity (sf and sc diet) Increased malic enzyme activity in liver (sf and sc diet) Adiol: Increased malic enzyme activity in liver (only tested with sf diet)	(Schmutzler et al., 2004)	2 (reliable with restrictions)

free food); Adiol is a supposed endogenous ligand for ERβ					
Repeated dose toxicity studies (OECD level 4)					
Repeated dose toxicity Beagle dogs Tolerability study Oral gavage dosing over 14 days One male and one female dog The two dogs were dosed with 20 mg/kg bw (day 1), 100 mg/kg bw (day 2), 500 mg/kg bw (day 3), 2500 mg/kg bw (day 4) and 500 mg/kg bw (days 5 – 14)	No effects on EAS-sensitive organs + thyroid	The male dog showed minimal activation of the thyroid (iso- and high-prismatic epithelium) No effects on T3, T4, and TSH reported but values were consistently higher after exposure and there was a gradual increase over time. TSH was detectable only at the end of the study. No effects on gross pathology and histology of the other investigated organs including male and female reproductive organs, mammary gland, adrenal, pancreas, parathyroid and pituitary No effects on food consumption, body weight, ECG, blood pressure, haematology, clinical chemistry Clinical signs: the male dog showed vomiting after administration of the high dose (2500 mg/kg bw/d)		(Merck, 2003) In IUCLID	2 (reliable with restrictions)
Repeated dose toxicity Beagle dogs Oral tolerability study Gavage dosing over 3 weeks Two male and two female dogs The four dogs were dosed	No effects on EATS-sensitive parameters	No effects on T3, T4, and TSH reported but values were consistently higher after exposure. No effectss on gross pathology and histology of the investigated organs including thyroid, male and female reproductive organs, mammary gland, adrenal, pancreas, parathyroid, and pituitary		(Merck, 2003) In IUCLID	2 (reliable with restrictions)

with 0 mg/kg bw (day 1), 20 mg/kg bw (day 4), 100 mg/kg bw (day 8), 500 mg/kg bw (days 11 - 21) Each dose escalation step was followed by a wash-out phase of 2 or 3 days					
Repeated dose toxicity 10 male and females Wi-AF/Han (SPF) albino rats Gavage dosing for 17 days 0, 30, and 300 mg/kg bw/d (vehicle: peanut oil) Histology only on thyroid gland	+ effects on EATS-sensitive parameters	Significantly increased TSH in males (1.9 fold) and females (7.5 fold) at 300 mg/kg bw/d (T3 and T4 were not measured) Significantly increased (absolute and relative) thyroid weight in males (absolute: 140%) and females (absolute: 160%) at 300 mg/kg bw/d Increased incidence of thyroid hypertrophy (4/20, 8/20, 16/20 at 0, 30, and 300 mg/kg bw/d; not seperately reported for males and females) Decreased (absolute and relative) prostate weight at 30 (absolute: 82%) and 300 mg/kg bw/d (absolute: 75%) Decreased (relative) thymus weight in females at 300 mg/kg bw/d Decreased adrenal weight in males at 300 mg/kg bw/d Discolouration of the renal cortex at 300 mg/kg bw/d in males and females Clinical signs after dosing: Restlessness, grooming, hypersalivation		(Merck 1983b) In IUCLID	2 (reliable with restrictions)
Repeated dose toxicity 10 male and females Wi-AF/Han (SPF) albino rats Gavage dosing for 4 weeks	+ effects on EATS-sensitive parameters	Increased T3 (96% in males and 28% in females), decreased T4 (30% in males and 23% in females) Increased absolute and relative thyroid	PTU: Decreased T3 in females (37%) and decreased T4 (61% in males and	(Merck 1983a) In IUCLID	2 (reliable with restrictions)

0, or 1000 mg/kg bw/d (vehicle: peanut oil) Positive control (propylthiouracil, PTU): 20 mg/kg bw/d (10 males and females) Histology only on thyroid gland No TSH measurement		weight (absolute; 1.9 fold) Thyroid hypertrophy/hyperplasia (mild – marked) in males and females Atrophy of the accessory sex glands Decreased prostate weight Decreased thymus weight (males and females) Increased weight of liver (males and females), kidney (females), adrenals (males and females)	77% in females) Incerased absolute and relative thyroid weight Thyroid hypertrophy/hyperplasia (excessive) lower food and water consumption, and lower body weight in males (27%) and females (23%); organ weight changes		
Repeated dose toxicity Similar to OECD TG 408 Exposure via diet for 13 weeks 20 male and females Wi-AF/Han (SPF) albino rats per group 0, 50, 125, 312 mg/kg bw/day (vehicle : feed) Half of the animals in each group were allowed a 1 month recovery Part 2 : 0, or: 25 mg/kg bw/d (20 males and females per group) Blood sampling for hormone measurements (N = 10 per group and sex) at week 7, at the end of the treatment,	+ effects on EATS-sensitive parameters	Increased TSH and T3 in all treatment groups; increased T4 in females at 50 and 312 mg/kg bw/d, and in males in the high dose recovery period; slight increases in T4 (not sign.) observed also in part 2 at 25 mg/kg bw/day Increased absolute and relative thyroid weights at ≥ 50 (males) and at ≥ 125 mg/kg bw/day (females); still apparent after recovery in the high dose group Thyroid hypertrophy/hyperplasia at ≥ 50 mg/kg bw/day (males and females); still apparent after recovery Decreased prostate and adrenal weights in the high dose group Increased liver weights at ≥ 50 (males) and ≥ at 125 mg/kg bw/day (females) Changes in haematology, clinical chemistry at ≥ 50 mg/kg bw/day		(Merck, 1984a) In IUCLID	1 (reliable without restriction)

