Substance Name: 4,4'-isopropylidenediphenol (Bisphenol A; BPA)

EC Number: 201-245-8

CAS Number: 80-05-7

MEMBER STATE COMMITTEE

SUPPORT DOCUMENT

FOR IDENTIFICATION OF

4,4'-ISOPROPYLIDENEDIPHENOL

(BISPHENOL A, BPA)

AS A SUBSTANCE OF VERY HIGH CONCERN BECAUSE OF ITS ENDOCRINE DISRUPTING PROPERTIES (ARTICLE 57(F)) CAUSING PROBABLE SERIOUS EFFECTS TO THE ENVIRONMENT WHICH GIVE RISE TO AN EQUIVALENT LEVEL OF CONCERN TO THOSE OF CMR[1] AND PBT/vPvB[2] PROPERTIES

Adopted on 14 December 2017

[1] CMR means carcinogenic, mutagenic or toxic to reproduction

[2] PBT means persistent, bioaccumulative and toxic; vPvB means very persistent and very bioaccumulative

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GLOSSARY

AR	androgen receptor
BPA	Bisphenol A
CSR	Chemical Safety Report
DEHP	Diethylhexyl phthalate
DHT	4,5-Dihydrotestosterone
DNMT	DNA methyltransferase
dpf/h	days post fertilization/hatch
DT_{50}	degradation half-life time
$DisT_{50}$	disappearance half-life time
20E	20-hydroxy-ecdysone
EATS	Estrogen/Androgen/Thyroidal/Steroidogenesis (modalities)
E2	17β-Estradiol
EE2	17α-Ethinylestradiol
EQS	Environment Quality Standard
ER	oestrogen receptor
ERR	oestrogen-related receptor
GPR30	G-protein-coupled receptor 30
GSI	Gonadosomatic index
hAR	human androgen receptor
hER	human oestrogen receptor
HEK293	Human Embryonic Kidney 293 cells
HELN	transfected (ER) human cervix adenocarcinoma cell line
Hpf	hours post fertilization
HPT	Hypothalamic-pituitary-thyroid (axis)
HSI	Hepatosomatic index
JH	Juvenile hormone
LOEC	Lowest Observed Effect Concentration
MOA	Mode of Action
MCF-7	breast cancer cell line (Michigan Cancer Foundation-7)
MF	methylfarnesoate (crustacean juvenile hormone)
NF	developmental stage according to Nieuwkoop, P.D. and Faber, J. (1994)
NOEC	No Observed Effect Concentration
NP	4-nonylphenol, branched and linear
OP	4-tert-octylphenol (EC No. 205-426-2)
PBT	persistent, bioaccumulative and toxic
PBL	Protein-bound lipid
PR	progesterone receptor
QSAR	Quantitative Structure-Activity Relationship
RBA	Relative binding affinity
REP	Relative estrogenic potency
RIC	Relative inhibitory concentration
rtER	oestrogen receptor of rainbow trout
SBP	Steroid-binding protein
SCCS	Scientific Committee on Consumer Safety
T_3	3,5,3'-triiodo-L-thyronine
T_4	L-thyroxine
TBT	Tributyltin
TGT	Technical Guidance Document on Risk Assessment
TH	thyroid hormone
TR	thyroid hormone receptor
TSH	thyroid-stimulating hormone (thyrotropin)
TTR	Transthyretin receptor
TWA	Time-weighted average
US EPA	Environmental Protection Agency of the United States of America
vPvB	very persistent and very bioaccumulative

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: 4,4'-isopropylidenediphenol (Bisphenol A, BPA)

EC Number: 201-245-8

CAS number: 80-05-7

- According to Article 57(f) of the REACH Regulation BPA is identified as a substance of equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of Regulation (EC) No 1907/2006 (REACH).

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

Bisphenol A (BPA) 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 the environment for which there is scientific evidence of probable serious effects to the environment which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of REACH Regulation.

The analysis of results for fish and amphibians according to the OECD Guidance Document for Endocrine Disrupters (OECD 2012b) reveals that BPA needs to be considered as an endocrine disruptor. It fulfils the WHO/IPCS definition of an endocrine disruptor as interpreted by the European Commission's Endocrine Disruptor Expert Advisory Group (JRC 2013) in their recommendations for a substance to be identified as an endocrine disruptor.

For BPA there is scientific evidence from good quality studies that the substance causes endocrine mediated adverse effects in several fish and amphibian species.

BPA clearly acts as an oestrogen agonist in fish.

In several **fish** species clear evidence that BPA acts as oestrogen agonist is provided:

- *In vitro* data unambiguously show that BPA binds to vertebrate (human and fish) oestrogen receptors in the µM range and modulates gene expression. BPA also competitively inhibits androgenic activity of a known AR agonist.
- The oestrogen agonist mode of action is unambiguously substantiated by *in vivo* data in several species. Diagnostic for the oestrogenic mode of action are the observed Vitellogenin (VTG) induction, changes in gonadal staging, testis ova, altered sex ratio, and reduced male secondary sex characteristics.

The effects observed are clearly adverse, such as the skewed sex ratio towards females. A direct link between the oestrogenic mode of action *in vivo* (e.g. VTG induction, testis, ova) and the adverse effects (sex ratio, reduced egg production) is provided for *O. latipes*, *D. rerio* and is very likely for *P. promelas*. Additionally, for six other fish species adverse effects which are known to be sensitive towards an oestrogenic mode of action were demonstrated, such as affected growth, behaviour and fertilisation success.

In addition, there is evidence that BPA potentially acts as oestrogen agonist in **amphibians**:

- An agonistic VTG induction was demonstrated in hepatocytes of *X. laevis* at 22.8 µg/L *in vitro*. Further evidence is provided by the *in vitro* studies demonstrating binding to the oestrogen receptor in other vertebrates.

- The oestrogenic mode of action is substantiated in *X. laevis* by a skewed sex ratio (23 µg/L), a delay of development, altered testicular structure (2.28 µg/) and the ability to induce Vitellogenin *in vivo* (22.8 µg/L) as well as similar results for E2.
- A direct link is provided between VTG induction *in vitro* and *in vivo* through a plausible binding to the oestrogen receptor and changes of the sex ratio and reproduction observed *in vivo* for *X. laevis* as well as three other species. These effects are considered adverse.

BPA clearly acts as a thyroid antagonist in <u>amphibians</u>:

- *In vitro* studies with amphibian, fish and mammal cells demonstrate that BPA is interfering with the HPT (Hypothalamic-Pituitary-Thyroid) axis (e.g. thyroid receptor, transport proteins).
- The endocrine mode of action is substantiated by *in vivo* data. Diagnostic for a thyroid mode of action in amphibians is the accelerated/asynchronous development or an abnormal histopathology, which could be demonstrated in 3 species. BPA inhibited the (TH induced and spontaneous) metamorphosis *in vivo*, leading to a delayed development and disturbed life-cycle in *R. rugosa, X. laevis* and *X. tropicalis*.
- Hence, a direct link between the *in vitro* and *in vivo* evidence can be shown. The observed in vivo effects (delayed development and disturbed life-cycle) are considered adverse.

In addition, there is some evidence that BPA also may act via a thyroidal mode of action in **<u>fish</u>**, although data is scarce. This is substantiated by *in vitro* studies, demonstrating an interference with the HPT axis and thyroid-related hormones in fish cells together with accelerated embryonic development in *O. latipes* which was blocked by the thyroid-antagonist amiadorone *in vivo*. The thyroid-mediated effects (accelerated development, earlier hatching and smaller individuals) are considered adverse.

Further support for endocrine-related effects of BPA

The analysis of **<u>invertebrate taxa</u>** revealed indications that adverse effects of BPA are possibly endocrine-mediated. It has to be kept in mind that there is still lack of an agreed guidance document which is clearly defining biological plausible links between endocrine modes of action and adverse effects for invertebrate taxa and that knowledge is still scarce in light of the large number and variety of invertebrates and their endocrine systems.

- In <u>molluscs</u>, characteristic adverse effects on reproduction and development were an increased egg production, mitigated by anti-oestrogens in two species *in vivo*, as well as the induction of superfemales, malformations of genital tissues (known for E2 and OP) in four species as well as embryo malformations in two species. BPA acts similar to known vertebrate-type (xeno-)oestrogens. A possible oestrogen receptor binding (*in vitro*, *in vivo*), mRNA expression and increased VTG or VTG-like protein levels were shown in three species.
- For arthropods such as insects and crustaceans ecdysteroids are known to regulate reproduction- and development-related processes. For <u>insects</u>, adverse effects of BPA were similar as for (xeno-)oestrogens (OP, NP, EE2), comprising a delayed development, reduced fecundity and emergence rates as well as increased weight/growth. *In vitro* evidence for antagonistic ecdysteroid receptor binding and changes in mRNA expression is provided for *Drosophila* and *Chironomus*. For <u>crustaceans</u>, adverse *in vivo* effects are associated with embryo malformations, developmental delay, molting disturbances and altered reproductive outcome (enhanced or reduced). Effects were similar to (xeno-)oestrogens and could be mitigated by ecdysteroids. Due to the close relationship to insects, a binding or interference with the ecdysteroid receptor and ecdysteroid related processes is possible.

For further invertebrate species, such as echinoderms, poriferans or cnidarians, data for BPA and knowledge of the endocrine systems is very fragmentary. However, developmental disturbances including embryo malformations are typical after BPA exposure and similar to the effects observed for other (xeno-)oestrogens in these groups.

Overall, Bisphenol A is clearly shown to disrupt steroid- (oestrogen) and thyroid mediated processes in fish and amphibians respectively, leading to adverse effects on the organisms which can affect population stability and recruitment. Endocrine-mediated effects occurred and at lower concentrations than acute, systemic or narcotic toxicity.

BPA is also identified as an SVHC according to article 57(f) for probable serious effects on human health due to its endocrine disrupting properties on the basis of data on mammals. There is a large degree of conservation of the primary amino acid sequences in proteins, which implies large commonalities between non-mammalian and mammalian vertebrate species in regard to hormones, enzymes and receptors involved in the EATS (Estrogen/Androgen/Thyroidal/Steroidogenesis) modalities (OECD 2017: Draft revised OECD Guidance No. 150). Evidence of endocrine disruptive properties of BPA on mammalian vertebrate species therefore provides further support for similar properties in non-mammalian vertebrates, in particular with regard to disruption of oestrogenic pathways.

Bisphenol A is considered as a substance giving rise to an equivalent level of concern due to its endocrine modes of action and the type of effects caused by these modes of action in wildlife species (fish, amphibians).

The assessment followed the same line of arguments as for previous SVHC-identifications according to 57(f) ED for the environment. Due to the amount of data available for BPA a large number of arguments for an equivalent level of concern can be provided. All arguments are used in a weight of evidence and as such, none of the arguments alone are decisive for the decision and not all of them are needed to conclude on the equivalent level of concern. We decided to present the available evidence to get a view on the overall picture on the data analyzed.

- BPA causes severe effects on reproduction- and development-related processes (including sexual development) in fish and amphibians, clearly linked to the endocrine mode of action. Results for fish demonstrate that BPA may cause a complete sex reversal resulting in all-female phenotype populations. In amphibians, thyroidal pathways, metamorphosis and development are disturbed, and additionally sex ratio skewed via a suspected additional oestrogen mode of action. Supporting evidence is provided by effects observed in invertebrates.
- BPA in particular causes severe effects on organisms when exposure took place during sensitive time windows or early life stages, also after short-term exposures when exposure later ceases. Many of these effects have to be regarded as irreversible, such as sex reversal or embryo or adult malformations which may have long-term consequences for the population. Moreover, some effects may only occur after exposure during particular seasons as shown for amphibians.
- BPA elicits long-term effects across generations and affects populations and communities. Transgenerational effects were observed for several fish species, where the following generations became much more sensitive to BPA exposure (after continuous as well as short-term exposures of the parental generation). Long-term effects were shown in one mesocosm study, where low BPA concentrations affected the fish population and changes in gonad morphology are likely endocrine-mediated.
- BPA affects a large variety of ecologically important species in different ecosystems, covering lentic, lotic, marine and terrestrial environments. BPA exposure is not restricted to certain environments but shown to be ubiquitously present. Certain fish (and also mollusc) species were shown to be particularly sensitive, but as data is only available for a small proportion of existing species, it is not possible to

exclude that further species are equally or even more sensitive. Also endangered species such as amphibians may be affected. It has to be kept in mind, that effects first become prominent in later life stages or in the next generation, even when organisms have migrated to uncontaminated regions.

- BPA has already, based on available data including a large number of results from studies on mammalian mainly rodent species, been concluded to be an endocrine disrupter of concern for human health according to Article 57 (f) of REACH. Whereas the available mammalian studies are relevant for human health, it is plausible, that they are also of relevance for other mammalian species including mammalian wildlife species. In relation to the environment, adverse effects concerning development and reproduction are generally regarded as endpoints of particular relevance because such effects are likely to manifest themselves at the population level. The effects observed in rats are of particular concern for mammalian wildlife species with a natural low reproductive output (including endangered species) as negative effects on reproduction have an even higher potential for causing long term negative effect at the population level for such taxa. However, it is unclear whether the effects observed for mammals in the human health assessment will lead to population level effects in mammalian wildlife species.
- Based on the current data and knowledge it appears difficult to derive and quantify a safe level of exposure for BPA, although it might exist. Effects on non-traditional endpoints and in specific species occurred at lower concentrations than those considered by standard OECD test guidelines. Moreover, as effects often occur in certain species, or after exposure during specific time windows and early life stages, some effects might be overlooked. Effects of BPA are presumably provoked via different modes of action and a greater variety of species could be affected.

In conclusion, there is scientific evidence that Bisphenol A causes probable serious effects in the environment which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of REACH Regulation.

Registration dossiers submitted for the substance: Yes.

Justification

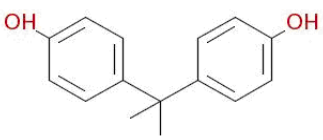
1 Identity of the substance and physical and chemical properties

1.1 Name and other identifiers of the substance

Table 1: Substance identity

EC number:	201-245-8
EC name:	4,4'-isopropylidenediphenol
CAS number (EC inventory):	80-05-7
CAS number: **Deleted CAS numbers:**	80-05-7
CAS name:	Phenol, 4,4'-(1-methylethylidene)bis-
IUPAC name:	2,2-bis(4-hydroxyphenyl)propane
Index number in Annex VI of the CLP Regulation	604-030-00-0
Molecular formula:	$C_{15}H_{16}O_2$
Molecular weight range:	228.28 g/mol
Synonyms:	Bisphenol A; Phenol, 4,4'-isopropylidenedi- (8CI); (4,4'-Dihydroxydiphenyl)dimethylmethane; 2,2-Bis(4-hydroxyphenyl)propane; 2,2-Bis(p-hydroxyphenyl)propane; 2,2-Di(4-hydroxyphenyl)propane; 2,2-Di(4-phenylol)propane; 2,2'-Bis(4-hydroxyphenyl)propane; 4,4'-(1-Methylethylidene)bisphenol; 4,4'-(Propane-2,2-diyl)diphenol; 4,4'-Isopropylidenebis[phenol]; 4,4'-Isopropylidenediphenol; 4,4'-Methylethylidenebisphenol; B 0494; BPA; BPA 154; BPA 157; BPA-M; Bis(4-hydroxyphenyl)dimethylmethane; Bis(p-hydroxyphenyl)propane; Dian; Diano; Diphenylolpropane; HT 3082; Hidorin F 285; Hidorin F 568; Ipognox 88; Isopropylidenebis(4-hydroxybenzene); NSC 1767; NSC 17959; Parabis; Parabis A; Pluracol 245; Rikabanol; p,p'-Bisphenol A; p,p'-Dihydroxydiphenylpropane; p,p'-Isopropylidenebisphenol; p,p'-Isopropylidenediphenol; β,β'-Bis(p-hydroxyphenyl)propane

Structural formula:



1.2 Composition of the substance

Name: 4,4'-Isopropylidenediphenol

Description: The detailed composition of the substance is confidential and provided in the technical dossier.

Substance type: mono-constituent

The identification of Bisphenol A as SVHC is based on the properties of the main constituent only. However, by definition all mono-constituent substances (real substances) with Bisphenol A as the main constituent will be covered. Therefore, other constituents and impurities are not relevant for the identification of Bisphenol A as SVHC.

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

Not relevant for the identification of the substance as SVHC in accordance with Article 57 (f) of REACH.

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

Not relevant for the identification of the substance as SVHC in accordance with Article 57 (f) of REACH.

1.5 Physicochemical properties

Table 2: Overview of physicochemical properties

Property	Value	Description	Reference
Physical state	white solid at 20 °C and 101.3 kPa	Value used for Chemical Safety Assessment (CSA)	Ashford RD 1994
Melting/freezing point	155 °C	Experimental result Method 10294, ASTM D4493, Method 58255B	
Boiling point	360 °C at 1013 hPa with decomposition 255°C at 17 hPa with potential decomposition		DIPPR 1994 CRC 1991
Vapour pressure	4.12E-09 hPa at 25 °C	Experimental result OECD Guideline 104 (Vapour Pressure balance)	
Water solubility	300 mg/L at 25 °C	Experimental result OECD Guideline 105 (flask method)	
Partition coefficient n-octanol/water (log value)	Log Kow: 3.4 at 21.5 °C and pH 6.4	Experimental result OECD Guideline 107 (Partition Coefficient (n-octanol / water), Shake Flask Method)	
Dissociation constant	pKa: 11.3		Zjawiony and Pasciak 1978

2 Harmonised classification and labelling

Bisphenol A is listed by Index number 604-030-00-0 in part 3 of Annex VI to the CLP Regulation as follows, as amended by Commission Regulation (EU) 2016/1179 (9th ATP):

Table 3: Classification according to Annex VI, Table 3.1 (list of harmonised classification and labelling of hazardous substances) of Regulation (EC) No 1272/2008

Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Spec. Conc. Limits, M-factors	Notes
				Hazard Class and Category Code(s)	Hazard statement code(s)	Pictogram, Signal Word Code(s)	Hazard statement code(s)	Suppl. Hazard statement code(s)		
604-030-00-0	Bisphenol A 4,4'-isopropylidenediphenol	201-245-8	80-05-7	Skin Sens. 1 Eye Dam. 1 STOT SE 3 Repr. 1B	H317 H318 H335 H360F	GHS07 GHS05 GHS08 Dgr	H317 H318 H335 H360F			

Concerning the environmental hazard classification, there is no harmonised classification available. From 1798 self-classifications, 1444 notifiers have no environmental hazard classification. The available ecotoxicity data for Bisphenol A is sufficient to propose a classification with Aquatic Chronic 1 (based on a LOEC of 0.372 µg/L for skewed sex ratio in *Danio rerio*, and a NOEC of 2.4 µg/L completely inhibited ovulation in *Salmo trutta*) (Lahnsteiner et al., 2005; Chen et al., 2015; reliability 2).

3 Environmental fate properties

3.1 Degradation

In the following key data is summarized. Study details and summaries are given in Annex I. The study descriptions and assessment of the studies are in line with the EU RAR (European Comission, 2010). Few new studies were added and the conclusions of the CSTEE, described in Bakker et al. (2014), taken into account.

3.1.1 Abiotic degradation

The physical and chemical properties of Bisphenol A suggest that hydrolysis and photolysis under environmental conditions is negligible (European Commission, 2003, 2008). An atmospheric half-life of 0.2 days was calculated (EUSES) based on the reaction kinetics of Bisphenol A with hydroxyl radicals (European Commission 2003, 2008).

3.1.2 Biodegradation in water

In three **screening tests** on ready biodegradation according to test guideline OECD 301F mineralisation levels for Bisphenol A were above the trigger value of 60%. Three screening tests show negligible degradation rates at the end of the test period. Bisphenol A therefore meets the criteria for rapid degradation.

Table 4: Summary of biodegradation screening test

Method	Test concentration	Results	Reference
OECD 301 F	7 mg/l and 25 mg/l	78.2 - 81.0% O_2 consumption after 28 days 10 day window fulfilled	(West et al., 2001)
OECD 301 F	unknown	87.8 ± 6.9% O_2 consumption after 28 days; 10 day window fulfilled	(Stasinakis et al., 2008)
OECD 301 F	100 mg/l	85-93 % O_2 consumption after 28 days 10 day window fulfilled	Registration Dossier (Katagiri, 2004)
OECD 301 C	unknown	0% after 14 days	(MITI, 1992)
OECD 301 D	8.9 mg/l	0% O_2 consumption after 28 days	(EC, 2010); Registration dossier (Stone and Watkinson, 1983)
OECD 301 B	8.9 mg/l	1-2% CO_2 evolution after 28 days	(EC, 2010); Registration dossier (Stone and Watkinson, 1983)

The results from the **tests in freshwater** demonstrate a rapid degradation of Bisphenol A under aerobic conditions with a half-life of 4 days (lag period 2-8 days) (Klečka et al., 2001; Kang and Kondo, 2002; Kang and Kondo, 2005; Suzuki et al., 2004b). Under anaerobic conditions in river water, 10% of the initial Bisphenol A concentration dissipated until day 10 (Kang and Kondo, 2002).

In **seawater**, the concentration of Bisphenol A did not change until day 30 or 40 (lag-phase), independent of temperature (4°C, 25°C or 35°C). After 60 days 30% primary degradation was observed at 4 °C and 80% primary degradation at 25°C and 35°C, respectively (Kang and Kondo, 2005). In another study (Ying and Kookana, 2003), only 17% Bisphenol A dissipated after 35 days, but was almost completely degraded (>90%) after 56 days. Under anaerobic conditions no primary degradation was observed until day 60 in seawater (Kang and Kondo, 2005).

Table 5: Summary of tests in fresh- and seawater

Conditions	Test concentrations	Results	Reference
Freshwater, aerobic (EPA OPPTS 835.3170; shake flask die-away)		DT_{50} = 0.5-1.4 days Lag period 2-8 days 65-80% $^{14}CO_2$ after 18 days Respirometry test: DT_{50}= 0.5-2.6 days Lag period = 2.3-4.4 days 59-96% CO_2 after 18 days	(Klecka et al., 2001)
Freshwater aerobic anaerobic (other method: shake flask die-away)	0.2 mg/L	Primary degradation: DT_{50} = 2-3 days DT_{50} > 10 days	(Kang and Kondo, 2002)

Freshwater, aerobic (each endpoint other method: shake flask die-away) Seawater, aerobic Seawater, anaerobic	1 mg/L	Primary degradation: DT_{50} = 4 and 3 days (25 °C and 35 °C) Lag period = 30 days (25 °C and 35 °C), 40 days (4 °C) after 60 days: 80% primary degradation at 25 °C and 35 °C 30% primary degradation at 4 °C no degradation until day 60	(Kang and Kondo, 2005)
Freshwater, aerobic (other method: shake flask die-away)	1 and 10 mg/L	Primary degradation > 90% in 6 days DT_{50} = 0.4-1.1 days Lag period 2-3 days	(Suzuki et al., 2004b)
Freshwater aerobic (other method: shake flask die-away)		Lag period = 2-5 days In 14 days: 34 of 44 river water microcosms: 40 - 90% removal 6 of 44 river water microcosms: 100% removal 4 of 44 river water microcosms: 0% removal	(Ike et al., 2000)
Seawater, aerobic (other method: shake flask die-away)	5 µg/L	Dissipation: ~17% after 35 days > 70% after 42 days > 90% after 56 days	(Ying and Kookana, 2003)

The available die-away studies indicate that Bisphenol A can be removed rapidly from freshwater environments.

Due to the limited information available it is difficult to conclude whether the measured rate of removal of Bisphenol A is the result of primary degradation dissipation from the test system.

In conclusion, Bisphenol A can be rapidly removed from surface waters.

3.1.3 Biodegradation in sediments

For the assessment of removal of Bisphenol A in sediment three reliable studies are available. A study with marine sediment determined a disappearance half-life time $DisT_{50}$ of 14.5 days (Ying and Kookana, 2003). A test using aquifer material (sediment and groundwater) showed no degradation within 70 days under aerobic conditions (Ying et al., 2003). Under anaerobic conditions (monitored with resazurin as a redox indicator in Ying and Kookana 2003) both studies showed no changes in the Bisphenol A concentration until day 70. In a study with estuarine sediment the half-life time DT_{50} was > 162 days (Voordeckers et al., 2002). Sarmah and Northcott (2008) observed a biphasic pattern with rapid initial dissipation (>90% in the first 4 days) of Bisphenol A and a smaller rate until the end of the test with trace amounts still remaining after 70 days.

Table 6: Summary of biodegradation tests in sediment

Conditions	Test concentrations	Results	Reference
Marine sediment (other method: shake flask die-away) aerobic anaerobic	1 µg/g	 $DisT_{50}$ = 14.5 days $DisT_{50}$ > 70 days	(Ying and Kookana, 2003)
Aquifer material (sediment and groundwater; other method: batch equilibrium) aerobic anaerobic	1 µg/g	 Primary degradation DT_{50} > 70 days DT_{50} > 70 days	(Ying et al., 2003)
Estuarine sediment (other method: shake flask die-away) anaerobic	200 µM (45.6 mg/L)	Primary degradation DT_{50} > 162 days	(Voordeckers et al., 2002)
River-water sediment (other method: shake flask die-away) Dissipation (whole system) Aerobic | anaerobic Groundwater-aquifer material (other method: shake flask die-away) Aerobic | anaerobic	100 µg/L	Dissipation > 90% after 4 days $DisT_{50}$ = 1.212 d | 1.38 d $DisT_{90}$ = 2.75 d | 4.901 d $DisT_{50}$ = 0.57 | 2.315 days $DisT_{90}$ = 2.26 | 306.9 days	(Sarmah and Northcott, 2008)

In conclusion, on the basis of this information, currently no definite conclusion on degradation of Bisphenol A in sediments is possible.

3.1.4 Biodegradation in soil

In soil radiolabelled Bisphenol A showed a rapid dissipation (DT_{50} < 3 days) (Fent et al., 2003). The major route of dissipation was the formation of non-extractable residues. After 120 days 76.0 - 81.6% non-extractable residues were formed in the four tested soils. Less than 20% of the applied radioactivity could be recovered as $^{14}CO_2$. A further study confirmed the rapid dissipation (DT_{50} = 7 days) of Bisphenol A in soil under aerobic conditions (Ying and Kookana, 2005), whereas under anaerobic conditions no degradation was observed within 70 days.

Table 7: Summary of biodegradation tests is soil

Conditions	Test concentrations	Results	Reference
Four different agricultural soils (two sandy loam and two loamy silt)	6 µg/100 g soil	after 120 days: $^{14}CO_2$ = 13.1-19.3% of the applied radioactivity Non-extractable = 76.0-81.6% of the applied radioactivity Extractable = 1.5-2% of the applied radioactivity Dissipation: $DisT_{50}$ < 3 days	(Fent et al., 2003)

(Laboratory simulation of soil die-away test, SETAC internal guideline)			
One soil (sandy loam) (Batch equilibrium method, no further specification of guideline)	1 µg/g	Aerobic: DT_{50} = 7 days Anaerobic: DT_{50} > 70 days	(Ying and Kookana, 2005)

In conclusion, under aerobic conditions, Bisphenol A is removed rapidly from soil. On the basis of the available conflicting data, currently no definite conclusion on removal of Bisphenol A in soil under anaerobic conditions is possible.

3.1.5 Summary and discussion of abiotic and biotic degradation

The physical and chemical properties of Bisphenol A suggest that abiotic degradation via hydrolysis and photolysis is negligible.The screening tests show that Bisphenol A can be rapidly removed from surface waters by biotic degradation processes and therefore is rapidly degradable.

In surface waters, on the basis of the information available, Bisphenol A is rapidly removed.

In sediments under aerobic and anaerobic conditions, on the basis of the available conflicting data, currently no definite conclusion on degradation of Bisphenol A is possible.

In soil, under aerobic conditions, Bisphenol A is removed rapidly. On the basis of the available conflicting data, currently no definite conclusion on removal of Bisphenol A in soil under anaerobic conditions is possible.

No information is available on environmental half-life in sediments or soils under standard test guideline conditions.

3.2 Environmental distribution

A core set of data is presented as background information.

3.2.1 Adsorption/desorption

The adsorption coefficients for environmental media were estimated using TGD methods and a log K_{ow} value of 3.40. The organic carbon-water partition coefficient (K_{oc}) was estimated with a value of 715 L/kg. Several experimental studies observed a K_{oc} value in the range of 251 to 1750 L/kg (outlier: 11,220 – 17,000). Bisphenol A is likely to be moderately adsorbed to solids (EC, 2010).

3.2.2 Volatilisation

Volatilisation is not considered to be a significant removal mechanism for Bisphenol A from water systems (Henry's Law constant = $4.03 \cdot 10^{-6}$ Pa·m³·mol^{-1}) (EC, 2010).

3.3 Environmental occurrence

Data from existing monitoring programs mainly in Germany and scientific publications across Europe of the years 2007-2012 were evaluated in an expert report committed by UBA (Fischer et al. 2014). BPA is ubiquitously and regularly found in all environmental compartments (freshwater, marine water, soil, biota, sediment, sewage sludge, air/dust), which gives evidence for continuous emissions of BPA. Data is mainly available for the aquatic compartment, in particular freshwater and groundwater. The most relevant environmental compartments are surface waters with measured concentrations in the ng/L and up to the low µg/L range. For freshwater, measurements are on average in the range between 10 and some 100 ng/l with mean maximum concentrations of 2000 ng/L for freshwater and above 8000 ng/L in marine waters with much higher peak concentrations, respectively. The highest concentrations were measured close to emission points. BPA is also detected in sediment samples (fresh and marine water).

During EQS deduction in 2016, data was compiled by JRC, which is summarized in Annex II.

Recent data furthermore demonstrates the presence of BPA in further biota and also remote areas. BPA has been detected in eggs of herring gull in a series of monitoring reports of an Urban Fjord in Norway (NIVA 2015, 2016). BPA was even detected in Arctic biota (Svalbard) in eggs of kittiwake and glaucous gull. Here, BPA was among the most quantitatively abundant compounds found in seabird eggs (Lucia, M. et al. 2016).

Hence, BPA reaches habitats continuously from various sources, has been shown to be continuously and ubiquitously present in surface waters and other compartments. Many organisms may therefore be exposed more or less continuously to Bisphenol A in the ng/L to low µg/L range and cannot avoid exposure.

3.4 Bioaccumulation

3.4.1 Bioaccumulation in aquatic organisms

Bioaccumulation was evaluated in the EU Risk Assessment Report of Bisphenol A (EC, 2010). The log K_{ow} of 3.4 indicates a moderate bioaccumulation potential. However, the available measured data indicated a low bioaccumulation potential (BCF ≤ 73.4 for fish and up to 144 for freshwater clams). Further details are summarized in Annex II.

3.4.2 Bioaccumulation in terrestrial organisms

The EU Risk Assessment Report of Bisphenol A (EC, 2010) calculated a bioconcentration factor for earthworms of 7.9 kg/kg based on QSARs (as implemented in EUSES).

3.4.3 Summary and discussion of bioaccumulation

As discussed in the RAR of Bisphenol A (EC, 2010), the bioconcentration factor for fish is estimated to be ≤ 73.4 and using QSAR methods a bioconcentration factor for earthworms was calculated to be 7.9 kg/kg.

Hence, Bisphenol A has a low potential for bioaccumulation in aquatic and terrestrial organisms (fish, earthworms).

4 Human health hazard assessment

Bisphenol A is already identified as SVHC owing to its reprotoxic effects according to Article 57(c) of REACH Regulation and additionally due to its endocrine disrupting properties with respect to human health according to Article 57(f).

The available data for mammals with respect to the effects of BPA on fertility, the mammary gland, neurodevelopment, metabolic effects and immune function in mammals was evaluated in the respective dossier leading to the identification of BPA as endocrine disrupter for human health. The conclusions are summarized in the supporting document published in June 2017 (ECHA, 2017), highlighting the importance and interference of BPA with estrogenic pathways in mammals:

"The dossier displays extensive evidence showing that Bisphenol A can affect several physiological functions and systems of mammal organisms through ED pathways. (…) BPA alters the reproductive function, mammary gland development, cognitive functions and metabolism through an ED MOA. (…) although the steps of the respective mechanisms of action are specific for each effect, the disruption of the estrogenic pathways is a common MoA consistently involved in each of the four effects. The primary target of BPA is however still not known with certainty. BPA binds to the estrogen receptors but with a weak affinity. In addition BPA binds also to other types of ER (…) with a higher affinity and receptors may also be involved in BPA-mediated effects, particularly at lower doses. The complexity of toxic responses also suggests different MoA that may interact. However, some evidence (…) enables to establish that the estrogenic pathway is central and common in the MoA. (…) Estrogens are known to be central in the regulation of sexual function and system but are also known to interact with many other physiological functions and developmental processes including neurobehaviour or metabolism." (ECHA 2017).

The present dossier focuses on the evaluation of the available ecotoxicological studies for fish, amphibians and invertebrates mainly in the aquatic environment. A thorough assessment of mammalian data from the human health data base is not provided. The assessment with respect to the environment also focuses on the protection of populations, communities, ecosystems composed by a large number of different species, and not a single species as for human health assessment. Still, the overview on *in vitro* data indicative for the possible different modes of action in vertebrates (chapter 5.3), comprises results from tests mainly conducted with mammalian cells/receptors but also from tests with fish and amphibian cells/receptors.

The adverse effects identified in the human health context with respect to mammalian vertebrates are relevant for effects on mammalian wildlife species in the environment (such as mice, rats). The data is also supportive for non-mammalian vertebrate species (fish, amphibians) with respect to the underlying mode of action and adverse effects due to the large degree of conservation of the primary amino acid sequences in proteins which implies large commonalities between non-mammalian and mammalian vertebrate species in regard to hormones, enzymes and receptors involved in the EATS modalities.

5 Environmental hazard assessment & endocrine disrupting properties

5.1 Standard ecotoxicological data

Bisphenol A was evaluated during the EU risk assessment for existing substances (EC, 2010) and a $PNEC_{water}$ of 1.5 µg/L was derived. Studies cited in (EC, 2010) were also used and discussed in this dossier. The EU RAR (EC, 2010) evaluated the studies with the focus on their suitability and validity for PNEC derivation and use for a SSD.

The database on ecotoxicological studies considered by the registrants is publicly available via the ECHA dissemination Website (https://echa.europa.eu/de/registration-dossier/-/registered-dossier/15752). Also here, the available ecotoxicological studies were evaluated with a focus on their suitability and validity for PNEC derivation and use for a SSD.

In 2016, an EQS[3] of 0.25 µg/L was derived based on the already existing and new data. For deriving the EQS, available information from standard ecotoxicological tests covering all taxa and results for either the most sensitive species or a species sensitivity distribution were used.

For fish and aquatic and terrestrial invertebrates short- and long-term ecotoxicity values are in the following ranges.

Short-term toxicity:

- Fish: 4.6 - 11.0 mg/L (96 h LC_{50}).
- Aquatic invertebrates: 3.9 - 16.0 mg/L (*Daphnia magna*, 48 h EC_{50}), but lower values for other crustaceans, e.g. 0.96 mg/L (*Acartia tonsa*, 72 h)
- Sediment organisms: 2.7 mg/L (*Chironomus tentans*, LC_{50} 96 h)

Long-term toxicity:

- Fish: 2.4 - 3640 µg/L (NOEC reproduction *Salmo trutta*, NOEC egg hatchability, *Pimephales promelas*, F2-generation; and NOEC 28d juvenile growth, *Oncorhynchus mykiss* respectively)
- Aquatic invertebrates: 0.038 - 3146 µg/L (EC_{10}, *Marisa cornuarietis*, 5 months, NOEC reproduction 21 d, *Daphnia magna* respectively)
- Sediment organisms: 0.1 µg/L (*Chironomus riparius*, NOEC growth and molting)
- Terrestrial invertebrates: 32 and 500 mg/kg (14 d NOEC mortality *Einsenia andrei* and 28 d NOEC reproduction *Folsomia candida* respectively)

A short summary of data is given in Annex III and in the respective assessments cited above.

5.2 General approach for the assessment of endocrine properties

To evaluate whether or not Bisphenol A fulfils the WHO/IPCS definition of an endocrine disruptor to the environment as interpreted by the EC ED EAG (JRC, 2013), both *in vitro* data and *in vivo* data were taken into account in order to demonstrate:

- Endocrine mode of action

[3] EQS (= Environmental Quality Standard under Water Framework Directive) are tools used for assessing the chemical status of waterbodies. In some cases, the PNEC from a risk assessment will be identical to the EQS. Reasons for deviations may be, that the concept of an overall threshold that protects all receptors and routes is a feature of EQS derivation that does not normally apply in chemical risk assessment and the underlying requirement of the WFD is to protect the most sensitive waters in Europe (European Commission (2011): Guidance Document No. 27 – Technical Guidance for Deriving Environmental Quality Standards)

- Adverse effects
- Plausible biological link between adverse effects and endocrine mode of action
- Environmental relevance

Non-testing, *in vitro* and mechanistic information obtained from *in vivo* studies were used to demonstrate the endocrine modes of action and pathways. The assessment of *in vivo* data focuses on the question whether adverse effects can be inferred to originate from the presumed modes of action or to be as a consequence of general systemic toxicity.

As the aim during the current assessment is an evaluation of the endocrine properties of BPA, the focus is different from the previous assessments where studies were evaluated with the aim to derive a PNEC value (such as for the EU RAR, the CSR or for the EQS). Hence, for the assessment of the underlying endocrine properties and related effects further studies are assessed with a focus on long-term ecotoxicity studies and endpoints relevant for identifying or explaining endocrine properties.

The structure and the assessment of *in vivo* data is mainly based on the OECD Guidance Document on standardised test guidelines for evaluating chemicals for endocrine disruption (OECD, 2012b). Furthermore, other guidance documents (e.g. OECD guidance document on the diagnosis of endocrine related histopathology in fish gonads (OECD, 2010a)) and information from literature (for vertebrates e.g. (IPCS, 2002; Kendall et al., 1998; Knacker et al., 2010; OECD, 2004)) are used.

Two different types of effects are assessed separately:

- Indicators of an endocrine mode of action (MoA)
- Effects on apical endpoints that provide evidence that a substance exerts adverse effects owing to its endocrine mode of action.

As the effects indicative for a MoA and the related apical effects differ between taxonomic groups, more specific information on the approach will be provided in the respective sections (5.5.1 and 5.6.1 for fish, 5.7.1 and 5.8.1 for amphibians, and in chapter 5.9 for invertebrates).

An overview on non-testing data (according to OECD conceptual framework level 1) is given in 5.3 and the available *in vitro* data (according to OECD conceptual framework level 2) for vertebrates is summarized for different MoA in 5.4. The available *in vivo* data (according to OECD conceptual framework level 3-5) is presented for each taxonomic group, in 5.5 and 5.6 for fish and 5.7 and 5.8 for amphibians. The results for invertebrates are evaluated subsequently for molluscs (5.10), insects (5.11), crustaceans (5.12), and further invertebrate species (5.13) covering both *in vitro* and *in vivo* effects as well as the plausible link between them. Chapter 5.14 summarizes the environmental relevance of the effects observed for fish, amphibians and invertebrates.

All studies were assessed with respect to their reliability according to the Klimisch score system. The reliability categories used 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 guidelines), 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.

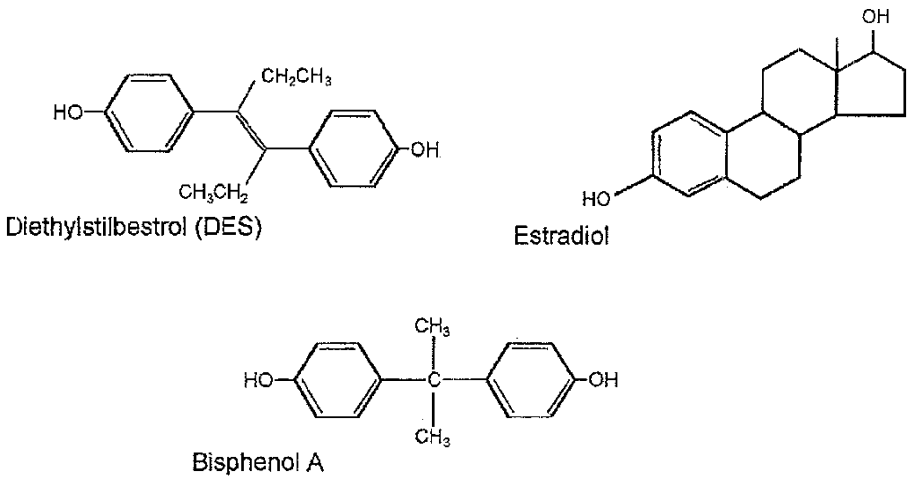
R3 Not reliable: Not all reliability criteria are fulfilled. The study has clear flaws in study design, performance and/or documentation.

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

5.3 Non-testing information

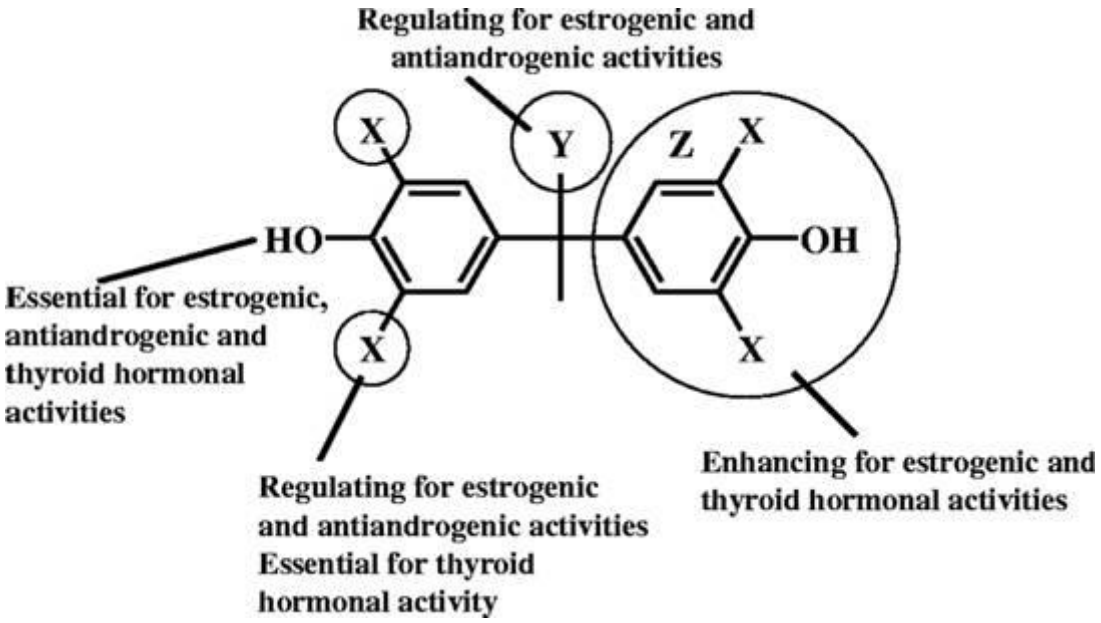
For Bisphenol A there is data available from docking and other QSAR models (data not shown in detail here), which points to a binding potential to oestrogen and androgen receptor proteins. Figure 1 compares the structure of Bisphenol A to known strong oestrogen receptor binding molecules. Based on these structural similarities several publications deal with the detailed analysis of the structural alerts necessary for receptor binding of Bisphenol A and analogous substances.

Figure 1: Chemical structure of BPA, DES and estradiol (Vandenberg et al., 2009)



Kitamura et al. (2005) e.g. summarized the structural alerts necessary for the different modes of action for BPA and other derivating compounds as shown in Figure 2.

Figure 2: Structural alerts in phenols important to exert different hormonal activities (Kitamura et al., 2005)



The following tendencies regarding receptor binding potential and specificity are deduced by Kitamura et al. (2005):

To exert estrogenic activity, an unhindered hydroxyl group on an aryl ring and a hydrophobic group on the *para*-position to the hydroxyl group is required (Blair *et al*., 2000; Elsby *at al*., 2000; Fang *et al*., 2000; Hong *et al*., 2002; Nishihara *et al*., 2000). For bisphenol derivates, this means the phenolic hydroxyl group. A hydroxyl group on one phenyl ring is also essential for an anti-androgenic activity of bisphenols.

Experimental data suggest that the distance between para hydroxyl groups and also the nature of the bridging carbon substituent modulate the estrogenicity. Furthermore, increasing polarity reduces the estrogenicity (Molina-Molina *et al*., 2013).

The thyroid receptor protein shows a higher substrate specificity compared to the estrogen and androgen receptors, because of the relatively small size of the active site (Wagner *et al*., 1995, 2001). Kitamura et al. (2005) demonstrated that a 4-hydroxyl group and double substitution by a halogen or methyl group at the 3,5-positions of the A-phenyl-group are essential for thyroid hormone activity of bisphenols.

Non-testing structure-activity data according to the conceptual framework level 1 indicates the potential of BPA to bind to the active site of estrogen and androgen receptor proteins.

5.4 *In vitro* information indicative for an endocrine activity in vertebrates

In vitro endocrine activity of Bisphenol A was assessed in different assays including binding assays, reporter gene assays, YES assays and assays analysing vitellogenin (VTG) induction in primary hepatocytes of *Cyprinus carpio, Salmo salar, Rutilus rutilus, Oncorhynchus mykiss* and *Xenopus laevis.*

Additionally, for *in vitro* assays based on mammalian cell systems there are many reviews and reports available e.g. EPA (2015), NTP-CERHR (2008), Vandenberg et al. (2013), Wetherill et al. (2007). As the investigated hormone receptors and their interaction with key enzymes are highly conserved among vertebrate species, this data can also be used to draw conclusions on possible endocrine modes of action in wildlife vertebrates, although binding affinity and hence adverse consequences may differ.

In the following sections, the available *in vitro* assays mainly cited from (EPA, 2014) are presented with respect to an oestrogen (5.4.1), androgen (5.4.2) or thyroidal (5.4.3).

5.4.1 *In vitro* tests for oestrogenic activity

With regard to the oestrogenic activity of Bisphenol A the following tests are available (see Table 8):

- 3 competitive ligand-binding assays using fish cytosol preparations and
  3 assays using recombinant human estrogen receptor α

Kloas et al. (2000), Olsen et al. (2005) and Segner et al. (2003b) assessed whether or not Bisphenol A is able to specifically bind to the carp or rainbow trout estrogen receptor α using cytosolic preparation of female trout liver. Bisphenol A showed a higher binding affinity to carp hepatic ER than the other alkylphenols assessed (nonylphenol and octylphenol) (Kloas et al., 2000). In comparison with the human estrogen receptor, Bisphenol A (as well as 4-n-nonylphenol) showed a higher relative binding affinity to the rtER (Olsen et al., 2005). Bisphenol A bound with a lower affinity to hER than nonylphenol and octylphenol (Kloas et al., 2000).

