Substance Name: 4,4'-sulphonyldiphenol

EC Number: 201-250-5

CAS Number: 80-09-1

MEMBER STATE COMMITTEE SUPPORT DOCUMENT FOR IDENTIFICATION OF

4,4'-SULPHONYLDIPHENOL

AS A SUBSTANCE OF VERY HIGH CONCERN BECAUSE OF ITS TOXIC FOR REPRODUCTION (ARTICLE 57C), ENDOCRINE DISRUPTING PROPERTIES (ARTICLE 57(F) - ENVIRONMENT), ENDOCRINE DISRUPTING PROPERTIES (ARTICLE 57(F) - HUMAN HEALTH) PROPERTIES

Adopted on 28 November 2022

CONTENTS

TABLES

FIGURES

ABBREVIATIONS

* : p<0.05
** : p<0.01
*** : p<0.001
Abs : absolute
AC50 : half-life activity concentration
ADME : absorption, distribution, metabolisation and excretion
AhR : Aryl hydrocarbon receptor
AO : Adverse outcome
AOP : adverse outcome pathway
ApoII : ApolipoproteinII
Approx. : approximately
AroB : aromatase B
ATE : acute toxicity estimate
AUC : Area under the curve
AVP : Arginine Vasopressin (gene)
AVT : Arginine vasotocin (hormone)
BAF : bioaccumulation factor
BCF : bioconcentration factor
BLYES : Bioluminescent Yeast reporter assay
BP : 2,2’-Biphenol
BPA : bisphenol A, 4,4’-isopropylidenediphenol
BPAF : bisphenol AF, 4,4'-(hexafluoroisopropylidene) diphenol
BPAP : bisphenol AP, 1,1-bis(4-hydroxyphenyl)-1-phenylethaneBPA quinone : 4,5-bisphenol-o-quinone
BPB : bisphenol B, 4,4'-(1-methylpropylidene)diphenol
BPE : bisphenol E, 4,4’-ethylidenediphenol
BPF : Bisphenol F, 4,4’-Methylenediphenol
BPP : bisphenol P, 4,4’-(1,4-phenylenediisopropylidene)diphenolBPPH: bisphenol PH, 5,5’-isopropylidenedi-2-biphenylol
BPS : Bisphenol S; 4,4’-sulphonyldiphenol
BPSIP : 4-hydroxyphenyl 4-isoprooxyphenylsulfone, also called D-8
BPZ: bisphenol Z, 4,4’-cyclohexylidenediphenol
Bw : body weight
Ca. : circa
CAR : constitutive androstane receptor
Cat. : category
CLP : Classification and labelling
CMC : carboxymethylcellulose
CMR : cancerogen, mutagen, reprotoxic
Conc. : concentration
Corr. : corrosity
Cre : creatinine
CYP19a1: Cytochrome P450 Family 19 Subfamily a Member 1 (gonad)
CYP19b : Cytochrome P450 Family 19 Subfamily b (brain)
d : day
D : development
DFO : double first order
Dgr : danger
DHEA : dehydroandrosterone
DIT : developmental immunotoxicity
DMSO : dimethylsulfoxide
DNT : developmental neurotoxicity
DOC : dissolved organic carbon
DPC : day post-coitum

Dpf : days post-fertilisation
Dph : days post hatching
DPP : day post-partum
DT50 : degradation half-life
Dw: dry weight
E2 : 17β-estradiol
EAS : estrogenic, androgenic and steroidogenic
EATS : estrogenic, androgenic, thyroidal and steroidogenic
EC10 : concentration producing effect in 10% of the test organisms
EC50 : concentration producing effect in 50% of the test organisms
ECHA : European Chemicals Agency
ED : endocrine disruptor
EDI : estimated daily intake
EDU : estimated daily uptake
EEC : European Economic Community
EEF : estradiol equivalence factor
EEQ : estradiol equivalence quantities
EFSA : European Food Safety Authority
ELoC: equivalent level of concern
EMPA: Eidgenössische Materialprüfungs- und Forschungsanstalt (Swiss Federal Laboratories for Materials Testing and Technology)
EOGRTS : Extended-One Generation Reproductive Toxicity Study
EPA : Environmental Protection Agency
ER : estrogen receptor
ErC50 : concentration affecting growth rate in 50% of the test organisms
ERE : estrogen-responsive element
ERK : extracellular signal-regulated kinase
ESI-MS: electrospray Ionisation Mass Spectroscopy
f : female
F : fertility
FG: full-grown oocytes
F.i.: for instance
Foll : follicle
FRST-50 : forest-50
FSH : follicle stimulating hormone
Fshr : follicle stimulating hormone receptor
G : gram
GC-MS : Gas Chromatography coupled to Mass Spectrometry
GD : gestational day
Geom. : geometric
GFP : green fluorescent protein
GH: growth hormone
GHS : Globally Harmonised System of Classification and Labeling of Chemicals
GLP : good laboratory practice
GnRH : gonadotropin-releasing hormone
GR : glucocorticoid Receptor
GSI: gonadosomatic index
H : hour
HCD : historical control data
hER : huma estrogen receptor
Hpf : hours post-fertilisation
HPG : Hypothalamic-pituitary-gonadal
HPI : Hypotalamis-pituitary-interrenal
HPLC: high-performance liquid chromatography
IC50 : concentration with 50% inhibition
ICI 182,780 : fulvestrant (CAS 129453-61-8)
Incl. : including

Irrit : irritation
ISO : International Organization for Standardization
JNK: c-Jun-N-terminal kinase
KE : key event
KER : key event relationship
kg : kilogram
L : liter
LBD : ligand binding domain
LC50 : Lethal concentration causing 50% death
LC-MS/MS : liquid chromatography coupled to mass spectrometry
LC-QTOF-MS : Liquid chromatography–Quadrupole Time of Flight Mass spectrometry.
LH : luteinising hormone
Ln : natural logarithm
LOAEL : lowest observed adverse effect level
LOD : limit of detection
LoE : level of effort
LogLED : logarithm of lowest effective doses
LOQ : limit of quantification
LOR : limit of reporting
m : male
Max : maximum
MCF : Michigan Cancer Foundation
Meas. : measured
Mg: milligram
MIE : Molecular initiating event
Min : minimum
MITI : Ministry of International Trade and Industry, Japan
ML : milliliter
MLOQ : Method limit of quantification
MoA : mode of action
MS : mass spectrometry
Muta : mutagenicity
NA : not applicable
Nb : number
NC : not classified
ND or n.d.: not detected
NER: non-extractable residues
Ng : nanogram
NOEC: No observed adverse effect concentration
NOErC : No observed adverse effect concentration for growth rate
NY : New York
OECD: Organisation for Economic Co-operation and Development
P/vP : persistent / very persistent
PBT : persistent, bioaccumulative and toxic
PC50 : response that is 50% of the maximal positive control response
PCA : protein-fragmentation complementation assay
PCOS : polycystic ovary syndrome
PDTC : pyrrolidine dithiocarbamate
PESU: polyethersulfone
PG: primary growth stage oocytes
Pg : picogram
PND : post-natal day
PPARγ : peroxisome proliferator-activated receptor gamma
PRL : prolactin
PSF-51 : Purdue Student Organic Farm-51
QSAR: Quantitative structure-activity relationship
RA : relative activity

RAC : Risk Assessment Committee
RBA : relative binding affinity
REACH : registration, evaluation authorisation, and restriction of chemicals
REC50 : 50% relative effective concentration
Rel. : reliability
REP : relative potency to E2
Repr. : reprotoxic
Resp. : respectively
RGC : radial glial cells
RNA : ribonucleic acid
RPF : relative potency factor
RPP : relative proliferative potency
SD : Sprague-Dawley
SDA: sea die-away
Sec : secondary
SFO : single first order
Sign : significantly
SML: specific migration limit
Sp. : species
SPE-HPLC-MS/MS: solid-phase extraction (SPE)−high-performance liquid chromatography (HPLC) −mass spectrometry (MS)/MS
SPE-LC-QTRAP-MS/MS solid phase extraction-liquid chromatography (SPE-LC) Quadrupole-Linear Ion Trap Mass Spectrometry (QTRAP-MS) /MS
SPM: suspended particulate matter
SR : social recognition
St. dev. : Standard deviation
Stat : statistically
STP : sewage treatment plant
SVHC: substance of very high concern
T: testosterone
TG : test guideline
TOC : total organic carbon
TOF : time-of-flight
Tox. : toxicity
TR : thyroid hormone receptor
TTP : time-to-pregnancy
TWA : time-weighted average
µg : microgram
UHPLC–MS/MS : Ultra High Performance liquid chromatography tandem mass spectrometry
uHPLC-TOF : ultra-high performance liquid chromatography mass spectrometry
US : United States
USA : United States of America
UV: ultraviolet
vPvB : very persistent very bioaccumulative
Vtg : vitellogenin (gene)
VTG : vitellogenin (protein)
WTP: water treatment plant
ww : weight by weight
WWTP : waste water treatment plant
ZEOGRT: Zebrafish Extended One Generation Reproduction Test
ZFL: zebrafish liver

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

Substance name: 4,4'-sulphonyldiphenol (Bisphenol S; BPS)

EC number: 201-250-5

CAS number: 80-09-1

- The substance is identified as a substance meeting the criteria of Article 57(c) of Regulation (EC) No 1907/2006 (REACH) owing to its classification in the hazard class toxic for reproduction category 1B[1], H360FD.

- The substance 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) according to Article 57(f) of REACH Regulation.

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

- 4,4'-sulphonyldiphenol, referred to hereinafter as BPS is covered by index number 604-098-00-1 of Regulation (EC) No 1272/2008 in Annex VI, part 3, Table 3 (the list of harmonised classification and labelling of hazardous substances)[2] and it is classified in the hazard class toxic for reproduction category 1B (H360FD).

  Therefore, this classification of the substance in Regulation (EC) No 1272/2008 allows its identification as substance of very high concern in accordance with Article 57(c) of REACH.

- In addition, BPS is identified as a substance of very high concern in accordance with Article 57(f) of Regulation (EC) 1907/2006 (REACH) because of its endocrine disrupting properties for which there is scientific evidence of probable serious effects to the environment and human health which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of the REACH Regulation.

Adverse effects

Human health:

BPS consistently affects the estrous cyclicity in female rodents, at different windows of exposure. All the available studies show irregular cycles, linked in most of them to a prolongation of the diestrus phase. The disturbance of estrous cycle is considered as EAS (estrogenic, androgenic and steroidogenic)-mediated.

In addition, effects that are sensitive to, but not diagnostic of, EATS (as potentially linked also to other Modes of Action) were also reported regarding rodent female reproduction. A statistically

[1] Classification in accordance with section 3.7 of Annex I to Regulation (EC) No 1272/2008.
[2] COMMISSION DELEGATED REGULATION (EU) 2022/692 of 16 February 2022 amending, for the purposes of its adaptation to technical and scientific progress, Regulation (EC) No 1272/2008 of the European Parliament and of the Council on classification, labelling and packaging of substances and mixtures (18th ATP)

significant decrease of the number of embryo implantation sites was observed in reproductive toxicity studies, resulting in decreased fertility and number of pups.

Other developmental and male reproductive adverse effects were observed in the available rodent studies supporting the endocrine disrupting properties of BPS. These include EAS-mediated effects such as reduced sperm count and motility at low doses and a high incidence of male rodent mammary gland multifocal atrophy. Additionally, adverse effects sensitive to, but not diagnostic of, EATS were observed including dose-dependent increased post-implantation loss in reproductive toxicity studies and higher adrenal glands weight, in particular in males, in several independent studies.

These adverse effects have been observed at doses showing neither maternal toxicity nor severe general toxicity. Moreover, since estrogen signalling is critical to reproductive success in all vertebrates including mammals, it is assumed that the observed adverse effects on fertility through disruption of estrogen signalling in rodents are relevant to humans.

The complexity of the effects sensitive to, but not diagnostic of, EATS observed following exposure to BPS suggests the interaction of multiple MoAs to produce the observed effects, increasing the concern for human health. For example, the consistent effects on the mammary gland in males in two rodent species provides an indication of hormonal disturbance and may have influence on e.g. human breast tumor development.

Environment:

There is evidence in literature that BPS affects sperm count and sex ratio in zebrafish (*Danio rerio*) after exposure in the µg/L range. In a ZEOGRT (OECD TG 240 adapted for zebrafish), the findings on sex ratio were not significant. However a similar trend towards feminisation was observed with the number of males close to or even below natural variation at low concentrations. These EAS-mediated effects were observed at concentrations below general toxicity.

In addition, effects that are sensitive to, but not diagnostic of, EATS (as potentially linked also to other Modes of Action) were also reported regarding reproductive effects: reduced fecundity, reduced hatching rate and altered oocyte maturation in fish.

Other important adverse effects on brain neurogenesis and behaviour were identified in fish. Experimental data on zebrafish demonstrated that these effects depend on BPS-induced changes in aromatase activity.

Effects on apical endpoints such as fecundity and altered sex ratio are considered to impair population stability and recruitment. Therefore, these effects are to be considered population relevant for the environment.

BPS induces adverse effects on development and reproduction in rodents and fish.

Endocrine activity

Bisphenols are known to target many endocrine pathways. Consistent *in vivo* and *in vitro* evidence is available on steroidogenesis and in particular on estrogenic activity.

- *Estrogenic activity*

In vitro ER binding assays demonstrate that BPS is capable of binding to the estrogen receptor, with IC50 ranging from 5.8 to 105 µM depending on the cell line used (rat and human). Several

in vitro literature studies using different cell cultures showed a weak increase in the estrogenic activity (ER reporter gene assays, proliferative assays and ER-regulated gene expression assays). *In vivo*, the increase in uterine weight, observed in all rodent uterotrophic assays, is a parameter diagnostic of estrogenicity.

Vitellogenin, a biomarker of estrogenic activity in fish, was induced in embryonic and adult male zebrafish. Literature data also reported a change in steroidal hormone balance with decreased testosterone and increased estradiol levels and an increased E2/T ratio in zebrafish.

BPS exhibits estrogenic activity.

- *Steroidogenesis*

In a range of *in vitro* assays investigating steroidogenesis following exposure with BPS, a clear trend towards decreased testosterone was observed. Furthermore, an increase in testis aromatase expression was observed in several studies following exposure to BPS. Several, but not all, *in vivo* studies, showed decrease in serum testosterone level in rodents.

Moreover, the impact on the synthesis of steroid hormones (decrease of testosterone and increase of estrogen) was clearly shown in *in vivo* studies with zebrafish. These findings were accompanied by an increased expression of genes involved in steroidogenesis and specifically in aromatase (CYP19a, CYP19b in testis and brain resp.).

BPS is shown to affect steroidogenesis.

Plausible link between adverse effects and endocrine activity

Human health:

Considering the results of all available experimental studies, there is strong evidence that the adverse effects on fertility in female rodents are due to the estrogenic activity of BPS. The increase in uterus weight (as seen in the available uterotrophic assays) is a strong diagnostic parameter for estrogenicity. Furthermore, the prolongation of the estrous cycle was consistently observed in the majority of the studies. In addition, the number of implantation sites was decreased in three reproductive studies, resulting in a decrease of both fertility and number of pups. All of these parameters are considered as either EATS-mediated or sensitive to, but not diagnostic of, EATS modalities. The different effects of BPS, in particular on the female reproductive system, can be plausibly linked to the estrogenic activity of the substance and could therefore explain the adverse impacts seen on fertility endpoints.

Other modes of action than those involving estrogenic activity and/or signalling pathways are likely. For example, altered testosterone production is probably linked to adverse effects on the male reproductive system (reduced sperm count and motility) or the male mammary gland. Despite the fact that these data give further indications of the endocrine activity of BPS, they are considered as supportive adverse human health effects.

In conclusion, the effects on the female reproductive organs and functional parameters are consistent with an estrogenic mode of action of BPS. The adverse effects on the estrous cycle are EATS-mediated, therefore, in the absence of information proving the contrary, the biologically plausible link is already pre-established based on existing scientific knowledge. There is strong evidence that the adverse effects on fertility and sexual function are plausibly linked to the estrogenic activity of the substance. BPS is therefore an endocrine disruptor according to the WHO/IPCS definition with regard to human health.

Environment:

Based on the weight of evidence approach and considering the results of all available studies there is evidence that the adverse effects of BPS on sperm count and sex ratio in zebrafish are due to the estrogenic activity and to disrupted steroidogenesis.

Skewed sex ratio is recognised as an EAS-mediated effect. Altered gametogenesis as reduced sperm counts has been also observed. Based on the existing knowledge in mammals and the similarities with fish gametogenesis, reduced sperm count is considered as EAS-mediated also in fish. The estrogenic activity of BPS is demonstrated in mammals and is further evidenced by vitellogenin induction in fish. Altered steroidogenesis may lead to the observed decreased sperm counts and altered oocyte maturation which, in turn, may lead to impaired hatchability of the eggs. Increased aromatase activity is consistently observed and is clearly responsible for effects on fish brain and behaviour. Impaired social behaviour may also result in reduced reproduction.

There is a large degree of conservation of the endocrine system, implying large commonalities between non-mammalian and mammalian vertebrate species in regard to hormones, enzymes and receptors involved in the EATS modalities. All mammalian data provide substantial evidence that BPS can disrupt particularly estrogenic pathways. Therefore, those data were also considered in the Weight of Evidence approach for the assessment of the ED properties in the environment and thus wildlife species. Considering all relevant and reliable information in a weight of evidence approach, it is concluded that BPS is an endocrine disruptor according to the WHO/IPCS definition with regard to environment.

Equivalent level of concern:

The effects of BPS due to its endocrine disrupting properties are considered to be of equivalent level of concern to CMR Cat. 1, PBT or vPvB substances as listed in Article 57 points (a) to (e) of the REACH Regulation.

Based on the scientific evidence, the effects on organisms and populations are considered to be severe and irreversible as effects on estrous cycle, sex ratio, etc. are observed following developmental exposure. Such effects are considered to impair population stability and recruitment. Moreover, a wide range of taxa in different ecosystems may be adversely affected due to conservation of the endocrine system. However, the difference between taxa concerning specific hormones affected, binding affinities and modes of action makes it difficult to determine the most sensitive species and thus to quantify a safe level of exposure with regard to the endocrine mediated effects.

Bisphenols are widely used and can be found together in the environment. It has been already recognised that bisphenols can act jointly in the environment by sharing the same mode of action resulting in additive effects. Bisphenols can also act together with chemicals other than bisphenols (sharing the same and/or a different MoA) occurring in the environment, at comparatively low concentrations, displaying the same and/or additional effects. This supports equivalent level of concern as endocrine disruptors with similar MoA but also chemicals with different MoA can act additively or even synergistically.

In conclusion:

Based on all available scientific evidence, it can be concluded that BPS fulfils the WHO/IPCS

(2002)[3] definition of an endocrine disruptor:

- It shows clear reproductive adverse effect in rodents and fish. The reproductive endocrine system is highly conserved not only between mammals, but also between mammals and other vertebrates like fish.

- It has endocrine modes of action: clear estrogenic mode of action and alteration of steroidogenesis.

- The adverse effects, including the recognised EAS-mediated effects (e.g. on estrous cycle and sex ratio) and effects sensitive, but not diagnostic of EAS (e.g. fecundity, fertility, implantation sites and number of pups), are a consequence of the endocrine modes of action.

The assessment performed demonstrates that there is scientific evidence of probable serious effects of BPS to the environment and human health due to its endocrine disrupting properties, which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of the REACH Regulation.

Registration dossiers submitted for the substance: Yes

[3] An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, its progeny or (sub)populations.

Justification

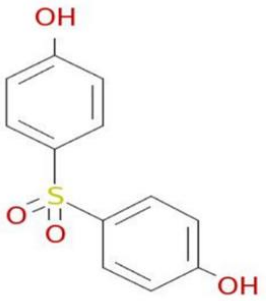
1. Identity of the substance and physical and chemical properties

1.1 Name and other identifiers of the substance

Table 1: Substance identity

EC number:	201-250-5
EC name:	4,4’-sulphonyldiphenol
CAS number (in the EC inventory):	80-09-1
CAS number:	80-09-1
IUPAC name:	4-(4-hydroxybenzenesulfonyl)phenol
Index number in Annex VI of the CLP Regulation	604-098-00-1
Molecular formula:	$C_{12}H_{10}O_4S$
Molecular weight range:	250.27 g/mol
Synonyms:	Phenol, 4,4'-sulfonylbis- (9CI) Phenol, 4,4'-sulfonyldi- (6CI, 8CI) 1,1'-Sulfonylbis[4-hydroxybenzene] 4,4'-Bisphenol S 4,4'-Dihydroxydiphenyl sulfone 4,4'-Sulfonylbisphenol 4-Hydroxyphenyl sulfone Bis(4-hydroxyphenyl) sulfone Bis(p-hydroxyphenyl) sulfone Bisphenol S BPS BPS 1 Diphone C p,p'-Dihydroxydiphenyl sulfone Phenol, sulfonylbis- Bis(hydroxyphenyl)sulphone Dihydroxydiphenyl sulphone Phenol, sulphonyldi- Sulfonyldiphenol-

Structural formula:



1.2 Composition of the substance

Name: 4,4'-sulphonyldiphenol (Bisphenol S; BPS)

Description: Solid

Substance type: mono-constituent

It should be noted that BPS is a mono-constituent with a purity ranging from 80 - 100%. SVHC identification is based on the properties of the main constituent only.

Furthermore, impurities did not contribute to the harmonised classification for reproduction (18th ATP)[4].

Table 2: Constituents other than impurities/additives

Constituents	Typical concentration	Concentration range	Remarks
4,4'-sulphonyldiphenol EC n° 201-250-5	/	≥ 80 - 100.0 % (W/W)	/

Table 3: Impurities

Impurities	Typical concentration	Concentration range	Remarks
Confidential	/	/	/

Table 4: Additives

Additives	Typical concentration	Concentration range	Remarks
None	/	/	/

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

Not relevant for the SVHC assessment of the substance.

[4] Committee for Risk Assessment (RAC) Annex 1 Background document to the Opinion proposing harmonised classification and labelling at EU level of 4,4'-sulphonyldiphenol; bisphenol S: https://echa.europa.eu/registry-of-clh-intentions-until-outcome/-/dislist/details/0b0236e182ed4414

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

Not applicable.

1.5 Physicochemical properties

Table 5: Overview of physicochemical properties[5]

Property	Description of key information	Value [Unit]	Reference/source of information
Physical state at 20 °C and 101.3 kPa	Visual inspection	A fine white odourless powder: solid at 20 °C and 101.3 kPa	Unpublished study report, 2012 (REACH registration dossier)
Melting/freezing point	No test guideline or method reported	245-248 °C	Beilstein, 2007 (REACH registration dossier)
Boiling point	OECD TG 103 Dynamic method	Decomposition at 315 °C before boiling	Unpublished study report, 2010a (REACH registration dossier)
Vapour pressure	Calculation according MPBPWIN v1.42	6.29 x10^{-10} hPa at 25 °C	Neely W.B. and Howard P.H., 1995 (REACH Registration dossier)
Density	No test guideline or method reported	1.4 g/cm^3 at 20 °C	Beilstein, 2007; Annaheim, 2007; Yaws C.L., 2009 (REACH registration dossier)
Water solubility	OECD TG 105	715 mg/l at 20 °C	Unpublished study report, 2015 (REACH registration dossier)
Partition coefficient n-octanol/water (log value)	OECD TG 117 HPLC method	log Kow= 1.2 at 23 °C	Unpublished study report, 2010b (REACH registration dossier)

[5] REACH registration dossier (https://echa.europa.eu/information-on-chemicals/registered-substances/-/disreg/substance/100.001.137)

2. Harmonised classification and labelling

BPS is covered by Index number 604-098-00-1 in part 3 of Annex VI to the CLP Regulation as follows:

Table 6: Classification according to Annex VI, Table 3 (list of harmonised classification and labelling of hazardous substances) of Regulation (EC) No 1272/2008

Index No	Chemical name	EC No	CAS No	Classification		Labelling			Spec. Conc. Limits, M-factors and ATEs[6]	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)		
604-098-00-1	Bisphenol S; 4,4'-sulphonyldiphenol	201-250-5	80-09-1	Repr. 1B	H360FD	GHS08 Dgr	H360FD			

[6] Acute Toxicity Estimate

3. Environmental fate properties

3.1 Degradation

Available studies and information on phototransformation (air and water), ready and inherent biodegradability, hydrolysis and simulation studies (seawater, soil) are summarised below.

Table 7: Summary of relevant information on rapid degradability[7]

Method	Results	Remarks	Reference
Phototransformation in air QSAR estimation Rel. 2	DT50= 26.5 h	EPIWIN SRC AOP v1.92	REACH registration dossier: Epiwin calculation, 2007
Phototransformation in water : Equivalent or similar to OECD draft guideline (Phototransformation of Chemicals in Water - Direct and Indirect Photolysis) GLP : not specified Rel. 2	DT50= 43.1 min	HPLC	REACH registration dossier : Cao *et al.*, 2012
Phototransformation in water : Equivalent or similar to OECD draft guideline (Phototransformation of Chemicals in Water - Direct and Indirect Photolysis) GLP : not specified Rel. 2	Phototransformation in water was readily Main product formed : p-hydroxybenzenesulfonic acid.	ESI-MS and GC-MS	REACH registration dossier: Wang *et al.*, 2014
Ready biodegradability OECD TG 301C (Ready Biodegradability: Modified MITI Test (I)) GLP : not specified Rel. 2	0% degradation within 28d (TOC meas.)	/	REACH registration dossier: Unpublished study report, 1998
Ready biodegradability OECD TG 301B (Ready Biodegradability: CO2 Evolution Test under enhanced conditions) GLP: yes Rel. 1	32% degradation within 59d	Duration of adaptation phase: 27d	REACH registration dossier: Unpublished study report, 2018a
OECD TG 302B (Inherent biodegradability: Zahn-Wellens/EMPA Test) GLP: no	88% degradation within 77d (TOC removal)	Study performed with a mixture of 4,4'-dihydroxydiphenylsulfone and 2,4'-	REACH registration dossier: Unpublished study report, 2006

[7] REACH registration dossier (https://echa.europa.eu/information-on-chemicals/registered-substances/-/disreg/substance/100.001.137)

Rel. 2	Role microbial adaptation: 67% elimination after 8d and up to 92% at day 105	dihydroxydiphenylsulphone (1:1)	
Aerobic biodegradation study using a modified Zahn-Wellens Test (similar to OECD302B) GLP: not specified Rel.2	47% degradation within 14d in nutrient-mineral rich medium, without any other source of organic carbon (half-life of 17.3 days) 36% degradation within 7d (nutrient-mineral rich medium) and 35% within 7d in nutrient-mineral rich medium, without any other source of organic carbon)	Assessment of biodegration kinetics during biological waste water treatment with activated sludge using GCMS/MS	REACH registration dossier Kovačič *et al*., 2021
Aerobic and anaerobic seawater simulation study TOC-Handai method Rel. 2	Aerobic : 0% degradation after 22 days Anaerobic : 60% at ca. day 80	/	REACH registration dossier Ike *et al.*, 2006
Simulation study : degradation in seawater TOC Handai (TOC, potential test) and river (sea) die-away (SDA, simulation test) method Rel. 2	No degradation observed after 60d	/	REACH registration dossier Danzl *et al.*, 2009
Aerobic biodegradation in soil Similar to similar to those methods described by Mashtare *et al.*, 2013 GLP not specified Rel. 2	DT50 (at 22 °C, PFO): Soil n°1: 0.935d Soil n°2: 0.649d 3 transformation products detected	2 surface clay loam soils from a forested area were used HPLC-MS/MS Analysis	REACH registration dossier Choi and Lee, 2017a
Aerobic degradation in soil	T1/2= 2.8d After 28d: biodegradation: 53.6% NER formation: 44.9%	^{14}C-BPS Soil from a Chinese paddy rice field: 46.7% clay, 37.9% silt and 15.4% sand	REACH registration dossier Cao *et al.*, 2020

3.1.1 Abiotic degradation

3.1.1.1 Hydrolysis

Phenols are generally regarded as stable towards hydrolysis (Harris, 1990). Additionally based on the conclusions of Kollig *et al.* (1993) and Boethling and Mackay (2000), it can be assumed that BPS is hydrolytically stable since BPS does not contain functional groups that are susceptible to hydrolysis.

3.1.1.2 Oxidation

Based on the results of a study performed according to EU method A.17 (Oxidising properties: solids), BPS is considered not having oxidising properties (Unpublished study report, 2010c; REACH registration dossier).

3.1.1.3 Phototransformation/photolysis

3.1.1.3.1 Phototransformation in air

Photodegradation was estimated by QSAR using EPIWIN SRC AOP v1.92.

A 24h-day, a concentration of OH radicals of 0.5 $x10^{6}$ OH/cm^{3}, a degradation rate constant of 14.5 $x10^{-12}$ cm^{3}/molecule*sec was assumed for calculating the half-life.

The substance is relatively fast photochemically decomposed once released to air with a DT50 value of 26.5h.

Phototransformation is, however, a non-relevant degradation pathway because the substance shows low potential for volatilisation.

3.1.1.3.2 Phototransformation in water

Cao *et al.* (2012), demonstrated photolysis of BPS in water under UV light. The rate of photolysis increased with light source intensity. DT50 was of 43.1min.

In another study (Wang *et al.*, 2014), p-hydroxybenzenesulfonic acid was identified as a major degradation product of BPS when phototransformed in water.

3.1.1.3.3 Phototransformation in soil

NA

3.1.1.4 Summary on abiotic degradation

BPS is hydrolytically stable. It photodegrades relatively fast in air (DT50= 26.5 h) but, due to the low volatilisation potential of BPS, it is considered as a non-relevant degradation pathway. Under UV-light, phototransformation in water is very fast (43.1 min) and the rate increases with increasing light intensity. However, it cannot be excluded that BPS might accumulate in deeper and thus darker water layers.

3.1.2 Biodegradation

3.1.2.1 Biodegradation in aqueous media or aqueous environment

3.1.2.1.1 Estimated data

NA

3.1.2.1.2 Screening tests

A modified MITI test was conducted according to OECD TG 301C (Unpublished study report, 1998; REACH registration dossier) over a period of 28d with the use of 30 mg/l non-adapted activated sludge taken from 10 different sites (municipal STPs, industrial STPs, lakes and rivers) in Japan. 0% degradation was observed after 28d (TOC).

In another study performed according to OECD TG 301B (unpublished study report, 2018a; REACH registration dossier) 32 % degradation of BPS was observed after 59 days with a duration of the adaptation phase of +/- 27 days.

Therefore, the substance is considered to be not readily biodegradable.

In the inherent biodegradability study of Kovačič *et al.* (2021), similar to OECD 302B (modified Zahn-Wellens test), biodegradation kinetics of BPS was examined during aerobic degradation of activated sludge from a biological wastewater treatment plant within 14 days. The biodegradation pathway and products are shown in Figure 1. Kinetics were determined using MS/MS and biotransformation products by using LC-QTOF-MS. Degradation rate (kt) for BPS was determined to be 0.04-0.16 with a half-life of 4.3-17.3 days. 47% degradation was reached within 14 days. The absence of any additional organic carbon source significantly slowed down degradation of BPS (lag phase on day 18 instead of day 7).

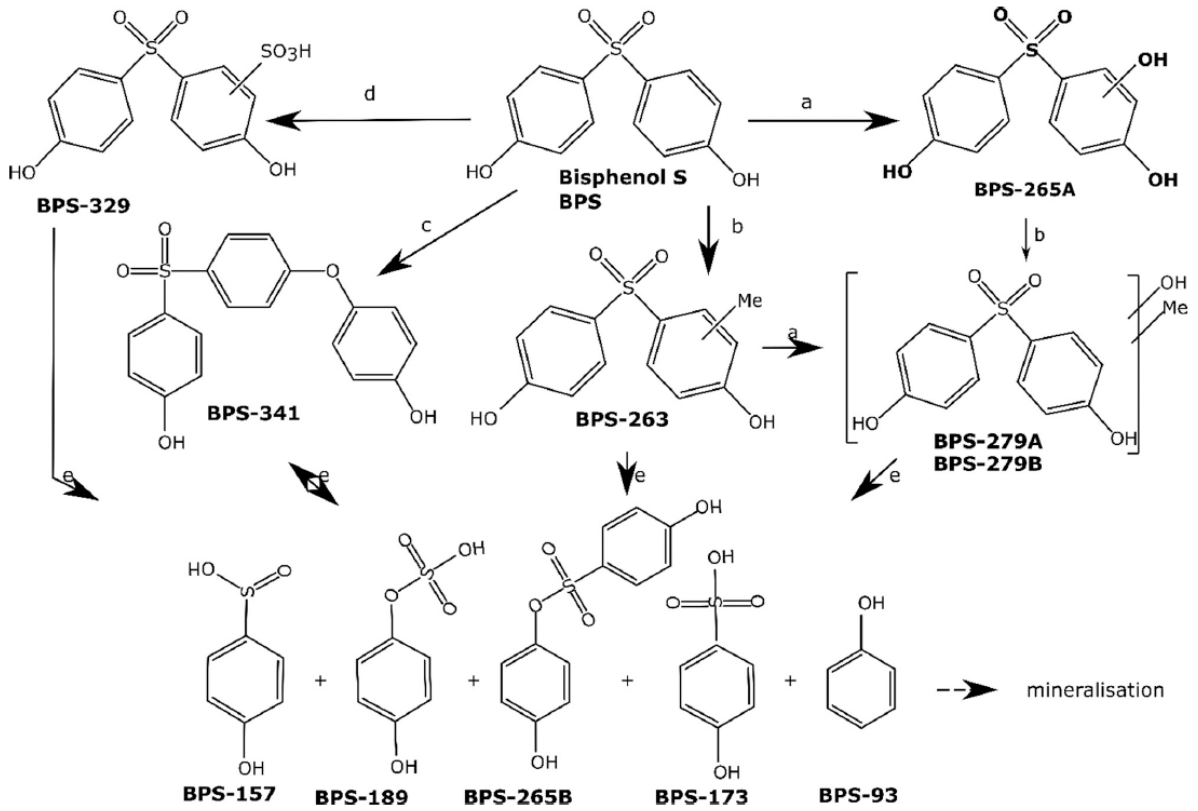


Figure 1: proposed biodegradation pathway of BPS in activated sludge
(a: hydroxylation, b: methylation, c: the coupling of smaller BPS moieties, d: sulphonation and e: cleavage of the S–C bond, dashed arrows; not identified) (from Kovačič *et al.*, 2021).