and after recovery					
Repeated dose toxicity Investigation of liver enzyme induction Male Wi-AF/Han (SPF) albino rats 4 week dietary exposure (312 mg/kg bw/d)	No effects on liver enzymes	Slightly reduced DNA content of the liver No effects on protein content, cytochrome P450 content, ribonucleic acid concentrations, and catalytic activity of glucuronyl transferase No clinical signs, no effects on food consumption, body weight, and liver weight Slightly increased water consumption (second week)		(Merck 1984c) In IUCLID	2 (reliable with restrictions)
Repeated dose toxicity OECD TG 411 Dermal exposure (semiocclusive) 10 male and females Wistar Han RCC (SPF) rats per group 0, 100, 400, 2000 mg/kg bw/day 10 males and females for 4-weeks recovery 9 males and females for toxicokinetic and hormone measurement	No effects on EATS-sensitive parameters	No effects on thyroid histology and levels of TSH, T3, and T4 No histological findings in any organ investigated Dermal irritation in the high dose group		(RCC, 2005) in IUCLID	1 (reliable without restriction)
Reproductive and developmental toxicity studies (OECD level 4 and 5)					
Screening study for reproductive and developmental toxicity Comparable to OECD TG 421 but without exposure of	No significant effects on EATS-sensitive parameters	Trend for higher T3 (high dose) and prolactin (mid and high dose) at day 21 of premating in F0 females. Lower T3 and TSH in F1 males (day 55 pp) - considered as inter-animal variation		(RCC, 2004) In IUCLID	2 (reliable with restrictions)

F0 males oral: gavage 0, 12.5, 25, 50 mg/kg bw/day (vehicle: hydroxypropyl methylcellulose) HanBrl:WIST SPF Exposure: 28 days premating, during mating, gestation and lactation No exposure of F0 males; Inclusion of neuro-behavioural and hormone measurements (day 55 pp) in F1 animals		Lower FSH, dose-dependent at ≥ 25 mg/kg bw/day in F1 males No effects on T4, LH, E2 and T in F0 females or in F1 animals. No effects on reproductive performance, weight or histology of reproductive organs and thyroid in F0 and F1 animals No effects on developmental indices and anogenital distances in F1 animals. No effects on learning or memory function General toxicity: higher water consumption, most pronounced at high dose			
Developmental toxicity Orienting test Rabbit (pregnant females, N = 3 per group) Gavage dosing from day 6-10 of pregnancy 0, 25, 50 and 100 mg/kg bw/day (vehicle : arachis oil)	Limited investigation EATS-sensitive prameters	Initial diarrhoea No effects maternal reproductive parameters No evidence for embryotoxicity or teratogenicity		(Merck 1984b) Not in IUCLID	4 – only short description available from SCCNFP (2004)
Developmental toxicity OECD TG 414 Pregnant Wistar (Wi-AF/Han SPF) rats (N = 25 per group) Gavage dosing from day 6-16 of pregnancy	Limited investigation EATS-sensitive prameters	Lower body weight gain in high dose dams No effects on maternal reproductive parameters Lower body weight of high dose foetuses Lower ossification of sternum in mid and high dose		(Merck 1988) In IUCLID	1 (reliable without restriction)

