

## **Annex XV report**

# **PROPOSAL FOR IDENTIFICATION OF A SUBSTANCE OF VERY HIGH CONCERN ON THE BASIS OF THE CRITERIA SET OUT IN REACH ARTICLE 57**

**Substance Name(s):** (±)-1,7,7-trimethyl-3-[(4-methylphenyl)methylene]  
bicyclo[2.2.1]heptan-2-one (4-methylbenzylidene  
camphor)

**EC Number(s):** 253-242-6

**CAS Number(s):** 36861-47-9

**Submitted by:** Germany

**Date:** 25 February 2016

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## GLOSSARY

AR	androgen receptor
3-BC	3-benzylidene camphor (1,7,7-trimethyl-3-(phenylmethylene)bicyclo[2.2.1]heptan-2-one, EC No. 239-139-9)
CMR	cancerogenic, mutagenic, toxic for reproduction
E2	17β-Estradiol
ER	estrogen receptor
GSI	Gonadosomatic index
hAR	human androgen receptor
hER	human estrogen receptor
HEK293	Human Embryonic Kidney 293 cells
HELN	transfected (ER) human cervix adenocarcinoma cell line
HSI	Hepatosomatic index
4-MBC	4-methylbenzylidene camphor ((±)-1,7,7-trimethyl-3-[(4-methylphenyl)methylene]) bicyclo[2.2.1]heptan-2-one, EC No. 253-242-6)
MCF-7	breast cancer cell line (Michigan Cancer Foundation-7)
NP	4-nonylphenol branched and linear
OP	4-tert-octylphenol (EC No. 205-426-2)
PBT	persistent, bioaccumulative and toxic
vPvB	very persistent and very bioaccumulative
PR	progesterone receptor
rtER	estrogen receptor of rainbow trout
SCCS	Scientific Committee on Consumer Safety
US EPA	Environmental Protection Agency of the United States of America
VTG	vitellogenin
WHO/IPCS	International Program on Chemical Safety of the World Health Organization

## PROPOSAL FOR IDENTIFICATION OF A SUBSTANCE OF VERY HIGH CONCERN ON THE BASIS OF THE CRITERIA SET OUT IN REACH ARTICLE 57

**Substance Name(s):** (±)-1,7,7-trimethyl-3-[(4-methylphenyl)methylene] bicyclo[2.2.1]heptan-2-one (4-methylbenzylidene camphor)

**EC Number(s):** 253-242-6

**CAS number(s):** 36861-47-9

- It is proposed to identify the substance as a substance of very high concern in accordance with Article 57(f) of Regulation (EC) 1907/2006 (REACH) because it is a substance with endocrine disrupting properties for which there is scientific evidence of probable serious effects to the environment which gives rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 REACH.

### Summary of how the substance meets the criteria set out in Article 57 of the REACH Regulation

The *in silico*, *in vitro* and *in vivo* data presented and discussed within this dossier as well as the weight of evidence from read-across to the structurally similar camphor substance 1,7,7-trimethyl-3-(phenylmethylene)bicyclo[2.2.1]heptan-2-one (3-BC), provide sufficient evidence to conclude that (±)-1,7,7-trimethyl-3-((4-methylphenyl)methylene) bicyclo[2.2.1]heptan-2-one (4-MBC, 4-methylbenzylidene camphor) acts via an endocrine mode of action and that this endocrine activity most likely leads to adverse effects in fish. Hence, 4-MBC fulfils the WHO/IPCS definition of an endocrine disruptor for the environment.

The specific mode of action of 4-MBC (estrogen receptor agonist and/or androgen receptor antagonist), the effects observed *in vivo* in fish and rodent species as well as the comparison of these effects with known endocrine disruptors and 3-BC acting via the same molecular mode of action provide strong evidence that the endocrine mediated effects of 4-MBC are of equivalent level of concern for the environment as those of PBT/vPvB and CMR substances. In detail, the following evidence of probable serious effects and reasons for their equivalent level of concern could be identified for 4-MBC:

- The identified main mode of action (estrogenic and/or antiandrogenic) of 4-MBC is comparable to that of 3-BC and known endocrine active substances like bisphenol A (EC No. 201-245-8) or ethinyl estradiol and already identified endocrine disrupting chemicals under REACH like nonylphenol branched and linear and 4-tert-octylphenol (EC No. 205-426-2).
- It may not be possible to derive a safe concentration limit of 4-MBC in the environment since 4-MBC acts via the same modes of action as many other environmentally relevant ED substances and hence mixture effects are very likely to occur. There are hints for further endocrine modes of action (antiprogesteric) at lower effect concentrations from *in vitro* studies.
- There is a high probability that 4-MBC can have irreversible and long lasting effects on populations and that even short term exposures during sensitive life stages of organisms can have adverse effects during the entire lifetime.

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- The specific mode of action (estrogenic and/or antiandrogenic) of 4-MBC and the data available for fish and rodent species point to a broad range of taxa that might be affected by exposure to 4-MBC in the environment. This is due to the fact that the estrogen and androgen receptor proteins are highly conserved across different species. Binding agonistically to the estrogen receptor and/or antagonistically to the androgen receptor was identified in various in vitro studies to be the molecular initiating event leading to the endocrine activity of 4-MBC. Mechanistic knowledge about invertebrate hormone receptors and a reproduction study with molluscs show that also invertebrate species might be affected by 4-MBC.
- There is a high likeliness that the effects are adverse not only for single organisms but also for populations and/or subpopulations in the environment.

Taking together the evidence presented in this dossier 4-MBC is a substance of very high concern according to REACH Art. 57 (f) owing to its endocrine disrupting properties, which lead to probable serious effects in intact organisms in the environment. The specific adversity of these effects demonstrates the equivalent level of concern compared to other substances of very high concern like PBT/vPvB and CMR chemicals. Hence, even though there are remaining uncertainties within the hazard assessment of 4-MBC the application of the precautionary principle is justified with regard to the probable serious effects to the environment from 4-MBC. Additionally, 4-MBC is not readily biodegradable and its relatively high log Pow of 5.92 fulfils the screening criterion for being bioaccumulative according to REACH Annex XIII. Hence there is also a probability for the occurrence of serious effects due to fate properties of 4-MBC (screening as potentially P and B).

**Registration dossiers submitted for the substance? No**

## PART I

### Justification

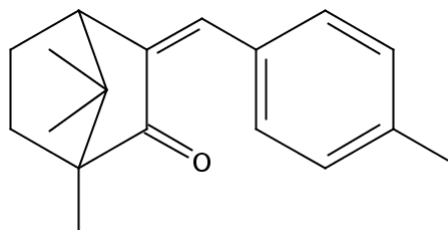
#### 1. Identity of the substance and physical and chemical properties

##### 1.1. Name and other identifiers of the substance

**Table 1: Substance identity**

<b>EC number:</b>	253-242-6
<b>EC name:</b>	(±)-1,7,7-trimethyl-3-[(4-methylphenyl)methylene]bicyclo[2.2.1]heptan-2-one
<b>CAS number (in the EC inventory):</b>	36861-47-9
<b>CAS number:</b> <b>Deleted CAS numbers:</b>	36861-47-9 ---
<b>CAS name:</b>	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-3-[(4-methylphenyl)methylene]-
<b>IUPAC name:</b>	(±)-1,7,7-Trimethyl-3-(4-methylbenzylidene)bicyclo[2.2.1]heptan-2-one
<b>Index number in Annex VI of the CLP Regulation</b>	---
<b>Molecular formula:</b>	C <sub>18</sub> H <sub>22</sub> O
<b>Molecular weight range:</b>	254.37 g/mol
<b>Synonyms:</b>	3-(4-Methylbenzylidene)-DL-camphor; 3-(p-Methylbenzylidene)-D,L-camphor; 4-MBC (4-methylbenzylidene camphor)

##### Structural formula:



## 1.2. Composition of the substance

**Name:** (±)-1,7,7-trimethyl-3-((4-methylphenyl)methylene)bicyclo[2.2.1]heptan-2-one

**Description:** organic

**Substance type:** multi-constituent including four stereoisomers

Table 2: Constituents

Constituents	Typical concentration	Concentration range	Remarks
(±)-1,7,7-trimethyl-3-[(4-methylphenyl)methylene]bicyclo[2.2.1]heptan-2-one EC: 253-242-6		80-100 % (w/w)	

## 1.3. Identity and composition of degradation products/metabolites relevant for the SVHC assessment

## 1.4. Identity and composition of structurally related substances (used in a grouping or read-across approach)

Table 3: Structurally related substance(s) identity

<b>EC number:</b>	239-139-9
<b>EC name:</b>	1,7,7-trimethyl-3-(phenylmethylene)bicyclo[2.2.1]heptan-2-one
<b>SMILES:</b>	<chem>C3=C(\C=C1/C2C(C(C1=O)(CC2)C)(C)C)C=CC=C3</chem>
<b>CAS number (in the EC inventory):</b>	15087-24-8
<b>CAS number:</b>	15087-24-8
<b>CAS name:</b>	-+
<b>IUPAC name:</b>	3-Benzylidene-1,7,7-trimethylbicyclo[2.2.1]heptan-2-one
<b>Index number in Annex VI of the CLP Regulation</b>	---
<b>Molecular formula:</b>	C17 H20 O
<b>Molecular weight range:</b>	240.35 g/mol
<b>Synonyms:</b>	3-Benzylidenecamphor (3-BC); 2-Bornanone, 3-benzylidene-; Benzylidenecamphor

**Substance type:** multi-constituent (four stereoisomers are possible)



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**Structurally related substance(s) formula:**

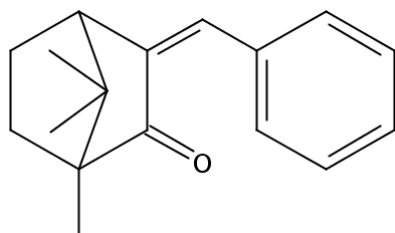


Table 4: Constituents of structurally related substance(s)

Constituents	Typical concentration	Concentration range	Remarks
1,7,7-trimethyl-3-(phenylmethylene)bicyclo[2.2.1]heptan-2-one, EC: 239-139-9		80-100 % (w/w)	

## 1.5. Physicochemical properties

Table 5: Overview of physicochemical properties

Property	Description of key information	Value	Reference/source of information
<b>Physical state at 20°C and 101.3 kPa</b>	Crystalline white powder		Material Safety Data Sheet from various suppliers
<b>Melting/freezing point</b>		121°C	Predicted data generated using the US Environmental Protection Agency's EPISuite (Mean or Weighted MP)
<b>Boiling point</b>		349.42°C	Predicted data is generated using the US Environmental Protection Agency's EPISuite™ (Adapted Stein & Brown method)
<b>Vapour pressure</b>		9.99E-5 Torr at 25 °C	Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02
<b>Density</b>		1.064±0.06 g/cm <sup>3</sup> at 20 °C, 760 Torr	Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02
<b>Water solubility</b>		0.1966 mg/l at 25 °C	Water Solubility Estimate from Log Kow (WSKOW v1.41)
<b>Partition coefficient n-octanol/water (log value)</b>		log Pow 5.92	KOWWIN v1.67 estimate

## **2. Harmonised classification and labelling**

The substance is not harmonised classified.

## 3. Environmental fate properties

### 3.1. Degradation

#### 3.1.1. Abiotic degradation

As the substance is not registered, no standard test on abiotic degradation of 4-MBC is available from registrations. Also from published studies no such data is available.

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 an hydroxyl concentration of  $1.5 \times 10^6$  hydroxyl radicals/cm<sup>3</sup>.

The atmospheric half-life of 4-MBC was estimated to be 1.443 hours, the overall hydroxyl rate constant was estimated to be  $8.897 \times 10^{-11}$  cm<sup>3</sup>/molec/sec<sup>3</sup>.

It is expected that photodegradation in air is not a relevant pathway for removal from the environment since it is assumed 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. Because of the adsorption potential of the substance it will predominantly bind to suspended organic matter and sediment which is supposed to decrease the tendency for photodegradation.

When used in sunscreen and other cosmetics, the substance is emitted directly to surface water and indirectly to wastewater, where the majority will adsorb to sewage sludge, which might be applied to agricultural fields. Only a negligible fraction will be available for photolytic degradation when the sludge is ploughed into soil.

#### 3.1.2. Biodegradation

As the substance is not registered, no screening or simulation tests for biodegradation are available from registrations.

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.

## **3.2. Environmental distribution**

### **3.2.1. Adsorption/desorption**

The following values for the soil adsorption coefficient of 4-MBC have been estimated by using KOCWIN v2.00 (US EPA, 2012): Koc: 12290 l/kg (Log Koc: 4.089) (MCI method) and Koc: 24840 l/kg (Log Koc: 4.395) (Kow method).

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

### **3.2.2. Volatilisation**

According to HENRYWIN v3.20 (US EPA, 2012) the Henry constant was determined to be 0.218 Pa×m<sup>3</sup>/mol indicating only little tendency for volatilisation.

### **3.2.3. 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.

## **3.3. Data indicating potential for long-range transport**

No data available.

## **3.4. Bioaccumulation**

The log Pow is estimated to be 5.92 (KOWWIN v1.68). 4-MBC therefore shows a high potential for bioaccumulation. This is supported by measured data in the environment (see Part II, section 9.1).

## 4. Human health hazard assessment

Not assessed. Supporting information for the environment hazard assessment is summarised in section 5.2

### ***PBT considerations regarding human health hazard assessment:***

Not relevant for this dossier

## 5. Environmental hazard assessment

### 5.1. Acute toxicity data - aquatic compartment

This chapter provides a short summary of acute toxicity test results in order to be able to compare between acute and chronic test results for 4-MBC.

There is an acute toxicity test with *Daphnia magna* according to OECD guideline 202 available. The five experimental concentrations ranged between 0.1 and 1000 µg/l. Fent et al. (2010) reports a 48-h-EC<sub>50</sub> of 0.56 mg/l (nominal). Sieratowicz et al. (2011) reports a 48-h-EC<sub>50</sub> of 0.80 mg/l (nominal) for an OECD 202 acute toxicity test with *Daphnia magna*. The concentrations used were 0.4, 0.8, 1.6, 3.2, 6.4 mg/l.

The exposure of *Desmodesmus subspicatus* with 4-MBC resulted in a growth inhibition with a 72h-E<sub>r</sub>C<sub>50</sub> of 7.66 mg/l and a 72h-E<sub>r</sub>C<sub>10</sub> of 0.81 mg/l (both nominal) (Sieratowicz et al. (2011)). The test was conducted according to OECD guideline 201 with concentrations of 0.5, 1.0, 2.0, 4.0, 8.0 mg/l.