In the Zahn-Wellens test, a level of 70% mineralisation (DOC removal) must be reached within 7 days, the log phase should be no longer than 3 days, and the percentage removal in the test before degradation occurs should be below 15% (pre-adaptation of the inoculum is not allowed) (ECHA guidance IR/CSA, 2017a; chapter R.11). Therefore, BPS can be considered not inherently degradable.

3.1.2.1.3 Simulation tests

3.1.2.1.3.1 Biodegradation in water/sediment

Two literature studies are available on the biodegradation of a variety of bisphenols, including BPS.

In the microcosm study conducted by Ike *et al.* (2006) biodegradability of BPS is examined in an aerobic (according to the TOC-Handai method (Nasu *et al.*, 1993), a kind of river-die away method) and an anaerobic (according to a method similar to that of Kleerebenzem *et al.*, 1999) simulation test. Natural river water from three sites of four streams running (aerobic system) over Osaka and sediment from one pond (Inukai Pond- anaerobic system) in Japan was used. Test results supported the findings of the ready biodegradability study.

Under aerobic conditions, the biodegradability of BPS is 0% after 22 days incubation (degradation in 0 out of the 24 microcosms tested).

Under anaerobic conditions, biodegradation (only primary degradation) reached about 60% at ca. day 80 (study end) after a lag phase of ca. 60 days.

Another study examined the aerobic degradation of 3 bisphenols (BPA, BPF and BPS) in seawater using modified TOC Handai (TOC, potential test) and modified river (sea) die-away (SDA, simulation test) method (Danzl *et al.*, 2009). The TOC method uses seawater microorganisms collected through filtration from natural seawater. The retained organisms are then dispersed into artificial seawater at original levels of cell density. The SDA method uses samples of indigenous seawater microorganisms in their natural seawater.

Degradation of BPS was not observed by any of these methods in aerobic conditions after 60d, suggesting that **BPS might accumulate and remain in the aquatic environment for a long time**.

BPS can be removed efficiently from municipal/industrial wastewater treatment with an average removal efficiency of 81.2% (Wang *et al.*, 2019a). In the study from Česen *et al. (*2018), BPS was found in the influent and sludge, but was below the detection limit in the effluent. Sun *et al.* (2017), concluded that based on the mass balance analysis, the mass loss can be attributed to biodegradation.

3.1.2.2 Biodegradation in soil

3.1.2.2.1 Simulation tests in soil

Choi and Lee (2017a) investigated the aerobic soil biodegradation of BPA alternatives BPS and BPAF for up to 180d. Two surface clay loam soils (100 µg/kg) were used: one from a forested area close to the Purdue campus (FRST-50) and one sampled from the Purdue Student Organic Farm (PSF-51) (Indiana, USA). The study was conducted similarly to those methods described by Mashtare *et al*. (2011).

Based on compound mass recovered from soils compared to the mass applied, BPS had short half-lives of <1 day in both soils: 0.649d (FRST-50) and 0.935d (PSF-51). These DT50s are Computer Aided Kinetic Evaluation (CAKE) outputs for the times (d) required for the time 0 concentration to decline by 50%. The data were fitted using both Single First order (SFO) and in parallel for comparison Double First Order (DFO) kinetic model: DT50 were determined to be 1.10 and 0.0661 days, respectively.

Metabolites were identified using uHPLC-TOF Analysis for Metabolites, an ultra-high performance liquid chromatography combined with a time-of-flight mass spectrometry, using non-target comparative screening. Three degradation products of BPS were detected.

Cao *et al.* (2020) examined the degradation and NER formation of BPS in aerobic soil. The study was performed with a soil collected from a paddy rice field in Jiangsu (China). The soil consisted of 46.7% clay, 37.9% silt and 15.4% sand.

A half-life of 2.8d was determined. After 28d, the dissipation of BPS could be attributed to biodegradation (53.6 +/- 0.2%) and NER formation (44.9 +/- 2.9%). NER formation was reached before maximum mineralisation (after +/- 6d vs +/- 11d resp.). Formation of NERs occurred mostly via physicochemical entrapment (more than half) and ester-linkages (one third). Formation of ester-linkages NERs was especially attributed to microbial activities while physicochemical entrapment was attributed to the abiotic aging of BPS in soil. These last NER-type were unstable and became again bioavailable when mixed with fresh soil.

Degradation of BPS to metabolites (degradation constant kPM = 0.186/d) was lower than mineralisation of the metabolites (Kmv = 0.773/d). Two major metabolites were formed. Both were detected at day 2 and increased until day 6. M1 remained stable thereafter, while M2 further decreased and was no longer detected at day 28.

3.1.2.3 Biodegradation in WWTP (Wastewater treatment plant)

Removal capacity/efficacy in WWTP was determined in several literature studies, although little information is available for Europe.

The mean concentration of eight bisphenols, including BPS (aqueous and suspended particulate matter combined) were measured in 5 Indian STPs by Karthikraj and Kannan (2017). Concentrations of BPS were found in the influent, effluent and sludge of the STPs (mean measured concentrations of 14.7, 2.4 and 185.7 ng/L resp.). The mean concentration of BPS in sludge was 10 times higher than that of BPA whereas, in the dissolved phase, the concentration of BPA was four-fold higher than that of BPS. These results suggest that BPS has a higher affinity for particulate matter/sludge than does BPA. Removal efficiency was calculated to be 77.7% (mean: range 69.6-96.7%).

Wang *et al.* (2019a) reviewed the occurrence and removal mechanisms of BPA and its analogues, including BPS, in municipal WWTPs from several countries/regions. BPS was measured in the influent of the municipal WWTPs with an average concentration of 54 ng/L and 5 ng/L in the effluent. The removal capacity of BPS in full-scale municipal WWTPs ranged between 3.6 to 100.0% (average 81.2%) which indicate a good removal performance of municipal WWTPs. Based on the relatively low Log Kow of BPS, it is suggested that biodegradation is likely to be an important route of removal. The concentration of BPS in the sewage sludge were in the range of not detected - 600 ng/g with an average concentration of 31.13 ng/g dw.

The study from Wang *et al*. (2019a) includes findings from Česen *et al.* (2018), for BPS in 5 municipal/industrial WWTPs in Slovenia. BPS was found above the LOD in 2 out of the 5 WWTP influent samples resulting in an average concentration of 21.3 ng/L, while in the effluent it was <LOD in all 5 samples, concluding a 100% removal capacity.

Also, findings from Sun *et al. (*2017), were included in this review. BPS was detected in the influent and the sludge of seven wastewater treatment plants in Xiamen (China) with a medium concentration of 48.0 ng/L and 1.01 µg/kg resp. The concentration of BPS was below the detection limit in the effluent. Total mass loads of BPS in the influent were 56.2 g with an adsorbed mass value of 1,19 g. After wastewater treatment, mass load in the effluent and sludge was 0.703 g and 0.259 g resp. The removal efficiency of BPS was 98.3% and based on the mass balance analysis it can be concluded that mass loss was mainly through biodegradation.

BPS was one of the most abundant bisphenols found in the influent, primary effluent and final effluent of 2 WWTPs in Albany, NY (USA) (Xue and Kannan, 2019). The detection rate was 44% in the influent (raw wastewater) with concentrations ranging from MLOQ-707 ng/L. BPS was not detected in the suspended particulate matter phase of the wastewater influent in one WWTP but had a detection rate of 6.3% in the 2nd WWTP. The geom. mean concentration in the sludge was between 7.76 and 15.8 ng/g dw, with detection rate of 77% and 73% resp. Removal efficiency of BPS in WWTP after primary treatment was between 6.4 and 24% and -11 and 1.1% after secondary treatment. No BPS was detected in ash of incinerated sludge, demonstrating that this is a good removal method for BPS in WWTP sludge.

3.1.2.4 Additional biodegradation data

Sakai *et al.* (2007) investigated biodegradation of BPA and related compounds by a specific microorganism. In this study, *Sphingomonas sp.* strain BP-7 was not able to degrade BPS, while BPA was degraded within 20h.

3.1.2.5 Summary and discussion on biodegradation

BPS is not readily biodegradable and shows low degradation in river water and seawater under aerobic conditions. The removal of BPS in sediment was circa 60% after 60 days under anaerobic conditions. BPS is quickly degraded in aerobic soils.

It can be concluded that BPS is not rapidly biodegraded under relevant environmental conditions.

Literature data confirm that BPS can be degraded by microbial organisms after acclimatisation. BPS was removed efficiently from municipal/industrial wastewater treatment plants mainly by biodegradation with an average removal of 81.2%. Also, the relatively low log Kow suggest that this is an important removal route.

However, it is most likely that at industrial sites microbial organisms in WWTP are adapted to BPS. Furthermore, BPS is a substitute for BPA and it became already the main developer used in thermal paper since the restriction of BPA in thermal paper is in force. BPS is found in several environmental compartments including water (Wu *et al.*, 2018). Therefore, it cannot be excluded that BPS ends up in municipal WWTP with unadapted microorganisms.

3.1.3 Field data

NA

3.1.4 Summary and discussion of degradation

BPS is hydrolytically stable and not readily biodegradable (0% degradation within 28d).

Simulation studies show low degradation in river water, seawater under aerobic conditions. The removal of BPS in sediment was circa 60% after 60 days under aerobic conditions. BPS is quickly degraded in aerobic soils, but mineralisation of the metabolites was slower.

Although photolysis in water is rapid (DT50= 43.1 min), it cannot be excluded that BPS might accumulate in deeper water layers and remain in the aquatic environment for a long time.

It can be concluded that **BPS is not rapidly degraded under relevant environmental conditions.**

Based on the above data, BPS fulfils the screening criteria for persistency.

A definitive conclusion cannot be drawn on P/vP in the absence of half-lifes determined under environmental relevant conditions and of studies performed according to standard test guidelines.

3.2 Environmental distribution

3.2.1 Adsorption/desorption

In the non-guideline study from Choi and Lee (2017b), the partitioning behaviour of BPS in soil-water and octanol water system was examined by using 4 soils with varying chemical and physical properties. A geom. log Koc of 2.82 (at 23°C) was determined.

In another non-guideline study, the partitioning of BPS was examined in water and sediment from Chinese rivers by Jin and Zhu (2016), which resulted in a log Koc of 3.5.

In a third study (Unpublished study report, 2016), performed according to the International Standard ISO 18749, Water Quality (Batch Test using specific analytical methods) <10% of DOC (mean value) was removed by adsorption onto the activated sludge after 72h of exposure.

3.2.2 Volatilisation

Henry's law constant was estimated to be 2.73 $x10^{-10}$ $Pa*m^3$/mole at 25°C by EPIWIN (SRC HENRYWIN v3.10) which suggest a low probability of partitioning from the aqueous system to the atmosphere.

3.2.3 Distribution modelling

3.2.3.1 Distribution

According to the information provided in the REACH registration dossiers[8] BPS is manufactured and used in closed systems and during industrial polymerisation process the substance is chemically bonded completely. The substance is ubiquitous in several environmental compartments (Wu *et al.*, 2018).

When 100% emission to water is considered when applying the Level III Fugacity Model (Episuite 4.1), BPS will remain mainly in the water (96.1%). However, when equal emission to the different environmental compartments is considered, BPS will partition mainly to soil (83%). Also, when emission to soil is considered, BPS is estimated to stay mainly in soil (99.8%).

[8] REACH registration dossier of 4,4'-sulphonyldphenol (BPS): Registration Dossier - ECHA (europa.eu)

3.2.3.2 Occurrence

3.2.3.2.1 Occurrence in articles and products

Several literature studies demonstrate the occurrence of BPS in different types of articles and products in Europe and worldwide (food and food contact material, thermal paper and other paper products, texiles, personal care products, dental sealants, medicinal material and medicines).

- **Occurrence in food and food contact material**

BPS has a European SML (specific migration limit) of 0,05 mg/kg for plastic materials and articles intended to come into contact with food (European Commission, 2011; EFSA, 2020). In China and the US, the highest concentrations of BPS were found in meat and meat products.

Data on BPS in foodstuffs and food contact materials in Europe:

- Different types of canned vegetables and their supernatant liquid were analysed for the presence of BPA, BPS and 2,2'-biphenol (BP). Cans, protected with an inner layer of epoxy lacquers, were collected from different manufacturers. Up to 36.1 ng/g of BPS was found in the food and up to 175 ng/ml in the liquid (Viñas *et al.*, 2010). Furthermore, migration of BPS was tested using food simulants according to Council Directive 85/572/EEC. Levels of BPS increased when acetic acid was used as simulant as well as temperature and contact time increased. Concentrations were up to 0.52 ng/mL (water, 25°C), 3.54 ng/mL (water, 80°C), 2.17 ng/mL (3% HAc, 25°C) and 5.36 ng/mL (3% HAc, 80°C) after 240h.

However, Gallart-Ayala *et al.* (2011) did not detect BPS in canned soft drinks collected in supermarkets in Barcelona (Spain) in July 2009. Analysis was done by on-line solid phase extraction fast liquid chromatography-tandem mass spectrometry.

- In Simoneau *et al.* (2011) the release of BPS from polyethersulphone baby bottles was found to be below the detection limit of the analytical methods used (0.1 µg/kg for the HPLC- and 0.3 µg/kg for the UPLC-MS method). Bottles were collected in the USA and 11 **European countries**: Belgium, Bulgaria, Germany, Denmark, Spain, France, Italy, Lithuania, Malta, Poland and United Kingdom.

- Canned and non-canned beverages purchased on the **Belgian** market between April and December 2014 did not contain detectable levels of BPS (Reguiero and Wenzl, 2015). LOD of non-alcoholic and alcoholic beverages was 17.6 ng/L and 19.4 ng/L resp., and the LOQ resp. 58.6 and 64.6 ng/L.

- García-Córcoles *et al.* (2018), examined the presence of seven bisphenols in 15 ready-to-eat plastic packed baby foods (powdered milk, cereals with milk, juices, yoghurt and homogenised fruit, meat and fish) from different brands purchased from local supermarkets in Granada, **Spain**. BPS was found the most abundant of the bisphenols with concentrations between 11.7 and 49.2 ng/g (LOD: 0.3 ng/g, LOQ: 1 ng/g). BPS was detected and quantified in 5 of the 15 samples.

- Levels of BPS were detected in food and beverage collected on a **Dutch** market (Van Leeuwen *et al.*, 2019). Concentrations in Tuna brine (juice accompanying the meat in the can), wiener sausage brine, carbonated soft drinks and apple juice were resp. 0.2 ng/mL, 0.1 ng/mL, <0.0007 and <0.001 ng/mL. BPS was lost during sample preparation in tuna meat, Wiener sausage, Corn, Tomato soup, pineapple and tomato puree.

- BPA-free reusable drinking bottles from five different brands were collected in **Austrian** stores during the summer of 2020 (Vienna) (Banaderakhshan *et al.*, 2022). Most of the bottles were made of Tritan and for usage by children and adults. Migration experiment shows that BPS was

detected in each leaching sample at levels between 0.013 µg/Land 0.26 µg/L (20°C) and 0.010 µg/L and 0.065 µg/L (60°C).

USA, Canada and China:

- The study of Liao and Kannan (2013), reports concentrations of several bisphenol analogues (including BPA, BPF, and BPS) in foodstuffs (n=267) collected in 2008, 2011 and 2012 from Albany (N.Y., USA), using HPLC-MS/MS. Foodstuffs were divided into nine categories: beverages, dairy products, fats and oils, fish and seafood, cereals, meat and meat products, fruits, vegetables, and "others". Concentrations of BPS were in general 1-2 orders of magnitude lower than those of BPA and BPF. BPS contributed <10% in the sum of the concentrations of the analysed bisphenols in the 9 food categories. BPS was most frequently (43.1%) and with the highest concentrations (in a geom. mean conc. of 0.609 mg/g, 95th percentile of 0.780 mg/g) found in meat and meat products.

- Another study (Liao and Kannan, 2014b) analysed 289 food samples collected in 2012 from nine cities in China. Significant positive correlations were found between BPA and BPS which suggest co-occurrence and similarity in sources in foods. BPS was most dominantly found in fish and seafood (frequency of 72.7%) in mean concentrations of 0.564 ng/g (95^{th} percentile of 2.53 ng/g). The highest concentration was however found in meat and meat products with a mean of 2.16 ng/g and a frequency of 30%. The EDI (estimated daily dietary intake) was estimated to be 9.55 ng/kg bw/day by adult males and 9.56 ng/kg bw/day by female adults.

- A novel liquid chromatography tandem-mass spectrometry (LC-MS/MS) was used by Hwang *et al.* (2018), to measure the migration of 8 bisphenols (BPA, BPS, BPF, BPB, BPP, BPAF, BPAP and BPZ) from 11 types of food contact materials. 234 articles were sampled from online kitchenware market and departments stores in Korea. No bisphenols were detected in any of the samples.

Also, in the study by Zhou *et al.* (2019), BPS was found dominantly in fish and shellfish (100% frequency), followed by meat (75% frequency) and canned fish (50% frequency) with resp. mean concentrations of 2.1 µg/kg, 9.2 µg/kg and 0.3 µg/kg. Highest concentrations were reported in fish.

- Concentrations of BPS were 2.34 µg/kg in pacifier and <LOQ in soy-bean milk, both purchased at a local market in China (Zhang *et al.*, 2019).

- After analysis of 23 dairy products from a local market in China, BPS was detected in milk beverages (0.4 µg/kg +/- 6.1) and yogurt (0.4-0.5 µg/kg) (Cheng *et al.*, 2017).

- Cao *et al.* (2019), did not detect BPS in canned food composite samples from a Canadian total diet study. BPS was however detected in nine food composite samples prepared from meat and meat products with concentration ranging between 1.2 and 35 ng/g.

- Zhang *et al.* (2019), examined the presence of BPS in soy-bean milk and pacifiers collected on the Chinese market. Pacifiers were soaked in hot water to investigate the solution behaviour of BPS and 7 other bisphenols. BPS concentrations were <LOQ in soy-bean milk, but could be found in pacifiers and their soaking solution with concentrations of 2.34 µg/kg and 0.417 µg/kg resp.

- **Occurrence in thermal paper and other paper products**

- The volume of BPS used as a developer in thermal paper manufactured and placed on the **EU** market has tripled from 64 kT in 2014 to 187 kT in 2019. The substance became the main developer used in thermal paper in 2019. If a 75% switch of BPA-based thermal paper to BPS is considered, it is estimated that 307 kT BPS will be used as developer in thermal paper in 2022, representing a growth of 376% for the period of 2014-2022 and a total share in thermal paper consumed in the EU market of 61% (ECHA, 2020).

- BPS was only detected in 4 out of 124 thermal paper receipts collected in **Switzerland** during 2013-2014 (Goldinger *et al.*, 2015). Concentrations ranged between 8.3 and 12.6 mg/g (mean: 10.2 mg/g).

- BPS was found in 31 out of 50 samples of thermal paper receipts collected on the **Italian** market from 2015-2016. Concentrations were determined by liquid chromatography to tandem fluorescence and ultraviolet detection and ranged between <LOQ to 357.989 µg/100 mg paper with a mean concentration of 41.97 µg/100 mg paper (Russo *et al.*, 2017).

- Molina-Molina *et al.* (2019), collected 112 thermal paper receipts in 2017 from Brazil (n=22), Spain (43) and France (47) and analysed them for the presence of BPA, BPS and BPF as well as for their estrogenic and anti-androgenic activity. In 9.1, 4.6 and 21% of the samples BPS was detected in the receipts from Brazil, **Spain** and **France** resp., and in concentrations between <0.03 and 13.29 mg/g.

- BPS was detected in the majority of the nine samples of thermal papers (cash receipts) collected in 2020 in the most common supermarkets and service providers in Vienna (**Austria**) (Banaderakhshan *et al.*, 2022). Concentrations of BPS were analysed using HPLC-MS/MS and were in the range of <LOQ (0.01 µg/L) and 38 µg/g. BPS and BPA were the most common bisphenols present in the examined samples.

- Liao *et al.* (2012b), obtained 111 samples of thermal paper receipts from 4 countries (USA, Japan, Korea and Vietnam), 52 currency bills from 21 countries (USA, Canada, Czech Republic, Russia, Turkey, Australia, Brazil, Egypt, South Africa, China, India, Japan, Korea, Kuwait, Malaysia, Philippines, Singapore, Thailand, Vietnam, and United Arab Emirates) and 105 of other paper products originating from 1 country (USA). The latter were divided in 14 categories (flyers, magazines, tickets, mailing envelopes, newspapers, food contact papers, food cartons, airplane boarding passes, luggage tags, printing papers, business cards, facial tissue, kitchen rolls and toilet paper).

BPS was present in all thermal paper receipts (100%) with concentrations ranging from 0.0000138 to 22 mg/g (geom mean 0.181 mg/g) which are in the same order of magnitude as BPA found in Liao *et al.* (2012b). Furthermore, a negative correlation was observed between BPS and BPA in those receipts: thermal paper receipts containing high concentrations of BPS contained low or non-detectable concentrations of BPA and vice versa.

Because of the structural similarity with BPA and the similar concentrations of BPA found in thermal receipts, migration of BPS from thermal receipts to paper currencies is therefore also assumed every time a receipt is placed near the currency in a cash register or wallet and thus considered an important source of BPS presence in paper currency. The same can be also the case when those thermal receipts come in contact with other products, which should be kept in mind when thermal receipt papers are recycled together with other paper products.

The detection rate of BPS in currency bills was of 94% and ranged between 0 and 100% in other paper products. Concentrations of BPS in currency bills and other paper products were much lower than in thermal paper receipts, resp. <LOQ−4.04 µg/g (geom mean: 0.0290 µg/g) and <LOQ−8.38 µg/L (geom mean: 0.0036 µg/g) (LOQ: 0.1 ng/g). The highest concentrations of BPS in other paper products were found in airplane luggage tags, boarding passes and mailing envelopes with a detection rate of 100%. Most probably due to a similarity in thermal printing process used with BPA.

Geometric mean estimated daily intake (EDI) of BPS via dermal absorption after handling of all paper products was 10.5 ng/day for the general population and 787 ng/day for occupational exposure, more specific for thermal papers 10.5 and 787 ng/day and paper currencies 0.0017 and 0.0168 ng/day. For the other 14 paper types, the EDI values for the general population and occupationally exposure were the same. The highest geometric mean EDIs were estimated for

airplane luggage tags (0.0518 ng/day), airplane boarding passes (0.435 ng/day), and tickets (0.339 ng/day).

Considering a body weight of 70 kg, the median and 95th daily intake for the general population and occupational exposure for thermal paper receipts was estimated to be 4.18 and 11.0 ng/kg bw/day and 312 and 821 ng/kg bw/day respectively. From Liao *et al*. (2012), it can be concluded that the major human exposure source to BPS among the analysed paper types comes from handling of thermal receipt papers (>88%).

Raw paper materials

- Samples of 6 different basic raw materials used for the production of paper packages including food and hygiene packages, collected in **Europe** in 2015, were analysed for the presence of BPA and its bisphenol analogues (Jurek and Leitner, 2018). Packaging products can be divided in virgin fibre samples (coated board, cellulose rolled and board primary bleached) and recycled samples (testliner white, coated recycled and recycled bleached). BPS was found in all samples in concentrations ranging from 0.19 to 99 µg/kg, with much higher concentrations in recycled samples (51-99 µg/kg) compared to virgin samples (0.11-13 µg/kg). A specific migration limit for BPS of 50 µg/kg exists for plastic food contact materials. Although 100% migration is not likely to occur, concentrations of BPS between 0.002 and 1.1 µg/kg (virgin samples: 0.002-0.23 µg/kg; recycled: 0.31-1.1 µg/kg) are calculated in such case.

Recycling

The highest concentrations of BPS were found in thermal paper receipts (210 µg/g) in paper in Denmark although in much lower concentrations than BPA (8300 µg/g) (Pivnenko *et al.*, 2015). BPS was found in 70% of the samples demonstrating the spreading in use or through wastepaper recycling. No significant difference was seen between source-segregated wastepaper intended for recycling and the mixed (residual) wastepaper. Nevertheless, in an earlier study performed by the Danish EPA (Miljøstyrelsen, 2011), BPS was identified in just 25% of the thermal paper receipts.

The second highest concentration (1.3 µg/g) was found in corrugated boxes, but in much lower concentrations than in paper receipts suggesting the spreading of BPS through paper recycling or the use of epoxy glues, in which BPS can be employed as a curing agent. Furthermore, it can be concluded that BPA was substituted by BPS because all samples with no or low BPA concentrations showed high BPS concentrations, confirming the negative correlation between BPA and BPS concentrations found by Liao *et al.* (2012b).

Based on the high affinity of BPS to both the water and solid phase (retention potential in paper fibers during wastepaper recycling), prediction of the behaviour of BPS in a recycling process is difficult. But if BPS is removed from the paper matrix via the water used in the paper recycling process, it might end up in the environment. Although this is expected to be in very small concentrations due to the optimalisation of paper production methods (recirculation of the water), it might be an important exposure source when considering the persistency of BPS (as presented in chapter 3.1.4).

Concentrations of BPS in all the analysed paper products ranged from <LOD (0.7 µg/g) to 8100 µg/g, with a median concentration of 7800 ng/g. There was no difference between recycled and virgin printerpaper : <LOD. Also, the concentration of BPS in non-carbon copy paper was below the LOD.

- **Occurrence in textiles**

In the study of Xue *et al.* (2017) investigating 77 textiles for babies (socks, fabric nappies, blankets, and bodysuits), concentrations of BPS ranging between 0.7 and 394 ng/g were reported.

Li and Kannan (2018) examined concentrations and profiles of 23 endocrine-disrupting chemicals, including BPS, in 74 pantyhose samples collected from 6 countries. This study revealed median concentrations of 1430 ng/g of BPS in almost all of the samples investigated (100% and 96% of the samples, respectively). Moreover, it was found that pantyhose made in Japan and China with 21–50% Spandex contained the highest concentrations of BPS (2.2 mg/g). Consequently, the authors calculated dermal exposure doses of 45 900 pg/kg bw/d.

In the study of Wang *et al.* (2019b), BPS was found in 43% of the samples of collected garments (n=93). Used clothes (n=49) were selected from wardrobes of 38 families in 3 Chinese cities, while new clothes (44) were purchased from online retailers and local stores in Tianjin (China). BPS concentration in the textiles ranged from <0.53 to 536 ng/g with a median and mean concentration of resp. 7.38 and 44.0 ng/g. Used clothes contained less BPS than new clothes (5.67 vs 12.3 ng/g, median concentration). Furthermore, very high concentrations of BPS were found in new textiles made of polyester and Spandex (536 ng/g).

Smit and Zoon published on their website figures of residual BPS in syntans used for leather retanning, which revealed concentrations of BPS ranging from >20 up to >28400 mg/kg (https://www.smitzoon.com/en/sustainability/chemicals-bisphenol/). The lowest detection limit was 20 mg/kg.

- **Occurrence in personal care products**

After BPA (13%), BPS was the second most frequently detected bisphenol (11%) analysed in 117 samples of personal care products collected from retail stores in China and US during 2012-2013. The highest detection rate was found in makeup (18.8%). The low detection frequency and concentrations of BPS in the analysed products suggest that personal care products are not the most dominant source of exposure to BPS (Liao and Kannan, 2014a).

Lu *et al.* (2018b) collected 150 samples of 11 categories of personal care products in retail stores and supermarkets in Guangzhou and Shenzen (China) in September 2015. BPS had a detection frequency of 33.3% in personal care products. BPS was most frequently found in toothpaste (75%), shampoos (86.7%) and face cleaners (55.6%). Mean concentrations measured for these categories were 2.6 ng/g, 2.79 ng/g and 1.79 ng/g, respectively. They also estimated the intake (EDI) and uptake (EDU) of BPS from personal care products by dermal contact. The daily dermal uptake was estimated by considering a dermal absorption rate of 10% (similar to BPA). Intake and uptake were very low with the highest EDI (5.18 $x10^{-2}$ ng/kg bw/d) and EDU (5.18 $x10^{-3}$ ng/kg bw/d) by use of sunscreens.

Gao and Kannan (2020) examined 77 feminine hygiene products from Albany, USA. The products were divided into 7 categories (pads, panty liners, tampons, wipes, bacterial creams and solutions, deodorant sprays and powders) and 24 chemicals were measured of which 8 bisphenols (BPA, BPF, BPP, BPS, BPZ, BPAP, BPAF and BPB). BPF, BPA and BPS were the major bisphenols found in these products.

The highest detection frequency for BPS was in wipes (17%) and panty liners (15%), but the highest concentration was found in bacterial creams and solutions with a mean of 0.18 ng/g.

- **Concentration in dental sealants**

BPS was not detected in dental sealants collected between June and August 2015 on the US market, originating from the US, Korea, **Liechtenstein**, **The Netherlands** and **Greece** (Xue *et al.*, 2018). However, authors measured BPS in the only analysed dental sealant sample from Japanese origin, with a concentration of 21.5 ng/g. The detection frequency was 1.4%. According to the authors, "leaching of Bisphenols into saliva can be a pathway of exposure to these chemicals by humans".

- **Medicinal material**

Shang *et al.* (2019) did not detect BPS in urine from Canadian men (pre- and post-surgery) suggesting that BPS is not present in medicinal device used in cardiac surgery in Canada as of May 2018 (see also human biomonitoring).

Zhang *et al.* (2019), determined concentration of 8 bisphenols via UHPLC–MS/MS in sodium chloride injection and glucose injection purchased at the Liaoning Province Tumor Hospital in China. BPS concentration were <LOQ in sodium chloride, but 0.051 µg/L in the glucose injections.

- **Concentrations in medicines**

BPS was detected in samples of commonly used over-the-counter medicines (OTC) manufactured in China, including western medicines and Chinese patent medicines for adults and children which were collected from local drugstores in China (n= 28) and 8 Chinese families in the USA (n= 58) in June 2014 (Jia *et al.*, 2021). BPS had a frequency of resp. 14% and 19% in pediatric and adult medicines, with a mean concentration of resp. 0.21 and 0.07 ng/g.

Point assessment and probabilistic assessment of estimated daily intake (EDI) for BPS in different age categories were:

Table 8: Point assessment and probabilistic assessment of EDIs for BPS in OTC medicines

		<1 year old	1-3 years old	Male	Female
Point estimation calculated with measured concentrations					
BPS	Mean (µg/kg bw/d)	0.77	0.33	-	0.01
	Median (µg/kg bw/d)	-	-	-	-
	95th	3.96	1.24	0.04	0.04
Probabilistic estimation from the Monte Carlo simulation					
BPS	Mean (µg/kg bw/d)	0.39	0.41	0.02	0.02
	Median (µg/kg bw/d)	0.10	0.08	0.01	0.01
	95th	0.83	0.81	0.04	0.05

Extracted from Jia *et al.*, 2021

3.2.3.2.2 Occurrence in indoor dust/air

BPS was found in indoor dust samples taken from 12 countries (China, Colombia, Greece, India, Japan, Kuwait, Pakistan, Romania, Saudi Arabia, South Korea, U.S., and Vietnam). Samples were collected from homes (n=284) and from other microenvironments (n=104, laboratories, offices, cars, air conditioner and e-waste workshop). BPS was detected in all samples from **Romania** (n=23) and 85% of the samples from **Greece** (n=28). The mean concentrations measured in house dust in Greece and Romania were 1500 and 380 ng/g resp (Wang *et al.*, 2015).

Larsson *et al.* (2017) examined the presence of phthalates, non-phthalate plasticisers and bisphenols in dust from 100 preschools from six areas of Stockholm (**Sweden**). Samples were collectes in two stages : 30 preschool samples between February and April 2015 and 70

additional preschool samples between September and November 2015. BPS was detected in concentrations ranging from <LOD (0.12 µg/g) to 22 µg/g dust, with a geometric mean of 0.26 µg/g. In 80% of the samples, concentrations were above the limit of detection.

Giovanoulis *et al.* (2019) selected 20 **Swedish** preschools (area of Stockholm) from the 100 preschools sampled in the previous study from Larsson *et al*. (2017). Dust samples were collected during January to February 2018 and results were compared with those of the previous study. The detection frequency of BPS increased from 80% in 2015 to 95% in 2018 as well as the median concentration in the dust, which increased with 93% from 0.255 µg/g to 0.626 µg/g. The median estimated daily intake from ingestion of preshool dust is 1.32 and 2.20 for intermediate and high exposure scenario resp. (mean EDI: 1.18 and 1.97 ng/kg bw/d resp.).

Dueñas-Mas *et al.* (2019) detected BPS in samples collected from public environments (electronic shops, clothing shops, sport clothing shop, decoration shop, bazaars and a cafetaria, n=10) in 2018 in Spain by using SUPRASs (simultaneous extraction/clean-up method based on the use of supramolecular solvents). BPS was found in a concentration range between the detection limit (1 ng/g) to 736 ng/g, with a mean and median concentration of 290 ng/g and 193 ng/g resp. BPS was detected in 70 percent of the samples (n=10).

The presence of BPS in indoor dust was detected in all samples (n=156) collected in China, Korea, Japan and US ranging from 0.8 to 26600 ng/g (340 ng/g geom mean) (Liao *et al.*, 2012b).