0, 10, 30 and 100 mg/kg bw/day (vehicle : arachis oil)		Delayed ossification of extremities in high dose males Dose-dependent increase of rudimentary lumbar ribs at ≥ 30 mg/kg bw/day			
Assessment of fertility : Male adult wistar rats (N = 10-12 per group) Subcutaneous injection of low doses of 4-MBC (2-20 mg/kg/day) Exposure : 5 days (2 days for 20 mg/kg/day group) LH and FSH serum levels	+EATS-mediated parameters	Significant decreases in the LH and FSH serum concentration, and decreases in hypothalamic GnRH release		(Carou et al., 2009a; Carou et al., 2008; Carou et al., 2009b)	2 (reliable with restrictions)
Developmental toxicity study Comparable to OECD TG 443 Long-Evans rats Oral exposure: 10 weeks pre-mating. Dams were exposed during pregnancy and lactation; offspring was exposed until adulthood (12 weeks of age) Experimant A: 0, 0.1, 0.33, and 0.66 g/kg chow (vehical: soy oil devoid of phytoestrogens according to manufacturer), corresponding to 0, 7, 24,	+ EATS-mediated parameters	Decreased litter size and survival rate at ≥ 24 mg/kg bw/day Delayed puberty in males at ≥ 7 mg/kg bw/day (0.7 mg/kg/day group not investigated); no body weight effect Decreased ventral and dorsal+lateral prostate weight (absolute and relative) at ≥ 7 mg/kg bw/day Increased growth of prostate and accessory sex glands at PND1 (7 mg/kg bw/d) Increased absolute (≥ 7 mg/kg bw/day) and relative (47 mg/kg bw/day) testis weight Decreased relative epididymis weight at		Different parts of the studies reported in different publications: (Durrer et al., 2007; Durrer et al., 2005; Faass et al., 2009; Hofkamp et al., 2008; Maerkel et al., 2007; Maerkel et al., 2005; Schlumpf et al., 2008a; Schlumpf et al., 2004a; Schlumpf et al., 2008b; Schlumpf et al., 2004b) In IUCLID: (Durrer et al., 2007; Durrer et al., 2005; Maerkel et al., 2007; Maerkel et al., 2005)	2 (reliable with restrictions)

or 47 mg/kg bw/d Experiment B: 0, 0.01, g/kg chow (vehical: soy oil deviod of phytoestrogens according to manufacturer), corresponding to 0, or 0.7 mg/kg bw/d Oestrogen challenge (injection of 10 or 50 µg/kg E2) in adult gonadectomized (at 10 weeks of age) offspring (12 weeks of age) Results published in several papers and reviewed in (Schlumpf et al., 2008a; Schlumpf et al., 2004b)		47 mg/kg bw/day Altered sexual behaviour of females at 7 and 24 mg/kg bw/day In uterus, prostate, ventromedial hypothalamus (VMH), and medial preoptic area (MPO): Changes in mRNA and/or protein expression of ERα, ERβ, AR, PR, IGF-1, SRC-1 4-MBC changed the sensitivity towards E2 challenge of mRNA and/or protein expression of ERα, ERβ, AR, PR, IGF-1, SRC-1 in uterus, prostate, ventromedial hypothalamus (VMH), and medial preoptic area (MPO) of gonadectomized animals Increased (absolute) thyroid weight at in males and females at 24 mg/kg bw/day) Increased TSH in males at 47 mg/kg bw/d Increased T3 in males at 24 mg/kg bw/d only, and in females at 47 mg/kg bw/d Decreased (males at ≥ 24 mg/kg bw/day) and increased (females at 47 mg/kg bw/d) thymus weight			

Summary of *in vivo* studies:

The available *in vivo* studies for fish only show some evidence for weak oestrogenic effects. In mammals, most uterotrophic assays demonstrate weak oestrogenic activity and there are indications that this is rather centrally mediated than a direct effect on the uterus. Additionally, an increase in uterine weight has been observed in three-month studies with ovariectomised rats combined with histopathological markers in uterus and vagina, pointing to an endocrine mode of action with some effects similar to, but others distinct from, the positive control E2 (Seidlova-Wuttke et al., 2006a; Seidlova-Wuttke et al., 2006b). Pre-/perinatal exposure in rats has been reported to induce alterations in reproductive organ weight (in particular the prostate) at birth, on day 14 and in adulthood, delayed sexual maturation, and altered gene expression in prostate and brain in male rats. Effects observed in females included increased uterus weights, changes in gene expression of oestrogen-regulated genes in brain and uterus, as well as strongly impaired sexual behaviour such as reduced proceptive and lordosis and increased rejection behaviour (see review by Schlumpf et al. (2008a)).

Furthermore, several studies in rats but also in dogs indicate an interference of 4-MBC with the thyroid hormone system (Merck 1983a; Merck 1983b, Merck 1984a, Merck 2003, Schutzler et al., (2004), Seidlova-Wuttke et al., (2006a), Maerkel et al., (2007)). This has been detailed in the background document for the identification of 4-MBC as an SVHC based on its endocrine-disrupting properties for human health (ECHA 2021).

Effects include increased thyroid weight and hypertrophy/hyperplasia accompanied with increased TSH levels, and frequently increased T3 and decreased T4 levels. The constellation of effects on the thyroid hormone system is distinct from propylthiouracil (Merck, 1983a) and the underlying mode of action remains unknown, although an interaction with TRs might play a role (Hofmann et al., 2009).