- One competitive ligand-binding assay assessed the binding to the plasma sex steroid-binding protein in fish cells

Kloas et al. (2000) also assessed the ability of Bisphenol A (and e.g. nonylphenol) to inhibit the binding of [^{3}H]17β-estradiol (E2) to the plasma sex steroid-binding protein (SBP). Bisphenol A (as well as nonylphenol) showed some ability to displace [^{3}H]E2 from the

plasma sex steroid binding protein from *Cyprinus carpio* but had a clearly lower SBP binding affinity compared to that of hepatic ER binding.

- Seven assays assessing the expression of estrogen-sensitive genes (vitellogenin VTG) in primary hepatocytes from carp, atlantic salmon, roach, rainbow trout and african clawed frog

One study derived the primary hepatocytes from genetically male and female carp (Smeets et al., 1999). The exposure to Bisphenol A resulted in a VTG induction with two fold greater inductions in male than in female hepatocytes. The other study with carp hepatocytes (derived from sexually immature females) conducted by Segner et al. (2003b) resulted in a VTG induction at Bisphenol A concentrations in the same order of magnitude. Segner et al. (2003b) also investigated the VTG induction in hepatocytes derived from rainbow trout with similar results. In the study by Tollefsen et al. (2003) using hepatocytes from male Atlantic salmon, Bisphenol A induced VTG protein expression at lower concentrations (EC_{50} is one order of magnitude lower compared to the other studies). In all studies, 4-nonylphenol was a little less potent than Bisphenol A. All of these results are also true for the test conducted by Mitsui et al. (2007) with *Xenopus* hepatocytes.

- Eight Reporter gene assays

Five yeast screening assays (YES) using recombinant human estrogen receptors are available as well as three assays analysing gene expression with the rainbow trout estrogen receptor α (rtER) and estrogen receptors of goldfish, bluegill, guppy, roach, carp, stickleback, zebrafish, medaka, and fathead minnow.

Miyagawa et al. (2014) and Tohyama et al. (2015) analysed the ligand- and species-specificity of nine different fish species in an *in vitro* ER α reporter gene assay. Medaka, stickleback, bluegill and guppy ERs showed higher sensitivities to Bisphenol A as well as to nonylphenol and octylphenol compared with cyprinid ER α.

The yeast estrogen screens with recombinant yeast expressing human estrogen receptor α conducted by Chen et al. (2002); Segner et al. (2003b); Schmitt et al. (2008) and Schreurs et al. (2005) showed evidence for Bisphenol A to exhibit oestrognic activity. The measured activity was five orders of magnitude lower than that of 17β-estradiol. Therefore, Bisphenol A was considered to be weakly estrogenic.

All tests described above indicate that Bisphenol A activates human and fish estrogen receptors. The relative potency was higher for fish ER than for human ER.

- Four MCF-7 cell proliferation assays (E-Screen)

Bisphenol A induced human breast cancer cell (MCF-7) proliferation (Olsen et al., 2005; Bonefeld-Jorgensen et al., 2007; Schlumpf et al., 2004 and Jimenez-Diaz et al., 2013) in a dose and time dependent manner.

Table 8: Summary of *in vitro* studies assessing the potential of Bisphenol A (BPA) to interact with the ER-mediated pathway*

Endpoint: Competitive ligand binding (IC_{50} is the concentration displacing 50% of [^{3}H]E2 from ER ligand binding pocket).					
Binding to ER					
Species	**Reference**	**Receptor origin and preparation**	**Endocrine mediated measurement parameters**	**Relative binding affinity (RBA) compared to 17ß-estradiol (= 1) (RBA was calculated as IC_{50}(E2)/IC_{50}(BPA))**	**Comment**
Cyprinus carpio carp	(Kloas et al., 2000)	Cytosolic preparation of female trout liver homogenates	IC_{50} = 26.62 µM	RBA = 0.0012	Comparison with 4-NP: IC_{50} = 68.86 µM
Cyprinus carpio Carp	(Segner et al., 2003b)	Cytosolic preparation of carp liver homogenates	EC_{50} = 26.62 µM	Relative potency compared to EE2: RBA = 0.0011	Comparison with 4-t-OP: EC_{50} = 32.05 µM RBA = 0.00094
Oncorhynchus mykiss Rainbow trout	(Olsen et al., 2005)	Cytosolic preparation of female trout liver homogenates	IC_{50} = 84,000 µM	RBA = 0.0058	Comparison with 4-n-NP IC_{50} = 36000 µM RBA = 0.00094
Human	See (EPA, 2014) METI, 2002	human estrogen receptor		RBA = 0.00195	
Human	See (EPA, 2014) Coleman, Toscano et al., 2003	Recombinant human estrogen receptor α		RBA = 0.0032	
Human	(Olsen et al., 2005)	Recombinant human estrogen receptor α	IC_{50} = 180 µM	RBA = 0.00315	Comparison with 4-n-NP IC_{50} = 1300 µM RBA = 0.02
Binding to the plasma sex steroid-binding protein					
Cyprinus carpio carp	(Kloas et al., 2000)		IC_{50} = 59.55 µM	0.0029 (RBA was calculated as IC_{50}(E2)/IC_{50}(BPA))	Comparison with 4-NP: IC_{50} = 29.54 RBA = 0.6
Endpoint: Expression of estrogen-sensitive genes					
Expression of vitellogenin					
Species	**Reference**	**Cell type and origin**	**Endocrine mediated measured parameters**	**Relative potency compared to 17ß-estradiol (= 1)**	**comment**
Cyprinus carpio	(Smeets et al., 1999)	Primary hepatocytes derived from	VTG induction LOEC (BPA) =	REP = 1E-5 REP was calculated as	

carp		genetically male and female carp	50 µM (in males 2fold greater than females)	threshold concentration of E2/ threshold concentration of BPA	
Cyprinus carpio Carp	(Segner et al., 2003b)	Hepatocytes derived from sexually immature or female carp	VTG induction EC_{50} = 29 µM	Relative potency compared to EE2: REP = 0.0038	Compared with 4-t-OP EC_{50} = 38.2 µM REP = 0.0029
Salmo salar Atlantic salmon	(Tollefsen et al., 2003)	Hepatocytes derived from saltwater-adapted male Atlantic salmon	VTG induction EC_{50}= 0.14 µM	REP = 0.00017 REP was calculated as threshold concentration of E2/ threshold concentration of BPA	Comparison with 4-n-NP EC_{50} = 0.81 µM REP = 0.0032
Rutilus rutilus Roach	(Gerbron et al., 2010)	Liver from caught male roach (plasma VTG conc. < 20 ng/mL)	VTG induction LOEC (BPA) = 100 µM (at 500 µM cytotoxic effects)	216% VTG produced under the stimulation with E2 100 nM (E2 control)	Comparison with 4-NP LOEC (4-NP) = 100 µM (also cytotoxic effects at 500 µM; 18% VTG induction compared to E2)
Oncorhynchus mykiss Rainbow trout	(Olsen et al., 2005)	Hepatocytes derived from juvenile rainbow trout	VTG induction EC_{50} = 350 µM	REP = 0.0029	Comparison with 4-n-NP EC_{50} = 0.008 µM REP = 0.0002
Oncorhynchus mykiss Rainbow trout	(Segner et al., 2003b)	Hepatocytes derived from sexually immature or female rainbow trout	VTG induction EC_{50} = 32.5 µM	Relative potency compared to EE2: REP = 0.002	Compared with 4-t-OP EC_{50} = 41.4 µM REP = 0.0016
Xenopus laevis African clawed frog	(Mitsui et al., 2007)	Primary-cultured *Xenopus* hepatocytes (ELISA) detection limit (VTG)= 0.06 ng/L	Agonistic VTG induction: LOEC = 0.100 µM Antagonistic VTG ind: LOEC = 0.8 µM	Relative potency with hER: 0.005 Relative potency (% TAM activity): 0.5, 1.1 % (2 assays)	Compared with NP Agonistic: LOEC = 0.1 µM Antagonistic: LOEC = 3 µM
Endpoint: Transcriptional activation of reporter genes under the control of the ER					
Transcriptional activation assay using recombinant yeast (yeast estrogen screen, YES)					
Species	**Reference**	**Cell type and origin**	**Endocrine mediated measured parameters**	**Relative potency compared to 17ß-estradiol (= 1)**	**comment**
Human	(Chen et al., 2002)	Yeast two-hybrid assay with estrogen receptor α and coactivator TIF2		REP = 1E-5	See (EPA, 2014): there are many more tests with similar results available

Human	(Segner et al., 2003b)	Recombinant yeast with human estrogen receptor	EC_{50} = 9.6 µM	Relative potency compared to EE2: REP = 0.000084	Comparison with 4-t-OP EC_{50} = 1.6 µM (REP = 0.00048)
Transcriptional activation assay using vertebrate cell lines					
Oncorhynchus mykiss Rainbow trout	(Kunz et al., 2006)	Recombinant yeast with rainbow trout estrogen receptor α	EC_{50}= 12.2 µM (27% effect compared to E2)	REP= 0.000148	
Carassius auratus Goldfish *Lepomis macrochirus* Bluegill *Poecilia reticulata* Guppy *Rutilus rutilus* Roach *Cyprinus carpio* Carp *Gasterosteus aculeatus* Stickleback *Danio rerio* Zebrafish *Oryzias latipes* Medaka *Pimephales promelas* Fathead minnow	(Miyagawa et al., 2014)	HEK293 cells used as host cells with the respective fish species ERα	goldfish EC_{50} = 1.895 µM bluegill EC_{50} = 0.425 µM guppy EC_{50} = 0.121 µM roach EC_{50} = 0.750 µM carp EC_{50} = 2.641 µM stickleback EC_{50} = 0.309 µM zebrafish EC_{50} = 0.664 µM medaka EC_{50} = 0.226 µM fathead minnow EC_{50} = 0.762 µM	Goldfish RBA = 0.00015 Bluegill RBA = 0.00059 Guppy RBA = 0.0012 Roach RBA = 0.00023 Carp RBA = 0.00013 Stickleback RBA = 0.00118 Zebrafish RBA = 0.00018 Medaka RBA = 0.00075 Fathead minnow RBA = 0.0003	Cyprinids such as carp and roach were less sensitive to BPA and other estrogenic EDCs (e.g. NP, OP) compared with other fish species tested
Rutilus rutilus Roach *Cyprinus carpio* Carp *Gasterosteus aculeatus* Stickleback *Danio rerio* Zebrafish *Oryzias latipes* Medaka	(Tohyama et al., 2015)	HEK293 cells used as host cells with the respective fish species ERα, ERβ1 and ERβ2	Roach Not-determinable because of weak oestrogenicity Carp EC_{50} (ERα) = n.d. EC_{50} (ERβ1) = n.d. EC_{50} (ERβ2) = 107 µM Stickleback EC_{50} (ERα) = 29.5 µM EC_{50} (ERβ1) = n.d. EC_{50} (ERβ2) = 174 µM Zebrafish EC_{50} (ERα) =	Roach Not-determinable because of weak oestrogenicity (n.d.) Carp REP (ERα) = n.d. REP (ERβ1) = n.d. REP (ERβ2) = 0.000075 Stickleback REP (ERα) = 0.10 REP (ERβ1) = n.d. REP (ERβ2) = 0.000049 Zebrafish REP (ERα) = 0.00017 REP (ERβ1) = 0.0000016 REP (ERβ2) = 0.000073 Medaka REP (ERα) = 0.00017 REP (ERβ1) = n.d. REP (ERβ2) = 0.000053	

			78.8 µM EC_{50} (ERβ1) = 182 µM EC_{50} (ERβ2) = 38.6 µM Medaka EC_{50} (ERα) = 78.8µM EC_{50} (ERβ1) = n.d. EC_{50} (ERβ2) = 61.1 µM		
Endpoint: MCF-7 cell proliferation assay (E-Screen) E-screen uses the proliferative effect of oestrogens on their target cells (cells of the female genital) as endpoint					
Species	**Reference**	**Cell type and origin**	**Endocrine mediated measured parameters**	**Relative potency compared to 17ß-estradiol (= 1)**	**comment**
Human	(Olsen et al., 2005)	MCF-7 cells	EC_{50} = 0.3 µM	REP = 0.002	Comparison with 4-n-NP EC_{50} = weak inducer (< 50% induction)
Human	(Bonefeld-Jorgensen et al., 2007)	MCF-7 cells	EC_{50} = 3.9 µM	REP = 0.0001	Comparison with 4-n-NP EC_{50} = 8.9 µM

* ER = estrogen receptor, E2 = 17β-oestradiol, TAM = tamoxifen, LOEC = lowest observed effect concentration, RBA = relative binding affinity, REP = relative estrogen potency

The competitive ligand-binding studies demonstrated that Bisphenol A is able to displace E2 from the ER ligand-binding pocket. In this context, Bisphenol A showed a higher relative binding affinity to the human ER than to fish ER.

Furthermore, there is evidence that binding of Bisphenol A to ER leads to activation of the ER-mediated pathway and consequently to transcriptional activation of typically estrogen-responsive genes. Modulation of ER-mediated gene expression by Bisphenol A was evidenced on the transcriptional, protein and cell physiological level.

In the MCF-7 cell proliferation assay, Bisphenol A acted like a full agonist (E2) of the hER. However, since a control using a known ER antagonist to repress the observed effects was not provided within the studies cited, it cannot be completely ruled out that the observed proliferative effects of BPA are based on other not ED mediated effects.

5.4.2 *In vitro* tests for androgenic activity

With regard to androgenic activity the following results are available (see Table 9):

- Six Reporter gene assays

Xu et al. (2005) investigated Bisphenol A and e.g. 4-nonylphenol for their agonistic and antagonistic activities using a human androgen receptor (hAR) reporter gene assay. Bisphenol A showed significant inhibitory effects on the dihydrotestosterone (DHT) induced AR-mediated transcriptional activity. 4-nonylphenol showed lower antiandrogenic activities

than Bisphenol A. The six other reporter gene assays conducted by Sohoni and Sumpter, (1998), Lee et al. (2003), Schreurs et al. (2005) and Ekman et al. (2012) showed comparable results.

Lee et al. (2003) showed that BPA was a stronger antagonist than NP in respect to the inhibition of androgen-induced AR transactivation.

Sohoni and Sumpter (1998) and Lee et al. (2003) furthermore demonstrated that BPA could compete with 4,5-dihydrotestosterone (DHT) for binding to the androgen receptor.

Thus, research results show that BPA can act as AR antagonist towards human and fish ARs.

Table 9: Summary of *in vitro* studies assessing the potential of Bisphenol A (BPA) to interact with the AR-mediated pathway*

Endpoint: Transcriptional activation of reporter genes under the control of the AR					
Transcriptional activation assay using recombinant yeast (yeast androgen screen, YAS)					
Species	**Reference**	**Cell type and origin**	**Endocrine mediated measured parameters**	**Relative potency**	**comment**
Human	(Sohoni and Sumpter, 1998)	Recombinant yeast with human androgen receptor	Inhibition of DHT induced AR activity		
Human	(Lee et al., 2003)	Yeast two-hybrid protein interaction assay (ARhLBD-ASCI cells)	0.05 µM BPA resulted in 40% inhibition of DHT		Compared to 4-NP: 0.005 µM NP resulted in 30% inhibition of DHT
Transcriptional activation assay using vertebrate cell lines					
Human	(Xu et al., 2005)	African monkey kidney cell line (CV-1) with human androgen receptor	Inhibition of DHT induced AR activity: IC_{50} = 80 µM		Compared to 4-NP: Inhibition: IC_{50} = 200 µM
Pimephales promelas Fathead minnow	(Ekman et al., 2012)	African monkey kidney cell line (CV-1) with fathead minnow androgen receptor	100 µM BPA inhibited all DHT (3 nM) induction of luciferase)		0.1 to 100 µM BPA resulted in a dose-dependent inhibition

* AR= androgen receptor, DHT= 4,5-dihydrotestosterone

5.4.3 *In vitro* tests for thyroidal activity

With regard to thyroidal activity the following results are available for BPA (see Table 10):

- Four reporter gene assays

In addition to eliciting oestrogenic and anti-androgenic activity as described in the previous sections, BPA has also been shown to bind to the thyroid hormone receptor in an antagonistic manner (Moriyama et al., 2002).

According to Canesi and Fabbri (2015) BPA was shown to be a weak ligand to liver TR in

rodents. BPA was shown to be a potent inhibitor of T_3 binding to human TH-binding proteins like the TTR protein. This suggests interference with the HPT axis also upstream of the TR binding.

The available *in vitro* assays show that Bisphenol A is capable of interfering with the TR and further HPT axis related proteins.

Table 10: Summary of *in vitro* studies assessing the potential of BPA to interact with the thyroid-mediated pathway*

Endpoint: Transcriptional activation of reporter genes under the control of the AR					
Transcriptional activation assay using recombinant yeast (yeast androgen screen, YAS)					
Species	**Reference**	**Cell type and origin**	**Endocrine mediated measured parameters**	**Relative potency**	**comment**
Human	See: (EPA, 2014) Kitagawa et al., 2003	Yeast two-hybrid protein interaction assay with TRα and coactivator TIF-2	No thyroid hormone receptor binding		
Transcriptional activation assay using vertebrate cell lines					
Human	See: (OECD, 2014) Sun et al., 2014	Vero cell (African green monkey) thyroid hormone reporter β	Weak activity Could not reach RIC_{20} No cytotoxicity		Compared to DEHP: same effects
Human	(Moriyama, et al., 2002)	TSA201 cells – derivate of HEK293	10 µM BPA resulted in a dose-dependent inhibition of the Gal4-TRα1 and Gal4-TRβ mediated transcription in the presence of 3 nM T_3		
Danio rerio Zebrafish	(Yang et al., 2015)	Hepatocyte cell line with epithelial-like morphology isolated from zebrafish (ZFL) with zebrafish TRβ	In co-exposure with 0.1 nM T_3 a significant luciferase activity was observed in a dose-dependent manner		
Xenopus laevis	(Kudo and Yamauchi, 2005)	^{125}I-T_3 binding assays using	Interference of BPA with the thyroid		In comparison, the IC_{50}s for 4-nonylphenol are

African clawed frog	See also: (Yamauchi et al., 2002) (similar ex periment)	TTR (TTR assay) and ^{125}I-T_3 binding to xTR LBD (TR assay)	receptor TTR Assay: IC_{50} (T_3) = 3.65 µg/L [5.6 +/- 1.3 nM]; IC_{50} (BPA) =**479 µg/L** [2100 +/- 200 nM]; TR assay: IC_{50} (T_3) = 0.43 µg/L [0.66 +/- 0.04 nM]; IC50(T4) = 2.72 µg/L [3.5 +/- 0.6 nM]; IC_{50} (BPA) = **5479 µg/L** [24000 +/- 3000 nM)]		1278 µg/L [5800 +/-600 nM] (TTR assay) and 3526 µg/L [16000 +/- 100 nM] (TR assay). Study mentioned in RAR, well-documented

* TR = thyroid receptor

5.4.4 Summary and Conclusions from *in vitro* data

The *in vitro* data presented above provide evidence that Bisphenol A can interfere with the endocrine system of vertebrate species. Bisphenol A shows oestrogen agonistic effects, androgen antagonistic effects as well as anti-thyroidal effects at the molecular and cellular level. Furthermore, other processes may be triggered via a molecular cross-talk between these pathways and the respective endocrine axes. Cross-talk is ubiquitous among endocrine signalling pathways (OECD, 2012a).

The *in vitro* data suggests that Bisphenol A may affect endocrine processes via different modes of action (oestrogen agonistic, androgen antagonistic and anti-thyroidal).

5.5 Fish: *In vivo* effects with regard to an oestrogen or androgen mediated endocrine mode of action

5.5.1 Approach used for assessing the oestrogen or androgen mediated adverse effects in fish

The assessment of *in vivo* data was focused on the question whether or not the results are in accordance with the presumed mode of action based on *in vitro* tests (presented above in chapter 5.4) or rather seem to be a consequence of systemic toxicity. The assessment is based on the OECD guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption (OECD, 2012b) and supplemented by further literature.

In general, two different types of effects are considered and analysed separately:

- Indicators of an endocrine mode of action *in vivo*
- Effects on apical endpoints that provide evidence that Bisphenol A treatment results in adverse effects owing to its endocrine mode of action.

Indicators of endocrine mode of action:

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

One of the most common **biomarkers** indicating an oestrogenic or androgenic endocrine mode of action is vitellogenin (VTG). Vitellogenin is naturally produced by female fish as a precursor of yolk proteins which are incorporated in eggs (IPCS, 2002). Induction of VTG in female and (more pronounced) in male fish is a known and accepted indicator of an oestrogen 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, 2010a), the following histological endpoints are diagnostic for endocrine activity:

- Male: increased proportion of spermatogonia (early sperm cells), presence of testis-ova (oestrogenic response especially in juvenile and adult Japanese medaka, but also in other differentiated gonochoristic species), increased testicular degeneration, interstitial (Leydig) cell hyperplasia/hypertrophy, retained peritoneal attachments/gonadal duct feminization of the testis (oestrogenic response in juvenile fathead minnow and zebrafish)
- 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 the GSI might be influenced by other modes of action too, reduction of GSI in male fish is regarded as a sensitive parameter in reproductive studies with oestrogenic substances (OECD, 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]

Apical endpoints providing evidence that adverse effects are owing to the endocrine MoA:

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

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

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

Change in sex ratio towards females is a known result of oestrogenic 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 oestrogen active substances (Baroiller et al., 1999; Piferrer, 2001).

Whether or not endocrine mediated effects are observable highly depends on the life stage tested. For example testis-ova might be induced in adult males as at least in some species gonads remain bipotent, but sensitivity is usually highest during sexual development (e.g. Nakamura et al., 1998). Differences in development of fish species must be considered. *O. latipes* for example is a differentiated gonochoristic species that naturally develops either male or female gonads and the 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 the life stage(s) under exposure need to be considered carefully while interpreting test results. If effects on gonadal staging are analysed, the reproductive cycle of a species should be considered. In particular, 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.

Alterations 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 oestrogen or anti-androgen specific. According to (OECD, 2012b) "no cases are known in which altered sex ratio was caused by a substance other than an ED."

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

Thus, in combination with indicators of endocrine activity, they provide evidence of oestrogen mediated effects but alone they are not diagnostic for this mode of action as

[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, G., Collette, B.B., and Facey, D.E. (1997). The Diversity of Fishes, 1st edn (Blackwell Science Ltd.). In repeated spawners, this process recurs and, as their spawning is usually not synchronized, individual gonadal growth differs in time.

they might also be influenced by other modes of action.

Table 11 summarises the endpoints and adverse effects that are considered to be indicators of oestrogenic and anti-estrogenic activity of Bisphenol A *in vivo*.

Table 11: Summary of endpoints indicating oestrogen activity considered during data analysis

Endpoints indicating an oestrogen receptor agonist mode of action (*in vivo* fish)	**Endpoint indicating an oestrogen antagonist MoA (*in vivo* fish)**
• Vitellogenin induction in males (only oestrogenic 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.	• Vitellogenin level reduced in females • Phenotypic sex ratio shifted towards males • Higher proportion of sexually undifferentiated fish

Table 12 summarizes the endpoints indicating androgenic and/or anti-androgenic activity and may be affected because of this activity of Bisphenol A *in vivo*.

Table 12: Summary of endpoints indicating androgen activity considered during data analysis

Endpoints indicating an androgen agonist mode of action (in vivo fish)	**Endpoints indicating an androgen antagonist mode of action (in vivo fish)**
• Masculinised secondary sex characteristics in females • Phenotypic sex ratio shifted towards males • Vitellogenin level reduced in females	• Depression of male secondary sex characteristics • Female biased phenotypic sex-ratio • Reduced or inhibited spermatogenesis • Delay of maturation of ovaries • Higher proportion of intersex fish • Vitellogenin level in females elevated

As oestrogens and anti-androgens have two very different modes of action but possible similar apical adverse effects on the reproductive organs, it is difficult to distinguish them. Both can skew the sex ratio towards females, reduce or inhibit spermatogenesis and delay maturation of ovaries, although only oestrogens induce VTG production in males.

5.5.2 *In vivo* effects with regard to an oestrogenic or androgenic mode of action in different fish species

In the following sections, we summarized the results of *in vivo* studies with respect to the diagnostic and supporting endpoints related to an oestrogen or androgen MoA for different fish species. An overview on the data is given in Table 14 at the end of this section.

Overall, for ten fish species *in vivo* data at different levels (biomarker, histology and apical endpoints) are available. This comprises two studies with *Cyprinus carpio*, two studies with *Gabiocypris rarus*, twelve studies with *Oryzias latipes*, five studies with *Danio rerio*, five studies with *Pimephales promelas*, one study with *Salmo trutta*, *Carassius auratus* and *Xiphophorus helleri*, two studies with *Oncorhychus mykiss*, and two studies with *Poecilia reticulate*.

5.5.2.1 Effects on carp (*Cyprinus carpio*) related to an oestrogen/androgen MoA

The EU Risk Assessment Report (European Commission, 2010) reports a study conducted by Bowmer and Gimeno (2001) (reliability 2) for which only an extended abstract was available. In this study the effects of Bisphenol A on the development of male carp reproductive tract when exposed during sexual differentiation were investigated. The fish was specifically genetically altered to be an all-male population. ***Males*** were exposed to nominal concentrations of 10, 32, 100, 320 and 1,000 µg/L Bisphenol A under flow-through conditions, during the period of sexual differentiation (from 45 to 55 days post hatch onwards). Two experiments were performed, the first conforming to the OECD principles of GLP. In both experiments, nominal concentrations were confirmed by analysis. In the first experiment 28- and 49-day NOECs for growth (wet weight) were >600 and 100 µg/L Bisphenol A; in the second experiment 28- and 56-day NOECs were both 226 µg/L. In the first experiment, 28- and 49-day NOECs for ***oviduct formation*** were 100 and 16 µg/L Bisphenol A while in the second experiment they were 60 and 17 µg/L. Therefore, the LOEC for the oviduct formation after 49-days was 32 µg/L. Oviduct formation is clearly an oestrogenic effect.

(Mandich et al., 2007) (reliability 1) conducted another study examining the reaction of *Cyprinus carpio* on exposure to Bisphenol A. One-year-old carps were exposed for 14 days to nominal concentrations of 1, 10, 100 and 1,000 µg/L Bisphenol A according to OECD Guideline 204 under flow-through conditions. A control and a solvent control were also used. The actual concentration of Bisphenol A using LC-MS/MS technique was 0.85, 7.34, 90.73 and 1,055.40 µg/L. A gonad histology was conducted to determine the stages of spermatogenesis and oogenesis and a sex steroid immunoassay (competitive ELISA) was performed. 9.1 and 42.9% of the ***male carps showed significantly elevated Vitellogenin concentrations*** (> 11,000 ng/mL) after 14 day exposure to 1 and 10 µg/L Bisphenol A. In this study, also effects on testis morphology were observed, starting from 1 µg/L Bisphenol A. At this concentration in 18.2% of ***males alteration of the interlobular connective tissue and granulocyte infiltration*** were observed. In the highest test concentration 36.4% of the males showed granulocyte infiltration and 72.7% showed interlobular tissue alteration. At the highest concentration, 27% carp, described as males by gross examination, exhibited ***intersex gonads*** characterized by the simultaneous presence of testicular tissue and small pre-vitellogenic oocytes. It is very interesting that these results were observed after only 14 days of exposure.

Vitellogenin induction in the male carp as well as the histopathological findings in both tests described above are endpoints for oestrogen- mediated agonistic activity according to the OECD guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption (OECD, 2010a; OECD, 2012b).

In summary, Vitellogenin induction in the male carps as well as the histopathological findings in both tests described above are endpoints for oestrogen-mediated agonistic activity according to the OECD guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption (OECD, 2012b). These results lead to a strong evidence for *in vivo* oestrogenic

activity of BPA in the fish species *Cyprinus carpio*.

5.5.2.2 Effects on Rare minnow (Gobiocypris rarus) related to an oestrogen/androgen MoA

There is also a test with another cyprinid fish species endemic in China, rare minnow *Gobiocypris rarus* conducted by Zhang et al. (2016a) (reliability 2). Rare minnow purchased from the Institute of Hydrobiology, Chinese Academy of Sciences were acclimatised. 5-month old male were exposed to 225 µg/L BPA (0.99 µM) in 30 L glass tanks for 1, 3 and 9 weeks. 54 male individuals were used per treatment group (18 fishes per tank in triplicate). The temperature was 25.0 ± 1.0 °C, and pH 8.01 ± 0.31. The concentrations were measured with HPLC, resulting in BPA concentrations from 172 to 210 µg/L. No significant difference was found between control and BPA groups in total length, standard length, body weight, testicular weight and GSI during exposure duration. Zhang et al. (2016a) found that after 3 and 9 weeks exposure, 225 µg/L BPA ***significantly increased apoptosis*** in rare minnow testis. Most apoptosis was focused on spermatocytes and here most of the apoptotic cells were primary and secondary spermatocyte. Finding a ***decrease of the genes ccna1*** (the essential promoter of meiotic division) ***and birc5*** (a cell cycle promoter) at both 1 and 9 weeks, it indicates that the intrinsic mitochondrial pathway played important roles in this process and the apoptosis was likely attributable to ***BPA-induced meiosis arrest***.

Zhang et al. (2016b) (reliability 2) exposed 6-month old female rare minnow to 1, 15, and 225 µg BPA/L or a solvent control (0.0001% DMSO) in 30 L glass tanks for 7 days. 30 individuals per treatment group were used (15 individuals per tank in duplicate). It was a semi-static test with water temperatures of 24.9 ± 0.8 °C, and pH 8.01 ± 0.31. The average measured BPA concentrations were 0.88 ± 0.11 µg/L, 13.07 ± 1.56 µg/L, and 192.76 ± 20.87 µg/L. Body weight and length were measured as well as Vitellogenin, 17β-oestradiol, testosterone and 11-ketotestosterone by ELISA method. ***Vitellogenin was significantly elevated*** in the 1 and 15 but not in the 225 µg /L BPA treatment group. Same result was measured for testosterone and similar for 17β-oestradiol. The ***ovarian weight increased*** up to the 15 µg /L BPA treatment (here it was significantly elevated). In the 225 µg /L BPA treatment it was on the same level as the control. In the 1 µg/L BPA treatment the ovarian weight was elevated compared to the control but not significantly.

In summary, also for rare minnow, there are biomarker (VTG) effects characteristically for oestrogen-mediated agonistic activity. Additionally, one test indicates that BPA induced meiosis arrest.

5.5.2.3 Effects on Medaka (Oryzias latipes) related to an oestrogen/androgen MoA

For Medaka, three Fish Sexual Development Tests, one Fish Screening Assay, a Full Life Cycle Test and a Partial life cycle test as well as four modified reproductive assays are available (see Table 13).

Yokota et al. (2000) (key study for this species) reported an extended early life stage (ELS) toxicity test (which is equivalent to a Fish Sexual Development Test) with *O. latipes* with test concentrations of 2.28, 13.0, 71.2, 355 and 1820 µg /L BPA (measured). The authors used semi-static exposures for the embryos and flow-through exposures for larvae. 60 eggs per treatment in four replicates were used. The test met the validity criteria of OECD 210 and 234 and, with the exception of some water quality parameters, all relevant parameters are reported (reliability 2). The parameters monitored were egg hatchability, time to hatching, cumulative mortality, growth (total length and body weight), secondary sex characteristics and gonadal histology. There was no effect observed on egg or embryo mortality, hatchability and time to hatch. At the highest test concentration, BPA reduced significantly the total length and body weight (growth) (LOEC 1820 µg/L). The sex ratios were determined from external secondary sexual characteristics. At the highest test concentration, ***all*** fishes were ***phenotypic females***. Through gonadal histological investigation it was determined that ***none*** of the fish ***developed testis*** (100 per cent

effect). Six of the phenotypic female fish showed ***testis-ova***. At the next lowest test concentration (355 µg/L), only 5 out of 20 fish (25%) showed male secondary sex characteristics. The sex ratio of males to females in the controls was 2:1. As the sexual characteristic results were not considered reliable indicators of sex, they were not treated statistically. Unfortunately, the test design did not include a concentration between 355 and 1820 µg/L. Therefore, the effects on sex ratio based on gonadal histology (LOEC) start between 355 and 1820 µg/L. Two fish in the 355 µg /L BPA concentration group had no distinguishable sex. Both, induction of testis-ova as well as change in sex-ratio towards females, are clear indicators of an oestrogenic mode of action according to the OECD guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption (OECD, 2012b).

Metcalfe et al. (2001) studied the effects of Bisphenol A on growth and sexual differentiation endpoints of *O. latipes* in an extended early life stage test (reliability 2). They started the tests with one day old fry and examined it on day 90 post-hatch histologically at concentrations of 5.9 – 29.8 – 59.6 – 119.2 µg/L (measured). At the lowest test concentration (5.9 µg/L), two out of 25 males showed ***testis-ova***. ***Male fish*** at higher concentrations (29.8, 59.6 and 119.2 µg/L measured) showed several ***morphological changes in testes*** (e.g. loss of testicular structure; increase in fibrotic tissue surrounding the testicular lobules; decrease in the number of sperm cells especially spermatozoa in males approaching breeding condition). The ***female fish*** at the highest concentrations had ***ovaries in advanced stages of oogenesis*** in comparison to control females. As medaka is a differentiated gonochorist, the sex of a fish is more stable than in undifferentiated gonochorists. Thus, no spontaneous intersex or sex reversal has ever been observed in medaka and could not be expected under the influence of Bisphenol A. The accelerated oogenesis in the female fish at 119.2 µg/L compared with the controls shows a clear endocrine effect of Bisphenol A but it is not known how this would affect the following generations.

This accelerated oogenesis in female fish was also found in the test conducted by Na et al. (2002) (reliability 2). They also studied the effects of Bisphenol A on sex differentiation and gonadal development in *O. latipes.* Na et al. (2002) took the fish from a stream in the wild and cultured it in laboratory for three months. The study started the exposure with nominal 50, 100 and 200 µg /L BPA with newly hatched larvae. At 70 days, ***female fish*** exposed to BPA had ***greater proportions of the later stages of oocytes***, including mature eggs, which were not present in the controls. The sexual development in male fish seems to be delayed as in ***males*** the proportions of the ***later stages of spermatogenesis were reduced*** at nominal 100 and 200 µg/L BPA. In the highest test concentration (nominal 200 µg/L) there were very few spermatocytes or spermatids. This disparity in the sexual maturation of female and male fish seems to lead to serious problems in mating. For length and weight (growth) the NOEC in this test is 100 µg/L (nominal) as the fishes in the highest test concentration were longer and heavier than those in the other groups.

Further information on endocrine relevant biomarkers is available from one additional partial life stage test (Japanese ministry of the environment 2006) and several fish screening assays (Kang et al., 2002; Kashiwada et al., 2002; Shioda and Wakabayashi, 2000; Tabata et al., 2001; Yamaguchi et al., 2005).

Kang et al. (2002) examined the effects of Bisphenol A on the reproductive capacity (fecundity and fertility) and oestrogenic response of adult *O. latipes* and studied the transgenerational effects (F1 generation growth and sex) of this substance on the F1 offspring. The first part of the test is comparable to OECD Guidance No. 230 (reliability 1). Sexually mature *O. latipes* at four months after hatching (300 mg body weight, 33 mm length) were acclimated to flow-through conditions for three weeks in 56 breeding pairs in individual 1-litre chambers. The fecundity of each pair was checked daily, and over the last week of the acclimation period eggs were collected daily, a few hours after deposition, counted and assessed for fertility. From these fish, 32 pairs were selected which spawned every day, with >15 eggs per day and mean fertility >90%. Kang et al. (2002) exposed these fish to Bisphenol A for three weeks, at nominal concentrations of 0, 1000, 2000 and 4000 µg/L. The concentrations in the exposure chambers were measured twice each week during the exposures. The levels varied, falling as low as 60% of the nominal concentration

on one day, but the average concentrations were 78-86% of nominal. The average measured levels were 837, 1720 and 3120 µg/L. There was ***no significant effect on fecundity or fertility*** in any treatment group. There were two cases of mortality in two different treatments which seemed not to be exposure related. The histological examinations of the gonads revealed ***intersex gonads*** (testis-ova) in ***male fishes*** from all exposure groups. One of the eight (12.5%) males in the 837 µg/L treatment, six of seven (86%) in the 1720 µg/L group and four of the eight males (50%) in the 3120 µg/L treatment had testis-ova. This effect is indicating an oestrogen agonist mode of action as summarised in Table 11 and fits well to the observed intersex gonads occurring at 1820 µg /L BPA reported by (Yokota et al., 2000) (no phenotypic males at this concentration observable).

O. latipes exhibits genetic sex determination, which occurs between day 5 and 7 post fertilization (dpf). Bhandari et al. (2015) conducted a test with the Hd-rR strain of medaka (*Oryzias latipes*) maintained in the USGS Columbia Environmental Research Center (CERC) exposing them from 8 hpf to 7 dpf. Fertilised eggs (F0) were collected from 6 to 10 breeding pairs, pooled, sorted, and assigned to 9 glass petri dishes. Each petri dish contained 50 fertilised eggs in 50 mL well water and each treatment group (only 1 test concentration for BPA) had three biological replicates. For the treatment groups, approximately 8 hpf (late blastula stage), well water was replaced by water containing the test chemical (83.7 µg BPA/L or 0.061 µg EE2/L – measured concentrations). The embryos were exposed for 7 days, which is the critical window of medaka sex determination. The control water and test chemical solutions were replaced daily until hatch. There were no further chemical exposures for these fish (F0) or any subsequent generations (F1, F2, F3, or F4). Newly hatched fry at 8–10 dpf were transferred to floating netted trays that were placed in 10 L aquaria with flowing water and aeration. At 30 dpf, all juveniles were transferred to aquaria and maintained. Culture of the three treatment lineages (control, EE2, and BPA) followed through production of F4 eggs and embryos. The water temperature was 26 ± 0.5 °C. All medaka in the test became sexually mature and spawned in an average of 120 dpf. A total of 6 adult pairs from the same treatment group were randomly chosen and mated to produce offspring for each subsequent generation. Fecundity (number of eggs per breeding pair), fertilization rate (fertilized eggs from total egg per day), survival rate (mean number of embryos survived in the pool of fertilized embryos before hatching), phenotypic abnormalities (examined microscopically) and gonadal changes (examined histologically) were examined. The exposure of medaka to 83.7 µg/L BPA did not affected the fertilisation rate of the eggs produced by F0 and F1 generation or the survival rate of the embryos of the F0, F1, and F2 generation. Significant reduced fertilization rates in the F2 and F3 generation and reduced embryo survival in the F3 and F4 generation occurred at 83.7 µg BPA/L in comparison with the control. This study provides evidence for transgenerational reproductive abnormalities in medaka caused by developmental exposure to BPA. However, the study on its own does not demonstrate whether these effects are clearly ED mediated for later generations.

Results observed in other tests substantiate an oestrogenic mode of action of Bisphenol A in Medaka:

- Induction of Vitellogenin, a clear indicator of oestrogenic activity, was observed in all tests measuring this endpoint (1 FLC, 1 partial life cycle tests, 5 screening tests with adults). Vitellogenin induction started at concentrations at which no mortality or effects on growth were observed in the tests, indicating that the induction was not a secondary side effect. Induction started between 470 µg/L (partial life cycle test) and 1179 µg/L if eggs were exposed (Japanese Ministry for the Environment, 2006) and thus at similar concentrations at which testis ova and changes in gonadal staging were observed (see below) and reached VTG concentrations similar to those observed in control females. In adults VTG induction started at 10 – 8000 µg/L (Kang et al., 2002; Metcalfe et al., 2001; Tabata et al., 2001; Yamaguchi et al., 2005).

- Similar to the results observed by (Yokota et al., 2000) at 1820 µg/L, testis-ova were observed in a full life cycle test and a partial life cycle test performed by the

Japanese Ministry of the Environment (2006, only abstract available) at 1179 µg/L and 890 µg/L and above respectively. Even after exposure of adults for 21 d testis-ova was observed at 837 µg/L and above by (Kang et al., 2002) (Klimisch 2).

- In addition, effects on gonadal staging were observed in two partial life cycle tests (Metcalfe et al., 2001, Klimisch 2) and (Na et al., 2002, Klimisch 2), which are considered diagnostic for endocrine activity by OECD (2010a). Histological evaluations revealed a decreased number of late spermatogenetic stages (starting at nominal 50 and 100 µg/L respectively) and an increased number of late oocytic stages (starting at nominal 200 and 100 µg/L respectively).

With regard to apical population relevant endpoints, the complete absence of phenotypic male fish at 1820 µg/L observed by Yokota et al. (2000) was among the most sensitive endpoints. Effects on growth were observed by the same author at the same concentration. Effects on survival, which again might or might not be endocrine mediated, were observed in a study by the Japanese Ministry of the environment at a slightly lower concentration (LOEC 1185 µg/L) in the fish full life cycle tests while no such effects were observed at this concentration in their partial life cycle test and in other tests (Yokota et al., 2000).

In summary, effects observed by Yokota et al. (2000) clearly show that Bisphenol A induces oestrogen mediated adverse effects in Medaka at 1820 µg/L: no male Medaka were observed at this concentration and the effect fits to the observed testis-ova at the same concentration. The effect of the absence of male fish at 1820 µg/L in Yokota et al. (2000) is strongly supported by the histopathological findings in Metcalfe et al. (2001) and Na et al. (2002). Metcalfe et al. (2001) and Na et al. (2002) found no effects on sex ratio as the test concentrations were significantly lower than in Yokota et al. (2000). Other studies conducted with *O. latipes* reported no effects on sex ratio. That could be because organisms were not exposed during their sensitive window of development but as adults, or the exposure duration was too short (Shioda and Wakabayashi 2000, Kang et al. 2002, Kashiwada et al. 2002, Tabata et al. 2001, Yamaguchi et al. 2005).

Indication is given for an asynchronous development of males and females: The sexual development for female fish was accelerated and for male fish was delayed under exposure of between 29.8 µg/L and 119.2 µg/L (measured) Bisphenol A in comparison with the controls (Metcalfe et al. 2001 and Na et al. 2002).

All other tests substantiate the oestrogen mode of action as Vitellogenin induction, testis-ova and changes in gonadal staging were observed at 5.9 and 470 µg/L (measured). VTG is among the most sensitive adverse endpoints for Medaka with only reduced growth in the same study and embryo mortality in one study occurring at the same concentration while no such effects were observed in other tests at these concentrations.

In addition, results observed by Shioda and Wakabayashi (2000), although of low reliability, indicate that short-term exposure to Bisphenol A might result in long-term effects. Exposure of groups of one adult and two females (at least 3 replicates, but appears to vary between test concentrations) for 2 weeks resulted in a decrease of number of eggs and a reduced hatchability at 2300 µg/L, if those previously exposed males were mated with unexposed females.

Sun et al. (2014) measured the transcriptional response of a set of genes associated with the hypothalamic–pituitary–gonadal (HPG) axis following BPA exposure during the early life stage of *Oryzias latipes*. Eggs spawned from stock females were collected within 4 h after fertilisation. Embryos were exposed to a control and 6, 20, 60, 200, and 600 µg /L BPA. Each treatment group contained 100 embryos divided into five replicates. Once hatched, the larvae were transferred to another beaker and exposed to either control or BPA-containing water until 44 days post fertilisation. The main recovery of BPA was 87.1 to 104.4% of the nominal values. The BPA exposure resulted in ***significantly reduced hatchability, total survival rate and in an increased body length and weight in female fish*** in treatment groups containing 200 and 600 µg /L BPA (NOEC of 60 µg/L). ***VTG1 mRNA level was significantly elevated*** in females (16.6 fold) and males (6.3 fold) exposed to 600 µg /L BPA. Similarly, ***VTG2 mRNA was increased*** in females and

males. For ***male*** larvae, the ***cytochrome P450 genes (CYP11A, 11B, 17A, 17B, 19A, 19B) showed a decreasing trend*** in a BPA concentration-dependent manner (significant from 20 µg /L BPA on). ***Transcription of ARα*** in females was ***decreased*** to the control level in the high exposure group, although a significant increase was found at 20 µg/L. ***In males***, BPA exposure significantly decreased the transcription of ARα at the high concentrations (200 and 600 µg/L). These changes in transcription show that BPA also acts as an androgen receptor antagonist in addition to an oestrogen receptor agonist.

In summary, Vitellogenin induction was observed in all tests measuring this endpoint. At similar concentrations, testis-ova and changes in gonadal staging were observed. A clear diagnostic adverse apical effect (sex ratios were skewed towards females) is shown after Bisphenol A exposure. Additionally, fertility success was reduced. The effects on testis-ova, skewed sex ratio towards females (with no males at all) and suppressed growth represent a direct plausible biological link that is characteristic of an oestrogen MoA and could all together be observed in the key study (Yokota et al., 2000). Hence, the studies described above provide clear evidence that Bisphenol A exposure leads to adverse effects in fish and that there is a biologically plausible link to an endocrine mode of action underlying these adverse effects.

5.5.2.4 Effects on Zebrafish (Danio rerio) related to an oestrogen/androgen MoA

With respect to *Danio rerio*, a Fish Full Life Cycle test, a two generations test and two (modified) screening assays are available (see Table 13). The FFLC tests and one Screening assay include endocrine specific biomarkers as well as apical endpoints.

Segner et al. (2003a) (key study for this species) (reliability 2) conducted a Fish Full Life Cycle test (reliability 2). In this study, 100 fertilised eggs of zebrafish per test vessel were exposed to 94 – 1500 µg/L BPA with an analytical confirmation of the exposure concentration in two replicates. After the first 42 days of treatment, the number of fish per tank was equated to 50 animals per vessel. At days 75 to 78, the number of fish was reduced again to 30 fish per vessel. The influence on apical endpoints such as fertilisation, time to first spawn and the growth of offspring were observed. The total number of eggs per aquarium was divided by the number of females in the respective tank to obtain the number of eggs per female and day. The number of females was determined histologically after termination of the study. The NOEC for ***Vitellogenin (VTG) induction*** was 188 µg/L (with a LOEC of 375 µg/L nominal). At the same concentration, ***changes in gonad histology (increased number of testis-ova)*** were reported. The LOEC for juvenile growth, time to spawning, mating behaviour, eggs per female and fertilisation success was 1500 µg/L (nominal concentration). No mortality occurred. The VTG induction in males which was observed in the Segner et al. (2003a) study indicates an oestrogen agonistic mediated activity. In addition, the histopathological finding of testis-ova is relevant for oestrogen agonistic mode of action.

Three other studies support the conclusion that Bisphenol A has an oestrogen agonistic mode of action for *Danio rerio* (Keiter et al., 2012; Van den Belt et al., 2003; Villeneuve et al., 2012), all showing ***VTG induction in males*** at lower or the same concentrations as Segner et al (2003a).