In the study of Xue *et al.* (2016), BPS was predominantly found in the vapor phase of indoor air samples from parking garages, auto repair shops, cars, barber shops, public places, homes, labs and offices in Albany (USA). Furthermore, it was the most frequently detected of the eight bisphenols analysed in the vapor phase, with a detection rate of 26.5%. Concentrations in bulk air (sum of particulate and vapor phase concentrations) ranged from <MLOQ to 0.94 ng/m^3, with a mean of 0.07 ng/m^3. The daily intake of BPS (total, geom. mean) was estimated to be 0.39 ng/day for infants, 0.60 ng/day for toddlers, 0.89 ng/day for children, 1.24 ng/day for teenagers and 1.13 ng/day for adults.

To measure the concentration of BPS in indoor dust, Liu *et al.*, 2019a took samples in Signaporean houses during November 2017 (n=32). BPS was detected in all dust samples ranging from 153 to 6491 ng/g with a geom.mean of 713 ng/g dust. BPS was detected in all samples.

3.2.3.2.3 Human biomonitoring data

It should be noted that most of the articles don't describe the form of BPS (free, conjugated or total form) that was measured (in biological samples). When information was available this was explicitly added for the literature studies examining European human biological samples.

1. Urine

Several biomonitoring studies are available, demonstrating the presence of BPS in human urine from general populations outside Europe [United States, China, India, Japan, Korea, Kuwait, Malaysia, Vietnam (Liao *et al.*, 2012a; Duan *et al.*, 2018) and Saoudi Arabia (Asimakopoulos *et al.*, 2016), from people living near a BPAF manufacturing plant in South China (Yang *et al.*, 2014), from adults in USA-Atlanta (Zhou *et al.,* 2014), from children in China (Yao *et al.*, 2018), from cashiers and non-cashiers in US-North Carolina (Thayer *et al.,* 2016) and in pregnant women in Australia (Heffernan *et al.,* 2016), China (Wan *et al.*, 2018; Zhang *et al.*, 2020) and USA (Ihde *et al.*, 2018) and Israel (Machtinger *et al.,* 2018)].

Less information is available for Europe:

Only 8 studies examined urine samples in Europe: in pregnant women (Gyllenhammar *et al.*, 2017; Philips *et al.*, 2018), in children (Larsson *et al*., 2017) and adolescents (Tkalec *et al.*,

2021), in cashiers and non-cashiers (Ndaw *et al.*, 2018) and in general population (Sakhi *et al.*, 2018; Husøy *et al.*, 2019; Balicco *et al.*, 2019).

Europe:

- In the Larsson *et al.* (2017) study, concentrations of BPS in urine (from 113 **Swedish** preschool children with age ranging between 40 and 58 months) ranged between 20-33 000 ng/L of and 30-50 000 ng/L for unadjusted and density adjusted levels resp. (geom mean conc. of resp. 190 and 200 ng/L, median conc of 170 and 160 µg/L). Before analysing the samples they were treated with ammonium acetate and glucorinidase. In this study samples were collected between March and May 2015 from a subset of 28 of the 100 selected preschools from six areas of Stockholm municipality. Furthermore, a time trend was studied by analysing the spot urine samples from longitudinal birth cohort BAMSE (Swedish abbreviation for Children, Allergy, Milieu, Stockholm, Epidemiology) collected between 1998 and 2000 from children in Stockholm. However due to the large number of samples below the LOD in 1998-2000 it was not possible to make a trend evaluation for BPS. Daily intake of BPS via dust was calculated to be 0.0004 µg/kg bw/d (geom. mean).

- Sakhi *et al.* (2018), collected urine samples from mothers and their children in spring 2012 in **Norway** and analysed them for the presence of environmental phenols. BPS was detected most frequently in the urine samples with a detection frequency of 42% in mothers with a Specific Gravity adjusted mean and geometric mean concentration of 0.18 ng/mL and 0.11 ng/mL resp. and of 48% in children with a Specific Gravity adjusted mean and geometric mean concentration of 0.45 ng/mL and 0.16 ng/mL resp.

- In the frame of a Norwegian biomonitoring study (part of the EU project EuroMix) levels of phenols and phthalates were measured between September 2016 and November 2017 in urine from **Norwegian** adults (Husøy *et al.*, 2019). Samples were deconjugated usting β-glucoronidase in ammonium acetate buffer.The detection rate of BPS in urine was 29% in all participants (n=144) and the Specific Gravity adjusted mean concentration and geometric mean were resp. 0.36 ng/mL and 0.19 ng/mL, with min 0.04 ng/mL and max 12.74 ng/mL.

- Bisphenol and phthalate concentrations were measured in first trimester spot urine samples of pregnant women (median gestational age of 12.9 weeks) in the **Netherlands** from February 2004 and July 2005 (Philips *et al.*, 2018). Urine samples were deconjugated with β-glucuronidase before extraction using a liquid-liquid extraction method. Median urinary BPS concentration was 0.35 ng/mL with an interquartile range of 0.17 ng/mL and 1.03 ng/mL. 29.5% of the values were below the LOD (0.05 ng/mL).

- In the 'Esteban cross-sectional study', urine from 500 **French** children and 900 adults (age between 6 and 74 years) was monitored between April 2014 and March 2016 (Balicco *et al.*, 2019). BPS was detected in almost all samples (resp. 99.9% and 100% for 'total BPS' and 'free BPS') and quantified in resp. in 99.9% and 56.2% of the samples. The geometric mean of the 'total BPS' impregnation was 0.444 µg/L (0.362-0.545 µg/L) and 0.442 µg/g creatinine (0.359-0.544 µg/g creatinine). The geometric mean for free BPS was not calculated due to a censoring rate > 40 %. The impregnation was higher in children than in adults. Although the conclusion of the causality of such cross-linking study should be treated with caution, concentrations in children were found to increase with the consumption of pre-packaged fish and a less regular ventilation of the dwelling, while in adults the increase was found to be due to the consumption of pre-packaged foods. (See https://www.hbm4eu.eu/hbm4eu-substances/bisphenols/)

- Tkalec *et al.* (2021) studied the presence of selected bisphenols, parabens and triclosan in first morning void from 246 children (6-9 years) and adolescents (11-15 years) in a rural region in **Slovenia**. Glucuronide and sulfate metabolites were deconjugated using β -glucuronidase/arylsulfatase. As a result of this study BPS was found in 27% of children samples and in 35% of samples taken from the adolescents (geom means of 0.30 and 0.36 µg/L, respectively). The highest measured concentration of BPS was 23 µg/L in adolescents.

The results obtained indicated also that despite of the effort put in substitution of BPA, this substance was still found in the highest concentrations throughout the studied population (99% of kids and 100% of adolescents with respective geom means: 2.1 and 1.9 µg/L).

- From urine samples from 17 **French** cashiers and 15 controls (none occupationally exposed workers), it can be concluded that the general population is exposed to BPS and that frequent contact with thermal paper can be responsible for an increase of BPS in urine of cashiers. Indeed, geom mean concentrations of total BPS found in controls were 0.72 µg/L (0.52 µg/g creatinine) and 2.48 µg/L (2.12 µg/g creatinine) in cashiers (Ndaw *et al.*, 2018). Unconjugated BPS was detected between 0.1 to 3.0 µg/L in cashiers.

- Urine and blood from 29 adults working in a hazardous waste incinerator in **Spain** were examined for the presence of 8 bisphenols (BPA, BPS, BPF, BPB, BPAF, BPZ, BPE and BPAP). Besides BPA and traces from BPB, no other bisphenols were detected neither in the urine nor in the blood (González *et al.*, 2019).

- Frederiksen *et al.* (2020) investigated amongst others exposure to BPS by monitoring of 100 urine samples in 2009, 2013 and 2017. The urine samples were collected from Danish men with a mean age of 20 years (range 18 – 30 years) and were deconjugated before analysis The authors observed significant increase of the urinary median osmolality adjusted concentration of BPS over the study period. In 2009 following values were obtained: 0.11 ng/mL (median); 0.21 ng/mL(75 percentile); 0.76 ng/mL (95 percentile), 4.62 ng/mL(max.), while in 2017: 0.18 ng/mL (median); 0.39 ng/mL (75 percentile); 2.78 ng/mL (95 percentile), 36 ng/mL (max.)). Interestingly, the urinary median osmolality adjusted concentration of BPA significantly decreased with 57% in from 2009 to 2017.

- In an Austrian children survey, a total of 85 elementary school children aged 6-10 years (45 girls and 40 boys) were examined for the presence of 130 compounds, amongst others bisphenol S (Hartmann, 2021). The study demonstrated an increase of exposure to BPA alternatives like BPS. BPS was found in urine with concentrations up to 4.2 µg/L (4.5 µg/g creatinine).

Outside Europe:

- In a study conducted by Liao *et al.* (2012a), total BPS (free and conjugated) was detected in 81% of the urine samples (n=315) analysed at concentrations ranging from below the LOQ of 0.02 ng/mL to 21 ng/mL (mean: 0.654 ng/mL, geom. mean: 0.168 ng/mL; median: 0.191 ng/mL) by high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). Creatinine adjusted concentrations were 0.598 µg/g (mean), 0.176 µg/g (geom mean), and 0.200 µg/g Cre (median). The urinary BPS concentration varied among countries, and the highest geom mean concentration of BPS was found in urine samples from Japan (1.18 ng/mL, 0.933 µg/g Cre), followed by the United States (0.299 ng/mL, 0.304 µg/g Cre), China, Kuwait and Vietnam. The higher values of Japan and US are in line with their substitution of BPA by BPS. There were no significant differences in BPS concentrations between genders (male versus female), or among age groups (≤19, 20-29, 30-39, 40-49, and ≥50) or races (Caucasian versus Asian). Daily intake was estimated, for all sampled countries, to be 0.930 µg/d (mean) and 0.248 µg/d (median). Due to the small sample sizes from the individual countries, association of BPS concentration with demographic features are to be mitigated.

- In urine samples of Saudi Arabian people (n= 130), BPS was found to be the most abundant of all the bisphenols analysed (Asimakopoulos *et al.*, 2016). Mean concentrations of BPS were 13,3 ng/mL and were even higher than those of BPA (mean 5,71 ng/mL).

- The median concentration found in the urine samples of non-occupational exposed U.S. adults (Atlanta) between 2009 and 2012 was 0.13 ng/mL with a range between <LOD (0.03 ng/mL) and 12.3 ng/mL (Zhou *et al.*, 2014). 100 urine samples were analysed by high performance

liquid chromatography isotope dilution tandem mass spectrometry and BPS was found in 78% of them.

- Concentrations of free BPS ranging from <LOQ and 0.058 µg/g creatinine (0.022 ng/mL-geom. mean), and total BPS ranging from <LOQ to 7.046 µg/g creatinine (0.028 ng/mL, geom. mean) were detected in the urine of people from South China (n=94), aged between 26 and 84 years and living near a BPAF manufacturing plant (Yang *et al.*, 2014). Measurement was performed by using liquid chromatography coupled to mass spectrometry (LC–MS/MS). Free BPS was detected in 9.4% of the samples, while total BPS in 40.4%.

- Duan *et al.* (2018), collected spot urine samples between May 2016 to June 2017 from controls and type 2 diabetes mellitus cases (T2DM) in China. BPS had a detection rate of 58% in total (n= 502) and 47.8% and 68.1% for controls (n= 251) and T2DM (n= 251) resp. Creatinine-corrected concentrations of the urine samples resulted in median concentrations ranging from not detected to 0.248 µg/g creatinine in controls. A median concentration of 0.199 µg/g creatinine (ranging from not detected to 0.563 µg/g creatinine) was measured in T2DM cases. BPS concentrations were significantly associated with T2DM (log transformed and categorical statistical models).

- The presence of 6 bisphenols and 10 phtalates in urine samples of men before and after cardiac surgery was investigated by Shang *et al.* (2019). They collected urine samples from the Jewish General Hospital (Montreal, Canada) and subsequently exposed mouse model with the measured concentrations in those urine samples. The concentration of BPS measured in the urine sample before surgery ranged from 0.17-1.8 µg/gm creatinine (median 0.29 µg/g (creatinine); 12h post-surgery from 0.17-1.0 µg/gm creatinine (median 0.27 µg/gm creatinine) and 24h post-surgery from 0.16-0.28 µg/gm creatinine (median 0.19 µg/gm creatinine). No increase in BPS was found suggesting that BPS was not present in medical devices used in cardiac surgery in Canada as of May 2018.

- BPS concentrations were measured in urine from healthy 22–37-year-old participants (n=33) from Signapore and ranged < dectection limit (0.007 ng/mL) and 1.93 ng/mL (specific gravity adjusted), with a geom. mean of 0.077 ng/ml (Liu *et al.*, 2019a). The detection frequency of BPS was 94%. For BPS analysis, urine samples were deconjugated with β-glucuronidase before extraction using a liquid-liquid extraction method.

Children

- Urine was sampled from 40 Chinese school children (8-11 years of age) and analysed using ultra-high-performance liquid chromatography coupled with triple quadrupole tandem mass spectrometry contained 0.25 to 50 ng/mL of BPS (Yao *et al.*, 2018).

Cashiers and non-cashiers

- Urine was sampled during 2011 and 2013 from cashiers and non-cashiers from North Carolina (US) after handling of thermal BPA, BPS and BSIP receipts (Thayer *et al.*, 2016). Each receipt contained 1–2% by weight of the paper of BPA, BPS or BPSIPS. A significantly higher concentration of BPS was detected in the urine post-shift compared to pre-shift in cashiers handling BPS receipts.

Table 9: Total BPS in urine (2011-2013) as reported by Thayer *et al.* (2016)

Type of receipt	Total BPS Urine (2011-2013)		
	Cashiers		Non-cashiers (n=21)
	Pre-shift µg/g creatinine (geom mean)	Post-shift µg/g creatinine (geom mean)	µg/g creatinine (geom mean)
BPA receipts	0.31 (n=33)	0.25 (n=33)	NA
BPS receipts	0.23 (n=31)	0.54*2 (n=31)	NA
BPSIP*1 receipts	0.38 (n=12)	0.28 (n=12)	NA
BPS in urine			0.41

*1BPSIP: 4-hydroxyphenyl 4-isoprooxyphenylsulfone, also called D-8
*2 $p < 0.001$, significant difference between pre-shift and post-shift

Pregnant women/first-time mothers

BPS was measured in the urine of **pregnant women** in Australia (Heffernan *et al.*, 2016), China (Wan *et al.,* 2018 and Zhang *et al.*, 2019), US (Ihde *et al.*, 2018) and in first-time mothers in the EU (Gyllenhammar *et al.*, 2017)

- For Europe, Gyllenhammar *et al.* (2017), examined the urine concentrations of BPS in first-time mothers (n=178) in **Sweden** (Uppsala) from 2009 to 2014. Ammonium acetate and glucuronidase wass added before the analysis. Lower concentrations of BPS (0.11 ng/L, mean value, density adjusted) were detected in comparison to what was found by Yang *et al.* (2014), and Zhou *et al.* (2014), in China and the U.S. resp. Also, no statistically significant temporal trend was observed for BPS, but such trends could be missed when single spot urine sample are taken and a relative short period is examined.

- In Australia, Heffernan *et al.* (2016) detected BPS in 10% of the urine samples from pregnant women in a concentration range <LOR - 8.1 ng/mL using automated online SPE-LC-QTRAP-MS/MS method. BPS was found at trace levels of 0.09 ng/mL in the average blank (n=4). The limit of reporting (LOR) was calculated as 10 times the signal-to-noise in low-level spiked synthetic urine or three times the average blank for compounds presenting procedural blanks – whichever gave the higher value. LOD of BPS was determined to be 0.067 ng/mL.

- BPS was found in a concentration of 0.17 µg/L (geom. mean, specific gravity adjusted) in urine of pregnant Chinese women at admission to labour (Wan *et al.*, 2018). The substance was detected in 93.7% of the spot urine samples (n=985). An increase of BPS in the urine with 1 ln-unit caused an increase of the pregnancy duration with 0.72d. Furthermore, when stratified for foetal sex, a significant correlation was found between a ln-unit increase of BPS in maternal urine and increased gestational age and increased odds of later term birth. Although not statistically significant, a trend was seen between the maternal urinary BPS concentration and decreasing birth weight in girls. No effect was seen on birth length.

- A novel mass spectrometric (MS) method was run by Ihde *et al.* (2018), on 30 paired maternal urine and foetal cord blood samples from mothers (New Jersey, USA) undergoing an elective Caesarean section. 60% of the mothers were tested positive for BPS. The median and mean concentration was 0.04 and 0.19 ng/mL resp. (range: 0.04-8.883 ng/mL).

- Zhang *et al.* (2020) examined concentrations of BPS in urine, serum and amniotic fluid samples collected from April 2017 to August 2017 from pregnant women originating from an e-waste dismantling area in South China. BPS was found with a detection rate of 100% in maternal urine with a geom. mean concentration of 0.05 ng/mL.

- Machtinger *et al.* (2018) investigated urine of 50 pregnant women (mean patient age: 34.4 ± 6.2 years; mean delivery week: 38 ± 1.1) in Israel in order to characterise exposure to selected phthalates, bisphenols and other chemicals in personal care products and to evaluate

associations between prenatal exposure to endocrine disruptors. The samples were collected on the same day or the day before scheduled elective cesarean section or upon admission to the delivery room. Additionally, questionnaires regarding patients' consumer habits during pregnancy were filled in. The study indicated that the BPS could have been found in 27% of the samples with a specific gravity adjusted concentration of 0.4 µg/L (90th percentile) and 0.7 µg/L (95thpercentile).

2. Blood serum

In a study conducted by Gély *et al.* (2021), the cord blood was collected in 44 pregnant women in **France** between June 2014 and October 2015. BPS-G (glucuronide) was determined in almost half of the cord plasma samples with concentration ranges nd-0.586 ng/mL.

Thayer *et al.* (2016) examined the concentration of BPS in blood serum and more particular in cashiers (pre- and post-shift) from North Carolina handling thermal paper.

Table 10: Total BPS in blood serum (2011-2013) as reported by Thayer *et al*. (2016)

Type of receipt	Total BPS in serum (2011-2013)	
	Cashiers	
	Pre-shift (number >LOD)	Post-shift (number >LOD)
BPA receipts	9/33 (27.3%)	5/33 (15.2%)
BPS receipts	5/32 (15.6%)	13/32 (40.6%)*[2]
BPSIP*[1] receipts	2/12 (16.7%)	1/12 (8.3%)

*[1]BPSIP: 4-hydroxyphenyl 4-isoprooxyphenylsulfone, also called D-8
*[2]p = 0.02, significant difference between pre-shift and post-shift.

Serum LOD BPS: 0.002–0.01 ng/mL.

BPS was found in human plasma from Chinese adults with a mean concentration of 0.15 ng/mL (Jin *et al.*, 2018). Mass fractions in plasma (mean of 0.78) indicate a strong partitioning of BPS to the plasma fraction.

Ihde *et al.* (2018), did not find BPS in foetal cord blood samples from US mothers undergoing an elective Caesarean section.

Blood samples collected from a hospital in China were analysed for BPA, BPF, BPS, BPB, BPAF, BPAP and BPZ by Tan *et al.* (2019) using pre-column derivatisation with high-performance liquid chromatography and tandem mass spectrometry. BPS had a detection frequency of 25%. Measured geom mean concentration was 0.06 ng/ml (<LOD~0.79 ng/mL).

In the study of Zhang *et al.* (2020) concentrations of BPS maternal serum and cord serum from pregnant women originating from an e-waste dismantling area in South China were respectively 0.01 ng/mL and 0.03 ng/mL. The detection rate of BPS was 30% in maternal serum and 63% in cord serum.

BPS was one of the most frequently detected of 10 bisphenols identified in serum of 181 pregnant women from China, with a detection rate of 72.4% and a median concentration of 0.113 ng/mL (Li *et al.*, 2020).

3. Other fluids

Europe:

Van Overmeire *et al.* (2019) detected levels of BPS in human placenta samples ranging from 0.8 to 1.3 ng/g. 71 samples were collected in 2014-2015 in the Hospital Oost-Limburg in Genk (**Belgium**) and analysed by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method.

Amar *et al*. (2020) detected levels of BPS glucuronide in follicular fluid samples collected from 59 **French** women who underwent *in vitro* fertilisation procedure. The concentration ranged from 0.5 to 12.6 nM, with an average of 4.4 ± 1.4 nM (1.9 ± 0.6 ng/mL). BPS glucuronide was detected in 11 of the 59 samples.

Banaderakhshan *et al.* (2022) examined the migration of BPS from reusable plastic bottles into artificial saliva by using HPLC-MS/MS. Bottles from five different brands were collected in Austrian stores during the summer of 2020. Depending on the type of bottle, BPS was detected in the saliva in concentrations between <LOQ (0.001 µg/L,) and 0.070 µg/L.

Outside Europe:

The detection rate of BPS in amniotic fluid samples from pregnant women originating from an e-waste dismantling area in South China was 67%, with a geom. mean concentration of 0.02 ng/mL (Zhang *et al.*, 2020).

3.2.3.2.4 Environmental occurrence

Occurrence in the environment

Data on the environmental occurrence of BPS is mostly available from the US and Asian countries (China, Japan, Korea, India). Data for Europe are scarce. Furthermore, due to the difference in production, uses and sources of discharge in the different countries, different concentrations and detection frequencies of BPS are observed.

- **Occurrence in indoor dust/air**

See section 3.2.3.2.2

- **Surface water**

In **Europe**, BPS was found in the river Meuse a few times a year at a concentration up to 3 µg/L (Kienhuis and Geerdink, 2000).

Several results are available for the Taihu lake (China). Jin and Zhu (2016) reported a mean concentration of BPS of 6.0 ng/L in samples of 2013, which is comparable to those found by Liu *et al.* (2017), in samples from 2016 (6.4 ng/L). Concentrations in Wang *et al.* (2017b), from samplings from 2015, were much higher with mean concentration of 27.6 ng/L. This is probably due to the inclusion of measurement of BPS in the SPM (suspended particulate matter). Mean contribution of the SPM-bound bisphenols to the total water concentrations was in the range of 2.52–53.6%. The detection frequency of BPS in those 3 studies was 100%.

From samples taken in 2013-2014, Yamazaki *et al.* (2015), reported concentrations of BPS up to 42 ng/L in Korean and 135 ng/L in Chinese rivers. They detected extremely high concentrations in Indian rivers (ND-6840 ng/L). Furthermore, river samples from 2016-2017 collected in South China contained up to 65.6 µg/L of BPS (Huang *et al.*, 2018).

In seawater from Tokyo Bay (Japan), concentrations of BPS were in the range of ND to 15 ng/L (mean 8.5 ng/L) (Yamazaki *et al.*, 2015).

Seawater samples were collected from the Pearl River Estuary in South China by Zhao *et al.* (2019). All samples contained BPS (100% detection frequency). The median concentration in the aqueous phase was 10.3 ng/L (1.60-59.8 ng/L), 1.6 ng/L in suspended particle matter (3.30-343 ng/L) and 12.3 ng/L in total aquatic phase (3.14-121 ng/L).

Zhao *et al*. (2021) evaluated the occurrence of Bisphenols in marine organisms (13 species; n = 74), as well as in seawater (n = 15) from the East China Sea. BPS concentration in seawater was 3.7 ± 2.8 ng/L (mean).

- **Sediment**

Liao *et al.* (2012d) found ranges of BPS from non-detectable to 1970 ng/g. An increase in the concentration of BPS was observed in sediments collected in USA, Japan and Korea from 2000 to 2012 probably due to the increased concentrations in wastewater treatment discharges and land-applied biosolids (Choi and Lee 2017b). However, Huang *et al.* (2018), recorded lower BPS concentrations in Chinese river sediment samples collected between 2016 and 2017: up to 45.4 ng/g (mean 7.25 ng/g, 100% detection frequency).

- **Sludge**

BPS was identified in 12 out of 15 sludge samples in China with an EEQ (estradiol equivalence quantities) between 0.00013 and 0.12 pg E/g dw (Ruan *et al.*, 2015).

In their review, Chen *et al.* (2016) reported median concentrations of BPS in sludge from waste water treatment plants in China, Korea and the US : 5.3 ng/g, 3.8 ng/g and 5.8 ng/g resp.

- **WWTP**

See section 3.1.2.3

- **Additional information**

Ruus *et al.* (2014), detected BPS in plankton (0.24-4.83 ng/g), bird eggs (not detected-44.2 ng/g), polychetes (0.06-2.35 ng/g), fish (<0.5-20.5 ng/g), prawns (1.34-2.87 ng/g) and mussels (<0.3-1.89 ng/g) of an urban fjord (Inner Oslofjord) in Norway in the frame of the "Environmental Contaminants in an Urban Fjord"-programme. However, in a follow-up study of 2016 and 2018, BPS was not detected in cod liver neither in blood and eggs from the herring gull resp. (Ruus *et al.*, 2017, and Ruus *et al.*, 2019, resp. However, in 2019 BPS was detected in one liver and 3 biles of cod (Liver: <1 – 1.52 ng/g w/w; Mean: 0.8 ng/g w/w; Bile: <1 – 1.58 ng/g w/w; Mean: 0.3 ng) (Ruus *et al.*, 2020).

3.2.4 Summary on occurrence and environmental distribution

Several literature studies demonstrate that BPS can be found worldwide and that it is ubiquitous in several environmental compartments (water, sediment, sewage sludge, indoor dust/air). Furthermore, BPS was found in biota of remote areas (see Section 3.3).

Besides indirect exposure via the environment, humans are exposed via several consumer products (food contact material, paper products, personal care products, leather and textile products). Furthermore, biomonitoring data demonstrate the presence of BPS in human urine and blood serum.

It can be concluded that BPS reaches diverse environmental compartments and biota of remote areas. Therefore, many environmental species and humans can be exposed more or less continuously to BPS and exposure can thus not be avoided.

3.3 Data indicating potential for long-range transport

BPS is found in the Arctic (Kongsfjorden, Svalbard) in detectable concentrations (<0.3-1.1 ng/g ww) in seabird eggs of black-legged kittiwake (n=5) and glaucous gull (n=5) and in artic char muscle (n=10) at concentrations between <0.3-1.3 ng/g ww (Lucia *et al.*, 2016).

3.4 Bioaccumulation

3.4.1 Bioaccumulation in aquatic organisms (pelagic & sediment organisms)

The log K_{ow} is determined by HPLC method according to OECD TG 117: log K_{ow} = 1.2, at 23 °C and pH 6.2. This value is supported by a calculated Log K_{ow} (KOWWIN v1.67) and reviewed database: 1.65 at 25 °C.

The bioaccumulation of the test substance was determined in *Cyprinus carpio* under flow through conditions following a method similar or equal to OECD TG 305C (Registration dossier: Unpublished study report, 1998). The study was run with at a concentration of 50 µg/l and 500 µg/l over a period of 6 weeks. The BCF for the test substance was measured to be very low: resp <2.2 and <0.2.

Wang *et al.* (2017b) examined the bioaccumulation and biomagnification of 9 bisphenols in aquatic organisms from Taihu Lake in China. BPS was found in all water samples and in 70.6% of the aquatic organisms (plankton, invertebrates and fish). Log BAF were between -1.70 and 0.650 in the aquatic organisms.

Wang *et al.* (2020a) determined BCFs in different tissues of *cyprinus carpio* after exposure to 8 bisphenols, including BPS. Both free and total forms of BPS were measured. The conjugated form was calculated based on the free and total forms. BPS free in the whole body was 20.9%, meaning that 79.1% was in the conjugated form.

BPS	Form	BCF (L/kg)
Blood	Free	0.2 ± 0.1
	Total	1.3 ± 0.3
Kidney	Free	0.2 ± 0.0
	Total	0.8 ± 0.1
Liver	Free	0.2 ± 0.0
	Total	0.3 ± 0.1
Muscle	Free	0.1 ± 0.0
	Total	0.1 ± 0.1
Whole body	Free	0.1 ± 0.0
	Total	0.3 ± 0.0

Extracted from Wang *et al.* (2020a)

Bioaccumulation of the test substance in aquatic organisms is not expected.

However, in the study of Li *et al.* (2021), a BCF was determined for the typical freshwater algae *Navicula sp*. At environmental-related concentration (0.5 mg/l), BCF in *Navicula sp.* increased sharply at 72h (157.83 ± 54.96 at 24 h to 2052.94 ± 732.13 at 72 h) but decreased to 72.22 ± 15.91 at 120 h. This BCF-variation in time may indicate a risk, after short exposure to BPS, for aquatic organisms predating on this species.

Bioaccumulation in *Chlorella vulgaris* was lower than in *Navicula sp* (Li *et al.*, 2021).

3.4.2 Bioaccumulation in terrestrial organisms (soil dwelling organisms, vertebrates)

No experimental studies are available.

The estimated log K_{oa}, using the experimental log K_{ow} of 1.2 and estimated Henry's Law Constant of 2.7 $x10^{-15}$ atm-m^3/mole (2.73 $x10^{-10}$ Pa-m^3/mole) was 14.157 (Epiwin, AEROWIN v1.00; eMSCA, 2018).

Although a high log K_{oa} was estimated, in combination with a log K_{ow} <2, this indicates that **BPS has a low potential to biomagnify in air-breathing (terrestrial) organisms.**

3.4.3 Field data

As already mentioned in Section 3.3, BPS is found in remote areas like the Artic in detectable concentrations (<0.3-1.1 ng/g ww) in seabirds eggs of black-legged kittiwake and glaucous gull and in artic char muscle (n=10) at concentrations between <0.3-1.3 ng/g ww (Lucia *et al.*, 2016). Furthermore, BPS was detected in several organisms in urban fjord (Inner Oslofjord) in Norway by Ruus *et al.* (2014). BPS was found in plankton (0.24-4.83 ng/g), bird eggs (ND-44.2 ng/g), polychetes (0.06-2.35 ng/g), fish (<0.5-20.5 ng/g), prawns (1.34-2.87 ng/g) and mussels (<0.3-1.89 ng/g). However, in a follow-up study of 2016 and 2018, BPS was not detected in cod liver neither in blood and eggs from the herring gull resp. (Ruus *et al.*, 2017, and Ruus *et al.*, 2019, resp.).

The presence of 10 bisphenols, of which BPS, in the northern pike (*Esox lucius*) were analysed by Tian *et al*. (2019). Fish were collected in late May to early June 2014 and 2015 from the St. Lawrence River, Canada, 4 km upstream (n =12) and 4 km downstream (n = 14) of the point of discharge of a major primary WWTP. BPS was not detected in the muscle tissues.

Zhu *et al.* (2019), determined the concentrations of 45 substances in urine samples of various bovine breeds. 183 samples were collected in rural and agricultural areas (without point sources in the vicinity) in China, India and US between March and November 2018. Bovines from China were housed permanently in shelters and fed with commercial food while those from India and US were allowed to graze in open pastured/grassland and fed with a combination of grain and grass. The detection frequency of BPS for the urine was 77%, 82% and 100% resp. with a median concentrations resp. <LOQ (ND-3.7 ng/mL), <LOQ (ND-4.0 ng/mL) and 0.40 ng/mL (<LOQ-1.7 ng/mL). LOQs for the 8 measured bisphenols (BPA, BPAF, BPAP, BPS, BPF, BPP, BPZ and BPB) was between 0.12 and 1.2 ng/mL.

Liao and Kannan (2019), collected 11 mollusks species between 2006 and 2015 from coastal areas of five cities located along the Bohai Sea (China). They determined the concentrations of 8 bisphenols and 5 benzophenones in 186 samples. BPS was detected in <5% of the samples. Concentrations of BPS ranged between not detected and 4.68 ng/g dw, with a geom. mean and median value of 0.146 and 0.141 ng/g dw resp.

Wild-caught marine organisms were gathered from fisherman in the Pearl River Estuary in South China and comprised shellfish (n=11) and fish (n=10) (Zhao *et al.*, 2019). Concentration of BPS in the marine organisms ranged between not detected and 328 ng/g, with a median concentration of 1.28 ng/g.

Zhao *et al.* (2021) evaluated the occurrence of Bisphenols in marine organisms (13 species; n = 74), as well as seawater (n = 15), from East China Sea. In marine organisms (without hydrolysis), BPA and BPS were the predominant bisphenols with concentrations of BPA of 3.8 ng/g mean (range 1.2−7.7 ng/g) and BPS of 1.5 ng/g mean (range: 0.19−6.1 ng/g). After enzymatic hydrolysis treatment, mean concentrations of BPS increased 1.8 times in marine organisms.

3.4.4 Summary and discussion of bioaccumulation

BPS has a log Kow of 1.2. The experimentally derived BCF in fish was determined to be <2.2. Therefore, no aquatic bioaccumulation is expected.

However, a sharp increase in BCF in *Navicula sp*. (freshwater algae) at 72h and environmental-related concentration (0.5 mg/L BPS) from 157.83 ± 54.96 at 24 h to 2052.94 (but BCF decreased to 72.22 ± 15.91 at 120 h.) may indicate a high risk for aquatic predators of this species (Li *et al.*, 2021).

There is no indication of bioaccumulation in air-breathing organism, although the estimated Log Koa is high (>5), the estimated log Kow was <2.

3.5 Summary and discussion of environmental fate properties

BPS is hydrolytically stable, not readily biodegradable and shows low degradation in river water and seawater under aerobic conditions. Therefore, BPS might accumulate and remain in the aquatic environment for a long time under relevant environmental conditions.

Primary degradation in soil was quick with reported DT50 between 0.0066 and 2.8 days.

Although, once released to air a relatively fast photodegradation is estimated (DT50 of 26.5h, EPIWIN SRC AOP v1.92), it is not expected to be a relevant degradation pathway seen the low Henry's law constant (2.73 x10^{-10} Pa*m^3/mole at 25 °C) of BPS.