Summary of evidence for endocrine disrupting effects

Considering all available information from *in vitro* and *in vivo* studies (incl. supporting studies with mammals), the following conclusion regarding endocrine disruption in the environment for 4-MBC can be drawn:

In vitro tests indicate that 4-MBC and/or its main metabolites can activate the human ERα and ERβ receptor in a dose-dependent manner. 4-MBC also shows antiandrogenic and antiprogestogenic activity and interferes with steroidogenesis in vitro. There is also *in vitro* evidence for an interaction of 4-MBC with TRs.

A variety of effects on EATS-sensitive parameters in mammalian studies substantiates the concern that 4-MBC is an ED and acts via multiple modes of action *in vivo*.

7.10.2. **Endocrine disruption - Human health**

The mammalian data was assessed as supporting information during the evaluation of the concern for ED properties in the environment. The eMSCA's conclusions in this regard are discussed above.

The Substance has been identified as an SVHC based on its ED properties for human health according to REACH Article 57(f), based on unanimous agreement of the MSC. 4-MBC was added to the candidate list by ECHA on 17 January 2022.

7.11. PBT and VPVB assessment

Not part of the substance evaluation

7.12. Exposure assessment

Not part of the substance evaluation

7.13. Risk characterisation

Not part of the substance evaluation

7.14. References

Ashby J., Tinwell H., Odum J., and Lefevre P. (2004): Natural variability and the influence of concurrent control values on the detection and interpretation of low-dose or weak endocrine toxicities. Environ Health Perspect 112 (8), 847-853. DOI: 10.1289/ehp.6862

Buser H.R., Balmer M.E., Schmid P., and Kohler, M. Occurrence of UV filters 4-methylbenzylidene camphor and octocrylene in fish from various Swiss rivers with inputs from wastewater treatment plants. Environ Sci Technol . 2006 Mar 1;40(5):1427-31. doi: 10.1021/es052088s.

Carou M.E., Deguiz M.L., Reynoso R., Szwarcfarb B., Carbone S., Moguilevsky J.A., Scacchi P., and Ponzo O.J. (2009a): Impact of the UV-B filter 4-(Methylbenzylidene)-camphor (4-MBC) during prenatal development in the neuroendocrine regulation of gonadal axis in male and female adult rats. Environmental Toxicology and Pharmacology 27 (3), 410-414. DOI: https://doi.org/10.1016/j.etap.2009.01.007

Carou M.E., Ponzo O.J., Cardozo Gutierrez R.P., Szwarcfarb B., Deguiz M.L., Reynoso R., Carbone S., Moguilevsky J.A., and Scacchi P. (2008): Low dose 4-MBC effect on neuroendocrine regulation of reproductive axis in adult male rats. Environmental Toxicology and Pharmacology 26 (2), 222-224. DOI: https://doi.org/10.1016/j.etap.2008.04.001

Carou M.E., Szwarcfarb B., Deguiz M.L., Reynoso R., Carbone S., Moguilevsky J.A., Scacchi P., and Ponzo O.J. (2009b): Impact of 4-methylbenzylidene-camphor (4-MBC) during embryonic and fetal development in the neuroendocrine regulation of testicular axis in prepubertal and peripubertal male rats. Exp Clin Endocrinol Diabetes 117 (9), 449-454. DOI: 10.1055/s-0028-1112153

Croce V., De Angelis S., Patrolecco L., Polesello S., Valsecchi S. (2005): Uptake and accumulation of sediment-associated 4-nonylphenol in a benthic invertebrate (Lumbriculus variegatus, freshwater oligochaete). Environmental Toxicology 2005, 24(5), 1165-1171.

Durrer S., Ehnes C., Fuetsch M., Maerkel K., Schlumpf M., and Lichtensteiger W. (2007): Estrogen sensitivity of target genes and expression of nuclear receptor co-regulators in rat prostate after pre- and postnatal exposure to the ultraviolet filter 4-methylbenzylidene camphor. Environ Health Perspect 115 Suppl 1, 42-50. DOI: 10.1289/ehp.9134

Durrer S., Maerkel K., Schlumpf M., and Lichtensteiger W. (2005): Estrogen target gene regulation and coactivator expression in rat uterus after developmental exposure to the ultraviolet filter 4-methylbenzylidene camphor. Endocrinology 146 (5), 2130-2139. DOI: 10.1210/en.2004-1272

ECHA (2021): Member State Committee Support Document for Identification of (±)-1,7,7-trimethyl-3-[(4-methylphenyl)methylene]bicyclo[2.2.1]heptan-2-one covering any of the individual isomers and/or combinations thereof (4-MBC) as a Substance of Very High Concern because of its Endocrine disrupting Properties (Article 57(f) - Human Health),

adopted on 29 November 2021. https://echa.europa.eu/documents/10162/6a6d37ea-30ea-5cc5-74f7-6c1f6e678fc0