## 5.2. Toxicity test results concerning endocrine disruption

### 5.2.1. General approach

As described in Art. 57 (f), a case by case assessment is needed to decide whether a substance is of equivalent level of concern due to its endocrine disrupting properties.

To be consistent with other Annex XV dossiers submitted so far for SVHC identification of endocrine disrupting substances, as a starting point the WHO/IPCs definition is used to describe whether 4-MBC is an endocrine disruptor. Thus, this chapter summarises data and presents a read-across scenario to the structurally similar compound 3-BC, which taken together provide evidence that 4-MBC acts via an endocrine mode of action and – as a consequence of this mode of action – exerts adverse effects in environmental species.

“An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations” (IPCS; cited in (European Commission, 1999)).

The information available is assessed based on the following questions:

- Does the substance influence the endocrine system?
- Are adverse effects observed likely to be a consequence of this alteration?

Information is summarised by organism groups in the following chapters, starting with a summary of available *in vitro* tests as supportive information.

The reliability categories used to assess the studies presented below are adapted from the Klimisch score. The reliability categories are defined as follows:

**R1** Reliable without restrictions: All reliability criteria are fulfilled. The study is well designed, performed and documented (not necessarily according to internationally adopted guide lines), and it does not contain flaws that affect its reliability.

**R2** Reliable with restrictions: The study is well designed and performed, but some minor flaws in the documentation are present.

**R3** Not reliable: Not all reliability criteria are fulfilled. The study has clear flaws in study design, performance and/or documentation.

**R4** Not assignable: Information needed to make an assessment of the study is missing (i.e. abstracts or secondary literature (books, reviews, etc.)).

### 5.2.2. *In silico* and *in vitro* tests

#### Non-Test Information

**Table 6: Non-test information concerning 4-MBC**

Method	Short Method description	Result	Description of results	References
<i>In silico</i>	Virtual screening of a 3d-structural database using pharmacophores of 17β-HSD3	+	Inhibits 17β-HSD2 at low micromolar concentration	Nashev et al. (2010)
QSAR prediction tool VirtualToxLab	Virtual screening of 3D structures against binding affinity to various	+ antiandrogenic	The prediction tool identified the AR as main target for 4-MBC	Vedani et al. (2009)

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	receptor proteins	(+) estrogenic	binding	
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Hypothalamus:Pituitary:Gonad (HPG) Axis

By virtually screening a 3D-structural database using pharmacophores of 17beta hydrosteroid dehydrogenase type 2 (17β-HSD2), Nashev et al. (2010)) has shown that 4-MBC is a potential inhibitor of 17β-HSD, an enzyme that metabolizes estrogens and androgens, in the low micromolar range.

***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 7: *In vitro* assays with 4-MBC**

(+ indicates a positive result; - indicates a negative result)

	Result	Description of results	Result positive control	References	Reliability
Estrogenicity:					
ERα and ERβ binding study (cellfree)	ERα: - estrogenic ERβ: + estrogenic	ERα: no binding observed up to 1mM ERβ: competitive binding (deliberation of radiolabeled E2) observed IC <sub>50</sub> = 35,3 μM	17β-Estradiol (E2): IC <sub>50</sub> = 2.1 nM	Schlumpf et al. (2004a)	2 – Acceptable, well-documented report
E-screen (MCF-7) (pS2 gene transcription)	+ estrogenic	Altering gene transcription: EC <sub>50</sub> = 1.90 μM	17β-Estradiol (E2): EC <sub>50</sub> = 4.80 pM = 0.0000048 μM	Heneweer et al. (2005)	4 – only short abstract available
E-screen (MCF-7)	+ estrogenic	REC <sub>10</sub> (Concentration showing 10 % of that of E2) = 6.3 μM	17β-Estradiol (E2) relative activity = 10 <sup>6</sup>	Matsumoto et al. (2005)	2 or 4 – publication in Japanese with tables in English
E-screen (MCF-7)	+ estrogenic	Stimulation of MCF-7 proliferation: EC <sub>50</sub> = 3.9 μM 50 % of E2 effect reached but no plateau was reached with highest test concentration	17β-Estradiol (E2): EC <sub>50</sub> = 0.00103 μM	Schlumpf et al. (2004a)	2 – Acceptable, well-documented report



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E-screen (MCF-7)	+ estrogen ic	Stimulation of MCF-7 proliferation: EC <sub>50</sub> = 3.9 µM  86 % of E2 effect is reached	17β-Estradiol (E2): EC <sub>50</sub> = 0.00103 µM  Estrogen antagonist ICI 182,780: Complete inhibition of the proliferative effect of 4-MBC	Schlumpf et al. (2001)	2 – Acceptable, well- documented report
E-screen (MCF-7)	+ estrogen ic	Pot. estrogenic activity or ability to stimulate cell proliferation: EC <sub>50</sub> = 24.14 µM	17β-Estradiol (E2)	Jimenez- Diaz et al. (2013)	2 – Acceptable, well- documented report

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	Result	Description of results	Result positive control	References	Reliability
Estrogenicity:					
hERα activation in a Yeast Estrogen Screen (YES) with additional enzymatic digestion of the yeast cells	+ estrogenic	Yeast hERα activation: EC <sub>50</sub> = 44.3 μM (8 % effect compared to E2)	17β-Estradiol (E2): EC <sub>50</sub> = 0.147 nM = 0.0000147 μM	Schmitt et al. (2008)	2 – Acceptable, well-documented report with clear dose-response relationship
Luciferase Gene Expression in HELN ERα cell lines	+ estrogenic	Binds to the estrogen receptor (ERα cell line) from 3 μM	17β-Estradiol (E2)	Gomez et al. (2005)	2 – Acceptable, well-documented report
Gene expression assay in stable ERα and ERβ reporter cell lines (HEK293cells)	+ estrogenic	Activation of transcription for hERα: IC <sub>50</sub> = 6.2 μM Activation for hERβ: IC <sub>50</sub> = 14 μM	17β-Estradiol Activation of transcription for hERα: IC <sub>50</sub> = 0.0021 μM Activation of transcription for hERβ: IC <sub>50</sub> = 0.083 μM	Schreurs et al. (2005)	2 – Acceptable, well-documented report
Gene expression assay in stable hERα and hERβ transfectants of HEK293 cells	? estrogenic	No induction of transcriptional activation		Schreurs and van der Burg (2002)	2 – Acceptable, well-documented report
Recombinant yeast systems carrying either a human estrogen (hERα) or androgen receptor (hAR)	- ? estrogenic	Very high concentrations used (up to 1000 μM)  Estrogenic response in the yeast hERα assay as well as Androgenic response in the yeast hAR assay: not detected  Anti-estrogenic response yeast hERα assay: EC <sub>50</sub> = 87.3 μM (181 % effect compared to 4-hydroxy-tamoxifen)  Antiandrogenic response in the yeast hAR assay: EC <sub>50</sub> = 0.118 μM (107 % effect of flutamide)	17β-Estradiol (E2) or 4,5-dihydro-testosterone (DHT)	Kunz and Fent (2006)	2 – Acceptable, well-documented report

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	Result	Description of results	Result positive control	References	Reliability
Androgenicity:					
AR-mediated gene-reporter activation assay in MDA-kb2 cells	- 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 <sub>50</sub> =0.136 nM  Antiandrogen: Flutamide in 0.5 nM DHT IC <sub>50</sub> = 3.62 µM	Ma et al. (2003)	2 – Acceptable, well-documented report
AR CALUX Bioassay	+ antiandrogenic	Repression of transpcription of hAR: IC <sub>50</sub> = 7.1 µM	Flutamide IC <sub>50</sub> = 0.5 µM	Schreurs et al. (2005)	2 – Acceptable, well-documented report
Progesterone activity:					
PR CALUX Bioassay	+ antiprogesterone	Repression of transpcription of hPR: IC <sub>50</sub> = 0.9 µM	RU486 IC <sub>50</sub> = 0.0049 µM	Schreurs et al. (2005)	2 – Acceptable, well-documented report
Human sperm: activation of the CatSper channel	+ antiprogesterone	EC <sub>50</sub> = 6.83 ± 2.26 µM (for comparison: 4-OP EC <sub>50</sub> = 5.93 ± 0.40 µM)	Inhibition by MDL12330A: 96.64 ± 2.83 %  (4-OP inhibition of MDL12330A: 62.49±23.49 %)	Schiffer et al. (2014)	2 – Acceptable, well-documented report
Thyroidal activity:					
TH-responsive luciferase-based reporter gene assay	+ thyroidal	Induction of T <sub>3</sub> -regulated gene expression: LOEC = 1 µM		Hofmann et al. (2009)	2 – Acceptable, well-documented report

## 1. Estrogenic activity

With regard to estrogenic activity the following tests are available:

- 5 MCF-7 cell proliferation assays analysing cell proliferation due to hER activation
- 2 gene expression test with human HEK293 cells transfected with hERalpha and hERbeta receptors
- 2 gene expression tests with yeast cells transfected with hERalpha
- 1 gene expression test with yeast cells transfected with rERalpha

Schlumpf et al. (2004a) conducted an E-SCREEN with MCF-7 human breast cancer cells. MCF-7 cells were trypsinized, plated into 96-well plates ((six wells)/concentration) at an initial density of 3000 cells per well in 100µL experimental medium and allowed to attach at 37 °C. After 24 h, another 100µL experimental medium containing either 4-MBC ( $10^{-4}$  to  $10^{-8}$  M, ethanol concentrations between 1.0 and 0.0001 %), or estradiol-17β (positive control, final concentrations  $10^{-8}$  to  $10^{-13}$  M; ethanol concentrations  $\leq 0.0001$  % (v/v)). No difference in proliferation rate was seen between control experiments with chemical free medium or with medium containing ethanol up to 1 %. Five independent experiments (with two plates per experiment) were run, each simultaneously with 3-BC, 4-MBC, and estradiol-17β as positive control. Experiments were terminated after 6 days of incubation by removing the media from the wells. 4-MBC activated cell proliferation in a dose-response manner, but test concentrations were not high enough to receive a full dose-response curve. The EC<sub>50</sub> was 3.99 µM and the maximum proliferation was 58 % of estradiol. The EC<sub>50</sub> for 17β-estradiol was 0.00103 µM and thus the relative potency of 4-MBC was 0.00026.

Schlumpf et al. (2001) conducted an E-SCREEN with MCF-7 human breast cancer cells. MCF-7 cells were trypsinized, plated into 24-well plates at an initial concentration of 40,000 cells/well. Cells were incubated with test compound (final concentrations 4-MBC:  $10^{-7}$ ,  $10^{-6}$ ,  $0.5 \times 10^{-5}$ ,  $10^{-5}$ ,  $0.5 \times 10^{-4}$  M), E2 as positive control ( $10^{-8}$ – $10^{-13}$  M), or chemical-free medium (control). Final concentrations of ethanol in culture medium were between 1.0 % and 0.001 % (v/v) with test compounds, and were  $\leq 0.0005$  % (v/v) with E2. No difference in the cell proliferation rate was observed in control experiments with chemical-free medium or medium with 1.0 % ethanol. Therefore, they used chemical-free medium as a control. They also tested antagonism by the pure antiestrogen ICI 182,780 (19) in MCF-7 cells exposed for 6 days to E2 (10 pM) or to 4-MBC (10 µM) in the presence or absence of 1, 10, or 100 nM ICI 182,780. They concluded that according to the maximum effect on cell proliferation in relation to E2, 4-MBC acted as partial agonist. The proliferative effects of 4-MBC and the positive control E2 were completely blocked by the pure estrogen receptor antagonist ICI 182,780.

Amongst others Gomez et al. (2005) conducted a Luciferase Gene Expression in HELN ERα cell lines. The human cervical epithelioid carcinoma HeLa cell line was chosen as the host cell line for the generation of stable reporter cells for screening substances that act via human ERα (hERα) and β (hERβ), as HeLa cells do not express ERs. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) without phenol red, supplemented with 6 % dextran-coated charcoal-treated fetal calf serum (DCC-FCS). DCC-FCS was prepared by mixing FCS with 1 % (w/v) charcoal Norit A and 0.1 % (w/v) dextran T70 for 30 min at 57°C. This same procedure was repeated once except that the temperature was only 40°C. Serum was then sterilized by filtration on a 0.22-µm filter (yield 95 %). Cells were seeded ( $10^4$  cells in 150 µl DCC-FCS-supplemented medium per well) in white opaque tissue culture 96-well plates. They were then incubated for 16 h with the tested chemicals. Blockage with the antiestrogen ICI was used to confirm the ER mediation of activities. For application on cell lines, organic solvents never exceeded

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0.1 % of the DCC-FCS supplemented culture medium. HELN constitutive cell line results were expressed relative to the 100 % value obtained in the presence of EtOH (0.1 %). The same value was obtained in the presence of E2 ( $10^{-8}$ M) or ICI 172.380 ( $10^{-8}$ M), as no ER receptor was present. Any variation in this reference activity was due to a nonspecific mechanism of activation or inhibition. Both HELN ER $\alpha$  and HELN ER $\beta$  cell lines exhibited transactivation of luciferase gene expression by E2. Transactivations were expressed relative to that obtained with  $10^{-8}$ M E2. The basal activities of HELN ER $\alpha$  and HELN ER $\beta$  were obtained with 0.1 % ethanol. These basal activities were slightly reduced in the presence of the pure antiestrogen ICI 172.380. The potency of E2 to activate reporter gene expression was similar for HELN ER $\alpha$  and ER $\beta$  cells, with their EC<sub>50</sub> being respectively 0.025 nM and 0.07 nM. As a result, 4-MBC, which had no effect on the HELN cell line, was estrogenic on the ER $\alpha$  cell line from 3  $\mu$ M.