Phototransformation in water is rather quick with a DT50 of 43.1 min. Although photolysis in water is rapid (DT50: 43.1 min), it cannot be excluded that BPS might accumulate in deeper water layers and remain in the aquatic environment for a long time. p-Hydroxybenzenesulfonic acid was identified as the major phototransformation product in water.

It can be concluded that BPS is not rapidly degraded under relevant environmental conditions.

With an experimental log Kow of 1.2 and BCF <2.2 in fish, BPS is not expected to bioaccumulate in aquatic organisms (fish). However, high BCF (2052.94) was reported for the algae *Navicula sp.* at 72h after exposure to 0.5 mg/L of BPS.

Estimated log Koa of 14.157 in combination with a log Kow of 1.2, indicate low potential of terrestrial bioaccumulation. However, the presence of BPS in aquatic and terrestrial organisms (ng/g) was demonstrated in several biomonitoring studies.

4. Human health hazard assessment

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

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

4.2 Acute toxicity

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

4.3 Irritation

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

4.4 Corrosivity

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

4.5 Sensitisation

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

4.6 Repeated dose toxicity

See section 4.9 Toxicity for reproduction

4.7 Mutagenicity

Not relevant for this dossier.

4.8 Carcinogenicity

Not relevant for this dossier.

4.9 Toxicity for reproduction

Bisphenol S has a harmonised classification[9] as Repr. 1B – H360FD, on the basis of treatment-related adverse effects on fertility, reproduction and pregnancy outcome (i.e. decreased number of implantation sites and prolonged estrous cycle) and treatment-related adverse effects on development (i.e. post-implantation loss) in several independent studies in animals. The RAC opinion (RAC, 2020) can be found on: *https://echa.europa.eu/documents/10162/03fac9dc-94e7-a81c-fed5-1c5008a1c1bc*.

[9] Commission Delegated Regulation (EU) 2022/692 of 16 February 2022 amending, for the purposes of its adaptation to technical and scientific progress, Regulation (EC) No 1272/2008 of the European Parliament and of the Council on classification, labelling and packaging of substances and mixtures (Text with EEA relevance): http://data.europa.eu/eli/reg_del/2022/692/oj

In its opinion of 10 December 2020, RAC adopted the following conclusions in support of classification of BPS as Repr. 1B, H360FD:

- **Fertility :**

"*Bisphenol S was shown to consistently and severely disturb reproductive parameters. Overall, RAC observes the following:*

a) Exposure to bisphenol S consistently resulted in a decrease in mean number of implantation sites at 300 mg/kg bw/day;

b) Exposure to bisphenol S consistently resulted in a prolongation of the estrous cycle at 300 mg/kg bw/day, as well as in an irregular estrous cycle with a decreased pro-estrous stage and an increased diestrous phase from 20 mg/kg bw/day onwards;

c) Exposure to bisphenol S resulted in a decrease in the fertility index of 58% and 80% at 300 mg/kg bw/day.

RAC concludes that the adverse effects of bisphenol S on the mean number of implantation sites, the decrease in fertility index, and the effect on the estrous cycle warrant classification as Repr. 1B; H360F.

RAC would like to emphasise that the dosing regimen in the guideline studies is also taken into account in the overall weight of evidence assessment for classification.

The top dose of 180 mg/kg bw/day, used in the EOGRTS, is not supported by adequate argumentation, and its correctness is questionable, especially in light of the effects seen in repeated dose studies where doses up to 1000 mg/kg bw did not exert severe toxicity in females, while 600 mg/kg bw/day did result in clear but not in severe toxicity in males."

- **Development :**

"*The classification of a substance in Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. Overall, RAC observes the following:*

a) Post-implantation loss was increased in two studies, from 60 mg/kg bw/day onwards. Furthermore, a decrease in the mean number of pups delivered per dam was consistently observed in three studies from 180 mg/kg bw/day onwards. This effect is a result of both increased implantation loss and post-implantation loss.

b) A consistent pattern of increased pup weight was observed in both sexes (up to 14/18% in f/m at 300 mg/kg bw/day), that is attributed to gestational bisphenol S exposure.

c) And although marginal, some specific neuro- and immuno-developmental effects were noted in the OECD TG 443 study. RAC considers these effects insufficient for classification as Repr. 1B; H360D on their own, but they contribute to the overall concern for effects on the developing organism and therefore also contribute to the weight-of-evidence in support of this classification. For considerations regarding the dose selection, see Adverse effects on sexual function and fertility

RAC concludes that the adverse effect of bisphenol S on the post-implantation loss and the mean number of pups delivered per dam warrant classification as Repr. 1B; H360D. The effects observed are severe and are not resulting from maternal toxicity.

RAC notes the difficulty in determining whether the effect on the mean number of pups delivered per dam is related to fertility or development. However, the significant increase in post-implantation loss in two studies is a consistent and severe finding, which increased with treatment. The CLP Regulation states that classification in Category 2 is appropriate when "there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility, or on development, and where the evidence is not sufficiently convincing to place the substance in Category 1". RAC considers that the evidence for bisphenol S on post-implantation loss is a clear effect on development, and therefore does not consider classification in Category 2 appropriate."

4.10 Endocrine disruption (Human Health)

4.10.1 Strategy and information sources for endocrine disruptor identification

Strategy

The strategy to assess whether a substance meets the endocrine disruptor criteria is outlined in the ED guidance (EFSA/ECHA ED Guidance, 2018). Therefore the identification of BPS as an endocrine disruptor has taken account of the ED Guidance. In this respect, all available relevant scientific data have been considered in the ED assessment and a weight of evidence approach is applied according to the ED criteria.

BPS, as other bisphenols, was shown to interfere with different hormone pathways (i.a. estrogen, androgen, steroidogenesis, thyroid, PPARγ) and to trigger various adverse effects on human health and environment. However, the ED guidance (EFSA/ECHA ED Guidance, 2018) explains that: "*The potential of a substance to elicit more than one MoA can obviously lead to difficulties in the interpretation of assay data. If there are indications that a substance may act via multiple MoAs then the investigations should start with the MoA for which the most convincing evidence is available*". Therefore, for the assessment of BPS and for the purpose of this document, the focus is on the estrogen disrupting activities of BPS resulting in affected female reproduction, as this is the most relevant pathway.

Information sources

In order to identify all studies from the open literature relevant for the assessment of endocrine disrupting properties of BPS in relation to human health and environment, a literature search was performed in PubMed and via Google search using the following key words/terms: "bisphenol S", "BPS", "4,4'-sulphonyldiphenol", "EC 201-250-5", "CAS 80-09-1". The literature search was conducted until 30 August 2021 except when stated otherwise. For the environmental part and human biomonitoring, a few additional articles relevant for the SVHC identification have been included after this date.

The selection of the studies was not restricted to specific levels of doses. Both "low doses" as well as "standard doses" in the standardised test guidelines were considered as relevant for the identification of adverse effects and the understanding of the MoA. It is however recognised that the MoA may have a different pattern and modulations across the whole range of doses.

It should be noted that a thorough scientific literature search for *in silico* data was not performed because sufficient amount of *in vitro* data on estrogen modality are available.

Additionally, although not considered as relevant for the identification of an adverse effect, studies performed in non-intact animals, namely in ovariectomised animals (i.e. a mimicking model of the menopausal status) were included for the understanding of the MoA.

Additional search on literature available for BPS was performed based on the references provided in the Support Documents for identification of BPA and BPB as substance of very high concern according to Art. 57(c) and (f) and Art. 57(f) respectively. These were also scrutinised for BPS related data in using the search equation mentioned above.

In a first step, all abstracts were systematically scrutinised. Then pending on the relevance of the retrieved studies, the full paper was analysed and considered in this review. Expert judgement was also used to determine whether a study was likely to provide information of relevance for the ED assessment.

Study reports available in the REACH registration dossier were assessed. Additionally, full study reports either available in the registration dossier, or obtained from the Registrants in the course of the substance evaluation were analysed. The single concept search strategy as suggested in the EFSA/ECHA Guidance (EFSA/ECHA ED Guidance, 2018) was followed.

Considerations related to the relevance of data

A very high number of *in vitro* studies on BPS investigating its estrogenic potential have been published so far. These studies cover many different mechanistic activities, assessed in many different types of assay (binding, transactivation, cell proliferation…) and a very large majority of them demonstrates an estrogenic activity of BPS (see LoE EAS activity, table X1 and X2). Accordingly, based on the coherence and reproducibility of the mechanistic data on the estrogenicity of BPS, only the reliability of the *in vitro* test guideline and test guideline-like studies were assessed.

However, in order to provide further support and transparency to the robustness of the conclusions, the *in vivo* experimental studies that were considered in the weight of evidence approach, were assessed with ToxRtool. This assessment tool was developed by the European Commission's Joint Research Center in 2009 (Segal *et al.*, 2015). It builds on Klimisch categories by providing additional criteria and guidance for assessing the reliability of (eco)toxicological studies. ToxRTool is applicable to various types of experimental data, endpoints and studies (study reports, peer-reviewed publications). The most informative studies were given a reliability score of 1 (reliable without restriction) or 2 (reliable with restriction). Oral and subcutaneous routes were considered relevant routes of exposure in human health. This final scoring reflects the overall quality of the study itself and its context.

4.10.2 Lines of evidence (LoE)- EAS[10] modalities

Recent experimental studies investigating alterations of the estrous cyclicity, implantation and fertility have been collected until 31.12.2021 and epidemiological studies evaluating the associations between exposure to BPS and female reproduction (i.e. fertility, endometriosis and on polycystic ovary syndrome (PCOS)) have been gathered until 30 August 2021 to produce the detailed analysis presented hereafter.

4.10.2.1 LoE Adversity – EAS

Considering that the effects of BPS on the reproductive function have been presented and discussed in an extensive way at EU level (see RAC opinion (Committee for Risk Assessment, 2020) stated in 4.9 Toxicity to Reproduction section), it was decided not to further present nor discuss the whole database in the main part of the present dossier. A short summary of the effects of BPS on toxicity for reproduction is presented below mainly based on the literature collected for the classification and labelling dossier.

The most relevant effects have been observed on female reproduction. Indeed several adverse effects have been observed in rodent experimental studies conducted according to OECD guidelines and numerous literature publications: (see Annex I, table lines of evidence)

- BPS affects the estrous cycle in female rodents, as shown in OECD TGs 443 (F0 and F1B), 421 and 422 and other literature studies (eg Shi *et al.*, 2019a), due to longer diestrus phases and irregular cycles.

[10] EAS – estrogenic, androgenic and/or steroidogenic

- BPS reduces the number of implantation of oocytes, in a statistically significant manner in the OECD TG 422 study (Unpublished study report, 2017), and as non-statistically significant trends in OECD TGs 421 and 443 studies (Unpublished study report 2000 and 2019).

- As a consequence, the number of born pups was reduced in a statistically significant manner in some studies, including OECD TGs 443, 422 and 421, and fertility index was affected, among others in OECD TGs 421 and 422. Fertility index was not modified in OECD TG 443, however the highest tested dose was only 180 mg/kg bw/d (compared to 300 mg/kg bw/d in OECD TGs 421 and 422).

- Very few epidemiological studies have evaluated the associations between exposure to BPS and female reproduction. However, there are indications that BPS exposure could be associated with prolonged time to get pregnant (Philips *et al.*, 2018) or polycystic ovary syndrome (PCOS) (Jurewicz *et al.*, 2021). Moreover, these results should be considered in the light of existing knowledge on the structurally similar substance BPA toxicity for which estrous cycle disturbance (shorter luteal phase), implantation failures, decreased ovarian function and reserve (oocyte count, peak of E2, antral follicle count), and increased risk of endometriosis and PCOS, were observed in woman (ECHA, 2017; *Hu et al.*, 2018; Laws *et al.*, 2021).

Some other adverse effects have been observed on male reproductive system, but not consistently:

- BPS modified serum hormone levels in males, showing an increase of estradiol level and a decrease of testosterone (Shi *et al.*, 2018 and 2019c; Ullah *et al*., 2016, 2018a, 2018b and 2019; also supportive screening study Shi *et al.*, 2017).

- BPS disrupted the spermatogenesis in rodents (Shi *et al*., 2018 and 2019c; Ullah *et al*., 2016, 2018a, 2018b and 2019; also supportive screening study Shi *et al.*, 2017).

- BPS affected the sperm count and motility (Shi *et al*., 2018 and 2019c; Ullah *et al*., 2019; also supportive screening study Shi *et al*., 2017). However, these effects on sperm were not confirmed in the OECD TG 443 (Unpublished study report, 2019), in which only a slightly reduced sperm motility was observed in P0 only.

Finally, BPS exposure resulted in a higher incidence of mammary gland atrophy in males in all OECD TG studies (OECD TGs 443, 422 and 408). A literature study reported low-dose BPS effects (2 to 200 µg/kg/d) on male mammary gland development with larger epithelial trees (Kolla *et al.*, 2019). Overall in male rats, mammary gland effects were of depressive nature while increased development was observed in male mice, making difficult to predict the human effect to expect. Knowing that the mammary gland is a very sensitive marker of endocrine activity, this remarkable consistency should be considered, even if the long-term outcome of this effect is yet unknown.

For the purpose of demonstrating that BPS exerts its effects on reproduction through endocrine disruption and, for the sake of clarity considering the extent of the database, the analysis will focus on a more specific well-established effect and for which the ED MoA is the most plausible.

With this aim, the following sections will focus on the ability of BPS to affect the female reproductive system and how this alteration is exerted through hormonal changes.

See also Annex I (Lines of evidence for adverse effects and endocrine activity) for more detailed information.

4.10.2.1.1 Estrogenic - Mediated adverse effects:

Alteration of estrous cyclicity

Seven experimental studies investigated the effects of exposure to BPS on estrous cyclicity (Unpublished study report, 2000; Unpublished study report, 2019; Unpublished study report, 2017b; Ahsan *et al.*, 2018; Shi *et al.*, 2019a and b; Tucker *et al.*, 2018). Their results are reported in Table 11 and in Annex I.

Among these seven studies, five studies showed an adverse effect on the estrous cycle, including prolonged and irregular cycles. In rats, prolonged estrous cycles were consistently observed in Sprague-Dawley rats after adult, perinatal and post-natal exposure by oral route in OECD TGs 421, 422 and 443 (respectively Unpublished study report, 2019; Unpublished study report, 2017b and Unpublished study report, 2000). The effect of BPS on the estrous cycle has been acknowledged by RAC during the classification procedure of BPS as toxic for reproduction Cat. 1b (see section 4.10.5.1 Postulation of MoA(s) for the extract of the RAC opinion (Committee for Risk Assessment, 2020)).

The EOGRTS (Unpublished study report, 2019), following OECD TG 443, revealed a statistically significantly higher mean duration of the estrous cycle in the parental generation (4.1* days at 180 mg/kg bw/d compared to 3.9 days in control group). A dose-dependent increase of the total time spent in diestrus over a period of 21 days preceding mating was evidenced in the F0 generation (6.3, 7.4, 7.7 and 9.0** days, respectively at 0, 20, 60 and 180 mg/kg bw/d, these means were calculated by the DS). In the F1B generation, slight but non significant increased mean duration of estrous cycle was also observed at the highest dose (4.1 days at 180 mg/kg bw/d *vs* 3.9 days in control group). However, the mean number of days spent in diestrus stage, over a period of 21 days, was also strongly and significantly increased (6.8, 8.4*, 9.2** and 11.8** days at 0, 20, 60 and 180 mg/kg bw/d, respectively; these means were calculated by the DS).

A reproductive toxicity study (Unpublished study report, 2000), performed according to OECD TG 421, showed a prolongation of the mean estrous cycle (5.57** at 300 mg/kg bw/d vs 4.08 days in control group). Five females out of 12 showed a prolonged diestrus period.

A range-finding study preceding the EOGRTS (Unpublished study report, 2017b), similar to OECD TG 422, revealed an increase of the mean duration of the estrous cycle which was of 5.16** days at the highest tested dose only (300 mg/kg bw/d) vs 4.02 days in control group.

As regard literature studies, disturbed estrous cycle was observed in three out of four studies. An increased occurrence of irregular cycles was observed after post-natal exposure in rats (Ahsan *et al.*, 2018), and in mice after gestational exposure (Shi *et al.*, 2019a) and in subsequent generations (Shi *et al.*, 2019b).

- Shi *et al.* (2019a) study assessed the effect of prenatal (GD 11 to birth via oral route) exposure to BPA, BPE and BPS on the reproductive function of female CD-1 mice. Irregular estrous cycles with longer estrus and diestrus phases were observed at all doses (0.5, 20 or 50 µg/kg bw/d of BPS) in the F1 generation.

- The same author reported an increased incidence of irregular estrous cycle with no impact on the % of days spent in each phase on F3 offspring issued from F0 animals exposed from GD 7 to birth at either 0.5 or 50 µg/kg/d (Shi *et al.*, 2019b).

- Ahsan *et al. (*2018) study examined the development of female reproductive system in rats after neonatal (PND 1 to PND 10) BPS exposure at doses of 0, 0.5, 5 and 50 mg/kg bw/d via subcutaneous route. Qualitative observations showed profound disturbancies of the cycle pattern.

- No changes in the number of estrous cycles were observed by Tucker *et al. (*2018), with the shortest period of exposure (GD 10.5 to GD 17.5) and higher doses.

Cyclicity is essential to reach successful ovulation. An alteration of cyclicity may therefore result in at least subfertility through disturbed (delayed or absent) ovulation. The hormonal regulation of the cycle also influences the maturation process of the oocytes and ovarian follicles.

This effect on fertility was recognised by RAC in its opinion in support of classification of BPS as Repr. 1B – H360F.

Table 11: Summary table of studies investigating the effects of BPS on the estrous cycle in animals

Reference	Species	Routes	Dose Exposure period	Age at observation	Effect on estrous cycle
Gestational exposure					
Shi *et al.*, 2019a	Mice (CD-1)	Oral (via pipette)	0, 0.5, 20 and 50 µg/kg bw/d (pipette tip containing selected dose in tocopherol-stripped corn oil). GD 11 to birth	Peripubertal	Irregular estrous cycles with longer estrus and diestrus phases at all doses.
Tucker *et al.*, 2018	Mice (CD-1)	Oral (gavage)	0, 0.05, 0.5 and 5 mg/kg (in sesame oil) twice a day from GD 10.5 to GD 17.5	PND 63-83	No significative difference on the number of estrous cycles.
Postnatal exposure					
Ahsan *et al.*, 2018	Rat (strain not specified)	Sub-cutaneous	0, 0.5, 5 and 50 mg/kg bw/d (castor oil). PND 1 to 10	From PND 60 to 70	Estrous cycle monitoring: Altered estrous cycle reported (irregular pattern).
Adult exposure					
OECD TG 421 Unpublished study report, 2000	Rat (SD)	Oral (gavage)	0, 10, 60 and 300 mg/kg bw/d Total of 40 to 46 d for females (from 14d premating to PND 3) 0.5% aqueous sodium CMC solution with 0.1 % Tween 80		Prolonged estrous cycle (4.08, 4.01, 4.14 and 5,57** days) ↗ nb of animals with longer diestrus (0, 0, 1 and 5*/12 females)
OECD TG 422 Unpublished study report, 2017b	Rat (SD)	Oral (gavage)	0, 30, 100 and 300 mg/kg bw/d (CMC) Males: 10w Females: From pre-mating until PND 21		Prolonged estrous cycle (4.2, 3.97, 4.01 and 5.16** days)
Multi-generation studies					
OECD TG 443 Unpublished study	Rat (SD)	Oral (via drinking water)	0, 20, 60 and 180 mg/kg bw/d in a 0.5% CMC suspension.		Statistically significant prolonged estrous cycle in P0 (3.9, 3.9, 3.9 and

report, 2019			P0: from 10w before mating to PND 21 F1A: for 13w F1B: to PND 21 F2A: for 11w F2B: for 3w F3: for 8w		4.1* days) andslightly increased in F1B (3.9, 4.0, 4.0 and 4.5 [11]days) No effect on F1A ↗ mean nb of days in diestrus stage in P0 (6.3, 7.4, 7.7 and 9.0 days**) and F1B (6.8, 8.4**, 9.2** and 11.2** days). Slight effect in F1A (4.5, 4.6, 4.8 and 5.4 days). Stat. sign. ↘ mean days of proestrus
Shi *et al.*, 2019b	Mice (CD-1)	Oral (pipette)	0, 0.5 and 50 µg/kg bw/d in tocopherol-stripped corn oil from GD 7 to PND 0 of F0 generation only.	Evaluation on F3 generation: Once vaginal opening occurred, estrous cyclicity was evaluated by examining vaginal smears daily for 30 days.	Irregular estrous cycle with several days in estrus, metestrus, or diestrus. Percentage of days in proestrus: No clear dose-response relationship with BPS.

4.10.2.1.2 Adverse effects 'sensitive to, but not diagnostic of, EATS':

Decrease of implantation sites:

Different experimental studies reported that female rodents exposed to BPS show a **reduction of the implantation sites** either in a statistically significant manner at the highest dose tested. Furthermore a trend was seen at lower doses in these studies (Unpublished study report, 2000; Unpublished study report, 2017b) and in the EOGRTS (Unpublished study report, 2019). The moderate decrease of the implantation sites number observed in this latter study might have been prevented by the choice of the highest tested dose levels (180 mg/kg bw/d) which was much lower than those used in Unpublished study reports 2017b and 2000 namely 300 mg/kg bw/d.

- The reproduction/developmental toxicity screening test (OECD TG 421; Unpublished study report, 2000) demonstrated that the mean number of implantation sites was severely reduced in females exposed to 300 mg/kg bw/d (10.7 compared to 15.9 in control group). The implantation index at this dose was of 64.89**% compared to 95.80% in control.

- The same trend was observed in the 28-day range-finding study preceding the EOGRTS (Unpublished study report, 2017b). In this study, the mean number of implantation sites was significantly lower at the highest dose (10.4** at 300 mg/kg bw/d vs 15.8 in control

[11] RAC (2020): *Note that this specific effect was initially mentioned to be 4.5 days at the highest dose tested in the CLH dossier and on IUCLID, but during the PC was noted that this effect size was incorrect and should be changed into 4.1 days. The information in IUCLID was updated to this regard as well (ECHA dissemination website consulted on 09-09-2020).*

group).

- In the EOGRTS (OECD TG 443; Unpublished study report, 2019), the mean number of implantation sites was moderately modified in the parental generation at the highest dose (14.3 compared to 15.3 in control group) and in the cohort 1B also at the highest dose (13.7 at 180 mg/kg bw/d vs 15.2 in control group). It is noteworthy that this dose was much lower than the effective ones in Unpublished study report, 2017b and 2000, studies.

Experimental studies investigating the effects of exposure to BPS on the decrease in the number of implantation sites are summarised in Table 12.

This effect on fertility was recognised by RAC in its opinion in support of classification of BPS as Repr. 1B – H360F.

Table 12: Summary table of studies showing a reduced implantation site number

Reference	Species	Routes	Dose Exposure period	Decrease in the number of implantation sites
OECD TG 443 Unpublished study report, 2019	Rat (SD)	Oral Drinking water	0, 20, 60 and 180 mg/kg bw/d (0.5% CMC) P0: from 10w before mating to PND 21 F1A: for 13w F1B: to PND 21 F2A: for 11w F2B: for 3w F3: for 8w	In the parental generation, the mean number of implantation sites was affected (15.3, 14.8, 14.9 and 14.3, resp at 0, 20, 60 and 180 mg/kg bw/d) Mean number of implantation sites was moderately modified in the cohort 1B at the highest dose (13.7 compared to 15.2 in control group).
OECD TG 421 Unpublished study report, 2000	Rat (SD)	Oral (gavage)	0, 10, 60 and 300 mg/kg bw/d (0.5 % aqueous sodium CMC solution with 0.1 % Tween 80) Total of 40 to 46d for females (from 14d premating to PND3)	Sign. ↘ at the highest dose (95.8, 80.84, 86.15 and 64.89** %)
OECD TG 422 Unpublished study report, 2017b	Rat (SD)	Oral	0, 30, 100 and 300 mg/kg bw/d (CMC) Males: 10w Females: From pre-mating until PND21	Mean nb of implantation sites sign. ↘ at the highest dose (15.8, 15.0, 15.5 and 10.4 **)

Decreased mean number of pups (litter size)

A lower mean number of pups delivered was observed in the reproductive toxicity study (Unpublished study report, 2000), as it was of 9.1 at 300 mg/kg bw/d compared to 14.2 in control group.

In the EOGRTS (Unpublished study report, 2019), exposure to BPS resulted in reduced mean number of pups delivered in parental cohort (12.7 pups after exposure to 180 mg/kg bw/d vs 14.9 pups in control group). The mean number of F2 pups delivered per F1B dam was statistically lower than the concurrent control values in the high-dose group (14.3, 13.8, 14.9 and 11.4** pups/dam, in control, 20, 60 and 180 mg/kg bw/d groups, respectively). Interestingly, a significant greater reduction of pups was also observed in the range-finding study preceding the EOGRTS (Unpublished study report, 2017b): in this study, the mean number of pups was of

10.8* after exposure to 300 mg/kg bw/d compared to 15.2 pups in control animals. Taken together, these two studies support a dose-dependent effect of BPS on the litter size.

Literature studies at much lower doses also provide evidence for an adverse effect of BPS on litter size. In Shi *et al. (*2019a), exposure of CD-1 mice to BPS from GD 11 to birth was associated to a decrease on the litter size at 9 months of age. Ahsan *et al.*, 2018 studied the impact of a short post-natal exposure (from PND 1-10) on F0 females rat to their reproductive performance. A marked decrease in the F1 litter size was retrieved at 50 mg/kg bw/d (5.33** compared to 8.80 in control group). In Shi *et al. (*2017), when treated mice were mated with untreated animals, no effect on the number of pups delivered was observed. In Shi *et al. (*2019b), after an exposure of the F1 generation in utero, the mean number of F4 pups delivered was not significantly affected.

Experimental studies investigating the effects of exposure to BPS on the number of pups are summarised in Table 13.

This effect on the number of pups was recognised by RAC in its opinion (RAC, 2020) in support of classification of BPS as Repr. 1B – H360F.

Table 13: Summary table of studies showing a reduced number of pups

Referen-ce	Species	Routes	Dose Exposure period	Number of pups delivered
Gestational exposure				
OECD TG 414 Unpublished study report, 2014	Rat (Wistar)	Oral (gavage)	0, 30, 100 and 300 mg/kg bw/d GD 6 – 19	Slightly ↘ mean nb of live foetuses (10.1 at the highest dose vs 10.6 in all other groups)
Gestational exposure and its impact on fertility at adulthood				
Shi *et al.*, 2019a	Mice (CD-1)	Oral	0, 0.5, 20 and 50 µg/kg bw/d GD 11 to birth	Trend decrease of nb of F2 pups in - 3 months old mice : 14.3, 12.0, 12.8 and 11.0 - 6 months old mice : 9.8, 7.8, 7.0 and 12.3 - 9 months old mice : 7.3, 2.0, 1.3* and 2.0
Postnatal exposure and its impact on fertility at adulthood				
Ahsan *et al.*, 2018	Rat (strain not specified)	Sub-cutaneous	0, 0.5, 5 and 50 mg/kg bw/d PND 1 to 10	Sign. ↘ of nb of pups born per female mated with untreated males (8.80, 8.80, 8.60 and 5.33**).
Shi *et al.*, 2017	Mice (CD-1)	Sub-cutaneous	0, 50 µg or 10 mg/kg bw From birth to PND 60	No effect on pups delivered when mated with non-treated females or males respectively (no data available)
Multi-generation studies				
OECD TG 421 Unpublished study report, 2000	Rat (SD)	Oral	0, 10, 60 and 300 mg/kg bw/d (in 0.5 % aqueous sodium CMC solution with 0.1 % Tween 80) Total of 40 to 46 d for females (from 14 d premating to PND 3)	↘ mean nb of pups delivered (14.2, 12.5, 13.5 and 9.1 (The highest dose was reported as not significant, no individual data available by DS))

OECD TG 422 Unpublished study report, 2017b	Rat (SD)	Oral	0, 30, 100 and 300 mg/kg bw/d (CMC) Males: 10 w Females: From pre-mating until PND 21	↘ Mean nb of pups delivered (15.2, 14.1, 14.5 and 10.8**)
OECD TG 443 Unpublished study report, 2019	Rat (SD)	Oral Drinking water	0, 20, 60 and 180 mg/kg bw/d (in 0.5% CMC) P0: from 10 w before mating to PND 21 F1B: to PND 21	P0 : ↘ Mean nb of pups delivered (14.9, 14.0, 13.5 and 12.7) F1B : ↘ Mean nb of pups delivered (14.3, 13.8, 14.9 and 11.4**)
Trans-generational studies				
Shi *et al.*, 2019b	Mice (CD-1)	Oral (pipette)	0, 0.5 and 50 µg/kg bw/d in tocopherol-stripped corn oil from GD 7 to PND 0 of F0 generation only.	No sign differences in F4 generation observed at any ages

Reduction of fertility index

Experimental studies demonstrated a **reduction of the fertility index** after exposure to BPS.

The reproductive toxicity study (Unpublished study report, 2000), OECD TG 421, revealed a severe decrease of the fertility index which was of 58.3% at 300 mg/kg bw/d compared to 91.7% in control group. In this study, 4 mice of the highest dose (out of 12) did not conceive at all.

This result was confirmed in the range-finding study preceding the EOGRTS (Unpublished study report, 2017b). At the highest dose (300 mg/kg bw/d), the fertility index was 60% while it was 100% in control group.

Few literature studies confirmed the reduction of the fertility index. In rats, Ahsan *et al. (*2018) revealed a lower fertility index at the highest dose (60% after exposure to 50 mg/kg bw/d compared to 100% after exposure to 0, 0.5 and 5 mg/kg bw/d). In mice, most of the studies available were conducted at much lower dose levels. Nevoral *et al. (*2018) showed a significant decrease of fertility in adult ICR mice treated at 10 µg/kg bw/d while fertility was increased at 100 µg/kg bw/d. The impact of a postnatal BPS exposure (from birth until PND 60) to fertility or mating performance was studied im CD-1 mice by Shi *et al. (*2017). No reduced fertility was observed, but both treated males and females needed more time to successfully mate, suggesting subfertility, namely some of the animals took over 10 days and up to 16 days to become pregnant. Lastly, in Shi *et al. (*2019a), after a prenatal exposure (GD 11 to birth), fertility was lower in 9-month-old mice (100, 66.7, 40 and 40%, at 0, 0.5, 20 and 50 µg/kg bw/d, respectively) but not in 3 or 6 months of age.

Experimental studies investigating the effects of exposure to BPS on fertility are summarised in

Table 14 presented below.

This effect on fertility was recognised by RAC in its opinion (RAC, 2020) in support of classification of BPS as Repr. 1B – H360F.

Table 14: Summary table of studies showing a reduced fertility

Reference	Species	Routes	Dose Exposure period	Fertility index
Gestational exposure and its impact on next generation / trans-generation				
Shi *et al.*, 2019a	Mice (CD1)	Oral	0, 0.5, 20 and 50 µg/kg bw/d GD 11 to birth	No impact on fertility in F1 in 3 and 6 months old mice. But fertility ↘ in 9 months old F1 mice (100, 66.7, 40 and 40 %)
Nevoral *et al.*, 2018	Mice (ICR) (adult)	Drinking water	0, 0.001, 0.1, 10 and 100 µg/kg bw/d 4 w	Sign. ↘ fertility at 10 µg/kg bw/d, but enhanced at 100 µg/kg bw/d
Postnatal exposure and its impact on F1-generation				
Ahsan *et al.*, 2018	Rat (strain not specified)	Sub-cutaneous	0, 0.5, 5 and 50 mg/kg bw/d PND 1 to 10	Sign. ↘ fertility at the highest tested dose (60% vs 100% in all other groups)
Shi *et al.*, 2017	Mice (CD-1)	Sub-cutaneous	0, 50 µg or 10 mg/kg bw From birth to PND 60	No reduced fertility, but both treated males and females need more time to successfully mate, suggesting subfertility (some of the animals took over 10 days and up to 16 days to become pregnant)
Multi-generation studies				
OECD TG 443 Unpublished study report, 2019	Rat (SD)	Oral Drinking water	0, 20, 60 and 180 mg/kg bw/d (in 0.5% CMC) P0: from 10w before mating to PND 21 F1B: to PND 21	P0 Fertility index not affected (96, 91, 100 and 96 %) F1B Fertility index not affected (100, 100, 96, 96 %)
OECD TG 421 Unpublished study report, 2000	Rat (SD)	Oral	0, 10, 60 and 300 mg/kg bw/d (in 0.5 % aqueous sodium CMC solution with 0.1 % Tween 80) Total of 40 to 46d for females (from 14 d premating to PND 3)	↘ Fertility index (91.7, 91.7, 100.0 and 58.3%) (Nb of pregnant females/nb of copulated females : 11/12, 11/12, 12/12 and 7/12)
OECD TG 422 Unpublished study report, 2017b	Rat (SD)	Oral	0, 30, 100 and 300 mg/kg bw/d (CMC) Males: 10 w Females: From pre-mating until PND 21	↘ Fertility index (100, 90, 100 and 60%)

4.10.2.1.3 Epidemiological data linking BPS exposure and female reproduction

Very few epidemiological studies have evaluated the associations between exposure to BPS and female reproduction: one on fertility (Philips *et al.*, 2018), one on endometriosis (Peinado *et al.*, 2020), and one on polycystic ovary syndrome (PCOS) (Jurewicz *et al.*, 2021).