Faass O., Schlumpf M., Reolon S., Henseler M., Maerkel K., Durrer S., and Lichtensteiger W. (2009): Female sexual behavior, estrous cycle and gene expression in sexually dimorphic brain regions after pre- and postnatal exposure to endocrine active UV filters. Neurotoxicology 30 (2), 249-260. DOI: 10.1016/j.neuro.2008.12.008

Fent K., Kunz P.Y., Zenker A., and Rapp M. (2010): A tentative environmental risk assessment of the UV-filters 3-(4-methylbenzylidene-camphor), 2-ethyl-hexyl-4-trimethoxycinnamate, benzophenone-3, benzophenone-4 and 3-benzylidene camphor. Marine Environmental Research 69 (SUPPL. 1), S4-S6. https://doi.org/10.1016/j.marenvres.2009.10.010

Gomez E., Pillon A., Fenet H., Rosain D., Duchesne M.J., Nicolas J.C., Balaguer P., and Casellas C. (2005): Estrogenic activity of cosmetic components in reporter cell lines: Parabens, UV screens, and musks. Journal of Toxicology and Environmental Health - Part A 68 (4), 239-251. https://doi.org/10.1080/15287390590895054

Heneweer M., Muusse M., van den Berg M., and Sanderson J.T. (2005): Additive estrogenic effects of mixtures of frequently used UV filters on pS2-gene transcription in MCF-7 cells. Toxicol Appl Pharmacol 208 (2), 170-177. DOI: 10.1016/j.taap.2005.02.006

Hofkamp L., Bradley S., Tresguerres J., Lichtensteiger W., Schlumpf M., and Timms B. (2008): Region-specific growth effects in the developing rat prostate following fetal exposure to estrogenic ultraviolet filters. Environ Health Perspect 116 (7), 867-872. DOI: 10.1289/ehp.10983

Hofmann P.J., Schomburg L., and Kohrle J. (2009): Interference of endocrine disrupters with thyroid hormone receptor-dependent transactivation. Toxicol Sci 110 (1), 125-137. DOI: 10.1093/toxsci/kfp086

Inui M., Adachi T., Takenaka S., Inui H., Nakazawa M., Ueda M., Watanabe H., Mori C., Iguchi T., and Miyatake K. (2003): Effect of UV screens and preservatives on vitellogenin and choriogenin production in male medaka (Oryzias latipes). Toxicology 194 (1-2), 43-50. DOI: 10.1016/s0300-483x(03)00340-8

Jimenez-Diaz I., Molina-Molina J.M., Zafra-Gomez A., Ballesteros O., Navalon A., Real M., Saenz J.M., Fernandez M.F., and Olea N. (2013): Simultaneous determination of the UV-filters benzyl salicylate, phenyl salicylate, octyl salicylate, homosalate, 3-(4-methylbenzylidene) camphor and 3-benzylidene camphor in human placental tissue by LC-MS/MS. Assessment of their in vitro endocrine activity. J Chromatogr B Analyt Technol Biomed Life Sci 936, 80-87. DOI: 10.1016/j.jchromb.2013.08.006

Jocsak G., Kiss D.S., Toth I., Goszleth G., Bartha T., Frenyo L.V., Horvath T.L., and Zsarnovszky A. (2016): Comparison of individual and combined effects of four endocrine disruptors on estrogen receptor beta transcription in cerebellar cell culture: The modulatory role of estradiol and triiodo-thyronine. International Journal of Environmental Research and Public Health 13 (6), 619

Klann A., Levy G., Lutz I., Muller C., Kloas W., and Hildebrandt J.P. (2005): Estrogen-like effects of ultraviolet screen 3-(4-methylbenzylidene)-camphor (Eusolex 6300) on cell proliferation and gene induction in mammalian and amphibian cells. Environ Res 97 (3), 274-281. DOI: 10.1016/j.envres.2004.07.004

Kunz P.Y. and Fent K. (2006a): Estrogenic activity of UV filter mixtures. Toxicol Appl Pharmacol 217 (1), 86-99. DOI: 10.1016/j.taap.2006.07.014

Kunz P.Y. and Fent K. (2006b): Multiple hormonal activities of UV filters and comparison of in vivo and in vitro estrogenic activity of ethyl-4-aminobenzoate in fish. Aquatic Toxicology 79 (4), 305-324. https://doi.org/10.1016/j.aquatox.2006.06.016

Kunz P.Y., Galicia H.F., and Fent K. (2004): Assessment of hormonal activity of UV filters in tadpoles of frog Xenopus laevis at environmental concentrations. Marine Environmental Research 58 (2-5), 431-435. https://doi.org/10.1016/j.marenvres.2004.03.027

Kunz P.Y., Galicia H.F., and Fent K. (2006): Comparison of in vitro and in vivo estrogenic activity of UV filters in fish. Toxicological Sciences 90 (2), 349-361. https://doi.org/10.1093/toxsci/kfj082

Liebig M., Moltmann J.F., Knacker, T (2005): Evaluation of measured and predicted environmental concentrations of selected human pharmaceuticals and personal care products. Environ Sci Pollut Res Int. 2006 Mar;13(2):110-9. doi:10.1065/espr2005.08.276.

