Substance Name: 1,7,7-trimethyl-3-(phenylmethylene)bicyclo[2.2.1]heptan-2-one (3-benzylidene camphor)

EC Number: 239-139-9

CAS Number: 15087-24-8

MEMBER STATE COMMITTEE

SUPPORT DOCUMENT FOR IDENTIFICATION OF

1,7,7-TRIMETHYL-3-(PHENYLMETHYLENE)BICYCLO[2.2.1]HEPTAN-2-ONE (3-BENZYLIDENE CAMPHOR)

AS A SUBSTANCE OF VERY HIGH CONCERN BECAUSE OF ITS ENDOCRINE DISRUPTING PROPERTIES WHICH CAUSE PROBABLE SERIOUS EFFECTS TO THE ENVIRONMENT WHICH GIVE RISE TO AN EQUIVALENT LEVEL OF CONCERN TO THOSE OF CMR[1] AND PBT/VPVB[2] SUBSTANCES

Adopted on 8 June 2016

[1] CMR means carcinogenic, mutagenic or toxic for reproduction
[2] PBT means persistent, bioaccumulative and toxic; vPvB means very persistent and very bioaccumulative

CONTENTS

TABLES

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	carcinogenic, mutagenic or 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

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

Substance Name: 1,7,7-trimethyl-3-(phenylmethylene)bicyclo[2.2.1]heptan-2-one (3-benzylidene camphor)

EC Number: 239-139-9

CAS number: 15087-24-8

- The substance is identified 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 of 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 provide sufficient evidence to conclude that 1,7,7-trimethyl-3-(phenylmethylene)bicyclo[2.2.1]heptan-2-one (3-BC) acts via an endocrine mode of action and that this endocrine activity leads to adverse effects in fish. Hence, 3-BC fulfils the WHO/IPCS definition of an endocrine disruptor for the environment.
The specific mode of action of 3-BC (estrogen receptor agonist and/or androgen receptor antagonist), the effects observed *in vivo* in fish and supporting information as reviewed by Hass et al. (2012) in rodent species as well as the comparison of these effects with known endocrine disruptors acting via the same molecular mode of action provide strong evidence that the endocrine mediated effects of 3-BC 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 3-BC:

- The identified main mode of action (estrogenic and/or antiandrogenic) of 3-BC is comparable to that of known endocrine active substances like bisphenol A (EC No. 201-245-8) or ethinylestradiol (EC No. 200-342-2) and already identified endocrine disrupting chemicals under REACH like 4-nonylphenol, branched and linear and 4-tert-octylphenol (EC No. 205-426-2). Based on *in vitro* data 3-BC also shows antiprogesteronic activity.
- It is probable that 3-BC causes irreversible and long lasting effects on populations and that even short term exposures during sensitive life stages of organisms have adverse effects during the entire life time.
- The specific mode of action (estrogenic and/or antiandrogenic) of 3-BC and the data available for fish and supporting information as reviewed by Hass et al. 2012 in rodent species point to a broad range of taxa that might be affected by exposure to 3-BC 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 3-BC. Mechanistic knowledge about invertebrate hormone receptors shows that also invertebrate species might

be affected by 3-BC.
- It is likely that the effects are adverse not only for single organisms but also for populations and/or subpopulations in the environment.
- Similar to certain other substances of very high concern it is difficult to quantify a safe level for 3-BC in the environment and therefore the risks using traditional risk assessment methods.

In addition to the endocrine disrupting properties, 3-BC shows the potential to be persistent and bioaccumulative at a screening level.

Taking together the evidence presented in this dossier, 3-BC 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 to those of other substances listed in points (a) to (e) of article 57 of REACH.

Registration dossiers submitted for the substance: No

Justification

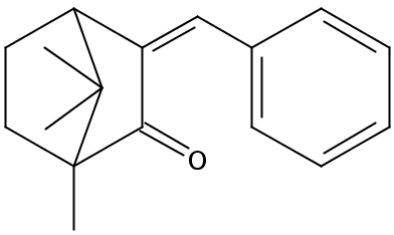
1. Identity of the substance and physical and chemical properties

1.1. Name and other identifiers of the substance

Table 1: Substance identity

EC number:	239-139-9
EC name:	1,7,7-trimethyl-3-(phenylmethylene)bicyclo[2.2.1]heptan-2-one
CAS number (in the EC inventory):	15087-24-8
CAS number: **Deleted CAS numbers:**	15087-24-8 -
CAS name:	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-3-(phenylmethylene)-
IUPAC name:	3-Benzylidene-1,7,7-trimethylbicyclo[2.2.1]heptan-2-one
Index number in Annex VI of the CLP Regulation	-
Molecular formula:	$C_{17}H_{20}O$
Molecular weight range:	240.35 g/mol
Synonyms:	*3-Benzylidenecamphor (3-BC);* *2-Bornanone, 3-benzylidene-;* *Benzylidenecamphor*

Structural formula:



1.2. Composition of the substance

Name: 1,7,7-trimethyl-3-(phenylmethylene)bicyclo[2.2.1]heptan-2-one

Description: organic

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

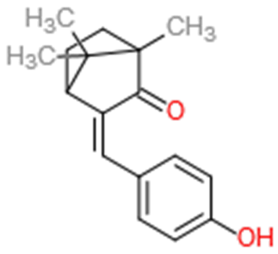
Table 2: Constituents

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)	

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

Regarding the metabolism of 3-BC, the Scientific Committee on Consumer Safety (SCCS) opinion summarises studies from hepatocytes, rats and humans indicating that 3-(4-hydroxybenzylidene) camphene is the major metabolite of 3-BC (SCCS, 2013). Thus, via metabolism a still lipophilic and phenolic 3-BC derivative is formed, which in analogy to known alkyl phenols (e.g. nonylphenol) contains a structural alert (phenyl structure) for binding to the active site of the hERα. The OECD QSAR toolbox identified this metabolite as a storng estrogen receptor binder. Consequently, this metabolite is taken into accoount during the following SVHC identification.

Structure of 3-(4-hydroxybenzylidene) camphene:



1.4. Identity and composition of structurally related substances

Table 3: Structurally related substance(s) identity

EC number:	253-242-6
EC name:	(±)-1,7,7-trimethyl-3-((4-methylphenyl)methylene)bicyclo[2.2.1]heptan-2-one
SMILES:	C1=CC(=CC=C1\C=C/2C(C3(CCC2C3(C)C)C)=O)C
CAS number (in the EC inventory):	36861-47-9
CAS number:	36861-47-9
CAS name:	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-3-[(4-methylphenyl)methylene]-
IUPAC name:	(±)-1,7,7-Trimethyl-3-(4-methylbenzylidene)bicyclo[2.2.1]heptan-2-one
Index number in Annex VI of the CLP Regulation	-
Molecular formula:	$C_{18}H_{22}O$
Molecular weight range:	254.37 g/mol
Synonyms:	3-(4-Methylbenzylidene)-DL-camphor; 3-(p-Methylbenzylidene)-D,L-camphor; 4-MBC

Substance type: multi-constituent (mixture of four possible stereoisomers)

Structurally related substance(s) formula:

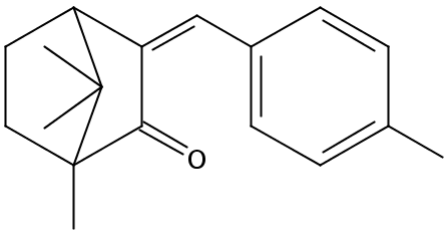


Table 4: Constituents of structurally related substance(s)

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

Since the substance is not fully registered under REACH so far, the given range of purity (80-100%) is considered to be just a standard substance description and no significant impurities are expected and taken into account for the following SVHC identification.

1.5. Physicochemical properties

Table 5: Overview of physicochemical properties

Property	Description of key information	Value	Reference/source of information
Physical state at 20°C and 101.3 kPa	White crystalline material		SCCS, 2013. OPINION ON 3-Benzylidene camphor. COLIPA No S61.
Melting/freezing point		88-90 °C	Zenker, Armin; Schmutz, Hansruedi; Fent, Karl, Journal of Chromatography A, Volume 1202, Issue 1, Pages 64-74, 2008
Boiling point		310 °C	Zenker, Armin; Schmutz, Hansruedi; Fent, Karl, Journal of Chromatography A, Volume 1202, Issue 1, Pages 64-74, 2008
Vapour pressure		4.45E-3 Pa at 25 °C	Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02
Density		1.079±0.06 g/cm3 at 20 °C, 1013 hPa	Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02
Water solubility		0.6893 mg/l at 25 °C	Water Solubility Estimate from Log Kow (WSKOW v1.41)
Partition coefficient n-octanol/water (log value)		log Pow 5.37	KOWWIN v1.67 estimate

2. Harmonised classification and labelling

The substance does not have harmonised classification.

3. Environmental fate properties

3.1. Degradation

3.1.1. Abiotic degradation

No standard test or published studies on abiotic degradation of 3-BC 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 OH-concentration of 1.5×10^6 OH-radicals/cm³. The atmospheric half-life of 3-BC was estimated to be 1.495 hours, and the overall OH-rate constant was estimated to be 8.588×10^{-11} cm³/molec/sec.

It is expected that photodegradation in air is not a relevant pathway for removal from the environment since it is assumed that that the largest part of 3-BC will be emitted directly from the use in sunscreens and indirectly via sewage treatment systems as well as possibly surface runoff into the aquatic compartment. Moreover, due to its very low vapour pressure the substance will not evaporate at ambient temperature. Therefore, photolytic degradation in air or aerosol binding is unlikely. However, testing data on photodegradation is missing and degradation on skin if 3-BC is applied in sunscreens cannot be excluded.

Photodegradation of 3-BC in water 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 largely bind to suspended organic matter and sediment so that the potential for photodegradation will be very limited.

When used in sunscreens and other cosmetics, the substance is released directly to surface water and indirectly to wastewater, where it is expected that a large fraction will adsorb to sewage sludge, which might be applied to agricultural fields. Only a negligible fraction will be available for photolytic degradation in soil when the sludge is ploughed into the soil. Additionally, the SCCS opinion on 3-BC (2013) mentions a UV stability study using a "non-ionic emulsion", which suggests that the substance rapidly forms a photostable isomer, followed by very slow irreversible degradation. This implies that UV degradation is unlikely to be rapid. However, the nature of this photostable isomer is unknown, and it remains unclear whether it might be formed in aquatic media under laboratory conditions.

3.1.2. Biodegradation

No screening or simulation tests or published studies for biodegradation of 3-BC are available.

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 0.0755 indicating that the substance is not rapidly biodegradable.