Kunz and Fent (2006) investigated many UV filters for multiple hormonal activities *in vitro* in human receptor systems. They systemically analysed the estrogenic, antiestrogenic, androgenic and antiandrogenic activity of 4-MBC *in vitro* at non-cytotoxic concentrations with recombinant yeast systems carrying either a human estrogen (hER $\alpha$ ) or androgen receptor (hAR). For the estrogenic and androgenic assay procedure yeast assays were carried out within a type II laminar flow. 96-well optically flat-bottomed microtitre plates sealed with plate sealers were used in which a positive control with either 17 $\beta$ -Estradiol (E2) or 4,5-dihydrotestosterone (DHT) in triplicates and the test compounds in quadruplicates. It contained also a blank row with ethanol. The assessment of antagonistic activities was similar to agonistic ones with the adaption that E2 or DHT was added to the medium of the appropriate assay at a concentration that produced 65 % of the maximal response, followed by the addition of the UV filter and the antagonistic standards (4-hydroxytamoxifen 4HT or Flutamide FT). Here it was measured to what extent the UV filter inhibited the colour change induced by the natural ligand. 4-MBC did not induce hER $\alpha$  receptor activation up to 10 mM but resulted in a complete inhibition of the activity of E2 at the highest test concentrations tested with an EC<sub>50</sub> of 87  $\mu$ M. The antiestrogenic response to the yeast hER $\alpha$  transactivation assay was 181 % of the effect of 4HT. An androgenic response to the yeast hAR transactivation assay compared with the effect of DHT was not detected. There was an antiandrogenic response to the yeast hAR transactivation assay with 107 % of the effect of FT.

Schreurs et al. (2005) observed in an *in vitro* gene expression assay in stable ER $\alpha$  and ER $\beta$  cell lines the (anti)estrogenic activity of 4-MBC using HEK293 cells with 96-well tissue culture plates (6000 cells/ well) at a volume of 200  $\mu$ L per well. After 48h the medium was changed and the compounds to be tested (dissolved in ethanol) were added directly to the medium in a 1:1000 dilution. They analysed the stably transfected cells with either hER $\alpha$  or hER $\beta$ , and a 3xERE-tata-Luc-reporter gene construct. To measure antiestrogenicity, cells were incubated with both the chemical to be tested and an E2 concentration of 3 and 100pM for hER $\alpha$  and hER $\beta$ , respectively. This E2 concentration was the approximate EC50, taken from the dose-response curves. As positive controls for ER antagonism, they used 4-hydroxytamoxifen (OHT) and ICI 182,780. Both compounds could completely inhibit E2-induced transactivation at both receptor subtypes. All UV filters showed agonism toward hER $\alpha$ , and additionally, a number were found to show agonism toward hER $\beta$  as well. None of the UV filters showed antiestrogenic effects. The EC<sub>50</sub> for the activation of transcription toward hER $\alpha$  was 6.2  $\mu$ M and toward hER $\beta$  was 14  $\mu$ M.

Schreurs and van der Burg (2002) conducted a gene expression assay in stable hER $\alpha$  and hER $\beta$  transfectants of HEK293 cells and this is described elsewhere (J.G. Lemmen, C.E. van den Brink, J. Legler, P.T. van der Saag and B. van der Burg, *submitted for publication*). The cells were trypsinized and suspended in phenol red-free DF medium containing 30 nM selenite, 10  $\mu$ g/ml transferrin and 0.2 % bovine serum albumin, supplemented with 5 % dextran charcoal stripped FCS. Cells were plated in 96-well tissue culture plates. After 48 h the medium was changed and the compounds to be

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tested (dissolved in ethanol) were added directly to the medium in a 1:1000 dilution. After 24 h, the cells were scraped in lysis solution. A 25- $\mu$ L portion of cell lysate was transferred to a black 96-well plate to which 25  $\mu$ L luciferine substrate was added. Luciferase activity was measured in a scintillation counter for 0.1 min per well. After 96 h, tissue lysates were prepared and light activity was measured. 10  $\mu$ M 4-MBC caused nearly 60 % of the maximum E2 induced activation of transcription of the human estrogen receptor  $\alpha$ . At the same concentration the activation of the transcription of the human estrogen receptor  $\beta$  was significant (with 10 % of the E2 induction; at 100  $\mu$ M: 30 % of the E2 induction).

Jimenez-Diaz et al. (2013) investigated the potential estrogenic, antiestrogenic and androgenic activity of different UV-filters in an *in vitro* test, amongst others 4-MBC. They used an E-Screen bioassay with MCF-7 cells in the estrogenicity test and PALM cells for the gene expression bioassay examining the agonistic activity of hAR. 4-MBC showed no cytotoxic activity in the concentration range tested (0.01 – 10  $\mu$ M). The potential estrogenic activity or ability to stimulate cell proliferation on MCF-7 cells of 4-MBC was characterised using the E-Screen bioassay. E2 was used as a positive control (0.1 – 1000pM) but results were not shown. 4-MBC increased the number of viable cells in a dose dependent manner, but test concentrations were not high enough to receive a full dose-response curve. The maximum increase was 2.8 fold, compared with control treated cells (hormone-free medium). The EC<sub>50</sub> was 24.1  $\mu$ M. In comparison, 3-BC resulted in a maximum increase by 4.5 fold (full dose-response curve) and an EC<sub>50</sub> of 1.7  $\mu$ M. As some reports suggested an anti proliferative activity for some UV filters, The authors examined also the potential antiestrogenic activity. All compounds tested failed to antagonise E2-induced proliferation in MCF-7 cells up to 10  $\mu$ M.

Schmitt et al. (2008) conducted a Yeast Estrogen Screen (YES) and two sediment assays with the freshwater invertebrates *Lumbriculus variegatus* and *Potamopyrgus antipodarum*. The Yeast Estrogen Screen (YES) was conducted in 96-well microtiter plates with eight replicates for each treatment. The plates included a blank, a negative control, a full concentration range of the positive control 17 $\beta$ -estradiol (E2, 3 pM – 0.1  $\mu$ M) and different test concentrations of 4-MBC (0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300  $\mu$ M). In the YES, 4-MBC resulted in only 8 % response at the two highest test concentrations. Although authors calculated an EC<sub>50</sub> value of 44.3  $\mu$ M this result should be questioned as it describes the half maximal activation compared to a maximal activation of 8 %.

**In summary** the cell free receptor binding studies seems to indicate that 4-MBC is not able to replace E2 at the hER $\alpha$  but does replace E2 at the ER $\beta$ . On the other hand cell based test results unambiguously show that 4-MBC – or its metabolites - is an ER agonist *in vitro* and that it activates both the human ER $\alpha$  and ER $\beta$  at almost similar concentrations. Regarding the metabolism of 4-MBC, the Scientific Committee on Consumer Safety (SCCS) opinion summarises studies from rats and humans indicating that 3-(4-carboxybenzylidene) camphor and 3-(4-carboxybenzylidene)-6-hydroxy camphor are the major metabolites of 4-MBC (SCCS, 2008). Thus, via metabolism two active compounds can be formed in cellular systems, which might modulate the observed estrogenic effects of the parent compound 4-MBC.

In combination with the available knowledge about the 4-MBC metabolism, the presented *in vitro* results indicate that the estrogenic activity of 4-MBC might be caused mainly by its two major metabolites, while 4-MBC itself may exhibit antiestrogenic activity by binding to the ER $\alpha$  at a different binding site compared to the E2 binding cavity however at moderate concentrations (EC<sub>50</sub> 83.3  $\mu$ M reported for antiestrogenic activity in Kunz et al. (2006)) .

All cell based tests showed positive results i.e. activation of the receptors or cell proliferation due to the activation of ER systems. The strength of effects differ between

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the tests and the cell systems used. This can be explained by the test design if metabolism is taken into account.

The highest receptor activation was observed in the MCF cell proliferation tests. All MCF cell tests showed full dose-response curves and Schlump et al. (2001) could show, by coinubation with a known ER antagonist, that the observed effect was ER mediated.

Similar results were obtained by Schreurs et al. (2005) in hER transfected HEK293 cells (gene expression test). Results of this test show that exposure to 4-MBC activates both ER $\alpha$  as well as ER $\beta$  at almost similar test concentrations (6.2 and 14  $\mu$ M, respectively). The slightly lower sensitivity compared to the MCF cells is in line with the observation that 4-MBC and/or its metabolites can bind to both ER $\alpha$  and ER $\beta$  as MCF cells exhibit both ER $\alpha$  and ER $\beta$  receptors and thus, proliferation is a result of activation of both types of receptors.

Both cell lines tested must be considered as highly metabolic active cells since they are derived from human cancer tissue. Thus, it is plausible that in these cell lines the metabolites of 4-MBC are produced in a higher rate compared to the yeast cells and thereby the difference in sensitivity of the test system can be explained.

Results by Kunz et al. (2006a) and Schmitt et al. (2008) using recombinated yeast cells indicate that yeast cell based assays are less sensitive than the assays using human cell lines, since EC<sub>50</sub> values of 4-MBC were higher in yeast cells compared to those obtained with HEK and MCF-7 cells. In addition, the maximal inducible effect compared to E2 was lower in the yeast assays (no and 8 % effect compared to E2, respectively) compared to the human cell lines (100 %).

This finding supports the assumption that the strong estrogenic activity of 4-MBC observed in the MCF-7 assays is mainly caused by its main metabolites and hence governed by the metabolic activity of the test system used. Due to the lower metabolic capacity of the yeast cells and the shorter test duration it is plausible that a lower percentage of metabolites is produced and that thus the overall activity is lower. This is in line with the significant antiestrogenic activity observed by Kunz et al. (2006a). The significantly submaximal dose-response curve observed by Schmitt et al. (2008) and the absence of an estrogenic effect reported by Kunz et al. (2006) may be explained by a combination of the estrogen-agonistic mode of action of the metabolites, which are not or only marginally formed in the yeast cells and the estrogen-antagonistic mode of action of 4-MBC itself.

The observed antiestrogenic activity of 4-MBC seem to be contradictory at a first glance. While Schreurs et al. (2005) found no antiestrogenic activity in transfected HEK cells, Kunz and Fent (2006) observed an inhibition of E2 induced receptor activation in a yeast system at 83.3  $\mu$ M and a very high efficacy (181 %) compared to the reference substance. This could be explained by the fact that HEK cells are metabolically more active than the yeast cells. Thus, one possible explanation could be, that the estrogenic activity is caused by the metabolites of 4-MBC, which are predominately active in the HEK cells while the antiestrogenic activity in the yeast cells is caused by a high concentration of unmetabolised 4-MBC itself.

In conclusion, 4-MBC itself seems not to be able to bind to the E2 binding site of hER $\alpha$  and cause its activation but it may have antiestrogenic activity although at moderate concentrations. Whether it shows estrogenic or antiestrogenic activity by binding to the E2 binding site of the hER $\beta$  remains unclear. Results from the studies described above support the hypothesis, that the observed estrogenic activity of 4-MBC is caused by its two main metabolites. Results show, that 4-MBC has estrogen receptor agonistic activity toward hER $\alpha$  and hER $\beta$  as well as towards rtER $\alpha$  after entering metabolically active cell systems.

## 2. Androgenic activity

With regard to androgen activity the following results are available:

- 2 AR-mediated gene-reporter activation assays in cells.
- 1 gene expression test with yeast cells transfected with hAR (AR CALUX Bioassay)

No androgenic or antiandrogenic effect *in vitro* was seen in a study by Ma et al. (2003). The studies conducted by Schreurs et al. (2005) and Jimenez-Diaz et al. (2013) revealed a strong antiandrogenic activity of 4-MBC.

In the following some studies are described in more detail:

Ma et al. (2003) studied the potential actions of 4-MBC on androgen receptors (AR) in the human breast carcinoma cell line MDA-kb2. MDA-kb2 cells were trypsinized and seeded into 96-well plates at a density of about  $1 \times 10^4$  cells/well with 100  $\mu$ L media/well using a multichannel pipettor. After the cells had attached, medium was removed and replaced by dosing medium. A negative as well as a solvent control (1 % ethanol) and 10nM DHT as a positive control (0.1 or 0.5 nM for testing AR antagonists) were used. 1 nM to 10  $\mu$ M 4-MBC showed no agonistic activity and did also not inhibit AR activation by DHT (no antiandrogenic activity).

Schreurs et al. (2005) observed *in vitro* the antagonistic activity of 4-MBC toward the androgen receptor (AR) and progesterone receptor (PR). They used AR and PR CALUX bioassays with 96-well tissue culture plates (6000 cells/ well) at a volume of 200  $\mu$ L per well. After 48h the medium was changed and the compounds to be tested (dissolved in ethanol) were added directly to the medium in a 1:1000 dilution. The  $IC_{50}$  value for repression of transcription of hAR in AR CALUX cells by 4-MBC was 7.1  $\mu$ M. The  $IC_{50}$  value for repression of transcription of hPR in PR CALUX cells by 4-MBC was 0.9  $\mu$ M. The authors also discussed differences between their findings and the results by Ma et al. (2003). They explained that Ma et al. (2003) did not find antiandrogenic effects for 4-MBC. This may be caused by the fact that Ma et al. (2003) used an MDA-kb2 cell line containing low endogenous AR and GR levels. This is compared to Schreurs et al. (2005) who used an U2-OS cell line overexpressing AR which is probably more selective and sensitive for measuring AR interaction.

The potential androgenic and antiandrogenic activity via hAR using PALM cells was also investigated by Jimenez-Diaz et al. (2013). In this cell line, the synthetic androgen R1881 exhibits strong androgenic activity. None of the studied UV-filters showed androgenic activity in the concentration range of 0.01 – 10  $\mu$ M. When antagonistic activity was tested, only 4-MBC proved to be a potent hAR antagonists at 10 $\mu$ M concentration, strongly inhibiting the luciferase activity induced by 0.2 nM of R1881.

In summary 4-MBC did not show any androgenic activity in the tests described above up to 10  $\mu$ M. With regard to antiandrogenic activity, results were ambiguous. While it showed antiestrogenic activity in two tests (Schreurs et al. (2005) and Jimenez-Diaz (2013)) with  $EC_{50}$  values of 7.1 and 10  $\mu$ M, respectively, it was not antiandrogenic in a third test up to 10  $\mu$ M (Ma et al. (2003)).