Ma R., Cotton B., Lichtensteiger W., and Schlumpf M. (2003): UV filters with antagonistic action at androgen receptors in the MDA-kb2 cell transcriptional-activation assay. Toxicol Sci 74 (1), 43-50. DOI: 10.1093/toxsci/kfg102

Maerkel K., Durrer S., Henseler M., Schlumpf M., and Lichtensteiger W. (2007): Sexually dimorphic gene regulation in brain as a target for endocrine disrupters: developmental exposure of rats to 4-methylbenzylidene camphor. Toxicol Appl Pharmacol 218 (2), 152-165. DOI: 10.1016/j.taap.2006.10.026

Maerkel K., Lichtensteiger W., Durrer S., Conscience M., and Schlumpf M. (2005): Sex- and region-specific alterations of progesterone receptor mRNA levels and estrogen sensitivity in rat brain following developmental exposure to the estrogenic UV filter 4-methylbenzylidene camphor. Environ Toxicol Pharmacol 19 (3), 761-765. DOI: 10.1016/j.etap.2004.12.055

Matsumoto H., Adachi S., and Suzuki Y. (2005): [Estrogenic activity of ultraviolet absorbers and the related compounds]. Yakugaku Zasshi 125 (8), 643-652. http://www.ncbi.nlm.nih.gov/pubmed/16079615

Merck (1983a): Pilot study of subacute toxicity in rats after oral treatment for 4 weeks. 4/16/83, date: 1983-05-05. Institute of Toxicology, E, Merck, Darmstadt. Unpublished study report. http://ec.europa.eu/health/ph_risk/committees/sccp/documents/out282_en.pdf

Merck (1983b): Pilot study of subacute toxicity (17 days) in rats after oral treatment. 4/17/83, date: 1983-05-05. Institute of Toxicology, E. Merck, Darmstadt. Unpublished study report. http://ec.europa.eu/health/ph_risk/committees/sccp/documents/out282_en.pdf

Merck (1984a): Subchronic toxicity study in rats (3 months feeding trial) with a 1-month, treatment-free follow-up period. Institute of Toxicology, E, Merck, Darmstadt

Merck (1984b): Eusolex 6300 : Orienting tests for embryotoxicity on rabbits after oral administration. Institute of Toxicology. E. Merck. Darmstadt

Merck (1984c): Eusolex 6300: Liver enzyme induction study in a 4-week feeding trial on rats. Institute of Toxicology. Merck. Darmstadt. Unpublished study report.

Merck (1988): Eusolex 6300, teratogenicity study with oral administration in rats. 4/43/88. Unpublished study report.

Merck (2003): Oral tolerability study in beagle dogs. 090006d18014fd61, date: 2003-01-27. Institute of Toxicology, Merck KGaA, Darmstadt. Unpublished study report. http://ec.europa.eu/health/ph_risk/committees/sccp/documents/out282_en.pdf

Minh S.D., Below S., Müller C., and Hildebrandt J.-P. (2008): Novel mammalian cell lines expressing reporter genes for the detection of environmental chemicals activating endogenous aryl hydrocarbon receptors (ArhR) or estrogen receptors (ER). Toxicology in Vitro 22 (8), 1935-1947. DOI: https://doi.org/10.1016/j.tiv.2008.09.002Morohoshi K., Yamamoto H., Kamata R., Shiraishi F., Koda T., and Morita M. (2005): Estrogenic activity of 37 components of commercial sunscreen lotions evaluated by in vitro assays. Toxicology in Vitro 19 (4), 457-469. DOI: https://doi.org/10.1016/j.tiv.2005.01.004

Mueller S.O., Kling M., Arifin Firzani P., Mecky A., Duranti E., Shields-Botella J., Delansorne R; Broschard T; Kramer PJ. Activation of estrogen receptor alpha and ERbeta by 4-methylbenzylidene-camphor in human and rat cells: comparison with phyto- and xenoestrogens. Toxicol Lett; 2003 Apr; 142(1-2):89-101. https://doi.org/10.1016/S0378-4274(03)00016-X

Nagtegaal, M., Ternes, T. A., Baumann, W. & Nagel, R. (1997) UV-filtersubstanzen in Wasser und Fischen. UWSF - Z. Umweltchem. Ökotox.9, 79–86.

Nashev L.G., Schuster D., Laggner C., Sodha S., Langer T., Wolber G., and Odermatt A. (2010): The UV-filter benzophenone-1 inhibits 17beta-hydroxysteroid dehydrogenase type

3: Virtual screening as a strategy to identify potential endocrine disrupting chemicals. Biochem Pharmacol 79 (8), 1189-1199. DOI: 10.1016/j.bcp.2009.12.005

IRB (2001): Eusolex 6300 : rodent uterotrophic assay. Istituto di Ricerche Biomediche "Antoine Marxer", I-Colleretto Giacosa. Unpublished report.