Philips *et al. (*2018) study included 877 participants enrolled in a Dutch mother-child cohort (the Generation R cohort) between 2004 and 2005. Exposure to BPS – among others bisphenols and phthalate metabolites – was assessed in spot urine samples and fecundability was estimated by time-to-pregnancy (TTP). BPS was detected in 70% of women urines with a median

concentration of 0.35 ng/mL. The study reported no association between any bisphenols and TTP in the whole study population (Fecundability Ratio, FR [95% confidence interval, CI] for BPS = 0.98 [0.94–1.02]) but observed differential effect according to folic acid supplementation status of the women. The sum of bisphenols A, S, and F, was associated with a significant increase in TTP (FR=0.88 [0.79; 0.99]) in the subgroup of women with inadequate folic acid supplementation, corresponding to a 12% decrease in the monthly chance of conception. Considering each bisphenol individually in this subgroup of women, a trend to longer TTP was observed for BPA and BPS, both with similar effect size (BPA: FR=0.93 [0.86–1.01]; BPS: FR=0.94 [0.87–1.01]), while BPF showed null association.

One Spanish case-control study explored the association between exposure to BPS and the risk of endometriosis (n=35 cases and n=89 controls). While a significant increased risk of endometriosis was reported in association with urine concentration of BPA, no association was observed with BPS (odds ratio [95% CI] = 1.4 [0.5–3.5]). However, BPS exposure level in this study population was low (detection rate =15%, geometric mean concentration = 0.1ng/mL) (Peinado *et al.*, 2020).

One Polish case-control study evaluated the association between exposure to bisphenols and the risk of PCOS (Jurewicz *et al.*, 2021). Higher urine concentration of BPS (but not BPA nor BPF) was observed in PCOS women compared to controls (geometric mean = 0.14 vs. 0.08 ng/mL, p = 0.02). The authors additionally reported a higher risk of PCOS in the lowest BPS exposure group (odds ratio [95% CI] = 1.21 [1.04; 3.46]) but the interpretation of this finding is questionable due to unclear reporting of the results.

In summary, there is a low number of epidemiological studies evaluating the effect of BPS on woman reproductive health. These results should be however considered in the light of existing knowledge on BPA toxicity for which estrous cycle disturbance (shorter luteal phase), implantation failures, decreased ovarian function and reserve (oocyte count, peak of E2, antral follicle count), and increased risk of endometriosis and PCOS, were observed (ECHA 2017; Hu *et al.*, 2018; Laws *et al.*, 2021).

4.10.2.2 LoE Endocrine Activity – EAS

See also Annex I for tabulated lines of evidence.

In vitro tests show that BPS can interfere with different hormone pathways. A clear estrogenic activity of BPS, via ER binding and ER activation is demonstrated. BPS has also been shown to disrupt steroidogenesis, impacting the production of testosterone and aromatase activity (see section 5.7.3.2.1 for more information).

Lastly, *in vitro* assays show that BPS can activate PPARγ receptors, has weak anti-androgenic activity and impact binding and activation of thyroid hormone receptors although effects are less obvious.

Most findings are related to estrogen and steroidogenic pathways. Estrogenic pathway is described below as responsible for the effects on female reproduction. while steroidogenesis pathway is described in section 5.7.3.2.1 *In vitro* mechanistic data (OECD level 1 and 2) as it is mainly related to adverse effects described in fish.

Estrogen modality

In vitro studies
In vitro studies demonstrate a clear estrogenic activity of BPS, via ER binding and ER activation.

- **ER-binding**

ER binding of BPS was evaluated in ER competitive binding assays using rat uterine cytosol (Blair *et al.*, 2000; Laws *et al.*, 2006) and hER (Hashimoto and Nakamura, 2000; Hashimoto *et al.*, 2001; METI, 2002; Yamasaki *et al.*, 2004; Akahori *et al.*, 2008; Molina-Molina *et al.*, 2013; Rajasärkkä *et al.*, 2014; Stossi *et al.*, 2014; Zhang *et al.*, 2018; Keminer *et al.*, 2020; Liu *et al.*, 2019b; Eilebrecht *et al.*, 2019).

BPS is capable of binding to the estrogen receptor, although weak affinity was observed with IC50 ranging from 5.8 (Zhang *et al.*, 2018) to 105 µM (Blair *et al.*, 2000) depending on the test system used. The relative binding affinity (RBA) ranged from 0.0055% (Yamasaki *et al.*, 2004 and METI, 2002) to no activity (Stossi *et al.*, 2014) for ERα, between 0.006% (Molina-Molina *et al.*, 2013) and 0.012% (Eilebrecht *et al.*, 2019) for ERβ, while Blair *et al.* (2000) reported a lower RBA of 0.0009% in rat uterine cytosol. The weak estrogen receptors binding was confirmed in fluorescence polarisation assays percentage (ERα: Hashimoto and Nakamura, 2000; Hashimoto *et al.*, 2001; ERβ: Eilebrecht *et al.*, 2019).

Furthermore, in a competitive binding assay using Human U251 glia cells transfected with zebrafish ERα, ERβ1 and ERβ2, it was demonstrated that BPS acts as a very weak estrogen receptor binder (Cano-Nicolau *et al.*, 2016).

These results shows that **BPS can bind the estrogen receptors α and β from rodent, fish and human.**

- **ER agonism**

ToxCast ER Pathway model.

The AUC score for ER agonist activity of BPS is 0.263, i.e. above the positivity threshold of 0.1.

ER reporter assays

Weak estrogenic activity was shown in several reporter gene studies (see Table X2 in Annex I) using different human cancer cell lines like MCF-7, MELN, T47D, VM7Luc4E2 (formerly BG1Luc4E2) and HELN -hERα and β, as well as other cell lines like zebrafish hepatic and Monkey kidney CV1. Estrogen-like response was induced with an EC50 of 1.1-12.10 µM in MCF-7 and MELN, 1.17-4.93 µM in VM7Luc4E2, 3.96-4.47 µM in HELN-hERα and 1.72 µM in HELN-hER β, 1.0 to 4.1 µM in zebrafish hepatic cells and 2.20 µM in Monkey CV1 cells, when reported. For comparison, IC50 values are 10 fold lower for BPA (Grimaldi *et al.*, 2019). In T47D-Kluc cells, Mesnage *et al.*, 2017, showed that BPS stimulated ERE-luciferase reporter gene expression with an EC50 of 1.5 µM, and that the addition of the anti-estrogen ICI 182,780 antagonised this effect, confirming its ER-dependancy. Furthermore, REC50 of 5.4 $x10^{-7}$ M were reported in a Luciferase reporter assay based on ERα and ERβ (Kojima *et al.*, 2018). Li *et al.,* 2018, demonstrated that ERE-mediated transcriptional activity of BPS was predominantly ERα-dependent.

The estrogen activity of BPS was also examined through 4 yeast two-hybrid assays performed according to the Nishikawa *et al.* method (1999) using an interaction of a nuclear hormone receptor (ERα) with a coactivator and the *lacZ* reporter gene (Nishihara *et al.*, 2000; Hashimoto *et al.*, 2001; Chen *et al.*, 2002; Hashimoto and Nakamura, 2000). Three out of the 4 studies showed a **positive estrogen activity, although weak** compared to E2. Only the study of Hashimoto *et al.* (2001), did not show estrogen activity, but after metabolic activation of BPS by rat liver S9-mix, significantly increased β-galactosidase activity was observed at concentrations of 10^{-3} M, suggesting a need for metabolisation of BPS before inducing estrogen activity. Furthermore, in addition to a dose-dependent estrogen activity without S9 mix (EC50= 6.97 x 10^{5} µM), Kang *et al*. (2014), demonstrated also the estrogenicity of BPS metabolites, since an increase of the **estrogen activity was induced** by adding rat liver S9 to the MVLN cell line

(MCF-7 cell line transfected with a luciferase reporter gene plasmid from X. laevis vitellogenin promotor region).

The estrogen acitivity of BPS was also examined in three YES assays (yeast estrogen screens) (Skledar *et al.*, 2016; Dvořáková *et al.*, 2016; Conroy-Ben *et al.*, 2018). In those studies, agonistic estrogen activity was observed with an EC50 between 84 to 588 µM. In another yeast reporter study of Rajasärkkä *et al. (*2014), a yeast strain containing a hERα was used and BPS was found to be two fold less potent than BPA. A BLYES study, an estrogen-inducible bacterial lux-based bioluminescent reporter assay, indicated activation of ER as BPS exhibited significant induction of the bioluminescence between 5 $x10^4$ and 1 $x10^6$ nM, while it was reduced in the presence of ICI 182,780, a strong antagonist blocking the ER (Ruan *et al.*, 2015). EC50 was calculated to be 4.13 $x10^5$ nM, EEF (estradiol equivalence factor) was determined to be 1.06 x 10^{-6}.

To determine the estrogen activity of BPS, ZELH-zFERs cell lines were used, i.e. zebrafish hepatic cell lines ZFL expressing the luciferase gene under the control of ERE and each of the three zebrafish estrogen receptors (zfERα, zfERβ1 and zfERβ2). BPS was weakly active in all cell lines, showing an EC50 of 4.058 µM in zfERα, 1.016 µM in zfERβ1 and 2.468 µM in zfERβ2. The relative estrogenic potency in comparison to E2 (REPE2) to each receptor was 3.7 $x10^{-5}$, 3.0 $x10^{-5}$ and 2.4 $x10^{-5}$, resp. (Le Fol *et al.*, 2017).

Proliferative assays

Four proliferations (E-screen) studies showed the **ability of BPS to promote cell growth in the hormone dependent human breast cancer cell line MCF-7** (Hashimoto and Nakamura, 2000; Hashimoto *et al.*, 2001; Kim *et al.*, 2017; Mesnage *et al.*, 2017). Furthermore, Mesnage *et al.* (2017) observed the same trend when using another hormone dependent human breast cancer cell line: **T47D**. The authors also examined the hormone independent and ER negative MDA-MB-231 human breast cancer cell line which showed no induction of proliferation suggesting that the effects are mediated by ER. They reported an AC50 of 1.33 µM.

Molina-Molina *et al.* (2013), performed an E-screen study with ERα-positive breast cancer MCF-7 cells and BPS from the termal paper extracts significantly induced the proliferation of MCF-7 cells at 10^{-5} M (approximately 3.7-fold compared to the control).

ER- regulated gene expression

Mesnage *et al.* (2017), performed a full transcriptome profiling on MCF-7 cells exposed for 48h to BPA and its analogues. It should be noted, BPS showed the most different profile, quite different from the other bisphenols, showing upregulation of cell cycle markers, progesterone receptor and estrogen receptor alpha.

Moreover, after exposure to 10^{-5} M BPS, significant upregulation of telomerase expression and activity has been observed in MCF7 cells, but not in MDA-MB-231 (ER-negative) cells, suggesting the involvement of ER (Awada *et al.*, 2019).

Regarding other species, BPS significantly upregulated, in a dose dependently manner, ApolipoproteinII (ApoII) and Vitellogenin (Vtg) mRNA levels in an *in vitro* chicken screening assay used together with the Avian Tox Chip polymerase chain reaction array (Ma *et al.*, 2015). ApoII and Vtg genes, both encoding egg yolk precursor proteins in birds and are associated with the sex steroid pathway and avian reproduction. At 300 µM ApoII and Vtg were upregulated by 30.7-fold and by 10.3-fold resp., compared to DMSO control.

Other test methods

Regarding non-genomic estrogen signalling, Viñas and Watson (2013), examined extracellular signal-regulated kinase (ERK)– and c-Jun-N-terminal kinase (JNK)–specific phosphorylations in

regard to the functional responses proliferation, caspase activation, and prolactin (PRL) release. BPS was able to activate ERK-activation, but did not significantly activate JNK.

All these results confirms that **BPS activates the estrogen receptors from rodent, fish and human.**

- **ER antagonism**

ToxCast ER Pathway model:

In the ToxCast ER Pathway model, the AUC score for ER antagonist activity of BPS is 0, *i.e.* considered inactive.

ER reporter assays

BPS showed no significant antagonistic estrogen activity in the available *in vitro* ER reporter studies using human breast cancer cells (Kitamura *et al.*, 2005; Dvořáková *et al.*, 2016; Simon *et al.*, 2016; Okazaki *et al.*, 2017).

Based on these results, there is **no indication that BPS is an ER antagonist.**

***In vivo* studies**

Uterotrophic assays

Three published uterotrophic assays show the **agonist estrogenic activity of BPS**. Two of them were performed following OECD TG 440 under GLP conditions, using immature non-ovariectomised rats exposed to BPS subcutaneously (Yamasaki *et al.*, 2004; Akahori *et al.*, 2008). In the third study, ovariectomised adult rats were exposed orally to BPS using a method similar to OECD TG 440 (Conley *et al.*, 2016). All studies showed a weak but significant increase of the uterine weight after BPS exposure.

In immature rats, the results obtained by Yamasaki *et al.* (2004) were not dose-dependent, the lowest and highest tested doses showing a significant increase of the wet and blotted uterus weight (at 20 mg/kg bw/d; $p<0.05$ and at 500 mg/kg bw/d; $p<0.01$) while the intermediate dose (100 mg/kg bw/d) did not show a significant effect. In the supportive study of Akahori *et al.* (2008) a weak estrogenic effect is reported (LogLED estrogenic 1.9 µmol/kg bw/d). OECD TG 440 recommends subcutanous administration to model dermal adsorption. As humans are exposed to BPS via skin (f.i. thermal paper) this route of administration is considered relevant.

In ovariectomised rats (Conley *et al.*, 2016), the wet uterus weight increased in a dose-dependent manner (31.6, 55.0, 63.0, 77.2, 139.5 and 156.7 mg, after exposure to 0, 50, 100, 200, 400 and 800 mg/kg bw/d, resp.), as well as the blotted uterus weight (25.4, 44.9, 54.8, 64.9, 107.2 and 121.4 mg, resp.). All results were significantly higher than the negative control ($p<0.05$). Dose-related histological changes, including increased epithelial and glandular cell height were also observed from 200 mg/kg bw/d.

All these results confirm clearly that **BPS shows agonistic estrogen activity.**

4.10.3 Lines of evidence - T[12] modality

Data on T-modality are available, but were not assessed further in detail in this report as a link is postulated between reproductive adverse effects and the estrogenic modality. Data on binding and activation of thyroid hormone receptor were not consistent across studies.

4.10.4 Lines of Evidence - Other modalities

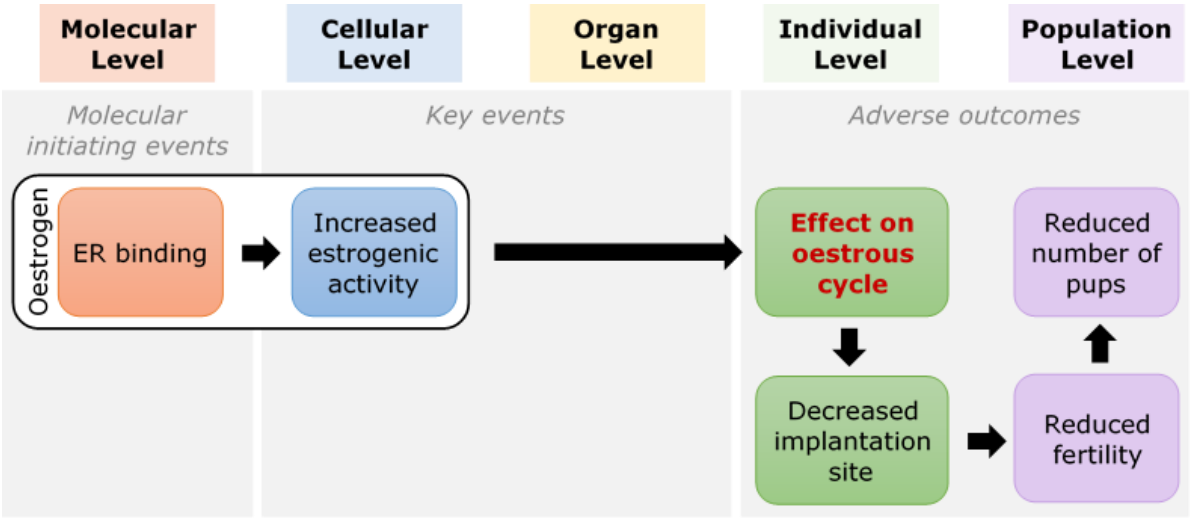
Several *in vitro* assays show that BPS can act on other modalities (AhR, CAR, GR, PPAR, …). These were not assessed further in detail as focus was put on Estrogen modality and Steroidogenesis. Some weak anti-androgenic activity has been observed *in vitro*, but the effect of BPS on this pathway is less obvious due to contradictory results.

4.10.5 Mode of Action (MoA) analysis

4.10.5.1 Postulation of MoA(s)

Postulated estrogenic MoA affecting female reproduction in mammals is presented in Figure 2. The MoA presented here does not describe every detail of the biology but instead focuses on describing critical steps, acknowledging that other activities could also influence each of the key events described.

Figure 2: Estrogenic MoA affecting female reproduction in mammals



Legend: EATS-mediated adverse effect / Sensitive but not diagnostic of EATS

Exposure to BPS leads to agonistic estrogenic activity

Several *in vitro* studies demonstrate a clear estrogenic activity of BPS via ER-binding and ER activation (see section 4.10.2.2 above).

Three different *in vivo* uterotrophic assays conducted with immature or ovariectomised rats (see section 4.10.2.2) show that BPS increases the uterus weight, which is a known marker of an estrogenic mode of action: *"Uterine tissues respond with rapid and vigorous growth to*

[12] T - thyroidal

stimulation by estrogens, particularly in laboratory rodents, where the estrous cycle lasts approximately 4 days. Rodent species, particularly the rat, are also widely used in toxicity studies for hazard characterisation. Therefore, the rodent uterus is an appropriate target organ for the in vivo screening of estrogen agonists and antagonists." (OECD TG 440).

As a consequence of estrogenic activity, BPS disturbs the estrous cycle.

"The estrous cycle is a very sensitive marker of endocrine disruption and a disturbed cyclicity is recognised as EATS-mediated parameter" (EFSA/ECHA ED guidance, 2018).

Several studies show a prolonged estrous cycle (see section 4.10.2.1.1).

The effect of BPS on the estrous cycle has been acknowledged by RAC (RAC, 2020) during the classification procedure of BPS as toxic for reproduction Cat. 1B (see below).

"The estrous cycle was prolonged in female rats in several studies (OECD TG 421; OECD TG 443; OECD TG 422) (see table below). Furthermore, results from the OECD TG 443 illustrated that females in the F0, F1A and F1B cohorts tended to be in the diestrus stage for a longer period and in the proestrus stage for a shorter period in all treated dose-groups (>20 mg/kg bw/day) compared to controls (see table below).

In the OECD TG 421 study, the incidence of females with an irregular estrous cycle was 0/12, 0/12, 1/12 and 5/12 at 0, 10, 60 and 300 mg/kg bw/day, respectively. Four out of five females in the highest dose group, which had a continued diestrus phase, did not conceive at all and consequently this led to a steep decrease of the fertilisation index of 58%.

The biological relevance and adversity of the effects on the estrous cycle, in the presence of indications of decreased fertility parameters (i.e. decreased mean number of implantation sites, decreased fertility index) is apparent. Taking this and the consistency of the effect among studies into account, RAC considers the effects on the estrous cycle as relevant for classification." (RAC opinion, 2020).

Table: Mean duration of the estrous cycle in days (RAC opinion, 2020)

Dose level (mg/kg bw/day)	0	10	20	30	60	100	180	300
OECD TG 421	4.08	4.01	-	-	4.14	-	-	5.57**
OECD TG 443 F0 F1A F1B	 3.9 4.1 3.9	 - - -	 3.9 4.1 4.0	 - - -	 3.9 4.1 4.0	 - - -	 4.1* 4.1 4.1[1]	 - - -
OECD TG 422	4.02	-	-	3.97	-	4.01	-	5.16**
HCD (mean; range): 4.2 (3.9-5.2)[a] HCD (mean; range): 4.9 (4.0-5.8)[b] HCD (mean; range): 4.5 (4.4-4.9)[c]								

[a] Historical control data (Charles River Ashland, Crl:CD(SD), OECD 412/422/443, 89/91 studies in a time period from 12/2000 to 08/2018)

[b] Historical control data (Janvier or Charles River France, RjHan:SD (Rats CD®), two-generation studies F0, time period 02/2016 to 04/2020)

[c] Historical control data (Janvier or Charles River France, RjHan:SD (Rats CD®), two-generation studies F1, time period 02/2016 to 04/2020)

[1] Note that this specific effect was initially mentioned to be 60% at the highest dose tested in the CLH dossier and on IUCLID, but during the consultation it was noted that this effect size was incorrect and should be changed into 80% fertility at the highest dose tested. The information in IUCLID was updated to this regard as well (ECHA dissemination website consulted on 18-08-2020). The RAC rapporteur was unable to verify the exact number as it had no access to the underlying study report. However, RAC wants to highlight that, either way, a dose-dependent decrease in fertility index is observed in this study.

Furthermore, as explained in the section 4.10.2.1.1, disturbed estrous cycle was observed in three out of four literature studies. An increased occurrence of irregular cycles was observed after post-natal exposure in rats (Ahsan *et al.*, 2018), and in mice after gestational exposure (Shi *et al.*, 2019a) and in subsequent generations (Shi *et al.*, 2019b). Moreover, alterations of folliculogenesis have also been reported jointly to altered estrous cycles in some studies. Even though the temporality of biological events is not a direct proof of a functional link between those events, these data suggest that a link might exist between estrous cyclicity modification and impaired oocyte/follicle maturation (Shi *et al*., 2019a). Additionally, BPS treatment induced a disturbed estrus jointly to reduced corpus luteum and antral follicles while atretic follicles were increased (Ahsan *et al*., 2018).

As a consequence of disturbed estrous cycle, BPS exposure leads to a decrease in the number of implantation sites.

The effect of BPS on implantation sites has been assessed in three different guideline (OECD TGs 421, 422 and 443) studies, showing all a reduced number of implantation sites from 180 mg/kg bw/d (Unpublished study report, 2000, 2017b and 2019). (see section 4.10.2.1.2).

This effect has been acknowledged by RAC (RAC, 2020) during the classification procedure of BPS as toxic for reproduction Cat. 1B (see below).

*"The mean number of implantation sites was affected in three different guideline studies (OECD TG 421, OECD TG 443, OECD TG 422) from 180 mg/kg bw/day onwards, with increasing severity at 300 mg/kg bw/day (see table below). Considering the size of the effect and the consistency of the effect among studies, RAC considers this effect as relevant for classification for sexual function and fertility" (**RAC opinion, 2020**).*

Table: Mean number of implantation sites per dam (RAC opinion, 2020)

Dose level (mg/kg bw/day)	0	10	20	30	60	100	180	300
OECD TG 421	15.9	13.3	-	-	14.8	-	-	10.7
OECD TG 443								
F0	15.3	-	14.8	-	14.9	-	14.3	-
F1B	15.2	-	14.6	-	15.4	-	13.7	-
OECD TG 422	15.8	-	-	15.0	-	15.5	-	10.4**
HCD (mean; range): 15.2 (12.3-17.8)[a] HCD (mean; range): 15.0 (13.8-16.0)[b] HCD (mean; range): 14.1 (12.1-15.3)[c]								

[a] Historical control data (Charles River Ashland, Crl:CD(SD), OECD 412/422/443, 89/91 studies in a time period from 12/2000 to 08/2018)

[b] Historical control data (Janvier or Charles River France, RjHan:SD (Rats CD®), two-generation studies F0, time period 02/2016 to 04/2020)

[c] Historical control data (Janvier or Charles River France, RjHan:SD (Rats CD®), two-generation studies F1, time period 02/2016 to 04/2020)

There may be intermediate steps between the disruption of the estrous cycle and the reduced number of implantation sites. Alterations of folliculogenesis have been reported in some studies, suggesting that a link might exist between estrous cyclicity modification and impaired oocyte/follicle maturation, affecting therefore the implantation success. For example, Ahsan *et al. (*2018), observed that BPS treatment induced a disturbed estrous cycle, and in parallel that the number of corpus luteum and antral follicles were reduced, while atretic follicles were increased. Normal oocyte maturation and meiosis and late folliculogenesis might result at least in part in implantation failure, post-implantation loss due at least in part to improper steroid environment (steroidogenesis is LH/FSH regulated at the level of follicles) and/or poor quality oocyte with deleterious consequences for the embryo.

The potential effects of disturbed hormone balance in ovaries, leading to disrupted folliculogenesis and resulting in post-implantation loss is discussed in chapter 4.10.5.5 Other supportive ED MoAs.

As a consequence of the decrease in the number of implantation sites, fertility is reduced.

Some studies investigated the impact of BPS exposure on fertility. At high dose, the effect is very concise in most cases. Rats exposed to 300 mg/kg bw/d showed a fertility index reduced to 58.3% in OECD TG 421 (Unpublished study report, 2000) and to 80% in OECD TG 422 (Unpublished study report, 2017b). This effect was also obtained at lower doses of exposure: rats exposed subcutaneously to 50 mg/kg bw/d in Ahsan *et al.* (2018), showed a reduced fertility to 60% and all mice exposed subcutaneously to up to 10 mg/kg bw/d in the study of Shi *et al*. (2017), successfully mated, but needed more time to become pregnant, suggesting subfertility. Only the OECD TG 443 (Unpublished study report, 2019) did not show any effect on fertility, but

the lower dose range (up to 180 mg/kg bw/d) via oral route of exposure could explain this.

At very low dose, the effects are less evident, but Nevoral *et al.* (2018), showed a significant reduced fertility after 4 weeks exposure to 10 µg/kg bw/d. Moreover, Shi *et al.* (2019a), showed a dose dependent decrease in fertility of 9-months-old mice (100%, 66.7%, 40% and 40%) that received in *utero* exposure to 0, 0.5, 20 and 50 µg/kg bw/d respectively. (see section 4.10.2.1.2)

This effect has been acknowledged by RAC (RAC, 2020) during the classification procedure of BPS as toxic for reproduction Cat. 1B (see below).

During the classification procedure of BPS as toxic for reproduction Cat. 1b, based on the OECD TG studies, RAC recognized that **BPS affects the fertility of rodents:**

"Both the OECD TG 421 and 422 studies show a dose-dependent decrease in fertility index at 300 mg/kg bw/day (see table below). In the OECD TG 421 study, most of the females at 300 mg/kg bw/day, which had a continued diestrus phase, were not fertilised. In the OECD TG 422 study, there were two females without implantation sites at 300 mg/kg bw/day. Considering the severity, RAC considers these effects relevant for classification." (RAC opinion, 2020)

Table: Fertility index (RAC opinion, 2020)

Dose level (mg/kg bw/day)	0	10	20	30	60	100	180	300
OECD TG 421	91.7%	91.7%	-	-	100%	-	-	58%
OECD TG 443 F0 F1B	 100% 100%	 - -	 100% 100%	 - -	 100% 100%	 - -	 100% 100%	 - -
OECD TG 422	100%	-	-	90%	-	100%	-	80%[1]

[1] Note that this specific effect was initially mentioned to be 60% at the highest dose tested in the CLH dossier and on IUCLID, but during the consultation it was noted that this effect size was incorrect and should be changed into 80% fertility at the highest dose tested. The information in IUCLID was updated to this regard as well (ECHA dissemination website consulted on 18-08-2020). The RAC rapporteur was unable to verify the exact number as it had no access to the underlying study report. However, RAC wants to highlight that, either way, a dose-dependent decrease in fertility index is observed in this study.

As a consequence of the decreased fertility, the number of pups is reduced.

The mean number of pups delivered was evaluated in four studies. In three of those (OECD TGs 421, 422 and 443), there was a dose-dependent decrease in the mean number of pups delivered (Unpublished study report, 2000, 2017b and 2019), with the exception of OECD TG 414 which showed only a slight decrease (10.1 vs 10.6 in control, non significant) (Unpublished study report, 2014). Ahsan *et al.* (2018), also observed a significant reduction of new-born pups per female (5.33 after exposure to 50 mg/kg bw/d vs 8.80 in control). In Shi *et al.* (2019a), a non-statistically significant decrease in new born pups was already observed after exposure to lower doses (see section 4.10.2.1.2).

This effect has been acknowledged by RAC (RAC, 2020) during the classification procedure of BPS as toxic for reproduction Cat. 1b (see below).

During the classification procedure of BPS as toxic for reproduction Cat. 1b, based on the OECD TG studies, **RAC recognised that BPS affects the fertility of rodents**.

"The mean number of pups delivered/mean number of live foetuses was evaluated in four studies. In three of those (the OECD TG 421, OECD TG 443, and the OECD TG 422), there was a dose-dependent decrease in the mean number of pups delivered (see table below). RAC considers this effect to be a direct consequence of the exposure to the substance but notes that it is difficult to discriminate whether this is due to fertility or developmental effects." (RAC opinion, 2020)

Table: Mean number of pups delivered (RAC opinion, 2020)

Dose level (mg/kg bw/day)	0	10	20	30	60	100	180	300
OECD TG 421	14.3	12.5		-	13.5	-	-	9.1
OECD TG 443								
F1	14.9	-	14.0	-	13.5	-	12.7	-
F2	14.3	-	13.8	-	14.9	-	11.4**	-
OECD TG 422	15.2	-	-	14.1	-	14.5	-	10.8**
OECD TG 414[a]	10.6	-	-	10.6	-	10.6	-	10.1
HCD (mean; range): 14.3 (12.1-15.9)[b]								

[a] Mean number of live foetuses

[b] Charles River Ashland, Crl:CD(SD), OECD 412/422/443, 89/91 studies in a time period from 12/2000 to 08/20

4.10.5.2 Assessment of biological plausibility of the link between endocrine activity and adverse effect(s)

Considering the results of all available studies, there is strong evidence that the adverse effects on fertility in females are due to the oestrogenic activity of BPS. The increase in uterus weight (as seen in all conducted uterotrophic assays) is recognised as a diagnostic parameter for oestrogenicity. Furthermore, prolongation of the estrous cycle was consistently observed in the majority of the studies. In addition, the number of implantation sites was decreased in reproductive toxicity studies, resulting in decreased fertility and number of pups. All of these parameters are considered as either "EATS-mediated" or "sensitive to, but not diagnostic of, EATS modalities" (OECD GD 150, 2018).

The different effects of BPS, in particular on the female reproductive system leading to adversity on apical fertility endpoints and litter size, can be plausibly linked to the oestrogenic activity of the substance. Bisphenols are known to target many different endocrine pathways. Available *in vitro* and *in vivo* studies show that BPS can interfere with the estrogen, androgen, steroidogenesis, thyroid, PPARγ, etc. pathways. The ability of BPS to interfere with the estrogen pathway is very strong. Different *in vitro* ER binding assays demonstrate that BPS is capable to bind to the estrogen receptor, with IC50 ranging from 5.8 to 105 µM depending on the source of ER used (rat and human). Several literature studies showed increased estrogen activity, although weak (ER reporter gene assays, proliferative assays and ER-regulated gene expression assays). This resulted in increased estrogen signalling, demonstrated in all available uterotrophic

assays (Yamasaki *et al.*, 2004; Akahori *et al.*, 2008; Conley *et al.*, 2016).

Therefore, a mode of action analysis was performed on the estrogen modality with focus on the female reproductive system (Table 15).

Table 15 : Analysis of mode of action

	Event	Supporting evidence
MIE	ER activation	**Strong evidence:** • Studies show binding of BPS to ER (Blair *et al.*, 2000; Yamasaki *et al.*, 2004; Laws *et al.*, 2006; Akahori *et al.*, 2008; Zhang *et al.*, 2018; Liu *et al.*, 2019b). • Several studies show an agonist activation of ER (among others Grignard *et al.*, 2012; Kang *et al.*, 2014; Dvorakova *et al.*, 2016; Le Fol *et al.*, 2017; Rosenmai *et al.*, 2014; Mesnage *et al.,* 2017; Kojima *et al.,* 2018; Pelch *et al.*, 2019).
KE1	Increased estrogenic activity	**Strong evidence:** • Uterotrophic assays show increase in uterus weight (Yamasaki *et al.*, 2004; Conley *et al.*, 2016). Supported also by Akahori *et al*. (2008).
KE2	Disturbed estrous cycle	**Strong evidence:** • 3/3 TG studies show disturbed estrous cycle (OECD TG 443, P0 and F1B, no effect on F1A; OECD TGs 421 and 422). Among them 2 studies show a prolongation of diestrus. • 3 additional studies report a disturbed cycle (Shi *et al.*, 2019a and 2019b; Ahsan *et al.*, 2018). The Shi studies describe a prolongation of diestrus and estrus phases.
KE3	Decreased implantation sites	**Strong evidence:** • Decreased number of implantation sites observed in 3/3 TG studies (significant in OECD TG 422; trend in OECD TG 443 P0 and F1B; and OECD TG 421)
AO1	Reduced fertility	**Strong evidence:** • Fertility rate reduced in 2/3 TG studies (OECD TGs 421 and 422; no effect in OECD TG 443) • Fertility affected in another 2 studies (Ahsan *et al.*, 2018, Nevoral *et al.,* 2018)
AO2	Decreased number of pups	**Strong evidence:** • Reduced number of pups in 3/4 TG studies (OECD TGs 443, 421 and 422, no effect in OECD TG 414) • Also observed in other studies (Shi *et al.*, 2019a; Ahsan *et al.*, 2018)

The hypothesised mode of action and the resulting AOs (Adverse Outcome) applies not only for

female human health, but also for mammals in general and has populational relevance in an ecotoxicological context (see chapter Environmental relevance 5.7.6.1). It should also be noted that estrogens exert a variety of effects on reproductive function including feedback on pituitary gonadotropins and hypothalamic neuropeptides as well as direct actions on reproductive organs.

There is strong evidence for ER binding and activation by BPS. Increased estrogen signalling is confirmed by findings in mechanistic *in vivo* studies (increased uterus weight in uterotrophic assays). Increased estrogen signalling disturbs estrous cycles, contributing to decreased number of implantation sites, and finally results in reduced fertility and consequently reduced litter size. The biological plausibility of the links between the different KEs and the AO is rated high based on knowledge on mammalian reproductive endocrinology and human contraception.