Keiter et al. (2012) investigated in a two-generation-test (reliability 2) the effects of Bisphenol A, PFOS and a mixture of both on VTG induction, fecundity and growth of *D. rerio*. In the study two replicates, each containing of 80 eggs 2-4 hpf, were exposed to 10, 200 and 400 µg/L BPA. At 30 dpf and 90 dpf the number of fish in each replicate test vessel was reduced by 35 and 25 individuals for the measurements of length, weight and VTG. At 90 dpf for each replicate test vessel a total of 10 males and 10 females were retained for reproduction experiments and breeding for the F_2 and F_3 generations. For the F_3-generation post-hatching survival was documented 14 dpf. The exposure with Bisphenol A resulted in a VTG induction in the F_1-generation (90 dpf) starting at nominal 400 µg/L, which was the

highest test concentration. In contrast, the VTG induction in the F_2-generation (180 dpf) started at the lowest concentration tested (10 µg/L nominal) and was significant for all concentrations tested. The exposure to Bisphenol A did not result in effects on growth in the F_1-generation but at 90 dpf all BPA treatment groups showed reduced length and weight in both males and females compared to the controls. For the adult males and females at 180 dpf the growth was significantly lower for the highest test concentration (400 µg/L nominal). As described in (IPCS, 2002; OECD, 2004, 2011) the growth of offspring is an endpoint considered to be sensitive to an oestrogen mode of action *in vivo*. The (Keiter et al., 2012) study shows for different endpoints (VTG induction and growth) that the sensitivity of the fish increases from one generation to the other.

Van den Belt et al. (2003) started their experiment with adult fish (reliability 2). Therefore, the concentration showing first effects in VTG induction was very high (nominal 1000 µg/L). The difference between this (highest) test concentration and the second highest one (nominal 200 µg/L) was very large, so the measured LOEC for VTG induction lies between them.

In contrast the result of a study conducted by Villeneuve et al. (2012) also investigating the effects of Bisphenol A exposure on (sexually mature) adult fish was a Vitellogenin-induction at a LOEC of 10 µg/L (nominal) (reliability 2).

Chen et al. (2015) investigated the effects of BPA exposure on *Danio rerio* (wild type AB strain) in a non-GLP two-generation study. It was a limit-test with a test concentration of 0.228 µg/L BPA (1 nM) (mean measured: 0.372 µg/L) and a solvent control (0.01% DMSO) (mean measured BPA: 0.032 µg/L) (reliability 2). The exposure concentration was analytically confirmed by HPLC analysis in fresh but not in expired solutions (personal communication with the author Jiangfei Chen, November 2017: equal to (Chen et al., 2017)). The test was conducted semi-static at 28°C (in the publication; personal communication of the registrant with the author: 22-25 ℃) with 14 hours light per day as recommended in OECD Test guideline 234. The oxygen supply was obtained by using an air pump system. The precise oxygen saturation was not measured. Zebrafish embryos were obtained from spawning adults in tanks overnight with a sex ratio of 1:1. Embryos were collected within 0.5 h of spawning and rinsed in an embryo medium. They were used to start the first generation (F1) for the solvent control and BPA-exposure. Adult F1 fish (150 d) within the same treatment group were mated (4 females x 4 males/replicate; 3 replicates) to produce F2 embryos. Mating was conducted in clean water. The F2 embryos obtained from the F1 fish exposed to BPA were exposed at 8 hpf to BPA again (B2 – exposed for two generations), or not exposed (solvent only – exposed for one generation) (B1). There was also a group without exposure to BPA at all (B0). According to (Chen et al., 2015) the whole experiment was repeated three times each starting with a new batch of embryos (with a minimum of 90 embryos per replicate). The sex ratio was checked visiually based on the morphological difference of the male and female zebrafish by an observer blind to the treatment. This was confirmed histologically on some fishes. Most were checked visually. There were no significant differences between the visually and histologically checked sex of fish (personal communication December 2017). The embryos were exposed from 8hpf in a petri dish. After 8-72 hpf the water once was changed. 5 dpf the fish was moved to a 2 L tank. From 5 to 150 dpf the water was changed every 5 days. At day 21 post fertilisation the fish was moved to 9 L tanks. 90 eggs were used from each replicate to evaluate the embryo development. Chen et al. (2015) used 30 eggs per replicate. Four replicates were used for the first generation and three for the second generation. The sex ratio of control fishes was 57% females (OECD 234: 30 to 70% males or females). The statistical analysis was performed using an ANOVA followed by Tukey's multiple comparison test. For gene expression test, an unpaired *t*-test with 5% FDR was performed. The control mortality was 2 to 16%, the control fish body weight was 280 mg (mean) and the control length was 18 mm (mean). This is well in line with OECD 234 (<25%). Chronic exposure to 1 nM BPA for only F1 generation or both F1 and F2 generation had no effect on adult fish survival nor were there any obvious malformations in parental F1 and F2 fish. The exposure resulted in a significantly ***altered sex ratio of the F1 and F2 population*** with ***more female*** in both F1 and F2 adults. BPA exposure also significantly

reduced sperm counts and quality of F1 and F2 males (reduced sperm density, sperm motility, sperm ATP production, and significant increase in sperm lipid peroxidation). Despite the observation that BPA exposure reduced sperm density and quality, they found ***no evidence of histological lesions in testes*** of exposed fish. There were no significant differences in egg production and fertilisation of F1 and F2 females or adverse effects of embryo hatching or survival in offspring from F1 parents. The results fit well to the fact that sperm cells that successfully fertilized eggs are more likely to be normal in motility because only one sperm is required to fertilize one egg. Also with less motile sperm there is still enough to fertilise a typical spawn of eggs from the females. Paternal BPA exposure had a ***significant adverse effect on malformation*** (e.g., uninflated swim bladder, pericardial oedema and bent body) ***and mortality at 8 dpf.*** For example, % malformation in larvae derived from females paired with males from B0 was in a range of 7 ~ 17%, which was increased to ~30% in those paired with males from B1 and ~46% in those paired with males from B2. Similarly, mortality increased from 2% to 18% in larvae derived from females paired with males from B0 to ~24% and ~44% in those paired with males from B1 and B2, respectively. It is ***possible*** that that this ***malformation*** and ***higher mortality results from an effect of BPA on sperm DNA.*** Chen et al. (2015) found reduced expression of *dnmt1, dnmt3, dnmt5 and sp3* in 5 d old larvae which may contribute to the paternal-specific reproduction failure. This is ***similar*** to the result from a study with ***rats*** (Doshi et al., 2012). Chen et al. (2015) state that further studies are needed. In Chen et al. (2017) further effects of BPA exposure (0.228, 2.28, and 22.8 µg/L) during different developmental stages (embryonic, larval, sexual mature) were examined. Exposure to 0.228 µg/L BPA during embryonic development increased malformations and mortality of offspring while egg production and fertilisation were reduced in higher concentrations (22.8 µg/L). Additionally, sperm quality (density, velocity, motility) and testis weight were decreased in F0 after embryonic exposure to 0.228 µg/L BPA.

In summary, in all studies conducted with *Danio rerio* VTG-induction was observed. The span in which the effects appeared was very broad, starting at 10 µg/L and ending at 1000 µg/L for the adult fish exposed. As stated above in chapter 5.5 VTG-induction in male fish is indicating an oestrogen agonist mode of action. To this conclusion leads also the appearance of testis-ova in the Fish Full Life Cycle test. Additionally, the change of the length and weight of the offspring, seen at a very low concentration of 10 µg/L in the two-generation test, is considered to be sensitive to an oestrogen mode of action *in vivo*. The female biased sex ratio is also indicative for an oestrogen receptor agonist and considered as a diagnostic adverse effect. Chen et al. (2015) reports this effect on sex ratio even at 0. 372 µg/L. No effects on sex ratio were observed in the two other multi-generation studies (Segner et al., 2003a, Keiter et al., 2012), which were performed at higher concentrations. It is not clear why. The key study (Segner et al., 2003a) provides a direct plausible biological link showing VTG induction in males, testis-ova and reduced fertilisation success but no effects on survival of fish.

Hence, for Bisphenol A on *Danio rerio*, there is evidence for an endocrine disrupting effect of BPA that fits to the oestrogen agonistic endocrine mode of action.

5.5.2.5 Effects on Fathead minnow (Pimephales promelas) related to an oestrogen/androgen MoA

For this fish species a multigenerational study (conducted in 2 parts and published in different publications), a partial life cycle test and a biomarker study exist.

The first part of the multigenerational study is the study from (Sumpter et al. 2001) (key study for this species), which is partly published as Sohoni et al. (2001) and Staples et al. (2011) (reliability 2). It examined effects of Bisphenol A in concentrations of 1, 16, 160, 640 or 1280 µg/L on the 122 d old adult F_0-generation and the F_1- and the F_2-generation

in a flow-through system. The study was started with 60 adult fish per treatment level. At day 42, eight breeding pairs per treatment were randomly selected and used to assess the fecundity of the F_0-generation. A subset of fertilised eggs were continually exposed and used for early life stage tests. The test of the F_2-generation was initiated using sexually mature (150 dph) F_1-adults obtained from the F_1 early life stage test. Eight breeding pairs per treatment were exposed to BPA. F_2-eggs obtained from a single female from the F_1 breeding trials were used to initiate two hatching trials per breeding pair. With the F_2-eggs two ELS tests were conducted. On day 444 all exposures ended. The ***Vitellogenin induction in males*** occurred in the ***F_0-generation*** at 160 µg/L. The next lower concentration was 10-fold lower, so it would be possible, that also in a concentration between these two an effect would occur (as observed in Rhodes et al. 2008 published in Mihaich et al., 2012). For the ***F_1-generation VTG-induction*** was also observed at a concentration of 160 µg/L but not only in ***males*** but also in ***females***. The ***size of the gonads of female fish of the F_0-generation*** was significantly greater than in the controls at 1µg/L on day 43. This changed during the course of the test and rose to ≥ 1280 µg/L and 640 µg/L (day 164). Effects on the different ***stages of male spermatozoa development*** were also observed at lower concentrations. In the F_0- and the F_1-generation the LOEC for the proportion of spermatogonia and spermatozoa lay already at the lowest test concentration of 1 µg/L. As described in European Commission (2010) it was agreed on that the study was designed to look for effects on reproduction, hatching and growth. So the experimental design was not optimised to look at effects on sperm cell types. Therefore, the effects on spermatogenesis were not used for PNEC derivation in the EU RAR. The ***hatchability of eggs*** of the ***F_1-generation*** was affected at 640 µg/L. The ***F_2-generation*** was ***more sensitive*** and showed effects at 160 µg/L. As the total number of eggs spawned by individual females varied in the controls, differing by more than 10-fold over the study period, the LOEC for egg production in the F_0- and F_1-generation was at higher test concentrations 1280 and 640 µg/L respectively, anyway showing that the fish become more sensitive with subsequent generations. Not only the Vitellogenin induction in males but also the increased proportion of spermatogonia indicates an oestrogen agonist mode of action of Bisphenol A in *Pimephales promelas*. For a couple of endpoints examined, this study provides evidence that the effects become stronger in the subsequent generations.

A study with similar duration and Bisphenol A concentrations to those in the F_0- and F_1-parts of the Sumpter et al. (2001)-study was a partial life cycle test conducted by Rhodes et al. (2008) published in Mihaich et al. (2012) (reliability 4 and 1 respectively). The measured Bisphenol A-concentrations were 1.19, 13.4, 52.8, 130 and 567 µg/L, which were 81 to 89% of the nominal (1.0, 16, 64, 160 and 640 µg/L). The methodology was similar to Sumpter et al. (2001) with some minor changes. The ***levels of Vitellogenin were significantly elevated*** compared to the control fish at concentrations of 64 µg/L and above ***in both males and females***. This concentration was not tested in Sumpter et al. (2001) but fits very well to the results of this study. There were ***histopathological lesions*** observed in ***male*** fish at 160 µg/L, which increased at 640 µg/L. At 640 µg/L this effect was also seen in females. A significant effect of Bisphenol A on the ***development of sex cell types in male fish*** was observed at 160 µg/L. In ***female*** fish, a ***shift to less mature cell types in the ovaries*** was observed at 640 µg/L.

The study conducted by Villeneuve et al. (2012) (reliability 2) supports the conclusion by reporting ***Vitellogenin-induction in male*** at lower concentrations than Sohoni et al. (2001) (LOEC = 100 µg/L; NOEC = 10 µg/L).

Ekman et al. (2012) exposed sexually mature (5-6 month old) fathead minnows to Bisphenol A either alone or in a binary mixture with 17ß-trenbolone, a strong androgen receptor (AR) agonist (reliability 2). They used a metabolomic approach. Female fish was exposed to BPA at two different nominal test concentrations: 10 µg/L and 100 µg/L or they were co-exposed with 500 ng/L 17ß-trenbolone. Ekman et al. (2012) reported a dose-dependent response in the hepatic metabolite profiles from the two Bisphenol A exposures with a larger impact by 100 µg/L Bisphenol A relative to the control and 10 µg/L Bisphenol A. There was greater similarity between the high Bisphenol A concentrations together with

17ß-trenbolone and the control than between the low BPA concentration and the control. The presence of the high BPA concentration in the co-exposition mixture with 17ß-trenbolone seems to mitigate the effects of 17ß-trenbolone on the hepatic metabolome in female fathead minnows, thus providing physiological evidence for androgen receptor antagonism.

Labadie and Budzinski (2006) investigated the alteration of the steroid hormone balance in juvenile turbot (*Psetta maxima*). Bisphenol A and p-nonylphenol exhibited the highest potency towards steroids dynamics, lowering the ratio of androgens to oestrogens in all three studied matrices. However, these two chemicals had different modes of action, because p-nonylphenol induced a decrease of androstenedione and 11-ketotestosterone levels, whereas BPA exposure led to an elevation of oestrone level (see also Peterson et al. 2015). Overall, these two chemicals seemingly disrupted the activity of some steroidogenesis enzymes, leading to serious hormonal imbalance in juvenile turbot.

In summary, there is strong evidence for endocrine disrupting activity of Bisphenol A in *Pimephales promelas.* The tests show the induction of Vitellogenin and increased proportion of spermatogonia (delay in male sex development).

The key study (Sumpter et al., 2001) provides a direct plausible biological link between biomarkers, histology and diagnostic apical effects showing effects on VTG production and a reduced egg production in the F0- and F1-generation with increasing sensitivity in subsequent generations and effects on growth.

5.5.2.6 Effects on Brown trout (Salmo trutta f. fario), Goldfish (Carassius auratus) and Rainbow trout (Oncorhynchus mykiss) related to an oestrogen/androgen MoA

Supporting the provided information above, for three other oviparous fish species effects of Bisphenol A were reported.

For *Salmo trutta f. fario* Lahnsteiner et al. (2005) described a 103 d-study starting with male and female fish during late pre-spawning and spawning period. The approximately three years old wild caught brown trout were exposed via a flow-through test system to 1.75, 2.40 and 5.00 µg BPA/L (one tank per test concentration). DMSO was used with concentrations following OECD recommendations. There is no indication for a chemical analysis but due to the flow-through system and DMSO use, no loss of BPA is expected. Lahnsteiner et al. (2005) also observed effects on ***egg production and semen fertility*** (LOEC= 5 µg/L; NOEC 2.4 µg/L) (reliability 2). At the highest exposure concentration (5.00 µg/L) no females gave eggs and the semen fertility was 28%. They observed also a delay in the time point of ovulation (LOEC = 1.75 or 2.4 µg/L) but with only 6 fishes for this endpoint no significance can be proven. Some issues (uncertainties about acclimatisation of test animals, appropriateness of statistics, possible influence of environmental factors and lack of tank replication) were raised in the transitional report (ECHA, 2009), so it was not used for PNEC derivation.

Hatef et al. (2012a) reported also effects on ***sperm quality*** (motility and velocity) in the male goldfish (*Carassius auratus* L.). They conducted a 30d-reproduction test. 2-3 years old male mature goldfish were exposed to 0.6, 4.5 and 11.0 µg BPA/L (initially measured concentrations). At 0.6 µg/L, which was the lowest concentration tested, the sperm motility was significantly decreased. In this test also the decrease of androgens and the increase of Vitellogenin were observed. These is evidence for anti-androgenic and oestrogenic modes of action of Bisphenol A. In summary, the observed apical endpoints semen production and development of ovaries are relevant for the viability of the population. According to Knacker et al. (2010) and OECD (2012b) effects observed on growth and development are known to be oestrogen sensitive (growth and abnormal development). Results of this test show that adverse effects which are considered endocrine sensitive start at 0.6 and 1.75 µg/L (LOEC).

Hatef et al. (2012b) exposed male goldfish (*Carassius auratus*) to 0.2 and 20 µg /L BPA

for up to 90 days. They also investigated the underlying molecular mechanism in measuring transcriptions of various reproductive genes in brain, liver, and testis. Volume, density, total number, motility, and velocity of sperm were measured to assess testicular function. At 0.2 µg/L, BPA reduced steroidogenetic acute regulatory protein and increased oestrogen receptors (ERs) messenger RNA (mRNA) transcript (ERβ1 in liver and ERβ2 in testis) after 90 d. At 20 µg/L, ***BPA increased mRNA transcript of androgen receptor in testis, brain- and testis-specific aromatase, and vitellogenin*** in liver after 90, 30, 60, and 60 d, respectively. Transcripts of ERs mRNA were increased after 30 to 60 d at 20 µg/L BPA; increase in ERβ1 mRNA was observed in testis after 7 d. Total number, volume, and motility of sperm were decreased in males exposed to 0.2 and 20 µg/L BPA, whereas sperm density and velocity were only reduced at 20 µg/L BPA. The results support the hypothesis that BPA may exert both anti-androgenic and oestrogenic effects, depending on concentration, leading to diminished sperm quality.

Overall, the results give clear indications for an oestrogen agonistic and androgen antagonistic endocrine mediated mode of action of Bisphenol A and subsequent adverse effects.

According to (OECD, 2012a) reduced semen quality may be a result of sertoli cell dysfunction which results from ER activation and AR suppression (see also Figure 3). This shows a logical connection between the observed reduced sperm quality and semen fertility in Lahnsteiner et al. (2005) and Hatef et al. (2012a).

Figure 3: Proposed cascade of events leading to testicular dysgenesis syndrome (OECD, 2012a)

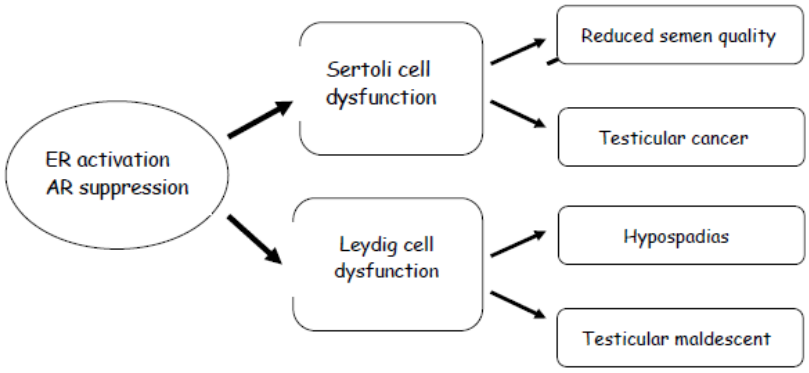


Figure 1-5. Proposed cascade of events leading to testicular dysgenesis syndrome.[12]

The other oviparous fish species tested is *Oncorhynchus mykiss*. For this species an OECD 215-test is available (Bayer AG 1999b) as well as a test with juvenile fish over 12 days (Lindholst et al., 2000). The test duration of the OECD 215-test is not adequate to reveal effects related to the mode of action. Here a NOEC for growth rate of 3640 µg/L is reported. Lindholst et al. (2000) describes an ***induction of plasma Vitellogenin*** at concentrations higher than 40 µg/L BPA. In this test also E2 (1 µg /L) was tested as the positive control which also increased the Vitellogenin level. This also hints to an oestrogenic mode of action of Bisphenol A. Unfortunately no apical endpoints were investigated.

In summary, for brown trout and goldfish there are hints from the tests described above, that Bisphenol A shows endocrine disrupting activity, as these tests show that Bisphenol A interferes with testicular function and reduces the sperm quality. It is recognised that issues were raised for one of these tests in the EU transition report, so this study has received lower weight in this assessment.

For rainbow trout, the VTG induction in males is an endpoint indicating an oestrogen agonistic mode of action. The growth rate of the offspring is considered sensitive also to an oestrogen mode of action.

5.5.2.7 Effects on viviparous fish species related to an oestrogen/androgen MoA

Also to support the results on effects on the above mentioned fish species, information on tests for two viviparous fish species of the family Poeciliidae are provided here (*Poecilia reticulate* and *Xiphophorus helleri*). There is one assay for *P. reticulate* (adult males) and one for *X. helleri* (juvenile growth test – Kwak et al. (2001), which include endocrine biomarkers as well as some apical endpoints. The VTG mRNA level was increased by BPA in a dose-dependent manner starting at 2 ppm. In a long-term test over 60 days (Kwak et al., 2001) exposed juvenile (30 day old) *Xiphophorus hellerie.* Normally male swordtails show a long fin – the "sword" – as secondary sexual characteristic. The ***sword growth was inhibited*** in a concentration-dependent manner beginning at 0.2 µg/L. At 20 µg/L almost no sword growth was seen. The significance of the changes in sword length is not understood, but it is thought that the length of the sword has an influence on mating success, with female fish preferring males with longer swords. There are indications for an endocrine effect but it is not clear what degree of change should be considered to be significant and therefore cannot be used as a definite proof (reliability 2, because of nominal concentrations. According to Benson and Basolo (2006) longer-sworded males experience greater competitive success. They confirmed that there is a relation between body size and competitive success for green swordtails, which means that larger males won more often than smaller males. However, small differences in body size were overlayed by the size of the sword lengths. This means that the sword length is most important in contests when males are closely matched for size. The relation between contest success and mating success has not been well explored, but in several systems, a link has been found (Andersson, 1994). In summary, increased Vitellogenin level as well as changes in secondary sex-characteristics clearly indicates an endocrine mode of action in *X. helleri*.

Kinnberg and Toft (2003) exposed sexual mature adult male *Poecilia reticulate* for 30 d (reliability 2). In the highest test concentration of nominal 5000 µg/L ***testis filled with spermatozeugmata and other changes in gonad histology*** occurred. As well, the LOEC for ***survival*** was 5000 µg/L as at this concentration 77% of the fish died (with testis filled with spermatozeugmata).

Another test with mature adult male *Poecilia reticulate* conducted by Haubruge et al. (2000) described a 21d-exposure with 274 and 549 µg /L BPA. Five treatment groups (control, 2 BPA concentrations, 11.2 and 22.4 ng /L TBT) were replicated three times each with five individuals in each replicate. Acetone was used as solvent. Body weight, number of spermatozeugmata and length of sperm per male were counted and measured. The exposure of the guppies with Bisphenol A as well as TBT resulted in a ***significant decline in sperm count*** in adult males by 40 to 75 %. This shows that exposure to 274 µg /L BPA disrupts male spermatogenesis. The result suggests that male fishes exposed to these concentrations of BPA may be reproductively compromised. As there was a decline in the number of active spermatogonia after only three weeks of exposure, it suggests disruption in the production process rather than a decline in the number of active spermatogonia generated from the germ line. This result is similar to the effect shown in Sumpter et al. (2001) for *Pimephales promelas* with ***reduced numbers of mature spermatogonia*** at 16 µg BPA/L.

In summary, for the viviparous fish species Swordtail and Guppy there exists clear evidence for an endocrine mode of action of Bisphenol A. The Vitellogenin level was elevated in male Swordtail fish and also histological changes were observed for Swordtail fish and for Guppy.

Table 13: Summary of oestrogenic or anti-androgenic effects of Bisphenol A in vivo in different fish species

Species	Life stage/ duration	Conc. [µg/L] / test condition	VTG	Histology	Fertility/ Fecundity	Sex ratio	Sec. sex characteristics	others	Positive control	Reference	Reliability
Cyprinus carpio Supporting study	Partial ELS Males during sexual differentiation (45-55 dph)/ 49 d	10 – 32 – 100 – 320 – 1000 µg/L (nominal)/ flow-through/ fish genetically altered to be an all-male population		LOEC (oviduct formation) = 32 µg/L				NOEC (growth) = 100 µg/L		(Bowmer and Gimeno 2001)	2 - only an extended abstract available Also used in (EC, 2010) for risk assessment
Cyprinus carpio Supporting study	1-year old adults/ OECD 204/ 14 d	1 – 10 – 100 – 1000 µg/L (nominal)/ flow-through	LOEC (males) = 10 µg/L	LOEC(gonadal and testis structure changes) = 1 µg/L						(Mandich et al., 2007)	1 – guideline study
Gabiocypris rarus Supporting study	5-month old male/ 36 d	225 µg/L (nominal) = 172 to 210 µg/L (measured)		LOEC (apoptosis in testis) = 225 µg/L (most apoptotic cells = primary and secondary sper-matocytes)				No effect on growth Decrease of genes ccna1 and birc5 → BPA may induce meiosis arrest		(Zhang et al., 2016a)	2
Gabiocypris rarus Supporting study	6-month old female/ 7d	1 – 15 - 225 µg/L (nominal) = 0.88 – 13.1 – 193 µg/L (measured)	Elevated at 1 and 15 µg/L but not at 225 µg/L	Ovarian weight increased at 1 (not sign) and 15 µg/L (sign.) but not 225 µg/L				Testosterone and 17β-estradiol elevated at 1 and 15 µg/L but not 225 µg/L		(Zhang et al., 2016b)	2

Species	Life stage/ duration	Conc. [µg/L] / test condition	VTG	Histology	Fertility/ Fecundity	Sex ratio	Sec. sex characteristics	others	Positive control	Reference	Reliability
Oryzias latipes Key study	FSDT extended ELS: fertilised eggs/ 60 d post hatch (~ 71 d)	2.28 - 13.0 - 71.2 - 355 - 1820 (measured) / semi-static and flow-through; equivalent or similar to OECD 210	-	6 of 19 fish (phenotypic females) with testis-ova at 1820 µg/L (32%)	-	LOEC (sex ratio skewed towards female) = 1820 µg/L (No phenotypic males and no fish with testis)	no males at 1820 µg/L (highest concentration), no significantly reduced number of males at 355 µg/L (but 25% effect already)	NOEC= 355 µg/L LOEC (total length ; weight) 1820 µg/L Hatchability, time to hatch, and embryo mortality were not affected	no	(Yokota et al., 2000)	2, some information on water quality missing, no. of eggs 60 instead of 120 compared to OECD 234
Oryzias latipes Supporting study	FFLC (fertilization/ 60 d post-hatch F1)	2 - 9.3 - 49.7 - 247 - 1179 (measured) / 20 fish per group	LOEC (males F1) = 1179 µg/L	LOEC (testis-ova F1) = 1179 µg/L No testis ova up to1179 µg/L in F0				No sign. dose-related effects on hatchability, time to hatch, body weight and length (F0 and F1) and mortality (F1) LOEC(mortality F0) = 1179 µg/L		Japanese Ministry of the Environment 2006	4 - only short abstract available; 2 - valid
Oryzias latipes Supporting study	Partial life –cycle test (fertilized eggs/ 60d post-hatch)	220-470-890-2120-4410 (measured) / 20 fish per group	LOEC (males) = 470 µg/L (at 890 and above comparable to control female level)	LOEC(testis-ova)= 890 µg/L (4/10), (3/4 and 5/6 males had testis-ova at 2120 and 4410 µg/L)				No effects on hatchability, mortality LOEC time to hatch 4410 µg/L Some – no dose-dependent sign. effects on body length and body weight (length at 470 and 890 µg/l sign. higher but not at higher concentrations, body weight at 220, 470		Japanese Ministry of the Environment 2006	4 - only short abstract available; 2 - valid

Species	Life stage/ duration	Conc. [µg/L] / test condition	VTG	Histology	Fertility/ Fecundity	Sex ratio	Sec. sex characteristics	others	Positive control	Reference	Reliability
								(increase) and 4410 µg/L (decrease))			
Oryzias latipes Supporting study	FSDT with deviations Fry 1 d ph/100 d	10 - 50 - 100 - 200 (nominal) WR 59.6%: 5.9 – 29.8 – 59.6 – 119.2 µg/L (measured) / semi-static		2 male fish out of 25 with testis-ova at 5.9 µg/L (measured) (nominal 10 µg/L) but not dose dependent Decreased number of spermatozoa in male fish, testicular fibrosis at 29.8 µg/L (nominal: 50 µg/L) and above Increased gonadal staging oocytes at 119,2 µg/L (nominal: 200 µg/L)		No change in sex-ratio		No significant effects on length and weight LOEC (condition factor – weight divided by total length)= 59.6 µg/L (measured) (nominal 10 µg/L)	E2: At 0.01 µg/L and above testis-ova LOEC sex-ratio 0.1 µg/l LOEC weight 0.0 µg/L EE2: At 0.1 µg/L testis-ova, LOEC sex-ratio 0.1 µg/L LOEC length 1 µg/L	(Metcalfe et al., 2001)	2 - acceptable, well documented publication
Oryzias latipes Supporting study	FSDT with deviations newly hatched larvae/ 70 d	50 - 100 - 200 (nom)/ semi-static		Effects on females at 100 and 200 µg/L (Greater proportions of the later stages of oocytes including		No change in sex-ratio. At 100 and 200 µg/L 3 of 50 fish undifferentiated		(LOEC (increased length, body weight) = 200 µg/L)	-	(Na et al., 2002)	2 - test organisms taken from wild, no analytic

Species	Life stage/ duration	Conc. [µg/L] / test condition	VTG	Histology	Fertility/ Fecundity	Sex ratio	Sec. sex characteristics	others	Positive control	Reference	Reliability
				mature eggs which were not present in the controls) Effects on males at 100 and 200 µg/L (reduced proportion of later stages of spermatogenesis in males, no mature spermatids)		based on histology (2 in controls)					
Oryzias latipes Supporting study	Extended screening assay: **adult** (sexually mature fish at 4 months ph)/ 21 d Eggs of exposed pairs were observed until 60d posthatch	837 - 1720 - 3120 (measured) WR 78-86%/ flow-through	LOEC (VTG induction in exposed adult males) = 3120 µg/L (comparable to female VTG concentration)	Testis-ova in adult exposed males in all concentration tested (837 µg/L: 1/8, 6/7 and 4/8 at 837, 1720 and 3120 µg/L respectively): LOEC = 837 µg/L	No effects on fertility or fecundity of exposed adults	No effects on sex-ratio of non-exposed offspring based on sec sex characteristics		No effects on survival and growth of exposed adults and non-exposed offspring		(Kang et al., 2002)	1 - reliable without restriction: guideline study (OECD 230); extended
Oryzias latipes Supporting study Hd-rR strain	8 hpf + 7dpf expo → F4 without treatment	50 fertilised eggs/ replicate; 3 replicates; 83.7 µg BPA/L; 26 ± 0.5°C			Reduced fertilisation rates in F2 and F3 generation			Reduced embryo survival in F3 and F4 generation	0.061 µg E2/L	(Bhandari et al., 2015)	1 - 2

Species	Life stage/ duration	Conc. [µg/L] / test condition	VTG	Histology	Fertility/ Fecundity	Sex ratio	Sec. sex characteristics	others	Positive control	Reference	Reliability
Oryzias latipes Supporting study	4 hpf	6, 20, 60, 200, 600 µg BPA/L; 100 embryos in 5 replicates	Elevated VTG level LOEC = 600 µg/L decreased transcription of ARα LOEC = 200 µg/L					Reduced hatchability, survival rate; increased body weight females: LOEC= 200 µg/L		(Sun et al., 2014)	2
Oryzias latipes Supporting study	Modified reproduction assay: Exposure: adult male medaka (10-15 months) for 14 d, afterwards reproduction of exposed males with unexposed females for one week.	68, 230, 680, 2300 µg/L) (n) Solvent: Acetone (<0.1 mL/L) Semi-static: water exchange every 2 days						LOEC (number of eggs and hatchability) = 10 µmol/L (2280 µg/L) NOEC= 680 µg/L	17 ß-estradiol, At 3 and 10 nmol/L sign. decrease in the number of eggs. At 3nmol/L significant effect on hatching.	(Shioda and Wakabayashi, 2000)	2
Oryzias latipes Supporting study	Modified reproduction assay: adult males/ 35 d	0.1 - 10 - 100 (nominal) WR 90 - 110%/ flow-through	Induction of female specific proteins including VTG in males at 10 µg/L (LOEC) after 4 weeks (at 100 µg/l after 2 weeks)						E2:Induction of female specific proteins including VTG in males at 0.005 µg/L and above after 3 and 4 weeks	(Kashiwada et al., 2002)	2- acceptable documentation

Species	Life stage/ duration	Conc. [µg/L] / test condition	VTG	Histology	Fertility/ Fecundity	Sex ratio	Sec. sex characteristics	others	Positive control	Reference	Reliability
Oryzias latipes Supporting study	Modified reproduction assay: Adult males (age 3 month)/ 35 d	0.1 mL/L DMSO - 0.1 – 10 – 100 µg/L/ 12 h light per day/ flow-through/ fed twice a day/ 20-23°C/ 7 males + 7 females	LOEC (14d) 100 µg/L LOEC (35d) 10 µg/L	LOEC 100 µg/L Abnormalities in gonads (1 male)				LC_{50} = 7.5 mg/L	17ß-estradiol: LC_{50} = 3.5 µg/L LOEC (VTG) = 0.005 µg/L LOEC (intersex) 0.1 µg/L	(Tabata et al., 2001)	2 – well-documented publication; no measured concentration
Oryzias latipes Supporting study	Modified reproduction assay: Adult males (age 3 month)/ 8h	800 – 8000 µg BPA/L – solvent carrier 0.01% DMSO;	LOEC 8000 µg/L						17ß-estradiol (E2): LOEC (VTG) = 0.1 µg/L	(Yamaguchi et al., 2005)	3 – no guideline followed; only 8h exposure
Danio rerio Key study	FFLC/ 75 dpf	94-188-375-750-1500 (nom)	LOEC (VTG induction) = 375 µg/L	LOEC (changes in gonad histology - increase in the number of testis-ova) = 375 µg/L	LOEC (eggs per female, fertilization success) = 1500 µg/L			No effects on survival LOEC (juvenile growth) = 1500 µg/L LOEC (altered mating behaviour) = 1500 µg/L		(Segner et al., 2003a)	2 – some missing experimental design details, lack of discussion on analytical details and sparse presentation of results
Danio rerio Key study	2-generations test 8 hpf eggs/ 308 d	0.228 µg/L (measured: 0.372 µg/L)/ 3 replicates; 90 embryos per replicate/		Reduced sperm counts and quality	No effects at 0.372 µg/L	Female biased sex ratio: LOEC= 0.372 µg/L		Parental expo resulted in malformation and mortality of larvae: LOEC= 0.372 µg/L		(Chen et al., 2015)	2 – only one concentration tested

Species	Life stage/ duration	Conc. [µg/L] / test condition	VTG	Histology	Fertility/ Fecundity	Sex ratio	Sec. sex characteristics	others	Positive control	Reference	Reliability
		0.01% DMSO/ 28 °C; pH 7.0 – 7.5; 14 h light per day									
Danio rerio Supporting study	2-full-generations test 2-4 hpf eggs/ 180 dpf	2 replicates; 160 individuals per treatment; 26°C; semi-static until 48 hpf; flow-through; external aeration; start with 80 eggs per replicate; 2 replicates; reduction of number of individuals at 30 dpf, 90 dpf…; pH 8.25-8.75/ 10 - 200 - 400 µg/L (nominal)	LOEC (VTG induction F0 90 dpf) = 400 µg/L – conc.-dependent increase (maybe result of higher loading rate) LOEC (VTG induction F1 90+180 dpf) = 10 µg/L	No deviation from controls for thyroid histopatology	LOEC (fecundity F0 + F1) ≥ 400 µg/L (no effects observed in the test)			LOEC (reduced growth of males and females F0 90 dpf) = 10 µg/L LOEC (reduced growth of males and females F2 180 dpf) = 400 µg/L		(Keiter et al., 2012)	2 - well documented; nominal conc. (in previous experiment no degradation was observed)
Danio rerio Supporting study	Fish Screening Assay adult/ 21 d	0.1% methanol - 40-200-1000 (nom)	LOEC (VTG induction) = 1000 µg/L					NOEC (mortality) = 1000 µg/L	LOEC (VTG induction) = 20 ng E2/L	(Van den Belt et al., 2003)	2

Species	Life stage/ duration	Conc. [µg/L] / test condition	VTG	Histology	Fertility/ Fecundity	Sex ratio	Sec. sex characteristics	others	Positive control	Reference	Reliability
Danio rerio Supporting study	sexually mature adult fish (6-7 month)/ 4 d	0.01 - 0.1 - 1 - 10 - 100 (nominal)	LOEC (VTG induction) = 10 µg/L (males)							(Villeneuve et al., 2012)	2 - very well-documented publication (additional methodological details are available via internet)
Pimephales promelas Key study	Adult fish at 120 dph/ Multigene-rational study over 431d	1-16-160-640-1280 (nom+measured)	***F0 Gen.:*** LOEC (VTG prod.) = 160 µg/L (males) ***F1 Gen.:*** LOEC (VTG prod.) = 160 µg/L (males + females)	***F0 Gen.:*** LOEC (higher proportion spermatocytes) = 1 µg/L; LOEC (reduced numbers of mature spermatogonia) = 16 µg/L; LOEC (more spermatogonia) = 640 µg/L ***F1 Gen.:*** dose-related effect on proportion of spermatogonia + inhibitory effect on proportion of the testes occupied by spermatozoa: LOEC (higher proportion of spermatogonia + spermatozoa) = 1 µg/L	**Egg pro-duction:** ***F0 Gen.:*** LOEC = 1280 µg/L ***F1 Gen.:*** LOEC = 640 µg/L **Egg hatchability:** ***F1 Gen.:*** LOEC= 640 µg/L ***F2 Gen.:*** 160 µg/L			***F0 Gen:*** LOEC (weight males) = 640 µg/L ***F2 Gen.:*** LOEC (survival, growth) = 160 µg/L		Sumpter et al. 2001 (Sumpter JP, Tyler CR, Sherazi A (2001). Bisphenol-A: Multigeneration study with the fathead minnow (*Pimephales promelas*). Brunel University.) (Sohoni et al., 2001)	2 - study design not optimised to look for effects relating to spermatogenesis (number of fish sampled, taking of tissue samples from the testes, preparation for counting, number of cells counted in each sample, statistical methods not appropriate)

Species	Life stage/ duration	Conc. [µg/L] / test condition	VTG	Histology	Fertility/ Fecundity	Sex ratio	Sec. sex characteristics	others	Positive control	Reference	Reliability
Pimephales promelas Key study	Adult fish at 120 dph/ Partial life cycle test over 164 d	1-16-64-160-640 (nom.) or 1.19-13.4-52.8-130-567 (measured) (WR 81-89%)/ equivalent or similar to EPA OPP 72-5 (Fish life Cycle toxicity); flow-through; freshwater	LOEC (Vitellogenin prod.) = 64 µg/L (males + females)	LOEC (gonadal cell distribution male fish) = 160 µg/L LOEC (histopathological lesions) = 160 µg/L (males) LOEC (histopathological lesions) = 640 µg/L (males + females)				LOEC (survival male fish) = 640 µg/L		Rhodes JE, Wolf JC and van der Hoeven N (2007). Bisphenol A: partial life-cycle toxicity test with the fathead minnow, Pimephales promelas. ABC Laboratories, Inc. and Experimental Pathology Laboratories, Inc., ABC Study number 48972 (in preparation, draft used). (RAR) → final: (Mihaich et al., 2012)	4 – only short abstract available 1 - valid
Pimephales promelas Supporting study	Adult fish (5-6 months)	10 and 100 µg BPA/L; 4 replicates; 25°C	Inhibition of androgenic activity of co-exosed 17β-trenbolone (TB) at 100 µg/L						17β-trenbolone (AR agonist) (TB),	(Ekman et al., 2012)	2

Species	Life stage/ duration	Conc. [µg/L] / test condition	VTG	Histology	Fertility/ Fecundity	Sex ratio	Sec. sex characteristics	others	Positive control	Reference	Reliability
Pimephales promelas Supporting study	sexually mature adult fish (5-6 month)/ 4 d	0.01 - 0.1 - 1 - 10 - 100 (nominal)/ flow-through; 3 replicate tanks with 3 male and 3 female fish	NOEC (VTG prod.) = 10 µg/L LOEC (VTG prod.) = 100 µg/L (males)							(Villeneuve et al., 2012)	2 - very well-documented publication (additional methodological details are available via internet)
Pimephales promelas Supporting study	21 d	56.6 – 344/ 4 tank replicates per treatment	↑ VTG: LOEC = 56.6 µg/L in males (2 mg/mL in 56.6 and 92.7 mg/mL in 344 µg/L)	↓ maturity of ovaries (↑ early stages + ↓ stage 3 oocytes)	Fecundity: NOEC = 56.6 µg/L Hatching percentage: 77% at 56.6 µg/L (control: 99%)			↓ E2: LOEC = 56.6 µg/L in males + females ↓ T and 11-KT: LOEC = 344 µg/L in males		(EPA, 2007)	4 – only abstract available
Salmo trutta f. fario brown trout Supporting study	male and female fish during late pre-spawning and spawning period/ 103 d	flow-through/ 1.75 - 2.40 - 5.00 (nominal)		LOEC (lower sperm quality and motility; reduced percent ovulation) = 1.75 µg/L LOEC (complete inhibition of ovulation in females) = 5µg/L				NOEC (weight) = 2.4 µg/L		(Lahnsteiner et al., 2005)	2 – nominal conc. Lead-Dossier: in discussion used – according to EU RAR of low relevance as there is a possibility of a problem with the reported exposure levels

Species	Life stage/ duration	Conc. [µg/L] / test condition	VTG	Histology	Fertility/ Fecundity	Sex ratio	Sec. sex characteristics	others	Positive control	Reference	Reliability
Carassius auratus L. Goldfish Supporting study	Male adults (2-3 years old)/ 30 d	Semi-static/ 18 – 20 °C; 12 h light per day; pH 7.2 – 7.8 Control – DMSO-control – 0.6 – 4.5 – 11.0 (measured)	LOEC (30 d) = 11.0 µg/L	LOEC (lower sperm motility exposed 30s or 90s post-activation; 30d) = 0.6 µg/L LOEC (lower sperm velocity exposed 90s post-activation) = 0.6 µg/L				Testosterone: LOEC (20 d) = 4.5 µg/L LOEC (30d) = 11.0 µg/L 11-KT: LOEC (20d) = 4.5 µg/L LOEC (30d) = 0.6 µg/L		(Hatef et al., 2012a)	2 – well documented study which meets basic scientific principles, measured concentra-tions
Oncorhynchus mykiss Supporting study	juvenile/ 28 d	flow-through; OECD 215; GLP/ 100 – 320 – 1000 – 3160 – 1000 µg/L (nominal)						NOEC (growth rate) = 3640 µg/L		Bayer AG 1999b (Bayer AG (1999b). Fish, juvenile growth test (O. mykiss) of Bisphenol-A. Study Number: 707 A/98FF.)	4 - only short abstract available (in UKRAR reliability 1); test duration not sufficient related to mode of action
Oncorhynchus mykiss Supporting study	juvenile/ 12 d	40 - 70 - 100 - 500 (nom) WR +- 0.3-11.2%/ flow-through	NOEC (induction of plasma VTG) = 40 µg/L						1 µg E2/L: increased VTG and zr proteins Possible hormone activity affected: increasing oestrogen activity	(Lindholst et al., 2000)	2 - no guideline used; acceptable, well-documented publication; measured concentra-tions
Xiphophorus helleri	male 30 d old	0.2 - 2 – 20 – 400 -	NOEC = 400 µg/L	NOEC (apoptotic			NOEC (reduction in swordtail			(Kwak et al., 2001)	2 - only nominal

Species	Life stage/ duration	Conc. [µg/L] / test condition	VTG	Histology	Fertility/ Fecundity	Sex ratio	Sec. sex characteristics	others	Positive control	Reference	Reliability
Swordtail Supporting study	juveniles/ 60 d	2000 – 10000 µg/L / static; 20 fish per conc.		cells) = 2000 µg/L			length (distal end of the middle rays of the caudal fin to the tip of the swordtail)) = 0.2 µg/L				concentration, static conditions, no information about solvent control, no data for pH, temperature, DO. The significance of the changes in sword length is not understood, but it is thought that the length of the sword has an influence on mating success, with female fish preferring males with longer swords. It is not clear what degree of change should be considered to be significant. The separation between exposure levels was an order of magnitude.

Species	Life stage/ duration	Conc. [µg/L] / test condition	VTG	Histology	Fertility/ Fecundity	Sex ratio	Sec. sex characteristics	others	Positive control	Reference	Reliability
Poecilia reticulata Guppy Supporting study	*sexually mature males/ 30 d*	5-50-500-5000 (nom) WR:+21%		LOEC = 5000 µg/L (pronounced effects, with testes filled with spermatozeugmata, some of which had ruptured resulting in free spermatozoa, and virtually no spermatogenic cysts)				NOEC (survival male fish) = 500 µg/L d21: at 5000 µg/L 77% of the fish died (testes filled with spermatozeugmata)		(Kinnberg and Toft, 2003)	2 - nominal conc.; no guideline
Poecilia reticulate Guppy Supporting study	Adult male 21 d			LOEC = 274 µg/L (reduced sperm counts – inhibited spermatogenesis)						(Haubruge et al., 2000)	

5.5.3 Summary on the plausible link between adverse effects and indications for the underlying oestrogenic/anti-androgenic MoA in fish

Overall, increased levels of VTG (a widely accepted biomarker for an oestrogenic mode of action) were determined in all studies analysing this endpoint[5]. The lowest NOEC was 0.1 µg /L BPA (Kashiwada et al., 2002) and the lowest LOEC was 10 µg /L BPA for female specific proteins in males (Kashiwada et al., 2002; Keiter et al., 2012; Tabata et al., 2001; Villeneuve et al., 2012; Hatef et al., 2012a; Mandich et al., 2007). Disruption of gametogenesis is another adverse reproductive outcome associated with BPA exposure in fish pointing to an oestrogenic MoA. In *P.promelas*, changes in spermatogenesis (increases in spermatogonia and decreases in spermatocyte or spermatozoa) were observed at 160 µg/L (700 nM) in (Mihaich et al., 2012) with an exposure duration of 164 days using adult fish. In *S.trutta f. fario*, a low level BPA exposure of 2.4 µg/L (10.5 nM) delayed the spermiation and decreased sperm density, motility and velocity (Lahnsteiner et al., 2005). In *C. auratus L.*, BPA exposure at 0.6 to 11 µg/L (2.6-48.2 nM) adversely affected sperm motility and velocity (Hatef et al., 2012a). *In vitro*, *P. fluviatilis L.* exposure of sperm to high concentrations of BPA (1 mM) affected sperm morphology, motility and velocity.