OECD (2018), *Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption*, OECD Series on Testing and Assessment, No. 150, OECD Publishing, Paris, https://doi.org/10.1787/9789264304741-en.

RCC (2004): Reproduction Toxicity Study in the Han Wistar Rat. Unpublished study report.

RCC (2005): EUSOLEX 6300 (4-MBC): 13-Week Dermal Toxicity (Semi-Occlusive) Study in the Rat followed by a 4-Week Recovery Period. Unpublished study report.

Rehfeld A., Dissing S., and Skakkebæk N.E. (2016): Chemical UV Filters Mimic the Effect of Progesterone on Ca2+ Signaling in Human Sperm Cells. Endocrinology 157 (11), 4297-4308. DOI: 10.1210/en.2016-1473 (last accessed 5/11/2020)

Rehfeld A., Egeberg D.L., Almstrup K., Petersen J.H., Dissing S., and Skakkebæk N.E. (2018): EDC IMPACT: Chemical UV filters can affect human sperm function in a progesterone-like manner. Endocrine Connections 7 (1), 16-25. DOI: 10.1530/EC-17-0156

SCCNFP (2004): 4-METHYLBENZYLIDENE CAMPHOR. SCCNFP/0779/04. THE SCIENTIFIC COMMITTEE ON COSMETIC PRODUCTS AND NON-FOOD PRODUCTS INTENDED FOR CONSUMERS.
https://ec.europa.eu/health/ph_risk/committees/sccp/documents/out282_en.pdf

Schiffer C., Muller A., Egeberg D.L., Alvarez L., Brenker C., Rehfeld A., Frederiksen H., Waschle B., Kaupp U.B., Balbach M., Wachten D., Skakkebaek N.E., Almstrup K., and Strunker T. (2014): Direct action of endocrine disrupting chemicals on human sperm. EMBO Rep 15 (7), 758-765. DOI: 10.15252/embr.201438869

Schlumpf M., Cotton B., Conscience M., Haller V., Steinmann B., and Lichtensteiger W. (2001): In vitro and in vivo estrogenicity of UV screens. Environ Health Perspect 109 (3), 239-244. DOI: 10.1289/ehp.01109239

Schlumpf M., Durrer S., Faass O., Ehnes C., Fuetsch M., Gaille C., Henseler M., Hofkamp L., Maerkel K., Reolon S., Timms B., Tresguerres J.A., and Lichtensteiger W. (2008a): Developmental toxicity of UV filters and environmental exposure: a review. Int J Androl 31 (2), 144-151. DOI: 10.1111/j.1365-2605.2007.00856.x

Schlumpf M., Jarry H., Wuttke W., Ma R., and Lichtensteiger W. (2004a): Estrogenic activity and estrogen receptor beta binding of the UV filter 3-benzylidene camphor. Comparison with 4-methylbenzylidene camphor. Toxicology 199 (2-3), 109-120. DOI: 10.1016/j.tox.2004.02.015

Schlumpf M., Kypke K., Vökt C.C., Birchler M., Durrer S., Faass O., Ehnes C., Fuetsch M., Gaille C., Henseler M., Hofkamp L., Maerkel K., Reolon S., Zenker A., Timms B., Tresguerres J.A.F., and Lichtensteiger W. (2008b): Endocrine active UV filters: Developmental toxicity and exposure through breast milk. Chimia 62 (5), 345-351. DOI: 10.2533/chimia.2008.345

Schlumpf M., Schmid P., Durrer S., Conscience M., Maerkel K., Henseler M., Gruetter M., Herzog I., Reolon S., Ceccatelli R., Faass O., Stutz E., Jarry H., Wuttke W., and Lichtensteiger W. (2004b): Endocrine activity and developmental toxicity of cosmetic UV filters--an update. Toxicology 205 (1-2), 113-122. DOI: 10.1016/j.tox.2004.06.043

Schmitt C., Oetken M., Dittberner O., Wagner M., and Oehlmann J. (2008): Endocrine modulation and toxic effects of two commonly used UV screens on the aquatic invertebrates Potamopyrgus antipodarum and Lumbriculus variegatus. Environmental Pollution 152 (2), 322-329. https://doi.org/10.1016/j.envpol.2007.06.031

Merck (2003): Oral tolerability study in beagle dogs. 090006d18014fd61, date: 2003-01-27. Institute of Toxicology, Merck KGaA, Darmstadt. Unpublished study report. http://ec.europa.eu/health/ph_risk/committees/sccp/documents/out282_en.pdf