4.10.5.2.1 Detailed analysis of the KE disturbance of the estrous cycle

In the postulated MoA, most of the adverse effects (reduced number of pups, reduced fertility and decreased implantation sites) are sensitive to, but not diagnostic of, EAS, except the disturbance of estrous cycle. Indeed "the estrous cycle is a very sensitive marker of endocrine disruption and a disturbed cyclicity is recognised as EATS-mediated parameter" (EFSA/ECHA ED guidance, 2018).

Estrous cycle disturbance is the central point of this postulated MoA. The other effects that are sensitive to, but not diagnostic of, EAS are consequence of this EAS-mediated effect. Therefore a detailed analysis of this KE and its relations to the other KEs/AOs has been developed:

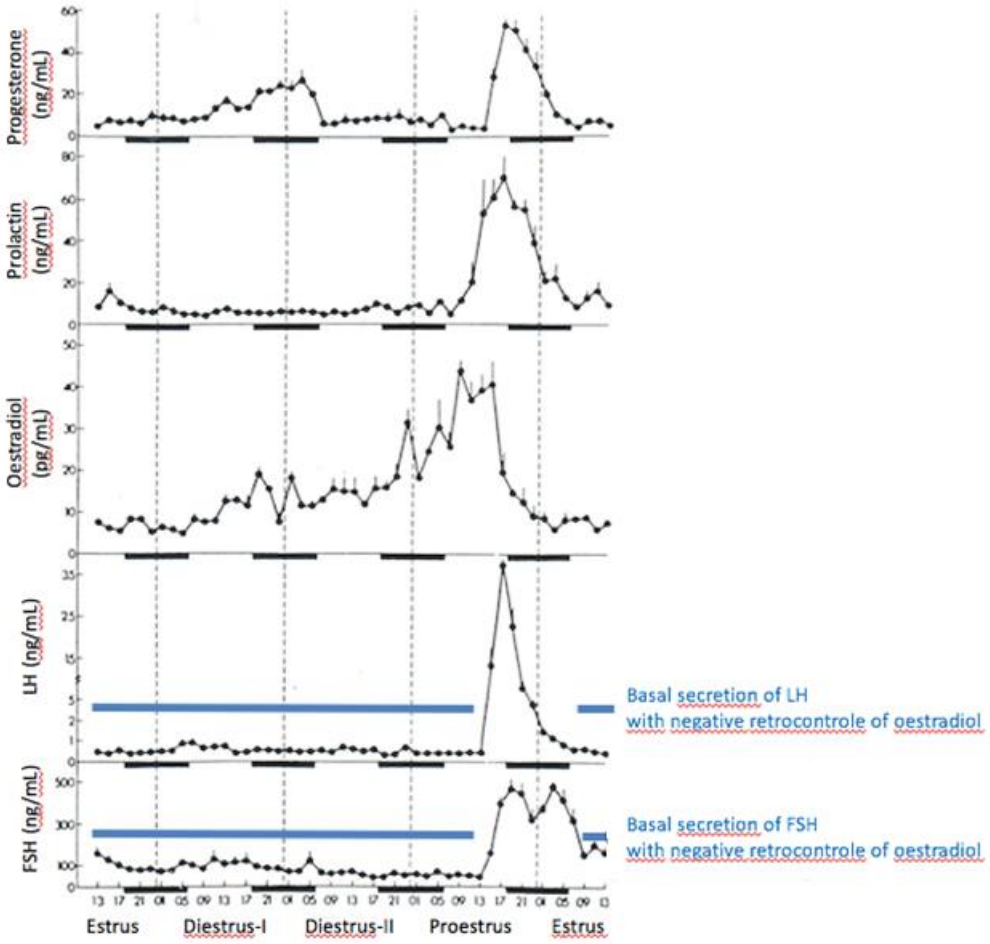
4.10.5.2.1.1 Impaired the estrous cycle in adult rodents

Background on hormonal regulation of the estrous cycle in rodents

In rat and mouse, the estrous cycle is characterised by the following sequential stages, called estrus, diestrus-1 (also named metestrus), diestrus-2 (also called diestrus) and proestrus; each of which lasting about one day. At the end of the follicle growth, ovulation occurs on estrus stage at 02:00hrs. During the night between proestrus and estrus, females are receptive for mating. After the ovulation, the follicle becomes the corpus luteum, which predominantly secretes progesterone until it becomes non-functional in the absence of mating/fertilisation. Maintenance of the corpus luteum function requires continued pituitary secretion of prolactin. Copulation during the estrus phase stimulates the daily release of prolactin by the anterior pituitary, which consequently disrupts normal estrous cyclicity by maintaining the corpus luteum in a functional state. Thus, the diestrus phase will be prolonged and the secretion of progesterone will be continued. In the absence of mating the corpus luteum regresses, prolonged diestrus will be terminated, and normal reproductive cyclicity will be resumed (Woldemeskel, 2017).

The time-related changes in hormonal secretions by the ovary and pituitary gland during the estrous cycle are well-known processes (Figure 3).

Figure 3: Concentrations of progesterone, prolactin, estradiol, LH and FSH in the peripheral plasma of the 4-days estrous cycle of the rat (adapted from Smith *et al*., 1975).



Mean values ± SE of 5-6 Sprague –Dawley rats are represented. Horizontal black bars represent the dark period in the animal room (18:00-06:00) of the 24-hr clock. Note that estradiol concentrations are expressed with pg/mL unlike all other hormonal concentrations which are expressed as ng/mL.

From the morning of estrus until 02.00h p.m of proestrus of the next cycle, the pool of recruited preantral and antral follicles grow and produce estrogens. Estrogen production results from a collaborative work inside the ovary: the theca-interstitial cells synthetise androgens from cholesterol and the granulosa cells, convert the androgens produced by theca-interstitial cells into estrogens as they specifically express cypP450arom (also called CYP19a1) encoding the aromatase, catalysing this conversion. The follicules grow by intense mitosis of granulosa cells and the production of estrogens is going increasing. This prepares the uterus for embryo implantation. During this period, LH and FSH levels in the plasma are relatively constant and this period is called the phase of basal secretion of gonadotrophins. LH and FSH stimulate follicular growth and steroidogenesis by acting on thecal cells and granulosa cells respectively. In addition of this hypothalamus-pituitary control of the ovary functions, there are also paracrine/autocrine regulation inside the follicle and the ovary. Oestrogens stimulate granulosa cells mitosis (and protect the follicle from atresia). Consequently, more and more cells produce estrogens. Furthermore, estrogens enhance the effects of LH and FSH on follicular cells. Thus, the levels of circulating estrogens increase throughout this period. Importantly during this whole period, the circulating levels of LH and FSH are strictly adjusted, mainly by the concentration of circulating estrogens which exerts a negative feed-back on the gonadotrope axis.

At 02.00 h pm of the proestrus, when plasma estrogens level reach a threshold, estrogens no longer exert a negative effect on the hypothalamus. On the contrary, they stimulate the hypothalamus-pituitary system to provoke the ovulatory surge of LH and FSH. Briefly, during

the proestrus phase, estradiol targets kisspeptin neurons in the hypothalamic preoptic area, which send projections to GnRH neurons. Activation of GnRH neurons by kisspeptin induces liberation of GnRH, which then triggers the ovulatory surge of LH by the pituitary. This surge induces oocyte expulsion of the oocyte out the follicle (at 02.00 a.m of the estrus), and luteinisation of the follicle. The *corpus luteum* predominantly produces progesterone. During the remaining phases of the cycle, the negative feedback exerted by estrogens at the hypothalamic level occurs through inhibition of kisspeptin neurons located in the hypothalamic arcuate nucleus.

In conclusion, the estrous cycle appears as a process basically controlled by sequential endocrine/paracrine and autocrine regulations. The key event is the endocrine dialogue between the hypothalamus-pituitary system and the ovarian follicles via the levels of estrogens that triggers a positive feedback leading to the ovulatory surge of LH, and exerts a negative control during the remaining duration of the estrous cycle.

Hormonal changes observed after BPS exposure

Except for two literature studies for which the result could not be considered as reliable as the reported concentrations were below the limit of sensitivity of the assays (Ahsan *et al.*, 2018 and Ijaz *et al.*, 2020), no studies evaluated hormonal levels following exposure of adult females to BPS including recently performed guideline studies.

Plausible link between BPS-induced endocrine changes and alterations of the estrous cycle

Based on general knowledge on the endocrine regulation of the estrous cycle, we can postulate that the well-established estrogenic activity of BPS may disturb estrous cyclicity in the case of adult exposure to BPS. Whether this disruption occurs at the ovarian, pituitary and/or hypothalamic level is not clear given the lack of available studies on that aspect.

4.10.5.2.1.2 Impaired the estrous cycle after developmental/peripubertal exposure

Background on neuroendocrine programming of estrous cyclicity

The sexual differentiation of the neuroendocrine function related to reproduction in rodents occurs during the perinatal (late gestational and early neonatal) and postnatal periods. This process is tightly regulated by gonadal hormones. More details about this hormonal-induced sexual differentiation are presented in Viguié *et al. (*2018). In females, the neuroendocrine pathways underlying the gonadotropic function are regulated by sex steroids with both positive and negative feedbacks exercised by estradiol during the estrous cycle. The positive feedback leading to the LH preovulatory surge is specific to females and the organisation of the neural structures involved in this estrogen-mediated regulation takes place during developmental periods. Briefly, in females, the expression level of kisspeptin in the anteroventral periventricular nucleus of the hypothalamus, the key region involved in the preovulatory surge of LH, is low at birth. It increases progressively during the postnatal period under the control of ovarian estradiol. Indeed, the ovarian production of estrogens, which starts around postnatal day 7, promotes kiss1 expression in this hypothalamic region. A maximal increase is observed during the prepubertal period and will be necessary for pubertal activation of GnRH/LH axis and initiation of estrous cyclicity and female reproduction.

Therefore, these periods are highly sensitive to hormonal changes. Exposure to exogenous factors exhibiting hormone-mimetic activities such as BPS could then interfere with these processes and induce long-term effects on the integrity of the gonadotropic axis and the estrous cyclicity.

Hormonal level changes observed after developmental BPS exposure

In the EOGRTS (Unpublished study report, 2019), rat F1 were constantly exposed from conception until sacrifice and observations were performed at adulthood. Prolonged estrous cycles were consistently observed with a clear and important prolongation of diestrus from the lower dose tested (i.e. 20 mg/kg bw/d). A slight decrease of estrus duration was also observed. F1 in EOGRTS is not an adequate model to investigate the specific effect of BPS on development because exposure occurs both during development and after development. The observed BPS effect in adulthood can be due either to a developmental alteration or to immediate actions or both. Nevertheless, the fact that disturbances in estrous cycle were more pronounced in F1 than in F0 shows that immediate and delayed final effects of BPS on estrous cycle are similar and additive.

Overall, three literature studies measured hormonal levels in adult females following postnatal (Ahsan *et al.*, 2018; Shi *et al.*, 2017) or peripubertal exposure to BPS (Ijaz *et al.*, 2020). In these studies, sampling was performed at the same stage of the cycle for all animals, to avoid any bias of hormonal level variation resulting from changes throughout the estrous cycle. However, the results on the hormone levels described in the three studies could not be considered as reliable for the reasons described below :

- In the study of Ahsan *et al*. (2018), rat female pups received 0, 0.5, 5 and 50 mg/kg bw/d BPS from PND 1 to PND 10. At adulthood, estrous cycle was irregular in all the groups, but no quantitative data are available to assess which estrus phase has been changed. Animals were sacrificed and blood was sampled in the morning of estrus. No changes were observed for estradiol levels while progesterone levels were found reduced for the highest BPS dose. For LH and FSH levels, the decreases reported (-10% and -16%, respectively) were too low and raise questions about the biological relevance and validity of these changes.

- In the study of Ijaz *et al*. (2020), performed in the same laboratory, peri-pubertal female rats were dosed intraperitoneally with 0, 0.05, 0.5, 5 and 50 mg/kg bw/d for 28 days (n=10 per group). Hormonal measurements were performed in adult females at the estrus stage. Estradiol and progesterone levels were below or at the limit of sensitivity of the assay as given by the assay provider. Changes in estradiol and progesterone levels were reported but the results are not reliable due to poor performance assay. Plasma LH and FSH levels were also measured and reported as reduced. However, as in the study of Ahsan *et al*. (2018), the extent of reduction was 15% and 23% for LH and 9% and 16% for FSH, at the two highest doses of 5 and 50 mg/kg bw/d, respectively. This raises again questions about the biological relevance of such changes. In addition, it is important to stress that the control levels of estradiol and progesterone were greatly different from those reported in Ahsan *et al*. (2018).

- In the study of Shi *et al*. (2017), increased estradiol levels were reported at the diestrus of adult females exposed to BPS during development. However, as mentioned above, the detected levels of estradiol were below or at the limit of sensitivity of the use assay for controls and treated groups. In addition, the weight of uterus, an estrogen-sensitive tissue, did not change between the treatment groups.

Given these limitations and inconsistencies, the reported results were not taken into account in the analyses of the MoA involved in the effects of developmental/peripubertal exposure to BPS on estrous cyclicity. However, based on the general background on the neuroendocrine programming of estrous cyclicity, the exposure to an estrogenic compound may interfer with these processes leading to long-term effects on estrous cyclicity at adulthood.

Plausible link between BPS-induced endocrine changes and alterations of the estrous cycle

The precise MoA underlying the alteration of the estrous cycle following developmental exposure to BPS is not yet known. In the absence of studies analysing the effects of developmental exposure to BPS on the whole gonadotropic axis including the hypothalamus (kisspeptin/GnRH system), pituitary (LH/FSH levels) and ovary, the exact mechanism of action remains to be determined. However, two recent studies from the same laboratory showed that developmental exposure to BPS increased the expression of genes encoding kisspeptin, GnRH3, FSHb and LHb as well as the expression of ERa, ERb and CYP19a (aromatase) genes in zebrafish larvae (Qiu *et al.*, 2016 and 2021). This shows that BPS has the ability to modify the expression levels of several actors of the gonadotropic axis. Given the conserved function of the gonadotropic axis in vertebrates, it is possible that developmental exposure to BPS alters the expression levels of hypothalamic neuropeptides and pituitary gonadotropins and consequently the levels of ovarian hormones.

Given the tight hormonal regulation of estrous cyclicity during adulthood and its neuroendocrine programming during developmental and pubertal periods, the estrogenic activity of BPS may induce changes triggering either immediate effects (for adult exposure) or long-term effects (for developmental exposure) on estrous cyclicity.

BPS endocrine disrupting activity impaired the estrous cycle in rodents.

4.10.5.3 Human relevance of MoA

Human relevance of MoA is assumed by default unless there is indication that this may not be the case. Hence, only if there is such indication, assessment of relevance for humans of the postulated MoA(s) needs to be carried out (see Sections 3.5.4.4 & 3.3.1.3 in EFSA/ECHA ED Guidance, 2018).

Estrogen signalling plays a key role in mammalian reproduction. There is no reason to assume that the observed adverse effects on fertility by disruption of estrogen signalling in rats and mice have no human relevance.

Moreover, *endocrine systems with respect to hormone structure, receptors, synthesis pathways, hormonal axes and degradation pathways are well conserved across vertebrate taxa especially in the case of estrogen, androgen and steroidogenesis* (OECD GD 150, 2018).

Specific investigation regarding human relevance of the effect on estrous cycle

In both primates and non-primate mammals, follicle selection, growth, and maturation, as well as ovulation, oocyte quality, and subsequent *corpus luteum* function, all depend on subtle sequential actions of gonadotropins and intraovarian regulators. Furthermore, the ovary and the hypothalamus-pituitary system are in permanent endocrine dialogue with each other. Consequently, any disturbances in the endo/para/autocrine activities of the ovary and/or the hypothalamus-pituitary system may lead to cycle disturbance.

In addition, the estrous cycle is a perfectly synchronised and timely regulated event that relies on specific neuroendocrine circuitries. In humans, the hypothalamic kisspeptin acts also upstream of GnRH neurones to coordinate GnRH and LH pulsatility (reviewed in Skorupskaite *et al.*, 2014). Kisspeptin has also been shown to mediate both negative and positive feedback exerted by sex steroids as presented above for rodents. The ovarian, pituitary and hypothalamic pathways differentiate during fetal life and pubertal periods are largely influenced by the steroid environment. Thus, either developmental/pubertal or adult exposure to steroidogenic compounds is very likely to result in estrous cycle disturbances.

Overall, the key principles of endocrine mechanisms of regulation of the cycle are the same between rodents and humans, despite some differences in circadian synchronisation and timing of ontogeny of the neuroendocrine axis (Viguié *et al.*, 2018). These general elements therefore bring support to the conclusion that the effects of BPS on disruption of cycles observed in rodents are relevant for humans.

In addition, as previously reported by Kortenkamp *et al. (*2012), an association between menstrual cycle characteristics and sub-fecundity and spontaneous abortion has been observed in humans and lifelong menstrual patterns have been associated with chronic diseases, including breast and ovarian cancer, uterine fibroids, diabetes and cardiovascular disease. Chronic anovulation is a well-established cause of female infertility. The few studies on menstrual cycle characteristics and fecundity have found that shorter cycles were less likely to be followed by conception, while both shorter and longer cycles were more likely to be followed by spontaneous abortion. Cycles with up to 4 days menstrual bleeding had lower fecundity, and spontaneous abortion was less likely after cycles with more than 5 days of menstrual bleeding (Small *et al*., 2006). Alteration of cyclicity is therefore considered as an effect fulfilling fully the criteria of adversity.

The results reported here clearly shows that exposure to BPS at the adult stage alters estrous cyclicity. Thus, we conclude that it is quite likely that **BPS may alter the ovarian cycle in humans**.

4.10.5.4 Conclusion on the Mode of Action analysis

Considering the results of all available experimental studies, there is strong evidence that the adverse effects on fertility in females are due to the estrogenic activity of BPS. The increase in uterus weight (as seen in all three uterotrophic assays) is a strong diagnostic parameter for estrogenicity. Furthermore, the prolongation of the estrous cycle was consistently observed in the majority of the studies. In addition, the number of implantation sites was decreased in three reproductive studies, resulting in decreased of both fertility and number of pups. All of these parameters are considered as either EATS-mediated or sensitive to EATS modalities (OECD GD 150, 2018). The different effects of BPS, in particular on the female reproductive system, can be plausibly linked to the estrogenic activity of the substance and could therefore explain the adverse impacts seen on fertility endpoints.

Other mode of action than those involving estrogenic activity and/or signalling pathways are likely. For example, altered testosterone production is probably linked to adverse effects on the male reproductive system or the male mammary gland. Despite the fact that these data give further indications of the endocrine activity of BPS, they were considered as supportive adverse human health effects.

In conclusion, the effects on the female reproductive organs and functional parameters are consistent with an estrogenic mode of action of BPS. While considering that effects on the estrous cycle are EATS-mediated (OECD Guidance Document 150), the causal link between the endocrine activity and the adverse effects is demonstrated. These adverse effects have been observed at doses showing neither maternal toxicity nor general toxicity.

There is strong evidence that the **adverse effects on fertility and sexual function are plausibly linked to the estrogenic activity of the substance. BPS is therefore an endocrine disruptor according to the WHO/IPCS definition (WHO/IPCS, 2002) with regard to human health.**

Table 16: Uncertainty analysis on the biological plausibility of the link between the adverse effect and the endocrine activity for the postulated MoA

	Key event relationships (KERs)			
	MIE to KE1	**KE1 to KE2**	**KE2 to KE3**	**KE3 to AE**
Biological plausibility for the KERs	STRONG ER binding → Estrogenic activity It is known that ER binding induced estrogen signalling and activity, as this is the base of uterotrophic assay (OECD TG 440)	STRONG Estrogenic activity → Disturbed estrous cycle Effects on estrous cycle are known as EATS-mediated, as the cycle is finely regulated through hormone levels and estrogenic activity	STRONG Disturbed estrous cycle → Decreased implantation Females with disturbed estrous cycle are known to have difficulty to conceive.	STRONG Decreased implantation → Reduce fertility and litter size Reduced implantation sites is a known marker of unfertility, resulting in reduced (or absence of) litter size.
Empirical support for the KERs	STRONG *In vitro* and *in vivo* results for ER transactivation confirmed as substance increases uterine weight in rats and mice, generally with dose-response and temporal concordance	STRONG Substance disturbs the estrous cycle in all studies with dose-response and temporal concordance	STRONG Females showing a disturbed estrous cycle show also a reduced number of implantation sites (dose-response and temporal concordance)	STRONG Females with reduced implantation had smaller litter size (dose-response and temporal concordance)

	MIE	**KE1**	**KE2**	**KE3**	**AE**
Essentiality of Kes		MODERATE – There are no stop-recovery studies available. But based on knowledge of the mammalian reproductive endocrinology and human contraception, an estrogenic activity affects definitely the estrous cycle and the fertility.			
Consistency	The KEs have been observed consistently in three different studies with different duration. The pattern of effects is consistent between the studies there are no conflicting observations. Consistency across species cannot be assessed because there are only rat studies available.				
Analogy	Other bisphenols and estrogenic compounds show similar MoA[13].				
Specificity	Effects have been observed in absence of general toxicity.				

Identified uncertainties	**Comments**
Uncertainty 1 : Prolongation of diestrus phase could be due to different effects on HPG axis	It is not possible to determine exactly the mechanism leading to prolonged diestrus phase in absence of analysis of all neuropeptides and hormones involved in the regulation of the HPG axis. But the female cyclicity is highly dependent upon hormonal fine regulation including estrogenic activity.

[13] European Chemicals Agency (ECHA) (2017b) : Support document for identification of 4,4'-isopropylidenediphenol (Bisphenol A, BPA) as substance of very high concern
European Chemicals Agency (ECHA) (2021) : Support document for identification of 4,4'-(1-methylpropylidene)bisphenol; (bisphenol B) as substance of very high concern

Uncertainty 2: Reduced litter size could be due to developmental toxicity	Both effects on fertility and development could explain the reduced litter size. But there are enough evidence supporting that the disturbance of estrous cycle and following reduction on implantation contribute to this effect.
Uncertainty 3: Other endocrine MoAs possible	Most of these adverse effects could be due to different MoAs (estrogenic, anti-androgenic, steroidogenesis…), and there are *in vitro* evidences that the substance interfere with these pathways. However, the interference with the estrogenic pathway is the most consistent one, including regarding the link between the different KE.

Overall conclusion on the postulated MoA

The overall biological plausibility is strong and substantiated by a strong empirical support for the majority of postulated KEs. The substance disturbs the estrous cycle through increased estrogenic activity, affecting the implantation and ultimately resulting in reduced fertility and litter size. It is considered likely that this is an endocrine MoA as no alternative non-endocrine mode of action has been identified.

4.10.5.5 Other supportive ED Adverse effects

As presented in section 4.10.1, the focus is on the estrogen disrupting activities of BPS affecting female reproduction, as this is the most relevant pathway. However, it is known that BPS, as other bisphenols, interferes with different hormone pathways (estrogen, androgen, steroidogenesis, thyroid, PPARγ, etc.) and triggers various adverse effects on health.

As supportive information, additional potential ED-related adverse effects are presented in this section. However, these effects were not assessed in detail , nor is a complete MoA provided for each of these endpoints.

EATS-mediated adverse effects

Effect on sperm count and motility

Sperm quality is a known sensitive marker of estrogenicity and anti-androgenicity. As EAS-mediated adverse effect, a change in sperm count and/or sperm motility is considered per se as indicative of an EAS modality (OECD GD 150, 2018).

All literature studies published so far reported that BPS exposure affects the production of sperm already at low dose, resulting in significantly reduced sperm count and sperm motility in rats (Ullah *et al.*, 2018b, 2019 and 2021). Less consistent effects have also been observed in mice (Shi *et al.*, 2017, 2018 and 2019c):

- In Ullah *et al*. (2018b), rats exposed to BPS from weaning until 48 weeks showed a significantly lower sperm count (53.3, 52.2, 50.3 and 48.2** $x10^6$, resp. at 0, 5, 25 and 50 µg/L), and a significantly reduced sperm motility (79.6, 78.1, 75.3 and 74.3** %). The sperm maturation was also affected, resulting in a significant decreased number of spermatogonia, spermatocytes and spermatids at highest dose, whereas the testis epithelium height was significantly reduced.

- Ullah *et al*. (2019) observed similar results after gestational exposure of SD rats, i.e. a significantly lower sperm count (73.4, 63.3, 62.3 and 61.3* $x10^6$, resp. at 0, 5, 25 and 50 µg/L), a significantly reduced sperm motility (79.6, 78.1, 75.3* and 74.3** %), and an affected spermatogenesis with significantly reduced count of spermatogonia,

spermatocytes and spermatids, as well as a reduced testis epithelium height, at highest dose.

- Ullah *et al*. (2021), repeated the chronic exposure for 48 weeks from 2018, including a lower dose and histopathological analysis. The results were similar to the previous studies, showing a significantly lower sperm count (52.0, 50.6, 49.7 and 47.3** $x10^6$, resp. at 0, 0.5, 5 and 50 µg/L) and a significantly reduced sperm motility (77.9, 78.2, 73.9, and 72.9* %). Again, the sperm maturation was affected, showing a reduced number of spermatocytes and spermatids, as well as reduced epithelial heights in testis after exposure to 5 and 50 µg/L BPS.

- In Shi *et al*. (2017), significantly lower sperm count was observed (6.4, 2.5** and 3.8** $x10^6$/ml, resp. at 0, 50 µg or 10 mg/kg bw/3 days), as well as a significantly reduce sperm motility (76.8, 67.2* and 63.1**%, resp. at 0, 50 µg or 10 mg/kg bw/3 days).

- Shi *et al*. (2018) examined sperm count and revealed a severe and significant lower sperm count (66** and 55***% resp. at 0.5 and 20 µg/kg bw/d expressed as % of control), but no effect was observed at the highest dose (50 µg/kg bw/d). In the same way, sperm motility was affected at 0.5 µg/kg bw/d, but not at the 2 highest doses.

- Shi *et al*. (2019c) examined transgenerational effects of BPS on the reproductive function of male mice. After prenatal exposure of F1 to BPS, F3 males showed alteration of the sperm parameters. Their sperm counts were significantly reduced, showing 40*** and 48***% sperm in comparison with the control group, at 0.5 and 50 µg/kg bw/day, resp. Sperm motility was also significantly reduced at 0.5 µg/kg bw/day (but not at higher dose). The spermatogenesis was also disturbed, showing significantly more testis tubules in stages I-VI, and significantly less tubules in stage IX. Again, this effect was only observed at 0.5 µg/kg bw/day and not at 50 µg/kg bw/day.

Among the TG studies, sperm count and sperm motility were assessed only in the EOGRTS (OECD TG 443, Unpublished study report, 2019), in which no reduction of sperm count has been observed in neither parental nor F1A cohorts. The sperm motility was slightly, but statistically significant reduced in F0 (88, 84*, 85* and 86*%, resp. at 0, 20, 60 and 180 mg/kg bw/d), although this was not observed in the F1A. The effect on sperm motility observed has to be considered as borderline, as the lowest value observed in F0 corresponds to the control value in F1A.

These effects of pre-, perinatal or adult BPS exposure on sperm count and sperm motility are remarkably consistent at low doses but were not observed at the high doses used for the EOGRTS (OECD TG 443, Unpublished study report, 2019).

Based on the general knowledge, these effects are indicative of endocrine activity. Moreover, several literature studies also showed that the spermatogenesis is disturbed after exposure to BPS in both mice and rats. Additionally, Shi *et al.* (2019c), reported that exposing mice during the pregnancy could affect the third generation, demonstrating that these effects could even be transgenerational. Histopathological analyses of rats exposed to BPS revealed that BPS modifies the structure of the testis (Ullah *et al.*, 2016, 2018b, 2019 and 2021).

These effects on sperm and spermatogenesis are probably due to a reduction in testosterone level, that has been reported in most of the literature studies (Shi *et al*., 2019c; Ullah *et al*., 2016, 2018a, 2018b, 2019, 2021), but not in all (Shi *et al*., 2017, 2018).

As a consequence of these effects on sperm, the male fertility could be reduced. Indeed, Shi *et al.*, 2017, reported a male subfertility (i.e. more time was needed to fertilise unexposed females). Additionally, the fertility was affected in OECD TGs 421 and 422 (Unpublished study report, 2000 and Unpublished study report, 2017b), but not in the EOGRTS (OECD TG 443, Unpublished study report, 2019). However, as in the TG studies both males and females have

been exposed to BPS, it is not possible to determine if the reduction of fertility is a consequence of the impact of BPS on the female or the male reproductive system, or both.

As conclusion, these data confirm that **due to its endocrine activity properties, BPS exposure affects the sperm production and quality at low doses**, resulting potentially in a reduced male fertility.

Table 17: Summary table of studies showing an affected sperm quality

Reference	Species	Routes	Doses Exposure period	Sperm quality (morphology and count)
Unpublished study report, 2019 OECD TG 443,	Rat (SD)	Oral (drinking water)	0, 20, 60 and 180 mg/kg bw/d (0.5% CMC) P0: from 10w before mating to PND 21 F1A: for 13w F1B: to PND 21 F2A: for 11w F2B: for 3w F3: for 8w	P0 : ↘ sperm motility (88, 84*, 85* and 86* % (St. dev. : 5, 7, 8 and 4) Other sperm parameters not affected F1A : No effect on sperm motility (84, 83, 84 and 83% (St. dev. : 10, 8, 7 and 8) Other sperm parameters not affected
Shi *et al.*, 2017	Mice (CD-1)	Subcutaneous	0, 50 µg or 10 mg/kg bw From birth to PND 60 (exposure every 3d)	↘ sperm count (6.4, 2.5** and 3.8** x10^6/ml (SEM : 0.2, 0.2 and 0.3) ↘ sperm motility (76.8, 67.2* and 63.1**% (SEM : 1.2, 1.7 and 2.1)
Shi et al., 2018	Mice (CD-1)	Oral	0, 0.5, 20 and 50 µg/kg bw/d GD 11 to birth	↘ sperm count (66** and 55*** % resp. at 0.5 and 20 µg/kg bw/d, but no effect at high dose). ↘ sperm motility at 0.5 µg/kg bw/d, but no effect at higher doses.
Shi *et al.*, 2019c	Mice (CD-1)	Oral	0, 0.5 and 50 µg/kg bw/d GD 7 to birth in F1 (effects in F3 males)	Transgenerational effects: ↘ sperm count (40%*** and 48%*** of reduction). Sign. ↘ sperm motility at 0.5 µg/kg bw/d, but not at higher dose
Ullah *et al.*, 2018b	Rat (SD)	Drinking water	0, 5, 25 and 50 µg/kg bw/d From PND 23 and for 48w	↘ sperm motility (79.6, 78.1, 75.3 and 74.3** % (SEM : 0.54, 0.51, 1.10 and 0.74) ↘ DSP (53.3, 52.2,

				50.3 and 48.2** $x10^6$ (SEM : 0.6, 0.5, 0.8 and 0.5) Nb of different cell types in testis : ↘ spermatogonia (65.7, 63.4, 63.6 and 61.6* (Sem : 0.62, 1.05, 1.15 and 0.87) spermatocytes (77.1, 74.7, 73.8 and 72.1* (SEM : 1.06, 1.30, 1.23 and 1.24) and spermatids (257.3, 250.0, 248.3 and 244.0** (SEM : 1.79, 2.77, 2.52 and 2.01)
Ullah *et al.*, 2019a	Rat (SD)	Drinking water	0, 5, 25 and 50 µg/kg bw/d GD 1 to 21	↘ sperm motility (79.6, 78.1, 75.3* and 74.3**% (SEM : 0.54, 0.51, 1.10 and 0.74) ↘ DSP (73.4, 63.3, 62.3 and 61.3* $x10^6$ (SEM : 0.6, 1.5, 0.2 and 0.6) Nb of different cell types in testis : ↘ spermatogonia (64.7, 62.4, 62.6 and 60.6* (SEM : 0.61, 1.04, 1.14 and 0.85), spermatocytes (76.1, 73.7, 72,8 and 71.1* (SEM : 1.05, 1.31, 1.22 and 1.23) and spermatids (256.3, 251.1, 247.3 and 243.0* (SEM : 1.77, 2.75, 2.51 and 2.03)
Ullah *et al.*, 2019b Similar to OECD TG 407	Rat (SD)		0, 5, 25 and 50 mg/kg bw/day 28 days	↘ daily sperm production (sign. at high dose) ↘ sperm motility trend (87.8, 85.1, 84.9 and 83.7%)
Ullah *et al.*, 2021	Rat (SD)	Drinking water	0, 0.5, 5 and 50 µg/l BPS From PND 23 and for 48w	↘ sperm motility (77.9, 78.2, 73.9 and 72.9* % (SEM : 1.28, 1.77, 2.13 and 1.06) ↘ daily sperm production (52.0, 50.6, 49.7 and 47.3** $x10^6$ (SEM : 0.78, 0.89, 0.93 and 0.36)

				↗ spermatogonia (36.4, 61.8, 61.3 and 59.4* (SEM : 1.12, 0.92, 1.51, 1.22) ↘ spermatocytes (75.5, 75.2, 70.1** and 68.3*** (SEM : 1.30, 1.35, 1.14 and 1.07) and spermatids (258.5, 258.2, 251.4* and 246.3** (SEM : 1.92, 2.68, 1.54 and 1.87)

Affected mammary gland morphology

Affected mammary gland morphology is a marker of endocrine activity in both female and males, and is recognised as EAS-mediated adverse effect (EFSA/ECHA ED Guidance, 2018).