In addition, the occurrence of testis-ova, as an indicator of an oestrogenic effect according to the OECD guidance document 123 (OECD, 2010a), was observed in all sexual development tests if examined, some testis-ova were observed even after short term exposure of adult males. The lowest LOEC value for this specific endpoint was 5.9 µg /L BPA (Metcalfe et al., 2001).

Apical effects observed fit to these indicators of an oestrogen mediated effect. The sex ratio was significantly skewed toward females in all assays where the Bisphenol A exposure started during sensitive life stages (before hatch). Based on sex ratio and secondary sex characteristics significant effects were observed at 0.228 µg/L (Kwak et al., 2001; Chen et al., 2015) and at concentrations higher than 355 µg BPA/L (Yokota et al., 2000). For *O. latipes* effects on sex ratio were only observed in one test due to deficiencies in the other tests.
Besides the observed oestrogenic effects, there is also evidence of an anti-androgenic mode of action of Bisphenol A in fish. As oestrogens and anti-androgens have two very different modes of action but similar effects on the reproductive organs, it is difficult to distinguish them. Both can skew the sex ratio towards females, reduce or inhibit spermatogenesis and delay maturation of ovaries, although only oestrogens induce VTG production in males. Ekman et al. (2012) assessed the androgenic mode of action by co-exposing fish with Bisphenol A and a known androgen receptor agonist. The result supports the evidence for the anti-androgenic mode of action of Bisphenol A. Labadie and Budzinski (2006) observed a decrease of androgens due to BPA exposure (["… p-nonylphenol induced a decrease of androstenedione and 11-ketotestosterone levels, whereas Bisphenol A exposure led to an elevation of oestrone level" (see also Petersen et al., 2015)). Knacker et al. (2010) describes that androgen-receptor antagonists affect the spawning and fecundity (number of eggs) of fish. Some studies conducted with Bisphenol A observed this effect in fish (Segner et al., 2003a; Sohoni et al., 2001; EPA, 2007). Bisphenol A also caused an increase of 11-ketotestosterone levels in fish (EPA, 2007; Hatef et al., 2012a), which indicate an androgen-receptor antagonist.

The effects described above show that Bisphenol A is an oestrogenic endocrine disruptor. Other effects described above also give some indication that Bisphenol A also has an anti-androgenic MoA.

[5] Some tests with *O.latipes* had deficiencies with regard to uncovering the endocrine mode of action of Bisphenol A on Medaka. Test concentrations were not high enough and the exposure started too late, missing the sensitive time-window of *O.latipes* (therefore only effects on sex ratio were observed in one test). Another deficiency was that the endpoints could not be analysed reliably (effects on weight at lower concentrations were not reliable because of a too high stocking rate resulting in an insufficient growth of the control fishes).

In Table 14 below, a summary on the plausible link between adverse effects and indications for an oestrogenic/anti-androgenic MoA in fish is given.

Table 14: Plausible biological link between oestrogenic/anti-androgenic MoA and adverse effects in fish

Species	Evidence for MoA	Apical effect	Comparison MoA and apical effect
Carp *Cyprinus carpio*	Vitellogenin induction in males at 10 µg/L (LOEC) Oviduct formation at 32 µg/L (LOEC)		According to (OECD, 2012b) indicator for an oestrogen agonist MoA.
Rare minnow *Gobiocypris rarus*	Vitellogenin induction in males at 1 and 15 but not 225 µg/L (LOEC) Increased apoptosis of primary and secondary spermatocyte in testis at 225 µg/L (LOEC)		According to (OECD, 2012b) indicator for an oestrogen agonist MoA.
Medaka *Oryzias latipes*	Vitellogenin induction at 10 to 3120 µg/L (LOEC) Testis-ova at 5.9 to 890 µg/L (LOEC)	Sex ratio skewed towards females (no males) at 1820 µg/L (LOEC) (diagnostic)	Direct causal link for oestrogen agonistic MoA.
Zebrafish *Danio rerio*	Vitellogenin induction at 10 to 1000 µg/L (adult) (LOEC) Turing more sensitive from one generation to the next (LOEC F1= 400; LOEC F2= 10 µg/L) Testis-ova at 375 µg/L (LOEC) Reduced sperm counts and quality at 0.372 µg/L (LOEC)	Sex ratio skewed towards females at 0.372 µg/L (diagnostic) Increased malformation and mortality of larvae (effect on sperm DNA?) at 0.372 µg/L Reduced embryo survival at 83.7 µg/L (F3 and F4) (LOEC) Reduced fertilization success at 83.7 (F2 and F3) to 1500 µg/L (LOEC)	Direct causal link for oestrogen agonistic MoA.
Fathead minnow *Pimephales promelas*	Vitellogenin induction at 64 and 160 µg/L (LOEC) Changes in gonadal staging (increased proportion of early sperm stages) at 640 µg/L (Histopathological lesions in males at 160 µg/L (LOEC) Inhibition of androgenic activity of co-exposed 17ß-Trenbelone at 100 µg/L (LOEC)	Reduced egg hatchability at 640 µg/L (F1) and 160 µg/L (F2) (LOEC) Reduced egg production at 1280 µg/L (F0) and 640 µg/L (F1) (LOEC) Suppressed growth at 640 µg/L (LOEC)	Direct plausible biological link for oestrogen agonistic MoA + increasing sensitivity with subsequent generations. Indication of anti-androgenic MoA.
Brown trout *Salmo trutta f. fario*	Reduced sperm quality and motility at 1.75 µg/L (LOEC)	Effects on reproduction (no eggs) at 5 µg/L (LOEC)	

Species	Evidence for MoA	Apical effect	Comparison MoA and apical effect
Goldfish *Carassius auratus*	Vitellogenin induction at 0.6 µg/L (LOEC) Increased mRNA transcript of AR in testis at 20 µg/L (LOEC) Reduced sperm motility at 0.2 and 0.6 µg/L (LOEC)		Indication of oestrogen agonistic MoA.
Rainbow trout *Oncorhynchhus mykiss*	Vitellogenin induction at 70 µg/L (LOEC)		Indication of oestrogen agonistic MoA.
Guppy *Poecillia reticulata*	Testis filled with spermatozeugmata at 5000 µg/L (LOEC) (NOEC = 500 µg/L) Changes in gonadal staging (decline of sperm count - disruption of male spermatogenesis) at 274 µg/L (LOEC)	Mortality at 5000 µg/L (LOEC) (because of filled testes)	
Green swordtail *Xiphophorus helleri*	Increased Vitellogenin mRNA level at 400 µg/L (LOEC) Induction of apoptotic cell death at 10000 µg/L (LOEC)	Depression of male secondary sex characteristics at 2 µg/L (LOEC) (diagnostic)	Hint for an oestrogenic MoA.

5.6 Fish: In vivo effects with regard to a thyroid mediated endocrine mode of action

5.6.1 Approach used for assessing the thyroidal mode of action in fish

Guidance on how to assess a thyroidal mode of action is at the moment only given for amphibians in the OECD Guidance Document on standardised test guidelines for evaluating chemicals for Endocrine disruption (OECD, 2012b).

The guidance document (OECD, 2012b) describes that an accelerated or asynchronous development in amphibians is considered to be diagnostic of thyroid active chemicals. This may also be true for fish: Terrien and Prunet (2013) summarised for zebrafish that a tri-iodothyroinine (T_3) exposure of the embryos up-regulates the T_3 receptor (TR) expression. This accelerates the developmental rate and hatching (Darras et al., 2011; Terrien et al., 2011) of the embryos.

The accelerated development may be mitigated by exposure to thyroid receptor antagonists.

Hence, during the following assessment, we take these evidence as well as accelerated development as endpoints indicating thyroid activity of Bisphenol A in fish.

Table 15: Summary of endpoints indicating thyroid activity considered during data analysis

Endpoints pointing to a thyroid activity (according to amphibian *in vivo* tests)
• Altered levels of thyroid hormones • Effects on accelerated development diminished by coexposure to thyroid receptor antagonist • Advanced development

5.6.2 In vivo effects with regard to a thyroidal mode of action in fish

Overall 2 studies were analysed with respect to a thyroid mode of action in fish (see Table 16).

The study conducted by Ramakrishnan and Wayne (2008) investigated the impact of BPA on the early embryonic development and reproductive maturation of medaka. They treated newly fertilised eggs (< 5 hpf) of medaka fish (*Oryzias latipes*) of the d-rR strain with initial concentrations of 20, 100 and 200 µg BPA/L. In all experiments, at least 16 embryos per group from at least five clutches of eggs were used. The fish were fed and maintained with a 14h-light: 10h-dark photoperiod at a temperature of 28°C. The embryos treated with 200 µg BPA/L showed an ***accelerated development*** starting 1 dpf (greater somite number and increased optic bud development compared to control). Also the eye development on 2 and 3 dpf in the 200 µg BPA/L-treatment showed an advanced development relative to controls. ***No difference in heart rate*** at 2 dpf was observed. Also the ***total numbers of surviving embryos*** from fertilisation to hatching did ***not differ*** significantly. The embryos exposed to 200 µg BPA/L hatched earlier and were consistently smaller than age matched controls from hatching until 25 dph. They also started significantly earlier with the production of viable fertilised eggs. BPA had no effect on the gonadosomatic index of sexually mature males in this study. The histological examination of testis revealed ***normal tissue***. Ramakrishnan and Wayne (2008) also exposed the newly fertilised medaka eggs to the thyroid-hormone receptor antagonist amiodarone in different treatments: amiodarone alone, together with 200 µg BPA/L or a vehicle control. As a result, the ***acceleration of early embryonic development caused by 200 µg BPA/L was blocked in the presence of 5 nM, 0.5 nM and 50 nM amiodarone***. The effect was not blocked at a lower dose of 0.05 nM amiodarone.

With the accelerated development, the study conducted by Ramakrishnan and Wayne (2008) shows a similar effect of Bisphenol A on medaka as described for amphibians for a thyroid active chemical.

Table 16: Summary of thyroidal effects of Bisphenol A *in vivo* in different fish species

Species	Life stage/ duration	Conc. [µg/L] / test condition	Hormone level	Histology	development	others	Positive control	Reference	Reliability
Oryzias latipes Key study	Newly fertilised eggs (< 5h pf)/ 49 d	20 – 100 - 200 µg/L (nominal)/ 16 embryos per group		Normal tissue	LOEC (embryo development) = 200µg/L	NOEC (survival of embryos, heart rate) ≥ 200 µg/L	accelerate development from BPA was blocked by amiodarone (thyroid hormone receptor antagonist	Ramakrishnan and Wayne (2008)	2
Danio rerio	Embryos/ 48 h	BPA in presence of T_3	Decreased Mineralocorticoid Receptor (MR) and Gluccorticoid Receptor					Terrien et al. (2011)	2

Terrien et al. (2011) exposed wild-type zebrafish embryos for 24 or 48 h to BPA in presence or absence of T_3. BPA decreased the Mineralocorticoid Receptor (MR) and the Gluccorticoid Receptor in comparison with the T_3-treatment. The study examined the potential crosstalk between thyroid hormone and corticosteroids.

The results indicate that BPA can interact with corticoid receptors, bind as agonist and may induce changes in corticosteroid signalling pathways independently of the thyroid hormone function. This results in the inhibition of T_3 effects by BPA, the inhibition of TR-alpha, TR-beta and TSH genes and shows the disrupting effect of BPA on the thyroid receptor signalling system.

In summary, also for fish, a disruption of throid signalling and an accelerated development are shown after BPA exposure. Very few studies were analysed for the thyroidal MoA of BPA in fish, but the results support the studies conducted with amphibians (see chapter 5.7) showing a thyroidal MoA.

5.6.3 Summary on the plausible link between adverse effects and indications for the underlying thyroidal MoA in fish

As there are only a few studies examining the thyroidal mode of action of Bisphenol A in fish, no plausible biological link but indications for a thyroidal effect can be concluded. These are summarized in table 17.

Table 17: Plausible biological link between thyroidal MoA and adverse effects in fish

Species	Evidence for MoA	Apical effect	Comparison MoA and apical effect
Oryzias latipes Medaka	accelerate development from BPA was blocked by amiodarone (thyroid hormone receptor antagonist)	Accelerated development	Indication for a thyroidal effect as known for amphibians.

5.7 Fish: other *in vivo* effects (neurodevelopmental effects)

The information on neurodevelopmental effects in fish was provided during public consultation. Due to the short time-frame for finalising the document, details on test conditions and detailed study discriptions could not be provide in the document. They are only summarised below.

There are studies indicating possible effects of Bisphenol A on neurodevelopment in fish. They seem to support the issue of increased neurodevelopmental disorders when organisms are exposed during brain developmental stages. Such effects of BPA were already described in the supporting document for SVHC-identification 57f for human health (ECHA 2017) with respect to effects on neurogenesis in humans and rodents.

Kinch et al. (2015) shows that exposure of zebrafish to very low doses of Bisphenol A (6.8 nM corresponds to 1.55 µg/L) during hypothalamic neurogenesis leads to changes in locomotor activity (i.e. hyperactive behaviour) of the larvae. This behavioural change could be an effect of a precocious neurogenesis linked to an increase of newly born neurons in the hypothalamus. The elements of the study support the hypothesis that Bisphenol A might act on neurodevelopment by the increase of AroB transcripton, as demonstrated by the reversal of the effects when gene expression of AroB is reduced. Aromatase B (AroB) is the key enzyme for local estradiol synthesis, which is expressed in hypothalamic progenitor cells.

There are other fish studies available as (Wang et al., 2015; Wang et al., 2013) showing effects of Bisphenol A on the behaviour of adult zebrafish. Zebrafishes were exposed for six months to low concentrations of Bisphenol A. The authors showed a lower adaptability to the environment and a modification of the circadian rhythm, which can have consequences for the survival of individuals. Wang et al. (2013) reported that Bisphenol A-exposed zebrafish shows altered neurodevelopment and locomotion suggesting potentially deleterious reflex losses for fish survival or to escape predator attack. More recently (Goundadkar and Katti, 2017) showed that fishes exposed for 75 days to 5 ng/L of Bisphenol A, exhibited reduced swimming time and increased freezing periods.

5.8 Amphibians: in vivo effects with regard to an thyroid mediated mode of action

5.8.1 Approach used for assessing the thyroidal mediated adverse effects in amphibians

In this chapter information about the potential endocrine mode(s) of action of Bisphenol A in amphibians (only anurans) is summarized.

While in fishes oestrogen- and/or androgen-mediated effects are the most commonly assessed

modes of action, in amphibians impact on the thyroid activity is a known potent endocrine mode of action which is linked to the thyroid-dependent process of amphibian metamorphosis.

According to the OECD guideline 231 for the amphibian metamorphosis assay (OECD, 2009a), the following effects indicate a ***thyroid mode of action***:

- Advanced development (according to development stages or hind limb length)
- Asynchronous development
- Remarkable histological effects

Delay in development may be induced by a thyroid antagonistic mode of action, but could also be influenced by systemic toxicity. Thus, this parameter should be regarded as indicative for an endocrine mode of action only, if no systemic toxicity (reduced growth, mortality) is observable. Similarly, increased body weight is often observed for substances negatively affecting normal development but should not be used alone.

According to (Brown and Cai, 2007) the thyroid hormone (TH) controls the formation of new organs and cell types for use by the frog after metamorphosis that either do not exist in the tadpole or have no function in tadpoles. These include the limbs, bone marrow, and skin and stomach glands. Not all frog organs require TH to develop. Raising tadpoles for many months on Methimazole (1mM), an inhibitor that blocks the synthesis of TH in the thyroid gland by inhibiting the thyroid peroxidase (TPO) and thus formation of T_4 and T_3 (Fini et al., 2007), a tadpole will arrest development spontaneously. Normally, gonads with visible eggs and sperm appear several months after metamorphosis. However, these arrested tadpoles form primary oocytes and spermatogonia and do not remodel their skeleton as usually done. TH action potentiates the liver to respond to oestrogen and synthesize Vitellogenin (VTG).

The sensitivity of tadpoles compared to frogs suggests a total inactivation of the TH sensitive state of the genome after metamorphosis.

TH-induced visible changes inhibited in animals of transgenic experiments are: tail resorption, gill resorption, intestinal remodelling, limb bud growth, and DNA replication in the brain, nose, and limb buds.

A functional thyroid gland is detected 10 days post fertilization (NF46). Endogenous TH concentration was not quantified before NF56 until recently. TH synthesis increases as the tadpole grows reaching a peak at the climax of metamorphosis (NF60 to 63).

The following endpoints observed in amphibians are considered specific for thyroid activity in the analysis of data provided below. These endpoints cover several different modes of action, including agonistic and antagonistic interactions, as well as substances interfering with thyroid hormone synthesis and transport.

Table 18: Summary of endpoints indicating thyroid activity considered during data analysis

Endpoints indicating thyroid activity (from amphibian *in vivo* tests)	
• Altered levels of thyroid hormones • Advanced development (NF stage 62) • Asynchronous development • Delayed development in absence of non-specific systemic toxicity • Thyroid histopathology (e.g. moderate or severe follicular hypertrophy and/or hyperplasia)	• Increased thyroid weight • Possible liver weight increase (in combination with other thyroid-related endpoints) • Histopathologic changes in thyroid • Serum T4, T3 decreased, TSH increased

5.8.2 *In vivo* effects with regard to a thyroidal mode of action in amphibians

For four species, eight tests were evaluated for the thyroidal mode of action of Bisphenol A (see Table 19 below).

Heimeier et al. (2009) (key study for this MoA) had the aim to identify the critical T_3 pathways that may disrupted by BPA. Therefore, they conducted a 21d-*in vivo* test (experiment 1) and a 4d *in vivo* test, which also included a RNA isolation and cDNA array analysis and real-time PCR quantification with *Xenopus laevis*. Premetamorphic tadpoles (stage 54) were used after acclimatisation to 23 to 24°C for 24h and fed before acclimatisation with spirulina. As, according to the authors, tadpoles undergoing metamorphosis or T_3 treatment do not feed, they were not fed to eliminate dietary influence on metamorphosis progression (in contrast to daily feeding in the OECD 231 AMA test guideline). This was a microinjection experiment, where a reporter construct, TRE-Luc, harboring the T_3-dependent X.laevis TRßA promoter driving the firefly luciferase reporter was microinjected (0.33 ng/oocyte) into the nuclei of X. laevis oocytes together with a plasmid harboring the control Renilla luciferase reporter. In vitro transcribed mRNAs encoding TRß and retinoid X receptor (RXR)-α were coinjected (1.15 ng/oocyte for TRß and RXRα) into the cytoplasm. After overnight incubation in the presence or absence of BPA and/or T_3, oocytes were assayed for luciferase activity. Both experiments were conducted semi-static with water changes every (exp.1) or every 2d (exp2). Groups of 10 tadpoles were exposed and replicated 3 times. For the first experiment, DMSO (0.1%), 1.3 µg/L T_3, 22.8 and 2280 µg/L BPA or a combination was used as treatment. This experiment resulted in no significant stage difference between control and BPA treated animals. At the end of experiment 1 (d 21), T_3-treated animals were at stage 64, the control animals at stage 56 and T_3+BPA-treated animals showed a delayed development compared with T_3-treated animals. The second experiment using DMSO, 1.3 µg/L T_3, 2280 µg/L BPA or a combination of T_3 and BPA, showed that BPA alone had no significant different intestine structure compared to the control. For the T_3 treated animals, an overall shortened length was observed. Histologically, the T_3-treated animals showed well-documented tissue remodelling responses to T_3 (increased thickness in muscle, connective tissue layers). The combined exposure with T_3 and BPA showed very little changes compared to control and BPA alone. Here it can be seen that BPA cancels the effect of T_3 on histology. This is also significant for gene expression with BPA blocking partially the regulation of established T_3-response genes. The findings of the test, the ***inhibition of TRβ-induced transcription and the inhibition of T_3-induced metamorphosis*** in a dose-dependent manner, strongly support that BPA acts as a T_3 antagonist *in vivo*. However, BPA alone did not show effects. Compared to the AMA no feeding was provided.

Also the test conducted by Iwamuro et al. (2003) (key study for this MoA) reveals the antagonistic action of BPA against T_3. At a LOEC of 5700 µg /L BPA (25 µM), the survival rate of the *Xenopus laevis* embryos was affected. In the experiment, ***T_3 stimulated the reduction of length of the tail sections*** for larvae stage 52-54 at 2300 and 23000 µg/L but ***Bisphenol A blocked*** this reduction in a dose dependent manner. The *in vitro* part of the study showed ***spontaneous inhibition and TH-induced metamorphic changes and suppression of TRß gene expressions*** at a Bisphenol A concentration of 2280 µg/L.

According to "The Amphibian Metamorphosis Assay (AMA)" (OECD, 2009a) a delayed development of the tadpoles (stage, HLL, BW; SVL) indicates an anti-thyroidal mechanism when there are no overt signs of toxicity or excessive mortality. As these results were detected in several studies, this strengthens the assumption that Bisphenol A acts as an anti-thyroidal substance.

In the AMA (OECD, 2009a) amongst others Methimazole (Thiamazole) was tested as a known inhibitor that blocks the synthesis of TH in the thyroid gland as an example. The exposure of tadpoles of *Xenopus laevis* and *Xenopus (Silurana) tropicalis* resulted in spontaneously arrested development. Similar results were reported by (Goto et al., 2006) for Bisphenol A and 1mM Metimazole *Xenopus (Silurana) tropicalis* in an *in vivo* experiment. Here premetamorphic tadpoles stage 57 were exposed to 228.28 µg /L BPA or 114.17 mg /L Methimazole (both 1 mM). The density was one organism per 50 mL. The exposure with BPA resulted in ***spontaneously***

inhibited metamorphosis. As only this single concentration of Bisphenol A was used, no NOEC derivation was possible.

Rana rugosa was also tested for the response to Bisphenol A exposure. Kashiwagi et al. (2008) reported an ***inhibition of the spontaneous metamorphosis by testing T_3 together with BPA*** (see also Goto et al., 2006)). The result was a NOEC (greater tails than corresponding T_3-group) of 2.28 µg/L BPA and a LOEC of 22.8 µg/L BPA. Three concentrations were tested (2.3 – 23 – 230 µg/L). After 5 d, T_3 was added to half of the test vessels with BPA and the control for one day. The thyroid hormone (T_3) influences the metamorphosis (amongst others tail shortening of tadpoles). It is a hormone of the thyroid, which accelerates the metamorphosis in the experiment with *Rana* sp. The exposure group BPA+T_3 in contrast shows a lesser degree of tail shortening – therefore it blocks the thyroid hormone receptor. For this reason, the metamorphosis happens more slowly than normal.

The results of several other tests, mainly with *Xenopus laevis* but also with *Xenopus* (*Silurana) tropicalis* and *Rana rugosa*, make it likely that Bisphenol A interferes with the thyroid system of amphibians as the effects observed are comparable with the ones described above (suppression of T_3-induced tail shortening, malformations, …).

Summing up all conducted studies, Bisphenol A shows a suppression of T_3-action (T_3-antagonistic action) when T_3 is co-exposed with a BPA concentration of 2.28 µg/L or above. This results in a delayed metamorphosis which e.g. becomes apparent in the experiments in amphibians with artificially T_3-induced metamorphosis through larger tails compared to T_3-groups and hence is evidence for a thyroid disrupting mode of action of BPA (anti-thyroidal) and a plausible biological link between the mode of action and the adverse effects can be established. These effects at once were seen in the two key studies but also separately in the supporting studies. It is not clear how environmentally relevant effects are from tests where artifically-induced metamorphosis was used.

Table 19: Summary of (anti-)thyroidal effects of Bisphenol A in Amphibians

Species	Reference	Test Conditions	Endpoints	Effect concentrations [µg/L]	Indication for ED	Reliability
Xenopus laevis Key study	(Iwamuro et al., 2003)	a) 60 – 100 stage 7 embryos; exposure for 72 h; survival of embryos after 120 h; morphological abnormalities 5-7 dpf; conc.: 2280 – 4600 – 5700 – 6850 – 11410 – 22800 µg BPA/L [10^{-5} or $2.0 \cdot 10^{-5}$ or $2.5 \cdot 10^{-5}$ or $3.0 \cdot 10^{-5}$ or $5.0 * 10^{-5}$ or 10-4M BPA] // b) Stage 52 tadpoles; groups of 10^{-12}; conc.: 10^{-7} M T_4 or 2280–5700 µg BPA/L [10^{-5} or $2.5 \cdot 10^{-5}$ M BPA]; exposure for 22 d; 23 °C; 12 h light/d; semi static → c) in vitro studies on tails removed from stage 52-54 tadpoles	a) survival rate of embryos b) head malformations, scoliosis, organogenesis suppression b+c) spontaneous inhibition and TH-induced metamorphic changes and suppression of TRß gene expressions both in vivo and in vitro	a) NOEC 4560; LOEC 5700; LD_{50} 4794 b) no apparent abnormalities in 2280 T_3 stimulates reduction of length of tail sections for larvae stage 52-54 → BPA 2300 and 23000 blocked this reduction concentration dependent c) LOEC 2280		2 – well-documented publication
Xenopus laevis Key study	(Heimeier et al., 2009)	Exp. 1: semi static; 23-24 °C; 10 premetaphoric tadpoles (stage 54) per replicate; 3 replicates; for 21 d; 12 h light per day/ conc.: 1.3 µg /L T_3 [2 nM] – 22.8 or 2280 µg /L BPA [0.1 or 10 µM] or the combination Exp. 2: 10 tadpoles stage 54 per replicate; 3 replicates; semi static; for 4 d; 23 - 24 °C12 h light per day/ conc.: DMSO – 1.3 µg /L T_3 [2 nM] - 2280 µg /L BPA [10 µM] RNA isolation and cDNA array analysis + real-time PCR quantification	Exp.1: Developmental stages; morphology Exp. 2: TR-dependent luciferase reporter (TRE-Luc) expression in vivo No effects on BPA alone	Exp.1: 22.8 Exp. 2: 2280 in the presence of T_3: BPA inhibited the transcriptional activation of the promoter T_3 → little effect in the absence of T_3	Findings strong support that BPA acts as a T_3 antagonist in vivo; Combined T_3+BPA: sign delayed metamorphosis compared with T_3–treated animals; BPA: no sign stage difference; Expression level of 3 early and 2 late response genes in combined T_3+BPA sign reduced compared with T_3 only → BPA inhibits the expression of known T_3-response genes	2 - very well documented study; artificially induced metamorphosis
Rana rugosa	(Goto et	tadpoles stage X acc. Taylor and	Tadpole Tail	LOEC = 22.8	T3 induces tail	2 – well documented

Species	Reference	Test Conditions	Endpoints	Effect concentrations [µg/L]	Indication for ED	Reliability
Supporting study	al., 2006) Also reported in (Kashiwagi et al., 2008)	Kollros/ 9 d; 2 replicates; densitiy 1 per 50 mL/ 2.3 - 23 - 230 µg/L (nominal) + 0.1% DMSO + at d5-6: T_3 + 2.28 – T_3 + 22.8 - T_3 + 228.28 µg /L BPA	shortening in T3-induced metamorphosis	NOEC = 2.28	shortening, this is suppressed by BPA (230 + 23 µg/L) in a dose-dependent manner See Xenopus laevis (Iwamuro et al., 2003) → there also blocked T3 induced tail shortening	study; artificially induced metamorphosis; test concentration differential is an order of magnitude.
Silurana tropicalis Supporting study	(Goto et al., 2006)	tadpoles stage 57/ 10 d 230 µg/L (nominal) or 1mM Methimazole (thyroid hormone synthesis inhibitor, also Thiamazole → inhibitor of the thyroid function)	effects on T3-induced metamorphic changes		Similar result with BPA and Methimazole → spontaneous metamorphose, tail shortening, elongation of the hind legs	2 - no guideline study; only one concentration was used
Rana nigromaculata Supporting study	(Yang et al., 2005)	tadpoles 5d ph; captured in the wilderness/ 60 d; 2 - 20 - 200 (nominal); semi-static; 23 - 24 °C; 10 tadpoles per 1-L tank; 12 h light per day/ T_3 – DMSO; also combined treatments with Nonylphenol	malformations of tail flexure	NOEC 20 LOEC 200 (16.7 % malformations) Same results for 4-Nonylphenols		2 – well documented study but without chemical analysis
Xenopus laevis Supporting study	(Fini et al., 2007)	Transgenic TH/bZip-eGFP X. Laevis; Stage 42 to 52 transgenic tadpoles (transgenic males with wild type females) → robust T_3 induction NF45 (app. 5 dph); ethanol; T_3; T4; GC-1 (TRβ isorom specific agonist); Methimazole; 228 µg/L (10-6 M) BPA; 6 well plates or 5 L aquaria for stage NF52; Measuring signals by MFA		BPA in presence of T_3 inhibited sign (20-30%) T_3-signaling at 228 µg/L	Similar result with TBBPA (LOEC= 544 µg/L)	1 – very well documented; in accordance with institutional and national guidelines (Sciences et Médecine des Animaux de laboratoire à l'ENVL 2005).

5.8.3 Summary on the plausible link between adverse effects and indications for the underlying thyroidal mode of action in amphibians

There is some evidence for an anti-thyroidal mode of action of BPA from 3 species. The evidence for a plausible biological link between the inhibition of thyroid hormone synthesis and inhibition of metamorphosis is summarized in Table 20.

Table 20: Plausible biological link between (anti-)thyroidal MoA and adverse effects in amphibians

Species	Evidence for MoA	Apical effect	Comparison MoA and apical effect
African clawed frog *Xenopus laevis*	Inhibition of thyroid hormone induced metamorphic changes at 2.28 µg/L Inhibition of T_3-induced gene expression at 2280 µg/L	Spontaneous inhibition of TH-induced metamorphic changes at 22.8 µg/L Blocking of T_3-stimulated tail length reduction at 2300 µg/L	According to (OECD, 2012b) there is evidence for *in vivo* anti-thyroid activity with potential adverse effects (developmental/ growth toxicity) in amphibians
Wrinkled frog *Rana rugosa* and Dark-spotted frog *Rana nigromaculata*		Inhibition of T_3-induced metamorphosis at 2.28 µg/L and 22.8 µg/L	See above

5.9 Amphibians: In vivo effects with regard to an oestrogen-like endocrine mode of action

5.9.1 Approach used for assessing the oestrogen-like adverse effects in amphibians

Like all vertebrates, reproduction in amphibians is under the control of the hypothalamic pituitary gonadal (HPG) axis (Kloas et al., 1999). Oestrogens and androgens are mediators of this endocrine system, directing the development and physiology of sexually-dimorphic tissues.

There are three distinct phases in the life cycle of amphibians when this axis is especially active: (1) gonadal differentiation during larval development, (2) development of secondary sex characteristics and gonadal maturation during the juvenile phase and (3) functional reproduction of adults. Each of these three developmental windows are likely susceptible to endocrine perturbation by certain chemicals such as oestrogens and androgens, ultimately leading to a loss of reproductive fitness by the organisms.

In order to identify whether or not Bisphenol A induces also ***oestrogen-like effects*** in amphibians, the effects observed are compared to effects observed after exposure to 17ß-estradiol (E2).

Although, no specific guidance is available on how to identify oestrogen-mediated effects and knowledge of vertebrate steroid hormones and their role in normal development and reproduction in non-mammalians is scarce (OECD, 2008; U.S.EPA, 2005) effects of E2 and/or EE2 on larval gonadal sex differentiation and sex ratio of several frog and toad species were shown in a number of studies summarized by (Kortenkamp et al., 2012).

To illustrate oestrogen- mediated effects relevant studies with the African clawed frog *Xenopus laevis* are summarized as examples in addition to the statements above: In a study by Hu et al. (2008) fertilized egg embryos were exposed 1-100 µg E2/L through metamorphosis and could develop in untreated media for two months after metamorphosis. Compared to controls, intersex gonads (11%) occurred and sex ratio skewed towards females at the lowest concentration (1 µg

E2/L with an increase to 99% females at 10 µg/L E2. Sharma and Patino (2010) used a similar design to study the effects of E2 on gonad structure and metamorphosis. Compared to controls 1 µg/L E2 (nominal) enhanced intersex gonads (10%) and sex ratio significantly skewed towards females. This confirms the findings of (Hu et al., 2008) that about half of would-be genetic males appeared to be partially or fully sex reversed by exposure to 1 µg/L E2.

Gyllenhammar et al. (2009) examined the effects of 17α-ethinylestradiol (EE2) on related frog species *Silurana (Xenopus) tropicalis*. Exposure of tadpoles to 1.8 ng/L EE2 was shown to lead to significant increase in females (72%) compared to controls.

Further "feminizing effects" of these oestrogens on larval reproductive development in other anuran species were summarized by Kortenkamp et al. (2012).

While oestrogens are not considered to lead to direct effects on the thyroid axes in amphibians, there is some evidence for hormone system cross-talk between sex steroids and thyroid axes as stated in a review by Pickford (2010). In addition, a delay in metamorphosis due to exposure to E2 was reported (Hu et al., 2008; Pickford et al., 2003; Sharma and Patino, 2010).

One Amphibian Metamorphosis Assay with E2 was performed during the validation process guideline development for the OECD 231 guideline. In this study, E2 did not affect developmental stage or thyroid histopathology. Nevertheless, a small but significant reduction in hind-limb-length relative to controls was found starting at concentrations of 2 µg/L (OECD, 2008). Even though the actual mechanism exerted by E2 is still unclear, there is a possibility of an amphibian specific hormone system cross-talk between sex steroids and thyroid axes.

Table 21: Summary of endpoints indicating oestrogen activity considered during data analysis (amphibians)

Endpoints indicating an oestrogenic MoA (from amphibian in vivo tests)	**Apical endpoints considered to be sensitive to an oestrogenic MoA (according to LAGDA)**
• Elevated levels of plasma VTG in males • Advanced development (NF stage 62) • Delayed development in absence of non-specific systemic toxicity	• Female biased sex ratio

5.9.2 In vivo effects with regard to an oestrogen-like mode of action in amphibians

With respect to an oestrogen-like mode of action of Bisphenol A, nine tests covering two species were assessed for amphibians (see Table 22). These studies support the oestrogen mode of action also observed in fish (see chapter 5.5).

Comparing the above-mentioned results obtained with E2 and other oestrogenic substances with results from studies performed with Bisphenol A, there is some evidence for an oestrogen-like effect of Bisphenol A. In the studies described below *in vitro* induction of Vitellogenin, *in vivo* intersex gonads and the shift in sex ratio results from the exposure of tadpoles to Bisphenol A. This is similar to the results with E2.

In vitro studies investigating oestrogen-like effects of Bisphenol A are (Kloas et al., 1999; Lutz et al., 2005; Mitsui et al., 2007; Suzuki et al., 2004a) as well as (Oehlmann et al., 2009). These studies observed a Vitellogenin induction and compared it with 17ß-estradiol (E2) and nonylphenol (NP) which showed similar effects.

In the studies conducted by Pickford et al. (2003) a NOEC value of 500 µg/L for *Xenopus laevis* (NF stage 43/45) after 90 d of exposure based on larval survival, adult growth and sex ratio was the result as it was the highest concentration tested.

The EU risk assessment reports a study with *Xenopus laevis* (NF stage 38/40) with a lower effect concentration of 7.3 µg/L for sex ratio and reproduction after 120 days of exposure (geometric mean of two experiments) (Levy, 2004).

As worked out in the RAR 2008, it is not clear why the results of (Pickford et al., 2003) and (Levy et al., 2004) are different. The experimental design is not the same in the test but also the true NOEC is uncertain. According to (Iwamuro et al., 2006) the only obvious difference between the (Pickford et al., 2003) and the studies of Kloas et al. (1999), to which also the studies of Levy (2004) belongs, was the stage of tadpoles examined. According to (Bogi et al., 2002) in Kloas' group, the level of 17β-estradiol in the whole body of *Xenopus* larvae at stage 38/40 was 10-fold that at stage 43/45 of Pickford's group. Therefore, this suggests that endogenous hormone levels of test animals are very important to assess endocrine disruption by BPA in tadpoles. Because of the accumulation of thyroid hormones in plasma, the tadpoles at stages from onset to end of metamorphosis can be affected easily by BPA, more than the tadpoles at earlier and later stages (Iwamuro et al., 2006). In the RAR 2010, a geometric mean was used, pointing towards a NOEC-value of 60.4 µg/L for sex ratio and reproduction.

Oehlmann et al. (2009) conducted a flow-through test starting with 25 stage 46 tadpoles per replicate and 4 replicates per treatment. The experimental concentrations were 0.228 – 2.28 – 22.8 – 228 µg Bisphenol A per L and in addition a negative and a positive (0.2 µg E2/L) control. No effects on gross morphological sex were determined but the histological analysis revealed effects on testicular structure in the same concentration range as the effect on growth and body weight (increase) (LOEC 2.28 µg/L).

As discussed above, in contrast to Pickford et al. (2003) Levy (2004) observed effects on sex ratio which was skewed towards females. Oehlmann et al. (2009) did not observe effects on sex ratio, but changes in testicular structure.

In summary, also in amphibians oestrogen like effects were observed. This is indicated by skewed sex ratios towards females and the induction of Vitellogenin in males, which is also observed for 17β-oestradiol. In one study (stage 43-45, 17ß-oestradiol lower) no effects on sex ratio were observed; in one study (stage 46, 17ß-oestradiol unknown) only effects on testicular structure but not gross-morphological sex were observed; one study (stage 38/40, 17ß-oestradiol higher) observed clear effects on sex ratio.

Table 22: Summary of oestrogen-like effects of Bisphenol A in Amphibians

Species	Reference	Test Conditions	Endpoints	Effect concentrations [µg/L]	Indication for ED	Reliability
Xenopus laevis Key study	(Kloas et al., 1999)	method developing study: 2-3 post-hatch tadpoles/ app. 84 d up to stage 38/40 (Nieuwkoop and Faber 1975) semi-static/ 23 - 2.3 µg/L (nominal)	sex ratio (increased number of female phenotypes)	NOEC 2.3 LOEC 23 (36f:64m) (Control 60f:40m) E2: 2.7 sex ratio 70% w	E2 caused VTG-mRNA induction: 0.27 (start) to 272 µg/L (saturation) (10-9 to 10-6 M) after 36h (dose-dependent) NP stimulated VTG-mRNA synthesis at conc. of 0.022 µg/L (10-8 M) BPA stimulated VTG-mRNA synthesis at conc. of 22.8 µg/L (10^{-7} M)	3 - relevant methodological deficiencies; nominal concentrations used; reproducibility? = only a method developing study
Xenopus laevis Key study	(Levy, 2004)	120 d, 2 experiments: semi-static; 2 replicates / 1) 2.3 - 23 (nominal) + 17ß-estradiol as positive control 2) additionally 230 (nominal) WR 70%	1) sex ratio (geometric mean) (sex reversal) 2) reproduction; feminization, reproduction	1) NOEC 2.3 both endpoints geo mean 1st + 2nd exp.: 7.3; LOEC 23 (70f:30m); (Control 48f:52m) 2) no significant differences in mortality, time to metamorphosis		follow-up (Kloas et al., 1999): 2 - well documented study; chemical analysis; deficiencies (replicates: 2 which were pooled; conc.: maybe not high enough tested)
Xenopus laevis Key study	(Pickford et al., 2003)	90 d flow-through; 4 replicates; 4d old larvae (NF stage 43)6; expo app. 2dph; according to: ASTM, 1991. (FETAX).	1) larval survival 2) sex ratio	1) NOEC 500 2) NOEC ≥ 500 no significant difference from expected 50:50 sex ratio; feminization in positive control		1 - guideline study; GLP; chemical analysis Repeat (Kloas et al., 1999)

Species	Reference	Test Conditions	Endpoints	Effect concentrations [µg/L]	Indication for ED	Reliability
		1 - 2.3 - 10 - 23 - 100 - 500 (nominal) + 17ß-estradiol as positive control	3) other	group; 3) no gross gonadal abnormalities; no sign difference in time to metamorphosis; no sign difference in total length…		
Xenopus laevis Key study	(Oehlmann et al., 2009)	according to (Lutz et al., 2008); flow-through; 4 replicates; 25 tadpoles per replicate/ 7dpf to 82 dpf (NF stage 46 to 66)/ control – 0.2 µg E2/L - 0.228 - 2.28 - 22.8 - 228 µg/L	1) gross morphological sex 2) detailed histological analysis of testes 3) growth and body weight in males increased 4) induction of Vitellogenin gene expression in liver	1) NOEC 228 E2-positive control 0.2 µg/L: 19% males, 72% females, 9% mixed sex Control: 45% males, 50% females 2) testicular lacunae: 15 – 20 – 10 – 25 % at 0.228 – 2.28 – 22.8 – 228 µg/L; oogonia: 40 – 60 – 55 – 60 % at 0.228 – 2.28 – 22.8 – 228 µg/L → NOEC 0.228 3) NOEC 0.228 LOEC 2.28 4) NOEC 22.8 LOEC 228	demonstrates oestrogenic effects supported by the induction of hepatic IGF-I expression at 2.28 µg/L and above	2 – well documented publication
Xenopus laevis Supporting study	(Sone et al., 2004)	1 per well; 24 well microplate; 3 replicates/ 1 - 2.5 - 5 - 10 - 15 - 20 - 25 - 30 µM (0.228 - 0.57 - 1.41 - 2.3 - 3.4 - 4.6 - 5.7 - 6.8 mg/L) nominal + <0.1% ethanol	abnormal gut coiling, oedema, microcephaly, and decreases in body length	NOEC 2280 LOEC 4560 sensitive window for BPA toxicity only at the earliest developmental stages esp. 3-12 h p.f. (stage 6-11); later than 12 h p.f. no gross morphological abnormalities found		2 – well-documented publication
Hyla japonica (Japanese tree frog) Supporting study	(Kohno et al., 2004)	Sexually immature frogs (snout vent length 22.7 mm); non-breeding season; 25 ± 3 °C; 12 h light/d; 120 µg/animal injected intraperitoneally	reduction of basal water absorption of male pelvic patches	120 µg/ animal	known oestrogenic response	2 – well-documented publication
Xenopus laevis Supporting study	(Suzuki et al., 2004a)	Competitive enzyme immunoassay (EIA) for Xenopus ERα Oestrogen	Relative binding affinity	IC_{50} (BPA) = 107.3 µg/L [470 nM]; IC_{50} (DES) = 1.2 µg/L [4.5 nM]	Relative binding affinity of BPA of 0.957%	2 – well documented study; Mentioned in EU RAR 2010

Species	Reference	Test Conditions	Endpoints	Effect concentrations [µg/L]	Indication for ED	Reliability
		binding assay using the kit: Ligand Screening System-ERα replacing the human ERα with Xenopus glutathione-S-transferase- ERα-ligand binding domain (GST- ERα-LBD)			compared with diethylstilbestrol (DES) (IC_{50} of DES divided by IC50 of BPA)	
Xenopus laevis Supporting study	(Lutz et al., 2005)	primary cultured hepatocytes	Time course of free ER determined by radioreceptorassay (RARA)	BPA, NP and E2 led to immediate drops in the free ER levels followed by significant increases BPA: sign increase at 10^{-7} M (23 µg/L)		2 – well documented study; Mentioned in EU RAR 2010
Xenopus laevis Supporting study	(Mitsui et al., 2007)	hepatocytes from adult male frogs; 0.04 nM 17ß-estradiol (E2), 0.03 nM EE2, 0.03 nM DES, 17 nM E1, 0.9 nM E3, 14 nM α-E2, 740 nM NP, 1800 nM OP, 530 nM BPA; control with 0.1% DMSO; 96-well tissue culture plates; 22°C; 6 days test duration; ELISA (measuring VTG and serum albumin) + second antibody [improved detection limit for Xenopus VTG = 0.06 ng/mL]	VTG induction Antagonistic activities in E2-dependent VTG induction	VTG-inducing (2 ng/mL) activity: LOEC 0.02 nM (E2, EE2, DES), 100 nM (BPA, NP), 200 nM (OP) Antagonistic activities in E2-dependent VTG induction: LOEC 800 nM (BPA), 3000 nM (NP), 5000 nM (OP)	Relative oestrogenic activity of BPA 0.008%, NP 0.005%, OP 0.002% of E2-activity	1 – very well documented; estimates for EE2, DES, BPA, NP, OP fit those in the yeast reporter gene assays BPA is a potent anti-thyroid hormone and anti-androgen hormone

5.9.3 Summary on the plausible link between adverse effects and indications for the underlying oestrogen-like MoA in amphibians

Tests with *Xenopus laevis, Xenopus (Silurana) topicalis, Rana rugosa* and *Rana nigromaculata* are available, supporting the conclusions on the oestrogen-like activity of Bisphenol A. Some of them (Kloas et al., 1999; Levy, 2004) show an increased number of female phenotypes in *Xenopus laevis* exposed from 2-3 dph tadpoles for over 90 d at concentrations of 2.3 and 23 µg /L BPA. The NOEC for sex ratio in this test is reported as 2.3 µg/L or by using the geometric mean from the first and second experiment (7.3 µg/L, see also chapter 3.2.1.4.2 of the complete Risk Assessment Report (European Commission, 2010)). The occurrence of more female phenotypes seems to be an oestrogen-mediated effect but as described above there is no specific guidance yet how to identify these effects exactly.

With vitellogenin induction and a sex ratio skewed towards females in one study, similar to the effects seen in fish species, there is some evidence for an oestrogen mode of action with a direct plausible biological link to adverse effects in amphibians (OECD, 2015).

Table 23: Plausible biological link between oestrogen-like MoA and adverse effects in amphibian

Species	Evidence for MoA	Apical effect	Comparison MoA and apical effect
African clawed frog *Xenopus laevis*	Vitellogenin induction at 22.8 to 228 µg/L Effect on testicular structure at 2.28 µg/L	Sex ratio skewed towards females at 23 to 228 µg/L	Some evidence for oestrogen MoA (OECD, 2015) with a direct plausible biological link to adverse effects in amphibians

5.10 Approach used for assessing the endocrine activity in invertebrates

Due to the large taxonomic variety, invertebrate groups differ in their endocrinology. Even though less is known for the endocrine systems of invertebrates than for vertebrates, progress has been made during recent years. Invertebrates are known to respond to vertebrate type sex steroids - although sometimes in higher concentrations than vertebrates (LeBlanc, 2007). Although some vertebrate hormones, such as Oestradiol, are found in invertebrate phyla covering cnidarians, crustaceans, molluscs, or echinoderms, the exact roles are not clear. Indeed, oestrogens are not the major class of hormones in invertebrates. A variety of other hormones regulate developmental and reproductive processes in invertebrates, such as e.g. the ecdysteroids and juvenoid hormones in arthropods.

There is no specific guidance document for evaluating chemicals for an endocrine mode of action in invertebrates yet. OECD standard test guidelines on partial or full-life cycle testing for invertebrate species are currently being developed (e.g. for molluscs, daphnids, copepods, chironomids and mysids), which are referred to in the OECD conceptual framework for testing and assessment of endocrine disrupters (GD 150).