## 3. Progesterone activity

With regard to progesterone activity the following results are available:

- 1 gene expression test with U2-OS cells containing a 3xPRE-TAT-Luc-reporter construct in combination with a hPR expression plasmid



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- 1 test with human sperm loaded with the  $\text{Ca}^{2+}$  indicator Fluo-4 and the  $\text{pH}_i$  indicator BCECF

Schreurs et al. (2005) studied the effects of 4-MBC and other chemicals like 3-BC at the human progesterone receptor by using the PR CALUX bioassay. ORG2058 was used as a stable PR agonist, while RU486 (Mifepristone) was used as a control for PR-antagonism. 0.03  $\mu\text{M}$  ( $\text{EC}_{50}$ ) ORG2058 was used for the measurement of antiprogestagenic activity. 4-MBC repressed the transcription of hPR in PR CALUX cells with an  $\text{IC}_{50}$  of 0.9  $\mu\text{M}$  (for RU486 the  $\text{IC}_{50}$  was 4.9  $\mu\text{M}$  with a similar dose-response curve as 4-MBC). There was no difference between 4-MBC and 3-BC concerning the potency. Fent (2015) reported in Table 3 of the publication some effects of RU486 on zebrafish: at 5 ng/l fecundity increase (21d, females) (Bluthgen et al. (2013a)), at 39 ng/l transcriptional effects in males after 21d, at 3 ng/l transcriptional effects in F1 embryos after 5d (Bluthgen et al. (2013b) and at 2 ng/l transcriptional effects on embryos after 48 to 144 h occurred (Zucchi et al. (2012)). The antagonistic effects of all of the compounds tested were reversed by coincubation with excess ORG2058 (100 times the  $\text{EC}_{50}$  value). This shows the specificity of the response. According to Fent (2015) progesterone receptor ligands eliciting progestonic activity may activate and/or interfere with genomic and non-genomic actions, including oocyte maturation and sperm motility (see also Murack et al. (2011)).

Schiffer et al. (2014) investigated the direct action of 4-MBC and also other chemicals like 3-BC and 4-octylphenol on (human) sperm through activation of the calcium-channels on sperm cells (CatSper). They used 384-microtiter plates for monitoring  $[\text{Ca}^{2+}]_i$  in human sperm. The injection of progesterone into the wells evoked a rapid, transient increase in  $[\text{Ca}^{2+}]_i$  followed by a slow, sustained elevation. Schiffer et al. (2014) demonstrated that the assay reliably differentiates between “active” and “inactive” chemicals. For instance bisphenol A (EC No. 201-245-8) did not affect  $[\text{Ca}^{2+}]_i$ . 4-MBC evoked a rapid biphasic  $\text{Ca}^{2+}$  increase at 0.1 and 1  $\mu\text{M}$ , whereas at 10  $\mu\text{M}$ , the  $\text{Ca}^{2+}$  signal was more sustained. The signal amplitude increased in a dose-dependent fashion. They also used the CatSper inhibitor MDL12330A to examine whether EDC-induced  $\text{Ca}^{2+}$  signals involve CatSper. MDL inhibited  $\text{Ca}^{2+}$  signals evoked by 4-MBC (and also by 3-BC and nonylparaben for instance). Therefore they concluded that 4-MBC acts primarily via activation of CatSper. According to Brenker et al. (2012) – in human sperm – progesterone and prostaglandins (two important ingredients of the oviduct) directly activate CatSper channels without involving classical nuclear receptors or G protein-coupled receptors (GPCRs). The sperm-specific CatSper channel controls the intracellular  $\text{Ca}^{2+}$  concentration and thereby the swimming behaviour of sperm.

Since progesterone is an endogenous ligand for the activation of calcium-channels on sperm cells, this is also relevant for other vertebrates than humans (e.g. fish). The interference of environmental chemicals with sperm motility through progesterone path has for example been demonstrated in several fish species (Murack et al. (2011); Thomas and Doughty (2004)). As demonstrated in Rurangwa et al. (2001) the reduction in sperm motility is correlated with decreased fertilisation rates in fish.

### 4. Thyroidal activity

With regard to thyroidal activity the following results are available:

- 1 TH-responsive luciferase-based reporter gene assay

4-MBC can also affect the thyroid system *in vitro*, by binding to the thyroid receptor (Hofmann et al. (2009)).

Hofmann et al. (2009) conducted  $\text{T}_3$  reporter gene assays. For each experiment,  $2.4 \times 10^7$  HepG2 cells were seeded into T150 flasks and grown overnight. The cells were

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transfected with 45 µg p(DR4)<sub>2</sub>-SV40-*luc*+ and 15 µg pRS-rTRα<sub>1</sub> or pRS-hTRβ<sub>1</sub> using the PolyFect reagent. After 8 h incubation the transfected cells (1×10<sup>5</sup> cells per well) were transferred into white 96-well luminescence plates and incubated for 16 h in DMEM/F12 containing 10 % FBS. Then cells were washed and incubated in DMEM w/o phenol red and containing no FBS for one hour. The medium was exchanged for fresh plain DMEM w/o phenol red and the cells of separate culture wells were incubated with different concentrations of test compounds ranging from 10<sup>-11</sup> to 10<sup>-5</sup>M, or with solvent control (0.05 % DMSO in DMEM w/o phenol red) for 24 h. The screening of ED was carried out in two types of assays, an activation assay and a T<sub>3</sub>-competition assay. For activation assays, the cells of different culture wells were exposed to a range of the test compound concentrations alone. In competition assays, the cells of separate culture wells were incubated with different concentrations of test substances in the presence of 1nM T<sub>3</sub>. Lysis of cells was done directly in culture plates by applying 20 ml of passive lysis buffer (Promega) and shaking on a microplate shaker for 15 min at ambient temperature. The luciferase assays were carried out using the luciferase assay system from Promega and luminescence of the culture wells was measured with a microplate luminometer. Each experiment was done at least in quadruplicate culture wells and repeated three times. The assay was optimized in terms of the cell model, culture conditions, transfection conditions, and assay duration. As a result of the T<sub>3</sub> reporter gene screening assay 4-MBC stimulated the reporter transcription with a LOEC of 1 µM and acted antagonistically in the competition assay at a concentration of 10 µM.

### Conclusion on the *in vitro* data:

**Estrogenic activity:** In summary all cell based tests showed positive and dose dependent estrogenic results i.e. activation of the receptors or cell proliferation due to the activation of ER systems after incubation with 4-MBC. One proliferation study using MCF-7 cells could furthermore demonstrate that the observed proliferative effect of 4-MBC was ER mediated. The strengths of the effects observed differ between the test systems but this can be explained with different metabolic activities of the cells used in the different tests and the literature supports the assumption that the two main metabolites of 4-MBC are formed within active cells and are responsible for the observed estrogenic effects.

**Androgenic activity:** There are contradicting results for antiandrogenic activity of 4-MBC with two studies showing a clear antiandrogenic effect and one study showing no effect up to the highest tested concentration of 10 µM of 4-MBC. This can be explained by the use of different cell lines with different levels of endogenous AR.

**Progesterone-like activity:** Two very different studies investigated the progestonic or progesterone-like activity of 4-MBC. In summary 4-MBC shows antagonistic activity, which also could be reversed by coincubating with a stable PR agonist. Similar to progesterone 4-MBC activates the calcium channels on sperm cells (CatSper) which affects their swimming behaviour. Reduced sperm motility is correlated with decreased fertilisation rates in fish.

Additionally, one study showed the potential of 4-MBC to interact with thyroidal pathways on the *in vitro* level. Transfected cells showed an increase in T<sub>3</sub> reporter gene transcription after a 8 h incubation with 4-MBC. The same study found that 4-MBC acted antagonistically in a thyroid receptor binding assay compared to the natural ligand T<sub>3</sub>. However, owing to the lack of further data the antiprogesteric *in vitro* effects as well as the *in vitro* hints for thyroid disrupting potential will not be taken into consideration during the following assessment of the effects observed *in vivo*. But in the overall conclusion this evidence for a possible multi pathway endocrine activity of 4-MBC will be taken into account.

### 5.2.3. *In vivo* tests

#### **Approach used for assessing the endocrine activity in fish:**

In this chapter mainly the effects of 4-MBC on fish were described. 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 on standardised test guidelines for evaluating chemicals for endocrine disruption (OECD, 2012). 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 and
- Effects on apical endpoints that are considered to provide evidence that a substance exerts adverse effects owing to its endocrine mode of action.

#### *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 estrogen mode of action.

One of the most common biomarkers indicating an estrogenic or androgenic endocrine mode of action is vitellogenin (VTG). Vitellogenin is naturally produced by female fish as a precursor of yolk proteins that are incorporated in eggs (IPCS, 2002). Induction of vitellogenin in female and (more pronounced) in male fish is a known indicator of an estrogen 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 may also be influenced by other modes of action.

Changes in the gonadosomatic index (GSI) may provide additional information about the gonad maturation and spawning readiness (OECD, 2004). 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 estrogenic substances (OECD, 2004). However, care must

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be taken as the GSI is highly dependent on the individual fish (frequent spawners) or seasonal gonadal stage (seasonal breeders)<sup>2</sup>.

In addition, the following apical endpoints are considered to be indicators of an estrogen receptor agonist mode of action according to the OECD guidance document (OECD, 2012).

- 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 estrogenic or antiandrogenic 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 uro-genital papillae in male zebrafish was shown to be significant after exposure to estrogenic substances (Kendall et al. (1998); OECD (2004)).

Change of sex-ratio towards females is a known result of estrogen 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 estrogen 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 gonochorist that naturally develops either male or female gonads and sex is naturally not changed after gonadal development. Hormonal influence (especially of female hormones) in this species starts very early during pre-hatch development (OECD, 2004) 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 (2004)).

Secondary sex characteristics and sex-ratio are apical endpoints that are considered to be estrogen specific.

Other endpoints such as growth, sexual maturity, reproduction and behaviour are known to be sensitive to estrogens or antiandrogens (IPCS, 2002; OECD, 2004; 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 estrogen receptor agonists in fish full life cycle tests (Knacker et al. (2010)).

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<sup>2</sup> The size of the sexual gonads (testis 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.

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Thus, in combination with indicators of endocrine activity they provide evidence of estrogen 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.** Table 8 summarises endpoints that are considered indicators of estrogenic activity and may be affected as a result of this activity *in vivo*.

**Table 8: Summary of endpoints that are considered during analysis of fish data**

Endpoints indicating an estrogen receptor agonist mode of action	Endpoint considered to be sensitive to an estrogenic mode of action <i>in vivo</i>
<ul style="list-style-type: none"> <li>• Vitellogenin induction in males</li> <li>• increased proportion of spermatogonia (early sperm cells), presence of testis-ova, increased testicular degeneration, interstitial (Leydig) cell hyperplasia/hypertrophy in males</li> <li>• increased oocyte atresia, perfollicular cell hyperplasia/hypertrophy, decreased yolk formation (aromatase inhibition), changes in gonadal staging in females</li> <li>• Depression of male secondary sex characteristics in fathead minnow or medaka and induction of female secondary sex characteristics such as uro-genital papillae in zebrafish</li> <li>• Female biased phenotypic sex-ratio during sexual development.</li> </ul>	<ul style="list-style-type: none"> <li>• Female biased phenotypic sex-ratio during sexual development especially in medaka</li> <li>• Reproduction (fecundity, fertility, number of males or females with reproductive success)</li> <li>• Spawning behaviour</li> <li>• Growth of offspring</li> </ul>

Table 9 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 activity.

**In vivo assays providing data about selected endocrine mechanisms/ pathways and adverse effects of 4-MBC:**

**Table 9: In vivo assays about the endocrine mechanism**

Species Life stage/ duration	Concentration/ test condition/ tested substance/ solvent	Vitellogenin	others	Result positive control	References	Reliability
<i>Oryzias latipes</i> 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)		positive control: 17β-Estradiol (E2)	Inui et al. (2003)	2 – Acceptable, well-documented report
<i>Pimephales promelas</i> 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 or 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 steel 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

Inui et al. (2003) examined also the estrogenicity of 4-MBC using adult male medaka (*O. latipes*) in regard to production of vitellogenin and choriogenin (CHG). They used a VTG enzyme-linked immunosorbent assay (ELISA) system exposing for 7 days the daily fed fish with 0.039 – 0.39 – 3.9 mM 4-MBC (9.92, 99.2 and 992 mg 4-MBC/L). They found an increase of VTG plasma concentration in medaka after the exposure to any concentration of 4-MBC and also increases in mRNA expression levels of VTG subtypes VTG-1 and VTG-2, and CHG subtypes CHG-L and CHG-H, in liver (compared with a non-treated control). They detected also increased mRNA expression levels of estrogen receptor (ER  $\alpha$ ). The LOEC in this study was 0.039 mM (9.9 mg/l).

Kunz et al. (2006) conducted a 14-day fish experiment using juvenile, sexually undifferentiated fathead minnows (*Pimephales promelas*), between 2 and 3 months of age. A 16/8 h light/dark cycle was used and the temperature constituted  $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ . 10 randomly selected fish were each placed in a 10L-stainless steel tank and exposed for 14 days. Two controls, solvent control (1mL ethanol in 10 L of water) and positive control for estrogenic activity (100 ng/l E2) were included. The nominal concentrations of 4-MBC were 10, 100, 500 and 1000  $\mu\text{g/l}$  (real: 9, 415 and 753 mg/l). The concentrations were analytically assured. The pH and oxygen saturation ranged between 7.2-7.9 and 6.5-8.3 mg/l, respectively, throughout the exposure period. Vitellogenin was analysed using a quantitative heterologous carp enzyme-linked immunosorbent assay (commercially available quantitative carp Vitellogenin ELISA kit (Biosense)). One result of the rER $\alpha$ -evaluation was that 4-MBC was inactive to  $2.5 \times 10^{-2}$  M. 4-MBC affected the survival in the highest test concentration of 753  $\mu\text{g/l}$  (2 fish died – survival 80 %) on day 8 in the *in vivo* test and the experiment was stopped. For all control, solvent control and E2 fish no mortality was observed. For 415 and 753  $\mu\text{g/l}$  of 4-MBC the length and weight gain was significantly dose-related decreased. 4-MBC showed no estrogenicity in this experiment.

#### Summary of *in vivo* endocrine relevant information in fish

In fish, 4-MBC at high concentrations induces estrogen-responsive gene products including vitellogenin (Inui et al. (2003)). Kunz et al. (2006) found no estrogenic activity of 4-MBC in a test with juvenile fish.

In summary, the available *in vivo* data in fish only provide evidence for a weak estrogenic mode of action. Interestingly, the data from Kunz et al. (2006) seem to show that 4-MBC and/or its metabolites in contrast to 3-BC cannot bind to the fish rER $\alpha$  in the concentration range tested. This difference in binding potential might explain the observed low effects in fish compared to the clear effects of 3-BC in the same studies.