- Biowin6 (MITI non-linear biodegradation probability) results in a value of 0.148 indicating that the substance is not readily degradable.
- Biowin3 (Survey model – ultimate biodegradation) results in a value of 2.2433 indicating that ultimate biodegradation is expected after months.

3-BC is therefore considered to be not readily biodegradable in the environment. In the key study discussed below (Kunz et al., 2006b) it was noted that there was substantial loss of 70-80% of the test substance from test media over 48 hours. The study authors discussed this in terms of adsorption to surfaces and organic matter as well as bioaccumulation in the fish, which seems plausible with respect to the relatively high log Po/w estimated for 3-BC. However, since no experimental data on the biodegradability of 3-BC are available, also biodegradation might contribute to the observed loss of the substance from the test media.

3.2. Environmental distribution

3.2.1. Adsorption/desorption

The following values for the soil adsorption coefficient of 3-BC have been estimated by using KOCWIN v2.00 (US EPA, 2012): Koc: 7659 L/kg (Log Koc: 3.884) (MCI method) and Koc: 12330 L/kg (Log Koc: 4.091) (Kow method).

It can be expected that the substance will adsorb to a certain degree 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 0.198 Pa×m³/Mol indicating only little tendency for volatilisation.

3.2.3. Distribution modelling

According to Mackay Level III Fugacity Model (EpiSuite v.4.11) 3-BC will be distributed as follows when emitted to water only: 0.02 % to air, 52.6 % to water, 0.01 % to soil, and 47.4 % to sediment. The results of this modelling indicate that the substance will largely adsorb to sediment, when considering that direct emission to soil is expected to be negligible regarding the uses of the substance.

3.2.4. Field data

There is only limited information on 3-BC concentrations in the environment. The IVL Swedish Environmental Research Institute Ltd. has performed a national screening programme and published a subreport on UV filter substances (Remberger et al., 2011). The detection frequency for 3-BC in the samples taken accounted for 57 % in sediment, 13 % in effluents of sewage treatment plants, and 13 % in sewage sludge. However, the substance was not detected in surface water.
3-BC is identified in krill (marine), mysis and smelt (aquatic) in a non-target Norwegian monitoring report (http://www.miljodirektoratet.no/no/Publikasjoner/2016/Februar-2016/Screening-program-2014/).
Goksoyr et al. (2009) detected 3-BC in surface water samples in the Pacific Ocean at concentrations of < 0.29 ng/l when collected with a passive semipermeable membrane device and 13 ng/l with an active surface layer.

3.3. Data indicating potential for long-range transport

No information is available indicating potential for long-range transport of 3-BC.

3.4. Bioaccumulation

No standard test on bioaccumulation is available for 3-BC. Based on the estimated log Pow of 5.37 (KOWWIN v1.68) 3-BC has potential for bioaccumulation. However, in a non-standard assay Kunz et al. (2006b) estimates fish BCF of 3-BC to be in the range of 102 – 493 depending on the exposure concentration. Since these measurements were done without using standard bioaccumulation test methods, artefacts might have influenced the results. Further data are needed to conclude on the bioaccumulation potential of 3-BC.

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 for 3-BC to enable the comparison to the chronic test results.

Fent et al. (2008) refers to a 96h-LC_{50} of 141 µg/l in fish (rainbow trout). The validity of the underlying study could not be evaluated as the reference is only a link to Scifinder Scholar 2006 (secondary literature) with no details.

The exposure of *Desmodesmus subspicatus* to 3-BC resulted in growth inhibition with a 72h-EC50 of 6.99 mg/l (nominal) in an OECD guideline 201 test (Sieratowicz et al., 2011) (reliability 1). It was conducted at a temperature of 20.5 ± 1 °C and a 24 h photoperiod with 6000 to 6500 lux. 5×10^4 cells per replicate with 5 replicates for the controls and 3 replicates for the solvent controls and each treatment group were used. The nominal test concentrations of 0.5, 1.0, 2.0, 4.0, and 8.0 mg/l were verified with HPLC and UV-SPE (LOQ = 0.5 µg/l).

Sieratowicz et al. (2011) also described a 48 h acute immobilisation test with *Daphnia magna* following OECD guideline 202 (reliability = 1). Four replicates per treatment group and 5 daphnids per replicate were used at 20 ± 1 °C and a photoperiod of 16:8h light:dark. The nominal test concentrations of 0.8, 1.6, 3.2, 6.4, and 12.8 mg/l were verified with HPLC and UV-SPE (LOQ = 0.5 µg/l). The exposition for 48h with 3-BC resulted in an EC_{50} of 3.61 mg/l (nominal).

5.2. Toxicity test results concerning endocrine disruption

5.2.1. General approach

As described in Art. 57(f) of REACH Regulation, 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 as endocrine disrupting substances, as a starting point the WHO/IPCS definition is used to describe whether or not 3-benzylidene camphor is an endocrine disruptor in the environment. Thus this chapter summarises data which provide evidence that 3-

benzylidene camphor 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 interact with the endocrine system?
- Are the adverse effects observed likely to be a consequence of this interaction?

Information is summarised by organism groups in the following chapters, starting with a summary of available *in vitro* tests as supportive information. Additionally, supporting information for the environmental hazard assessment originating from human health studies is summarised at the end of this section.

Since the water solubility of 3-BC is rather low (estimated to be around 0.689 mg/L) most of the studies cited used solvents to increase the apparent concentration of 3-BC in the aquatic test media. Where available from the literature cited, the use of solvents and solvent control is indicated in the text. Additionally, information on nominal and/or measured effect concentrations is included within the study description. In cases where only nominal values are given it can be assumed, owing to the poor water solubility and the estimated high adsorption potential of 3-BC, that the effects might occur at lower real concentrations in the assays. Hence, the effect values reported might underestimate the effect potential of 3-BC in these cases.

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 3-BC

Method	Short Method description	Result	Description of results	References	Reliability
Computational Chemistry	Virtual screening of a 3d-structural database using pharmacophores of 17β-HSD3	+	Inhibits 17β-HSD2 at low micromolar concentration	Nashev et al. (2010)	2 – Acceptable, well-documented report
QSAR prediction tool VirtualToxLab	Virtual screening of 3D structures against binding affinity to	+ estrogenic	The prediction tool identified the ERβ subtype as	Vedani et al. (2009)	2 – Acceptable, well-documented

	various receptor proteins		main target for 3-BC binding		report

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 3-BC to inhibit 17β-HSD, an enzyme that metabolizes estrogens and androgens.

Additionally, Vedani et al. (2009) identified the human ERβ subtype as the main target for binding of 3-BC using a quantitative 3D computational screening model. Among various endocrine related receptor proteins (e.g. AR, ERα/β, TRα/β, PPAR) binding of 3-BC to the ERβ was calculated to be significant and a comparatively high toxic potential via this molecular binding event is predicted by the authors.

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

For 3-BC some *in vitro* assays are available. They are summarised in Table 7. Whereafter the descriptions of the different studies are provided.

Table 7: *In vitro* assays with 3-BC (+ indicates a positive result; - indicates a negative result)

Short Method description	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_{50} = 11,8 µM	17β-Estradiol (E2): IC_{50}= 2.1 nM	Schlumpf et al. (2004a)	2 – Acceptable, well-documented report
E-screen (MCF-7)	+ estrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 0.68 µM Full dose-response	17β-Estradiol (E2): EC_{50}= 0.00103 µM	Schlumpf et al. (2004a)	2 – Acceptable, well-documented report
E-screen (MCF-7)	+ estrogenic - antiestrogenic	Stimulation of MCF-7 proliferation: EC_{50}= 1.70 µM Full dose-response no antagonism of E2 induced proliferation up to 10 µM	17β-Estradiol (E2)	Jimenez-Diaz et al. (2013)	2 – Acceptable, well-documented report
Gene expression assay in stable ERα and ERβ reporter cell lines (HEK293cells)	+ estrogenic	Activation of transcription for hERα: IC_{50}= 13 µM Activation for hERβ: IC_{50}= 10 µM Maximal activation: 50% of 17ß-estradiol	17β-Estradiol Activation of transcription for hERα: IC50= 0.0021 µM Activation of transcription for hERβ: IC_{50}= 0.083 µM	Schreurs et al. (2005)	2 – Acceptable, well-documented report
hERα activation in a yeast Estrogen Screen (YES) with additional enzymatic degestion of the yeast cells	+ estrogenic	hERα activation: EC_{50}= 44.2 µM (maximal activation 80% of E2)	17β-Estradiol (E2): EC_{50}= 0.147 nM = 0.0000147 µM	Schmitt et al. (2008)	2 – Acceptable, well-documented report with clear dose-response relationship
Recombinant yeast systems carrying a human estrogen (hERα)	(+) estrogenic (+) antiestrogenic	hERα activation: EC_{50}= 310 µM (21% effect compared to E2) hERα inhibition : EC_{50}= 8460 µM (134 % effect compared to 4-hydroxy-tamoxifen)	17β-Estradiol (E2)	Kunz and Fent (2006)	2 – Acceptable, well-documented report but only submaximal effects

Recombinant yeast system carrying either a rtERα or hERα	(+) estrogenic	rtERα activation: EC_{50}= 12.2 µM (maximal activation 27 % of E2) hERα activation: EC_{50}= 310 µM (21 % effect compared to E2)	17β-Estradiol (E2) rtERα activation: EC_{50}= 18.1 nM = 0.00181 µM 17β-Estradiol (E2) hERα activation: EC_{50}= 0.291 nM = 0.0000291 µM	Kunz et al. (2006a)	2 – Acceptable, well-documented report but only submaximal effects
Androgenicity:					
Recombinant yeast systems carrying a human androgen receptor (hAR)	- androgenic + antiandrogenic	hAR activation: EC_{50}= nd hAR inhibition: EC_{50}= 18.5 µM (143 % effect of flutamide)	4,5-dihydro-testosterone (DHT)	Kunz and Fent (2006)	2 – Acceptable, well-documented report but only submaximal effects
PALM cells (hAR)	- androgenic	no androgenic activity: 0.01-10 µM	Synthetic androgen R1881	Jimenez-Diaz et al. (2013)	2 – Acceptable, well-documented report
AR-mediated gene-reporter activiation assay in MDA-kb2 cells	- androgenic or antiandrogenic	No agonistic or antagonistic (in 0.5 or 0.1 nM DHT) action on AR 0.001 to 10 µM	Androgen agonist: 4,5-dihydro-testosterone (DHT) EC_{50}=0.136 nM Antiandrogen: Flutamide in 0.5 nM DHT IC_{50}= 3.62 µM	Ma et al. (2003)	2 – Acceptable, well-documented report
AR CALUX Bioassay	+ antiandrogenic	Repression of transpcription of hAR: IC_{50}= 4.6 µM	Flutamide IC_{50}= 0.5 µM	Schreurs et al. (2005)	2 – Acceptable, well-documented report
Progesterone activity:					
PR CALUX Bioassay	+ antiprogesterone	Repression of transpcription of hPR: IC_{50}= 0.4 µM (coincubation with excess ORG2058 (100 times the EC_{50} value))	RU486 IC_{50}= 0.0049 µM	Schreurs et al. (2005)	2 – Acceptable, well-documented report
Human sperm: activation of the CatSper channel	+ antiprogesterone	EC_{50}= 1.73 ± 1.36 µM (for comparison: 4-OP EC_{50}= 5.93 ± 0.40 µM)	Inhibition by MDL12330A: 95.88 ± 1.63% (4-OP inhibition of MDL12330A: 62.49±23.49%)	Schiffer et al. (2014)	2 – Acceptable, well-documented report