Comprehensive overviews on the endocrine systems, hormones and possibilities for an interference of endocrine mediated pathways in invertebrates are (among numerous others) for example given in de Fur et al. (1999), Oetken et al. (2004), LaFont (2000) or Oehlmann and Schulte-Oehlmann (2003). A special edition of Ecotoxicology (Volume 16 (1): 1-238) on endocrine disruption in invertebrates comprises reviews for crustaceans (LeBlanc, 2007; Tatarazako and Oda 2007), non-genomic pathways (Janer and Porte 2007), and more general aspects (Gourmelon and Ahtiainen 2007, Hutchinson 2007, Weltje and Schulte-Oehlmann 2007).

Detailed OECD review papers on life-cycle toxicity testing exist for molluscs (no. 121, (OECD, 2010b) and arthropods (no. 55, (OECD, 2006), which both focus on developmental, reproductive and consider endocrine effects.

Specifically for Bisphenol A, a summary on proposed modes of action of Bisphenol A and effects on invertebrates can be found in a review of (U.S.EPA, 2005), however, not being comprehensive and not including results of more recent studies. In addition to a multitude of original research papers addressing the modes of action and observed adverse apical effects, several review papers summarise the observed effects of Bisphenol A on invertebrate organisms (Flint et al., 2012; Kang et al., 2007; Segner et al., 2003a; Zou 2010, Grineisen 1994, LaFont 2000, Subramoniam 2000, Fingerman et al. 1998).

The analysis aims at clarifying

1) whether BPA influences the endocrine system of invertebrates and provide evidence for an endocrine mode of action, and
2) whether observed adverse effects on invertebrate species are likely to be a consequence of this alteration and are therefore assumed to be endocrine mediated.

The *in vivo* studies provide evidence for the adverse effects on organisms and also may indicate an endocrine mode of action. Apical endpoints which may indicate a possible underlying endocrine mode of action and may lead to severe adverse effects on the organism and population level are in particular effects in the life-cycle on developmental and reproductive processes such as e.g. embryo malformations, a delayed development or moulting, an impaired reproduction or altered sex ratios, depending on the organism group. Linked to *in vitro* or biomarker studies, as e.g. receptor-binding studies, gene expression profiles, measurement of hormone titers (e.g. OH-ecdysone) or enzyme activities/protein levels (such as the yolk protein vitellogenin), and information from a co-exposure to known or suspected antagonists mitigating the effects, or a comparison with (xeno-)oestrogens, this may give an indication whether the adverse effects are a consequence of an alteration of the endocrine system.

In the following analysis, we subsequently evaluate the available data for molluscs, crustaceans, insects and further invertebrate groups and give specific details on the respective approach there. For all taxa the available in vitro and biomarker data is analysed as well as the available *in vivo* data.

5.11 Molluscs: in vitro and in vivo effects with regard to an endocrine mode of action

5.11.1 Reference documents and data basis for molluscs

For molluscs, a detailed OECD review paper with a focus on full life cycle toxicity testing (OECD 2010) is available and two OECD test guidelines (*Potamopyrgus antipodarum* OECD 242 and *Lymnea stagnalis* OECD 243) were adopted in 2016. Furthermore, overviews are given e.g. in (DeFur et al., 1999; Janer and Porte 2007; Zou 2010). In total, 17 studies were analysed covering seven different species. The two prosobranch snails *Marisa cornuarietis*, and *Potamopyrgus antipodarum* were the most extensively studied.

5.11.2 Indicators of an endocrine mode of action: *in vitro* and biomarker studies for molluscs

The available *in vitro* receptor binding, gene/protein expression or biomarker studies are summarised in Table 24.

Snails respond to chemicals that are oestrogen agonists (as well as androgens) in vertebrates. Although, beside neuroendocrine hormones, vertebrate-type steroids have been shown to be present and synthesized in molluscs, their specific role and site of action is not resolved (e.g. de Fur 1999). Recently, oestrogen receptor orthologues have been identified in several mollusc taxa (as diverse as snails, bivalves and octopus), which

however failed to bind to vertebrate estrogens *in vitro* (e.g. Thornton et al. 2003, Keay et al. 2006, Kajiwara et al. 2006, Matumoto et al. 2007). Hence, the actual functionality of this ER receptor orthologue *in vivo* still needs clarification.

Still, it was shown for *Marisa cornuarities* in a competitive receptor displacement experiment, that Bisphenol A binds to an oestrogen-specific binding site and displaces 17ß-estradiol with a greater affinity than the natural ligand 17ß-oestradiol (Oehlmann et al., 2006). Furthermore, the observed *in vivo* effects of BPA are antagonised by coexposure with known oestrogen receptor antagonists (Oehlmann et al., 2006). In the same species, Bannister et al. (2013) showed an increased reporter gene expression after BPA exposure at the highest tested concentration (2.2 mg/L) in an *in vitro* screening test with an orthologue of oestrogen-related receptor (mcERR) of *Marisa cornuarietis*, although other potential ligands (such as EE2, OP) failed in other tests to interact with the receptor *in vitro* and exposure of E2 *in vivo* did not significantly affect gene transcription levels.

Also in *Potamopyrgus antipodarum* an oestrogen receptor analogue was identified and its expression was shown to be transitory affected after exposure to 40 µg/L Bisphenol A (increase of ER-mRNA). This was linked to an increase of embryo numbers *in vivo* (Stange et al., 2012). Similar *in vivo* effects were also found for EE2 (Stange et al., 2012).

There has been some dispute about the possible presence of ER-like receptors in mollusc. It has to be kept in mind that other receptors and also other endocrine pathways (e.g. neuropeptides, non genomic MoA) are involved in the endocrine regulation in molluscs (e.g. de Fur 1999, Janer and Porte 2007). Hence, vertebrate-type (xeno-)oestrogens may interfere with the endocrine systems in mollusc via other pathways. The clear functioning of putative receptors and their interaction with chemicals is still unclear.

In two studies, effects of BPA on VTG-like proteins were studied. Gagnaire et al. (2009) observed an increase in VTG-like protein levels after 14 d at 100 µg/L BPA (and 1 µg/L OP) in *Potamopyrgus antipodarum* depending on the dose and duration of exposure. Effects on necrosis in ovo-testes were observed for another *Valvata piscinalis* (Gagnaire et al. 2009). In the bivalve Mytilus edulis, an induction of VTG levels was observed together with malformations of gonads at 50 µg/L (Aarab et al., 2006).

Effects of BPA on gene regulation were analysed in *Haliotis diversicolor supertexta* exposed to BPA by Zhou et al. (2011). An altered expression of three genes involve in steroid metabolism, cell cycling and proten phosphorylation were discerned as well as cellular disturbances, but no clear indications for a specific mode of action leading to the observed effects in vivo (embryo malformations, developmental delay) (Zhou et al. 2011).

Table 24: Results from in vitro and biomarker studies for Bisphenol A supporting the evidence for the MoA in molluscs

Species	Reference	Test conditions	Test type/endpoints	Effect/concentration	MoA Indication	Comment/reliability
Marisa cornuarietis	(Oehlmann et al., 2006)		Receptor displacement experiments in cytosolic extracts	BPA displaces 17ß-estradiol from binding sites with a greater affinity than 17-ß oestradiol. +androgen specific binding	Oestrogen antagonist possess different receptors responding towards oestrogen and testosterone	4 Study results described within article, no full documentation of methodological details
Marisa cornuarietis	(Bannister et al., 2013)	10^{-4} to 10^{-10} M BPA	Ligand screening in vitro tests with mollusc oestrogen related receptor (mcERR)	Increased reporter gene expression at 10^{-5} M BPA (relatively high conc. 2.2 mg/L)	Results suggests that natural and anthropogenic ligands for receptors exists	2 Additional experiment in this study, focus was on gene transcription after OP, E2 exposure
Potamopyrgus antipodarum	(Stange et al., 2012)	Real time PCR of exposed snails to 40 µg/L BPA (sublethal effects on reproduction)	Identification and expression of oestrogen receptor (mRNA) Reproductive output	An ER-like transcript was identified, with an aminoacid identity of 84% of the human ERa, and 38% of ligand binding domain Increase of ER-mRNA Increase in embryo numbers	Receptor and expression identified after BPA exposure, linked to *in vivo* effects	2, no analytics EE2 also increased expression of ERmRNA, while antagonist methyltestosterone decreased it
Valvata piscinalis (hermaphrodite) *Lithoglyphus naticoides* (sexual) *Potamopyrgus antipodarum* (asexual)	Gagnaire et al. 2009	ALP technique, PBL assay and gradient gel electrophoresis 14 and 28 d exposure to BPA, OP, TBT	VTG-like and yolk proteins analysed, species specific bands	Increase in VTG-like protein levels after 14 d at 100 µg/L BPA (1 µg/L OP) in P. Significant necrosis in ovo-testes of V. for all compounds, but not for P.		2 Method development to track changes in Vg-like proteins
Haliotis diversicolor supertexta	(Zhou et al., 2011)	0.05 – 0.2 - 2 - 10 mg/L 9 h embryo test with fertilized eggs	physiological parameters Expression of reference genes	Disturbance of cellular ionic homeostasis and osmoregulation, oxidative damage. Downregulation of the PC1 gene and upregulation of CB and CDK1 genes, responsible for cellular endocrine regulation	Gene regulation affected, possibly affecting cellular endocrine regulation (see *in vivo* results)	2 - well-documented study but no guideline used
Mytilus edulis	(Aarab et al., 2006)	50 µg/L (3 w flow-through)	alkali-labile phosphate assay vitellogenin-like protein levels	female mussels had slightly increased VTG levels, males had similar levels to the controls	MoA unclear, but VT affected, linked to effects *in vivo* (gonadal damage)	2, but only 1 conc. Discussed in RAR (See also Tab. 25)

5.11.3 Indicators for adverse effects and endocrine mode of action: *in vivo* studies for molluscs

In vivo data indicating adverse apical effects in molluscs (snails and bivalves) that are possibly endocrine mediated are summarised in Table 25. Here, also co-exposure with vertebrate oestrogen-antagonists or a comparison with known effects for oestrogens were taken as supporting indicators.

Typical apical effects in gastropod and bivalve molluscs that are similar to effects of (xeno-)oestrogens are:

- The interference with reproductive processes associated with an imbalance of steroid hormones
- enlarged sex organs ("superfemales")
- a stimulation of oocyte/embryo production at lower concentrations or reduction at higher concentrations
- embryo abnormalities

Characteristic effects that might indicate an oestrogen-like mode of action in snails include an observed "super-feminisation" (i.e. a hypertrophy of the uterus and capsular gland) leading to a higher mortality, an increased egg production or spawning mass, enlarged reproductive tracts and glands or an advanced timing of oogenesis (Oehlmann et al., 2006). As pointed out by U.S. EPA (2005), the effects of Bisphenol A exposure in snails are consistent with feminization effects and, therefore, could be the result of effects on oestrogen or oestrogen-analogue signalling.

The most extensive studies revealing effects of BPA and their underlying modes of action were performed with *Marisa cornuarietis* by Oehlman and co-workers (Oehlmann et al., 2007; Oehlmann et al., 2006; Oehlmann et al., 2000; Schulte-Oehlmann et al., 2001). These studies revealed extremely low effect concentrations in the ng/L range, well below 1 µg/l. Exposure to Bisphenol A in a semi-static test leads to an overproduction of eggs as well as malformations of gonads and induction of "superfemales" at concentrations of < 0.048, 1 µg/L,EC_{10} 0.0139 µg/L respectively, as calculated by Oehlmann et al. (2000). The effect on egg production was only observable during a seasonal period with natural low egg production and effects were not observed during the seasonal reproduction period when egg production is naturally high. Results on egg production were confirmed by Oehlmann et al. (2006) with again extremely low effect concentrations (EC_{10} 0.0148-2.1 µg/L depending on statistics). The studies by Oehlmann et al. (Oehlmann et al., 2006; Oehlmann et al., 2000; Schulte-Oehlmann et al., 2001) have been discussed extensively (European Commission, 2008; Dietrich et al. 2006 and Oehlmann et al. 2006b) with regard to the uncertain origin of the breeding population (strain optained from Aquazoo Düsseldorf, with unclear inbreeding), the test design (breeding groups instead of breeding pairs), the difficulties in estimating measured concentrations due to a decrease in test concentrations between renewal cycles in the semi-static test design and the statistical analysis performed. Although the studies and in particular the extrapolated estimates of effect concentrations have to be handled with care due to the statistical deficiencies. The studies (Oehlmann et al., 2006; Oehlmann et al., 2000; Schulte-Oehlmann et al., 2001) may indicate that this species responds extremely sensitively to Bisphenol A. Definite effects occur at ≤ 0.25 µg/L (nominal) and EC_{10} values may be as low as 0.0053 µg/L (embryo production, based on TWA conc.) according to a recalculation of the data (Ratte, 2009). A simultaneous exposure of Bisphenol A and an anti-oestrogen completely antagonized the stimulatory effect of BPA on egg production in this study, which provides indications that the mode of action is similar to known (xeno-)oestrogens. Moreover, the observed additional female sex characteristics, enlargement of female sex glands and oviduct malformations leading to a higher mortality of females give evidence for the disruption of sexual development and adverse effects, which cannot be neglected. Overall, the effects observed in the succeeding studies of Oehlmann et al. were reproducible,

differences between different laboratory tests are explainable, the studies support the hypothesisfor effects occurring at low concentrations, and indicate similar effects than known (xeno-)estrogens.

Later studies (Forbes et al., 2007a and b, Forbes et al. 2008) with the freshwater snail *Marisa cornuarietis* were carried out with a high standard of quality with a suitable level of replication and test design under flow-through conditions but could not replicate the effects observed by Oehlmann et al. (2000, 2006 and 2007). The study revealed only effects at higher concentrations (NOEC juvenile growth 25 µg/L) and no effects on fecundity at 22°C (tested additionally at 1 test concentration) and 25°C. The evaluated endpoints only comprised mortality, adult fecundity, hatchability and juvenile growth. It is not fully comparable with the Oehlmann studies and could not reveal the potential increase of egg production as an endocrine mediated effect. Reasons for this may be that it was performed with another (tropical) strain and did not consider the seasonality of reproduction. Seasonality is very important as the increase in egg production is only significantly observable during a season of low spawning behaviour and thus was masked in the study of (Forbes et al., 2007a and b). Moreover, reproductive traits may be more variable in this species at lower temperatures, which could explain the differences of the studies. It has also been questioned whether an active metabolite of Bisphenol A (MBP, 4-methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene) that shows a much higher oestrogenic activity than BPA in fish (medaka) in studies of Ishibashi et al. 2005 (cited in Kang et al., 2006) could have elicited the high toxicity and effects in the studies by Oehlmann et al. as the degradation and formation of metabolites could have been more prominent due to the semi-static exposure design and not been detected in the studies under flow-through conditions of Forbes et al.. However the toxicity of the metabolite to snails is still unknown.

Another important and extensively studied species is the prosobranch mud snail *Potamopyrgus antipodarum.* For this species an OECD test guideline was adopted in 2016. (Duft et al., 2003) observed a stimulation of embryo production and estimated a NOEC of < 1µg/kg for the species exposed via sediment. Also when exposed via water the effects on embryo production were demonstrated and a low NOEC of 1 µg/L and significant effects at 5 µg/L (nominal) were estimated by (Jobling et al., 2003), corrected 2004). (Jobling et al., 2004) showed in studies with an oestrogenic sewage effluent (EE2) that embryo production was enhanced at lower, but decreased at higher concentrations and revealed the same U-shaped concentration-response relationship for BPA on snails as observed for OP and EE2 and similar effects as for fish (fathead minnow). Also (Sieratowicz et al., 2011) observed a stimulation of embryo numbers in *Potamopyrgus antipodarum* after exposure to low concentrations (5-20 µg/L). An additional study of Benstead et al. (2008), discussed in the transitional RAR of UK (ECHA 2009), reported a significant stimulatory effect on the reproductive output (embryo numbers) of *Potamopyrgus antipodarum* after 28d exposure at concentrations of 0.2 µg/L (nominal, measured: 0.168 µg/L). Athough the study had some drawbacks with respect to analytics (i.e. BPA was detected in the controls, but test concentrations were maintained during the 28d exposure and test concentrations where first effects were detected were 5 times higher than in the controls), it supports stimulatory effects at low effect concentrations. An increased embryotoxicity was also observed for the pulmonate freshwater snail *Physa acuta* by (Sanchez-Arguello et al., 2012). However, this species was first affected in much higher concentrations (LOEC 500-1000 µg/L) compared to other species.
For the marine snail *Nucella lapillus* Oehlmann et al. (2000) observed an enhanced oocyte development and enlarged sex glands at low concentrations (NOEC < 1 µg/L nominal, no analytical confirmation).

For the marine sea snail *Haliotis diversicolor supertexta* (Liu et al., 2011) observed a failure of embryo development and malformed larvae at low concentrations (EC_{50} values of 0.18 and 1.02 µg/L for reaching trochophore stage and metamorphosis respectively, nominal, but analytics confirmed -3+8%). The disturbances of embryonic development (malformations and developmental delay) are supported by (Zhou et al., 2011) for the same species in higher - but still comparatively low - concentrations (NOEC 50 µg/L). The effects were related to an observed down-/up-regulation of genes responsible for the

cellular endocrine regulation.

For bivalves less data is available. In one study with *Mytilus edulis* (Aarab et al., 2006), increased vitellogenin levels in females were demonstrated after exposure to 50 µg/L BPA (only one concentration tested) in vivo. Aarab et al. (2006) also observed an induction of spawning in males and females and a damage of ovarian follicles and oocytes in females.

Table 25: Summary of apical effects of BPA on molluscs from in vivo tests (including indications for underlying mechanisms/modes of action if evaluated in the respective studies)

Species	Reference	Test Conditions	Endpoints	Effect concentrations	Indication for ED	Comment/reliability
Marisa cornuarietis	(Oehlmann et al. 2000)	1 – 5 – 25 - 100 µg/L, semi-static 20-22 °C 5 m - 12 m Parental and full life cycle	Increased egg production Malformations of gonads	LOEC egg prod. <0,048 µg/L Malformations < 1 µg/L (EC_{10} 0,0139 µg/L) Superfeminisation (enlarged accessory pallial sex glands, gross malformations, palial oviduct section), at all conc., increased female mortality; stimulation of oocyte and spawning mass production; conc. dependent increase of cumulative number eggs	overproduction of eggs during a seasonal period with natural low egg production (effects are not observed during the seasonal reproduction period when egg production is natural high)	2 No analytics discussed in RAR
Marisa cornuarietis	(Schulte-Oehlmann et al., 2001)	0.05-1 µg/L 180d exposure	Egg production Superfemales mortality	7.9 ng/L NOEC (egg production) 13.9 ng/L EC10 0.05 µg/L superfeminisation Causing mortality	Superfeminisation, increased egg production	2 Analytics (only 15-48% of nominal concentrations) discussed in RAR
Marisa cornuarietis	(Oehlmann et al. 2006)	0.25 – 0.5 – 1.5 - 5 µg/L nominal, semi static, 5m 20 (and 27) °C Coexposure with oestrogen receptor antagonists Tamoxifen and Faslodex (ICI 182,780)	Increase egg production, decrease in penis length of males at 20°C	Egg prod. LOEC 0.25 EC_{10} 0.0148 µg/L-2.1 µg/L	Simultaneous exposure to BPA and an anti-oestrogen, stimulatory effect of BPA on egg production was completely antagonized.	2 Well-documented. Analytics, but rapid loss of BPA in test system. Statistical deficiencies: EC_{10} dependent on statistics, possibly overfitting, high SDs Key study in RAR (EC_{10} 0.0148 µg/L only considered together with recalculation and other study)

Species	Reference	Test Conditions	Endpoints	Effect concentrations	Indication for ED	Comment/reliability
						Recalculations of van der Hoeven 2005: EC10 2.1 µg/L, recalculations of UBA (Ratte, 2009): EC_{10} 0.038 µg/L).
Marisa cornuarietis	(Forbes et al., 2007 a and b)	Static renewal/flow-through Nominal: 0.1, 1, 25, 640 µg/L (25 °C) 181 d	Juvenile growth, hatchability, adult fecundity	NOEC juvenile growth (90d) 25 µg/L (15.5 µg/L meas.); LOEC 640 µg/L (429 measured) NOEC (57d) hatchability 640 µg/L NOEC (181d) fecundity 25 µg/L	Not investigated.	2 Analytics. Different strain. GLP, but may have missed effects as the seasonality of reproduction was not considered, and reproductive output was maximized Discussed in RAR (NOEC 25 µg/L used together with Oehlmann study for PNEC)
Marisa cornuarietis	(Forbes et al. 2008)	Static renewal/flow-through 84 d, 25 µg/L nominal (22°C)	Mortality, adult fecundity	NOEC (84d): No effects on fecundity at 25 µg/L	Not investigated.	2-3, see above (single conc., study conducted at 22°C to support results of previous study (Forbes et al. 2007a and b))
Marisa cornuarietis	(Schirling et al. 2006a)	0-50-100 µg/L 14 d, water renewal, petri dishes	Formation of eyes/tentacles, heart rate, hatching, weight after hatching	Increase of weight of newly hatched snails and decrease of heart rate at 100 µg/L, NOEC 50 µg/L	Compared with 10 µg/L EE2	2 Few conc., study for test development. Discussed in RAR, tentative NOEC 50 µg/L usable)
Potamopyrgus antipodarum	(Duft et al., 2003))	1-10-30-100-300 µg/kg sed (8 w) 15 °C	Embryo production Number unshelled embryos	stimulation of embryo production at all test concentrations ≥ 30 µg/kg dw (NOEC < 1 µg/kg) after 8 w	Stimulation embryo production	2 No analytics of sediment possible as test conc. below LOD (5

Species	Reference	Test Conditions	Endpoints	Effect concentrations	Indication for ED	Comment/reliability
				increased production of un-shelled embryos after 2 w 30 µg/kg dw (nominal)		mg/kg) Discussed in RAR (not used for PNEC/SSD, but indicator low effect conc.)
Potamopyrgus antipodarum *Marisa cornuarities* *Nassarius reticulates (all prosobranch snails)*	Duft et al. 2007	P: static, 15 °C, 8 w, lab culture M: semi-static, 22 °C, 5 m, lab culture N: static, 14 °C, 4 w, wild caught	Stimulation of egg production and embryo production, increased weight of glands.	P: EC_{10}: 0.19 µg/kg dw, NOEC water 1 µg/L (8 w) M: EC_{10}:13.9 ng/L (5m) N: NOEC 100 µg/kg dw (4w)	Stimulation of embryo production	2 Guideline development No analytics possible Reference to Duft et al. 2003, also OP; NP, EE2 tested
Potamopyrgus antipodarum	(Jobling et al., 2004) corrected version	Water 1-5-25-100 µg/L Nominal	Increased Embryo production	NOEC of 1 µg/l (63d, nominal) LOEC 5 µg/L (5w) LOEC 5 µg/L (63d, slight effects at 1 µg/L) (100 µg/L: decrease)	inverted U-shaped response (5 and 25 higher but 100 µg/L lower than control), similar to that seen with EE2 (at ng/l levels) no effects on survival	2 No analytics Discussed in RAR, not used for PNEC, but indicator for effects at low conc.
Potamopyrgus antipodarum	Benstead et al. 2008 (cited in ECHA 2009)	Water 0 - 0.2 – 2 – 20 µg/L Nominal (measured: 0.036 - 0.168 – 1.08 – 10.3 µg/L) 28d exposure	Increased Embryo production	LOEC: embryo numbers 0.2 µg/L nom (0.168 µg/L meas) (28d) Effects at 2 and 20 µg/L not significant (high variability, lower offspring numbers at 20 µg/L	Stimulation of embryo production	4 (Not assignable as original report not at hand, only ECHA 2009) Drawbacks in analytics (measured BPA in control) Discussed in RAR, not used for PNEC
Potamopyrgus antipodarum	(Sieratowicz et al., 2011)	5-10-20-40 µg/L 28 d exposure Different temperatures	Increase of embryo numbers, seasonal fluctuations	At 16 °C number of embryos increased NOEC 20 µg/L, at 7 and 25 °C NOEC: 5 µg/L	Stimulation of embryo production	2 Pre validation of OECD test proposal. Analytics, TWA concentrations
Physa acuta	(Sanchez-Arguello et al., 2012)	0.1-0.5-1-2.5-5-10 mg/L	Genotoxicity: micronucleus test	LOEC hatchability: 0.5 mg/L	embryotoxicity	2 Analytics.

Species	Reference	Test Conditions	Endpoints	Effect concentrations	Indication for ED	Comment/reliability
		21 d embryo toxicity tests in microtiterplates (start with 24-96 h egg masses) Micronucleus test, 1w	Embryotoxicity: abnormal development and mortality	LOEC MN induction: 10 mg/L Clear dose response for embryotoxicity (1.5-2.5 µg/L) but not for genotoxicity		
Haliotis diversicolor supertexta	(Liu et al., 2011)	12-96 h embryotest (fert-metamorph). Nominal conc., analytics (3+8%).	embryo development, failure of metamorphosis, malformations (completion of larval metamorphosis indicates survival of embryos, malformed larvae did not survive the stage),	12h EC_{50} (trochophore): 30.7 µg/L 96 h EC_{50} (metamorphosis): 1.02 µg/L 12h HC5 0.18	Developmental malformations	2 - not according to a guideline but very well documented study, analytics, BPA more toxic than NP
Haliotis diversicolor supertexta	(Zhou et al., 2011)	0.05 – 0.2 - 2 - 10 mg/L 9 h embryo test with fertilized eggs	Embryonic development (% abnormalities, hatching, metamorphosis) physiological parameters	NOEC (all parameters): 50 µg/L LOEC: 200 µg/L	Malformations, developmental delay. See also effects on Gene regulation (in vitro)	2 - well-documented study but no guideline used
Nucella lapillus	(Oehlmann et al. 2000)	wild caught, 3 m Nominal 1-25-100 µg/L Sea water, 14°C	enlarged pallial sex glands; enhancement of oocyte production; no oviduct malformations; gonad resorption	NOEC < 1 µg/L, LOEC 1 µg/L	Oocytes enhanced	2 No analytics discussed in RAR (additional evidence)
Mytilus edulis	(Aarab et al., 2006)	3 w, flow-through, one conc.: 50 µg/L	Gonadal development, vitellogenin-like protein levels	Expression of phosphor proteins in females, VTG levels induction of spawning in males and females, damage of ovarian follicles + oocytes in females (LOEC 50 µg/L)	Gonadal damage.	2, but only 1 conc. Discussed in RAR but not used for PNEC, VTG not certain

5.11.4 Summary and indications for a possible link between endocrine mode of action and adverse effects in molluscs

A summary of the studies analysed above is given in Table 26 providing an overview on the indications for a possible link between the observed effects *in vitro* and *in vivo*. In particular for the two prosobranch snails a link between an endocrine MoA and increased embryo production *in vivo* is possible. The apparent sensitivity and observed effects of these two snails towards BPA is supported by studies with other mollusc species.

Bisphenol A acts similar to (xeno-)oestrogens in molluscs. For *Marisa cornuarities* and *Potamopyrgus antipodarum* a BPA may bind to an oestrogen-like receptor that triggers mRNA expression and exerts oestrogen-like in vivo effects were mitigated by anti-oestrogens. The characteristic effects elicited comprise an increase of egg numbers, the induction of superfemales and malformations of the genital tissues. Similar effects on enlarged sex glands and an enhanced oocyte production, and VTG induction and malformed gonads were observed for the marine snail *Nucella lapillus* and the marine bivalve *Mytilus edulis* respectively. In the pulmonate freshwater snail *Physa acuta* and the marine abalone *Haliotis diversicolor supertexta,* an abnormal development of embryos (delay, malformations) and a high embryotoxicity became evident.

It is possible that the observed adverse effects are endocrine mediated, although the precise mechamnism is not clarified yet. Oestrogen-like receptors were shown to play a role, although further endocrine regulated pathways are known to be prominent in molluscs. For two species studies have observed *in vitro* findings together with in vivo effects. Here, consistent effects, i.e. increased reproduction and abnormal reproductive organs were observed after exposure to BPA as well as to oestrogens. Moreover, an abnormal embryonic development (delay, malformations) was obsereved in three different species. The disturbed embryonic development and reproduction may involve an endocrine mode of action of Bisphenol A. Effects are associated with reproductive and developmental disturbances, which may lead to unpredictable population effects in the long term.

The two prosobranch snails *Marisa cornuarities*, and *Potamopyrgus antipodarum* are in particular sensitive towards Bisphenol A. Molluscs were the most sensitive organism group analysed, although the determination of the lowest effect concentrations is challenging due to statistical difficulties and was already controversially discussed during previous assessments. The lowest observed effect concentrations were as low as 0.0053 µg/L (EC_{10} embryo production, based on TWA) with definite effects at ≤0.25 µg/L (nominal). The extremely low effect concentrations below 1 µg/L, as well as the high variety between studies reveal that effects might be highly dependent on certain life stages, time windows, seasons and specific concentrations at specific time points during signal transduction which may indicate an endocrine mode of action.

Table 26: Indications for a possible link between the endocrine MoA and adverse effects in molluscs

Species	Indications for MoA	Histology	Apical effect
Marisa cornuarietis	Receptor binding oestrogen related receptor 2.2 mg/L (Bannister et al., 2013; Oehlmann 2006) Stimulatory effect of BPA on egg production antagonized by anti-oestrogen Oehlmann 2006)	Malformations gonads 1 µg/L superfemales (Oehlmann 2000, 2006)	Increased embryo production 0.25 µg/L/0.048 µg/L (Oehlmann 2000, 2006*) Juvenile growth 640 µg/L, no effects on fecundity at 25 µg/L (Forbes et al., 2007a and b)
Potamopyrgus antipodarum	Oestrogen receptor expression after exposure 40 µg/L BPA (Stange et al., 2012) VTG-like proteins increase at 100 µg/L (Gagnaire et al 2013)		Increased embryo production 30 µg/kg (Duft et al., 2003), 40 µg/L (Stange et al., 2012), 5-20 µg/L (Sieratowitz et al. 2011) , 5 µg/L (Jobling et al. 2004) 2 µg/L (Benstead at al. 2008)
Nucella lapillus	-	Sex glands enlarged (Oehlmann et al. 2000)	Oocyte production enhanced 25 µg/L (Oehlmann et al. 2000)
Physa acuta	-		Hatchability and Embryo malformations 500 µg/L (Sanchez-Arguello et al., 2012)
Haliotis diversicolor supertexta	Gene regulation affected (unspecific) (Zhou et al., 2011)		Developmental delay, embryo malformations, failure metamorphosis 1-30 µg/L EC_{50}, 200 µg/L (Liu et al., 2011; Zhou et al., 2011)
Bivalve *Mytilus edulis*	VTG induction (Aarab et al., 2006)	Malformations of gonads 50 µg/L (Aarab et al., 2006)	

5.12 Insects: In vitro and in vivo effects with regard to an endocrine mode of action

5.12.1 Reference documents and data basis

For Chironomus, OECD standard test procedures are available. An OECD detailed review paper (2006) on life-cycle toxicity testing for arthropods is available as well as some overviews (e.g. de Fur 1999, Grieneisen 1994).

For insects, seven studies covering two species were considered for the assessment.

5.12.2 Indicators of an endocrine mode of action: in vitro and biomarker studies for insects

For arthropods the most prominent hormones belong to the group of ecdysteroids (e.g. OH-ecdysone, ponasterone). Ecdysteroids are known to mediate various processes such as embryogenesis, development, molting, energy metabolism and reproduction in arthropods (Oberdörster and Cheek, 2000). Furthermore, juvenile hormones are of major influence for sex determination, metamorphosis and development.

Studies with indications for the modes of action of BPA in insects are summarised in Table 27. As general indicators of the mode of action in insects, receptor binding/expression and biomarker studies were analysed as well as comparisons to (xeno-)oestrogens or co-exposure to mitigating substances.

An antagonistic receptor binding (it competes with ecdysteroids at the ligand binding site) of Bisphenol A has been shown *in vitro* with a Drosophila BII in vitro assay for insects at relatively high concentrations (10^{-4} M)(Dinan et al., 2001). Same results were also shown for other oestrogen-like chemicals such as nonylphenol, although in higher concentrations than those for natural ecdysteroids. No significant effects were measured with oestradiol.

Recently, a 2-fold increase of the mRNA level of the ecdysteroid receptor (EcR) was shown for the midge *Chironomus riparius* after 12 and 24 h exposure to 3 mg/L and after 72h exposure to 0.5 mg/L BPA compared to the controls (Planello et al., 2008). These were similar concentrations where VT induction was detected by (Hahn et al., 2002), who showed a reduction of vitellogenin levels for males at all concentrations, for females at 3 mg/L, but may have been influenced by cross-reactivity of the immunoassay used. An interaction with the ecdysteroid receptor, which is directly induced by ecdysone, triggering transcriptional activity was proposed by Planelló et al. (2015). Interestingly, the sequence of an oestrogen-related receptor (ERR) and significantly increased mRNA levels were identified in *Chironomus riparius* after exposure ≥5 µg/L BPA by (Park and Kwak, 2010) for females, but not males.

It has been discussed that vertebrate-type oestrogen receptor agonists may act as ecdysteroid receptor antagonists in invertebrates (LeBlanc, 2007; Zou and Fingerman, 1997; Dinan et al., 2001). Hence, for arthropods, in particular insects, a possible mode of action of BPA is an interference with ecdysteroid signaling, in particular a blocking of the ecdysteroid receptor.

Table 27: Results from in vitro and biomarker studies for Bisphenol A indication possible underlying MoA in Insects

Species	Reference	Test conditions	Test type/parameters	Effect/Concentration	MoA	Comment/reliability
Drosophila melanogaster	(Dina et al., 2001)	BII bioassay with Ecdysteroid responsive cell line to detect EcR (ant)agonists		BPA competes with ecdysteroids (20-OH ecdysone) for the ligand binding site at 1x 10^{-4} M BPA (EC_{50}) (22.83 mg/L)	Ecdysteroid antagonist	2, established assay, also DEP, NP, 17ß-ethinylestradiol at higher conc., in general higher conc. than natural ecdysteroids proposed
Chironomus riparius (larvae)	(Planello et al., 2008)	mRNA extracts	0-12-24 h Exposure of 4th instar larvae to 3 an 0.5 mg/L BPA (sublethal)	mRNA level of ecdysone receptor (EcR) increased 2 fold after 12 and 24 h exposure to 3 mg/l and after 72 h exposure to 0.5 mg/L compared to controls	Interaction with ecdysteroid receptor, EcR directly induced by ecdysone, triggering transcriptional activity	2 Same concentrations were VT induction was detected (Hahn et al., 2002) No comparison with Ecdysone exposure, no analytics
Chironomus riparius	(Park and Kwak, 2010)	Larvae extracts after exposure	Exposure of 4th instar larvae to 5-50-500 µg/L (Sublethal) for 24 or 96 hours and 0.2 µg/L	cDNA sequence for oestrogen-related receptor (ERR) gene was identified level of mRNA of ERR expressed during all life stages, but not in adult males, gene expression significantly upregulated after 24 h and 96 h at all conc. (5-500 µg/L), not at 0.2 µg/L	Nuclear receptors are primary targets, Induction may be due to indirect or direct binding of the receptor	2 Also for NP at 1-10-100 µg/L after 24 h, well documented
Chironomus riparius	(Hahn et al. 2002)	semi static 1-100-3000 µg/L	yolk protein content, vitellogenin	significant reduction of VTG in males at all test conc. (1-1000), in females only at 3000 µg/L		3 – no analytics, method in development (possibility for cross-reactivity in the immunoassay) discussed in RAR but not used for PNEC

5.12.3 Indicators for adverse effects and endocrine mode of action: *in vivo* studies for insects

In vivo studies with indications for endocrine mediated adverse effects from partial or full life cycle tests in insects are summarised in Table 28. Apical effects that give an indication for anti-ecdysteroidal effects are often developmental abnormalities or a molting delay. Efects from exposure to vertebrate oestrogens are e.g. induction of vitellogenin, embryo mortality or an altered development (delay, increase). According to the overview by US EPA (2005), the *in vivo* effects of Bisphenol A exposure on insects comprise a delay of hatching or mouthpart deformities.

(Watts et al., 2001a) reported a delay in second generation adult insect emergence (*Chironomus*) after very low concentrations of Bisphenol A (from 0.078 -750 µg/L). However, (Watts et al., 2003) showed a delay in time to the first molt at the highest test concentration (1000 µg/L), and a decrease of the weight of first instar larvae and specific mouthpart deformities (mentum) at low concentrations from 0.01 µg/L with highest effects between 0.1-1 µg/L). The results were similar after EE2 exposure. The specific mouthpart deformities of the mentum have also been shown for nonylphenol (100 µg/L) (Meregalli et al., 2001). Another study (Lee and Choi, 2007) observed an increase in dry weight at concentrations below 1 µg/L, a decrease in total adult emergence rate at 10 µg/L and an increased male/female sex ratio at 1 µg/L (indicating that females were more sensitive). Also Staples et al. (2016) observed effects on development and emergence, but first at concentrations starting from 110 mg/kg dw and no emergence at all at 490 mg/kg dw after 28d standared exposure. However, although a relationship between the proposed mode of action, malformations and effects on development is possible for chironomids, the ecological consequences remain unclear.

Also the sexual development of the earwig *Euborellia annulipes* (Rankin and Grosjean, 2010) was affected by BPA exposure via drinking water (at 1 µg/L inhibition of testis growth, after 100 µg/L an inhibited ovarian growth). When BPA was injected, 0.12 µg/L triggered a reduced weight gain, increased testis and seminal vesicle size in males, and an enhanced clutch size in females, at higher dosages embryogenesis duration was affected. Although the dosing method by injection is not environmentally relevant and the effects differ from the drinking water exposure situation, both exposure scenarios point towards some alterations of sexual development.

BPA also affects development and reproduction of *Drosophila melanogaster* with a delay of pupation and maturation and reduced offspring numbers at concentrations from 0.1 mg/L (Atlı and Ünlü, 2012). Although the possibility for an anti-ecdysteroid mode of action due to the observed receptor binding in the studies of Dinan et al. and effects similar to those of Nonylphenol were discussed, no direct link is provided in the study. The effect on fecundity was also supported by Atli (2013) with same effect at the same concentrations also found for OP and NP. Weiner et al. (2014) found an increase in larval growth at 0.1 mg/L BPA (but not at 10 mg/L) as well as an increased body size 48-96 h after egg laying, associated with increased food intake and earlier onset of pupation as well as the known increased activity of insulin growth factor (GF) signalling.

Hence, for insects there are some signs for developmental disturbances as well as biochemical and mode of action studies that reveal an effect of BPA on the receptor level although at high concentrations. A link between the proposed MoA as an anti-ecdysteroid and the associated effects is possible.

Table 28: Summary of apical effects of BPA on insects from in vivo Tests (including evidence for underlying mechanisms/modes of action if evaluated in the respective studies)

Species	Reference	Test Conditions	Endpoints	Effect concentrations	Indication for ED	Comment/reliability
Chironomus riparius	(Watts et al., 2001a)	1st instar larvae, spiked sediment with 6 aqueous solutions between 10 ng/L and 10.4 mg/L	Emergence time, number of emerged adults, sex ratio, egg production, egg viability, emergence of F1	F0: Emergence time and number affected, but not sex ratio and offspring numbers F1: Emergence of males and females sign. delayed at 0.078 – 750 µg/L BPA, number of egg ropes varied, but no CRR	Development affected (at low conc. only), for ethinylestradiol opposite: earlier emergence.	2 Analytics of solutions and overlying water, (82% of nominal), but not sediment well-documented Standard test for sediment tox. as basis (not used in RAR for PNEC as exposure conc. in sediment not determinable)
Chironomus riparius	(Watts et al., 2003)	0.01-0.1 -1-100-1000 µg/L all aquatic life-cycle stages (eggs-larvae-pupae) start with 1st instar larvae, test solutions with filter paper as substrate, daily renewal of water	larval molting larval mouthpart structure (mentum, mandibles, pectin epipharyngis) wet weight 2d after 4th instar molt	Delayed time to first moult by 24h for 1st molt and 72h for 3rd molt and reduced mean wet weights of 1st instar larvae at 1000µg/L. Mouthpart deformities at 0.01-100 µg/L: In particular mentum deformities (0.1-1µg/L highest incidence). Pectin epipharyngis less affected, little evidence for mandible deformities; but not at 1000µg/L	Delay (1-3 d!) of molting at 1 mg/L. Specific mouthpart deformities at low concentrations, highest level of deformities at low/intermediate concentrations Interaction with ecdysteroids/receptor is proposed, indications for developmental disturbances Similar results for EE2, mentum deformities also known after exposure to NP (cited)	1 / 2 Analytics only for 1 mg/L test solution (830 µg/L measured, 83% of nominal) Standard test for sediment tox. as basis key study in RAR for PNEC (with 100 µg/L NOEC), although no clear concentration-response-relationship. Ecological consequences of deformities unclear, but clear developmental disturbances
Chironomus riparius	(Lee and Choi, 2007)	0.001 – 0.01 – 0.1 – 1 – 10 – 100 µg/L 4th instar larvae (50 per conc.)	Enzyme activities (catalase, peroxidase, glutathione-S-transferase) after 24 h Growth (dry and fresh weight) after 48 h Emergence during 20 d Sex ratio of emerged adults	Acute LC_{50} 6.03 mg/L Increased catalase, decreased peroxidase, increased glutathione-S-transferase activities Increase in dry weight at all low conc. below 1 µg/L (fresh weight only at 0.001 and 0.1 µg/L)	Enzyme activities not related to endocrine MoA (but homeostasis, antioxidant, detoxification).	2 Analytics with ELISA, BPA conc. (10 µg/L) decreased rapidly in first 24 h (10% of start) data not sufficient to establish a plausible biological relationship

Species	Reference	Test Conditions	Endpoints	Effect concentrations	Indication for ED	Comment/reliability
				Decrease in total adult emergence rate at 10 µg/L Increased male/female sex ratio at 1 µg/L (females more sensitive)		
Chrionomus riparius	Staples et al 2016	(Overlying water dosed), in sed: 50 mg/kg, 100 mg/kg, 200 mg/kg, 400 mg/kg, and 800 mg/kg dry wt	Emergence, developmetal rate	28 d LOEC 110 mg/kg emergence No emergence at 490 mg/kg dw	Effects on development/emergence, but it was not am to detect ED effects	1 / 2 standard test
Drosophila melanogaster	(Atli and Ünlü 2012)	0.1, 1 and 10 mg/L, larvae	Time larvae to pupae Time pupae to adult Mean offspring numbers	Delayed pupation and maturation of larvae, offspring numbers reduced at 100 µg/L	Anti-ecdysteroid MoA and similar effects as for NP are discussed	2, no analytics
Drosophila melanogaster	(Atli 2013)	0.1, 1 and 10 mg/L, larvae	Mean offspring numbers	Decreased fecundity in all conc. from 0.1 mg/L	Same effects observed for NP and OP, hypothesized that reduced fecundity might be due to increased ecdysteroid levels	2, no analytics
Drosophila melanogaster	(Weiner et al. 2014)	0.1, 1, 10 mg/L, first instar larvae exposed, 120 h after egg laying	Larval growth Body size Onset pupation	Increase in larval growth at 0.1 mg/L (but not at 10!) Increased body size 48-96 h after egg laying associated with increased food intake and earlier onset of pupation (increased activity of insulin growth factor (GF) signalling known)	growth and development governed by interaction between insulin growth factor (GF) and ecdysone signalling, but definite MoA unclear	2 – but no analytics, non monotonic dose-response
Euborellia annulipes (earwig)	(Rankin and Grosjean, 2010)	0-0.12-1.2-12 µg/L injected over 6 d in 0 d old males, 7 d old females; Exposure via drinking water of 1-10-100 µg/L over	Testis size, seminal vesicle size, duration of egg development of females, body weight	0.12 µg/L reduced weight gain, increased testis and seminal vesicle seize, higher dosages not or less effective in males; enhanced clutch size at 0.12 µg/L, at higher	Not clarified, oestrogen, ecdysteroid receptor interference assumed, but no proof	2, with respect to exposure via water, 3, with respect to exposure via injections as not realistic no analytics

Species	Reference	Test Conditions	Endpoints	Effect concentrations	Indication for ED	Comment/reliability
		1-2 weeks (from day 0)		dosages embryogenesis duration affected, Via drinking water: 1 µg/L inhibition of testis growth, 100 µg/L inhibited ovarian growth		

5.12.4 Summary and indications for a possible link between endocrine mode of action and adverse effects in insects

A summary of the studies analysed above is given in Table 29 providing an overview of the indications for a possible biological link between the observed effects in vitro and in vivo.

BPA was shown to antagonistically bind to the ecdysteroid receptor of *Drosophila melanogaster in vitro.* Furthermore, mRNA expression of the ecdysone receptor was evident for *Chironomus riparius* (from 0.5 mg/L). An ecdysteroid antagonistic mode of action is therefore suspected in insects.

The indications for the underlying anti-ecdysteroid mode of action from *in vitro* studies may be linked to the adverse effects, supporting a possible endocrine mediated action of Bisphenol A.

However, only few apical effect studies are available. *In vivo* data was only available for three insect species. The observed *in vivo* effects comprise i.e. characteristic malformations of mouthparts (Mentum) in Chironomus which is also shown to be elicited by oestrogens, malformations of the reproductive organs as well as a delayed moulting and hatching. The F1 generation was more sensitive, possibly due to exposure during embryonic development. Furthermore, malformations of gonads and abnormal development were observed for an earwig in one study. In Drosophila, a delayed development and reduced offspring were observed, a link is possible due to the known receptor binding but not demonstrated.

Chironomus was affected in concentrations as low as 0.01 µg/L (mouthpart deformities), 0.078 µg/L (delayed emergence F1), 1 µg/L (sex ratio), 10 µg/L (decreased emergence); *Drosophila sp.* at 0.1 mg/L. The ecological consequences for the population level remain unclear.