#### **Supporting information from mammalian toxicity tests:**

A summary of *in vivo* studies examining mammals is cited from Hass et al. (2012), page 11 and 35:

*"The evidence of estrogenic activity from short term in vivo studies is conflicting, however increases in uterine weights and histopathological effects in uterus and vagina have been observed after longer exposure scenarios. Furthermore a large number of endocrine sensitive endpoints such as reproductive organ weights, timing of sexual maturation and impaired sexual behaviour have been shown to be affected in the developmental studies. Also, changes in LH, FSH and GnRH levels have been observed."*

4-MBC has been shown to exert estrogenic effects *in vivo* in the uterotrophic assay (Schlumpf et al. (2001; 2004a); Tinwell et al. (2002)). However, when Ashby et al. (2004) tried to repeat their finding of estrogenic activity in the uterotrophic assay reported in Tinwell et al. (2002), they were not able to do so, and concluded that the

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effects they had observed previously were due to low uterine weight in their previous control group. Increases in uterine weights have also been observed after longer exposure scenarios, as three months of dosing of adult female rats caused significant increases in uterine weights (Seidlova-Wuttke et al. (2006a,2006b)). The effects on uterus weight were not very marked but histopathological effects in uterus and vagina were observed, and the 4-MBC dosing also decreased T4 levels and increased TSH and LH in serum. Schmutzler et al. (2004) showed that 4-MBC could also affect the thyroid system as T4 levels were decreased and TSH levels increased after 3 months of dosing. Carou et al. (2008) treated adult male rats with sc injections of 4-MBC at low doses (2-20 mg/kg/day) and found that significant decreases in the LH and FSH serum concentration, and decreases in hypothalamic GnRH release. They also performed developmental studies where doses of 100 mg/kg/day caused changes in LH, FSH and GnRH levels and earlier vaginal opening in females.

A number of studies examining the developmental toxicity of 4-MBC have also been performed by Schlumpf and coworkers. Different parts of the studies have been reported in different publications (Schlumpf et al. (2004; 2008 a,b); Durrer et al. (2005 ; 2007 ); Maerkel et al. (2005; 2007); Hofkamp et al. (2008); Faass et al. (2009)), making it difficult to evaluate exactly how many studies have been performed and when which doses and endpoints have been investigated.

The effects of perinatal 4-MBC seen in these studies have in male offspring included alterations in reproductive organ weight at birth, on day 14 and in adulthood, delayed sexual maturation, and altered gene expression in prostate and brain, while effects observed in females include increased uterus weights, changes in gene expression of estrogen regulated genes in brain and uterus, as well as strongly impaired sexual behaviour. The LOAEL for most of these effects was 7 mg/kg/day, and the NOAEL 0.7 mg/kg/day.

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

Schlumpf et al. (2001) performed two uterotrophic assays with immature 21-day old LE rats (n=4-19). In the first assay, they were dosed with 66, 119, 211, 337 or 402 mg/kg/day in the feed for four days. In the other assay 4-MBC was dissolved in olive oil, and applied dermally to the skin of hairless rats for 6 days (n=4-11). In the feeding study a significant increase in uterine weight was seen from 119 mg/kg/day and above and the ED<sub>50</sub> was 309 mg/kg/day. A dermally applied dose of which was calculated to correspond to 37 mg/kg/day), significantly increased uterine weight in the other assay.

Tinwell et al. (2002) conducted two uterotrophic assays in immature Wistar rats. 19-20 day old rats (n=12) were either dosed orally with 500 or 800 mg/kg/day for three days or subcutaneously using doses of 500 or 1000 mg/kg/day for three days. Increased uterine weight was apparent in both studies at both dose levels, making 500 mg/kg/day a LOAEL while no NOAEL was found.

Ashby et al. (2004): An attempt to repeat their previously reported uterotrophic effects of 4-MBC, (reported in the paper by Tinwell et al. (2002) failed. Further evaluation led the authors to conclude that 4-MBC is uterotrophic only when the control uterine weights are at the low end of their normally encountered range.

Siedlova-Wuttke et al. (2006): Adult ovariectomized SD rats (n=11) were treated with 4-MBC in the feed for 12 weeks at doses of approximately 223 and 1023 mg/kg/day. The high dose increased uterine weight and in the uterus and vagina both doses of 4-MBC affected histopathology. In the bone, 4-MBC shared the antiosteoporotic effects of E2 but the mechanism of action of 4-MBC appeared to be different than that of E2.



**Table 10: Supporting *in vivo* assays with mammals**

Short Method description	Result	Description of results	Result positive control	References	Reliability
<p>Uterotrophic assay (immature LE rats)</p> <p>OECD 440</p> <p>21-d old LE rats</p> <p>66, 119, 211, 337, 402 mg/(kg*d) in feed/ duration: 4 days</p>	+ estrogenic	<p>Increased uterine weight</p> <p>ED<sub>50</sub>= 309 mg/kg/d; LOEC= 119 mg/kg/d</p> <p>Maximum increase as percent of EE: 35.5 %</p> <p>No signs of general toxicity</p>	<p>Ethinylestradiol-17α (EE):</p> <p>increased uterine weight</p> <p>ED<sub>50</sub>= 0.000818 mg/(kg d); LOEC= 2 mg/(kg d)</p>	Schlumpf et al. (2001)	2 – Acceptable, well-documented report
<p>Uterotrophic assay (immature LE rats)</p> <p>19-20-d old female Alpk:APfSD rats</p> <p>500 or 800 mg/(kg*d) in feed/ duration: 3 days OR 500 or 1000 mg/(kg*d) subcutaneous</p>	+ estrogenic	<p>Increased uterine weight</p> <p>LOAEL = 500 mg/kg/d</p>	DES (5 µg/kg)	Tinwell et al. (2002)	2 – Acceptable, well-documented report
<p>Uterotrophic assay (immature LE rats)</p>	+/- estrogenic	<p>Increase in uterine weight was only significant when controls are at the low end of their normally encountered range</p>		Ashby et al. (2004)	2 – Acceptable, well-documented report
<p>Developmental toxicity study:</p> <p>LE rats</p> <p>Expo: parents 10 weeks before mating + dams throughout gestation and lactation + offspring until</p>	<p>+ estrogenic</p> <p>+ thyroidal activity</p>	<p>Proliferative effect on prostate growth: LOEC = 7 mg/kg/d</p> <p>↓ Relative testis weight: LOEC = 7 mg/kg/d</p> <p>Delayed start of puberty in males: LOEC = 7 mg/kg/d (no nutritional effects)</p> <p>↓ Prostate weights and altered gene expression in prostate and hypothalamus</p>		<p>Different parts of the studies reported in different publications: (Durrer et al. (2005;2007) Faass et al. (2009); Hofkamp et al. (2008); Maerkel et al. (2005;2007);</p>	4 – Only summary of various studies is provided.

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<p>adulthood</p> <p>0.7, 7, 24 and 47 mg/kg/day per feed</p>		<p>Adult females: ↑uterus weights and changes in gene expression of estrogen regulated genes in brain and uterus</p> <p>Strongly impaired sexual behaviour of females offspring: LOEC = 7 mg/kg/d</p> <p>↑ Thyroid weights, altered levels of TSH and T<sub>3</sub></p>		<p>Schlumpf et al. (2004a;2004b; 2008a; 2008b;))</p>	
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A number of developmental studies of 4-MBC from the same research group have been reported in the following publications: Durrer et al. (2005; 2007); Faass et al. (2009); Hofkamp et al. (2008); Maerkel et al. (2005; 2007); Schlumpf et al. (2004a; 2004b; 2008a; 2008b;).

It is difficult to evaluate exactly how many studies have been performed, when which doses and endpoints have been investigated and when data from different studies has been investigated separately or together. However, the following experimental setup was used in each case. Male and female LE rats were dosed with the compound by adding it to the feed. The parental generation was exposed for 10 weeks before mating, exposure of dams continued throughout gestation and lactation, and the offspring were further dosed until adulthood. The following doses have been investigated: 0.7, 7, 24 and 47 mg/kg/day. A dose of 70 mg/kg was also used initially, but was discontinued because it was too high for the offspring and resulted in reduced postnatal survival. Unfortunately, the number of litters used in the studies, has not been stated in some of the publications which report many of the important results used for evaluating endocrine disrupting effects (Schlumpf et al. (2004; 2008a)), but in the papers that do include this information the number of litters in each dose group was between 5-8 (Maerkel et al. (2007); Faass et al. (2009)) and in some publications data from more studies has been analyzed together yielding litter numbers between 12-17 (Durrer et al. (2007)). The dose of 7 mg/kg/day caused proliferative effect on prostate growth. On PND 1 an increase of 70 % in accessory sex glands and prostate volume was seen, caused by increasing number and volume of ducts in the prostate (Hofkamp et al. (2008)). On PND 14 decreased relative testes weight was seen from the dose of 7 mg/kg and higher (Schlumpf et al. (2008b)), and the same was true for delayed start of puberty in males (Durrer et al. (2007)). Body weight at puberty was normal in males, indicating that the delay of puberty did not result from nutritional effects (Durrer et al. (2007); Schlumpf et al. (2008b)). In adulthood, males from this group had decreased prostate weights and altered gene expression in the prostate (Durrer et al. (2007); Schlumpf et al. (2008 a,b)) and in the hypothalamus (Faass et al. (2009)), while adult females showed increased uterus weights (Schlumpf et al. (2008b)) and changes in gene expression of estrogen regulated genes in the brain (Faass et al. (2009)) and uterus (Durrer et al. (2005)).

Sexual behaviour of female offspring exposed to 7 and 24 mg/kg was also strongly impaired as a decrease in both proceptive and receptive behavior was observed (Faass et al. (2009)) At the dose of 24 mg/kg/day effects were also seen on litter size and survival rate, decreased thymus and increased thyroid weights, altered levels of TSH and T<sub>3</sub> (Maerkel et al. (2007)), whereas the highest dose of 47 mg/kg/day further caused increased testes weights in adulthood (Schlumpf et al. (2004; 2008b)). Furthermore, 4-MBC caused alterations in gene expression in sexually dimorphic areas of the brain in all dose levels (Maerkel et al. (2005; 2007); Schlumpf et al. (2008); Faass et al. (2009)). Timing of sexual maturation of the female offspring was not affected by any dose of 4-MBC, nor was oestrous cyclicity (Faass et al. (2009); Schlumpf et al. (2008)).

**Summary of *in vivo* studies:** The available *in vivo* studies for fish only show some evidence for weak estrogenic effects. In mammals equivocal estrogenic activity and effects on endocrine sensitive endpoints in developmental studies were found. The uterotrophic assays by Schlumpf et al. (2004) and Tinwell et al. (2002) demonstrated estrogenic effects, whereas Ashby et al. (2004) could not confirm these results. Additionally, an increase in uterine weight has been observed in a three month study performed by Seidlova-Wuttke et al. (2006) combined with histopathological markers in uterus and vagina pointing to an endocrine mode of action. Perinatal studies showed alterations in reproductive organ weight at birth, on day 14 and in adulthood, delayed sexual maturation, and altered gene expression in prostate and brain in male rats, while effects observed in females included increased uterus weights, changes in gene expression of estrogen regulated genes in brain and uterus, as well as strongly impaired

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sexual behaviour such as reduced proceptive and lordosis behaviour and increased rejection behaviour. Furthermore, some studies indicated thyroid mediated effects in mammals including increased TSH levels and decreased T4 levels as well as increased thyroid gland weights.

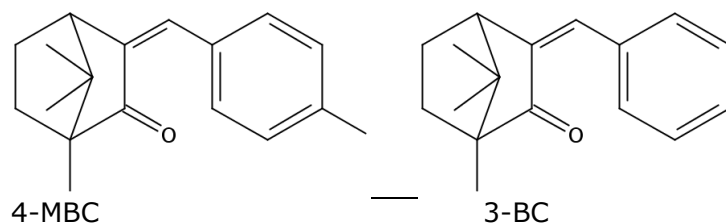
#### **5.2.4. Read-across from 3-BC to 4-MBC**

In this section a qualitative one-to-one read-across for 4-MBC to the structurally highly similar substance 3-BC is performed. The aim of this read-across is to show in a weight of evidence approach that owing to the high chemical (molecular structure and interaction potentials) and biological similarity (target proteins and molecular initiating events) of both camphor substances 4-MBC can act via the same endocrine modes of action as 3-BC. It is discussed why the same adverse effects in the environment, observable for 3-BC, can be expected for 4-MBC as well and that consequently 4-MBC should also be considered as an endocrine disruptor in the environment according to the WHO/IPCS definition with an equivalent level of concern to 3-BC. For 3-BC more *in vivo* data are available to show apical adverse effects on organisms and the identification as a substance of very high concern according to REACH Art. 57 (f) is justified in a separate dossier.

In respect to the guidance for performing weight of evidence approaches and to the recently published Read Across Assessment Framework (RAAF) by the ECHA, the comparison and transferability of data from 3-BC to 4-MBC will be performed within the following subsections addressing the issues of chemical and biological similarity. It should be mentioned here that this analysis is qualitative and focused on the key issues needed to demonstrate similarity between both camphor substances with respect to the aim mentioned above.

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Structural similarity



Both substances, sharing the same chirality and hence isomeric pattern as well as the same 3D structure, only differ structurally in one methyl group at the aromatic ring system. Thus, looking at the molecular interaction potential and its spatial orientation both substances are nearly identical whereas 4-MBC shows a slightly higher lipophilicity owing to the methyl group introduced at the benzene ring system. This leads to the conclusion that uptake and the receptor binding potential for both substances is in the same order of magnitude, modulated only by the small difference in lipophilicity. Hence, the potential for eliciting the same molecular initiating events leading in the end to apical adverse effects in organisms is comparable. However, the methyl group added in the 4-MBC structure might have consequences for the metabolic activation of 4-MBC compared to 3-BC, which will be discussed below.

In vitro effects of 3-BC and 4-MBC

That this structural and chemical similarity of both camphor substances leads to comparable activity on the molecular level can be demonstrated by comparing the available *in vitro* data for 3-BC and 4-MBC. The following table provides a summary and brief description of the *in vitro* data pointing to the mode of action for both camphor derivatives.