1. Estrogenic activity

With regard to estrogenic activity the following tests are available:

- 1 receptor binding study using either uterine cytosolic estrogen receptors or recombinante human ERα (hERα) and ERβ (hERβ)
- 2 MCF cell proliferation assays analysing cell proliferation due to hER activation
- 1 gene expression test with human HEK293 cells transfected with hERα and hERß receptors
- 2 gene expresion tests with yeast cells transfected with hERα
- 1 gene expression test with yeast cells transfected with rainbow trout ERα (rtERα)

Fent et al. (2008) summarises and gives an overview of the published studies on the hormonal activity of UV filters *in vitro* (part one) – see also Kunz and Fent (2006); Kunz et al. (2006a) - and *in vivo* in fish (part two) – see also Holbech et al. (2002); Kunz et al. (2006a); Kunz et al. (2006b).

Schlumpf et al. (2004a) conducted an estrogen receptor ligand binding assay (ER-LBA) with recombinant human ERα and ERβ and a porcine cytosolic uterine estrogen receptor preparation. 3-BC was tested in competition experiments with 16α^{125}I-estradiol and estradiol as positive control. In the cytosolic ER preparation, 3-BC displaced the radioligand in a concentration-dependent manner. In contrast, in the binding experiment with recombinant human ERα, 3-BC did not displace the radioligand up to a concentration of 1mM. However, 3-BC was able to displace 16α^{125}I-estradiol from the ERβ (IC_{50} = 11.8 µM) in a concentration-dependent manner.

Schlumpf et al. (2004a) also conducted an E-SCREEN with MCF-7 human breast cancer cells. MCF-7 cells were trypsinized, plated into 96-well plates at an initial density of 3000 cells per well in 100µL experimental medium and allowed to attach at 37 °C. After 24 h, another 100 µL experimental medium containing either 3-BC (stock solution:10^{-2} M; final concentrations 10^{-5} to 10^{-9} M; final ethanol concentrations between 0.1 and 0.00001 % (v/v)), 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 ≤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, containing six wells per concentration) 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. 3-BC activated cell proliferation with a full dose-response curve, yielding an EC_{50} of 0.68 µM. The maximum proliferation observed for 3-BC was similar to the maximum proliferation of estradiol (102 % of estradiol). The EC_{50} for 17ß-estradiol was 0.00103 µM and thus the relative potency of 3-BC was calculated to be 0.0015.

Jimenez-Diaz et al. (2013) investigated the potential estrogenic and antiestrogenic as well as the androgenic activity of different UV filters in an *in vitro* test, and amongst others 3-BC was tested. The potential estrogenic activity or ability to stimulate cell proliferation on estrogene sensitive MCF-7 cells of 3-BC was characterised using the E-Screen bioassay. 3-BC showed no cytotoxic activity in the concentration range tested (0.01 – 10 µM). E2 was used as a positive control (0.1 – 1000 pM) but results were not shown. 3-BC was the most active compound (EC_{50}= 1.70 µM) increasing the number of viable cells by 4.5-fold, compared to the control treated cells (hormone-free medium). 3-

BC induced significantly the proliferation of the MCF-7 cells resulting in a full dose response curve. As some reports suggested an antiproliferative activity for some UV filters, Jimenez-Diaz et al. (2013) examined also the potential antiestrogenic activity. All compounds tested failed to antagonise E2-induced proliferation in MCF-7 cells up to 10 µM.

Schreurs et al. (2005) observed in an *in vitro* gene expression assay in stable ERα and ERβ cell lines (anti)estrogenic activity of 3-BC using HEK293 cells with 96-well tissue culture plates (6000 cells/ well) at a volume of 200 µL 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 hERα or hERβ, and a 3xERE-tata-Luc-reporter gene construct. To measure antiestrogenicity, cells were incubated with both the chemical to be tested and E2 concentration of 3 and 100 pM for hERα and hERβ, respectively. This E2 concentration was the approximate EC_{50}, 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. 3-BC showed agonism towards hERα and towards hERβ as well. No full dose-response-curve was obtained for either hERalpha or hERß due to too low test concentrations. The maximum activation was 50 % compared to the positive control, but no plateau was observed. None of the UV filters showed antiestrogenic effects. The EC_{50} for the activation of 3-BC induced transcription of hERα was 13 µM and of hERβ was 10 µM.

Schmitt et al. (2008) conducted a Yeast Estrogen Screen (YES) and two sediment assays with 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. An additional digestion step was included at the end of the 24h incubation period using the enzyme lyticase. The plates included a blank, a negative control, a full concentration range of the positive control 17ß-estradiol (E2, 3 pM – 0.1 µM) and different test concentrations of 3-BC (0.01, 0.03, 0.1. 0.3, 1, 3, 10, 30, 100, 300 µM). After 23h of incubation with the test compounds, the absorbance was measured and after that, 100 µL of the lyticase stock solution containing CPRG was added and absorbance was measured in intervals of 30 min. In the YES, 3-BC exhibited a clear dose-response relationship with a maximal response of 80 % of E2 and significant higher responses at the three highest concentrations compared to the negative control.

Kunz and Fent (2006) investigated many UV filters for multiple hormonal activities *in vitro* in human estrogen and androgen receptor systems. They systemically analysed the estrogenic, antiestrogenic, androgenic and antiandrogenic activity of 3-BC *in vitro* at non-cytotoxic concentrations with recombinant yeast systems carrying either a human estrogen (hERα) 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 there was a positive control with either 17β-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 observed with E2 treatment, 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. 3-BC caused receptor activation in a full dose-response manner. However the maximal activation observed at several test concentrations was only 21 % of the maximal activation observed for E2. 3-BC completely inhibited the activity of E2 at the highest concentrations tested in a full dose-response manner but very high concentrations were used which were potentially cytotoxic. An androgenic response to the yeast hAR transactivation assay was not

comparable to the effect caused by DHT . There was an antiandrogenic response to the yeast hAR transactivation assay with 143% of the effect of the positive control Flutamide.

Kunz et al. (2006a) provides another evaluation of the endocrine activity of 3-BC. They determined the estrogen activity of 23 UV filters using recombinant yeast carrying the estrogen receptor of rainbow trout (rtERα) and made comparisons with yeast carrying the human hERα for receptor specificity. The rtERα is based on transactivation of rtERα and induction of β-galactosidase leading to a color change. The induction is strictly dependent on the presence of rtERα and estrogens. When an active ligand (i.e., 17β-estradiol or an estrogenic UV filter) binds to the receptor, β-galactosidase is synthesized and secreted into the medium, leading to acatalytic hydrolysis of o-nitrophenyl-β-galactopyranoisd (ONPG) and resulting in the development of a yellow color, which was measured as absorbance at 405 nm. The assay was performed using 96-well microplates. Three rows contained serially diluted positive control E2, one row the ethanol blank and four rows contained the UV filter in quadruplicates with increasing concentrations. Results described for hERα are identical to those published in (Kunz and Fent, 2006) with an EC_{50} of 310 µM and a submaximal dose response curve resulting in maximal 21 % activation. Also for the rtERα 3-BC caused receptor activation in a full dose-response manner. But similar to the hERα, the maximal activation (27 %) observed at several tests concentrations was far away from the maximal activation of E2. However, with an EC_{50} of 12.2 µM 3-BC was much more potent at the rtERα than at the hERα.

In summary the cell free receptor binding studies indicate that 3-BC is not able to replace E2 at the hERα but that it does replace E2 at the ERβ. On the other hand cell based test results unambigously show that 3-BC – or its metabolites - is an ER agonist *in vitro* and that it activates both the human ERα and ERß at similar concentrations. Results indicate that fish ERα might be more susceptible to 3-BC than human ERα. Regarding the metabolism of 3-BC, the Scientific Committee on Consumer Safety (SCCS) opinion summarises studies from hepatocytes, rats and humans indicating that 3-(4-hydroxybenzylidene) camphene is the major metabolite of 3-BC (SCCS, 2013). Thus, via metabolism a still lipophilic and phenolic 3-BC derivate is formed, which in analogy to known alkyl phenols (e.g. nonylphenol) contains a structural alert for binding to the active site of the hERα.

In combination with the available knowledge about the 3-BC metabolism, the presented *in vitro* results indicate that the estrogenic activity of 3-BC might be caused mainly by its main phenolic metabolite. However, 3-BC itself may exhibit antiestrogenic activity by binding to the ERα at a different binding site compared to the E2 binding cavity but at very high concentrations only. Additionally, it has to be taken into account that the test substance might be a mixture of stereoisomers, which could have different biological activties. From the studies description it is not possible to conclude on the isomeric pattern used and analytical data that could provide information on the exact identity of the test substances used are not available.

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 the effects differ between 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. Both MCF cell tests showed full dose-response curves with EC_{50} values of 0.68 µM and 1.7 µM respectively (Jimenez-Diaz et al., 2013; Schlumpf et al., 2004a). Results by Schlumpf et al. (2004a) showed that exposure of MCF-7 cells to 3-BC results in an induction of a proliferation rate comparable to that of E2 although at higher concentrations (relative potency ($EC_{50\ E2}/EC_{50\ 3\text{-}BC}$) = 1.5×10^{-3} (0.0015). (No positive control was tested by

Jimenez-Diaz et al., 2013).

Similar results were obtained by Schreurs et al. (2005) in hER transfected HEK293 cells (gene expression test). Results from this study show that exposure to 3-BC activates both ERα as well as ERß at similar test concentrations (13 and 10 µM, respectively). The slightly lower sensitivity compared to the MCF cells is in line with the observation that 3-BC binds to both ERα and ERß as MCF cells exhibit both ERα and ERß receptors and thus proliferation is a result of activation of both types of receptors.