Table 29: Indications for a possible link between the endocrine MoA and adverse effects in insects

Species	Indications for MoA	Apical effect	Comparison with known ED/hormone
Midge *Chironomus riparius*	Ecdysone receptor (EcR) expression increased at 0.5-3 mg/l (Planello et al., 2008) Oestrogen-related receptor (ERR) expressed from 5 µg/L (Park and Kwak, 2010)	Delayed emergence, F1 more sensitive 0.078 – 750 µg/L (Watts et al., 2001a) Delayed moulting 1 mg/L, Mouthpart deformities (Mentum!) 0.01-100 µg/L (Watts et al., 2003) Increase in weight <1 µg/L, decreased emergence 10 µg/L, less females 1 µg/L (Lee&Choi 2007) emergence and development affected (Staples et al. 2016)	similar results for EE2, Mentum deformities also known after exposure to NP
Drosophila melanogaster	BPA competes with ecdysteroids in BII cell in vitro receptor binding assay (Dinan et al., 2001)	Delayed pupation and maturation of larvae, offspring numbers reduced at 100 µg/L (Atli and Ünlü 2012, Atli 2013) Increase larval growth	Similar effects as seen for NP, OP

*E*arwig *Euborella annulipes*		inhibition of testis growth 1µg/L and ovarian growth 100 µg/L (when injected: increased testis, enhanced clutch size) (Rankin and Grosjean, 2010)	

5.13 Crustaceans: *in vitro* and *in vivo* effects with regard to an endocrine mode of action

5.13.1 Reference documents and data basis

For crustaceans, there are standard test procedures available. Test guidelines for *Daphnia magna* and *Acartia tonsa* are under development and may reveal effects on development and reproduction, although not yet explicitly for ED effects (except the existing extension of the OECD standard guideline TG 211 for juvenoid screening via male induction in daphnids). An OECD detailed review paper (2006) is available for life-cycle toxicity testing for arthropods. Furthermore, knowledge on the endocrine system of crustaceans has been reviewed in a multitude of studies and overviews (LeBlanc, 2007; de Fur 1999, Tatarazako and Oda 2007, Zou 2010, Subramoniam 2000, Fingerman et al. 1998, Chang 1993, Hassold 2009).

For crustaceans, 15 studies were considered for the assessment covering seven different freshwater, marine and terrestrial species.

5.13.2 Indicators of an endocrine mode of action: *in vitro* and biomarker studies for crustaceans

Due to their close taxonomic relationship crustaceans use similar endocrine pathways as insects. For both taxonomic groups the most prominent hormones that regulate developmental and reproductive processes belong to the group of ecdysteroids (molting hormones, e.g. OH-ecdysone, ponasterone) as well as juvenoid hormones. The most prominent juvenoid hormone in crustaceans is methylfarnesoate, which is of major influence for sex determination, metamorphosis and development.

General indicators of the mode of action in crustaceans are receptor binding/expression studies as well as comparison to (xeno-)oestrogens, co-exposure of co-mitigating chemicals and biomarker studies. Only two *in vitro* studies on gene or protein expression profiles were available for crustaceans, and no receptor binding studies. Studies with indications for the possible modes of action of BPA in crustaceans are summarised in Table 30.

Due to the close taxonomic relationship to insects in the arthropod taxon the same mechanism of an anti-ecdysteroid action, i.e. a disruption of ecdysteroid signalling (blocking of the ecdysteroid receptor) for BPA is also discussed for crustaceans (see data for insects in chapter 5.12). It is discussed that some vertebrate oestrogen receptor agonists interfere with ecdysteroid-related processes and may act as ecdysteroid receptor antagonists in arthropods, such as e.g. Nonylphenol or Diethlystilbestrol (Dinan et al., 2001; LeBlanc, 2007; Zou and Fingerman, 1997). A possible ecdysteroid-related MoA of BPA is indicatedby studies with co-mitigators (Dahl and Breitholtz, 2008; Hutchinson, 2002; Mu and LeBlanc, 2004) such as 20-hydroxy-ecdysone as ecdysone agonist, or testosterone as ecdysone antagonist (Baldwin and LeBlanc, 1994; Oberdörster et al., 2001). But also other hormones such as methylfarnesoates may be affected and indirectly

affect ecdysteroid pathways as is shown for daphnids. In daphnids, the possible mode of action is well-described in experiments of (Mu et al., 2005) as described below in detail (*in vivo* studies).

Jeong et al. (2013) demonstrated for *Daphnia magna*, that the gene expression pattern was altered after 48 h exposure to 10 mg/L BPA (at concentrations where reproduction was significantly reduced) and putative up/down regulated genes were related to molting and reproduction processes (cuticular, chitin binding, and vitellogenin proteins).

(Lemos et al., 2009) measured total ecdysteroid (20-hydroxyecdysone, 20E) titers during the intermoult period in adult males of the terrestrial isopod *Porcelio scaber* after exposure to BPA and detected an increase of 20E after 28d at 10, 300 and 1000 mg/kg dw soil. Together with the observed incomplete ecdysis resulting in an enhanced mortality, an enhanced molting at lower concentrations and a skewed sex ratio this indicates disturbances of ecdysteroid-regulated pathways. Also protein expression in males was analysed in different organs after BPA exposure and concentration-response relationships as well as a differential pattern for different EDCs were established (Lemos et al., 2010c). An upregulation of two proteins in hepatopancreas was observed at the lowest and highest concentration (10 and 1000 mg/kg dw soil) as well as an upregulation of one protein in the gut at concentrations from 300 mg/kg dw soil and an upregulation of three proteins in testes from 100 mg/kg dw soil. It was postulated that these changes may lead to effects at the individual level as these were especially relevant for gonads (*see in vivo* studies).

Table 30: Results from *in vitro* and biomarker studies for Bisphenol A supporting the indications for the MoA in Crustaceans (see also indications for Insects in table 27)

Species	Reference	Test conditions	Test type/parameters	Effect/Concentration	Indication for MoA	Comment/reliability
Daphnia magna	(Jeong et al. 2013)	48 h exposure to 10 mg/L Micro-array experiment	Gene expression pattern	Expression pattern was altered due to BPA, putative genes related to molting and reproduction processes (cuticular, chitin binding, and vitellogenin proteins) In vivo effects: LC_{50} (48h): 10.4 mg/L, reproduction sign. reduced at 10 mg/L 21 d (slight reductions at 6.67 mg/L)	Certain genes up/downregulated in response to BPA that might influence reproduction and molting, but definite MoA unclear	2 Responsive genes proposed as biomarkers in response to BPA exposure
Porcelio scaber	(Lemos et al., 2010c)	Male isopods exposed to 10-30-100-300-1000 mg/kg dw soil	Protein expression gut, hepatopancreas and testes	up-regulation of two proteins in hepatopancreas at 10 and 1000 mg/kg dw soil) up-regulation of one gut protein from 300 mg/kg dw soil up-regulation of three testes proteins from 100 mg/kg dw soil.	BPA affected specific and concentration-dependent protein expression changes which may lead to effects at the individual level as especially relevant for gonads	2 Analytics (HPLC-PDA). All values within ±5% of nominal concentrations Organ specific effects of BPA Supportive for in vivo studies
Porcellio scaber	(Lemos et al., 2009)	Adult males: 10 weeks to 10-30-100-300 and 1000 mg/kg dw soil BPA	total ecdysteroid (20E) concentration during intermoult	Increase of 20E after 28d at 10, 300 and 1000 mg/kg dw soil	Enhanced mortality result of incomplete ecdysis related to increased ecdysteroid titers, sex ratio skewed, molting stimulated	2 Analytics (HPLC-PDA). All values within ±5% of nominal concentrations Supportive for in vivo studies

5.13.3 Indicators for adverse effects and endocrine mode of action: *in vivo* studies for crustaceans

In vivo studies with indications for endocrine mediated adverse effects in crustaceans are summarised in Table 31.

A disruption of ecdysteroid mediated pathways may cause disrupted embryo development, delayed molting, altered offspring production or onset of reproduction and may have effects on population development (LeBlanc, 2007). In particular effects on molting may be indicative for a disruption of ecdysteroid mediated pathways (Fingerman et al., 1998; LeBlanc, 2007; Lee and Buikema jr., 1979). According to the overview by the US EPA (2005), the effects of Bisphenol A exposure for crustaceans are an increase in egg production (as observed for copepods). Hence, apical effects that may give an indication for anti-ecdysteroidal effects in crustaceans are for example developmental abnormalities, a delay in development or molting, a disruption of embryogenesis, embryo malformations, a delay or enhancement of the molting cycle or development, the number of offspring (decrease or increase).

Daphnids only respond in high concentrations to Bisphenol A, but there are indications that the observed effects may be endocrine mediated. Mu et al. (2005) observed a prolongation of the first intermolt duration (>5 mg/L), embryo abnormalities depending on the embryonic age (>10 mg/L) and a reduction of fecundity (>1.3 mg/L) due to abnormally developed offspring. A coexposure to 20-OH ecdysone had no protective effect on molting and embryo abnormalities, but resulted in enhanced embryotoxicity. Together with methylfarnesoate, Bisphenol A had an enhancing effect on the male stimulation effect of methylfarnesoate, but no effect alone. Hence, it was concluded, that in daphnids, Bisphenol A may not directly act as an anti-ecdysteroid but enhance the activity of methylfarnesoates which then may inhibit ecdysteroid-mediated processes resulting in a delay in molting and embryo malformations (Mu et al., 2005). Juvenoid hormones and ecdysteroids are proposed to be closely coordinated (Wang et al., 2005), with juvenoid hormones modulating ecdysteroids (but not in the opposite way). This possible mode of action is supported by a previous study of (Mu and LeBlanc, 2004) which demonstrated that juvenoid hormones can regulate ecdysteroid activity reducing mRNA levels of the ecdysone receptor in daphnids. Further support is provided by a mechanistic study of (Wang et al., 2005) who screened BPA for juvenoid male inducing effects and again observed a BPA induced potentiation of methylfarnesoate effect on male production, but no effect on this endpoint of BPA alone. Hence, Bisphenol A elicits ecdysteroid antagonistic effects, but possibly via an indirect mode of action. Multiple modes of action are also likely as proposed by (Mu et al., 2005). In addition to this mechanistic evidence, further studies support the effects of BPA on embryonic development and reproduction of daphnids which seem to be endocrine-mediated.

(Hassold, 2009) observed embryonic malformations among offspring of exposed mother animals with a LOEC at 9.9 mg/L and a NOEC at 4.4 mg/L (% embryo malformations), similar to embryo abnormalities observed for the ecdysone antagonist testosterone (unextended shell spines, underdeveloped first antennae) as also observed by Mu and LeBlanc (2004). Adverse effects on reproductive output were observed at similar concentrations with a LOEC of 9.9 mg/L with a slight increase of the number of offspring after 21 d below 2.2 mg/L. Molting was only slightly delayed (LOEC 10.3 mg/L) and associated with death without any molt after 48 h (for control individuals the first molt was completed after 24h) at the highest concentration tested (14.8 mg/L). Furthermore, the second generation was tested at one single concentration (10.7 mg/L, corresponding to the EC50 reproduction). Exposure of the embryos in the mother with recovery (without exposure) during 21d further development resulted in reduced offspring production, reduced number of broods and delayed developmental time. These effects were stronger (in comparison with daphnids only exposed from neonate age on or only during embryonic development) when exposed during the whole life cycle and resulted in a 35% inhibition

of reproduction. Hence, exposure during embryonic development seems to be in particular sensitive.

This is supported by a study of (Brennan et al., 2006) who estimated a LC_{50} value of 806 µg/L (LOEC 600 µg/L) for mortality of the first generation of *Daphnia magna*. In the same study they found an increased mortality with a LC_{50} of 600 µg/L (LOEC 200 µg/L) for the second generation pointing towards a higher sensitivity of the F1 generation. However, molting and reproduction were not significantly affected after 21 d in concentrations up to 1000 µg/L. Further studies support the observed effects on reproduction. Olmstead and LeBlanc (2004) observed a reduced fecundity at slightly lower concentrations (EC_{05} 5 mg/L, EC_{50} 7.3 mg/L), but no effects on life-span and growth. Tatarazako et al. (2002) estimated a LOEC of 1.88 mg/L for the parameter reproduction after 7 d exposure during adult development (NOEC 940 µg/L). An early study by Caspers (1998) could not discern any effects on molting at the tested concentrations of 0.316 and 3.16 mg /L BPA, estimated a NOEC above the highest concentration tested of <3.16 mg/L and concluded that BPA does not have any endocrine effects on daphnids. However, the more recent studies mentioned above demonstrated that Bisphenol indeed may exert endocrine mediated effects in daphnids, although in relatively high concentrations at around 1 mg/L, which are not environmentally relevant.

In copepods, an increase in egg production of BPA exposed *Acartia tonsa* was observed and the same effects were shown after exposure to 17ß-estradiol (Andersen et al., 1999). In addition, (Andersen et al., 2001) investigated larval development of *Acartia tonsa* and estimated an EC_{10} for developmental inhibition at 100 µg/L (EC_{50}: 550 µg/L). Similar effects were observed for E1, E2, juvenile hormone III and the endocrine disrupter octylphenol but not for OH-ecdysone which would fit to an oestrogenic or anti-ecdysteroid mode of action. Also in the copepod *Tigriopus japonicas* a developmental delay was observed for the parental generation at concentrations starting from 0.1 µg/L (Marcial et al., 2003). The F1 generation tested, which already was exposed during embryonic development in the same study, was affected at much lower effect concentrations starting from 0.01 µg/L (Marcial et al., 2003). Hence, although the authors concluded a low impact on the demographic profile of the copepod, the next generation was more sensitive and also for copepods the embryonic development seems to be in particular sensitive.

This seems also to be true for the terrestrial isopod *Porcellio scaber* where increased abortions of embryos were reported at 10 and 1000 mg/kg (Lemos et al., 2010b). However, Lemos et al. (2009) also observed a stimulated and not inhibited molting in males (delaying effect of solvent mitigated in concentration dependent manner at all conc.), and an enhanced mortality as a result of incomplete ecdysis (10 w LC_{50} males 990 mg/kg dw soil, no mortality for juveniles during 16w exposure) and a sex ratio skewed towards females at ≥ 10 mg/kg dw soil (1male:2 females), which was attributed to an increase of ecdysteroid titers (20E) after 28 d at 10, 300 and 1000 mg/kg dw soil. A decreased growth was observed by (Lemos et al. 2010a): at 10 and 1000 mg/kg dw soil in males exposed as adults, in concentrations ≥ 25 mg/kg for males exposed as juveniles and in concentrations ≥ 10 mg/kg for females exposed as juveniles; as well as an induced molting at 300 mg/kg dw soil in juveniles. Here also an increased chronic toxicity for juvenile stages and females was indicated (Lemos et al. 2010a).

Gammarids seem to respond more sensitively towards Bisphenol A. Ladewig et al. (2006) tested *Gammarus fossarum* in a 103 d pulse-dose exposure scenario in artificial indoor streams. An EC_{10} of 17 µg/L (nominal, confirmed analytics 101-122%) is estimated for the proportion of reproductive females in the fourth brood and an EC_{10} of 5 µg/L for a decrease in brood size in the fourth brood, although for the first three broods they observed an increase in brood sizes at the highest Bisphenol A concentration. Schirling et al. (2006b) also observed possible effects on gonad histology (enhanced oocyte maturation, reduced number and size of early vitellogenic oocytes) in the same experimental setup. However, (Watts et al., 2001b) reports effects on precopulary behaviour of *Gammarus pulex* at much higher concentrations of 830 µg/L (analytics confirmed). Johnson et al. (2005) exposed *Gammarus pulex* for 14 d and could not discern effects on molting and reproduction. However, lethal effects were observed at 1 mg/L after 96 h (LC_{50}) exposure.

Similar effect concentrations were reported for the mysid *Americamysis bahia* in a study of (Hirano et al., 2004), who reported a 96h-LC_{50} of 1.03 mg/L.

Although concentrations are comparatively high for most crustacean species and often close to lethal toxicity (mg/L range) as seen for daphnids, this gives at least indications that effects of BPA also in crustaceans may be endocrine mediated and related to the ecdysteroid (as well as methlyfarneosates) pathways.

Table 31: Summary of apical effects of BPA on crustaceans from in vivo Tests (including indications for underlying mechanisms/modes of action)

Species	Reference	Test Conditions	Endpoints	Effect concentrations	Indication for ED	Comment/reliability
Acartia tonsa	(Andersen et al., 1999)	0.2 – 2 – 20 µg/L (10 replicates), start with 24 h eggs, 11 d Semi-static	Mortality Egg production at day 10	Mortality: EC_{10} 290 µg/L, EC50 960 µg/L (72h Immob.) Enhanced egg production at 20 µg/L (10 d), but not at days 9 and 11	stimulated maturation of ovaries leading to enhanced egg production. Also observed for 17ß oestradiol, but not for negative control 2,3 dichlorophenol.	2 No analytics but well documented Discussed in RAR, but not used for PNEC due to lack of analytics
Acartia tonsa	(Andersen et al., 2001)	Exposure from 24 h eggs to copepodite stage 6-8 test conc. For acute tests, min. 5 for chronic Semi-static, 5d exposure or until metamorphosis	Mortality Larval development (% reaching copepodite stage)	48h LC_{50}: 4.2 mg/L Development inhibition: EC_{10} 100 µgL, EC_{50} 550 µg/L	Effects on development could be indicative, similar to E2, E1, but BPA less potent than EE2 and OP, (no effects of testosterone and progesterone), juvenile hormone III had effect, but not OH ecdysone (-> no ecdysone agonistic effect)	2 No analytics short exposure, but sensitive endpoint Discussed in RAR, but not used for PNEC due to lack of analytics
Tigriopus japonicas (marine copepod)	(Marcial et al., 2003)	48 h acute tests 21 d at 25 °C F0 and F1 generation 0.01 —0.1 — 1.0 — 10 µg/L Daily renewal	Mortality Development (days to copepodite stage and sexual maturity) Fecundity Sex ratio	48h LC_{50} 4.32, NOEC: 3.5 mg/L Parental: delay to reach nauplii stage from 0.1 µg/L, maturity from 1 µg/L In F1 already from 0.01 and 0.1 µg/L respectively; fecundity, sex ratio and survival not affected	No effect of OH ecdysone. Embryonic development especially sensitive	2 No analytics but well documented Discussed in RAR
Daphnia magna	(Wang et al., 2005)	10,000 µg/L 10 d exposure of mothers, 3rd brood analysed Coexposure with 10 or 22 µg/L methyl farnesoate (nominal)	Screen for juvenoid effects (=production of male offspring under female favouring conditions): %broods containing males; % males/brood. Screen potentiating (+	10 mg/L BPA alone: no effect (males) Together with methyl farnesoate (potent agonist) effect on male stimulation potentiated compared to MF alone	Possibly BPA inhibits the enzyme that degrades methyl farnesoate BPA may potentiate the activity of endogenous MF No juvenile hormone (JH) agonist effect.	2 Mechanistic study. Only one conc. Discussed in RAR, not used for PNEC as only indications for ED

Species	Reference	Test Conditions	Endpoints	Effect concentrations	Indication for ED	Comment/reliability
			agonist MF) juvenoid stimulated male offspring		Also observed for NP	
Daphnia magna	(Mu et al. 2005)	48 h acute 21d 0-5-10 mg/L Semi-static < 1h at test start	Mortality fecundity Intermolt duration Embryonic development Male offspring	Acute EC_{50}: 16 mg/L NOEC fecundity 1.3 mg/L (but could be higher, ca. 5 mg/L) Longer intermolt at conc > 5 mg/L Increased percentage of abnormal embryos at 10 mg/L	Propose anti-ecdysteroidal MoA No JH activity (no male stimulation)	2, no analytics Discussed in RAR, but not used for PNEC due to lack of analytics
Daphnia magna	(Hassold, 2009)	21d F0 (7 test concentrations µM) Additional molting experiments (21-65 µM= 4.8-14.8 mg/L), 4 test conc., up to 96h (3 juvenile molts)	Reproduction Molting delay Embryo malformations	Reproduction LOEC and EC10 10.1 mg/L, slight increase of reproduction below 2.2 mg/L, EC50 12.6 mg/L % embryo malformations NOEC 4.4 mg/L, LOEC 9.9 mg/L, EC10 12.1 mg/L Molting: death without ecdysis after 48h (controls: 1. molt at 24 h) at 14.8 mg/L, slight delay at 7.1 and 10.3 mg/L (Mortality LC50 14.5 mg/L)	Malformations (unextended shell spine, antennae) anti-ecdysteroid mechanism proposed steep conc.-response-curves (difficult to fit due to slight increase of offspring at low conc.)	2 21d Standard test, 3 independent tests, but n=5 for treatments, 15 for controls, 7 test conc.), nominal concentration, analytics of stock solution
Daphnia magna	(Hassold, 2009)	21 d repro test with F1 generation that was either exposed to 47 µM = 10.7 mg/L BPA (ca. EC50 fecundity	Reproduction after 21d, Developmental delay Malformations	Exposure during embryonic development only: delay to reach maturation/first brood compared to unexposed controls after 21d. Exposure during whole life cycle: reduced	F1 exposed to BPA in the mother during embryonic development affected (embryonic development important, F1 sensitive)	2 Only 1 test concentration, but 4 different scenarios tested, n=10, no analytics Extremely high variability, only trends Effects lower than intended

Species	Reference	Test Conditions	Endpoints	Effect concentrations	Indication for ED	Comment/reliability
		reduction F0 21 d) and unexposed in mater respectively (<12 h old). 21d exposure to 47 µM or recovery without exposure to BPA		fecundity, delay to reach maturation/first brood, compared to organisms only exposed for 21d from neonate age on.		(based on EC50) at tested concentration (rather increase of offspring)
Daphnia magna	(Caspers 1998)	21d standard test 3.9 ppm/µg/L – 0.039 ppm Molting at 0.316 and 3.16 µg/L (ppm)	Reproduction Molting (preadult and adult molts)	21 d NOEC > 3.16 reproduction Pre adult and total molts not affected		2 GLP study, measured concentrations, but not well documented (detailed data for each test concentration missing). Too low concentrations to detect effects, only 2 conc. tested for molting. Discussed in RAR
Daphnia magna	(Brennan et al., 2006)	48 acute tests up to 10.5 mg/L 21d exposure of F0 and F1 to 200 -400 – 600 – 800 – 1000 µg/L >24h at test start	48 h Survival and Molting 21d Survival, molting and reproduction	Mortality: (immobilization) EC50 48h: 7.75 mg/L Molting not sign. affected by BPA up to 10.5 mg/L after 48h Reproduction and molting (21 d) only affected at1000 µg/L F0: 21d NOEC 400, LOEC 600, LC50 806 µg/L F1: increased mortality - LOEC 200 , LC50 600 µg/L (100% death at 1 mg/L)	E2, DES and NP affected molting after 48 h, E2 and BPA not after 21d! NP decreased offspring in F0 and F1, DES only in F2 Second generation more sensitive!	2 - No analytic but well documented. Discussed in RAR (molting not affected, but older individuals than in Mu et al. 2005)
Daphnia magna	(Olmstead and LeBlanc, 2004)	3 brood test (17-19d) 50 test conc. (10	Life span (survival) Growth rate Fecundity (offspring	Lifespan and growth rate not affected (EC50 >8200)	None proposed Fecundity: steep concentration-response-	2 Data obtained for a heuristic mixture model

Species	Reference	Test Conditions	Endpoints	Effect concentrations	Indication for ED	Comment/reliability
		replicates for control) 5 – 8200 µg/L	number)	Reduced fecundity EC05: 5000 µg/L, EC50 7300 µg/L	curve Brood sizes are smaller (-> 3 brood test)	3 brood test does not cover delay in development No analytics
Ceriodaphnia dubia	(Tatarazako et al., 2002)	7d test adults 0.94 – 1.88 – 3.75 – 7.5 – 15 – 30 mg/L	Reproduction	NOEC 940 µg/L (LOEC 1880) reproduction		3 Paper about styrene dimers, BPA only for comparison
Americamysis bahia *Daphnia magna*	(Hirano et al., 2004)	48 h acute	Mortality	Americamysis: 1.34 mg/L Daphnia: 12.8 mg/L	Not usable for ED, only acute (No effects of ecdysteroids tebufenozide and JH analogue methoprene at tested conc.)	2/3, inly acute tests, no analytics, not used for ED Americamysis used in RAR for saltwater PNEC
Gammarus pulex	(Watts et al., 2001b)	24 h and 10d 14.6 ng/L – 53.8 ng/L – 0.36 µg/L – 5.1 µg/L, 56 µg/L – 0.83 mg/L – 8.4 mg/L	Mortality Reproduction Molting precopulary behaviour	LC50 24 h: 1.49 mg/L Precopulary behaviour only disturbed at 830 µg/L.	Similar to EE2 (ethinylestradiol), too short exposure time to detect effects	2 - Confirmed analytics
Gammarus pulex	(Johnson et al., 2005)	14 d semi-static 1-10-100-1000 µg/L	precopula pairs 14 d, molting survival number offspring	Reduced survival at 1000 µg/L No sign. effect on molting or reproduction results variable		2 Concentration measured at start, but not documented
Gammarus fossarum	(Ladewig et al., 2006)	5-50-500 µg/L 103 d pulse-dose exposure scenario in artificial indoor streams (nominal, confirmed analytics 101-122%)	Brood size, reproductive females	EC10: 17 µg/L proportion of reproductive females in the fourth brood EC10 5 µg/L decrease in brood size in the fourth brood increase in brood sizes of 1st -3rd brood at the highest Bisphenol A conc.	Effects on population level, underlying endocrine mode of action unclear	2 Confirmed analytics, well-documented but no standard tests

Species	Reference	Test Conditions	Endpoints	Effect concentrations	Indication for ED	Comment/reliability
Gammarus fossarum	Schirling et al. 2006b)	5-50-500 µg/L 103 d pulse-dose exposure scenario in artificial indoor streams (nominal, confirmed analytics 101-122%)	Stress proteins, gonad histology	Accelerated maturation of oocytes (non-sign. trend 50-500 µg/L after 34d, at 5-500 µg/L after 69d), decline in number of early vitellogenic oocytes (after 34/96d exposure) Reduced stress protein levels (hsp90 known to play role in vertebrate sex steroid signal transduction) Mortality elevated after 96d	Effects related to (sexual) development, but only trends due to high variability	2 confirmed analytics, histological effects trends only
Porcellio scaber	(Lemos et al., 2010b)	56 d	Reproductive cycle	Increased abortions at 10 and 1000 mg/kg	Aborts possibly due to embryotoxicity	2 Analytics (HPLC-PDA). All values within ±5% of nominal concentrations
Porcellio scaber	(Lemos et al., 2009)	Adult males: 10 weeks to 10-30-100-300 and 1000 mg/kg dw soil BPA Juveniles: 16 weeks to 10-25-50-150 and 300 mg/kg dw soil	Males: Molting and total ecdysteroid (20E) concentration during intermoult Juveniles: Sex ratio	Males: 10 w LC50 (990 mg/kg dw soil), no mortality for juveniles during 16w exposure Molting stimulated (earlier) in males (delaying effect of solvent mitigated in concentration dependent manner at all conc.) Increase of 20E after 28d at 10, 300 and 1000 mg/kg dw soil Sex ratio skewed towards females at ≥ 10 mg/kg dw soil (1 male:2 females)	Enhanced mortality result of incomplete ecdysis related to increased ecdysteroid titres Molting Sex ratio shift	2 Analytics (HPLC-PDA). All values within ±5% of nominal concentrations Methanol used as solvent but evaporated changed physical soil properties (delayed molting in solvent control) Effects not seen for Vinclozolin
Porcellio scaber	(Lemos et al.,	Adults: 10 weeks,	Molting	Decreased growth at 10	Molting perturbations	2

Species	Reference	Test Conditions	Endpoints	Effect concentrations	Indication for ED	Comment/reliability
	2010a)	10-30-100-300 and 1000 mg/kg dw soil BPA Juveniles: 16 weeks to 10-25-50-150 and 300 mg/kg dw soil	Growth Juveniles, males, females	and 1000 adult males, LOEC of 25 mg/kg for males exposed as juveniles, LOEC 10 mg/kg females exposed as juveniles BPA induced molting at 300 mg/kg juveniles Increased chronic toxicity for juveniles, Females more sensitive		Analytics (HPLC-PDA). All values within ±5% of nominal concentrations

5.13.4 Summary and indications for a possible link between endocrine mode of action and adverse effects in crustaceans

A summary of the studies analysed above is given in Table 32 providing an overview of the indications for a possible link between the observed effects *in vitro* and *in vivo*.

Ecdysteroids are the most prominent hormones responsible for developmental and reproductive processes and are in strong interaction with other arthropod hormones, such as e.g. juvenoid hormones. For daphnids it was shown that BPA possibly elicits its anti-ecdysteroidal effects indirectly via an interaction with the juvenoid hormone methylfarnesoate (crustacean juvenoid hormone). For the analysed crustacean species, no explicit in vitro studies are available examining receptor binding of BPA (a receptor binding was only demonstrated for Insects), but protein and gene expression studies are available for 1 species after exposure to BPA. The indications for an ecdysteroid receptor and an antagonistic binding of Bisphenol A from studies with insects may support the MoA and a receptor binding in crustaceans due to the close taxonomic relationship. Measurements of increased ecdysteroid titres in isopods associated with incomplete ecdysis leading to death, sex ratio shift towards females and malformations may at least indicate an interference with ecdysteroid-related processes.

Adverse effects on daphnids are associated with embryo malformations, delay of development and adverse effects on the reproductive outcome. In particular the embryonic development was shown to be sensitive leading to a higher sensitivity of the F1 generation. This was shown for daphnids and copepods. In the copepod *Acartia tonsa*, as well as to some degree at low concentrations for daphnids a stimulatory effect on embryo production (as characteristic for molluscs) became evident. A sex ratio shift as well as embryo malformations, increased molting and incomplete ecdysis were observed in isopods.

Even if the majority of studies first show effects at high test concentrations and some effects (e.g. for daphnids) were indeed close to lethal toxicity and characterized by extremely steep concentration-response-relationships, someevidence for an underlying endocrine mode of action is provided. Some crustacean species, such as copepods, in particular a marine copepod (Marcial et al. 2003) seem to be more sensitive with effect concentrations also in the low µg/L range, although not as sensitive as molluscs. It has to be noted, that the second generation was affected at lower concentrations as indicated for copepods and daphnids and exposure during embryonic development seems critical. Long-term effects were studied in gammarids which indicated effects on population development at low concentrations, although the underlying reasons remain unclear in this case. Effects in terrestrial isopods were observed at 10 mg/kg dw soil.

Table 32: Indications for a possible link between the endocrine MoA and adverse effects in crustaceans

Species	**Evidence for MoA**	**Apical effect**	**Comparison with known ED/hormone/Link**
Water flea *Daphnia magna*	Ecdysteroidal activity by enhancing activity of methylfarnesoate 20-OH-ecdysone enhanced embryotoxicity (Mu et al., 2005; Wang et al., 2005) Gene expression pattern altered (Jeong et al. 2013)	Longer intermolt 5 mg/L Abnormalities 10 mg/L (younger more sensitive) Fecundity reduction 1.3 mg/L, abnormalities. (Mu et al., 2005; Wang et al., 2005) Increased mortality in F1 (Brennan et al., 2006) Reproduction 10 mg/L (slight increase <2 mg/L). Embryo malformations 9.9 mg/L F1 more senstive (Hassold, 2009)	(Mu et al., 2005) + (Wang et al., 2005) mechanistic studies provide a good link
Copepod *Acartia tonsa*		Enhanced egg production at 20 µg/L Development inhibition at 100 µgL (Andersen et al. 1999/2001)	Also observed for EE2. Similar to E2, E1, EE2 and OP (Andersen et al. 1999/2001)
Copepod *Tigriopus japonicus*		Delay to reach nauplii stage 0.1 µg/L, Maturity 1 µg/L F1 more sensitive (0.01/0.1µg/L) (Marcial et al., 2003)	
Gammarus pulex		Disturbed precopulary behavior 8.4 mg/L (short exposure) (Watts et al., 2001b)	Disturbed precopulary behaviour similar to EE2 (unclear)
Gammarus fossarum		Decrease reproductive females 17 µg/L, decrease in brood size 5 µg/L (4th brood); Increase in brood sizes 1st -3rd brood 500 µg/L (Ladewig et al., 2006) as well as a trend for accelerated oocyte development (Schirling et al. 2006)	MoA/effect unclear
Isopod *Porcelio scaber*	Ecdysteroid titres (20E) increased, protein expression pattern changed	Increased abortions at 10 and 1000 mg/kg (Lemos et al., 2010b) Incomplete ecdysis, Sex ratio shift (Lemos et al. 2009)	Increased ecdysteroid titres together with incomplete ecdysis, sex ratio shift, enhanced molting, altered protein expression patterns (Lemos et al. 2010c)

5.14 Further invertebrate species: in vitro and in vivo effects with regard to an endocrine mode of action

5.14.1 Reference documents and data basis

Knowledge on the endocrine system of invertebrates has been reviewed for example by DeFur et al. (1999), Zou (2010) or Janer (2007) and was taken as a basis.

Data from seven studies for ten different species, covering two poriferans, two cnidarians, two annelids and five echinoderms were available. The studies for these invertebrates are summarized in this chapter. The respective taxa were not analysed in depth as data for these taxonomic groups is very fragmentary. It is to be noted that the discussed taxa differ widely with respect to their taxonomic relationship.

5.14.2 Indicators of an endocrine mode of action: in vitro and biomarker studies

The endocrine system of the further invertebrate taxa is comparatively less understood and fewer studies are available, which are summarized in the following. The underlying modes of action are not clear, although effects on developmental and reproductive processes are observed for a range of species. It needs to be considered that also the endocrine systems may differ considerably. Indications from studies with mitigators or comparison to hormones with known ED effects are given below in the next section.

5.14.3 Indicators for adverse effects and endocrine mode of action: in vivo studies

Studies with indications for the modes of action of BPA as well as *in vivo* data indicating possible endocrine mediated adverse effects in further invertebrate species (annelids, cnidarians, poriferans and echinoderms) are summarised in Table 33.

Annelida

The annelid *Lumbriculus variegatus* (Ladewig et al., 2006) showed a reduced population growth at extremely low concentrations with an 103 d EC_{10} of 2 µg/L (nominal but analytics provided), although the underlying endocrine mode of action remained unclear. Biggers and Laufer (2004) estimated an EC_{50} of 11.5 µg/L (0.05 µM) for the marine polychaete *Capitella capitata* for the number of larvae that settled and completed metamorphosis after 1h.

Cnidaria

For cnidarians two studies are available. Pascoe et al. (2002) investigated the structure and physiology of polyps of *Hydra vulgaris* and observed an inhibition of the regeneration of digestive cells at concentrations >460 µg/L and an alteration of polyp structure >42 µg/L. Similar effects were observed for EE2 at concentrations of 150 µg/L and > 58 µg/L, and in addition effects on sexual reproduction at high concentrations of EE2 which were not observed for BPA. Fukohori et al. (2005) observed a suppression of testis formation and an induction of budding reproduction at test concentrations (1000 µg/L) and a stimulation at higher concentrations. Although, the underlying modes of action are not clarified, Bisphenol A seems to cause disruption of development in cnidarias.

Porifera

(Hill et al., 2002) observed developmental abnormalities and an inhibition of germination in both poriferan species *Heteromyenia sp.* and *Eunapius fragilis* at concentrations as low as 16 and 80-160 µg/L. The abnormalities lead to a reduced growth rate at 160 µg/L. The underlying MoA is unknown but similar results were observed for nonylphenol.

Echinodermata

Echinoderms, which are deuterostomes more closely related to vertebrates, are known to respond to vertebrate sex steroids, which possibly play a role in reproductive processes (Kropp et al., 2005). Effects of BPA were shown in two studies with sea urchins. Roepke et al. (2005) investigated the morphology of larvae of *Strongylocentrotus purpuratus* and *Lychtechinus anamesus* and observed abnormal development after BPA exposure (EC_{50} 226.5 µg/L). Adding the ER agonist Tamoxifen diminished the abnormal development, whereas the estrogen receptor antagonist ICI 182, 780 increased the potency of BPA. A similar developmental delay was observed for OP and E2 but not for EE2. Kiyomoto et al. (2005) showed for *Hemicentrotus pulcherrimus* and *Strongylocentrotus nudus* a suppression of growth suppression (at 22.8 – 228.3 µg/L) and slight abnormalities (570 µg/L). Exposure during early embryonic development (0-12 h) was in particular sensitive. The underlying mode of action is unclear, the synthetic EE2 had different effects, promoting growth and causing a higher ratio of abnormalities at lower concentrations. Also effects on embryotoxicity and spermiotoxicity were observed by Özlem and Hatice (2008) in *Paracentrotus lividus after exposure of s*perm and eggs at concentrations ≥300 µg/L (calc. EC_{10}: 138 µg/L for spermiotoxicity and 420 µg/L for embryotoxicity, respectively). Growth was reduced at early life stages and an increase in larval malformations observed.

Table 33: Summary of apical effects of BPA on other organism groups from in vivo Tests (including indications for underlying mechanisms/modes of action if evaluated in the respective studies)

Species	Reference	Test Conditions	Endpoints	Effect concentrations	Indication for ED	Comment/reliability
Annelida						
Lumbriculus variegates	(Ladewig et al., 2006)	5-50-500 µg/L 103 d pulse-dose exposure scenario in artificial indoor streams (nominal, confirmed analytics 101-122%)	Population growth	Reduced population growth with an 103d EC_{10} of 2 µg/L BPA (nominal)	Effects on population level, underlying endocrine mode of action unclear	2 Confirmed analytics, well-documented but no standard tests
Capitella capitata (marine polychaete)	(Biggers and Laufer, 2004)	Metamorphosis assay 1h	Number of larvae that settled and completed metamorphosis after 1h	EC_{50} 11.5 µg/L (0.05 µM)	No indication, unclear.	3 Focus of article on lobster, no analytics, very short-term exposure
Cnidaria						
Hydra vulgaris	(Pascoe et al., 2002)	72 h (10 ng/L-5 mg/L)	Structure and physiology of polyps Inhibition of regeneration of digestive cells	Polyp structure: >42 µg/L Inhibition regeneration >460 µg/L	EE2: same effect >58 µg/L, 150 µg/L (+ sexual reproduction affected only 500 µg/L EE2)	2 - Analytics of stock solutions and actual test concentrations for low conc. Used in RAR for PNEC and SSD
Hydra oligactis	(Fukohori et al 2005)	1000-2000-3000-4000 µg/L 35 d	testis formation asexual reproduction	1000-4000 µg/L Supression of testis formation Induction of budding reproduction at low (1000) conc., reduction at higher conc.	Developmental disruptions	2 – Analytics Discussed in RAR
Porifera						
Heteromyenia sp *Eunapius fragilis*	(Hill et al., 2002)	long term (9 d) 0.16-1.6-16-80-160 µg/L	Developmental abnormalities Growth rate	Both species: Developmental abnormalities 16 µg/L inhibition of germination	Mode of action unknown, similar effects and abnormalities also	2 (key study in RAR for risk based assessment) Analytics for highest conc.

Species	Reference	Test Conditions	Endpoints	Effect concentrations	Indication for ED	Comment/reliability
			germination	80 and 160 µg/L Reduced growth rate due to abnormalities at 160 µg/L	observed for nonylphenol	
Echinodermata (Sea urchins only)						
Strongylocentrotus purpuratus *Lychtechinus anamesus*	(Roepke et al., 2005)	Embryos 1.25-125-250-500-750-1000 µg/L	Morphology of larvae at the Pluteus stage (normal, delayed, different malformations, hatched)	Abnormal development EC_{50} 226.5 µg/L	Adding the ER agonist Tamoxifen diminished abnormal development. Receptor antagonist ICI increases abnormalities and potency of BPA OP, BPA, E2 all excert a similar developmental delay (EE2 different).	2 no analytics, good documentation, comparison with known modulators discussed in RAR
Hemicentrotus pulcherrimus *Strongylocentrotus nudus*	(Kiyomoto et al., 2005)	48h 1-10 µM (=228.3 -2283 µg/L H.p. exposed 80 d to 114 µg/L for growth)	Embryo development/metamor-phosis (unhatched, stages, abnormal, hatched) Juvenile growth	Growth suppression at 22.8 – 228.3 µg/L Only slight abnormalities at 570 µg/L, exposure during early development (0-12 h) in particular sensitive (12-48 h not) Smaller size H.P. after 80d exposure NOEC (Strongylocentrus nudus) 0.71 mg/L	EE2 had different effects than BPA (higher rate of malformations at 1-1.25 µM, growth was promoted) BPA affects early development, life stage dependent	2, no analytics Discussed in RAR, not suitable for PNEC
Paracentrotus lividus	(Özlem and Hatice 2008)	Sperm and eggs exposed to 300 – 3500 µg/L (7 conc.), static, 6 replicates 72 h old pluteus larvae used for embryotoxstudies	Successful sperm fertilisation Larval malformations Developmental arrest Embryonic/larval mortality	Spermiotoxic and embryotoxic effects at concentrations ≥ 300 µg/L (calc. EC_{10}: 138 and 420 µg/L respectively) Growth reductions at early life stages Increase in larval malformations	Reproduction and development affected, embry malformations	2, no analytics

5.14.4 Summary and indications for a possible link between endocrine mode of action and adverse effects in further invertebrates

A summary of the studies analysed above is given in Table 34, providing an overview on the observed effects *in vivo* as well as comparisons with known EDs.

The underlying modes of action are not clear. This is in particular true for the annelids, cnidarians and poriferans. Still, similar effects as for known oestrogen (mimics) were observed.

In echinoderms, studies with BPA and co-exposure with oestrogen modulators point towards effects similar to (xeno-)oestrogens. The observed adverse effects of BPA exposure comprise embryo malformations and alterations of development and reproduction with early developmental periods as a sensitive time window and are comparable to the effects of known (xeno-)oestrogens (OP, E2).

In particular sea urchins, cnidarians and poriferans are very sensitive when compared with other invertebrate species and affected in the low to mid µg/L range.

Table 34: Effects in further invertebrate taxa (Cnidaria, Porifera, Echinodermata, and Annelida) and comparison with (xeno-)oestrogens.

Taxa	**Apical effects**	**Comparison with EDs/hormones**
Annelida *Lumbriculus variegatus, Capitella capitata*	Population growth reduced at 2 µg/L 103 d (Ladewig et al., 2006) Settling and metamorphosis of larvae at 0.05 µg/L EC_{50} (Biggers and Laufer, 2004)	Underlying MoA and endocrine action unclear
Cnidaria *Hydra vulgaris*	Polyp structure at 42 µg/L (72h) (Pascoe et al., 2002) Testis formation supressed and induction of reproduction at 1 mg/L, reduced at higher conc. (35 d) (Fukohori 2005)	Underlying MoA unclear, but Polyp structure and reproduction similar for EE2
Porifera *Heteromyenia sp., Eunapius fragilis*	Embryo abnormalities at 16 µg/L → reduced growth at 160 µg/L (Hill et al., 2002)	Underlying MoA unclear, but similar effects for nonylphenol
Echinodermata *(Strongylocentrotus purpuratus/nudus, Lychtechinus anamesus, Hemicentrotus pulcherrimus, Paracentrotus lividus)*	Abnormal development at 226 µg/L (Roepke et al., 2005) Growth supressed at 22.8 µg/L , abnormalities at 570 µg/L , exposure during early development sensitive (Kiomoto et al. 2006) Larval malformations, embryotoxicity	Underlying MoA unclear, as oestrogen Antagonist Tamoxifen reduces abnormalities Similar effects for OP, E2

5.15 Environmental relevance of endocrine mediated effects in fish, amphibians and invertebrate taxa

5.15.1 Fish: environmental relevance of endocrine mediated effects

Bisphenol A causes severe adverse effects on a number of fish species, which are considered population relevant as they affect population stability and recruitment.

These are for example effects on reproduction resulting in no male fish at all at higher concentrations. The exposure of *O. latipes* with 1820 µg BPA/L in an extended early life stage toxicity test (similar to FSDT) caused all fishes to develop into phenotypic females (Yokota et al., 2000). Gonadal histological investigations revealed that none of the fish developed testis. Also in the next lower concentration (355 µg/L) only 25% showed male secondary sex characteristics. For *D.rerio* a skewed sex ratio towards females even at 0.372 µg/L was observed (Chen et al., 2015).

The effects on secondary sex characteristics of *X.helleri,* resulting in an inhibition of sword length growth is likely to influence the mating success of males as the larger males are preferred by females. For the green swordtail, the sword is the predominating element for the size because the body fish itself is very small compared to the sword. The significance of the changes in sword length is not understood, but it is thought that the length of the sword has an influence on mating success, with female fish preferring males with longer swords. There are indications for an endocrine effect but it is not clear what degree of change should be considered to be significant and therefore cannot be used as a definite proof.

There are hints from several studies that the sensitivity of the fish increases from one generation to the other, demonstrating that further generations may be affected at even lower concentrations (e.g. Keiter et al., 2012; Sumpter et al., 2001; Bhandari et al., 2015).

Effects on fish were observed in the low µg/L range (e.g. 0.23 µg/L larval malformations).

5.15.2 Amphibians: environmental relevance of endocrine-mediated effects

In amphibians, Bisphenol A acts via two different endocrine modes of action, i.e. as thyroid antagonist and as oestrogen agonist. Different species were affected.

With respect to in vivo effects linked to the thyroidal mode of action, Bisphenol A inhibits the TH-induced and also the spontaneous metamorphosis, thus the development and life cycle of amphibians is disturbed (*X. laevis, R. rugosa, S. tropicalis*). Ultimately, this leads to a loss of reproductive fitness of the organisms and to a destabilisation of the population.

With respect to the oestrogen mode of action of Bisphenol, a skewed sex ratio towards females in amphibians similar to fish, surely affects the population development.

Effects on amphibians were observed in the low µg/L range (e.g. 7.3 µg/L sex ratio, 0.23 µg/L increased growth/body weight).

5.15.3 Invertebrates: environmental relevance of endocrine-mediated effects

For invertebrates, the observed effects are in particular related to an interference with developmental and reproductive processes, including a high embryotoxicity with embryo abnormalities as well as malformations of the reproductive organs and affected development. These may lead to unpredictable population effects in the long term. The F1 generation was often shown to be more sensitive (e.g. shown in crustaceans). Possible long-term effects on population development were indicated for gammarids in one study. Effects seem to partly depend on certain time windows, with early life stages being in particular sensitive.

In molluscs, BPA may exert oestrogen-related effects similar to (xeno-)oestrogens and the characteristic effects comprise an increase in egg numbers, the induction of superfemales and malformations of the genital tissues. Molluscs were shown to be extremely sensitive, affected in concentrations well below 1 µg/L (e.g. 0.0053 µg/L EC10).
In insects, BPA is proposed to disturb ecdysteroid-mediated pathways and effects are associated with delayed development and malformations, indicating consequences for population stability and recruitment. Also insects were already affected at concentrations below 1 µg/L (e.g. 0.01 µg/L mouthpart deformities, 0.078 µg/L delayed emergence F1 in Chironomus).

In crustaceans, BPA is also assumed to disturb ecdysteroid-mediated pathways, possibly indirectly via methylfarnesoates. Effects are associated with incomplete ecdysis leading to death, a sex ratio shift towards females, malformations, a delay of development and molting and effects on the reproductive outcome, with the F1 generation becoming more sensitive. Copepods were shown to be most sensitive with effects in the low µg/L range. Effects in terrestrial isopod were observed at 10 mg/kg dw soil.

Also for further invertebrate species adverse effects comprise embryo malformations and alterations of development and reproduction with early developmental periods as a sensitive time window, although the underlying mode of action is unclear. Sea urchins, cnidarians and poriferans are comparatively sensitive and affected in the low to mid µg/L range.