**Table 11: Mode of action summary for 4-MBC and 3-BC**

	<b>Result 4-MBC</b>	Description of results	<b>Result 3-BC</b>	Description of results
ER $\alpha$ and ER $\beta$ binding study (cellfree) Schlumpf et al. (2004a)	ER $\alpha$ : - estrogenic ER $\beta$ : + estrogenic	ER $\alpha$ : no binding observed up to 1mM ER $\beta$ : competitive binding (deliberation of radiolabeled E2) observed IC <sub>50</sub> = 35,3 $\mu$ M	ER $\alpha$ : - estrogenic ER $\beta$ : + estrogenic	ER $\alpha$ : no binding observed up to 1mM ER $\beta$ : competitive binding (deliberation of radiolabeled E2) observed IC <sub>50</sub> = 11,8 $\mu$ M
E-screen (MCF-7) (pS2 gene transcription) Heneweer et al. (2005)	+ estrogenic	Altering gene transcription: EC <sub>50</sub> = 1.90 $\mu$ M	Not investigated	
E-screen (MCF-7) Matsumoto et al. (2005)	+ estrogenic	REC <sub>10</sub> (Concentration showing 10 % of that of E2) = 6.3 $\mu$ M	Not investigated	

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	<b>Result 4-MBC</b>	Description of results	<b>Result 3-BC</b>	Description of results
E-screen (MCF-7) Schlumpf et al. (2004a)	+ estrogenic	Stimulation of MCF-7 proliferation: EC <sub>50</sub> = 3.9 µM  50 % of E2 effect reached but no plateau was reached with highest test concentration	+ estrogenic	Stimulation of MCF-7 proliferation: EC <sub>50</sub> = 0.68 µM  100 % E2 effect
E-screen (MCF-7) Schlumpf et al. (2001)	+ estrogenic	Stimulation of MCF-7 proliferation: EC <sub>50</sub> = 3.9 µM  86 % of E2 effect is reached	Not investigated	
E-screen (MCF-7) Jimenez-Diaz et al. (2013)	+ estrogenic	Pot. estrogenic activity or ability to stimulate cell proliferation: EC <sub>50</sub> = 24.14 µM	+ estrogenic	Pot. estrogenic activity or ability to stimulate cell proliferation: EC <sub>50</sub> = 1.7 µM
hERα activation in a Yeast Estrogen Screen (YES) with additional enzymatic digestion of the yeast cells Schmitt et al. (2008)	+ estrogenic	Yeast hERα activation: EC <sub>50</sub> = 44.2 µM (8 % effect compared to E2)	+ estrogenic	Yeast hERα activation: EC <sub>50</sub> = 44.3 µM (80 % effect compared to E2)
Luciferase Gene Expression in HELN ERα cell lines Gomez et al. (2005)	+ estrogenic	Binds to the estrogen receptor (ERα cell line) from 3 µM	Not measured	
Gene expression assay in stable ERα and ERβ reporter cell lines (HEK293cells) Schreurs et al. (2005)	+ estrogenic	Activation of transcription for hERα: IC <sub>50</sub> = 6.2 µM  Activation for hERβ: IC <sub>50</sub> = 14 µM	+ estrogenic	Activation of transcription for hERα: IC <sub>50</sub> = 13.0 µM  Activation for hERβ: IC <sub>50</sub> = 10.0 µM
Gene expression assay in stable hERα and hERβ transfectants of HEK293 cells Schreurs and van der Burg (2002)	-estrogenic	No induction of transcriptional activation	Not measured	

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	<b>Result 4-MBC</b>	Description of results	<b>Result 3-BC</b>	Description of results
Recombinant yeast systems carrying either a human estrogen (hERα) or androgen receptor (hAR) Kunz and Fent (2006)	- estrogenic - androgenic	Very high concentrations used (up to 1000 μM)  Estrogenic response in the yeast hERα assay as well as Androgenic response in the yeast hAR assay: not detected  Anti-estrogenic response yeast hERα assay: EC <sub>50</sub> = 87.3 μM (181 % effect compared to 4-hydroxy-tamoxifen)  Antiandrogenic response in the yeast hAR assay: EC <sub>50</sub> = 11.8 μM (107 % effect of flutamide)	+ Estrogenic - estrogenic - androgenic	Estrogenic response in the yeast hERα assay  EC <sub>50</sub> = 310 μM (21% of E2)  Androgenic response in the yeast hAR assay: not detected  Anti-estrogenic response yeast hERα assay: EC <sub>50</sub> = 8460 μM (134 % effect compared to 4-hydroxy-tamoxifen)  Antiandrogenic response in the yeast hAR assay: EC <sub>50</sub> = 18 μM (143 % effect of flutamide)
AR-mediated gene-reporter activation assay in MDA-kb2 cells Ma et al. (2003)	- androgenic - antiandrogenic	No agonistic or antagonistic (in 0.5 or 0.1 nM DHT) action on AR 0.001 to 1 μM	- androgenic - antiandrogenic	No agonistic or antagonistic (in 0.5 or 0.1 nM DHT) action on AR 0.001 to 1 μM
AR CALUX Bioassay Schreurs et al. (2005)	+ antiandrogenic	Repression of transcription of hAR: IC <sub>50</sub> = 7.1 μM	+ antiandrogenic	Repression of transcription of hAR: IC <sub>50</sub> = 4.6 μM
PR CALUX Bioassay Schreurs et al. (2005)	+ anti-progesterone	Repression of transcription of hPR: IC <sub>50</sub> = 0.9 μM	+ anti-progesterone	Repression of transcription of hPR: IC <sub>50</sub> = 0.4 μM
Human sperm: activation of the CatSper channel Schiffer et al. (2014)	+ anti-progesterone	EC <sub>50</sub> = 6.83 ± 2.26 μM  (for comparison: 4-OP EC <sub>50</sub> = 5.93 ± 0.40 μM)	+ anti-progesterone	EC <sub>50</sub> = 1.73 ± 1.36 μM  (for comparison: 4-OP EC <sub>50</sub> = 5.93 ± 0.40 μM)
TH-responsive luciferase-based reporter gene assay Hofmann et al. (2009)	+ thyroidal	Induction of T <sub>3</sub> -regulated gene expression: LOEC = 1 μM	Not measured	

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Summing up the *in vitro* biological similarity of 3-BC and 4-MBC, both substances show evidence for an estrogenic as well as antiestrogenic mode of action. Both substances do not bind directly in a cell free system to the hER $\alpha$  receptor protein but to the hER $\beta$  pointing to a similar mode of estrogenic activity. Furthermore, the data suggests that for both substances the major metabolites exhibit the estrogenic activity observed in the cellular systems by activating the hER $\alpha$ . From a structural point of view the metabolite of 3-BC should exhibit the stronger ER binding potential, which is reflected in the mostly lower effect concentrations observable for the 3-BC treatment of cells. The observed antiestrogenic activity might be mediated via direct ER $\beta$  and ER $\alpha$  (not at the E2 binding site) binding of the parent compounds and/or by partial antagonism of the metabolites. The degree of metabolisation and the inherent binding potential of the metabolites formed seem to modulate the estrogenic and antiestrogenic effects of 3-BC and 4-MBC leading to 4-MBC showing the same mode of action but with lower estrogenic potential than 3-BC.

Furthermore, both substances exhibit a comparable and high antiandrogenic activity as well as antiprogesteric activity, which also points to the same biochemical binding behavior of both camphors.

For 4-MBC additionally some *in vitro* evidence for thyroidal modes of action was identified in the available literature.

### *In vivo* effects of 3-BC and 4-MBC

For 3-BC all tests described provide clear evidence of an endocrine mode of action in fish. All tests resulted in significant *in vivo* induction of vitellogenin in juveniles and males. 3-BC caused vitellogenin induction in a clear dose-response manner and induction was at least 3-fold compared to controls. Results by Kunz et al. (2006b) show that vitellogenin concentrations in males reached levels comparable to those observed in females. In addition, the complete loss of male secondary sex characteristics after 21d of exposure of adult fish was observed. According to OECD, 2012 and the OECD Guideline (No. 234) for the fish sexual development test changes in phenotypic sex-ratio should be considered as clearly diagnostic for an estrogen receptor agonistic or androgen receptor antagonistic mode of action. In this case adult males were visually not discernible from females at the highest concentration tested. Thus, even after such a short exposure of adult fish, the phenotypic sex changed from male to females. This corresponded with the complete cessation of spawning and is considered to be an adverse apical effect.

For 4-MBC two studies in fish are available. At high concentrations 4-MBC also induces estrogen-responsive gene products including vitellogenin (Inui et al. (2003)). Kunz et al. (2006) found no estrogenic activity of 4-MBC in a test with juvenile mixed-sex fish. In summary, the available *in vivo* data for fish only provide evidence for a weak estrogenic activity of 4-MBC. Interestingly, *in vitro* data from Kunz et al. (2006) show that 4-MBC and/or its metabolites in contrast to 3-BC cannot bind to the fish rER $\alpha$  in the concentration range tested. This effect modulation might be explained by the *in vivo* metabolism of 3-BC and 4-MBC and by the possibly different composition of the test compounds regarding their specific isomers.

### *In vivo* metabolism of 3-BC and 4-MBC

As presented in the dossier for 3-BC studies in hepatocytes, rats and humans identified 3-(4-hydroxybenzylidene) camphor as the major metabolite of 3-BC. The introduction of a phenolic ring system with the hydroxyl group orientated in para position represents a strong structural alert for binding to hER $\alpha$  and hER $\beta$ . Thus, it is inferred from the chemical similarity that the main metabolite of 3-BC is an ER agonist.

As indicated in section 5.2.2. above, 4-MBC has two major metabolites when entering metabolically active cells or organisms. These two metabolites are 3-(4-carboxybenzylidene) camphor and 3-(4-carboxy)-6-hydroxy camphor. Both metabolites



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share the feature of an aromatic carboxy group. The formation of a phenolic moiety is inhibited here by steric hindrance. The 3-(4-carboxy)-6-hydroxy camphor additionally includes a hydroxy group located at the camphor ring structure. The aromatic carboxyl group introduces a polar interaction potential in para position of the ring system as well as a positive charge under physiological conditions. Looking at the E2 binding site of the human ER receptor proteins there is a structural alert for a specific binding interaction via the positively charged arginine residue at the active center. However, this interaction should be weaker compared to a phenolic hydroxy compound since also repulsive interactions with the Glu residue at the binding pocket have to be considered. This leads to the conclusion that the metabolites of 4-MBC can bind to ER proteins but with a lower affinity than the 3-BC metabolite.

Conclusion for the read-across scenario

The chemical and biological similarity of 3-BC and 4-MBC on the *in vitro* and *in vivo* level clearly demonstrates that both camphor compounds most likely act via the same endocrine modes of action and that this activity leads to comparable effects in organisms. The following table provides an overview of this similarity for both substances.

**Table 12: Comparison of the biological similarity of 4-MBC and 3-BC**

<b>3-BC</b>	<b>4-MBC</b>
++ estrogenic <i>in vitro</i>	+ estrogenic <i>in vitro</i>
(+) antiestrogenic <i>in vitro</i>	+ antiestrogenic <i>in vitro</i>
++ antiandrogenic <i>in vitro</i>	++ antiandrogenic <i>in vitro</i>
++ antiprogesteronic <i>in vitro</i>	++ antiprogesteronic <i>in vitro</i>
- thyroidal activity <i>in vitro</i>	+ thyroidal effects <i>in vitro</i>
++ estrogenic/antiandrogenic effects in fish	(+) estrogenic/antiandrogenic effects in fish

The *in vitro* antiandrogenic activities of the substances are comparable as well as the observed antiprogesteronic activities investigated in two studies. With respect to the estrogenic *in vitro* activity 4-MBC shows consistently a lower potency than 3-BC. This modulation of the activity might be explained by differences in metabolism (see above) and/or differences in the isomeric pattern of the test solutions.

At the *in vivo* level, 3-BC shows clearly estrogenic and/or antiandrogenic modes of action leading to adverse apical effects in fish species. For 4-MBC the available data in fish point to an estrogenic mode of action at a much lower potency but no clear apical adverse effects can be found in the two fish studies available. Again this might be explained by metabolic issues or the isomeric pattern of the test solutions if the isomers differ in their biological activity.

Taking together the *in vitro* and *in vivo* similarity showing obviously the same molecular targets it can be concluded that 4-MBC acts via the same mode of endocrine action in wildlife animals as does 3-BC. The observed differences in potency can be explained via

the different metabolism of both compounds in different species and/or differences in the isomeric composition of the tested solutions. Thus, also for 4-MBC adverse effects in intact organisms via an endocrine mediated pathway are expected. This conclusion is supported by the *in vivo* data obtained from the above presented rodent studies pointing to estrogen and/or antiandrogen mediated adverse effects in rodents.

### 5.2.5. Summary of evidence for endocrine disrupting effects

Considering all available information from *in vitro* and *in vivo* fish studies supported by mammalian toxicity studies and the read-across from 3-BC as an endocrine active substance in fish and 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 are able to activate the human ER $\alpha$  and ER $\beta$  receptor in a dose dependent manner. 4-MBC also shows antiandrogenic and antiprogesterone like activity as well as possible thyroidal activity. The read-across from 3-BC demonstrates that 4-MBC acts via the same modes of action and differences can be explained by differences in metabolism altering the potency but not the mode of action.

Endocrine effects (increase in uterine weight, decrease in testis weight, delayed pubertal development in males) observed in mammalian *in vivo* tests substantiate these modes of action and indicate that 4-MBC acts via an estrogenic or antiandrogenic mode of action *in vivo*. The fish studies presented alone do not allow for a conclusion on the endocrine disrupting potential of 4-MBC, but the conclusions drawn from the read-across regarding the mode of action and differences in potency together with the overall evidence gained from the *in vivo* studies point to an endocrine disrupting effect of 4-MBC in wildlife species.

## 5.3. Aquatic invertebrates

Schmitt et al. (2008) conducted additionally to a Yeast Estrogen Screen (YES) (see chapter **Error! Reference source not found.**) two sediment assays with the freshwater invertebrates *Lumbriculus variegatus* and *Potamopyrgus antipodarum*. The 56-day 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 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  $\pm$  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<sub>50</sub> of 4.60  $\mu$ M 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 mudsnail, which is an apical effect.

Fent et al. (2010) reports on 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 guideline 211. At the highest concentration of 4-MBC, 50  $\mu$ g/l, reproduction and body length were reduced. Apparently 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 apparently no effects were seen on the sex ratio of the offspring.

## 5.4. Other aquatic organisms

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Kunz et al. (2004) investigated whether 4-MBC interferes with the thyroid and sex hormone system of the tadpoles of frogs *Xenopus laevis* during metamorphosis. At concentrations of nominal 1, 5 and 50 µg/l 4-MBC had no effects 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 do not negatively affect the thyroid system and sex ratio of frogs at the tested concentrations. The tested concentrations are, however, lower than the LOEC for vitellogenin induction in fish.

### 5.5. Sediment organisms

Schmitt et al. (2008) conducted a 28-day sediment test with *L.variegatus* according to the Draft-OECD 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 unspiked 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 4-MBC /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)).