Both cell lines must be considered as metabolically active cells since they are derived from human cancer tissue. Thus it is plausible that in these cell lines, 3-(4-hydroxybenzylidene)-camphene is produced as the main metabolite of 3-BC.

Studies 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_{50} values of 3-BC were much higher in yeast cells compared to those obtained with HEK and MCF-7 cells. In addition, the maximal inducable effect compared to E2 was lower in the yeast assays (27 and 80 %, respectively) compared to the human cell lines (100 %).

This finding supports the assumption that the strong estrogenic acitvity of 3-BC is mainly caused by its main metabolite 3-(4-Hydroxybenzylidene)-camphene 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 3-(4-Hydroxybenzylidene)-camphene is produced and that thus the overall activity is lower. This is in line with the antiestrogenic activity observed by Kunz et al. (2006a) (see below). The submaximal dose-response curve may be explained by a combination of the estrogen-agonistic mode of action of the metabolite and the estrogen antagonistic mode of action of 3-BC itself.

Results by Kunz et al. (2006a) comparing rtERα and hERα activation indicate that rainbow trout receptors may be more sensitive to 3-BC compared to hERα (EC_{50} 12.2 µM compared to 310 µM) although these results should be considered with care due to the submaximal inhibition observed here.

The results of the studies on the antiestrogenic activity of 3-BC 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 very high concentrations (EC_{50} 8460 µM) and a very low relative activity compared to the reference substance 4HT (4.2×10^{-5}). 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 metabolite 3-(4-Hydroxybenzylidene)-camphene which is predominately active in the HEK cells while the antiestrogenic activity in the yeast cells is caused by a high concentration of unmetabolised 3-BC itself.

In conclusion, 3-BC itself seems not to be able to bind to the E2 binding site of hERα and cause its activation but it may have antiestrogenic activity, although at high concentrations. Whether it shows estrogenic or antiestrogenic activity by binding to the E2 binding site of the hERβ remains unclear. Results from the studies described above support the hypothesis that the observed estrogenic activity of 3-BC is caused by its main metabolite 3-(4-Hydroxybenzylidene)-camphene. Results show that 3-BC has estrogen receptor agonistic activity toward hERα and hERβ as well as towards rtERα in metabolically active cell systems.

2. Androgenic activity

With regard to androgen activity the following results are available:

- 1 AR-mediated gene-reporter activation assay in MDA-kb2 cells.
- 1 gene expresion test with yeast cells transfected with hAR (AR CALUX Bioassay)
- 1 gene expresion test with PALM cells (hAR)

Jimenez-Diaz et al. (2013) used PALM cells for the gene expression bioassay examining the agonistic activity of hAR. 3-BC showed no cytotoxic activity in the concentration range tested (0.01 – 10 µM). In this cell line, the synthetic androgen R1881 exhibits strong androgenic activity. None of the studied UV filters, including 3-BC, showed androgenic activity in the concentration range of 0.01 – 10 µM.

Schreurs et al. (2005) observed *in vitro* antagonistic activity of 3-BC 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 µL 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 used a U2-OS cell line overexpressing AR. This is probably more selective and sensitive for measuring AR interaction than other cell lines like an MDA-kb2 cell line containing low endogenous AR and GR levels. The IC_{50} value for repression of transcription of hAR in AR CALUX cells by 3-BC was 4.6 µM. This reveals clear antiandrogenic effects of 3-BC.

Ma et al. (2003) studied the potential actions of 3-BC on androgen receptors (AR) in the human breast carcinoma cell line MDA-kb2 which expresses functional endogenous AR and was transfected with a luciferase reporter plasmid. MDA-kb2 cells were trypsinized and seeded into 96-well plates at a density of about 1×10^4 cells/well with 100 µL 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 antagnoists) were used. 1 nM to 10 µM 3-BC showed no agonistic activity and did not inhibit AR activiation by DHT either (no antiandrogenic activity). This is probably because of the low endogenous AR and GR levels of this cell line.

In summary 3-BC did not show any androgenic activity in the tests described above up to 10 µM. With regard to antiandrogenic activity, the results were ambiguous. While 3-BC showed antiandrogenic activity in two tests (Schreurs et al., 2005 and Kunz and Fent, 2006) with EC_{50} values of 4.6 and 18.5 µM respectively, it was not antiandrogenic in a third test up to 10 µM (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
- 1 test with human sperm loaded with the Ca^{2+} indicator Fluo-4 and the pH_i indicator BCECF

Schreurs et al. (2005) studied the effects of 3-BC and other chemicals like 4-MBC 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 µM (EC_{50}) ORG2058 was used for the measurement of antiprogestagenic activity. 3-BC repressed the transcription of hPR in PR CALUX cells with an IC_{50} of 0.4 µM (for RU486 the IC_{50} was 4.9 pM with a similar dose-response curve as 3-BC). 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 EC_{50} value). This shows the specifity 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 3-BC and also other chemicals like 4-MBC and 4-tert-octylphenol on (human) sperm through activation of the calcium-channels on sperm cells (CatSper). They used 384-microtiter plates for monitoring $[Ca^{2+}]_i$ in human sperm. The injection of progesterone into the wells evoked a rapid, transient increase in $[Ca^{2+}]_i$ 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 did not affect $[Ca^{2+}]_i$. They also used the CatSper inhibitor MDL12330A to examine whether ED-induced Ca^{2+} signals involve CatSper. MDL supressed Ca^{2+} signals evoked by 3-BC (and also by 4-MBC and nonylparaben for instance). Therefore they concluded that 3-BC 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 Ca^{2+} concentration and thereby the swimming behaviour of sperm.

In a recent poster presentation at the ENDO conference (April 2016) Rehfeld et al. reported that 3-BC and 4-MBC interfere with human sperm function by mimicking progesterone (see https://endo.confex.com/endo/2016endo/webprogram/Paper24339.html for the poster abstract).[3]

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 pathways 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.

Conclusion on the *in vitro* data:

Estrogen 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 3-BC. 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 supported assumption that the main metabolite 3-(4-Hydroxybenzylidene)-camphene is formed within active cells and responsible for the observed estrogenic effects.

Androgen activity: There are different results for antiandrogenic activity of 3-BC in two studies showing a clear antiandrogenic effect and in one study showing no effect up to

[3] We are aware of the fact that this conference report does so far not represent a peer reviewed publication and hence can only be used as supportive information. Thus, this citation is just given to complete the recent picture of possible *in vitro* effects of 3-BC and was not considered among the studies ranked according to their Klimisch score for reliability.

the highest tested concentration of 10 µM of 3-BC. 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 progesterone or progesterone-like acitivity of 3-BC. In summary 3-BC shows antagonistic acitivity which also could be reversed by co-incubating with a stable PR agonist. Similar to progesterone, 3-BC activates the calcium channels on sperm cells (CatSper) which affects their swimming behaviour. Reduced sperm motility is correlated with decreased fertilisation rates in fish.

5.2.3. *In vivo* tests

Approach used for assessing the endocrine acitivity in fish:

In this chapter mainly the effects of 3-BC on fish are described. Additionally, a summary of available supporting studies, analysed by Hass at al. (2012), with mammals potentially indicating endocrine disrupting properties of 3-BC is presented.

The assessment of whether 3-BC 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 the 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 an 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 estrogenic mode of action.

One of the most common biomarkers indicating an estrogen or androgen 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 and accepted indicator of an estrogen receptor agonistic 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 without further specifying the exact endocrine mode of action:

- 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 modes of action other than endocrine mediated.

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 be taken as the GSI is highly dependent on the individual fish (frequent spawners) or seasonal gonadal stage (seasonal breeders)[4].

In addition, the following apical endpoints are considered to be indicators of an estrogen receptor agonist or antiandrogenic 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 estrogenic or antiandrogenic 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

[4] 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.

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 or antiandrogen 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).

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.

Table 8 summarises endpoints that are considered indicators of estrogenic or antiandrogenic 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 or anti-androgenic mode of action	Endpoint considered to be sensitive to an estrogenic mode of action *in vivo*
• Vitellogenin induction in males (only estrogenic mode of action) • increased proportion of spermatogonia (early sperm cells), presence of testis-ova, increased testicular degeneration, interstitial (Leydig) cell hyperplasia/hypertrophy in males • increased oocyte atresia, perifollicular cell hyperplasia/hypertrophy, decreased yolk formation (aromatase inhibition), changes in gonadal staging in females • Depression of male secondary sex characteristics in fathead minnow or medaka and induction of female secondary sex characteristics such as uro-genital papillae in zebrafish • Female biased phenotypic sex-ratio during sexual development.	• Female biased phenotypic sex-ratio during sexual development especially in medaka • Reproduction (fecundity, fertility, number of males or females with reproductive success) • Spawning behaviour • Growth of offspring

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 acivity.

***In vivo* assays providing data about selected endocrine mechanisms/ pathways and adverse effects of 3-BC:**

Table 9: *In vivo* assays with 3-BC – part 1

Species Life stage/ duration	Concentration/ test condition/ tested substance / solvent	Vitellogenin	others	Positive control	Reference	Reliability
Oncorhynchus mykiss juvenile (102 ± 13 g)/ duration: exp.1 10d + exp.2 14d	**intraperitoneal injections** of 3-BC on day 0, 3 and 6 and in experiment 2 the injections were given on day 10 also **exp. 1: 27, 205 or 410 mg 3-BC/kg/injection** exp. 2: 2.7, 8.2, 14, 27, 68 or 137 mg 3-BC/kg/injection	LOEC < 27 mg/kg increase (measured per ELISA) At 205 and 410 mg/kg/injection: VTG levels comparable to E2 LOEC = 27mg/kg VTG level at 68 and 134 mg 3-BC/kg/injection: 10,000 µg VTG/ml	No effects on HSI	17β-Estradiol (E2) (5 fish with 683 µg E2/kg/injection): exp.1: comparable to 205 and 410 mg 3-BC/kg/ injection 2: 683 µg E2/kg/injection (positive control)	Holbech et al. (2002)	2 – acceptable, well – documented report Exposition route unrealistic (injection)
Pimephales promelas Mixed-sex juvenile (2-3 months, 19 to 27 mm body length)/ duration: 14d	Solvent control (0.1 mL ethanol/L), 10, 100, 500 or 1000 µg 3-BC/L (nominal) or 9, 435, 953 µg 3-BC/L (real)	LOEC 435 µg/l Dose-related sign VTG induction (measured per ELISA) at 435 and 953 µg 3-BC/l (407 and 1,753 µg VTG/ml)	LOEC = 435 µg/l (length)	100 ng/l E2 (1,000 µg VTG/ml) 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. (2006a)	2 – acceptable, well-documented report
Pimephales promelas Juvenile/ duration: 14 days	6 concentrations and also mixtures with benzophenone-1 and -2	Induction of VTG in fish LOEC = 420 µg/l (r), NOEC = 215 µg/l (r)		Positive control: Ethinyl estradiol (EE2) (100 ng/l)	Kunz and Fent (2009)	4 – only abstract available