5.16 Summary and discussion of the environmental hazard assessment

In summary, *in vitro* data and *in vivo* data show that Bisphenol A is a clear endocrine disruptor in fish with an oestrogen agonist mode of action. In amphibians, there is clear evidence for a thyroid antagonist mode of action. Furthermore, there are indications that BPA also may act via thyroid-related pathways in fish and oestrogen agonist in amphibians. Also in different invertebrate taxa it is possible that adverse effects of BPA are a result of endocrine modes of action, and BPA possibly acts similar to (xeno-)oestrogens in molluscs and interfering with ecdysteroid-related processes in arthropods. However, knowledge on the endocrine systems and data is still fragmentary and the possible link less clear, so that data is supportive. The effects of BPA go clearly beyond systemic toxicity and are severe. The endocrine-mediated effects are environmentally relevant as they affect population stability and recruitment in a number of taxa with certain particularly sensitive species.

In fish, endocrine-mediated reproduction and development related effects were mainly linked to the effects of BPA on oestrogen regulated pathways. BPA acts as oestrogen agonist. Oestrogenic effects occurred at different levels including an altered sex ratio skewed towards females. Further effects were reduced quality of sperm, malformations of reproductive organs, delayed spermatogenesis, delayed or enhanced oogenesis, and higher incidence of embryo malformations and larval mortality, and altered secondary sex characteristics with negative consequences for mating success and successful reproduction.

Reduced sperm quality was observed for *Danio rerio* (Chen et al. 2015) and *Salmo trutta* (Lahnsteiner et al. 2005). This may reduce the probability for reproductive success, depending on the number and mobility of sperm cells. Indeed, in *Salmo trutta* sperm quality was already reduced at 1.75 µg/L and only 28% fully functional sperms were left at 5 µg/L. In addition, reduced sperm quality could also imply genetic alterations and lead to further effects in the offspring. Some evidence is given for *Danio rerio*, where sperm quality was linked to increased number of embryo malformations and larval mortality, which was supported by another study (Chen et al. 2015, Bhandari 2015).

In fish, the development of germ cells was often altered. A trend for a delayed or inhibited

ovulation was observed in fish (*Salmo trutta*) in concentrations from 1.75 µg/L with a total inhibition at 5 µg/L (Lahnsteiner et al. 2005). An accelerated oogenesis was observed for example in *O. latipes* at 100 µg/L (Na et al. 2002 and Medcalfe et al. 2001). In male fish, spermatogenesis was delayed (diagnostic histological endpoint for ED effects) in *O. latipes* at 100 µg/L (Na et al. 2002), *P. promelas* at 1 µg/L (Sumpter et al. 2001), *P. reticulate* (Kinnberg and Toft 2003) and *Gabiocypris rarus (*Zhang et al 2016a). If reproductive processes such as the development of germ cells do not occur synchronized for females and males, serious mating problems might occur, which was discussed by Na et al. (2002) and Medcalfe et al. (2001).

An *altered sex ratio leading to feminisation* (apical endpoint providing evidence for an oestrogen agonist/androgen antagonist MoA) was observed for *Orizia latipes* starting at concentrations of 0.228 µg/L with complete sex reversal at 1820 µg/L, where all fishes developed into phenotypic females and no male fish occurred any more among the tested individuals (Yokota et al. 2000). Histology revealed that none of the fish developed testis. Also at 355 µg/L only 25% showed male secondary sex characteristics. In fish, feminisation is linked to increased Vitellogenin levels in males. A drastic reduction of male fishes may definitely impair population development on the long-term (e.g. Shioda and Wakabayashi 2000). In addition, also altered secondary sex characteristics may have consequences for mating success. BPA inhibited the sword length growth in the green swordtail *Xiphophorus helleri*, which did not subsequently develop in a normal manner (Kwak et al., 2001). This is very likely to influence mating success as males with larger swords are preferred by female fish.

For fish, no studies were available that directly evaluated the reproductive outcome or number of offspring. However, a *disturbed hatchability* was described for *Pimephales promelas* and *Oryzias latipes* (Sumpter et al. 2001, Bhandari et al. 2015). Moreover, as stated above, embryo malformations and higher larval mortality were found in fish. Altogether, Bisphenol A causes developmental and reproductive disturbances in different fish species that most probably will lead to decreased offspring numbers and hence altered population development.

For the thyroid-related MoA in fish, there are only few indications. Similar to the assessment for amphibians, for fish an accelerated development was observed at 200 µg/L, which was blocked when the known thyroid hormone receptor antagonist amiodarone was co-exposed.

In amphibians, endocrine-mediated development and reproduction related effects are linked to the disruptive effects of BPA on thyroidal pathways, leading to effects on metamorphosis and sex ratio respectively.

BPA inhibited the TH-induced as well as spontaneous metamorphosis in *X. laevis*, *R. rugosa* and *S. tropicalis* starting at concentrations of 22.8 µg/L, leading to a delayed development and disturbed life cycle (Iwamuro et al., 2003; Heimeier et al., 2009; Goto et al., 2006, Kashiwagi et al., 2008; Fini et al., 2007). Ultimately, this may lead to a loss of reproductive fitness and definitely affects population development.

In addition some indications are available for an oestrogen-related MoA and adverse effects in ampibians. Similarly to fish, also in amphibians, such as *Xenopus sp.* the sex ratio was skewed towards more female phenotypes at concentrations starting from 23 µg/L as a consequence of the oestrogenic mode of action. This also may lead to negative consequences for mating, reproduction and hence directly affect population development.

Also for amphibians no studies were available that directly evaluated the effects on reproductive outcome or the number and health of offspring. However, the developmental and reproductive disturbances observed may certainly lead to consequences at the population level.

In invertebrates, effects were associated with altered egg or offspring-production, higher incidences for embryo malformations, malformations in the organism itself, altered development, a skewed sex ratio towards females as well as reduced offspring numbers. Due to the high variety of taxa, differences in the endocrine systems, underlying modes of

action and related effects between the different taxonomic families are evident. The effects of Bisphenol A are similar to effects of (xeno-)oestrogens in molluscs. Bisphenol A may interfere with ecdysteroid-related processes in arthropods (insects and crustaceans) as described in the respective chapters.

Altered egg development or offspring production was typically observed in molluscs and crustaceans (daphnids and copepods). Stimulatory effects such as an increase in oocytes, eggs or embryos was observed in the snails *Marisa cornuarietis*, *Potamopyrgus antipodarum* (0.0048 µg/L up to 40 µg/L), the crustacenas *Acartia tonsa* (20 µg/L) and *Daphnia magna* (exposed during the embryonic development, 1 mg/L), as well as in cnidarians (1 mg/L). Further, an increased growth or weight was observed in *Drosophila* and *Chironomus* (the latter: <1 µg/L). Indeed, BPA reduced offspring production has been observed for example in insects (*Drosophila* 100 µg/L) and crustaceans (daphnids at high concentrations) and gammarids (5-17 µg/L). BPA also often caused developmental or embryo malformations or aborts, as observed for molluscs (*P. acuta,* 500 µg/L and *H. d. supertexta,* 1-30 µg/L), crustaceans (daphnids at 10 mg/L, isopods at 10 mg/kg), insects (*Chironomus,* 0.01 µg/L), as well as in poriferans, echinoderms and cnidarians. Malformations of reproductive organs, such as enlarged sex glands (e.g. "superfeminisation") were characteristically observed in 3 snail species (1-50 µg/L, leading to death in *Marisa)*. BPA also caused skewed sex ratios towards females in the terrestrial isopod *Porcelius scaber* (10 mg/kg dw soil) and in snails, intersex was observed. BPA caused developmental delays in several species, which will directly affect reproduction and population growth, such as in mussels (*Haliotis d. supertexta*), insects (*Chironomus* 1-10 µg/L and *Drosophila* 100 µg/L), custaceans (copepod 0.1 µg/L, daphnids 10 mg/L) and annelids. In contrast, a stimulated molting and incomplete ecdysis, resulting in death was observed in isopods related to disturbed ecdysteroid-related pathways.

Taking all the observations for invertebrates together, it becomes clear that the embryo-toxic effects, malformations, or delays in development directly affect the number and health of living individuals of the next generation and will lead to a reduced reproductive outcome with effects on the population level. Also increased offspring numbers may at first glance appear positive for the population, but imply higher energy costs at a possibly "wrong time point" and may decrease the offspring production again in the long-term. Only few studies evaluated the long-term effects, but effects on the population level, e.g. reduced population growth could for example be shown for annelids as well as gammarids after 103 d exposure to BPA in low concentrations.

The following table summarizes the endocrine disrupting effects of Bisphenol A including indications for the underlying endocrine MoA, the apical adverse *in vivo* effects and provides indications for a plausible biological link.

Table 35: Summary of endocrine disrupting effects of Bisphenol A (indications for the endocrine MoA, apical adverse effects and the plausible biological link) in the different taxonomic groups

Taxon	# Species and studies	Indications for the endocrine MoA	Apical adverse effects	Plausible link between MoA and effects & conclusion
Fishes (Chapter 5.5)	10 species (6 apical): *Cyprinus carpio* *Danio rerio* *Gobiocypris rarus* *Oryzias latipes* *Pimephales promelas* *Salmo trutta* *Carassius auratus* *Oncorhynchus mykiss* *Poecilia reticulate* *Xiphophorus helleri* total of 33 studies evaluated	Yes, evidence for a clear oestrogen agonist/ androgen antagonist MoA observed in all 10 species: *In vitro*: • able to activate the oestrogen receptor *in vitro* • competitive binding of Dihydrotestosterone at AR *In vivo (histology/biomarker)* • increased Vitellogenin level in males (6 of 9 species) • changes in gonadal staging • changes in sperm stages and quality in males • testis-ova • secondary sex characteristics	Yes, evidence with respect to oestrogen agonist/androgen antagonist-related effects in all 6 species with tested apical endpoints. Most sensitive adverse endpoints: • Sex ratio (*O.latipes*, *D.rerio*) • Fertilisation success (*O.latipes*, *D.rerio*, *P.promelas*) • Growth (*D.rerio*, *S.trutta*, *O.mykiss*) Most sensitive LOEC: • 0.372 µg/L (sex ratio skewed towards females in F1+F2 generation, *D.rerio*) • 83.7 µg/L (sign. reduced fertilisation rates in F2+F3 generation, reduced embryo survival in F3+F4 generation, *D.rerio*) • 0.6 µg/L other effects: semen quality *C.auratus* and *S.trutta* and sec. sex characteristics *X.helleri*	Yes, **strong *evidence***, plausible link between MoA indications and effects for 5 fish species (*O. latipes, D. rerio, P. promelas, X. helleri)* that ***BPA is an oestrogen agonist/ androgen antagonist.*** Effects observed in all (10) species support the conclusion that there is "strong evidence for adverse effects in fish by an endocrine mechanism". They substantiate the endocrine mode of action and are known to be oestrogen sensitive.
Fishes (Chapter 5.6)	2 species *Oryzias latipes* *Danio rerio* (2 studies)	Evidence for a thyroid MoA from 1 species • altered levels of thyroid hormones • accelerated development caused by 200 µg/L BPA blocked by amiodarone	• Accelerated early embryonic development (1-3 dpf) caused by BPA in *O. latipes* • Additional effects: embryos hatched earlier, were smaller, started sign. earlier with egg production	***Indications for a thyroidal MoA*** In one species accelerated development and altered levels of thyroid hormones occurred, similar to effects known from amphibians.

Taxon	# Species and studies	Indications for the endocrine MoA	Apical adverse effects	Plausible link between MoA and effects & conclusion
		• disruption of thyroid signalling pathways: BPA can interact with corticoid receptors resulting in inhibition of T_3 effects	• effects at 200 µg/L	
Amphibians (Chapter 5.7)	3 species *Xenopus laevis* *Rana rugosa* *Rana nigromaculata* 8 studies	Yes, clear evidence for thyroid antagonistic endocrine activity *In vitro* • Inhibition of T_3-induced gene expression (2.28 mg/L) *In vivo* Inhibition of thyroid hormone induced metamorphic changes in *X. laevis* (2.28 µg/L).	Yes, clear in vivo evidence in 3 species: • Inhibition/delay of metamorphosis *for X.laevis*, *Rana nicromacrulata*, and *R.rugosa at* 2.28 and 22.8 µg/L (in absence of non-specific systemic toxicity) • Blocking of T3 stimulated tail length reduction in *X. laevis* at 2300 µg/L Most sensitive LOEC with regard to a thyroid antagonistic MoA • 2.28 µg/L (metamorphosis)	***Clear evidence for endocrine (thyroid antagonistic) mediated adverse effects*** Effects observed on delayed development (metamorphosis) in absence of non-specific systemic toxicity in 3 species (*X.laevis*, *S.tropicalis*, *R.rugosa)* are diagnostic for an anti-thyroidal mode of action.
Amphibians (Chapter 5.8)	2 species *Xenopus laevis* *Hyla japonica* 9 studies	Clear evidence for an oestrogen-agonist MoA *In vitro* • oestrogen receptor binding *in vivo* • Vitellogenin induction at 22.8 and 228 µg/L in *X. laevis* • Reduction of basal water absorption at 120 µg/L in *H.japonica*	Yes, evidence in 2 species • Sex ratio (more female phenotypes) starting at 23 µg/L (*X. laevis*) (NOEC 2.3 µg/L) Most sensitive LOEC with regard to an oestrogen mode of action: • 7.3 µg/L (geomean of two experiments - sex ratio in *X.laevis*)	***Indications for an oestrogen agonist-like activity*** Effects observed on sex ratio in *X.laevis* and also the reduction of basal water absorption of male pelvic patches in *Hyla japonica* point to an oestrogen mediated mode of action.

Taxon	# Species and studies	Indications for the endocrine MoA	Apical adverse effects	Plausible link between MoA and effects & conclusion
Molluscs (Chapter 5.9)	6-9 species *Marisa cornuarietis* *Potamopyrgus antipodarum* *Physa acuta* *Nucella lapillus* *Haliotis diversicolor supertexta* *Mytilus edulis* *(Valvata piscinali, Lithoglyphus naticoides, Nassarius reticulates)* Studies evaluated: 13 for *in vivo*, 6 for in vitro/biomarker (17 in total)	*In vitro*/biomarker: • Indications for oestrogen receptor binding (2 mg/L) and mRNA expression (40 µg/L) in *Marisa* and *Potamopyrgus* • Increased Vg-like proteins and VTG (*Mytilus and Marisa,* 100 µg/L) *In vivo* • Stimulatory effect of BPA on egg production antagonized by anti-oestrogen • Effects are consistent with effects of estradiol	*In vivo*/apical: • Increase in reproduction (oocytes, embryos) in three species (0.0048-40 µg/L) • Superfeminisation (leading to death) in three species (1-50 µg/L) • Malformations of reproductive organs/tissues at 1 µg/L • Enlarged sex glands • malformed embryos in (*P. acuta* and *H. d. supertexta*) (500, 1—30 µg/L) Lowest effect concentrations • below 1 µg/L (0.048 µg/L embryo production Marisa)	***Similar effects as for (xeno-)oestrogens*** due to in vitro/biomarker studies (receptor binding, mRNA expression, VTG) and the observed *in vivo* effects (superfeminisation, enhanced reproduction, malformations), which are similar to those of the oestrogen estradiol and furthermore co-mitigated by anti-oestrogens. Link provided for at least four species.
Insects (Chapter 5.10)	3 species: *Chironomus riparius* *Drosophila melanogaster* *Euborellia annulipes* (studies evaluated: 3 *in vitro*/biomarker, 7 *in vivo*, 10 in total)	• Ecdysteroid receptor binding in two species (0.5-3 mg/L) and mRNA expression • Oestrogen related receptor (ERR) in Chironomus expressed from 5 µg/L	• Delayed development in two species (emergence, maturation and molting) at 1-10 µg/L • Reduced fecundity, decreased emergence (100 µg/L Drosophila 10 µg/L Chironomus). • F1 more sensitive (emergence Chironomus from 0.078 µg/L), less females 1µg/L • Characteristic mouthpart deformities (Mentum) 0.01 µg/L Chironomids • Enhanced larval growth (Drosophila), weight	Proposed ***disruption of ecdysteroid-related pathways*** In vitro an ecdysteroid receptor binding/expression was demonstrated, the in vivo effects such as mouthpart deformities and delayed development are similar to effects of known (xeno-)oestrogens such as NP Chironomus is sensitive, responds at low concentrations, relatively few in vivo studies available.

Taxon	# Species and studies	Indications for the endocrine MoA	Apical adverse effects	Plausible link between MoA and effects & conclusion
			(Chironomus, < 1µg/L) • Malformations of reproductive organs (testis and ovarian growth Euborella, injected)	
Crustaceans (Chapter 5.11)	8 species: *Daphnia magna* *Ceriodaphnia dubia* *Acartia tonsa* *Tigriopus japonicas* *Americamysis bahia* *Gammarus pulex* *Gammarus fossarum* *Porcelio scaber* Studies evaluated: 14 for *in vivo*, 3 for *in vitro* effects, 16 in total)	• Increased ecdysteroid levels in isopods • Affects methlfarnesoates and thereby indirectly ecdysteroids in daphnids • effects mitigated by 20-OH-Ecdysone in daphnids • Gene and protein expression pattern related to reproduction or molting in daphnia and isopoda	• Delay/Enhancement of molting cycle and development (copepods 0.1 µg/L, 10 mg/L daphnids) • Reproduction and brood sizes reduced (Gammarus 5-17 µg/L) • Also stimulation of offspring production in copepods (20 µg/L), daphnids (10 mg/L) • F1 generation more sensitive (copepods development, 0.01 µg/L), daphnids • Embryo malformations (9.9 mg/L daphnids), abortions (10 mg/kg isopods) • incomplete ecdysis, skewed sex ratio and stimulated molting related to increased ecdysteroid titres in isopods (from 10 mg/kg)	Proposed ***disruption of ecdysteroid-related pathways*** Evidence from in vitro studies (ecdysteroid receptors known from related insects, changes in ecdysteroid titres in crustaceans, alternative action via methylfarnesoates for daphnids) fit to the observed effects in vivo (malformations, delayed development, but also increase of offspring production) and partly demonstrated in same study (e.g. increased ecdysteroid titres related to incomplete ecdysis, skewed sex ratio and stimulated molting in isopods). Effects are mitigated/diminished by known ecdysteroids or similar to effects of known EDs. Malacostracan crustaceans more sensitive.

Taxon	# Species and studies	Indications for the endocrine MoA	Apical adverse effects	Plausible link between MoA and effects & conclusion
Further Inverte-brates (Chapter 5.12)	**2 Porifera** *(1 study)* *Heteromyenia sp.* *Eunapius fragilis* **1 Cnidaria** (2 studies) *Hydra vulgaris* **Annelida** (2 studies) *Lumbriculus variegatus* *Capitella capitata)* **5 Echinodermata** (3 studies) *Strongylocentrotus purpuratus* *Strongylocentrotus nudus* *Lychtechinus anamesus* *Hemicentrotus pulcherrimus* *Paracentrotus lividus*	No *in vitro* studies available, but comparison to known (xeno-)oestrogens: • Porifera: Similar embryo abnormalities than known for NP • Cnidaria: similar effects on polyp structure and reproduction than EE2 • Annelida: no evidence • Echinoderms: effects mitigated/enhanced by known modulators, similar effects on development as OP and E2, but effects on malformations different from EE2	• Developmental malformations, embryotoxicity in Porifera (16 µg/L), Echinodermata (EC_{10} 142-570 µg/L, Cindaria (42 µg/L Cinidaria) • Spermiotoxicity Echinodermata (138 µg/L EC_{10}) • Developmental disturbances (0.05 µg/L EC_{50} metamorphosis delayed Annelida larvae, reproduction enhanced at 1 mg/L Cnidaria) • Growth reduced (160 µg/L Porifera, 22.8 µg/L Echinodermata) • Population growth reduced in Annelida (2 µg/L after 103 d)	***Underlying modes of action are unclear.*** Similar effects as observed for known (xeno-)oestrogens but data is very fragmentary for the different taxa Echinoderms: similar effects as for known EDs (embryo abnormalities, abnormal development) Porifera and Cnidaria: similar effects (abnormalities, polyp structure) as for known EDs Mechanism unclear for Annelida.

6 Conclusions on the SVHC Properties

6.1 CMR assessment

Not relevant for the identification of the substance as SVHC in accordance with Article 57 (f) REACH.

6.2 PBT and vPvB assessment

Not relevant for the identification of the substance as SVHC in accordance with Article 57 (f) of REACH Regulation.

6.3 Assessment under Article 57(f)

Bisphenol A is assessed in order to identify, if it is a "substance 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" (Article 57(f) of REACH). Hence, two questions need to be answered:

- Does Bisphenol A have endocrine disrupting properties? (6.3.1)
- Is there scientific evidence of probable serious effects to the environment which give rise to an equivalent level of concern compared to CMR or PBT substances? (6.3.2)

The fulfilment of this requirement with regard to the seriousness of effects and the equivalency of concern is considered employing a weight of evidence approach.

6.3.1 Summary of the data on the hazardous properties

A summary on the indications for an endocrine MoA and apical adverse effects for the analysed taxonomic groups is given in the following paragraphs, which follow the structure of the hazard assessment section, where details are given (chapter 5.5. – 5.13, summary in 5.15). It has to be noted, that the focus is on the vertebrates species (fish and amphibians). The results for invertebrate taxa are considered as supportive due to the still fragmentary data, knowledge and the lack of agreed guidance for regulatory purposes to conclude on ED properties – these data only support and complete the overall picture. The observed adverse effects in mammalian vertebrates are relevant for effects on mammalian wildlife species in the environment (such as mice, rats) and supportive for non-mammalian vertebrate species (fish, amphibians) with respect to the underlying mode of action and adverse effects.

6.3.1.1 Fish

The mode of action of BPA as an **oestrogen agonist/androgen antagonist** in fish is supported by a number of *in vitro* studies, demonstrating that it is able to bind to and activate the oestrogen receptor of mammals and fish *in vitro* in the low µM-range, and showing competitive inhibition of androgenic activity of a known AR agonist (Dihydrotestosterone) at the AR in mammalian and fish cells in concentrations between 0.1-100 µM.

Diagnostic for the oestrogen agonist mode of action in fish according to OECD Guidance No. 150 (OECD, 2012b) are a receptor binding, Vitellogenin induction in males (and females), changes in gonadal staging (increased proportion of early sperm stages and changes in oocyte stages) and testis-ova according to the OECD Guidance on Histology Assessment (OECD, 2010), as well as a female biased phenotypic sex ratio and a depression of male secondary sex characteristics. These effects were observed for BPA in high quality studies.

For the following species, there is a biologically plausible link between the mode of action and adverse effects: *Oryzias latipes* (Medaka) (VTG induction in males at similar concentrations at which testis ova and changed gonadal staging occurred without secondary side effects such as mortality or growth, skewed sex ratio towards females), *Danio rerio* (Zebrafish) (VTG induction,

testis-ova and sex ratio skewed towards females, reduced fertilisation success). For one species (*Pimephales promelas* - Fathead minnow) it is likely that the observed effects (VTG induction and reduced egg production) are endocrine mediated, although effects were first observed at higher concentrations, where survival was affected (16 µg/L). For six other fish species (*Cyprinus carpio, Gabiocypis rarus, Salmo trutta, Carassius auratus, Oncorhynchus mykiss*, *Poecillia reticulata, Xiphophorus helleri*) results show that Bisphenol A is endocrine active *in vivo*. Vitellogenin was induced (*C. carpio, G. rarus, C. auratus, O. mykiss, X. helleri*), oviduct formation was observed in males (*C. carpio*), and effects on sperm quality up to disruption of spermatogenesis occurred (*G. rarus, S. trutta, C. auratus, P. reticulata*). In females the complete inhibition of ovulation (*S. trutta*) was observed.

Additional endpoints that are potentially sensitive but not diagnostic with respect to an oestrogen MoA in fish are growth, survival, behaviour, time to first spawn, fecundity, and fertilisation success. For example, in *Danio rerio* fertilisation rates and embryo survival were affected, also in the following generations (F3-F4), and also growth was affected in the FSDT.

The lowest endocrine mediated effects observed for fish were below 1 µg/L, i.e. the phenotypic skewed sex ratio in F1 and F2 generation of *D. rerio* at 0.372 µg/L (Chen et al., 2015). Semen quality and secondary sex characteristics were affected in *C. auratus* and *X. helleri* at 0.6 µg/L (Hatef et al. 2012a).

According to OECD GD 150 a substance is almost certain to be an ED causing endocrine mediated effects if the sex ratio is biased towards females and effects observed at other levels (*in vitro*, histology) fit to this observation. There is a biological plausible link that Bisphenol A alters the function of the endocrine system in fish leading to adverse effects in the intact organism, its offspring and populations. Endocrine mediated effects in fish are among the most sensitive endpoints for the species tested and occur at concentrations below 1 µg/L (e. g. female biased sex ratio) and in several cases below 10 µg/L. The lowest NOECs for non-specific systemic toxicity are much higher. The NOEC used for PNEC derivation in the EU RAR (EC, 2010) was 16 µg/L.

Thus, there is strong evidence from high quality experimental studies that Bisphenol A acts as a sexual endocrine disruptor in a variety of fish species via an oestrogen agonist (androgen antagonist) mode of action.

Additionally, there is some evidence for a possible **thyroidal action** of Bisphenol A in fish. In *in vitro* studies with mammalian, amphibian and fish cells BPA interacts with proteins (e.g. TR, TTR) related to the HPT axis. Diagnostic for a thyroidal mode of action in amphibians is an accelerated development *in vivo* together with increased levels of thyroid hormones. In fish, i.e. *O. latipes* early embryonic development was enhanced by BPA at 200 µg/L BPA (and blocked by amiadorone a known thyroid antagonist (inhibitor of deiodinases)). Additionally embryos hatched earlier, were smaller and started significantly earlier with egg production. Unfortunately, for fish no study reported measured hormone levels. However, the mitigation experiments with a known thyroid antagonist, which diminished the effects induced by BPA clearly support the thyroid acion of BPA.

Hence, there are some indications for a second mode of action in fish, a thyroidal action similar to amphibians.

6.3.1.2 Amphibians

The **thyroidal mode of action** in amphibians is supported by a number of *in vitro* studies with amphibian and mammal cells, which demonstrate that BPA is able to replace T_3 from the TTR transport protein as well as from the thyroid receptor (TR) and hence acts as a T_3-antagonist.

Diagnostic for a thyroid related mode of action are accelerated development (developmental stages or hind limb length), asynchronous development or abnormal thyroid histopathology according to OECD TG 231 (Amphibian Metamorphosis Assay (AMA)) and the OECD Guidance No. 150 (2012).

For BPA, *in vivo* evidence is available showing a delayed metamorphosis and altered development/malformations. Several studies mainly with *X. laevis* but also with *X. tropicalis* and

R. rugosa reported inhibition of T_3 and delayed metamorphosis when exposed together with T_3. The results are very similar to the effects of the known thyroid antagonist Methimazole (Thiamazole), which blocks the synthesis of TH in the thyroid gland and also spontaneously inhibits metamorphosis in the "Amphibian Metamorphosis Assay" (OECD, 2009b).

Hence, for amphibians, there is clear evidence that Bisphenol A acts as thyroid antagonist.

In addition, indications for a possible oestrogen-like mode of action - similar to fish - is given in amphibians. Indicators for an **oestrogen-like activity** of BPA in fish are "feminising effects" such as a sex ratio skewed towards females (intersex gonads) and a delay in metamorphosis according to Kortenkamp et al. (2012) and OECD No. 91 (2008) as well as the ability to induce Vitellogenin. For amphibians no agreed guideline is available with respect to oestrogenic effects. However, evidence that BPA also exerts oestrogen-agonist effects in amphibians is provided for three species:

In vitro results for one species (*X. laevis*) indicate that Bisphenol A induces Vitellogenin expression by binding to the oestrogen receptor in concentrations starting at 22.8 µg/L. Adverse effects in a study with *X. laevis* (change in sex ratio and reproduction) are comparable to effects observed for 17ß-estradiol providing indication that effects may be caused by an oestrogen-like mode of action. A skewed sex ratio towards females was observed in concentrations starting at 23 µg/L. This oestrogen-like effect was also found in three other frog species (*X. tropicalis*, *R. rugosa* and *R. nigromaculata*) where sex reversal towards females was shown to start in low µg/L concentrations in reliable studies.

Hence, there are indications that BPA may additionally act via an oestrogen mode of action in amphibians.

6.3.1.3 Molluscs

Although no specific internationally accepted guidance or specific test protocols (except OECD TG 242 and 243 for Potamopyrgus and Lymnea, which have not been validated for stimulatory effects) to determine endocrine effects in molluscs exist, there are indications from review studies for possible endocrine related modes of action and related effects as well as comparisons to effects of known oestrogens. Still, knowledge on the endocrinology and available studies for different mollusc taxa is fragementary.

The possible **action similar to (xeno)oestrogens** is supported by binding to an identified oestrogen-related receptor (2 mg/L), mRNA expression (40 µg/L) in *M. cornuarietis* (*in vitro*) and *P. antipodarum* (*in vivo*), increased VTG-like protein and VTG levels in *M. edulis* and *M. cornuraietis* (100 µg/L) as well as by the mitigation of characteristic effects on egg production by anti-oestrogens in vivo for two species (*M. cornuarietis* and *P. antipodarum*). There is still discussion on the functionality of estrogen receptors in molluscs and further endocrine pathways are possibly involved and could further substantiate the underlying mode of action of the known effects of oestrogens and oestrogen-like substances in molluscs.

The characteristic effects in molluscs were an increase of oocytes and embryos (0.0048–40 µg/L), the induction of superfemales (1-50 µg/L), and malformations of the genital tissues (1 µg/L), observed at very low concentrations for three snail species and one bivalve. These effects are known to be caused by oestradiol. For one other snail species and a bivalve species, there is evidence for embryotoxicity with malformed embryos and developmental disturbances (the snail *Physa acuta* at 500 µg/L and the abalone *Haliotis diversicolor supertexta* at 1-30 µg/L).

Although the determination of the lowest effect concentrations are statistically difficult, molluscs were the most sensitive invertebrate group analysed with effects around 1 µg/L depending on statistics. Overall, the studies support the finding that snails are affected at low concentrations and effects are associated with reproductive and developmental disturbances, which may lead to unpredictable population effects in the long term.

6.3.1.4 Insects

Ecdysteroids (such as e.g. OH-ecdysone) are the most prominent hormones responsible for developmental and reproductive processes in arthropods. As ecdysteroids are steroid hormones and very similar to oestrogens, it is possible that these may be affected by BPA. The possible **anti-ecdysteroid action** and **interference with ecdysteroid-related** processes is substantiated by an antagonistic ecdysteroid receptor binding of Bisphenol A in *Drosophila melanogaster* at 22 mg/L and mRNA expression after BPA exposure in *Chironomus riparius* at 0.5-3 mg/L. In addition the presence of an oestrogen-related receptor (ERR) was identified for chironomids and expressed at 5 µg/L BPA, but the function of the receptor is not clear. Also here knowledge and available studies are still fragmentary.

The apical effects related to an interference with ecdysteroids in Chironomus and Drosophila comprise a delayed developmental time (emergence, maturation, molting) at 1-10 µg/L, reduced fecundity and decreased emergence at 10 and 100 µg/L respectively, and enhanced larval growth in Drosophila and increased weight in Chironomus. In Drosophila the reduced fecundity was shown for BPA at the same concentrations as observed for OP and NP in the same studies. In Chironomus the characteristic mouthpart deformities, known for the (xeno-) oestrogens (EE2, NP), occurred at 0.01 µg/L BPA. For Chironomus, the F1 generation proved to be more sensitive (emergence 0.078 µg/L, less females 1 µg/L). Additionally, indications for an abnormal development of testis and ovaries were provided for the earwig *Euborella annulipes*, although test conditions were artificial.

Although very few *in vivo* studies are available for insects, the possible interference with ecdysteroid related processes and action as an ecdysteroid antagonist is supported by *in vitro* studies as well as the similarity to effects from (xeno-)oestrogens. Effects are also comparable to those observed for the other arthropod taxon crustaceans (see below). It is to be noted, that in particular the second generation of Chironomus was shown to be adversely affected in low concentrations (µg/L range).

6.3.1.5 Crustaceans

Similar to insects there are indications for a **disruption of ecdysteroid-related pathways** in crustaceans. Although no receptor studies are available for crustaceans, the possible existence of such receptor and binding was demonstrated for the closely related insects. For daphnids it was shown that BPA probably elicits anti-ecdysteroidal effects indirectly via an interaction with the crustacean juvenoid hormone methylfarnesoate. Further indications are provided by increased ecdysteroid levels in isopods related to *in vivo* effects as well as gene and protein expression patterns related to reproduction or molting in daphnia and isopods. Additionally, co-mitigation experiments with known hormones (e.g. with OH-ecdysone in daphnids) substantiate the underlying mode of action.

Apical effects are associated with embryo malformations, a delay of development and molting and effects on the reproductive outcome. A delay of the molting cycle and development was observed in copepods at 0.1 µg/L while offspring production was enhanced at higher concentrations (20 µg/L). An increase of offspring production was also observed for daphnids (10 mg/L), but decreased due to a high incidence of embryo abnormalities (from 9.9 mg/L). Abortions have also been observed for isopods (10 mg/kg). Furthermore, the second generation was more strongly affected in both copepods (0.01 µg/L) and daphnids. In malacostracan crustaceans a possible link to the underlying mode of action and apical effects may only be provided for isopods. In isopods, increased ecdysteroid levels were related to skewed sex ratio towards females, embryo malformations, incomplete ecdysis leading to death, and an enhanced molting (from 10 mg/kg). For gammarids, less information is available. However, reproductive parameters were affected at low concentrations (5-17 µg/L) in a long-term (103 d) experiment with *G. fossarum*.

While daphnids only responded in the mg/L range close to lethal toxicity, these studies provide indications for a possible underlying endocrine mode of action. Some crustacean species, such as copepods are very sensitive with effect concentrations in the low µg/L range, although not as sensitive as molluscs.

6.3.1.6 Further invertebrate taxa

Only very fragmentary data and knowledge is available for the diverse set of further invertebrate taxa, covering echinoderms, poriferans, cnidarians and annelids. There are some indications for possible endocrine-mediated effects due to comparisons to known (xeno-)oestrogens. However, no clear link to the underlying modes of action for the different taxonomic groups can be provided. In Porifera, similar embryo abnormalities as known for NP and in Cnidaria similar effects on polyp structure and reproduction compared to EE2 were found. For Echinoderms similar effects on development were observed as for OP and E2, but different effects on malformations compared to EE2.

The adverse *in vivo* effects comprise embryo malformations and alterations of development and reproduction with early developmental periods as a sensitive time window. Evidence for developmental disturbances, embryotoxicity and malformations is available for Porifera (16 µg/L), Echinodermata (EC_{10} 142-570 µg/L, 138 µg/L spermiotoxicity), Cindaria (42 µg/L development, 1 mg/L reproduction) and Annelida (0.05 µg/L EC_{50} metamorphosis delayed). Growth was reduced for Porifera (160 µg/L) and Echinodermata (22.8 µg/L).

Hence, for echinoderms similar effects as for known (xeno-)oestrogens were observed (embryo abnormalities, abnormal development). For Porifera and Cnidaria, less indications (similar effects on abnormalities, polyp structure as for known (xeno-)oestrogens) are provided. Possible mechanisms remain unknown for Annelida.

6.3.1.7 Conclusions on the hazardous properties

Based on the presented data for fish and amphibians, Bisphenol A meets the WHO/IPCS definition of an endocrine disruptor: *"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)"* as interpreted by the EC ED EAG (JRC, 2013).

As summarized above, Bisphenol A unambiguously acts as endocrine disruptor in fish and anuran amphibians with different modes of action: oestrogen agonistic/androgen antagonistic in fish and thyroid antagonistic in amphibians. Here, clear indicative effects as well as clear plausible links between the MoAs and adverse effects are provided for fish and amphibians. The endocrine-mediated effects occurred at lower concentrations than acute, systemic or narcotic toxicity.

Additionally, there is evidence for a possible anti-thyroidal mode of action in fish as well as an oestrogen agonstic mode of action in amphibians leading to adverse effects.

The observed adverse effects in mammalian vertebrates are relevant for effects on mammalian wildlife species in the environment (such as mice, rats) and supportive for non-mammalian vertebrate species (fish, amphibians) with respect to the underlying mode of action and adverse effects.

The evidence for vertebrates is further supported by the indications for adverse effects of Bisphenol A in invertebrates, which are possibly endocrine-mediated. The observed *in vivo* effects together with studies on receptor binding, gene expression, and comparisons with known EDs and natural hormones, point towards an endocrine-related MoA similar to (xeno-)oestrogens in molluscs and an interference with ecdysteroid-related pathways in arthropods (crustaceans and insects). Data for further invertebrate taxa is still fragmentary.

Overall, Bisphenol A clearly disrupts oestrogen-mediated processes in fish (also observed in amphibians) and thyroid-mediated processes in amphibians (also observed in fish). This is supported by evidence for adverse effects in invertebrates, which are possibly endocrine-mediated.

6.3.2 Equivalent level of concern assessment for the environment

The effects of Bisphenol A on fish and amphibians are considered to be of equivalent concern to those of PBT or vPvB substances due to the severity and irreversibility of the effects on organisms

and populations and the difficulties to quantify a safe level of exposure with regard to the endocrine mediated effects.

The assessment follows the same line of arguments as for previous SVHC-identifications according to Article 57(f) ED for the environment. Due to the amount of data available for BPA a large number of arguments for an equivalent level of concern can be provided. All arguments are used in a weight of evidence and as such, none of the arguments alone are decisive for the decision and not all of them are needed to conclude on the equivalent level of concern. We decided to present the available evidence to get a view on the overall picture on the data analyzed.

BPA severely affects reproduction- and development-related processes in organisms

Apical effects for BPA were associated with developmental and reproductive disturbances, malformations, or embryo-toxic effects at the organism level (see chapter 5 and 6.3.1). These effects are considered severely affecting population stability and recruitment. Effects are not reversible, but still persistent when exposure ceases. Such irreversible effects are for example embryonic or adult malformations and sex reversal. As observed in the studies, reproduction is often not only affected by lowering the number of offspring, but caused by a delay in developmental time, increased incidences of malformed embryos, malformed sexual organs and may also cover abnormal increased offspring production. The long-term effects of these alterations in normal functioning are difficult to predict. The severity of these effects and significance for the population level have already been demonstrated for different alkylphenols with an oestrogenic mode of action and similar apical effects.

In fish, adverse effects clearly related to the oestrogen agonist mode of action included an altered sex ratio leading to a feminisation which was observed for *O. latipes* and *D. rerio* in concentrations starting at 0.372 µg/L (Chen et al., 2015). At higher concentrations (up to 1280 µg/L) a complete sex reversal where all fishes developed into phenotypic females occurred (Yokota et al., 2000). Such a drastic reduction of male fishes may definitely impair population development in the long-term. The development of germ cells was altered in several species, so that sperm and oocytes did not develop in a synchronized way for males and females as seen for *O. latipes* (100 µg/L) (Tabata et al., 2001) which may lead to serious mating problems. Further effects included altered secondary sex characteristics with consequences for mating success or a reduced quality of sperm in *D. rerio* and *S. trutta* (1.75–5 µg/L) (Chen et al., 2015 and Lahnsteiner et al., 2005).

In amphibians, adverse effects are clearly related to a thyroidal mode of action. The inhibited metamorphosis (TH-induced as well as spontaneous) in *X. laevis*, *R. rugosa* and *S. tropicalis* (22.8 µg/L) lead to a delayed development and disturbed life cycle (Heimeier et al., 2009 and Goto et al. 2006). Ultimately, this may also lead to a loss of reproductive fitness. Additionally, there is some evidence for a skewed sex ratio towards more female phenotypes, related to an additional oestrogen mode of action in *Xenopus sp.* at low concentrations (23 µg/L) (Levy et al., 2004). Here seemingly contradicting results (no effects) at 500 µg/L exist (Pickford et al., 2003), but Levy et al. (2004) has not tested higher than 23 µg/L and U-shaped dose-response curves were also seen in experiments with other organisms. None of the studies directly evaluated the effects on reproductive outcome or number and health of offspring, but the developmental and reproductive disturbances found are considered to lead to consequences at the population level.

Further support is gained from the group of ***invertebrates***, where adverse effects potentially fit to an endocrine activity of BPA (proposed oestrogen-like action in molluscs, anti-ecdysteroid-related in arthropods). Endocrine-related effects were associated with altered egg or offspring-production in molluscs (0.0048 µg/L up to 40 µg/L, e.g. Oehlmann et al. 2006, Duft et al. 2003, Jobling et al. 2004) and crustaceans (copepods at 20 µg/L, Andersen et al. 1999). Increased offspring numbers may at a first glance be positive for a population, but imply higher energy costs at a possibly "wrong time point" and may lead to reduced reproduction in the long-term. Furthermore, higher incidences of malformations in snails (enlarged sex glands at 1-50 µg/L, leading to death) or chironomids (mouthpart deformities at 0.01 µg/L) as well as malformed embryos in snails (1-30 and 500 µg/L) and crustaceans (daphnids at 10 mg/L, isopods at 10 mg/kg) as well as echinoderms became prominent. In the terrestrial isopod *Porcelius scaber*

skewed sex ratios towards females were observed (10 mg/kg dw soil). Taken together, these effects may directly affect the number and "quality of living" for individuals of the next generation. Decreased or delayed reproduction after BPA exposure was observed for example in crustaceans and insects at variable concentrations (0.1–10 mg/L and 1-100 µg/L, respectively).

BPA causes severe effects also after short-term exposures (in particular during sensitive time windows)

Exposure of organisms to Bisphenol A may cause delayed or long-term effects. Here, a short time exposure may be sufficient to provoke long-term effects even if exposure ceases. This is in particular the case when exposure occurs during critical time windows, life stages during development or during specific seasons. This is in line with our knowledge about the endocrine system. Endocrine modulation is a very complex feedback process that is set up during critical life stages. As summarized in IPCS (2002) disturbance of this set up may result in effects during the entire life-time. The disturbances of a transient exposure during sensitive life stages is irreversible and results in effects during the entire life and even in the next generation with long-term consequences at the (sub)population level.

In fish, a short-term exposure (7 d) of *O. latipes* embryos to 87 µg/L BPA in a critical time window for sex determination in the F0 generation only resulted in reproductive abnormalities in the F2 generation (reduced fertilisation rates) and the F3 generation (survival of embryos), but not in the parental F0 or the F1 generation (without any further exposure to BPA) (Bhandari et al., 2015). Several studies with fish, i.e. *Danio rerio* (Chen et al., 2015) and *O. latipes* (Shioda and Wakabayashi, 2000), indicate stronger effects on the next generations and that parental exposure influences the health of offspring. In *O. latipes*, a two-week exposure of adult males lead to a reduced fecundity and hatching success of offspring. In *D. rerio*, the proportion of malformed and dead offspring increased if parents were exposed to BPA.

This is supported by the studies with invertebrates, where the second generation was affected to a higher degree or at lower concentrations in crustaceans (copepods: factor 10 and daphnids) as well as insects (chironomids). In different taxa early life stages and the embryonic development were in particular affected by BPA, visible in the high embryo-toxicity and malformations in several invertebrate species.

Furthermore, effects may only become prominent under certain seasonal conditions. This was for vertebrates shown in amphibians (e.g. Kloas et al., 1999). In snails the influence of seasonality was demonstrated for *Marisa cornuarietis*. While significant effects occurred during periods of low spawning (Oehlmann et al., 2006), effects were masked during the main spawning period in another study (Forbes et al. 2007 and b).

BPA elicits long-term effects across generations affecting populations and communities

Effects on the following generations or population development are usually not covered with standard test protocols and data availability is scarce. However, some studies demonstrate trans-generational effects of Bisphenol A, supporting the arguments in the previous paragraph related to the impact of effects on sensitive early life stages on reproduction and development and the long-term consequences for further generations and the population and even community level.

Different studies showed that the sensitivity of fish increases from one generation to the other when continuously exposed. In *Pimephales sp.,* the LOEC for the parameters egg production and hatchability decreased from one generation to another generation by a factor of 4 (LOEC(F1) 640 µg/L, LOEC(F2) 160 µg/L)) (Sumpter et al. 2001). For *Pimephales promelas* the effect on VTG induction in males was stronger in the subsequent generation (Sumpter 2001, Sohoni et al. 2001). For *Danio rerio* effects on Vitellogenin levels in males increased from F1 to F2 generation by a factor of 40 (F1: LOEC 400 µg/L, F2: LOEC 10 µg/L) (Keiter et al. 2012).

Sensitivity even increased in the following generations when only the parental generation was exposed. This was for example shown for *Oryzias latipes*, where the LOEC for the parameter fertilisation rate decreased in the F2 and F3 generations and embryo survival decreased in F3

and F4 generations after exposure of the F0 generation only (83.7 µg/L, 7 d during critical window for sex determination) (Bhandari et al., 2015).

In general, studies directly investigating the effects on the population or the community level are scarce. Long-term effects of Bisphenol A were observed by de Kermoysan et al. (2013) in a long-term (165 d) lotic mesocosm study for macrophytes, macroinvertebrates (the wandering snail *Radix balthica*, *Chironomus sp.,* turbellarians *Dugesia sp*. (plathelminthes), the leech *Glossiphnia complanata*) and fish (the three-spined stickleback *Gasterosteus aculeatus*). Significant effects on three trophic levels were shown at 100 µg/L: BPA directly affected macrophyte community structure, and directly and indirectly affected macro-invertebrate abundances and community structure as well as fish population structure. In particular, probable endocrine-mediated effects on fish were already identified at low concentrations: gonad morphology was affected at ≥1 µg/L for female and ≥10 µg/L for male fish. This substantiates the hypothesis, that the indications for endocrine disrupting effects in fish (via an oestrogen MoA) are also manifested in long-term experiments that are rather close to real environments.

BPA affects a large variety of species in different ecosystems

Bisphenol A adversely affects a high variety of different ecologically important species in different ecosystems. In the present analysis effects were demonstrated in nine fish species, six amphibian species, as well as a high number of invertebrate species. Presumably a higher number of organisms is affected, because of the assumed different modes of action and target sites of Bisphenol A. As only representatives for different organism groups and thus only a small proportion of the existing species are considered during the assessment, potential effects on further organisms remain unknown and there is a potential that a number of further species may be very sensitive to an exposure of Bisphenol A. Adverse effects are thus not restricted to certain taxonomic groups or species.

It is evident that effects are not restricted to certain environments. Although fish and amphibians mainly belong to freshwater habitats, species in lentic and lotic freshwaters, marine waters, sediments, and terrestrial environments are affected. Moreover, BPA has an ubiquitous occurrence and enters the environment more or less continuously via emissions from various sources, which is supported by monitoring studies (see section 3.3). Hence, BPA is not restricted to certain environmental compartments, local sites or specific time points. Therefore migratory species may be more or less continuously exposed to low concentrations, cannot escape from exposure and effects may become pronounced later as was described in the previous paragraphs.