Schmutzler C., Gotthardt I., Hofmann P.J., Radovic B., Kovacs G., Stemmler L., Nobis I., Bacinski A., Mentrup B., Ambrugger P., Grüters A., Malendowicz L.K., Christoffel J., Jarry H., Seidlovà-Wuttke D., Wuttke W., and Köhrle J. (2007): Endocrine disruptors and the

thyroid gland--a combined in vitro and in vivo analysis of potential new biomarkers. Environ Health Perspect 115 Suppl 1 (Suppl 1), 77-83. DOI: 10.1289/ehp.9369

Schmutzler C., Hamann I., Hofmann P.J., Kovacs G., Stemmler L., Mentrup B., Schomburg L., Ambrugger P., Gruters A., Seidlova-Wuttke D., Jarry H., Wuttke W., and Kohrle J. (2004): Endocrine active compounds affect thyrotropin and thyroid hormone levels in serum as well as endpoints of thyroid hormone action in liver, heart and kidney. Toxicology 205 (1-2), 95-102. DOI: 10.1016/j.tox.2004.06.041

Schreurs R.H.M.M., Sonneveld E., Jansen J.H.J., Seinen W., and van der Burg B. (2005): Interaction of Polycyclic Musks and UV Filters with the Estrogen Receptor (ER), Androgen Receptor (AR), and Progesterone Receptor (PR) in Reporter Gene Bioassays. Toxicological science 83, 264-272. DOI: 10.1093/toxsci/kfi035

Schreurs R.L., Peter; Seinen Willem; and van der Burg B. (2002): Estrogenic activity of UV filters determined by an in vitro reporter gene assay and an in vivo transgenic zebrafish assay. Arch Toxicol 2002 (76), 257–261. DOI: 10.1007/s00204-002-0348-4

Seidlova-Wuttke D., Christoffel J., Rimoldi G., Jarry H., and Wuttke W. (2006a): Comparison of effects of estradiol with those of octylmethoxycinnamate and 4-methylbenzylidene camphor on fat tissue, lipids and pituitary hormones. Toxicol Appl Pharmacol 214 (1), 1-7. DOI: 10.1016/j.taap.2005.11.002

Seidlova-Wuttke D., Jarry H., Christoffel J., Rimoldi G., and Wuttke W. (2006b): Comparison of effects of estradiol (E2) with those of octylmethoxycinnamate (OMC) and 4-methylbenzylidene camphor (4MBC)--2 filters of UV light - on several uterine, vaginal and bone parameters. Toxicol Appl Pharmacol 210 (3), 246-254. DOI: 10.1016/j.taap.2005.05.006

Sieratowicz A., Kaiser D., Behr M., Oetken M., and Oehlmann J. (2011): Acute and chronic toxicity of four frequently used UV filter substances for Desmodesmus subspicatus and Daphnia magna. J Environ Sci Health A Tox Hazard Subst Environ Eng 46 (12), 1311-1319. DOI: 10.1080/10934529.2011.602936

Song M., Song M.K., Choi H.S., and Ryu J.C. (2013): Monitoring of deiodinase deficiency based on transcriptomic responses in SH-SY5Y cells. Archives of Toxicology 87 (6), 1103-1113. DOI: 10.1007/s00204-013-1018-4

Tinwell H., Lefevre P.A., Moffat G.J., Burns A., Odum J., Spurway T.D., Orphanides G., and Ashby J. (2002): Confirmation of uterotrophic activity of 3-(4-methylbenzylidine)camphor in the immature rat. Environ Health Perspect 110 (5), 533-536. DOI: 10.1289/ehp.02110533

7.15. Abbreviations

4-MBC	(±)-1,7,7-trimethyl-3-[(4-methylphenyl) methylene] bicyclo [2.2.1] heptan-2-one
AP	Alkaline phosphatase
AR	Androgen receptor
BCF	Bioconcentration factor
CALUX	Chemical Activated LUciferase gene eXpression assay
DES	Diethylstilbestrol
DHT	Dihydrotestosterone
E2	17β-Estradiol
ED	Endocrine disruptor/endocrine disruptive
EE	17α-Ethinylestradiol
ELISA	Enzyme-linked immunosorbent assay
eMSCA	Evaluating member state competent authority
ER	Estrogen receptor
FET	Fish Embryo Acute Toxicity Test
FSH	Follicle-stimulating hormone
HDL	High-density lipoprotein
hER/hAR	Human estrogen/androgen receptor
IGF	Insulin-like growth factor
LDL	Low-density lipoprotein
LH	Luteinising hormone
LOEC	Lowest observed effect concentration
MPO	Medial preoptic area
PR	Progesterone receptor
SCR	Steroid receptor coactivator
TH	Thyroid hormone
TPO	Thyreoperoxidase
TSH	Thyroid-stimulating hormone
VMH	Ventromedial hypothalamus
VTG	Vitellogenin
YAS	Yeast androgen screen
YES	Yeast (o)estrogen screen