Table 10: *In vivo* assays with 3-BC – part 2

Species *Life stage/ duration*	Concentration / test condition/ tested substance / solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sec. sex characteristics	others	Positive control	Reference	Reliability
Pimephales promelas Age: 8-9 months mature/ short-term reproduction assay similar to OECD 229/ 21 days	0.5, 3, 33, 74 and 285 µg/l, (1ml DMF in 10 l of water/ 48h static-renewal procedure/ 3 replicates with 4 females + 2 males each/	**Dose-dep. VTG induction in male: LOEC= 74 µg/l (5,272 to 18,020 µg VTG/ml – control males: 15 µg VTG/ml)**	Testis: starting at 3 µg/l: dose-dep. inhibition of spermatogenesis → ↑spermatocytes and spermatides Ovaries: starting at 3 µg/l: ↑number of artretic follicles + ↓early and late VTG stages	↓cumula-tive number of eggs spawned: LOEC= 74 µg/l No egg production at 285 µg/l in females	**74 µg/l** and higher: **male** fish had dose-dependent **less tubercle (numbers) and they seemed less pronounced**. At 285 µg/l males were visually not discernible from females, all but one had lost all tubercles	no toxic side effects (i.e., lethargy, uncoordinated swimming, loss of equilibrium, hyperventi-lation	Testis: histological response of 3-BC much like that observed with E2 and EE2: full inhibition of testicular develop-ment	Kunz et al. (2006b)	2 – guideline study with deviations (semi-static instead of flow-through)

Holbech et al. (2002) conducted an *in vivo* fish assay with juvenile rainbow trout *Oncorhynchus mykiss* in two experiments. The temperature during the experiments was 15 ± 1°C and the photoperiod 12 h light per day. 80 L stainless steel tanks were used. In experiment 1 the trout were given intraperitoneal injections of 3-BC on day 0, 3 and 6 and in experiment 2 the injections were given on day 10 also. Experiment 1 contained 5 groups of 10 fish each except in the positive control 17β-Estradiol (E2) (5 fish with 683 µg E2/kg/injection). In experiment 1: 27, 205 or 410 mg 3-BC/kg/injection were injected with peanut oil (vehicle control). In experiment 2: 8 groups of 10 fish were used. The trout were injected with peanut oil (vehicle control), 683 µg E2/kg/injection (positive control), 2.7, 8.2, 14, 27, 68 or 137 mg 3-BC/kg/injection. The experiment 2 was conducted until day 14. The vitellogenin concentration was quantified by a direct non-competitive sandwich ELISA. All three doses of injected 3-BC in experiment 1 resulted in significant vitellogenin responses. There were no significant differences among the hepatosomatic indexes (HSI) from any of the groups. Experiment 2 was conducted to derive a LOEC. On day 0 vitellogenin values did not differ among groups. A clear relationship between the doses of injected 3-BC and measured vitellogenin was seen on day 3 after one injection. Significant differences from the control group, i.e. LOEC on day 3, 6, 10 and 14 were obtained at doses of 68, 27, 27 and 27 mg 3-BC/kg/d. At these concentrations vitellogenin concentration was twice the highest concentration observed in all groups on day 0 and in the control group throughout the experiment. The ED_{50} was 16 mg 3-BC/kg/injection on day 3 and 20. 2 mg 3-BC/kg/injection on day 14. Vitellogenin concentration for 68 and 134 mg 3-BC/kg/injection and 683 µg E2/kg/injection on day 14 was 10,000 µg VTG/ml plasma (day 0: 0.100 µg VTG/ml plasma).

Kunz et al. (2006a) performed 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 was 25 ± 1 °C. Ten randomly selected fish were placed in a 10L-stainless steel tank and exposed for 14 days. Two controls, solvent control (0.1mL ethanol per L water) and positive control for estrogenic activity (100 ng/l E2) were included. The analytically assured nominal concentrations of 3-BC were 10, 100, 500 and 1000 µg/l (real: 9, 100, 435 and 953 µg/l). 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)). The exposure with 3-BC in the *in vivo* test resulted in the death of one fish on day 12 at 953 µg/l. For all control, solvent control and E2 fish no mortality was observed. For 435 and 953 µg/l 3-BC the length gain was significantly decreased in a dose-related manner. Dose-dependent vitellogenin induction occurred also at 3-BC concentrations of 435 and 953 µg/l. Vitellogenin induction was more than 3-fold compared to the controls and in a similar range compared to E2. 435 and 953 µg/l 3-BC resulted in 407 and 1753 µg VTG/ml respectively and 100 ng/l E2 in 2600 µg VTG/ml compared to controls with 0.3 µg VTG/ml.

Kunz et al. (2006b) conducted a short-term reproduction assay with *Pimephales promelas*. They used mean measured concentrations of 0.5, 3, 33, 74 and 285 µg/l (nominal 1,10,100, 250 and 500 µg/l) of 3-BC as well as a control and a solvent control (1 mL DMF in 10 l of water) in the 21-day-test. They used reproductively mature fathead minnows between 8 and 9 month of age, which had not been held in a culture situation conductive to routine spawning before the onset of the experiment. They used fish from a cultivator and adapted them for a minimum of 14 days. The photoperiod was 16-h light per day and the temperature 25 ± 1°C. The 3-BC exposure started once the fish started successfully spawning, generally after 14-21 d. Survival, appearance and behaviour of the fish, reproductive behaviour, secondary sex characteristics and fecundity (cumulative number of spawned eggs) were determined. The experimental procedure was similar to OECD Guideline 229 with regard to test conditions, endpoints measured and analytical

procedures (e.g. VTG assessment) with the following exceptions: In line with OECD 229 four females and two males were assigned to the replicate stainless steel tanks (10 l). However, 5 instead of 3 concentrations were chosen with 3 instead of 4 replicates. They used a 48h static-renewal procedure, renewing the total of aquaria water (10 l). To minimise the disturbances to ensure normal reproductive performance of the fish, they chose a static-renewal regime of 48h instead of 24h.

Results: There were no toxic side effects (i.e., lethargy, uncoordinated swimming, loss of equilibrium, hyperventilation) observed. Neither in males nor in females was GSI altered. Measured test concentrations were close to nominal concentrations at the beginning of the test but decreased to 19 – 29 % of the nominal concentration before water renewal.
3-BC caused a dose-dependent VTG induction in male fathead minnows, which was significant at 74 and 285 µg/l. At these concentrations VTG concentrations were similar to those observed in females and 2-3-fold higher than in control males. In female fish, no significant VTG induction occurred. 3-BC caused depression of male secondary sexual characteristics. At 74 µg/l and higher male fish had less tubercles in a dose-dependent manner (numbers) and they seemed less pronounced. At the highest exposure concentration all but one males had lost all tubercles. In females the inhibition of oogenesis started at 3 µg/l and was indicated histologically by an increase of atretic and a decrease of early and late vitellogenic follicles in ovaries. 3-BC affected the gonadal histology of male fish causing a dose-dependent inhibition of spermatogenesis in the testis and therefore increased spermatogonia. According to the authors, histological changes were significant at 3 µg/l and above. However, as no information is provided on how statistics were applied for this endpoint, these results are used in a qualitative rather than quantitative way.

The test revealed dose-dependent effects of 3-BC inhibiting fertility and reproduction (number of spawns, number of eggs per spawn and the number of eggs per female per day) in fathead minnows significantly starting at 74 µg/l. At 285 µg/l females stopped egg production. As described in OECD guideline 229, variation of cumulative number of eggs spawned during pre-exposure was observable. Two treatments (3 and 285 µg/l) showed a lower number of cumulative eggs during pre-exposure compared to controls and thus a mean lower number of eggs/female/day. One replicate at 285 µg/l stopped spawning before the start of exposure. However the mean number of eggs/spawn was very close to those of controls. Despite this variation, effects of 3-BC exposure are very distinct. At 285 µg/l females stopped egg production immediately after exposure in all replicates. At 74 µg/l one replicate stopped reproduction immediately and two replicates spawned only once after the onset of exposure. At 33 µg/l effects were very distinct too although – due to high variations – not significant: 2 replicates stopped spawning after 4 and 7 days and the third replicate showed reduced spawning activity. Even at 3 and 0.5 µg/l slight but not significant effects on the number of spawns and the number of eggs/female/day were observed in a dose-dependent manner. The fact that effects observed at these concentrations are not statistically significant fits to the observation described in OECD 229 that the fish reproduction assay may have a very low statistical power with the result that only very pronounced effects (i.e. more than 60 %) may be detected as statistically significant. Thus in summary, effects observed at 74 and 285 µg/l are clearly significant effects despite the low statistical power and variations in fecundity. Effects observed at lower concentrations indicate that 3-BC may reduce fecundity even at lower test concentrations but effects were not statistically significant due to the low statistical power of the test.

Summary of *in vivo* endocrine relevant information in fish

In summary all the three tests described above provide clear evidence of an endocrine mode of action in fish.

All three 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. According to the OECD guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption (OECD, 2012) vitellogenin induction is a clear diagnostic endpoint for an estrogenic mode of action. Vitellogenin induction occurred in the absence of systemic toxicity in Kunz et al. (2006b). Growth was reduced in the test by Kunz et al. (2006a) at concentrations inducing vitellogenin in males. As discussed by e.g. Knacker et al. (2010) changes in growth rates can be evoked by estrogenic substances. For fish it was shown that the sexual endocrine system and the growth regulation are highly interrelated and that growth, as an apical endpoint, can be as sensitive as reproduction responding to an exposure against estrogens. Thus, together with the observed increase in male VTG levels this might be a further hint for the proposed estrogenic activity of 3-BC in fish that can lead to adverse effects in organisms. Additionally, in accordance with (OECD, 2012), it is unlikely that increased vitellogenin will be caused by systemic toxicity.