Moreover, it is to be kept in mind that BPA acts jointly with other chemicals occurring in the environment, so that exposures at comparatively low concentrations may lead to effects. Typical examples are sewage plant effluents, e.g. from paper mills, where Bisphenol A occurs jointly with other chemicals. These may be known EDs with similar MoA but also chemicals with different MoA that act additively or even synergistically.

Difficulties to derive and quantify a safe level of exposure for BPA

The endocrine properties of Bisphenol A as shown in several studies lead to a very high uncertainty with respect to a definite toxicity threshold in the environment. On the basis of the available data it appears difficult to derive a safe level in the environment, although it might exist. Reasons were already mentioned in previous paragraphs and are in summary:

It is difficult to determine definite low effect concentrations as effects may only be observed in certain life stages, time windows or seasons as was shown for fish and amphibians, but also crustaceans. The impact is very difficult to predict as sensitive time windows during development are very different for organism groups and also individuals. This may be a reason why effects sometimes are observed, sometimes are "overlooked" or do not become prominent in experiments as discussed above.

Moreover, concentration response-relationships are often not monotonic. For BPA, this could for example be observed in fish where sperm quality was affected at very low concentrations and very high concentrations but not inbetween. In invertebrates (crustaceans), egg production was sometimes stimulated at lower but reduced at higher concentrations. An explanation for these

contradicting effects is that the hormonal receptors are sensitive to certain trigger-concentrations, or that different modes of action are triggered.

It was shown that BPA elicits effects via different modes of action in fish and amphibians (oestrogenic and thyroid mode of action) as well as in invertebrates (anti-ecdysteroid action in arthropods) and that BPA affects a great variety of organisms possibly due to these different "points of attack".

Moreover, certain species are in particular sensitive and affected at low concentrations (even below or around 1 µg/L), levels which are indeed measured in the environment. This is true for fish and amphibians, but also for snails. In particular, endangered species such as amphibian species are affected (artificially T_3-induced metamorphosis was inhibited at 22.8 µg/L) (Heimeier et al., 2009 and Goto et al., 2006).

It was demonstrated, that non-standard test species and non-traditional endpoints may be much more sensitive than endpoints usually considered in OECD standard test protocols as. A great variety of taxa was shown to be affected by BPA, making it probable that certain – up to now unknown – species could be affected. It was demonstrated that a wide range of species with different functions in ecosystems is affected.

Although the endocrine system with its hormones and functioning is conservative among species, the affected specific hormones, binding affinities and modes of action differ between taxa. Bisphenol A is able to act via slightly different but still related endocrine pathways. Owing to the lack of in depth knowledge of their endocrine system and the lack of test systems it is difficult to estimate which species are most sensitive and therefore difficult to establish a concentration which could be regarded as safe for the environment.

6.3.3 Conclusion on the hazard properties and equivalent level of concern assessment

Bisphenol A (BPA) 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 the environment for which there is scientific evidence of probable serious effects to the environment which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of REACH Regulation.

The analysis of results for fish and amphibians according to the OECD Guidance Document for Endocrine Disrupters (OECD 2012b) reveals that BPA needs to be considered as an endocrine disruptor. It fulfils the WHO/IPCS definition of an endocrine disruptor as interpreted by the European Commission's Endocrine Disruptor Expert Advisory Group (JRC 2013) in their recommendations for a substance to be identified as an endocrine disruptor.

For BPA there is scientific evidence from good quality studies that the substance causes endocrine mediated adverse effects in several fish and amphibian species.

BPA clearly acts as an oestrogen agonist in fish.

In several **fish** species clear evidence that BPA acts as oestrogen agonist is provided:

- *In vitro* data unambiguously show that BPA binds to vertebrate (human and fish) oestrogen receptors in the µM range and modulates gene expression. BPA also competitively inhibits androgenic activity of a known AR agonist.
- The oestrogen agonist mode of action is unambiguously substantiated by *in vivo* data in several species. Diagnostic for the oestrogenic mode of action are the observed Vitellogenin (VTG) induction, changes in gonadal staging, testis ova, altered sex ratio, and reduced male secondary sex characteristics.

The effects observed are clearly adverse, such as the skewed sex ratio towards females. A direct link between the oestrogenic mode of action *in vivo* (e.g. VTG induction, testis, ova) and the adverse effects (sex ratio, reduced egg production) is provided for *O. latipes*, *D. rerio* and is very likely for *P. promelas*. Additionally, for six other fish species adverse effects which are known to

be sensitive towards an oestrogenic mode of action were demonstrated, such as affected growth, behaviour and fertilisation success.

In addition, there is evidence that BPA acts as oestrogen agonist in **amphibians**:

- An agonistic VTG induction was demonstrated in hepatocytes of *X. laevis* at 22.8 µg/L *in vitro*. Further evidence is provided by the *in vitro* studies demonstrating binding to the oestrogen receptor in other vertebrates.
- The oestrogenic mode of action is substantiated in *X. laevis* by a skewed sex ratio (23 µg/L), a delay of development, altered testicular structure (2.28 µg/) and the ability to induce Vitellogenin *in vivo* (22.8 µg/L) as well as similar results for E2.
- A direct link is provided between VTG induction *in vitro* and *in vivo* through a plausible binding to the oestrogen receptor and changes of the sex ratio and reproduction observed *in vivo* for *X. laevis* as well as three other species. These effects are considered adverse.

BPA clearly acts as a thyroid antagonist in amphibians:

- *In vitro* studies with amphibian, fish and mammal cells demonstrate that BPA is interfering with the HPT (Hypothalamic-Pituitary-Thyroid) axis (e.g. thyroid receptor, transport proteins).
- The endocrine mode of action is substantiated by *in vivo* data. Diagnostic for a thyroid mode of action in amphibians is the accelerated/asynchronous development or an abnormal histopathology, which could be demonstrated in 3 species. BPA inhibited the (TH induced and spontaneous) metamorphosis *in vivo*, leading to a delayed development and disturbed life-cycle in *R. rugosa, X. laevis* and *X. tropicalis*.
- Hence, a direct link between the *in vitro* and *in vivo* evidence can be shown. The observed in vivo effects (delayed development and disturbed life-cycle) are considered adverse.

In addition, there is some evidence that BPA also may act via a thyroidal mode of action in **fish**, although data is scarce. This is substantiated by *in vitro* studies, demonstrating an interference with the HPT axis and thyroid-related hormones in fish cells together with accelerated embryonic development in *O. latipes* which was blocked by the thyroid-antagonist amiadorone *in vivo*. The thyroid-mediated effects (accelerated development, earlier hatching and smaller individuals) are considered adverse.

Further support for endocrine-related effects of BPA

The analysis of **invertebrate taxa** revealed indications that adverse effects of BPA are possibly endocrine-mediated. It has to be kept in mind that there is still lack of an agreed guidance document which is clearly defining biological plausible links between endocrine modes of action and adverse effects for invertebrate taxa and that knowledge is still scarce in light of the large number and variety of invertebrates and their endocrine systems.

- In molluscs, characteristic adverse effects on reproduction and development were an increased egg production, mitigated by anti-oestrogens in two species *in vivo*, as well as the induction of superfemales, malformations of genital tissues (known for E2 and OP) in four species as well as embryo malformations in two species. BPA acts similar to known vertebrate-type (xeno-)oestrogens. A possible oestrogen receptor binding (*in vitro*, *in vivo*), mRNA expression and increased VTG or VTG-like protein levels were shown in three species.
- For arthropods such as insects and crustaceans ecdysteroids are known to regulate reproduction- and development-related processes. For insects, adverse effects of BPA were similar as for (xeno-)oestrogens (OP, NP, EE2), comprising a delayed development, reduced fecundity and emergence rates as well as increased weight/growth. *In vitro* evidence for antagonistic ecdysteroid receptor binding and changes in mRNA expression is provided for *Drosophila* and *Chironomus*. For crustaceans, adverse *in vivo* effects are associated with embryo malformations, developmental delay, molting disturbances and altered reproductive outcome (enhanced or reduced). Effects were similar to (xeno-

)oestrogens and could be mitigated by ecdysteroids. Due to the close relationship to insects, a binding or interference with the ecdysteroid receptor and ecdysteroid related processes is possible.

For further invertebrate species, such as echinoderms, poriferans or cnidarians, data for BPA and knowledge of the endocrine systems is very fragmentary. However, developmental disturbances including embryo malformations are typical after BPA exposure and similar to the effects observed for other (xeno-)oestrogens in these groups.

Overall, Bisphenol A is clearly shown to disrupt steroid- (oestrogen) and thyroid mediated processes in fish and amphibians respectively, leading to adverse effects on the organisms which can affect population stability and recruitment. Endocrine-mediated effects occurred and at lower concentrations than acute, systemic or narcotic toxicity.

BPA is also identified as an SVHC according to article 57(f) for probable serious effects on human health due to its endocrine disrupting properties on the basis of data on mammals. There is a large degree of conservation of the primary amino acid sequences in proteins, which implies large commonalities between non-mammalian and mammalian vertebrate species in regard to hormones, enzymes and receptors involved in the EATS (Estrogen/Androgen/Thyroidal/Steroidogenesis) modalities (OECD 2017: Draft revised OECD Guidance No. 150). Evidence of endocrine disruptive properties of BPA on mammalian vertebrate species therefore provides further support for similar properties in non-mammalian vertebrates, in particular with regard to disruption of oestrogenic pathways.

Bisphenol A is considered as a substance giving rise to an equivalent level of concern due to its endocrine modes of action and the type of effects caused by these modes of action in wildlife species (fish, amphibians).

The assessment followed the same line of arguments as for previous SVHC-identifications according to Article 57(f) ED for the environment. Due to the amount of data available for BPA a large number of arguments for an equivalent level of concern can be provided. All arguments are used in a weight of evidence and as such, none of the arguments alone are decisive for the decision and not all of them are needed to conclude on the equivalent level of concern. We decided to present the available evidence to get a view on the overall picture on the data analyzed.

- BPA causes severe effects on reproduction- and development- related processes (including sexual development) in fish and amphibians, clearly linked to the endocrine mode of action. Results for fish demonstrate that BPA may cause a complete sex reversal resulting in all-female phenotype populations. In amphibians, thyroidal pathways, metamorphosis and development are disturbed, and additionally sex ratio skewed via a suspected additional oestrogen mode of action. Supporting evidence is provided by effects observed in invertebrates.
- BPA in particular causes severe effects on organisms when exposure took place during sensitive time windows or early life stages, also after short-term exposures when exposure later ceases. Many of these effects have to be regarded as irreversible, such as sex reversal or embryo or adult malformations which may have long-term consequences for the population. Moreover, some effects may only occur after exposure during particular seasons as shown for amphibians.
- BPA elicits long-term effects across generations and affects populations and communities. Transgenerational effects were observed for several fish species, where the following generations became much more sensitive to BPA exposure (after continuous as well as short-term exposures of the parental generation). Long-term effects were shown in one mesocosm study, where low BPA concentrations affected the fish population and changes in gonad morphology are likely endocrine-mediated.
- BPA affects a large variety of ecologically important species in different ecosystems, covering lentic, lotic, marine and terrestrial environments. BPA exposure is not restricted

to certain environments but shown to be ubiquitously present. Certain fish (and also mollusc) species were shown to be particularly sensitive, but as data is only available for a small proportion of existing species, it is not possible to exclude that further species are equally or even more sensitive. Also endangered species such as amphibians may be affected. It has to be kept in mind, that effects first become prominent in later life stages or in the next generation, even when organisms have migrated to uncontaminated regions.

- BPA has already, based on available data including a large number of results from studies on mammalian mainly rodent species, been concluded to be an endocrine disrupter of concern for human health according to Article 57 (f) of REACH. Whereas the available mammalian studies are relevant for human health, it is plausible, that they are also of relevance for other mammalian species including mammalian wildlife species. In relation to the environment, adverse effects concerning development and reproduction are generally regarded as endpoints of particular relevance because such effects are likely to manifest themselves at the population level. The effects observed in rats are of particular concern for mammalian wildlife species with a natural low reproductive output (including endangered species) as negative effects on reproduction have an even higher potential for causing long term negative effect at the population level for such taxa. However, it is unclear whether the effects observed for mammals in the human health assessment will lead to population level effects in mammalian wildlife species.
- Based on the current data and knowledge it appears difficult to derive and quantify a safe level of exposure for BPA, although it might exist. Effects on non-traditional endpoints and in specific species occurred at lower concentrations than those considered by standard OECD test guidelines. Moreover, as effects often occur in certain species, or after exposure during specific time windows and early life stages, some effects might be overlooked. Effects of BPA are presumably provoked via different modes of action and a greater variety of species could be affected.

In conclusion, there is scientific evidence that Bisphenol A causes probable serious effects in the environment which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of REACH Regulation.

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Annex I - Environmental fate properties

Degradation

Abiotic degradation

The physical and chemical properties of Bisphenol A suggest that hydrolysis and photolysis is likely to be negligible (EC, 2010). An atmospheric half-life of 0.2 days was calculated (EUSES) for the reaction of Bisphenol A with hydroxyl radicals (EC, 2010).

Biodegradation in water

The ready biodegradability of Bisphenol A was evaluated in a Manometric Respirometry Test (OECD 301F) at 22°C (West et al., 2001). The initial concentrations of Bisphenol A used in this study were 7 and 25 mg/L (test material) which is lower than specified than the necessary concentration in the test guideline. As inoculum, activated sludge from a municipal wastewater treatment plant was used which was rated to be not adapted to the test substance. After a lag-phase of 4.7 days (7 mg/L Bisphenol A) and 5.2 days (25 mg/L Bisphenol A) the extent of biodegradation reached 78.2 to 81.0% at day 28 based on O_2 consumption and 76.3 to 81.2% based on CO_2 formation. The 10-day window was fulfilled and the substance was rated as readily biodegradable in this test.

In a second OECD 301F test, Bisphenol A with an initial concentration of 100 mg/L and activated sludge from a municipal wastewater treatment plant was used (Katagiri et al., 2004). The lag phase ended in the time between day 7 and day 14. Bisphenol A was mineralised with 85-93% (O2 consumption) at day 28. From these results the substance might be rated as readily biodegradable.

In a further OECD 301F test, Bisphenol A with an initial concentration of 35 mg/L (88.4 mg/L as ThOD) and 30 mg/L activated sludge from a municipal wastewater treatment plant was used (Stasinakis et al., 2008). In addition 10 mg/L allythiourea was added for preventing nitrification. After a lag phase of 4.3±0.3 days, Bisphenol A was mineralised with 87.8±6.9% at day 28. Degradation reached 10% by day 4.5 and exceeded 60% by day 6.2. From these results the substance might be rated as readily biodegradable.

The study of Stone and Watkinson (1983) was discussed in the Risk Assessment Report of Bisphenol A, which has been copied here in italic letters (EC, 2010):

Stone and Watkinson (1983) studied the biodegradation of Bisphenol-A in the OECD 301D Closed Bottle Test and the OECD 301B Modified Sturm Test. They also conducted an inhibition test on the growth of Pseudomonas fluorescens. The theoretical oxygen demand (ThOD) was calculated as 2.53 mg O2/mg and the theoretical carbon dioxide evolution (ThCO2) as 2.90 mg CO2/mg.

In the Closed Bottle Test the initial test concentration used was 3 mg/l (test substance). The oxygen concentration in the bottles was measured at 5, 15, and 28 days. At the end of the test no degradation was observed. Inhibition of microbial activity was negligible under the test conditions.

In the Modified Sturm Test the initial concentration of bisphenol-A used was 20 mg/l (test substance). The test medium was dispensed into the Sturm vessels, inoculated and aerated with CO2 free air. The extent of biodegradation was measured at 3, 7, 11, 18, 25, 27, and 28 days by titrating the total carbon dioxide released from the incubation. On day 27 the medium was acidified to release the total carbon dioxide by day 28. At the end of the test no degradation was observed.

In the microbial inhibition test the IC50 for the inhibition of growth of Pseudomonas fluorescens by Bisphenol-A was 54.5 mg/l.

An OECD 301C test confirmed the results of Stone and Watkinson (1983). No biodegradation was observed after 14 days (MITI, 1992).

A registration dossier listed a further Manometric Respirometry Test according to OECD 301 F (Katagiri, 2004). This test was carried out with an unknown concentration of non-adapted domestic activated sludge and 100 mg/L initial Bisphenol A. Until day 7 no degradation was observed. But after 14 days the 10 day window was fulfilled. Finally, 85-93 % O2 consumption was observed after 28 days.

Table 36: Summary of screening test

Test system	Test concentrations	Results	Reference
OECD 301 F	7 mg/L and 25 mg/L	78.2 - 81.0% O_2 consumption after 28 days 10 day window fulfilled	(West et al., 2001)
OECD 301 F	Unknown	87.8 ± 6.9% O_2 consumption after 28 days; 10 day window fulfilled	(Stasinakis et al., 2008)
OECD 301 F	100 mg/L	85-93 % O_2 consumption after 28 days 10 day window fulfilled	Registration Dossier (Katagiri, 2004)
OECD 301 C	Unknown	0% after 14 days	(MITI, 1992)
OECD 301 D	8.9 mg/L	0% O_2 consumption after 28 days	(EC, 2010); Registration dossier (Stone and Watkinson, 1983)
OECD 301 B	8.9 mg/L	1-2% CO_2 evolution after 28 days	(EC, 2010); Registration dossier (Stone and Watkinson, 1983)

The degradation of Bisphenol A was examined in surface water by Klečka et al. (Klečka et al., 2001). The water samples from seven different rivers across the United States and Europe were collected upstream and downstream from wastewater treatment plants known to treat wastewater containing Bisphenol A. Two different methods were conducted: River-die-away studies for ^{14}C- Bisphenol A (initial Bisphenol A concentrations 50-5500 µg/L) and respirometry studies (initial Bisphenol A concentration 5000 µg/L). The test vessels were incubated at 20 ± 2°C in the dark and were continuously stirred with 100 rpm. Bisphenol A was not detected in the river samples prior to the addition of the test compound. Negligible losses of Bisphenol A were observed in autoclaved controls, indicating abiotic degradation. There was no significant difference between the tests conducted with different river waters or river waters upstream or downstream from wastewater treatment plants. The results indicated rapid biodegradation of Bisphenol A after an initial lag phase. In the ^{14}C river die-away studies lag periods of 2 - 8 days were observed and half-lives between 0.5 and 1.4 days were estimated. Degradation of ^{14}C-Bisphenol A resulted in mineralization with an average yield of 65-80% $^{14}CO_2$ at the end of the test period (18 days). In the respirometry studies 59 - 96% CO_2 was formed after 18 days. The estimated half-lives ranged from 0.5 to 2.6 days (lag period 2.3 - 4.4 days).

(Kang and Kondo, 2005) studied the primary degradation of Bisphenol A in river water under aerobic and anaerobic conditions (Kang and Kondo, 2002). Three river water samples were spiked with 1 mg/L Bisphenol A and incubated at 30°C. In the river water

samples no Bisphenol A was detected (LOD = 0.005 mg/L using HPLC analysis). Under aerobic conditions Bisphenol A was rapidly primarily degraded with half-lives of 2 - 3 days. After 10 days the concentration was below the LOD. Under anaerobic conditions less than 10% primary degradation was observed until day 10.

The same authors investigated primary degradation in seawater and in river water under aerobic and anaerobic conditions at different temperatures (4°C, 25°C, and 35°C) (Kang and Kondo, 2005). The seawater samples were taken from five sites over one kilometre away from a junction of river and sea. The river water samples were collected from three rivers. All water samples were spiked with 1 mg/L Bisphenol A. In river water (aerobic conditions), half-lives were 4 and 3 days at 25°C and 35°C, respectively. In seawater lag periods of 30 days (25°C and 35°C) and 40 days (4°C), respectively, were observed. At the end of the experiment (60 days) the initial concentration decreased to ~200 µg/L at 25 °C/35 °C and ~700 µg/L at 4 °C. In autoclaved seawater, no degradation was observed over 60 days, indicating no abiotic removal process. Under anaerobic conditions no changes were observed until day 60.

Suzuki et al. investigated the primary degradation of Bisphenol A in river water under laboratory conditions (Suzuki et al., 2004b). The river water was collected from a site which was influenced by effluent from a wastewater treatment plant. Hence, inoculum was probably adapted to Bisphenol A. As initial concentrations, 1 or 10 mg/L Bisphenol A were added to the water samples. The flasks were incubated under aerobic conditions at 15°C for 14 days in the dark. After a lag period of 2-3 days Bisphenol A decreased rapidly. After 6 days more than 90% of the initial concentrations of Bisphenol A were primary degraded at both 1 and 10 mg/L. The half-lives were estimated with 0.4 days at 1 mg/L and 1.1 days at 10 mg/L.

The biodegradation potential of Bisphenol A in 44 river water microcosms were investigated (Ike et al., 2000). The river water samples were collected from 15 sites of seven rivers with water quality ranging from clean to heavily polluted. A Bisphenol A solution was added to the systems to give a final TOC concentration of 20 mg/L. The systems were incubated at 28 °C with rotary shaking and dark conditions. 34 of the 44 river water systems showed TOC removal of 40 to 90% after 14 days. Six microcosms could completely remove TOC and four microcosms showed no TOC removal within the test period. The removal of Bisphenol A in microcosms with unpolluted and less polluted river water is lower than in microcosms spiked with heavily polluted river water. Two metabolites were identified: 2,3-bis(4-hydroxyphenyl)1,2-propanediol and p-hydroxyphenacyl alcohol. These metabolites cannot be removed by Bisphenol A degrading bacteria.

Ying and Kookana studied the dissipation (loss from the test system) of Bisphenol A in marine environment using seawater taken from a coastal area near Adelaide, Australia (Ying and Kookana, 2003). The initial concentration of Bisphenol A in water was 5 µg/L (incubation at 20 ± 3 °C). Only little dissipation was observed until day 35. But after 42 days more than 70 % Bisphenol A was dissipated.

Table 37: Summary of simulation tests in water

Conditions	**Test concentrations**	**Results**	**Test system**	**Reference**
Freshwater, aerobic (EPA OPPTS 835.3170; shake flask die-away)		DT_{50} = 0.5-1.4 days Lag period 2-8 days 65-80% $^{14}CO_2$ after 18 days Respirometry test: DT_{50}= 0.5-2.6 days Lag period = 2.3-	(EPA OPPTS 835.3170; shake flask die-away)	(Klečka et al., 2001)

		4.4 days 59-96% CO_2 after 18 days		
Freshwater aerobic anaerobic (other method: shake flask die-away)	0.2 mg/L	Primary degradation: DT_{50} = 2-3 days DT_{50} > 10 days	(other method: shake flask die-away)	(Kang and Kondo, 2002)
Freshwater, aerobic (each endpoint other method: shake flask die-away) Seawater, aerobic Seawater, anaerobic	1 mg/L	Primary degradation: DT_{50} = 4 and 3 days (25 °C and 35 °C) Lag period = 30 days (25 °C and 35 °C), 40 days (4 °C) after 60 days: 80% primary degradation at 25 °C and 35 °C 30% primary degradation at 4 °C no degradation until day 60	(each endpoint other method: shake flask die-away)	(Kang and Kondo, 2005)
Freshwater, aerobic (other method: shake flask die-away)	1 and 10 mg/L	Primary degradation > 90% in 6 days DT_{50} = 0.4-1.1 days Lag period 2-3 days	(other method: shake flask die-away)	(Suzuki et al., 2004b)
Freshwater aerobic (other method: shake flask die-away)		Lag period = 2-5 days In 14 days: 34 of 44 river water microcosms: 40 - 90% removal 6 of 44 river water microcosms: 100% removal 4 of 44 river water microcosms: 0% removal	(other method: shake flask die-away)	(Ike et al., 2000)

Seawater, aerobic (other method: shake flask die-away)	5 µg/L	Dissipation: ~17% after 35 days > 70% after 42 days > 90% after 56 days	(other method: shake flask die-away)	(Ying and Kookana, 2003)

Biodegradation in sediments

The dissipation of Bisphenol A under aerobic and anaerobic conditions was investigated in marine sediment from a coastal area near Adelaide, Australia (Ying and Kookana, 2003). Sediment (5 g) and seawater (5mL) samples were spiked with 1µg/g Bisphenol A and incubated under 20°C for 70 days. Under aerobic conditions Bisphenol A dissipated with a half-live of 14.5 days. Under anaerobic conditions no dissipation was observed within 70 days.

Ying et al. carried out degradation experiments using aquifer material from South Australia (Ying et al., 2003). 5 g sediment (153-154 m deep) and 5 ml groundwater were taken from the same aquifer and spiked with 1 µg/g Bisphenol A (incubation temperature 20°C). No significant change in Bisphenol A concentration was observed over the test period of 70 days both under aerobic and anaerobic conditions.

Voordeckers et al. confirmed the above-mentioned results under anaerobic conditions (Voordeckers et al., 2002). No significant change in Bisphenol A concentration was observed under methanogenic, sulphate-, iron(III)- or nitrate reducing conditions within 162 days.

The dissipation of Bisphenol A was examined in river-water sediment and groundwater-aquifer under aerobic and anaerobic conditions (Sarmah and Northcott, 2008). The results showed a biphasic pattern with rapid initial dissipation (> 90% in the first four days) and a much smaller rate until the end of test period (trace amounts still remaining at day 70). For Bisphenol A no degradation product was monitored. The measured data were modelled using a first-order double exponential decay (FODED) model. The dissipation values range from 0.57 to 1.21 days (DisT90 = 2.26-2.75 days) for aerobic conditions and 1.38 to 2.315 days (DisT90 = 4.901-306.9 days) for anaerobic conditions. The authors noted incongruent results compared to previous studies which found limited or no biodegradation of Bisphenol A under anaerobic conditions. Sarmah and Northcott suggest that lower test concentrations (100µg/L) and variations in experimental protocol could be the reasons for the incongruence. Additionally, abiotic processes and irreversible sorption possibly contributed to the overall dissipation of Bisphenol A.

Table 38: Summary of biodegradation tests in sediment

Conditions	Test concentration	Results	Test method	Reference
Marine sediment (other method: shake flask die-away) aerobic anaerobic	6 µg/100 g soil	 $DisT_{50}$ = 14.5 days $DisT_{50}$ > 70 days	(other method: shake flask die-away)	(Ying and Kookana, 2003)

Aquifer material (sediment and groundwater; other method: batch equilibrium) aerobic anaerobic	1 µg/g	 Primary degradation DT_{50} > 70 days DT_{50} > 70 days	(other method: batch equilibrium)	(Ying et al., 2003)
Estuarine sediment (other method: shake flask die-away) anaerobic		 Primary degradation DT_{50} > 162 days	(other method: shake flask die-away)	(Voordeckers et al., 2002)
River-water sediment (other method: shake flask die-away) Dissipation (whole system) Aerobic | anaerobic Groundwater-aquifer material (other method: shake flask die-away) Aerobic | anaerobic		 > 90% after 4 days $DisT_{50}$ = 1.212 | 1.38 days $DisT_{90}$ = 2.75 | 4.901 days $DisT_{50}$ = 0.57 | 2.315 days $DisT_{90}$ = 2.26 | 306.9 days	(other method: shake flask die-away)	(Sarmah and Northcott, 2008)

Biodegradation in soil

Fent et al. studied the fate of ^{14}C-Bisphenol A in four different agricultural soils from Germany (Fent et al., 2003). The degradation studies were carried out according to SETAC design. For each soil 12 test systems were set up. 6µg ^{14}C-Bisphenol A /100g dry soil was applied. At different sampling intervals (until day 120), the test systems were analyzed for the amount of extractable, non-extractable, and volatile radioactivity, as well as the amount of ^{14}C-Bisphenol A still remaining in the test system. In contact with soil ^{14}C-Bisphenol A rapidly forms non-extractable residues. After 1 hour 19-59% of the applied radioactivity was non-extractable under normal conditions (extraction with methanol containing 5% acetic acid). After 3 days the non-extractable amount increased to 84.7-88.6%. After a following hot reflux extraction an additional 2.8% of the applied radioactivity was extractable. The combined total extractability using both techniques was below 7.4%. After 120 days, less than 2% of the applied radioactivity was extractable. Dissipation of ^{14}C-Bisphenol A was independent of the soil type. Neither ^{14}C- Bisphenol A, nor significant metabolites were detected after 3 days. The major route of dissipation of ^{14}C-Bisphenol A in soil was the rapid formation of bound, non-extractable residues. The bound residues (^{14}C-Bisphenol A and/or transformation products) were degraded very slowly to $^{14}CO_2$. After 120 days 13.1-19.3% of the applied radioactivity (depending on the soil) was recovered to $^{14}CO_2$. No other volatile radioactivity was observed.

Ying and Kookana investigated the degradation of Bisphenol A under aerobic and anaerobic conditions in a sandy loam soil (Ying and Kookana, 2005). 1µg/g Bisphenol A was added to 5g soil (additional 5 ml creek water for anaerobic experiment) and incubated at 20°C for 70 days. Under aerobic conditions more than 90% Bisphenol A was degraded within 15 days (DT50 = 7 days). Little or no degradation occurred in the sterile control, which indicates that the degradation of Bisphenol A resulted from microbial activity. Under anaerobic conditions no elimination of Bisphenol A occurred within 70 days.

Table 39: Summary of biodegradation tests in soil

Study	Results	Test method	Reference
Four different agricultural soils (two sandy loam & two loamy silt) (Laboratory simulation of soil die-away test, SETAC internal guideline) Dissipation:	after 120 days: $^{14}CO_2$ = 13.1-19.3% of the applied radioactivity Non-extractable = 76.0-81.6% of the applied radioactivity Extractable = 1.5-2% of the applied radioactivity $DisT_{50}$ < 3 days	(Laboratory simulation of soil die-away test, SETAC internal guideline)	(Fent et al., 2003)
One soil (sandy loam) (Batch equilibrium method, no further specification of guideline)	Aerobic:DT_{50} = 7 days Anaerobic: DT_{50} > 70 days	(Batch equilibrium method, no further specification of guideline)	(Ying and Kookana, 2005)

Environmental occurrence

Monitoring results were evaluated during a research project of UBA in the context of the substance evaluation for Bisphenol A in 2012 (Fischer et al. 2014).

Also during deduction of an UQN for BPA in 2016 measured concentrations were compiled from various summarizing sources. These are shown for the different compartments in the following table:

Table 40: Compiled measured concentrations of Bisphenol A in fresh and marine waters, fresh and marine sediments, water treatment plant effluents and biota

Compartment	Measured environmental concentration (MEC)	Master reference
Freshwater	PEC1: 0.178µg**.L^{-1}** PEC2: 0.143µg**.L^{-1}** (see attached data sheet for further information)	INERIS, 2010
	Data from 13 EU countries (DE, IT, NL, UK, ES, etc) Weighted observations: 848 Weighted observations below detection limit: 415 Median Concentration: 0.01 µg.L^{-1} Mean Concentration: 0.13 µg.L^{-1} Standard Deviation: 1.5 µg.L^{-1} 95th percentile: 0.35 µg.L^{-1}	E.C., 2008a, pp. 43
	Data from monitoring programmes in the EU and from scientific publications published between 2007 and 2012. Ranged from 9.40 ng/l in CH to 1,057 ng/l in river water downstream of a waste water treatment plant in IT	Fisher et al. (2014)
Marine water	Data from 13 EU countries (DE, IT, NL, ES) Weighted observations: 115 Weighted observations below detection limit: 58 Median Concentration: 0.0016 µg.L-1 Mean Concentration: 0.017 µg.L-1 Standard Deviation: 0.052 µg.L-1 95th percentile: 0.088 µg.L-1	E.C., 2008a, pp. 43
	Data from monitoring programmes in the EU and from scientific publications published between 2007 and 2012. Range from 1.25-42.3 ng.L-1	Fisher et al. (2014)
Freshwater sediment	Data from 9 EU countries (CZ, DK, DE, IT, NL, etc) Weighted observations: 249 Weighted observations below detection limit: 75 Median Concentration: 16 ng/g dw Mean Concentration: 60 ng/g dw Standard Deviation: 134 ng/g dw 95th percentile: 256 ng/g dw	E.C., 2008a, pp. 43

	Data from monitoring programmes in the EU and from scientific publications published between 2007 and 2012. The median BPA concentrations fresh water sediment: 10.43 to 14.1 ngg-1	Fisher et al. (2014)
Marine sediment	Data from 5 EU countries (DK, NO, ES, SE, UK) Weighted observations: 67 Weighted observations below detection limit: 44 Median Concentration: 8.5 ng/g dw Mean Concentration: 75 ng/g dw Standard Deviation: 209 ng/g dw 95th percentile: 566 ng/g dw	E.C., 2008a, pp. 43
	Data from the Arctic Neither BPA nor TBBPA were detected in any samples of sediment, fish or seabirds analysed	SFT, 2009
	Data from monitoring programmes in the EU and from scientific publications published between 2007 and 2012. The median BPA concentrations marine sediments: 5.69 to 19.7 ngg-1)	Fisher et al. (2014)
WWTP effluent	PNECWWTP : (P. fluorescence) 320mg.L-1	E.C., 2008a, p. 62
	Data from monitoring programmes in the EU and from scientific publications published between 2007 and 2012. Mean effluent BPA concentrations indicated in the publications ranged from 5.00-635 ng.L-1	Fisher et al. (2014)
Biota	Freshwater fish: Range of species, several locations, wet weight, whole fish and muscle. Range: 1.0 to 14.1ng/g wet weight Marine species: Cod, liver, range from below LoD to 62ng/g wet weight See E.C.2008ª for detail Data from the Arctic	E.C., 2008a, p. 60 + 61
	Neither BPA nor TBBPA were detected in any samples of sediment, fish or seabirds analysed.	SFT, 2009
	Data from monitoring programmes in the EU and from scientific publications published between 2007 and 2012. Mussels sampled in Greece had levels of BPA ranging from <115 to 626 ng.g-1 dry weight, while in mussels from the Baltic Sea levels were considerably lower (<1 to 3.30 ng.g-1 BPA. BPA concentrations found in fish muscle ranged between <0.60 to 3.90 ng.g-1. In fish liver higher	Fischer et al. (2014)

	BPA concentrations were detected than in muscle samples (2.17 to 8.49 ng.g-1). BPA concentrations in fish bile caught in Sweden were reported to range from <30 to 700 ng.g-1, which was the highest value found for BPA concentrations in fish in the UBA review.	

Recent data furthermore demonstrates the presence of BPA in further biota and also remote areas. BPA has been detected in eggs of herring gull in a series of monitoring reports of an Urban Fjord in Norway (NIVA 2015, 2016). BPA was even detected in Arctic biota (Svalbard) in eggs of kittiwake and glaucous gull. Here, BPA was among the most quantitatively abundant compounds found in seabird eggs (Lucia, M. et al. 2016).

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Annex II - Bioaccumulation in aquatic organisms

Bioaccumulation was evaluated in the Risk Assessment Report of Bisphenol A (EC, 2010):

The available measured data suggested that bisphenol-A has a low potential for bioaccumulation in fish, in contrast to the moderate potential indicated by the log Kow value. A slightly higher potential was indicated by the measured bioconcentration in freshwater clams (up to 144). Measured data are preferred over calculated values when the studies are valid. A BCF of 67 for fish was therefore used in the published risk assessment, and the accumulation in clams was considered in the risk characterisation (EC, 2003).

Information from 2003 to 2010 was also evaluated in (EC, 2010), but *the new BCF values for fish are generally similar to that used in the published risk assessment and so no change is necessary.* One representative study from (EC, 2010) is described below.

Lindholst et al. (2003) studied the metabolism of bisphenol-A in zebrafish (Danio rerio) and rainbow trout (Oncorhynchus mykiss). Adult zebrafish were exposed to 100 µg/l bisphenol-A in a flow through system for 168 hours. Exposures took place in a 100 l aquarium, with a flow rate of 8 replacement volumes per day, and 150 fish. The bisphenol-A concentration was measured every two days; the actual concentration found was 97.5±5.2 µg/l. Fish were sampled to a system to which bisphenol-A was not added and kept for the same length of time, with sampling at the same intervals. Zebrafish tissue samples were analysed for Bisphenol A, Bisphenol A glucuronic acid (BPAGA) and Bisphenol A sulphate (BPAS).

Rainbow trout were exposed under similar conditions for eight days to 100 µg/l bisphenol-A (actual concentration from 2-day samples 107.3±6.3 µg/l). After eight days, gall bladder and blood samples were taken, and the bile fluid and blood plasma analysed for the same three substances (Bisphenol A, BPAGA and BPAS). Uptake and excretion rates for fish were calculated by fitting data to exponential uptake and decay models (much of the data for rainbow trout came from earlier publications). Uptake was fitted to a first order model, excretion to a first or second order model depending on the goodness of fit. Bisphenol A was detected in zebrafish after two hours' exposure, and steady sate was reached by 24 hours. Steady state concentrations were 569 ng/g for Bisphenol A, 12.6 µg/g for BPAGA and 39.3 ng/g for BPAS. The whole body uptake rate for zebrafish was calculated as 0.23; tissue specific values from rainbow trout plasma, liver and muscle were 0.73, 0.11 and 0.16, so the rates were similar between the two species despite the different matrices. Elimination from zebrafish was fitted to a second order model; the first compartment had a half life of <1.1 hours, the second compartment half life was 139 hours. The three trout tissues had elimination half-lives of 3.7, 1.8 and 5.8 hours for plasma, liver and muscle respectively, as first order elimination. The authors suggest that in zebrafish Bisphenol A is rapidly removed from tissues, metabolised by the liver and excreted primarily as BPAGA into the gall bladder (compartment 2). Elimination from the tissues in zebrafish is much more rapid than from trout tissues. Zebrafish have a lower sensitivity to Bisphenol A than trout when considering vitellogenin synthesis. It is suggested that this may be due to the more rapid metabolism resulting in lower Bisphenol A concentrations and a reduced response. Data on specific tissue concentrations in the liver for Bisphenol A and metabolites was needed to confirm this.

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Annex III – Summary of standard ecotoxicological data

Reliability assessment

This chapter provides a short summary of the systemic toxicity results which were also used for $PNEC_{water}$ derivation in the EU Risk Assessment Report (EC, 2010).

The reliability categories according to Klimisch score were used to assess the studies presented below. 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.)).

Aquatic compartment (including sediment)

Fish: Short-term toxicity

Several acute data for fish are available, though some of them are use with care studies. For fish, acute toxicity test results (96 h LC_{50}) are in the range of 4.6 to 11.0 mg/L.

Table 41: Fish toxicity data used for PNEC derivation in the EU Risk assessment report (EC, 2010)

Species	Effect	Duration	L(E)C_{50} [mg/L]	Analytical measurement	Reference	Reliability
Danio rerio	Hatching success	96 h	5.25	N	Chan & Chan 2012	2
Danio rerio	Hatching success	72 h	13.8	Y	Duan et al. 2008	2
Danio rerio	Survival	5 dph	5	N	McCormick 2011	2
Danio rerio	Survival	14 d	3.2	Y	Bayer AG 1999a	2
Pimephales promelas	Survival	96 h	4.6	Y	Alexander et al. 1988	2
Xiphophorus helleri	Survival	14 d	17.93	N	(Kwak et al., 2001)	2
Rhinella arenarum	Survival	168 h	7.1	N	Wolkowicz et al. 2011	2
Cyprinodon variegatus	Survival	22 h	1.24	N	Matsushima et al. 2013	2
Cyprinodon variegatus	Survival	96 h	11	n.a.	Sayers 2009	Valid
Menidia menidia		96 h	9.4	n.a.	Springborn Bionomics (1985a)	valid

					and Alexander et al (1988)	

Fish: Long-term toxicity

Some of the tests and endpoints used for the PNEC derivation are also used in this dossier to evaluate the endocrine disrupting properties of Bisphenol A. On the other hand, some test procedures used for PNEC derivation are not suitable to show endocrine disrupting properties e.g. because the tests starts too late and does not cover the sensitive life cycle step for this effect in the organism. Therefore, some of the results in the table can also be found in connection with the assessment of the endocrine disrupting properties of Bisphenol A.

Table 42: Fish toxicity data used for PNEC derivation in the EU Risk assessment report (EC, 2010)

Species	Endpoint	Result [µg/L]	Analytical measurement	Reference	Reliability
Cyprinus carpio	49-d growth NOEC	100	Y	Bowmer & Gimeno (2001)	1
Danio rerio	Full life-cycle multiple endpoint NOEC	750	Y	(Segner et al., 2003b)	2
Oncorhynchus mykiss	28-d juvenile growth NOEC	3640	Y	Bayer AG (1999b)	1
Pimephales promelas	Multi-gneration F_2 egg hatchability NOEC	16	Y	Sumpter et al. (2001)	1
Oryzias latipes	Multi-generation multiple endpoint NOEC	247	Y	Japanese Ministry of the Environment (2006)	2
Poecilia reticulate	30-d survival NOEC	500	Y	(Kinnberg and Toft, 2003)	2

Aquatic invertebrates: short-term toxicity

Several acute data for aquatic invertebrates are available, though some of them are to be used with care. For invertebrates, the results (48h-EC_{50}) are between 0.96 and 16 mg/L.

Table 43: Short-term toxicity data for aquatic invertebrates

Organism	Effect	Duration	L(E)C_{50} [mg/L]	Analytical measurement	Reference	Reliability (a)
Daphnia magna	Immobilisation	24 h	13.8	Y	Jemec et al. 2012	2
Daphnia magna	Immobilisation	48 h	10.2	N.A.	Alexander et al. 1988	Valid
Daphnia magna	Immobilisation	48 h	10.4	N	Jeong et al. 2013	2
Daphnia magna	Immobilisation	48 h	9.9	N	Mansilha et al. 2013	2
Daphnia magna	Immobilisation	48 h	3.9	N	Stephenson 1983	2

Daphnia magna	Immobilisation	48 h	10	N	Chen et al. 2002	2
Daphnia magna	Immobilisation	48 h	16	N	(Mu et al., 2005)	2
Daphnia magna	Immobilisation	48 h	7.75	N	Brennan et al. 2006	2
Gammarus pulex	Survival	120 h	1.5	Y	(Watts et al., 2001b)	2
Dugesia japonica	Survival	48 h	8.3	N	Li 2013	2
Americamysis bahia	Survival	96 h	1.1	Y	Alexander et al. 1988	1
Americamysis bahia	Survival	96 h	1.05	N	(Hirano et al., 2004)	2
Artemia franciscana	Survival	48 h	34.7	N	Castritsi-Catharios et al. 2013	2
Tigriopus japonicas	Survival	48 h	4.32	N	(Marcial et al., 2003)	2
Acartia tonsa	Immobilisation	72 h	0.96	N	(Andersen et al., 1999)	2

Aquatic invertebrates: long-term toxicity

Table 44: Invertebrate toxicity data used for PNEC derivation in the EU RAR (EC, 2010)

Species	Endpoint	Duration	Result [µg/L]	Analytical measure-ment	Reference	Reliability
Daphnia magna	Reproduction NOEC	21 d	3146	n.a.	Bayer AG (1996)	Valid
Hyalella azteca	Reproduction NOEC	42 d	490	n.a.	Springborn Smithers (2006b)	valid
Brachionus calyciforus	Intrinsic rate of increase NOEC	48 h	1800	n.a.	Springborn Smithers (2006a)	valid
Marisa cornuarietis	Egg production EC_{10}	5 month	0.038	Y	Ratte (2015) recalculated from Oehlmann et al. (2006)	valid
Hydra vulgaris	Polyp structure NOEC	6 week	42	Y	(Pascoe et al., 2002)	1, valid
Heteromyenia sp.	Growth NOEC	9 d	1600	Y	(Hill et al., 2002)	2, valid
Paracentrotus lividus	Development					

Algae and aquatic plants

Table 45: Algae and aquatic plant toxicity data

Species	Endpoint	Duration	Result [µg/L]	Analytical measure-ment	Reference	Reliability
Pseudokirchneriella subcapitata	Biomass EC_{50} Cell count EC_{10}	96 h	2730 1360	n.a.	Alexander et al. (1985b & 1988)	valid
Lemna gibba	Growth NOEC	7 d	7800	n.a.	Putt (2003)	valid
Skeletonema costatum	Biomass EC_{50}	96 h	1100	n.a.	Alexander et al. 1988	Valid
Cyclotella caspia	Biomass EC_{50}	96 h	7960	N	Li et al. 2008	2
Navicula incerta	Growth rate EC_{50}	96 h	3730	Y	Liu et al. 2010	2

Sediment organisms

Table 46: Sediment toxicity data

Organism	Effect	Duration	Effect [µg/L]	Analytical measurement	Reference	Reliability
Chironomus tentans	Survival EC_{50}	96 h	2700	Y	Sayers 2005	1
Chironomus riparius	Time to moult and growth NOEC	Life-cycle	0.1	n.a.	(Watts et al., 2003)	valid

Other aquatic organisms

Table 47: Toxicity data for other aquatic organisms

Organism	Effect	Duration	Effect [µg/L]	Analytical measurement	Reference	Reliability
Rhinella arenarum	Survival EC_{50}	168 h	7100	N	Wolkowicz et al. 2011	2
Xenopus laevis	Survival EC_{50}	72 h	4800	N	Iwamuro et al. 2003	

Terrestrial compartment

Toxicity to soil macro-organisms

Table 48: Terrestrial toxicity data used for PNEC derivation in the EU RAR (EC, 2010)

Organism	Effect	Duration	Effect [mg/kg]	Analytical measurement	Reference	Reliability
Folsomia candida	Number of juveniles NOEC	28 d	500	Y	ECT, 2007a	valid
Eisenia andrei	Mortality NOEC	14 d	32		(Johnson et al., 2005)	Valid

Enchytraeus crypticus	NOEC	28 d	≥ 100		ECT, 2007b	valid

Toxicity to terrestrial plants

Table 49: Terrestrial toxicity data used for PNEC derivation in the EU RAR (EC, 2010)

Organism	Effect	Duration	NOEC [mg/kg]	Analytical measure-ment	Reference	Relia-bility
Corn (*Zea mays*)	Dry shoot weight Percent emergence	21 d	130 320	Y	Springborn Smithers, 2007	valid
Oats (*Avena sativa*)	Dry shoot weight Percent emergence	21 d	47 800	Y	Springborn Smithers, 2007	valid
Wheat (*Triticum aestivum*)	Dry shoot weight Percent emergence	21 d	47 300	Y	Springborn Smithers, 2007	valid
Cabbage (*Brassica oleracea*)	Dry shoot weight Percent emergence	21 d	50 130	Y	Springborn Smithers, 2007	valid
Soybean (*Glycine max*)	Dry shoot weight Percent emergence	21 d	320 800	Y	Springborn Smithers, 2007	valid
Tomato (*Lycopersicum esculentum*)	Dry shoot weight Percent emergence	21 d	20 130	Y	Springborn Smithers, 2007	valid

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