## 6. Conclusions on the SVHC Properties

### 6.1. CMR assessment

Not relevant for environmental hazard assessment.

### 6.2. PBT and vPvB assessment

No information from standard tests is available for 4-MBC, which allows to conclude on the PBT/ vPvB properties of the substance.

4-MBC is predicted not to be readily biodegradable and has a calculated log Pow of 5.92. Therefore, the substance shows a high potential for bioaccumulation.

### 6.3. Hazard and equivalent level of concern assessment under Article 57(f)

According to article 57(f), substances having endocrine disrupting properties, for which there is scientific evidence of probable serious effects to the environment which give rise to an equivalent level of concern to those of PBT/vPvB and/or CMR substances might be substances of very high concern, identified on a case by case basis.

Although article 57(f) provides no clear criteria for identifying an “equivalent level of concern”, starting from the legal text two questions seem to be relevant:

- Does 4-MBC have endocrine disrupting properties, i.e. does 4-MBC act via an endocrine mediated mode of action and does this mode of action causes adverse effects?
- Is there scientific evidence of probable serious effects, evoked by these endocrine disrupting properties, to the environment which give rise to an equivalent level of concern compared to CMR and/or PBT substances?

The information available for 4-MBC is structured in the following along these two questions in order to facilitate a conclusion.

#### 6.3.1. Endocrine disrupting properties of 4-MBC

Endocrine disrupting properties are one example of inherent properties that might, if scientific evidence of probable serious effects is available, give rise to an equivalent level of concern as exerted by CMR and/or PBT/vPvB substances.

Although the term “endocrine disrupting properties” is not equivalent to the term “endocrine disruptor” the definition of an endocrine disruptor provided by WHO/IPCS (WHO/IPCS, 2002) is used as starting point to analyse the endocrine disrupting properties of 4-MBC:

*“An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations” (WHO/IPCS, 2002).*

Hence, the information presented in section 5.2. including the read-across results will be analysed in the following in the sense of a weight of evidence approach. Starting from *in silico* and *in vitro* results up to the available *in vivo* data it will be shown that 4-MBC can act via endocrine modes of action and that these intrinsic properties might result in adverse effects in an intact organism. This examination is based on the criteria set out in the OECD guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption (OECD, 2012). How these effects can subsequently be interpreted to demonstrate an equivalent level of concern compared to PBT and vPvB as

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well as CMR substances will be discussed in subsection 6.3.2..

As described in section 5.2.2. there is one *in silico* study that predicts a significant binding potential of 4-MBC to the ligand binding site of the human AR and a weaker binding to the human ER $\beta$ . Another *in silico* study predicts the inhibition of a certain enzyme that is involved in the homeostasis of estrogens and androgens in vertebrate species. Thus, there is some evidence on the *in silico* level that 4-MBC might interact with estrogen and androgen mediated pathways in organisms.

The *in vitro* results described under section 5.2.2. point to four modes of endocrine action of 4-MBC. Cell based test systems (human MCF-7 and HEK239 cells as well as yeast cells) show a dose related binding of 4-MBC to both subtypes of the estrogen receptor via proliferation and gene expression analysis. Further studies are investigating the androgenic/antiandrogenic potential of 4-MBC. Using cell based (mammalian cells and transfected yeast cells) gene expression studies no androgenic responses could be observed. However, two of the studies show significant antiandrogenic effects with IC<sub>50</sub> values in the low micromolar range. Further, two studies could demonstrate antiprogesteron effects of 4-MBC using a reporter gene assay to detect direct binding to the progesterone receptor and an indirect assay for progesteronic activity based on a human sperm ion channel. Finally, one gene expression study shows the induction of T3 genes pointing to thyroidal modes of action of 4-MBC. Hence, the available *in vitro* data show the potential of 4-MBC to interfere in an agonistic and antagonistic way with at least three different endocrine related receptor families and the thyroid related genes providing evidence on a molecular basis for possible estrogenic, antiandrogenic, antiprogesteron and thyroidal effects.

As described in chapter 5.2.3. the *in vivo* data available for fish support the conclusions drawn about the mode of endocrine action from the *in vitro* evidence. However, clear evidence for apical adverse effects cannot be demonstrated in the available two fish studies. Therefore a qualitative read-across to 3-BC, where adverse effects could be demonstrated in fish, was performed. As discussed in section 5.2.5. the high chemical and biological similarity of 4-MBC to 3-BC justifies the conclusion that 4-MBC acts via the same modes of endocrine action and hence shows comparable effects in intact organisms to 3-BC. Thus, the apical adverse effects shown for 3-BC are very likely to occur also in the environment after exposure to 4-MBC.

### *Overall summary of endocrine disrupting effects of 4-MBC*

In summary, available information from mechanistic *in vitro* and *in silico* studies as well as results from *in vivo* experiments and the read-across from 4-MBC to the structurally similar camphor compound 3-BC show that 4-MBC can act as an endocrine disruptor in fish most likely via an estrogenic and/or antiandrogenic mode of action. The observed antiprogesteron effects at even lower effect concentrations in two *in vitro* studies as well as the interference with thyroidal gene activation suggests that there are further endocrine pathways with which 4-MBC can interfere. However, so far there is no causal link between these further modes of action and the observed effects in fish or other species and the evidence is too low to conclude on this mechanistic data at this stage. Additionally, there are some hints from rodent studies that 4-MBC has endocrine activity in mammalian species as well.

### 6.3.2. Equivalent level of concern based on probable serious effects in the environment

As described in article 57 (f), an endocrine disruptor should be regarded as of very high concern if the probable serious effects to the environment are of equivalent level of concern compared to CMR and/or PBT/vPvB substances (REACH, Art. 57 f). The seriousness of effects and the equivalent level of concern need to be analysed case by case employing a weight of evidence approach.

Thus, in the following it will be analysed for 4-MBC how the above described, and by an endocrine mode of action mediated, effects are of equal concern for the environment as those evoked by CMR and/or PBT/vPvB substances. It should be noted here that this analysis focuses on the question whether the distinct nature of the effects (e.g. irreversibility, critical windows of exposure that lead to long lasting effects, interference with background contaminants acting via the same mode of action) is of very high concern and urges for regulatory action whether or not there are some uncertainties remaining. In other words, it will be discussed below why the seriousness of effects justifies the application of the precautionary principle and hence the SVHC identification of 4-MBC. Owing to the structural and biological similarity of 4-MBC to 3-BC the same arguments in demonstrating the equivalent level of concern are used in the following. To structure the discussion on the seriousness of the effects the following issues will be addressed:

- Mode of action
- Irreversibility of effects and long term adverse outcomes
- How many species are at risk?
- Population relevance of the effects

#### *Mode of action*

The *in vitro* and *in vivo* data available for 4-MBC provide strong evidence that 4-MBC can act via an estrogenic and/or antiandrogenic mode of action. Additionally, some hints for an antiprogesteric pathway and interference with thyroidal pathways are available. Thus, it could be shown that the effects observed in fish are endocrine mediated and hence 4-MBC must be considered as an endocrine disruptor in the environment. The severity of these endocrine effects can be shown by comparing the mode of action of 4-MBC with effects observed with known endocrine disrupting chemicals acting via the same specific molecular mechanism.

Estrogen receptor agonists are known to interfere with reproduction parameters as well as sexual development (including changes in sex-ratio) and growth. Specific life stages and endpoints such as sexual development and sexual maturation are especially sensitive to the influence of estrogen receptor agonists (Kendall et al. (1998); IPCS (2002)). Effects are considered relevant as they impair population stability or recruitment. A known ED substance that acts, like inferred for 4-MBC, via an ER agonistic and AR antagonistic mechanism is nonylphenol branched and linear (NP). With NP comparable effects on fecundity (number of spawned eggs and effects on spermatogenesis) were observed at the same order of magnitude when comparing the LOEC values of NP and 3-BC. Furthermore for NP a fish full life cycle test is available that shows effects on sex ratio at a LOEC of 23.5 µg/l (Seki et al., 2003). For 3-BC (from which there is read-across proposed to this substance) no full life cycle test is available, but it was found that the male secondary sex characteristics decrease starting from a tested concentration of 33 µg/l. Concomitant with this change of phenotypic secondary sex characteristics there was a decrease/cessation of spawning observed. Thus, potential indirect effects via behaviour that may lead to the same adverse effect than change in the (genetic) sex ratio (e.g. endocrine mediated impact on mating behaviour of males or no recognition of phenotypically abnormal males by females as sexual partner) can be

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expected for 3-BC. Comparable effects are observed with further known ER agonistic substances like ethinyl estradiol, bisphenol A (EC No. 201-245-8) and 4-tert-octylphenol (EC No. 205-426-2). Since the ER and AR pathways are highly conserved between vertebrate species further endpoints affected by these known and data rich endocrine active substances can also be expected to be triggered and influenced by 4-MBC.

From the *in vitro* studies of 4-MBC presented in section 5.2. it can be concluded that beside ER and AR binding further endocrine modes of action (antiprogesteric) of 4-MBC must be considered at concentrations one order of magnitude lower than those observed for the estrogenic and antiandrogenic activity *in vitro*.

Finally, it must be considered that under environmental conditions the background burden of substances acting like 4-MBC via an ER agonistic and/or AR antagonistic mode of action does not allow for setting a safe threshold for 4-MBC in the environment for different species owing to specific mixture effects. While this holds true for many environmental contaminants the specific severity (see below) of the endocrine mediated effects demands special attention here.

### *Irreversibility of effects and long term adverse outcomes*

Endocrine modulation is a very complex feedback process that is set up during critical life stages. Disturbance of this set up may result in effects during the entire life (IPCS, 2002). Effects may result in a substantial failure of recruitment and almost disappearance of populations even after cessation of exposure, as observed in the wild for ethinylestradiol, a known ER agonist, in fathead minnow (Blanchfield et al. (2015)). Even transient exposure during sensitive life stages may result in severe effects on populations later on. Changes in male reproduction capacity might influence genetic variability of populations in the long-term, as only a part of the males may be capable of reproduction (Sumpter and Johnson (2008)). The complete loss of nuptial tubercles as an important secondary sex characteristics in male fish after exposure to 3-BC points to such severe effects on reproduction. As pointed out by e.g. Segner et al. (2003) effects of ethinyl estradiol, bisphenol A (EC No. 201-245-8) and 4-tert-octylphenol (EC No. 205-426-2) on the reproduction of *D. rerio* are irreversible. Exposure of nonylphenol branched and linear in one generation resulted in effects in the next generation even if that generation was not directly exposed. In *O. mykiss* VTG induction and increased level of sexual steroids were observable in 3 year old unexposed progeny of parents exposed as adults. Viability of eggs and hatching rate of unexposed progeny was reduced (Schwaiger et al. (2002)). Results obtained for *O. latipes* after exposure to 4-tert-octylphenol (EC No. 205-426-2) in a sexual development tests indicate that exposure during a very short window (pre-hatch) adversely influences the endpoints on sexual development and sex-ratio (Seki et al., 2003). Given the same underlying endocrine modes of action there is a strong concern that 4-MBC can lead to the same long-term effects in fish.

For invertebrates, there are also indications of a high sensitivity of the early life stages. For instance, larval stages of crustaceans have been shown to be highly sensitive to exposure to various substances of very high concern (reproduction toxicants as well as endocrine disruptors) under REACH. In molluscs, arthropods, amphibians, alligators, turtles and birds, estrogen agonists, such as 17 $\beta$ -estradiol and ethinylestradiol, influence the endocrine system by causing adverse changes in development, reproduction and behaviour.

Finally, migration is a common pattern in species such as birds, amphibians, mammals and fishes. It includes long-distance migration of migratory birds or of fish species, such as salmonids and eels. Thus, exposure in one area might influence population stability in another area (e.g. exposure during development of flatfish in coastal area may result in population changes in the open sea, or exposure of adult salmonids in estuarine areas during migration might influence sperm quality and fertilization success at the reproduction sites in rivers). Due to the potentially long lasting effects, as well as the wide variety of species potentially affected it is again very difficult to estimate which species are most sensitive and which concentration should be regarded as safe for the

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environment. Since 4-MBC most likely like 3-BC shows endocrine mediated adverse effects in fish such scenarios must also be taken into account when assessing the equivalent level of concern especially with regard to PBT/vPvB substances.

### *How many species are at risk?*

Vertebrate hormone receptors are highly conserved through evolution in a broad range of taxa but extent and type of effects may differ between fish species. Hence, extrapolation of effect concentrations between fish species is difficult. The studies available for 3-BC point to effects in at least two different teleost fish species. Additionally, the developmental toxicity data for rodents presented in section 5.2. show that effects on estrogen and/or antiandrogen sensitive endpoints are also present in mammals. Thus, 4-MBC like 3-BC, due to the highly conserved mode of estrogenic and/or antiandrogenic action, might affect a broad range of wildlife animals and not only fish.

Vertebrate-type sex steroids and related receptors have been detected in a range of invertebrate taxa. However, there are still substantial gaps with regard to our knowledge on sex steroid receptors in many invertebrate phyla. As emphasised by OECD (2012), structurally related molecules may have other functions in invertebrates than in vertebrates. For instance, in the rotifer *Brachionus manjavacas* progesterone appears to induce the transition from asexual to sexual reproduction. Hence, this hormone seems to be conserved over a wide range of phyla, yet with a changed function (Stout et al., 2010). The fact that ecdysteroids are structurally similar to steroid estrogens explains that the latter may affect moulting in crustaceans. Testosterone and a number of known estrogen receptor agonists (e.g. bisphenol A (EC No. 201-245-8) and 4-nonylphenol) appear to function as antiecdysteroids in crustaceans (LeBlanc (2007)).

Endocrine systems of invertebrates differ substantially from those of vertebrates. In addition – given that invertebrate species are extremely diverse in their biology and physiology – there are also considerable differences between the endocrine systems of various invertebrate taxa. Nevertheless, some conclusions can be drawn from the evaluation of the data compiled for the model substances bisphenol A (EC No. 201-245-8), 4-tert-octylphenol (EC No. 205-426-2), tributyltin (EC No. 215-958-7) and triphenyltin (EC No. 211-358-4). Effects of the estrogen receptor agonists bisphenol A (EC No. 201-245-8) and 4-tert-octylphenol (EC No. 205-426-2) on invertebrates were observed at similar or even lower concentrations than effects on fish. Highest toxicity was observed in molluscs, copepods and echinoderms, i.e. species that are not yet part of the OECD testing framework for endocrine disrupters (echinoderms) or that have only recently been included (copepods, molluscs). Thus, owing to the comparable mode of action of 4-MBC to other xenoestrogens like bisphenol A (EC No. 201-245-8), nonylphenol branched and linear and 4-tert-octylphenol (EC No. 205-426-2) effects of 4-MBC on invertebrate species are likely and they may occur at lower effect concentrations than it is inferable from the available fish data. Generally, having in mind our current knowledge on endocrine disruption and the underlying endocrine processes in invertebrates, it is difficult to predict which invertebrate taxa or species will be most strongly affected by which endocrine mechanism of action and hence, which concentration can be assumed to be sufficiently safe in the environment.