Remarkably, almost all OECD TG studies reported a dose-dependent increased incidence of male mammary gland atrophy after BPS exposure (see Table 18):

- 7/10 and 10/10 after exposure to 100 and 1000 mg/kg bw/d in OECD TG 408 (Unpublished study report, 2014),
- 10/10 after exposure to 300 mg/kg bw/d in OECD TG 422 (Unpublished study report, 2017b),
- 2/20 and 7/20 after exposure to 60 and 180 mg/kg bw/d in F1A in OECD TG 443 (Unpublished study report, 2019).

In scientific literature study, only one study investigated the effect of BPS on the male mammary gland, affecting morphology in mice exposed pre- and perinatally to 200 µg/kgbw/day (Kolla *et al*., 2019).

The mammary gland is known to be estrogen-sensitive, particularly in males (EFSA/ECHA ED Guidance, 2018). Indeed, estrogenic compounds have been shown to promote the growth of the male mammary gland (Szabo and Vandenberg, 2021). Furthermore, the authors concluded "that growth of the male mammary epithelium in the mouse might be similar to changes in anogenital distance, another outcome that does not cause mortality itself, but is rather associated with diminished health and increased risk of male reproductive diseases.". However, the atrophy of male mammary gland has been described as related to an anti-androgenic activity (Rudmann *et al.*, 2012).

Overall, the effects observed with BPS treatment was of depressive nature in male rats while increased development was observed in male mice at 100-fold lower doses (Kolla *et al*., 2019), supporting therefore an anti-androgenic modality at high dose levels or a pro-estrogenic effect at lower dose levels. However, the *in vitro* assays assessing the potential anti-androgenic activity of BPS were not conclusive, showing inconsistency between the different studies, some indicating an anti-androgenic activity (among others Park *et al.*, 2020, Molina-Molina *et al.*, 2013 and Conroy-Ben *et al.*, 2018), whereas others did not show any AR binding and/nor anti-androgenic activity (among others Rosenmai *et al.*, 2014, Skledar *et al.*, 2016, Eilebrecht *et al.*, 2019). Similar pattern of effects (atrophy versus increased mammary gland development) was also

observed with Bisphenol AF (ECHA RAC Opinion BPAF, 2019).

Therefore, despite uncertainties regarding the modality (anti-androgenic or pro-estrogenic), these results confirm that **BPS endocrine disrupting properties affect the male mammary gland** in rodents.

Table 18: Summary table of studies showing an affected mammary gland morphology

Reference	Species	Routes	Doses Exposure period	Mammary glands
Unpublished study report, 2019 OECD TG 443,	Rat (SD)	Oral (drinking water)	0, 20, 60 and 180 mg/kg bw/d (0.5% CMC) P0: from 10w before mating to PND 21 F1A: for 13w F1B: to PND 21 F2A: for 11w F2B: for 3w F3: for 8w	Atrophy of male mammary gland in F1A (0, 0, 2 and 7 animals affected of 20 animals per group) No effect in P0 nor F1B
Unpublished study report, 2017a RF(28d) for OECD TG 443	Rat (SD)	Oral (drinking water)	0, 100, 300 and 600 mg/kg bw/d 28d	↗ incidence of diffuse atrophy (3 at mid dose and 4 at highest dose)
Unpublished study report Range finding study for OECD TG 443, 2017b OECD TG 422	Rat (SD)	Oral	0, 30, 100 and 300 mg/kg bw/d Males: 10w Females: From pre-mating until PND21	Atrophy of male mammary gland (0, 0, 0 and 10 animals affected of 10 animals per group)
Unpublished study report, 2014 OECD TG 408	Rat (Wistar)	Oral	0, 100, 300 and 1000 mg/kg bw/d (for males, the highest dose changed to 600 mg/kg bw/d onwards 70 days) 90d	Atrophy of male mammary gland (0, 0, 7 and 10 animals affected of 10 animals per group) Furthermore, severity increased (at mid dose, 7 of grade 1 while at the highest dose 4 of grade 2, 2 of grade 3, 3 of grade 4 and 1 of grade 5)
Kolla *et al.*, 2019	Mice (CD-1)	Oral	0, 2 and 200 µg/kg bw/d GD 9 to PND 2 or 20	Changed morphology of the mammary gland in adult males (larger epithelial trees)

Adverse effects sensitive, but not diagnostic of EATS

Increase of post-implantation loss

Almost all OECD TG studies reported a strong dose-dependent increase of post-implantation loss (see Table 19):

- In the EOGRTS (unpublished study report, 2019), BPS exposure increased significantly the mean number of post-implantation loss in the parental generation with 0.5, 0.8, 1.3* and 1.5* after exposure to 0, 20, 60 and 180 mg/kg bw/d (corresponding to 3.1, 5.9, 9.4* and 10.5*%). Similar effect has been observed in cohort F1B, with 0.9, 0.8, 1.1 and

3.3** after exposure to 0, 20, 60 and 180 mg/kg bw/d BPS (corresponding to 6.4, 5.3, 11.1 and 24.6*%).

- This effect was even stronger in the range-finding study similar to OECD TG 422 (Unpublished study report, 2017b), showing 3.6, 5.2, 6.5 and 34.6*% of post-implantation loss at 0, 30, 100 and 300 mg/kg bw/d, resp.).

- In a rat developmental toxicity study (unpublished study report, 2014), a very slight increase in percentage of post-implantation loss was observed.

The difference in the percentage of post-implantation loss across these studies can be explained by the difference in test design of these studies. The reproductive toxicity studies have a much longer duration of exposure from pre-mating, mating, gestation until lactation (TG 443, Unpublished study report, 2019 and TG 422, Unpublished study report, 2017b) compared to developmental toxicity study (OECD TG 414) with an exposure limited to gestation (GD 6-GD 19) only.

Moreover, the increase of post-implantation loss is dose-dependent among the studies, and cannot be explained by maternal toxicity, as the general condition of the dams were not affected.

Post-implantation loss could be the consequence of different eventsamong various endocrine MoA. It might be due to an event occurring prior implantation (e.g. decreased quality of oocyte by altered hormone levels) that results in post-implantation loss. Some literature studies support this hypothesis, showing that BPS exposure reduced the embryonic development after *in vitro* fertilisation of oocytes collected in untreated animals (Desmarchais *et al*., 2020; Sabry *et al*., 2021). Moreover, oocytes collected in mice exposed *in vivo* to BPS showed an inhibition of embryo development already at 2-cell stage (85.9, 83.3, 77.6, 67.6*, 65.5*, and 61.9* after exposure to 0, 1, 5, 10, 50 and 100 µg/kg bw/d) (Nourian *et al*., 2020).

However, other causes cannot be excluded with the actual data set, such as impact on the progesterone levels (that are essential to maintain the gestation), the placenta of the dams (producing the gestational hormones), etc. Therefore, these data show that **BPS exposure increases the post-implantation loss, maybe due to the endocrine activity of BPS**, resulting in a reduced number of pups.

Table 19: Summary table of studies showing an increase in post-implantation loss

Reference	**Species**	**Routes**	**Dose** **Exposure period**	**Post-implantation loss**
Unpublished study report, 2019 OECD TG 443,	Rat (SD)	Oral Drinking water	0, 20, 60 and 180 mg/kg bw/d (0.5% CMC). P0: from 10w before mating to PND 21 F1A: for 13w F1B: to PND 21 F2A: for 11w F2B: for 3w F3: for 8w	P0 : ↗ mean nr of post-implantation loss (0.5, 0.8, 1.3* and 1.5**, corresponding to 3.1, 5.9, 9.4* and 10.5** %). F1B : ↗ mean nr of post-implantation loss (0.9, 0.8, 1.1 and 3.3**, corresponding to 6.4, 5.3, 11.1 and 24.6** %).
Unpublished study report, 2017b OECD TG 422	Rat (SD)	Oral	0, 30, 100 and 300 mg/kg bw/d (in CMC). Males: 10w Females: From pre-mating until PND21	↗ % of post-implantation loss (3.6, 5.2, 6.5 and 34.6** %).
Unpublished study report, 2014 OECD TG 414	Rat (Wistar)	Oral (gavage)	0, 30, 100 and 300 mg/kg bw/d. From GD6 to GD19	Very slightly ↗ at the highest dose (4.7, 3.9, 3.9 and 6.3 %).

Increased weight of adrenal glands in male

Almost all OECD TG studies reported a dose-dependent increase of relative adrenal gland weight in males (see Table 20):

- In the EOGRTS (Unpublished study report, 2019), BPS exposure induced a statistically significant increase of the relative weight of adrenal glands in the parental generation with 0.010, 0.011, 0.011* and 0.012* after exposure to 0, 20, 60 and 180 mg/kg bw/d (corresponding to 54.0, 56.0, 58.8* and 60.6* mg in absolute weight). Similar effect was observed in cohort F1A with 0.014, 0.014, 0.014 and 0.016** (corresponding to 65.0, 63.2, 63.6 and 70.5 mg in absolute weight), and in cohort F1B, with 0.011, 0.012, 0.012* and 0.013** after exposure to 0, 20, 60 and 180 mg/kg bw/d BPS (corresponding to 59.8, 62.6, 67.7** and 64.7 mg in absolute weight)

- In the 28-days repeated dose study (TG 407, Unpublished study report 1999), SD rats exposed to 0, 40, 200 and 1000 mg/kg bw/d BPS showed a statistically significant higher relative weight of adrenal gland at the highest dose, with 0.018, 0.019, 0.018 and 0.033** (corresponding to 70, 71, 66 and 101** mg). At the highest dose, a hypertrophy of the adrenal glands was detected in almost all male animals (5/6). The increase of adrenal glands weight was still observable at the end of the 2-weeks recovery period (relative weight: 0.021** after exposure to 1000 mg/kg bw/d vs 0.014 in control; absolute weight: 80** mg vs 59 in control)

- In the 90-days repeated dose study (TG 408, Unpublished study report 2014), a similar increase of adrenal glands relative weight was observed, with 0.016, 0.016, 0.018 and 0.029** after exposure to 0, 100, 300 and 1000 (600 from D70) mg/kg bw/d (corresponding to 64.5, 59.1, 63.7 and 90.1** mg in absolute weight). In this study also, hypertrophy of the adrenal glands was found in male animals exposed to the highest dose (8/10).

- In the reproductive study performed as dose-range finding study for the EOGRTS (TG 422, Unpublished study report 2017b), a slight non-statistically significant increase of adrenal glands relative weight was reported, with 0.015 after exposure to 300 mg/kg bw/d vs 0.014 in control.

- In the EOGRTS non-TG dose-range finding study similar to 28-day repeated dose (Dose Range Finding Study, Unpublished study report 2017a), a higher relative adrenals weight was reported with an increase of 18 % and 35 % after exposure to 300 and 600 mg/kg bw/d, compared to the control group. Moreover, minimal hypertrophy/hyperplasia in the adrenal cortex was observed in 3/5 males exposed to 600 mg/kg bw/d.

Interestingly, the effect on females is less pronounced, with only one study showing an increased weight of adrenal glands in females with 0.030, 0.030, 0.036* and 0.039* % after exposure to 0, 100, 300 and 1000 mg/kg bw/d (corresponding to 65.6, 64.5, 74.6 and 80.4** mg in absolute weight) (OECD TG 408, Unpublished study report, 2000).

Increased weight of adrenal glands, or adrenocortical hypertrophy, is generally considered as a response to stress (Harvey and Sutcliffe, 2010). However, it could also be a consequence of a disturbed hypothalamic-pituitary-adrenal (HPA) axis. Indeed, it is known that a disturbed steroidogenesis reducing the production of glucocorticoid provokes the loss of feedback regulation in this axis. This leads to a compensatory over secretion of adrenocorticotrophic hormone (ACTH), promoting the growth/hypertrophy of adrenal gland weight, that could result in adrenal insufficiency (Harvey, 2016).

Whereas other modalities cannot be excluded, there are indications that the adrenal gland weight

increased following an endocrine disrupting cause. Indeed, two literature studies demonstrated that BPS reduced the production of cortisol in H295R cell line (human adrenocortical carcinoma) by 79% after exposure to 70 µM, compared to the control (Feng *et al.*, 2016) and up to 74% after exposure to 50 µM compared to the control (Rosenmai *et al.*, 2015). In this latter study, the level of corticosterone was also reduced by 70%.

Accordingly, despite many uncertainties there are indications that the **adrenal gland is affected by BPS exposure, maybe through its endocrine disrupting activity**. This effect seems to be more sensitive in male rodents.

Table 20: Summary table of studies showing an increase of adrenal glands weight

Reference	Species	Routes	Doses Exposure period	Adrenal glands
Unpublished study report, 2019 OECD TG 443,	Rat (SD)	Oral Drinking water	0, 20, 60 and 180 mg/kg bw/d (0.5% CMC) P0: from 10w before mating to PND 21 F1A: for 13w F1B: to PND 21 F2A: for 11w F2B: for 3w F3: for 8w	P0: In males: ↗ abs weight (54.0, 55.958, 58.75* and 60.625 mg) and ↗ rela weight (0.01, 0.011, 0.011* and 0.012* %) In females: no significant modifications (abs: 80.208, 70.391, 77.208 and 71.625 mg ; rela: 0.029, 0.025, 0.028 and 0.026 %) F1A: In males: abs weight not sign modified (65.0, 63.2, 63.6 and 70.5 mg) while rela weight ↗ at the highest dose (0.014, 0.014, 0.014 and 0.016** %) In females: no significant modifications (abs: 69.05, 69.15, 71.5 and 76.737 mg; rela: 0.029, 0.029, 0.029 and 0.031 %) F1B: In males: abs weight ↗ at the mid dose (59.792, 62.625, 67.708** and 64.708 mg) while rela weight ↗ at the 2 highest doses (0.011, 0.012, 0.012* and 0.013** %) In females: no significant modifications (abs: 76.708, 72.792, 77.435 and 80.083 mg; rela: 0.026, 0.026, 0.025 and 0.026 %) No histopathological changes observed
Unpublished study report, 1999 OECD TG 407	Rat (SD)	Oral	0, 40, 200 and 1000 mg/kg bw/d for main groups 0, 200 and 1000 mg/kg bw/d for recovery groups 28d	At the end of the exposure period: ↗ abs and rela weight in males at the highest dose (abs: 70, 71, 66 and 101** mg; rela: 18, 19, 18 and 33** %) Histopathology: ↗ inc of hypertrophy at the highest dose (in 5 males out of 6) In females: no significant modifications (abs weight: 72, 74, 65 and 74 mg) At the end of the recovery period: ↗ abs and rela weight in males at the highest dose (abs: 59, 60 and 80** mg; rela: 14, 14 and 21** %) In females: no significant modifications (abs weight: 71, 69 and 75 mg)
Unpublished study report, 2014 OECD TG 408	Rat (Wistar)	Oral	0, 100, 300 and 1000 mg/kg bw/d (for males, the highest dose changed to 600 mg/kg bw/d onwards 70 days)	In males: ↗ abs and rela weights at the highest dose (abs : 64.5, 59.1, 63.7 and 90.1** mg; rela: 0.016, 0.016, 0.018 and 0.029** %) At microscopic examination: hypertrophy/hyperplasia was noted in 8 males out of 10 exposed to the highest dose

			90d	In females: ↗ abs weight at the highest dose (65.6, 64.5, 74.6 and 80.4** mg) while rela ↗ at the 2 highest doses (0.03, 0.03, 0.036* and 0.039* %)
Unpublished study report, 2017b OECD TG 422	Rat (SD)	Oral	0, 30, 100 and 300 mg/kg bw/d (in CMC). Males: 10w Females: From pre-mating until PND21	relative adrenals weight not modified: 0.014, 0.014, 0.014 and 0.015 % in males and 0.027, 0.029, 0.03 and 0.03 % in females
Unpublished study report, 2017a RF for OECD TG 443	Rat (SD)	Oral (drinking water)	0, 100, 300 and 600 mg/kg bw/d 28d	relative adrenals weight was higher in males (+18 and +39 % resp. at 300 and 600 mg/kg bw/d, compared to the control group). Minimal hypertrophy/hyperplasia in the adrenal cortex was observed in 3 males of the highest dose

4.10.6 Overall conclusion on endocrine disruption with regards to human health

Based on all available scientific evidence, it can be concluded that BPS fulfils the WHO/IPCS (2002) definition of an endocrine disruptor with regard to the human health:

- It shows clear adverse effect on reproduction and development in mammals.
- It has endocrine mode of action: clear estrogenic mode of action.
- The adverse effects are considered EAS-mediated effects and are thus a consequence of the endocrine mode of action.

4.11 Other effects

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

4.12 Summary and discussion of human health hazard assessment

- The substance is identified as a substance meeting the criteria of Article 57(c) of Regulation (EC) No 1907/2006 (REACH) owing to its classification in the hazard class toxic for reproduction category 1B, H360FD.
- The substance(s) is identified as a substance(s) 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) according to Article 57(f) of REACH Regulation.

5. Environmental hazard assessment

5.1 Aquatic compartment (including sediment)

5.1.1 Fish

5.1.1.1 Short-term toxicity to fish

The acute toxicity in fish was tested according to OECD TG 203. The 96-hour LC50 value was determined to be above the limit dose of 100 mg/L (Registration dossier: Unpublished study report, 2010d).

A supporting study "Testing methods for industrial wastewater" performed according to Japanese Industrial Standard JIS K 0102-1986-71 under semi-static conditions with *Oryzias latipes* resulted in a 96-hour LC50 value above the limit dose of 100 mg/L (>500 mg/L) (Registration dossier: Unpublished study report, 1998).

5.1.1.2 Long-term toxicity to fish

- A non-GLP chronic toxicity study (Registration dossier: Unpublished study report, 2018b) with zebrafish was performed according to OECD TG 210 (Fish, Early-Life Stage Toxicity Test).
  The study deviated from the test guideline:

  - A reduced number of replicates per treatment group (2) was used
  - 8 concentrations + control were used
  - Growth assessment was measured at day 33/34 instead of 30 dph

  The 34d NOEC was determined to be 34d NOEC ≥10 mg/L (nom.) for hatch rate, post hatch survival and growth.

- Literature studies (Naderi *et al.*, 2014 and Ji *et al.*, 2013) showed reduced egg production and sperm count (tested concentrations were resp. 0.1, 1, 10 and 100 µg/L in Naderi *et al.* (2014) and 0.5, 5 and 50 µg/L in Ji *et al.* (2013), for more details on these 2 studies see section 5.7). Effects on development were also observed: among others, decreased hatchability in F0 and F1, increased malformation rates in exposed F1 embryos and reduced body weight in male F0 adults. Moreover, a skewing of phenotypic sex ratio was observed, which is a clear ED specific adverse effect.

- In the frame of the Substance Evaluation a Zebrafish Extended One Generation Reproduction test (ZEOGRT- adapted OECD TG 240, Unpublished study report, 2020) was performed to elucidate the environmental endocrine adverse effect as well as to determine a NOEC (Registration dossier: Unpublished study report, 2020).

  Zebrafish were exposed to nominal concentrations of the test substance of 2, 10, 50, 250 and 1250 µg/L. No effects were seen on survival, hatching, growth and reproduction in F0 and on survival in F1.

  Although length (females and males) was significantly affected in F1 at ≥10 µg/L after day 35, the effect was transient and no longer observed at day 65. The lowest NOEC of 250 µg/L was determined for male body length of F1 at the end of the study (day 125-128), which was significantly different from control at 1250 µg/L.

  In this study, a non-significant decrease of the sex ratio was shown for all concentrations

except for the treatment with 250 µg/L BPS. Nevertheless, the sex ratio at the 10 µg/L concentration fell below natural variation with only 29% of males. It should however be noted that, similar to the range-finding study, the percentage of males in the control group was very low (41%), impacting the sensitivity of the observations.

Histological analysis showed a dose-dependent decrease in the number of females with mature oocytes at the end of the experiment (total exposure duration of 170 days, ±150 d for F1) for all concentrations (calculated by the dossier submitter using Cochrane-Armitage trend analysis) and was significantly affected at 1250 µg/L (calculated by the dossier submitter using Fisher exact test).

Furthermore, F1 showed a decrease in fecundity for all concentrations (reduced by 43, 21, 36, 21 and 7%, resp. at 2, 10, 50, 250 and 1250 µg/L) and the reduction was significant at 2 and 50 µg/L (Wilcoxon test-one sided). Additionally, the preliminary study (range-finding test) resulted in a (non-significant) decrease in fecundity for concentrations of 3.2, 10 and 32 µg/L with reductions of 36, 70 and 50% resp. However, the statistical outcome is questionable as a Jonckheere-Terpstra-test was applied while the treatment mean had a non-ordering hypothesis (non-monotonic). Re-calculation using a Wilcoxon test showed, on the contrary, that fecundity was significantly reduced (by 70%) at 10 µg/L.

Furthermore, it should be noted that also fertility (non statistically significant) decreased in the range-finding study but also here the Jonckheere-Terpstra-test was applied (the Jonkheere-Terpstra test is a trend test, i.e. it will test whether there is a monotonic dose-response relationship between increasing doses and the effect studied). The reduction compared to the control was 91, 62, 64, 78 and 78%, resp at 3.2, 10, 32, 100 and 320 µg/L).

In the main study no effect on fertility in F1 was observed. While in the F2-generation, although minor, a statistically significant difference was determined on the hatching success (0-4 days) at 10 (94*%), 250 (95*%) and 1250 µg/L (94*%).

5.1.2 Aquatic invertebrates

5.1.2.1 Short-term toxicity to aquatic invertebrates

The acute toxicity to *Daphnia magna* was tested according to OECD TG 202 under static conditions (2001). The 48-hour EC50 value was determined to be 55 mg/L (REACH registration dossier: Chen *et al.*, 2002).

The 48-hour EC50 value was determined to be 100 mg/L in a second study (OECD TG 202, Unpublished study report, 2010d) with a *Daphnia sp*.

In a non-guideline study (US EPA report, 2014), a 96h EC50 of 45 mg/l was determined, supporting the results found in the key study. Information regarding the measured test substance concentration was not reported.

5.1.2.2 Long-term toxicity to aquatic invertebrates

The chronic toxicity to *Daphnia magna* was tested according to OECD TG 211 (21d Reproduction Test). The 21 days NOEC was determined to be 2.65 mg/L (meas. TWA). Measured values (time-weighted average values) were adopted for calculation of each effect concentration, because

some measured concentrations of the test substance exceeded ±20% of nominal values (Registration dossier: Unpublished study report, 2010d).

5.1.3 Algae and aquatic plants

The study examined the effect of BPS on green alga (*Desmodesmus subspicatus*) for 72 hours in a growth inhibition test under static conditions. The study was conducted in accordance with the OECD TG 201 and all validity criteria were fulfilled. The cultures were exposed to nominal concentrations of 0, 1.02, 3.2, 10.2, 32, 102, 320 mg/L. Since the analytically determined concentrations of the test substance in the test solutions were within ±20% of the nominal concentrations, the effect concentration was expressed relative to the nominal concentration. The 72-hour NOErC value was determined to be 10.2 mg/L and the ErC50 to be 106 mg/L (Registration dossier: Unpublished study report, 2010d).

In a second study, also conducted in accordance with OECD TG 201, the toxicity of BPS was examined in green algae (species not specified) and resulted in a 72h ErC50 of 65 mg/L and a 72h NOErC of 4.6 mg/L (Registration dossier: Unpublished study report, 2010e).

5.1.4 Sediment organisms

No studies available.

5.1.5 Other aquatic organisms

No studies available.

5.2 Terrestrial compartment

5.2.1 Toxicity to soil macro-organisms

No studies available.

5.2.2 Toxicity to terrestrial plants

No studies available.

5.2.3 Toxicity to soil micro-organisms

No studies available.

5.2.4 Toxicity to other terrestrial organisms

No studies available.

5.3 Atmospheric compartment

See section 3.2.3.2.2

5.4 Microbiological activity in sewage treatment systems

The toxicity of BPS to microorganisms was assessed in an activated sludge respiration inhibition test according to OECD TG 209 (Registration dossier: Unpublished study report, 2009). The EC10 and EC50 (3h) were 200 and 390 mg/L, resp.

5.5 Toxicity to birds

No studies available.

5.6 Mammalian wildlife

Mammalian data can be found in section 4. There is a large degree of conservation of the endocrine system, which implies large commonalities between non-mammalian and mammalian vertebrate species in regard to hormones, enzymes and receptors involved in the EATS' modalities (OECD GD 150, 2018). Therefore, evidence of endocrine disruptive properties of BPS on mammalian vertebrate species provides further support for similar properties in non-mammalian vertebrates, in particular with regard to disruption of estrogenic pathways.

5.7 Endocrine disruption (Environment)

5.7.1 Information sources and strategy for endocrine disruptor identification

Strategy and information sources

The same strategy and information sources as described for human health have been used for the ED assessment in the environment (see section 4.10.1 for more details).

The evaluation of BPS for the environment is based on fish data used for the environmental assessment and supported by available *in vivo* mammalian tests and adverse outcome pathways. Human health data can be used for the environmental ED assessment as mammals are also environmental organisms and those tested organisms are representatives for all other terrestrial and marine mammals (see section 5.7.2).

Considerations related to the relevance of data

The same considerations on the relevance of data described for human health apply also to the environment (see section 4.10.1 for more details).

The reliability of the *in vitro* studies on steroidogenesis (incl. aromatase activity) was not checked as there are reliable *in vivo* studies (with zebrafish) available examining steroid hormone synthesis and transcription of genes involved in steroidogenesis.

Both exposure via the medium/water and food were considered relevant exposure routes for environment.

5.7.2 Approach applied for Environment

The environmental endocrine assessment in this dossier is based on the Weight of Evidence approach in which both fish and rodent data are considered. Available fish data on observed adverse effects and modalities are discussed in section 5.7.3, while section 5.7.6 assesses the biological plausibility of the link between endocrine activity and adverse effects. Furthermore, as presented in section 4.10.5, there is strong evidence that the adverse effects on fertility and sexual function in rats, particularly in females, are plausibly linked to the estrogenic activity of the substance. BPS therefore is an endocrine disruptor according to the WHO/IPCS definition (WHO/IPCS, 2002) with regard to human health.

Additionally, EFSA/ECHA ED Guidance (2018) states that: "*effects on growth, development, reproduction in single species are generally regarded relevant for the maintenance of the wild population".* Such changes in both fish and mammals associated with endocrine modalities might pose unacceptable risks to the environment. It should be noted that effects observed in rats are of particular concern for wildlife species with a natural low reproductive output, including top predators and other mammals (including endangered species) as negative effects on reproduction have an even higher potential for causing long term negative effects at the population level for such taxa.

Reasons for using mammalian data (rodents) to support the endocrine properties of BPS for the environment:

- Conservation of the endocrine system and cross-species extrapolation

As outlined in the EFSA/ECHA ED Guidance (2018), mammalian data are always relevant for environmental ED assessment:

"*According to the Commission Delegated Regulation (EU) No 2017/21003 and Commission Regulation (EU) No 2018/6054, the conclusions as to whether the ED criteria are met need to be drawn separately with respect to humans and non-target organisms. However, it should be highlighted that there may be data available on non-target organisms relevant for the assessment of the ED properties with regard to humans. Furthermore because of the high level of conservation of the endocrine system across taxonomic groups, the mammalian data may also be relevant for other vertebrates (OECD, 2018b). Therefore, data on mammals and other taxa are considered together in a holistic approach as part of the available evidence, but also for identifying potential data gaps when assembling lines of evidence for endocrine activity and/or endocrine-related adversity. This means, for example, that information on endocrine effects in fish/amphibians, could be used to investigate the mammalian data set with heightened scrutiny for similar effects and to target potential requests for the generation of further mammalian information, or vice versa*.".

"It is recognised that the standard information requirements for BPs and PPPs currently require more studies which may be informative on ED properties with regard to human health and mammals than for other taxonomic groups. Thus, in line with the general principle of desired reduction of unnecessary animal testing, the assessment strategy aims at making the most efficient use of the available data set to reach a conclusion. Therefore, it is recommended to strive for a conclusion on the ED properties with regard to humans and in parallel, using the same database, to strive for a conclusion on mammals as non-target organisms. Only where, based on this assessment, the criteria are not met for mammals as non-target organisms, would the assessment need to proceed to the other taxonomic groups, which may require the generation of additional data. It is sufficient that the substance meets the ED criteria in one taxonomic group in order to conclude that a substance meets the ED criteria for non-target organisms."

Following the OECD Revised Guidance Document no 150 (2018), cross-species extrapolations should be considered during data assessment:

"*Endocrine systems with respect to hormone structure, receptors, synthesis pathways, hormonal axes and degradation pathways are well conserved across vertebrate taxa especially in the case of estrogen, androgen and thyroid hormones and steroidogenesis. In invertebrates, many systems are distinct from those in vertebrates and are not fully understood; however, the retinoic acid system is also relevant in many species (OECD, 2017b). When interpreting data for endocrine assessment, this conservation should be borne in mind as results from tests using human in vitro or non-human mammalian (in vitro and in vivo) systems may be highly relevant for vertebrate wildlife species and vice versa.*

In addition, results from non-human mammalian studies are also highly relevant for mammalian wildlife species. Caution should be exercised, however, when extrapolating in this way, as species differences in exposure pathways, ADME, organ physiology, effects of hormones at different life stages across taxa/classes and other differences should be considered. The consequences of the action of a hormone may be different in different species, even if the molecular initiating event is the same."

Moreover, the OECD Guidance no. 150 clearly indicates that: "*The in vitro screens in question (although at present based largely on mammalian receptors and/or enzymes) are generally capable of providing information applicable to both humans and vertebrate wildlife (OECD,*

2010d). Such extrapolation of in vitro information is generally qualitative (…)". Additionally, EFSA/ECHA ED Guidance (2018) states that: *effects on growth, development, reproduction in single species are generally regarded relevant for the maintenance of the wild population.* Therefore, in the view of the dossier submitter all information available from *in vitro* and *in vivo* (from both mammalian and fish) studies should be taken into account in the overall WoE approach.

Furthermore, EFSA/ECHA ED Guidance (2018) further specifies that:

- the same database can be used to conclude on the endocrine disrupting properties for human health and the environment: "*The information needed to assess ED properties for humans and non-target organisms may overlap. Mammalian data are always relevant for ED assessment on non-target organisms. Furthermore, there may be information on non-target organisms that could be relevant also for the ED assessment for humans*." and "[…] *it is recommended to strive for a conclusion on the ED properties with regard to humans and in parallel, using the same database, to strive for a conclusion on mammals as non-target organisms.*".
- *"effects on growth, development, reproduction in single species are generally regarded relevant for the maintenance of the wild population."*

Therefore, information available from *in vitro* and *in vivo* (from both mammalian and fish) studies should be taken into account in the overall WoE approach.

There is scientific evidence that BPS shows severe and irreversible effects on the reproduction of mammalian species. Such effects are considered to impair population stability and recruitment. Taking into account 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 (OECD GD 150, 2018) evidence of endocrine disruptive properties of BPS on mammalian vertebrate species therefore provides evidence for similar properties in non-mammalian vertebrates. For BPS, parameters strongly diagnostic of estrogenicity were seen in *in vivo* mammalian experimental studies (increase in uterus weight in uterotrophic assays and prolongation of the oestrus cycle). Adversity is proven by significant effects on fecundity and fertility in mammals (such as reduced number of implantation sites, reduced fertility index) leading to a reduced number of offspring. 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, as indicated in the EFSA/ECHA ED Guidance (2018). Hence, those can be used in the WoE approach to identify BPS as ED for the environment. The adverse effects are biologically plausibly linked to the ED activity as described in the support document and therefore BPS fulfils the WHO/IPCS definition of an endocrine disruptor for both human health and environment.

- <u>Rodents: their presence, their role and the impact on population/community level in the environment</u>

Rodents represent approx. 42% of all mammalian species, with approx. 2277 rodent species and therefore they can be considered the largest order of mammals in the world (Witmer and Shiels, 2017).

They play an important role in the environment in the dispersal of seed and spore, pollination, seed predation, energy and nutrient cycling, modification of plant succession and species composition and are a food source for many predators (Witmer and Shiels, 2017).

The adverse effects on the reproduction of mammalian species (rodents) due to exposure to BPS, described in section 4.10, can be plausibly linked to the estrogenic pathway and are considered to impair population stability and recruitment. Therefore, these effects are to be considered population relevant for human health as well as for the environment.

5.7.3 Lines of evidence (LoE)- EAS[14] modalities

5.7.3.1 LoE Adversity

A summary of the effects of BPS on toxicity for reproduction in fish is presented below:

- Reduced reproductive success

BPS exposure leads to a significant decrease in egg production which was seen both in literature studies as well as in an ZEOGRT (adapted OECD TG 240, Unpublished study report, 2020). In the latter, BPS impaired fecundity at low concentrations, similar to those for which an effect was seen in the literature studies.

BPS exposure impairs gametogenesis by decreasing sperm production and oocyte maturation, which was also observed in rodents. As a consequence of this bad egg quality, BPS impacted hatchability by increasing the time to hatch and decreasing the hatching success.

BPS exposure alters social behaviour.

- Altered sex ratio

BPS alters the sex ratio leading to feminisation. This was clearly demonstrated in a literature study (Naderi *et al.*, 2014). Also a trend towards feminisation was observed in the ZEOGRT (Unpublished study report, 2020), where the sex ratio at low concentrations (comparable to literature concentrations) was close to or even fell below natural variation.

Effects on sex ratio is considered an EATS-mediated effect and thus providing evidence for both endocrine activity and adverse effect. In fish, sperm count is not assessed in the current standardised OECD TG and is therefore not listed as EATS-mediated effect in the guidance document (EFSA/ECHA ED Guidance, 2018). However, based on the existing knowledge in mammals and the similarities with fish gametogenesis, this effect could be considered as EATS-mediated also in fish.