With regard to test concentrations resulting in vitellogenin induction, it is not possible to compare the three tests. Holbech et al. (2002) used repeated intraperitoneal injections of the test compound. Thus, this test provides evidence that 3-BC is able to induce estrogenic activity in rainbow trout once uptaken. However, due to the unrealistic exposure regime, it does not provide information as to whether the substance is actually taken up from water /the gastro-intestinal tract and about external water concentrations that cause induction. In addition, juvenile fish were used and thus a life stage at which fish may not react particularly sensitively towards estrogen exposure with vitellogenin induction. Similar aspects hold true for results observed in a study by Kunz et al. (2006a). Their study clearly shows that 3-BC enters the fish body and causes estrogen mediated modulations of the endocrine system. However, due to the usage of juvenile fish test concentrations causing such effects may be underestimated as adult fish might be much more susceptible. Hence, the only test providing information about the test concentration causing vitellogenin induction in fish is provided by Kunz et al. (2006b).

Results by Kunz et al. (2006b) substantiate an endocrine mode of action in fish. Depression of male secondary sex characteristics is an endpoint clearly diagnostic for an endocrine activity either through an estrogen agonistic or androgen antagonistic mode of action.

Some histological changes observed are primary diagnostic criteria for an endocrine mode of action according to the guidance document on the diagnosis of endocrine related histopathology in fish gonads (OECD, 2010) (i.e. increased oocyt atresia and changes in gonadal staging in females). Other effects observed are considered as of secondary diagnostic interest as they fit to an endocrine mode of action but may be caused by other mode of actions too (i.e. changes in gonadal staging, increased number of spermatides and decreased spermatocytic stages). In summary, histological changes are diagnostic for an endocrine mode of action or fit to such a mode of action.

Results observed by Kunz et al. (2006b) on fertility and fecundity fit to such an endocrine mode of action as well. Effects were observed at concentrations at which other endpoints, diagnostic for an endocrine mode of action were observed too. Although results were obtained with a short term reproduction assay which is considered to have a low statistical power, the result must be considered as clearly adverse and thus of population relevance.

In addition, the disappearance of male secondary sex characteristics (nuptial tubercles) after 21d of exposure of adult fish observed by Kunz et al. (2006b) should be recognized. Even after such a short exposure the phenotypic appearance of adult males changed in a way that they were visually not discernible from females at the highest concentration tested. This corresponded with the complete cessation of spawning and

should be considered as an adverse apical effect. Even though other factors (e.g. stress) cannot be ruled out definitively to be responsible for the loss of tubercles, we think that this loss in male fish here is a direct response to the increasing level of vitellogenin. A significant concentration-dependent loss of tubercles starts exactly at the 3-BC concentration where a significant raise in the VTG concentration in males is observed. In addition, it could be shown for other estrogenic acting chemicals like Bisphenol A that a loss of tubercles in male fish can occur during a period of 14 days (see e.g. Ankley et al., 2010, Aquatic Toxicology).

In summary, effects observed clearly show that 3-BC has an endocrine mode of action in fish. Effects observed on gonadal changes and secondary sex characteristics are diagnostic for an estrogen receptor agonist or androgen receptor antagonist mode of action while the observed vitellogenin induction is a clear estrogen receptor agonist mode of action. Adverse effects observed on fertility and fecundity fit to both modes of action. Although in principle they could be caused by systemic toxicity or other non-endocrine modes of action too, effects observed at other levels such as vitellogenin induction and the absence of other systemic toxicity clearly indicate that it is probable that 3-BC or its metabolites cause adverse effects due to an estrogen receptor agonistic and/or androgen receptor antagonistic mode of action.

Supporting information from mammalian toxicity tests

As the dossier focuses only on endocrine disruption in wildlife with the focus on fish, the mammalian toxicity tests are not assessed in detail. However, as supporting information, an analysis of the available mammalian toxicity tests provided by Hass et al. 2012, is given in the citation below and the key mammalian studies are summarised in table 11.

Table 11: Summary of key mammalian toxicity tests as described by Hass et al. (2012)

Method	Short Method description	Result	Description of results	Result positive control	References
OECD 440 but some details are missing: GLP, physical and chemical characterisation of the product, the choice of dosage (9 doses ranging from 0.8 to 300 mg/kg bw/d) and even the choice of animal strain (SCCS 2013).	Uterotrophic assay (immature LE rats)	+ estrogenic	Increased uterine weight ED_{50}= 45.3 mg/(kg d); LOEC= 2 mg/(kg d) Maximum increase as percent of EE: 70% No signs of general toxicity	Ethinylestradiol-17α (EE): increased uterine weight ED_{50}= 0.000818 mg/(kg d); LOEC= 2 mg/(kg d)	Schlumpf et al. (2004a)
	Developmental toxicity	+	Male offspring: Delayed sexual maturation and decreased relative epididymis		Different parts of the studies reported in different publications: Schlumpf et al. (2004b; 2008a;

			and seminal vesicle weights in adulthood Females: irregular estous cyclicity and strongly impaired sexual behaviour		2008b); Schmutzler et al. (2004)

Citation from (Hass et al., 2012), page 28:

"*In an* in vivo *screening study for estrogenic effect, 3-BC has been shown to increase uterine weight in immature LE rats (Schlumpf et al., 2004a). The study was performed in 21-23 days old female rats, which were dosed for 3 days by oral gavage (n=4-9 in the dose groups and 24 in the control). 9 doses of the compound were tested (0.8, 2, 4, 9.4, 18.75, 37.5, 75, 150 and 300 mg/kg/day, and significant increases in uterine weight were seen from between 2-4 mg/kg and above, depending on the statistical analysis performed. The ED50 in this study was 45 mg/kg, indicating that this compound is more potent in the uterotrophic assay than many other tested UV-filters.*

Some reproductive/developmental toxicity studies of 3-BC have also been performed. Different parts of the studies have been reported in different publications (Schlumpf et al. 2004b; 2008a; 2008b; Hofkamp et al. 2008; Faass et al. 2009), making it difficult to evaluate exactly how many individual studies have been performed and which dose levels have been tested. The endocrine disrupting effects of perinatal 3-BC exposure on male offspring was delayed sexual maturation and decreased relative epididymis and seminal vesicle weights in adulthood, while females showed irregular estrous cyclicity and strongly impaired sexual behaviour. Depending on which endpoints were chosen, the LOAEL value for 3-BC was between 0.24 and 2.4 mg/kg/day. At the dose of 2.4 mg/kg many results pointing in the direction of endocrine disruption were seen, whereas the only effect seen at 0.24 mg/kg was irregular oestrous cycles in females. As the study was performed with only about 3 litters per dose group, and it is recommended that a group size of 20 is used for examination of oestrous cyclicity (Cooper and Goldman, 1999) the significant change seen at this very low dose may not reflect a real biological effect. Therefore the LOAEL in this evaluation of 3-BC is set at 2.4 mg/kg and the NOAEL at 0.7 mg/kg/day.

Detailed summary of the methods and findings described in the following publications (Schlumpf et al. 2004b; 2008a; 2008b; Hofkamp et al. 2008; Faass et al. 2009), are presented below:

The following experimental setup was used in each study. 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 has continued throughout gestation and lactation, and the offspring were further dosed until adulthood. The following doses have been investigated: 0.07, 0.24, 0.7, 2.4 and 7 mg/kg/day. The number of dams in each dose group was unfortunately not stated in the publications by Schlumpf et al., where most of the results on endocrine sensitive endpoints are reported. However, in papers by (Hofkamp et al. 2008) and (Faass et al. 2009) it is stated that between 3-7 litters per dose group were used, and this was likely also the case for the studies described in Schlumpf et al. (2004; 2008 a,b).
Doses of 0.24 mg/kg/day and above all caused irregular oestrous cycles in the adult female offspring (Schlumpf et al. 2008b; Faass et al. 2009), however only between 5-11

female rat offspring were used for this study, and they only represented 3 litters each. Adult prostate weights were reduced in the 0.24 mg/kg dose group but not in any of the higher groups, indicating that this might be a chance finding. At the dose of 2.4 mg/kg/day decreased postnatal survival rate and delayed sexual maturation in male offspring was observed. Body weights at puberty were normal in dosed males, indicating that the delay of puberty did not result from nutritional effects (Schlumpf et al. 2008b). In (Schlumpf et al. 2004) it was reported that a dose of 0.24 mg/kg/day also caused delayed puberty in males. However in a later publication from this group, delayed preputial separation was only reported to occur in the 2.4 and 7 mg/kg/day groups (Schlumpf et al. 2008 a,b). Timing of sexual maturation of the female offspring was not affected by any dose of 3-BC (Schlumpf et al. 2004b). The dose of 2.4 mg/kg/day also caused decreased relative epididymis and seminal vesicle weights in adult males. These effects were however not seen at the higher dose of 7 mg/kg, and might therefore be a chance finding. Adult testes weights were not affected at any dose levels and no effects on volume of accessory sex glands or prostate were seen on PND 1 (Hofkamp et al. 2008)). Thyroid gland weights were not reported, and it is unclear whether they were not measured or whether no significant effects were seen. The immune system of the animals was probably not affected by 3-BC exposure, as thymus weights were not different from controls. Decreased adult body weight was seen in females at the dose of 2.4 mg/kg and in adult males at 7 mg/kg. The highest dose further caused decreased litter size. Female sexual behaviour, measured both as proceptive and receptive behaviour was strongly impaired in offspring exposed to 2.4 and 7 mg/kg (Schlumpf et al. 2008b; Faass et al. 2009), while this endpoint was not investigated in any other dose groups. Furthermore, 3-BC caused alterations in gene expression in the uterus as well as in sexually dimorphic areas of the brain on PND 6 in all dose groups (Schlumpf et al. 2008b; 2008a; Faass et al. 2009).”

Summary of in vivo results: In fish, the available *in vivo* studies show strong evidence of estrogenic effects. 3-BC has been shown to induce vitellogenin and cause significant effects on reproduction. In support, according to the analysis provided by Hass et al. (2012) in mammals significant estrogenic activity and developmental toxicity were observed. In a screening study for estrogenic effects, 3-BC has been shown to increase uterine weight in immature rats. In reproductive studies, testing the developmental toxicity of 3-BC by exposing the parental generation for 10 weeks before mating, dams continuously throughout gestation and lactation, and the offspring further until adulthood, the perinatal 3-BC exposure has been shown to cause delayed sexual maturation, decreased relative epididymis and seminal vesicle weights in adult male offspring, while female offspring showed irregular oestrous cyclicity and strongly impaired sexual behaviour as reduced proceptive and lordosis behaviour as well as increased rejection behaviour.

5.2.4. Conclusion concerning endocrine Disruption

Considering all available information from *in vitro* and *in vivo* fish studies supported by mammalian (rodent) reproductive toxicity studies, the following conclusion regarding endocrine disruption in the environment (wildlife vertebrates such as fish and rodents) for 3-BC can be drawn:

In vitro tests indicate that 3-BC and/or its main metabolite is able to activate the human ERα and ERβ receptor and the rainbow trout ERα receptor in a dose dependent manner. 3-BC also shows antiandrogenic and possibly antiprogesterone like activity.