### *Population relevance of the effects*

To demonstrate the severity of effects that might be evoked by 4-MBC for the environment, in the following subsection the possible adverse outcome of an exposure to 4-MBC on the level of populations and/or subpopulations will be discussed.

Delays in male sexual development, reproductive behaviour and reproduction have often been observed upon exposure to estrogen receptor agonists (e.g. Schäfers, 2003). Furthermore, there is evidence of an only incomplete recovery of effects on the reproductive capacities of populations in cases where exposure started during early life stages (Scholz and Klüver, 2009). For the known ER agonists and AR antagonists it could



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be shown that a transient short term exposure during sensitive life stages may result in life long effects even in following generations. Since 4-MBC was shown to act via the same mechanism as 3-BC, and at comparable effect concentrations as known ER agonists and AR antagonists, there is a science based concern of probable serious effects on the population level for 4-MBC.

Finally, as described in section 3, 4-MBC is not readily biodegradable and its relatively high log Pow of 5.92 (KOWWIN v1.68 estimate) fulfils the screening criterion for being bioaccumulative according to REACH Annex XIII. Hence there is a probability for the occurrence of serious effects due to fate properties of 4-MBC (screening as potentially P and B).

### *Summary of the hazard and equivalent level of concern assessment of 4-MBC*

The *in silico*, *in vitro* and *in vivo* data presented and discussed within this dossier as well as the weight of evidence from read-across to the structurally similar camphor substance 1,7,7-trimethyl-3-(phenylmethylene)bicyclo[2.2.1]heptan-2-one (3-BC), provide sufficient evidence to conclude that (±)-1,7,7-trimethyl-3-((4-methylphenyl)methylene) bicyclo[2.2.1]heptan-2-one (4-MBC, 4-methylbenzylidene camphor) acts via an endocrine mode of action and that this endocrine activity most likely leads to adverse effects in fish. Hence, 4-MBC fulfils the WHO/IPCS definition of an endocrine disruptor for the environment.

The specific mode of action of 4-MBC (estrogen receptor agonist and/or androgen receptor antagonist), the effects observed *in vivo* in fish and rodent species as well as the comparison of these effects with known endocrine disruptors and 3-BC acting via the same molecular mode of action provide strong evidence that the endocrine mediated effects of 4-MBC are of equivalent level of concern for the environment as those of PBT/vPvB and CMR substances. In detail, the following evidence of probable serious effects and reasons for their equivalent level of concern could be identified for 4-MBC:

- The identified main mode of action (estrogenic and/or antiandrogenic) of 4-MBC is comparable to that of 3-BC and known endocrine active substances like bisphenol A (EC No. 201-245-8) or ethinyl estradiol and already identified endocrine disrupting chemicals under REACH like nonylphenol branched and linear and 4-tert-octylphenol (EC No. 205-426-2).
- It may not be possible to derive a safe concentration limit of 4-MBC in the environment since 4-MBC acts via the same modes of action as many other environmentally relevant ED substances and hence mixture effects are very likely to occur. There are hints for further endocrine modes of action (antiprogesteric) at lower effect concentrations from *in vitro* studies.
- There is a high probability that 4-MBC can have irreversible and long lasting effects on populations and that even short term exposures during sensitive life stages of organisms can have adverse effects during the entire lifetime.
- The specific mode of action (estrogenic and/or antiandrogenic) of 4-MBC and the data available for fish and rodent species point to a broad range of taxa that might be affected by exposure to 4-MBC in the environment. This is due to the fact that the estrogen and androgen receptor proteins are highly conserved across different species. Binding agonistically to the estrogen receptor and/or antagonistically to the androgen receptor was identified in various *in vitro* studies to be the molecular initiating event leading to the endocrine activity of 4-MBC. Mechanistic knowledge about invertebrate hormone receptors and a reproduction study with molluscs show that also invertebrate species might be affected by 4-MBC.
- There is a high likeliness that the effects are adverse not only for single organisms but also for populations and/or subpopulations in the environment.

Taking together the evidence presented in this dossier 4-MBC is a substance of very high concern according to REACH Art. 57 (f) owing to its endocrine disrupting properties,

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which lead to probable serious effects in intact organisms in the environment. The specific adversity of these effects demonstrates the equivalent level of concern compared to other substances of very high concern like PBT/vPvB and CMR chemicals. Hence, even though there are remaining uncertainties within the hazard assessment of 4-MBC the application of the precautionary principle is justified with regard to the probable serious effects to the environment from 4-MBC. Additionally, 4-MBC is not readily biodegradable and its relatively high log Pow of 5.92 fulfils the screening criterion for being bioaccumulative according to REACH Annex XIII. Hence there is also a probability for the occurrence of serious effects due to fate properties of 4-MBC (screening as potentially P and B).

## Part II

### 7. Manufacture, import and export

4-MBC has not been registered yet, although preregistrations indicated registrations in 2010 and 2013. The substance has been preregistered 64 times: 1 x 2010, 3 x 2013, 58 x 2018, 2 x not specified. The number of individual notifications in ECHA's C&L Inventory database<sup>3</sup> leads to the conclusion that 4-MBC is commercially relevant inside Europe (total number of notifiers: 271). The substance has not been self-classified by 4 notifiers and self-classified according to the CLP Regulation EC No. 1272/2008 by other notifiers as follows: 200 x Aq. Acute 1, Aq. Chronic 1; 41 x Aq. Chronic 2; 23 x Repr. 2; 2 x H361 (only Labelling Haz. St. Code); 1 x Skin Irrit. 2, Eye Irrit. 2, Aq. Acute 1, Aq. Chronic 1.

The European Existing Substances Information System (ESIS)<sup>4</sup> reports 4-MBC as a Low Production Volume Chemical (LPVC), i.e. the substance has been produced or imported into the EU with a tonnage > 10 t/a but never more than 1000 t/a.

4-MBC is mainly used in sunscreens and other cosmetics. 4-MBC is listed in the REGULATION (EC) No 1223/2009 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 30 November 2009 on cosmetic products, ANNEX VI: List of UV filters allowed in cosmetic products with a maximum concentration in ready for use preparations of 4 %. In a conclusion of the Scientific Committee on Consumer Products (SCCP) 4-MBC is considered to be safe for the use in cosmetic products up to a concentration of 4 % (SCCP, 2008). Therefore, it is assumed that the trend in used amounts remains stable.

No information is available for other potential uses, e.g. the structurally similar substance 3-BC is stated to be used among others in textiles (Kunz et al. (2006b)).

The annual production volume of UV filters in total is estimated to be in the hundreds of tons range (Buser et al. (2006)).

### 8. Information on uses of the substance

4-MBC is a chemical UV filter which is used in sunscreens and other cosmetics. According to Kunz et al. (2006b) the structurally similar substance 3-BC is a constituent of skin and hair care products, household products and textiles for UV protection. Due to the lack of registrations no detailed information is available on used volumes and other uses than in cosmetics.

<sup>3</sup> <http://echa.europa.eu/information-on-chemicals/cl-inventory-database> (last accessed 11.05.2015)

<sup>4</sup> <http://esis.jrc.ec.europa.eu/> (last accessed 11.05.2015)

## 9. Release and exposure from uses

### 9.1. Introduction

#### Emissions

When used in sunscreens and other cosmetics, 4-MBC is mainly distributed to surface waters as well as sediments and agricultural soils (Petersen et al., 2007). UV filters can enter the aquatic environment from bathing/showering, wash-off, washing (laundry) indirectly via wastewater treatment plants, and directly from recreational activities such as swimming and bathing in lakes and rivers (Balmer et al., 2005). Another contamination route might be the leaching from surfaces exposed to UV-radiation e.g. from car polish or textiles (Díaz-Cruz et al., 2008).

Disposal methods for sewage sludge significantly differ among EU Member States. The application of sludge in agriculture may lead to deposition of UV-filters in agricultural soils (Plagellat et al., 2006). In EU Member States with high application rates of sludge to land, relevant environmental releases to soil and indirect emissions into water can be expected.

#### Monitoring data

The use of 4-MBC in cosmetics and especially sunscreens leads to direct and indirect emissions to the environment as can be shown by various measured data.

Balmer et al. (2005) investigated the occurrence of organic filter compounds in waste water, surface waters, and in fish in Switzerland. In lakes they found 4-MBC in concentrations up to 28 ng/l. No UV filters were detected in remote mountain lake (without recreational activities). Higher concentrations of 4-MBC were measured during summer reflecting an increased use of UV filters. In Sweden 4-MBC could be detected in all investigated samples from surface waters and the concentration ranged from low ng/l up to 430 ng/l (Remberger et al., (2011)). The same report found seasonal peaks in summer and early autumn in the concentration profile of 4-MBC.

This was also reported for the coastal zone in Norway by Langford and Thomas (2008) who found reduced concentrations in winter (nd-2.6 ng/l) compared to summer (nd-262.1 ng/l) and very high measured levels in the bathing areas due to washing off directly from the skin. Highest concentrations of 4-MBC (798.7 and 262.1 ng/l) were taken where children were swimming and it was expected that these levels decrease further from the swimming area. The substance was also found in surface sea water of the Pacific (Goksoyr et al. (2009)).

Wastewater treatment plants (WWTPs) are an important indirect source of 4-MBC-emissions.

Balmer et al. (2005) detected 4-MBC in influents and effluents of wastewater treatment plants (WWTPs) in the range 600-6500 ng/l and 60-2700 ng/l, respectively. Fent et al. (2010) detected 4-MBC at almost all sampling sites of the rivers Glatt and Rhine in concentrations up to 100 ng/l. They also report effluents of WWTPs to be an important source of 4-MBC. The substance was not found below the outflow of Lake Greifen, but along the river stretch it was first detected downstream of the first WWTP.

Plagellat et al. (2006) investigated sludge from 14 WWTPs. They found concentrations of 4-MBC up to 4980 ng/g dry matter. Additionally, Remberger et al. (2011) found 4-MBC concentrations up to 1300 ng/g dry matter in sludge from WWTPs in Sweden. Since sewage sludge application in agriculture is common practice in many countries it can be expected that beside the aquatic compartments also agricultural soils will be exposed to 4-MBC.

4-MBC can also be found in biota. Nagtegaal et al. (1997) were the first researchers studying the occurrence of 4-MBC-residues in fish. In 1991 they detected 68 ng/g in

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roach and 930 ng/g lipid in perch.

Balmer et al. (2005) found that the lipid-based concentrations of 4-MBC in roach from different lakes was considerably higher than the concentrations of the water samples from the respective lakes. In roach up to 94 ng/g lipid and in perch 166 ng/g lipid were measured.

Buser et al. (2006) determined concentrations of 4-MBC in the muscle tissue of brown trout from seven small rivers receiving inputs from WWTPs. The substance was found in all river fish at concentrations from 50-1800 ng/l lipid, which are higher than previously found in lake fish. The authors attribute these higher levels to the large contribution from waste water.

However, Fent et al. (2010) did not detect the substance in biota which they ascribe to the decreasing trend in use of 4-MBC.

**Table 13: Measured levels of 4-MBC in the environment**

Country/ region	Samplin g year	WWTP (ng/l)	Sewage sludge (ng/g dry matter)	Water (ng/l)	Biota (ng/g lipid)	Referenc e
Switzerlan d	2006/ 2007			River Glatt and Rhine: constantly detected at almost all sampling sites (by POCIS: up to 100)	Macrozoobenthos, fish, cormorants: not detected	Fent et al. (2010)
Pacific	2006			Surface sea water by SPDM: < 0.29 by ML: 18; 30		Goksoyr et al. (2009)
Norwegian coastal zone	(year not specified ; before 2008)			samples collected amongst swimmers: 798.7;  Range: summer (nd- 262.1) winter (nd-2.6)		Langford and Thomas (2008)
Switzerlan d	2003				Brown trout ( <i>Salmo trutta fario</i> ) from small rivers receiving input from WWTPs: 50-1800 (average: 420)	Buser et al. (2006)
Switzerlan d	2001/ 2003		Sewage sludge from 14 WWTPs: Range: 150-4980 Mean: 178 0 Median: 1580			Plagellat et al. (2006)

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Switzerland	2002/ 2003	Influent : 600- 6500; effluent: 60-2700		Lakes -Jörisee (remote mountain lake): <2; Zürichsee: 8, 4, 11, 6, 2; Greifensee: 12,10; Hüttnersee: 7, 28	Whitefish ( <i>Coregonus spec.</i> ): - Thunersee: nq - Pfäffikersee: nq  Roach ( <i>Rutilus rutilus</i> ): - Zürichsee: 80, 73 - Greifensee: 94, 60 - Hüttnersee: 44  Perch ( <i>Perca fluviatilis</i> ): - Hüttnersee: 166	Balmer et al. (2005)
Germany	1991/ 1993				Meerfelder Mar (lake)  Perch ( <i>Perca fluviatilis</i> ): 930  Roach ( <i>Rutilus rutilus</i> ): 68	Nagtegaal et al. (1997)

## **10. Current knowledge on alternatives**

Possible alternatives regarding the use in sunscreens and other cosmetics are listed in Annex List VI of REGULATION (EC) No 1223/2009 on cosmetic products (List of UV-filters allowed in cosmetic products). It has to be noted that for certain UV filter substances regulatory activities have been initiated or they are subject to substance evaluation due to environmental concerns which need to be clarified.

Detailed information on other potential uses than sunscreens and other cosmetics is not available yet. It is expected that other UV filter substances can be used for these applications.

## **11. Existing EU legislation**

4-MBC is mainly used in sunscreens and other cosmetics. 4-MBC is listed in the REGULATION (EC) No 1223/2009 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 30 November 2009 on cosmetic products, ANNEX VI: List of UV filters allowed in cosmetic products with a maximum concentration in ready for use preparations of 4 %.

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