Effects observed in the *in vivo* fish tests support this mode of action and show that 3-BC acts via an estrogenic or antiandrogenic mode of action *in vivo*:

- Vitellogenin induction, observed in three studies, clearly show that 3-BC causes *in vivo* endocrine activity by an estrogenic mode of action.

- Histological changes observed in males and females fit to an estrogenic or antiandrogenic mode of action and some of the changes observed are diagnostic for an endocrine mode of action.

- The observed depression of male secondary sex characteristics is indicative for an estrogenic or antiandrogenic mode of action.

Effects observed by (Kunz et al., 2006b) show, that 3-BC not only alters the function of the endocrine system but consequently causes adverse effects in fish.

- Effects on fecundity observed by (Kunz et al., 2006b) fit to an estrogenic and/or antiandrogenic mode of action.

- No other systemic side effects were observed which could indicate that observed effects are caused by systemic toxicity.

This conclusion is summarised in table 12.

Table 12: Summary of evidence for endocrine disrupting effects of 3-BC in fish based on the OECD Guidance document (OECD, 2012). Only results from tests with at least reliability 2 have been considered.

Indication of hormonal activity?	Apical endpoints positive?	Indication that apical endpoints fit to mode of action
Yes, - Positive *in vitro* tests (estrogenic and antiandrogenic) - VTG induction, in juveniles and males (estrogenic) - Decreased male secondary sex characteristics (estrogenic and antiandrogenic) - Histological changes - Effects observed in mammal toxicity studies (estrogenic) Lowest LOEC 33 µg/l for histological changes	Yes - Reduced fecundity - Complete change of male secondary sex characteristic of males resulting in fish not discernible from females Lowest LOEC 74 µg/l	Yes - Effects observed on fecundity fit to the mode of actions observed *in vivo* and *in vitro* - Complete change of male secondary sex characteristics must be considered as apical endpoint (change of sex-ratio) which is diagnostic for an estrogenic or antiandrogenic mode of action although this is usually observed during fish sexual development tests Lowest LOEC 74 µg/l

Based on the OECD Guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption (OECD, 2012) the following conclusions can be drawn:

Effects observed in modified 21 day fish screening tests (Holbech et al. 2002; Kunz et al. 2006a, Kunz et al., 2006b) together with positive *in vitro* testing on endocrine activity result in the conclusion that 3-BC is a probable ED *in vivo*. As adult instead of juvenile fish have been used in the studies by Holbech et al. and Kunz et al. it is probable that not the most sensitive live stage has been tested and effects in adult fish might occur at higher concentrations than observed in these tests.

Hence, the effects observed by (Kunz et al. 2006b) in the modified fish short term reproduction test and classically reported in an OECD 229 guideline study (VTG, fecundity) provide, together with positive results of the *in vitro* tests, strong evidence for *in vivo* endocrine activity resulting in adverse effects in fish. Thus in conclusion 3-BC is an ED *in vivo* in fish according to the WHO/IPCS definition. In addition, 3-BC is estimated to have a slow degradability in the environment.

Below a more detailed analysis of the results by Kunz et al. (2006b) is provided:

The fish short term reproduction test (OECD guideline 229) is considered as a

level 3 test according to the OECD conceptual framework – and thus a screening test rather than a test resulting in reliable EC50 values - due to its low statistical power for reproductive endpoints. The effects observed allow to conclude, that adverse effects occur, which are in line with the assumed estrogenic/antiandrogenic mode of action. Effects observed are very pronounced (total inhibition of reproduction directly after the start of exposure at 285 µg/l and total inhibition after 5 days at 74 µg/l) and clearly dose-response related. Thus, taking into account the endpoints such as fecundity and histolopathological endpoints together with VTG induction in male fish and the observed influence on male secondary sexual characteristics this OECD level 3 assay demonstrates adverse effects in a weight of evidence manner.

Thus considering the aspects described above it can be concluded, that 3-BC alters the function of the endocrine system most possibly via an estrogenic and/or antiandrogenic mode of action and consequently causes adverse effects in fish.

Laboratory study results from reproductive toxicity studies with rodents, as reviewed by Hass et al. 2012, further support that adverse effects as consequence of an estrogenic or anti-androgenic mode of action may also occur in mammalian species.

Hence available evidence shows that the endocrine mode(s) of action of 3-BC may lead to adverse effects in vertebrate species (i.e. fish and probably rodents) causing very high concern for endocrine disruption in vertebrate wildlife species supported by the estimated slow degradability of the substance in the environment.

5.3. Aquatic invertebrates

Schmitt et al. (2008) conducted additionally to a Yeast Estrogen Screen (YES) (see chapter 5.2.2) two sediment assays with the freshwater invertebrates *Lumbriculus variegatus* (see section 5.4) and *Potamopyrgus antipodarum*. The 56-day sediment test with *P.antipodarum* started with the exposure of adult snails in a static system. Glass beakers of 10 cm diameter served as test vessels, containing artificial sediment and reconstituted water. Test treatments comprised 3-BC at 0.06, 0.28, 1.72, 6.33, and 31.6 mg/kg sediment (dw) as well as control and solvent control. All treatments were run in duplicate with 80 snails with a shell height of 4.0 ± 0.5 mm. After 14 days of exposure, 3-BC increased the occurrence of unshelled embryos (increase in reproduction rate) significantly at the lowest test concentration (0.06 mg/kg sediment dw), whereas it inhibited reproduction at the highest concentration (31.6 mg/kg sediment dw) significantly. A similar effect was observed after 56 days of exposure to 3-BC, where a significant increase in the occurrence of unshelled embryos (increase in reproduction rate) was detected in the second lowest (0.28 mg/kg sediment dw) and second highest (6.33 mg/kg sediment dw) concentration. The decrease in reproduction rate, observed after 14 days of exposure to the highest concentration, was not detected anymore after 56 days. After 56 days of exposure the mortality was significant at 6.33 and 31.6 mg/kg sediment dw. Overall, the study shows that exposure to 3-BC increased the occurrence of unshelled embryos of *P.antipodarum* at lower concentrations whereas at higher exposure concentrations 3-BC resulted in significant mortality. An abnormal enhancement of reproduction rate disturbs the natural reproduction cycle of the animals and interferes with the annual variances. The reproduction of *P.antipodarum* follows annual fluctuations and it is also regulated to prevent the population from becoming too big, which could be impacted by the presence of 3-BC.

5.4. Sediment organisms

Schmitt et al. (2008) conducted a 28-day sediment test with *L.variegatus.* The study was performed according to the Draft OECD Guideline 218 with minor modifications. The sediment was spiked with 3-BC concentrations of 0.05, 0.26, 1.49, 6.47, and 29.1

mg/kg sediment dw dissolved in ethyl acetate. In addition to an unspiked control also a solvent control was used. Measured concentrations in all tests conducted were in the range of 55.1 to 108 % of the nominal concentrations. In the lowest test concentration 3-BC was below the limit of quantification. The validity criterion of the test "increased average number of living worms per replicate in the controls by a factor of at least 1.8" was well met with a factor of 3.98 which was two times higher than requested. 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, solvent control and also in the two lowest test concentrations of 3-BC (mean 29.0 to 39.8 worms per test vessel) the reproduction started to decrease to an average of 21 worms per test vessel at 1.49 mg/kg sediment dw of 3-BC. At 6.47 and 29.1 mg 3-BC/kg sediment dw reproduction was significantly lower compared to the solvent control. An EC_{10} of 19.2 µg/kg dw and an EC_{50} of 1.43 mg/kg dw were calculated for 3-BC. 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 filters (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, oligocheates 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 filters found in fish (Buser et al., 2006; Nagtegaal et al., 1997).

5.5. Other aquatic organisms

Kunz et al. (2004) investigated whether 3-BC interferes with the thyroid and sex hormone system of the tadpoles of *Xenopus laevis* frogs during metamorphosis. At nominal concentrations of 1, 5 and 50 µg/l 3-BC had no effects on the rate of metamorphosis and no obvious differences were observed in body length and tail length compared to controls. 3-BC also did not affect the sex ratio of *X. laevis* tadpoles.

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 3-BC, which would allow to conclude on the PBT/ vPvB properties of the substance.

3-BC is predicted at a screening level not to be readily biodegradable and has a calculated log Pow of 5.37. Thus, 3-BC has the potential of being bioaccumulative and persistent. However, in a non-standardised test (Kunz et al. 2006b) the BCF value of 3-BC is below the B criterion. Further experimental data are needed to definitively conclude on the bioaccumulation potential in aquatic organisms.

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 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 concern", starting from the legal text two questions seem to be relevant:

- Does 3-BC have endocrine disrupting properties, i.e. does 3-BC act via an endocrine mediated mode of action and does this mode of action cause 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 3-BC is structured in the following along these two questions in order to facilitate a conclusion.

6.3.1. Endocrine disrupting properties of 3-BC

Endocrine disrupting properties are an example of inherent properties that might, if scientific evidence of probable serious effects is available, give rise to an equivalent level of concern to those 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 3-BC:

"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. is analysed in the following using a weight of evidence approach. Starting from *in silico* and *in vitro* results and proceeding to the available *in vivo* data it will be shown that 3-BC can act via endocrine modes of action and that these intrinsic properties might cause adverse effects in intact organisms. 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 well as CMR substances is 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 3-BC to the ligand binding site of the human ERβ. 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 3-BC might interact with estrogen and androgen mediated pathways in organisms.

The *in vitro* results described under section 5.2.2. point to three modes of endocrine action of 3-BC. There are six studies using cell based test systems (human MCF-7 and HEK239 cells as well as yeast cells) showing a dose related binding of 3-BC to both subtypes of the estrogen receptor via proliferation and gene expression analysis. A further four studies investigated the androgenic/antiandrogenic potential of 3-BC. 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_{50} values in the low micromolar range. Finally, two studies could demonstrate antiprogesteronic effects 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. Hence, the available *in vitro* data show the potential of 3-BC to interfere in an agonistic and antagonistic way with three different endocrine related receptor families providing evidence on a molecular basis for possible

estrogenic, antiandrogenic and antiprogesteronic effects.

As described in chapter 5.2.3. the *in vivo* data available for fish substantiate the *in vitro* evidence for an estrogenic and/or antiandrogenic mechanism of action of 3-BC and provide evidence that this endocrine activity results in population-relevant adverse effects:

Four studies with two different fish species (*P.promelas* and *O.mykiss*) showed consistently a significant and dose related increase in vitellogenin level in males and females. The maximum inducible increase in vitellogenin levels was comparable to those obtained with the positive control EE2.
One of the studies (Kunz et al., 2006b) cited in section 5.2.3. showed, using an OECD 229 conforming short term reproduction protocol, that 3-BC is endocrine active in fish and could cause population-relevant adverse effects, which are clearly linked to the endocrine activity. Besides the dose-related increase in vitellogenin levels in males this study observed the following adverse effects on histology, fecundity and secondary sex characteristics:

- *Histological changes*: Dose dependent inhibition of spermatogenesis, an increase in spermatocytes and spermatids and an increase in the number of artretic follicles in the female ovaries.
- *Effects on fertility/fecundity*: Significant and dose dependent decrease in the cumulative number of eggs spawned (LOEC: 74 µg/l), complete inhibition of egg production in females at a concentration of 285 µg/l.
- *Secondary sex characteristics*: Significant and dose related decrease in male nuptial tubercles (LOEC 74 µg/l). At 285 µg/l 3-BC males lost all tubercles and were visually not any more discernible from females.

Thus, there is strong evidence from high quality data that 3-BC actually acts as an endocrine disruptor in fish i.e. that the substance alters the function of the endocrine system and consequently causes adverse, population-relevant effects. Comparable effects observed in two fish species show that this holds most likely true for a variety of different fish species and potentially also for other vertebrate species.

With regard to invertebrates there is one study presented in section 5.2.3., describing a 56-day sediment test carried out with *P.antipodarum*. After 14 days of exposure to 3-BC, a significantly increased occurrence of unshelled embryos (increased reproduction rate) was seen at the lowest test concentration (0.06 mg/kg sediment dw), whereas reproduction at the highest concentration (31.6 mg/kg sediment dw) was significantly inhibited. A similar effect was observed after 56 days of exposure to 3-BC, where the occurrence of unshelled embryos was significantly increased in the second lowest (0.28 mg/kg sediment dw) and second highest (6.33 mg/kg sediment dw) treatments. The study demonstrates that 3-BC increased the occurrence of unshelled embryos of *P.antipodarum* at low concentrations. This observation fits to an underlying estrogenic mode of action since estradiol is known to exert the same effect. An abnormal enhancement of reproduction rate disturbs the natural reproduction cycle of the animals and hence is of relevance for the whole population. However, so far specific test systems are missing to unravel endocrine modes of action in invertebrates and hence it is difficult to establish a causal link between the effects observed here and an underlying endocrine mode of action.

Overall summary of endocrine disrupting effects of 3-BC

In summary, available information from mechanistic *in vitro* and *in silico* studies as well as results from *in vivo* experiments show that 3-BC acts as an endocrine disruptor in fish via an estrogenic and/or antiandrogenic mode of action. The observed antiprogesteronic effects *in vitro* suggest that there are further endocrine pathways 3-BC can interfere

with. Additionally, there are indications from one study which suggest that 3-BC may be endocrine active via an estrogenic pathway in invertebrate species too, but no clear conclusion can be drawn due to the lack of precise knowledge about the endocrine system of invertebrates.

The following table gives an overview of the presented evidence that 3-BC can act via an estrogenic and/or antiandrogenic mode of action and that this endocrine activity can result in apical adverse effects in fish.

Table 13: Summary of evidence for endocrine disrupting properties of 3-BC in fish.

Indication of hormonal activity?	Apical endpoints positive?	Indication that apical endpoints fit to mode of action
Yes, - Positive *in vitro* tests (estrogenic and antiandrogenic) - VTG induction, in males (estrogenic) - Decreased male secondary sex characteristics (estrogenic and antiandrogenic) - Histological changes - Effects on fecundity and fertility	Yes - Reduced fecundity - Complete change of male secondary sex characteristics resulting in fish not visually discernible from females Lowest LOEC 74 µg/l	Yes - Effects observed on fecundity fit to the mode of actions observed *in vivo* and *in vitro* - Complete change of male secondary sex characteristics must be considered as apical endpoint (change of sex-ratio) which is diagnostic for an estrogenic or antiandrogenic mode of action although this is usually observed during fish sexual development tests Lowest LOEC 74 µg/l

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 a substance 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 equivalency of concern need to be analysed case by case. Thus, it will be analysed in the following for 3-BC in a weight of evidence approach how the above described endocrine mode of action mediated adverse effects are of equivalent level of concern for the environment to those evoked by CMR and/or PBT/vPvB substances. It will be discussed below why the seriousness of effects substantiate the SVHC identification of 3-BC. 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
- Can many species be adversely affected?
- Population relevance of the effects

Mode of action

The *in vitro* and *in vivo* data available for 3-BC provide strong evidence that 3-BC can act via an estrogenic and/or antiandrogenic mode of action. Additionally, some hints for an antiprogesteronic pathway are available. Thus, it is shown that the effects observed in fish are endocrine mediated and hence 3-BC 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 3-BC 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 3-BC, via an ER agonistic and AR antagonistic mechanism is 4-nonylphenol, branched and linear (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 no full life cycle test is available, but it was found that the male secondary sex characteristics decrease starting from a tested concentration of 74 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 as 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 expected for 3-BC. Comparable effects are observed with further known ER agonistic substances like ethinylestradiol (EC No. 200-342-2), 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 ED substances can also be expected to be triggered and influenced by 3-BC.

From the *in vitro* studies of 3-BC presented in section 5.2. it can be concluded that beside ER and AR binding further endocrine modes of action (antiprogesteronic) of 3-BC must be considered at concentrations one order of magnitude lower than those observed for the estrogenic and antiandrogenic activity *in vitro*.
Furthermore, according to the analysis provided within the review by Hass et al. (2012), experimental study results of reproductive toxicity studies with rodents suggest that there might be adverse effects related to estrogenic or anti-androgenic modes of action in mammalian wildlife exposed to 3-BC.

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). As presented in section 5.2.3 there is clear evidence of endocrine activity linked to reproductive effects in fish.

As pointed out by e.g. Segner et al. (2003) effects of ethinylestradiol (EC No. 200-342-2), 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 4-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 three sexual development tests indicate that exposure during a very short window (pre-hatch) adversely influences the endpoints on sexual development and sex-ratio. Given the same underlying endocrine modes of action there is a strong concern that 3-BC can lead to the same long-term effects in fish.

For invertebrates, there are also indications of a high sensitivity during the early life stages. For instance, larval stages of crustaceans have been shown to be highly sensitive to exposure to various substances identified as substances of very high concern (reproduction toxicants as well as endocrine disruptors) under REACH. In molluscs, arthropods, amphibians, alligators, turtles and birds, estrogen receptor agonists, such as 17ß-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 environment. Since 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 those of PBT/vPvB substances.

Can many species be adversely affected?

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 comparable effects in at least two different teleost fish species. Additionally, the developmental toxicity data for rodents as reviewed by Hass et al. (2012) presented in section 5.2. suggest that effects on estrogen and/or antiandrogen sensitive endpoints are also present in mammals. Thus, 3-BC, due to the highly conserved mode of estrogenic and/or anti androgenic action, might affect a broad range of wildlife animals and not only fish.

Vertebrate-type sex steroids and the 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 (Stout et al. 2010). The fact that ecdysteroids are structurally similar to steroidal estrogens explains that the latter may affect moulting in crustaceans. Testosterone and a number of known estrogen receptor agonists (e.g. bisphenol A and 4-nonylphenol) appear to function as anti-ecdysteroids in crustaceans (LeBlanc, 2007).

Endocrine systems of invertebrates differ substantially from those of vertebrates. 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. Thus, owing to the comparable mode of action of 3-BC 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 3-BC on invertebrate species cannot be excluded.

Population relevance of the effects

To demonstrate the severity of effects that are evoked by 3-BC for the environment, in the following subsection the possible adverse outcome of an exposure to 3-BC 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 only partial recovery from the 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 be shown that a transient short term exposure during sensitive life stages may result in life long effects even in following generations. Since 3-BC was shown to act via the same mechanism and at comparable effect concentrations, there is a science based concern of probable serious effects on the population level for this substance.

Finally, on a screening level 3-BC shows the potential of being persistent in the environment and the estimated log Pow of 5.37 points to the potential of bioaccumulation. Hence, the concern for endocrine disruption in the environment is supported by the estimated slow degradability of 3-BC in the environment and by the indication that 3-BC may be bioaccumulative.

Summary of the hazard and equivalent level of concern assessment of 3-BC

The *in silico*, *in vitro* and *in vivo* data presented and discussed within this dossier provide sufficient evidence to conclude that 1,7,7-trimethyl-3-(phenylmethylene)bicyclo[2.2.1]heptan-2-one (3-BC) acts via an endocrine mode of action and that this endocrine activity leads to adverse effects in fish. Hence, 3-BC fulfils the WHO/IPCS definition of an endocrine disruptor for the environment.

The specific mode of action of 3-BC (estrogen receptor agonist and/or androgen receptor antagonist), the effects observed *in vivo* in fish and supporting information as reviewed by Hass et al. (2012) in rodent species as well as the comparison of these effects with known endocrine disruptors acting via the same molecular mode of action provide strong evidence that the endocrine mediated effects of 3-BC 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 are identified for 3-BC:

- The identified main mode of action (estrogenic and/oranti androgenic) in fish of 3-BC is comparable to that of known endocrine active substances like bisphenol A (EC No. 201-245-8) or ethinylestradiol (EC No. 200-342-2) and already identified endocrine disrupting chemicals under REACH like 4-nonylphenol, branched and linear and 4-tert-octylphenol (EC No. 205-426-2). Based on in vitro data 3-BC also shows antiprogesteronic activity.
- It is probable that 3-BC causes irreversible and long lasting effects on wildlife populations and that even short term exposures during sensitive life stages of such organisms have adverse effects during the entire life time.
- The specific mode of action (estrogenic and/or antiandrogenic) of 3-BC and the data available for fish and supporting information as reviewed by Hass et al. 2012 in rodent species point to a broad range of taxa that might be affected by

exposure to 3-BC 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 3-BC. Mechanistic knowledge about invertebrate hormone receptors shows that also invertebrate species might be affected by 3-BC.

- It is likely that the effects are adverse not only for single organisms but also for populations and/or subpopulations in the environment.
- Similar to certain other substances of very high concern it is difficult to quantify a safe level for 3-BC in the environment and therefore the risks using traditional risk assessment methods.

In addition to the endocrine disrupting properties, 3-BC shows the potential to be persistent and bioaccumulative at a screening level.

Taking together the evidence presented in this dossier, 3-BC 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 to those of other substances listed in points (a) to (e) of article 57 of REACH.

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