5.7.3.1.1 *In vivo* EAS-mediated parameters

- *Gametogenesis: sperm count*

Sperm count

In a developmental toxicity study (Naderi *et al.*, 2014), similar to OECD TG 234, 2 hpf embryos of zebrafish (*Danio rerio*) were exposed to 0 (solvent control: 0.01% acetone), 0.1, 1, 10 and 100 µg/l of BPS for 75 days under semi-static conditions. Both a solvent control and water control were present. Although not mentioned in the article, authors confirmed that results of the water control and the solvent control were the same. A statistically significant dose-dependent reduction in sperm count was observed at ≥10 µg/l BPS. Sperm morphology and sperm motility were not examined in this study. After 75 days of exposure, statistically significant mortality was

[14] EAS – estrogenic, androgenic and/or steroidogenic

observed at 100 µg/L (results made no distinction between males and females). Nevertheless, sperm count was already affected before overt toxicity took place.

Sperm count, sperm morphology and sperm motility were not examined in any other literature or guideline fish study. However, sperm count, sperm motility and spermatogenesis have been described in rodent literature studies (see section 4.10.5.5 Other supportive ED MoAs Male reproductive functions for further details).

Using a weight of evidence approach, results in fish and rodents show that **BPS affects the male reproductive system**.

- *Sex ratio*

In Naderi *et al*. (2014), the phenotypic sex ratio in adults (F0) skewed towards females in a dose-dependent manner. Respectively 58.8% and 66.7% females were observed at concentrations of 10 and 100 µg/l BPS, compared to 46.3% in the solvent control (X2-test, X2= 12.14, p = 0.016). As shown, this adverse effect occurred already at a concentration below that at which statistically significant mortality was observed (at 100* µg/L: +/-30% mortality compared to +/-5% in the control group).

In the ZEOGRT (Unpublished study report, 2020), a non-significant decrease of the phenotypic sex ratio in F1 was demonstrated after exposure to 2, 10, 50, 250 and 1250 µg/L of BPS in absence of mortality. This increasing trend in females was observed at all concentrations except at 250 µg/L (see table below).

Table 21: Phenotypic sex ratio in F1

Concentration (µg/L)	0	2	10	50	250	1250
Females (%)	59	68	71	67	51	70
Males (%)	41	31	29	33	49	30

It is noteworthy that the percentage of males fell below natural variation (30/70%) at 10 µg/L.

Therefore, available studies demonstrate that **BPS can alter the sex ratio in zebrafish**.

5.7.3.1.2. *In vivo*: parameters 'sensitive to, but not diagnostic of, EATS'

- *Reproduction (fecundity,fertility)*

Four experimental studies investigated the effects of exposure to BPS on reproduction (Naderi *et al*., 2014; Ji *et al*., 2013; ZEOGRTS (Unpublished study report, 2020); Qin *et al.*, 2021). Their results are reported in Table X3 in Annex I.

In Ji *et al.* (2013), performed according to OECD TG 229, adult zebrafish were exposed to 0.5, 5 and 50 µg/L of BPS and 0.1% Methanol for 21 days. No effect on survival was observed. Egg production statistically significantly decreased at ≥0.5 µg/L BPS and was half of that of the control at the highest concentration tested. From the additional information provided by the authors of this article, a dose-dependent significant decrease in egg production was observed: 19.36 at 0.5 µg/L, 15.99 at 5 µg/L, 12.69 at 50 µg/L vs 26.72 (solvent control) and 26.32 eggs/female/day in control). No data on fertility are reported by the authors.

In the developmental toxicity study of Naderi *et al.* (2014), egg production also decreased statistically significantly and in a dose-dependent manner in fish exposed to ≥10 µg/l BPS (+/-

half of the egg production compared to the solvent control). At 100 µg/l a statistically significant difference was seen in the survival rate of zebrafish exposed for 75d to BPS.

In the ZEOGRT (Unpublished study report, 2020) after exposure to 2, 10, 50, 250 and 1250 µg/L, fecundity (egg count from day 97-118) decreased in F1 at all concentrations in a non-dose dependent manner (resp. by 43, 21, 36, 21 and 7%) in absence of mortality. A statistically significant difference with control was seen at environmentally relevant concentrations using the Wilxocon test (one-sided), with respectively 8 and 9 eggs/ female reproduction day at 2 and 50 µg/L vs 14 eggs/female reproduction day in control. This effect was not statistically significant in the F0 generation although a slight decrease was seen in the 2 and 10 µg/L groups (by 9 and 19% resp.).

Also in the range-finding study of the ZEOGRT (Unpublished study report, 2020), which deviated from the requested test method OECD TG 210[15], fecundity decreased non-significantly between test day 111 and 128 at BPS concentrations of 3.2, 10 and 32 µg/L by 36, 70 and 50 % respectively in absence of mortality. Statistical analysis was performed according to a Jonckheere-Terpstra test. It should be noted that the mean values of the treatment had a non-ordering hypothesis (non-monotonic) which makes this statistical method not fit for observing significant effects in this case. A recalculation using the Wilcoxon test showed, on the contrary, that fecundity was drastically and statistically significantly decreased (by 70%) at 10 µg/L. A 36 and 50% decrease in fecundity was also noted after exposure to 3.2 and 32 µg/L, showing repeatedly the non-monotonic effect on fecundity.

Furthermore, in the range finding study, using the Jonckheere-Terpstra method, a non-statistically significant decrease in fertility was reported (compared to the control, reduction with 9, 38, 36, 22 and 22% at 3.2, 10, 32, 100 and 320 µg/L resp.). In the main study no effect on fertility in F1 was observed. While in the F2-generation, although minor, a statistically significant difference was determined on the hatching success (0-4 days) at 10 (94*%), 250 (95*%) and 1250 µg/L (94*%).

In contrast to the above studies, Qin *et al.* (2021), observed a statistically significant dose-dependent increase in the number of eggs/breeding tank/day after chronic exposure (2 hpf - 240 dpf) to 1 and 100 µg/L of BPS. All eggs were fertilised and resp. 75% and 82% survived at 1 and 100 µg/L after 24hpf compared to the solvent control (87% survival). However, the result is doubtful (number of eggs produced per female per reproduction day e.g. +/- 300 eggs/breeding tank/day at 100 µg/l BPS) . It is unclear in this study how many females were assigned per breeding tank.

Therefore, except for the Qin *et al.* (2021), all studies point toward an **impairement of fish fecundity** when exposed to environmentally relevant concentrations of BPS (with the lowest reported effect seen at 0.5 µg/L).

- *Oocyte maturation*

In the ZEOGRT (Unpublished study report, 2020), histological analysis showed a dose-dependent decrease in the number of F1 females with mature oocytes at the end of the experiment (total exposure duration of 170 days, ±150 d for F1) for all concentrations (2, 10, 50, 250 and 1250 µg/L of BPS) (calculated by the dossier submitter using Cochrane-Armitage trend analysis) and was affected in a statistically significant manner at 1250 µg/L (calculated by the dossier submitter using Fisher exact test). No effect on survival was seen in F1. This study was performed according to the adapted OECD TG 240 (protocol described in the Decision on substance evaluation for 4,4'-sulfonyldiphenol: https://echa.europa.eu/information-on-chemicals/evaluation/community-rolling-action-plan/corap-table/-/dislist/details/0b0236e180686aaf).

[15] The range finding study was performed according to OECD TG 210 followed by OECD TG 234 and subsequently followed by OECD TG 229.

Qin *et al.* (2021), reported a statistically significant decrease in primary oocytes and contrary to the observations in the ZEOGRT (Unpublished study report, 2020), an increase in full grown stage oocytes and a statistically significant increase of follicle- and vitellogenic-stage oocytes after a 240-d exposure of 2 hpf zebrafish to 1 and 100 µg/L of BPS. However, the results are unclear in this study as the increase in mature oocytes is expressed in percentage, not taking into account the atretic oocytes which were reported to increase in number. From this study, however, it can be concluded that BPS promoted the excessive use of lipids as an energy source for oocyte maturation as a result of elevated VTG content, disrupted lipid metabolism and lipid accumulation in the ovary and thus leading to a change in the early stage development (reduced hatching success at 1 µg/L).

For the determination of the oocyte maturation, Park *et al.* (2022)[16], divided oocytes in 3 stages: immature oocytes (incl. perinuclear oocytes and yolk vesicle oocytes), mature oocytes (incl. yolk globule stage oocytes) and regressed oocytes (observed oocyte degeneration). Adult male and female zebrafish were exposed to 0 (solvent control, 0.1% DMSO), 8, 40, 200 µg/mL BPS for 21 days. Concentrations were chosen such that they were below the EC15 of 250 µg/mL. Compared to the solvent control (no statistically significant difference with water control in reproductive parameters) and the 8 µg/ml exposure group where females mainly contained mature oocytes, regressed oocytes were found at the 40 and 200 µg/ml of BPS which are characterised by excess of oocytes atresia. It should be noted that the concentration range applied in this study is an order of magnitude higher than in most other studies.

However, it should be noted that this effect is in line with the one described in rodents, where oocyte maturation is affected after BPS exposure (decrease in secondary follicles (Shi *et al.*, 2019a; Zhang *et al.*, 2020; Liu *et al.*, 2021), preantral follicles (Nevoral *et al.*, 2018) and antral follicles (Ahsan *et al.*, 2018; Nevoral *et al.*, 2018; Zhang *et al.*, 2020; Liu *et al.*, 2021), increase in atretic follicles (Ahsan *et al.*, 2018; Ijaz *et al.*, 2020)).

Therefore, except for Qin *et al.* (2021), all studies demonstrate that **BPS impairs oocyte maturation in fish as well as in rodents.**

- *Hatching*

In the study of Ji *et al.* (2013), adult zebrafish were exposed to 0.5, 5 and 50 µg/L of BPS for 21 days. Eggs were collected on day 16 and futher examined with or without subsequent exposure to BPS. Time to hatch was statistically significantly and dose-dependently prolonged at all doses when eggs and parents were exposed, and statistically significantly prolonged when only parents were exposed to 50 µg/L BPS (Ji *et al.*, 2013). Also hatchability was statistically significantly and dose-dependently reduced at all doses when eggs and parents were exposed, but only statistically significantly reduced at 5 and 50 µg/L if only parents were exposed.

No significant effect on hatching success is observed in the F1 generation of the ZEOGRT study. However, survival in F2 decreased significantly at 10, 250 and 1250 µg/L at all concentrations (95%, 94%*, 95%, 95*% and 94%* at 2, 10, 50, 250 and 1250 µg/L).

In the study of Wei *et al.* (2018), performed in compliance with OECD TG 210 and 230 with minor modifications, freshly fertilised embryos were exposed to 1, 10 and 100 µg/L. A statistically significant reduction in hatching was reported at all concentrations compared to the solvent control (0.002% w/w DMSO) in F1 after 60 and 72hpf.

Naderi *et al.* (2014), reported a statistically significantly decreased hatching rate in F1 (~-40%) at 10 and 100 µg/L and an increased hatching time (~+10%) after exposure of embryos for 75 dpf. This effect on hatching is supported by the findings of Qin *et al.* (2021). Furthermore, Moreman *et al.* (2017) noted an increased hatching time at 72hpf and decreased hatchability

[16] Park *et al.*, 2022: this article was retrieved after 30 August 2021 (systematic review).

(non-significant, but dose-dependent) after exposure of freshly fertilised embryos to 10, 20, 50, 200 and 300 mg/L.

Zhang *et al.* (2017) reported a statistically significantly decreased hatchability after embryonal exposure at 30 µg/L of BPS (solvent control (0.01 v/v DMSO), 1, 3, 10 and 30 µg/L: at 72hpf: 81.2 ± 2.65, 79.5 ± 4.04, 73.3 ± 3.76, 74.1 ± 3.2 and 62.3 ± 5.55* %, at 96hpf: 94.5 ± 1.2, 93.3 ± 1.0, 90.8 ± 1.4, 91.2 ± 1.7 and 90.8 ± 1.9 %).

Maternal exposure to 1 and 100 µg/l of BPS resulted in a statistically significant decrease in hatchability in F1 at 1 µg/L at 60 and 72 hpf and at 100 µg/L at 48 hpf (Qin *et al.*, 2021).

Mu *et al.* (2018) (at high concentrations of BPS: 3, 6, 12.5, 25 and 50 mg/L) did not see any significant effect on hatching rate. Also Lee *et al.* (2019) did not observe a significant effect on hatchability in F1 after maternal exposure to relatively high concentrations of BPS (87.5 ± 16.0, 83.3 ± 0.0, 79.2 ± 16.0, 95.8 ± 83 and 87.5 ± 16.0 % resp. at solvent control (< 0.1% v/v DMSO), 0.4, 2, 10 and 50 mg/L). However a non-signifcant increase in time to hatch was determined (2.09 ± 0.11, 2.90 ± 0.12*, 2.51 ± 0.37, 2.38 ± 0.34, 2.51 ± 0.26 days).

Qiu *et al.* (2018b and 2021) measured a statistically significant increase in hatching rate after embryonal exposure. Significant effects were observed at 48 and 54h (1*, 10* and 100* µg/l) but no effect at 1000 µg/L in Qiu *et al.* (2018b) and at 48h at 100 µg/L (dose dependent increase: 10, 100* µg/L) in Qiu *et al*. (2021). This effect was however not observed in their 2016 publication where embryos were exposed to 100 µg/L of BPS for 25hpf as survival rate was not significantly affected (Qiu *et al.*, 2016).

Therefore, except for Qiu *et al.* (2018 and 2021), all studies point towards impaired hatching at low concentrations (range of 10 to 100 µg/L), therefore it can be concluded that **BPS affects hatching.**

- *Gonadosomatic index*

The Gonadosomatic index (GSI) was statistically significantly decreased in males at the highest concentration tested (50 µg/L), while in females a decrease was already observed at ≥ 0.5 µg/L by Ji *et al*. (2013). In both male and females, the decrease was dose dependent. Furthermore, these findings were seen in the absence of mortality. Also Naderi *et al*. (2014) observed a dose-dependent effect of the GSI in males and females compared to the solvent control, however males were more sensitive than females (statistically significant decrease at 10 and 100 µg/L in males and only at 100 µg/L in females). It should be noted that statistically significant mortality was observed at 100 µg/L in this study.

Park *et al*. (2022), found no effect on the GSI in adult males exposed to 0, solvent control (0.1% DMSO), 8, 40, 200 µg/mL BPS for 21 days, while the GSI in adult females increased in a statistically significant manner at 40 µg/mL (16.06%) and decreased at 200 µg/mL (5.17%) compared to the solvent control. This was observed in absence of mortality.

No effect on GSI was measured in the ZEOGRT in any generation (Unpublished study report, 2020).

Altogether, based on these diverging results no overall conclusion can be drawn. It should be noted that GSI is a rather general parameter for gonadal development. However, histological analysis of the gonads is the most accurate method for maturity staging.

- *Behaviour*

Several studies reported an effect of BPS exposure on zebrafish behaviour, mainly on social behaviour, memory and locomotion.

Kinch *et al.* (2015), reported the induction of precocious hypothalamic neurogenesis in embryonic zebrafish after exposure to BPS, more specifically during the neurogenic window. This was associated with hyperactive behaviour in zebrafish larvae. BPS exposure (6.8 nM=1.7µg/L) resulted in a 240% increase in neuronal birth in the rostral hypothalamus at 24 hpf. Furthermore, a significant increase (160%) in locomotor activity was observed after BPS exposure. This increase in locomotor activity was reduced by transient knockdown of aromatase B, but not by treatment with 1 µM ICI 182,780 (an estrogen receptor antagonist). Together, these data imply that BPS influences hypothalamic development and locomotor activity. In zebrafish, the hypothalamus participates to the regulation of locomotor activity (McPherson *et al.*, 2016), suggesting that the increased hypothalamic neurogenesis was possibly responsible of the observed increased activity. In addition, the increased locomotor activity was related to changes in the hypothalamic production of neuroestrogens since transient knockdown of brain aromatase expressed specifically in hypothalamic progenitor cells counteracted these effects.

Exposure of zebrafish embryos from 2 hpf until 5 dpf, to 0.001 µM (~0.25 µg/L) lead to thigmotaxis, an anxious behaviour where animals have the tendency to remain close to the walls of an arena (Naderi *et al*., 2022)[17]. Fish exposed to low and high concentrations (0.001 µM; 0.1 µM) showed signs associated with social deficits (e.g. decreased inter-fish distance), while fish exposed to a high concentration of 0.1 µM of BPS showed object recognition memory disorders (e.g. decrease in exploration ratio) at 21 days of age. Co-exposure to the aromatase inhibitor fadrozole reversed the anxiogenic effects of BPS, except for object recognition memory, which was the only effect reversed by co-administration with the ER inhibitor ICI 182,780. C Moreover, high and low BPS concentrations had opposite effects on the expression of *it*-genes (sign. increased at 0.001µM, sign. decreased at 0.1 µM). Furthermore, 0.001µM BPS statistically sign. increased the expression of esr1 and esr2a genes, while expression of esr2b was statistically sign. reduced at mid and high dose. BPS induced a biphasic change in the mRNA expression levels of nkcc1, while the mRNA expression of kcc2 in the larval brain was upregulated after exposure to low BPS concentration. Furthermore, it was shown that E2 exposure upregulated the transcription of both genes in larval fish. At higher concentrations of BPS nkcc1 and kcc2b were downregulated, probably due to the impact of BPS on sex steroid hormone levels or estrogen signaling. Those genes allow the excitatory/inhibitory switch of GABAergic neurons in the brain, a switch that is associated with neuronal developmental defects when affected. Moreover, Song *et al.* (2017) demonstrated that GABA (via $GABA_B$ receptors) regulates GnRH3 neurons in a developmentally dependent manner in zebrafish.

Salahinejad *et al.* (2020) investigated the social behaviour (shoaling, social preference and locomtory activity) in zebrafish by exposing 9 months old male and female fish for 75 days to a solvent control (0.01% v/v) 1, 10 and 30 µg/L BPS and to 1 µg/L E2 (positive control). They reported a statistically significant increase in inter-individual distances (decreased shoal cohesion) at 30 µg/L BPS and 1 µg/L of E2, while excursion behaviour increased non-significantly. At all BPS concentrations and 1µg/L of E2, social preference for conspecifics (group preference) was statistically significantly and dose-dependently affected, as fish swam farther away from each other. These changes in social behaviour were observed together with altered gene expression of isotocin (*it*) and arginine vasotocin (*avt*). Both are neuropeptides of the brain involved in regulation of non-reproductive social behaviours like group preferences, shoaling behaviour and social approaches, which are crucial for mating. BPS and E2 upregulated the transcription of the *it*-gene in a non-monotonic manner (sign. at 30 µg/L BPS) in males while it was downregulated in females (non-monotonic, sign. at 30 µg/L BPS). *itr*-expression was dose-dependently upregulated in males (sign. at 30 µg/L BPS and 1 µg/L E2) and non-significantly, not-dose dependently downregulated in females. Furthermore, BPS and E2 also altered the expression levels of *avt* in a dose-dependent manner (sign. at 30 µg/Lin males and females; sign. at 1 µg/L E2 in females only). While an up-regulation in the expression of *avt* gene was found in males and females, E2 and BPS decreased the transcript levels of AVT-receptors in both sexes. It is suggested that changes in IT and its receptors may be due to the estrogenic activity of BPS in males, as males have lower estrogen levels than females. In females, however, it might be due to a direct interference with the estrogen receptors or by a negative feedback

[17] Naderi *et al*., 2022 : this article was retrieved after 30 August 2021 (systematic review).

mechanism. Furthermore, the authors concluded that the shift in balance from IT towards AVT is possibly the most important underlying mechanism for alteration of the social behaviour in zebrafish. No effect on locomotory activity was observed.

Naderi *et al.* (2020) showed that social recognition was significantly affected in female adult fish (9 months old) after 120 days exposure to 1,10 and 30 µg/L BPS. Fish exposed to 10 and 30 µg/L BPS spent statistically significantly less time to explore unfamiliar conspecifics than the control group (social recognition). No changes were observed after 1 µg/L E2-treatment. A statistically significant lower exploration was also observed when the object was moved to a new location in the object placement test at 30 µg/L BPS, while in the object recognition test significant less time was spent (decreased exploration ratio) with a novel object already at ≥ 10µg/L BPS.

Furthermore, Salahinejad *et al*. (2022)[18] exposed 9 months old female zebrafish for 60 days to 1, 10 and 30 µg/L BPS and 1 µg/L E2. After mating with non-exposed males, no BPS could be detected above the detection limit of ~0.25 µg/L in the collected eggs. Maternal exposure to BPS affected the inter-individual distance, among shoal members of 6 months old male offspring, which was significantly decreased at 10 µg/L. Maternal treatment with 30 µg/L BPS also significantly increased the number of excursions that the offspring undertook from the shoal. Fish group preference was reduced compared to the control in a not dose-dependently manner and was only significant after maternal exposure to 1 µg/L BPS. Moreover, maternal exposure to 1 and 30 µg/L BPS and 1 µg/L E2 caused a significant decrease in the anxiety response as less time was spent at the bottom half of the tank by male offspring. Also here, no significant effect on locomotion was recorded.

Maternal exposure induced a non-dose-dependent increase in AVT-levels in the male offspring brain (only sign. at 1 µg/L BPS) while no significant effect was observed on IT and its receptors. *avp*-genes from the AVT receptor on the other hand were significantly upregulated at 1 and 10 µg/L BPS and non-significantly downregulated at 30 µg/L.

Wang *et al*. (2020b) exposed adult male and female zebrafish of six months old to a blank control, a solvent control, and three concentrations of BPS (1, 10 and 100 µg/L). In shoaling behavioural experiments, female shoaling was weakened, as the time ratio spent in the area close to other fish was significantly decreased ($p<0.05$), and the time ratio in the far area was significantly increased in the female 100 µg/L BPS group ($p<0.05$) compared to the solvent control. Female fish from BPS-treated groups also showed altered external features. Depigmentation (a significant decrease in melanin) of the body color was observed for the 1 µg/L BPS group ($p<0.05$) and for the 10 and 100 µg/L BPS groups (both $p<0.01$) when comparing to the solvent control group. Histological examination of ovaries showed that the total proportion of late vitellogenic and spawning oocytes in ovaries was 28.12 in the solvent control and reduced to 11.46 (1 µg/L BPS), to 17.89 (10 µg/L BPS) and to 13.68 (100 µg/L BPS). It was also clear that during mating behaviour, untreated males spent significantly less time close to the BPS-treated females (1, 10 and 100 µg/L BPS; $p<0.05$), when comparing with the solvent control group. The authors linked the lower attraction potential of BPS-treated females to the fact that untreated females had a higher proportion of late vitellogenic and spawning oocytes, and to a changed body color pattern of BPS-treated females.

These new intergenerational approaches, or exposure of embryonic stages, to environmental doses show that several behavioural alterations can be detected in non-target organisms, which might cause population alterations (and have an impact on mating).

Taken together, these data imply that **BPS influences hypothalamic development and mainly acts through aromatase, but estrogenic mechanism cannot be excluded as well.**

[18] Salahinejad et al., 2022 : this article was retrieved after 30 August 2021 (systematic review).

5.7.3.1.3. Population relevance of adverse effects

Effects on growth, development, reproduction in single species are generally regarded relevant for the maintenance of the wild population (European Commission, 2011 in EFSA/ECHA ED guidance, 2018). Behavioural changes and impaired ability to cope with additional stress are factors implicitly covered by the WHO definition of adversity, since they would affect the reproductive performance and the development. Furthermore, endpoints related to growth (body weight and length), and reproduction such as fecundity, sex ratio, hatching success and offspring survival, measured in several in vivo ecotoxicology fish tests, are generally considered to be population relevant (Marty *et al.*, 2017; Table 1 of this publication). Therefore, it can be concluded that BPS can affect population stability by altering population relevant parameters like fecundity, growth, sperm numbers, hatching rate, sex ratio and social behaviour.

Furthermore, clear EATS-mediated effects were seen in rodents (see section 4.10.5 MoA Human health for BPS). 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 modalities (OECD GD 150, 2018). Therefore, observed effects for human health are also considered population relevant for mammalian wildlife.

Although male fertility in rodents was affected it was not considered adverse. It should be noted however that fertility in rodents is not considered a sensitive indicator of testicular toxicity.

5.7.3.2 LoE Endocrine Activity – EAS

See also Annex I for tabulated lines of evidence.

5.7.3.2.1 *In vitro* mechanistic data (OECD level 1 and 2)

In vitro tests show that BPS can interfere with different hormone pathways. A clear estrogenic activity of BPS, via ER binding and ER activation is demonstrated. BPS has also been shown to disrupt steroidogenesis, impact the production of testosterone and aromatase activity.

Moreover, *in vitro* assays show that BPS can activate PPARγ receptors, show weak anti-androgenic activity and impact binding and activation of thyroid hormone receptors although effects are less obvious.

Most apparent findings are related to estrogen and steroidogenic pathways. For estrogenic pathway see section 4.10.2.2 LoE Endocrine Activity – EAS while steroidogenesis pathway is described below.

Estrogen modality

For tabulated lines of evidence see Annex I.

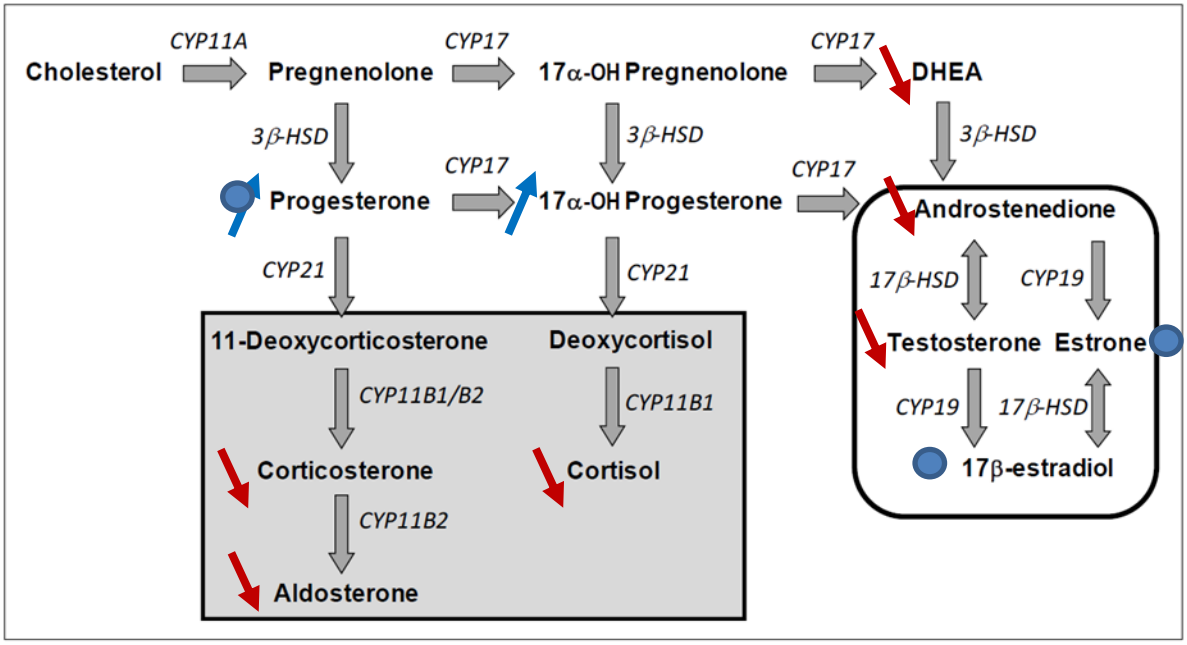
Two *in vitro* studies are available with zebrafish cells. These are described in section 4.10.2.2.

Steroidogenesis

For tabulated lines of evidence see Annex I.

Steroidogenesis of BPS was examined in three H295R Steroidogenesis Assays (human adrenocortical carcinoma cell line) (Rosenmai *et al.*, 2014; Goldinger *et al.*, 2015; Feng *et al.*, 2016). Only one of those studies was performed following OECD TG 456 (Goldinger *et al.*, 2015). All studies showed clear decrease in testosterone levels but did not detect a significant change in estradiol concentration. Rosenmai *et al.,* 2014, reported also a significant lower level of the male hormones dehydroandosterone (DHEA) and androstenedione. In both non-guideline studies, an increase in progesterone was observed, together with a decrease in cortisol levels. Furthermore, exposure of BPS lead to a decrease in corticosterone (Rosenmai *et al.*, 2014) and aldosterone (Feng *et al.*, 2016).

Figure 4: Steroidogenic pathway in H295R cells (figure from OECD TG 456)



no effect ; increase; inconsistent result; decrease

Several studies assessed changes in hormone levels in testis explants of rats, mice and humans (Eladak *et al.*, 2015; Ullah *et al.*, 2016; Desdoits-Lethimonier *et al.*, 2017; Ullah *et al.*, 2018a).

In Eladak *et al.* (2015), BPS significantly reduced testosterone secretion in mouse fetal testis explants exposed to 100 nM. Moreover, BPS was found to be more potent than BPA (decrease observed at 1000 nM). In human fetal testis explants, the dose-response was also non-monotonic, as seen by Desdoits-Lethimonier *et al.* (2017). In adult rat testis explants, the measured testosterone concentration was reduced, although not statistically significant (Ullah *et al.*, 2016 and Ullah *et al.*, 2018a).

In the study of Roelofs *et al.,* 2015, using MA-10 mouse Leydig cells, no effect on testosterone was observed. However, BPS induced a significant increase of pregnenolone and progesterone

at 10^{-5} M. Indeed, it is known that Leydig cells are missing 17α-hydroxylase/C17–20 lyase. It is therefore not surprising that no effect on testosterone can be observed.

Although it was observed at physiological irrelevant concentration (100 µM, highest dose tested), estradiol concentration was increased to about 4-fold in bovine granulosa cells after BPS exposure. However, in the presence of FSH, no effect has been observed. Furthermore, androstenedione concentration was not affected in bovine theca cells after BPS exposure, in presence or absence of LH. Progesterone concentration was also unaffected in both cell types (Campen *et al.*, 2018a). A significant increase in estradiol expression was also seen in ovine granulosa cells, together with a reduction in progesterone secretion. (Téteau *et al.*, 2020).

Furthermore, several authors assessed *in vitro* the expression of genes involved in steroidogenesis:

BPS (as BPA) tended to reduce the expression of fetal Leydig cell-specific genes involved in testosterone biosynthesis (Eladak *et al.*, 2015).

In the study of Feng *et al.* (2016) no disruption was reported in H295R cells for genes involved in progesterone production (StAR, FDX-1, CYP11A1 and HSD3B2), but downregulation of single genes involved in testosterone/17β-estradiol production (CYP17A1) were observed.

In mouse spermatocytes exposed to 10^{-10} or 10^{-8} M, BPS increased StAr, CYP11a1, Hsd17b3, CYP17a1 and CYP19a1 gene expression (Sidorkiewicz *et al.*, 2018).

Furthermore, Williams and Darbre (2019) evaluated aromatase in three different human breast cell lines (MCF-7, ZR-75-1 and HMF3A). In all cell lines a significant increase of CYP19A1 mRNA synthesis and aromatase activity was shown as well as a significant increase in 17β-estradiol synthesis.

Based on the above findings, it can be concluded that **steroidogenesis is impaired after exposure to BPS**. The divergence in the *in vitro* results might be explained (among others) for example by the difference in exposure concentrations or cell lines used.

5.7.3.2.2 *In vivo* mechanistic data in fish (OECD Level 3/4/5)

In several fish studies, endocrine activity was reported after exposure to BPS. ES-mediated activity is described underneath (Ji *et al.*, 2013; Naderi *et al.*, 2014; Kinch *et al.*, 2015; Qiu *et al.*, 2016; Cano-Nicolau *et al.*, 2016; Wang *et al.*, 2017a; Moreman *et al.*, 2017; Le Fol *et al.*, 2017; Mu *et al.*, 2018; Qiu *et al.*, 2018b; Anjali *et al.*, 2019; ZEOGRT, Unpublished study report, 2020; Qin *et al.*, 2021; Qiu *et al.*, 2021).

Estradiol receptor binding, aromatase induction, change in hormone balance (steroids, gonadotropins) and steroidogenesis modulation

- Zebrafish

It should be noted that hormone balance is not included in the OECD TG 240 (MEOGRT) as a biological endpoint to be measured. Therefore no results are available in the ZEOGRT (Unpublished study report, 2020).

Ji *et al. (*2013), performed according to OECD TG 229, reported a statistically significant dose-dependent increase of plasma E2 in male and female adult fish and a statistically significant decrease of plasma testosterone in males at the highest dose after exposure for 21 days to 0.5, 5 and 50 µg/L of BPS and 0.1% methanol. These findings were observed in the absence of mortality. Furthermore, there was no significant difference between the water and solvent control.

The transcription of genes involved in the HPG axis was affected by exposure to BPS. Gnrh3, gnrhr1, and gnrhr2 genes were up-regulated in male fish suggesting that BPS could modulate concentrations of GnRHs in fish, which could subsequently affect production of gonadotropin hormones. In vertebrates, gonadotropin-releasing hormone (GnRH) has a crucial role on the control of reproduction through HPG axis and regulates the synthesis and release of gonadotropin hormone. In Zebrafish, two types of GnRH (GnRH2 and GnRH3) and four different GnRH receptors (GnRHR) exist. The up-regulation of fshβ, lhβ, fshr and lhr genes in male, and the decrease of fshβ in female observed in this present study, supports the fact that BPS can indirectly affect gonadotropin hormones.

In a developmental study (Naderi *et al.*, 2014) where 2 hpf embryos of zebrafish (*Danio rerio*) were exposed to 0 (solvent control : 0.01% acetone), 0.1, 1, 10 and 100 µg/l of BPS for 75 days, plasma levels of 17β-estradiol (E2) were statistically significantly and dose-dependently increased in both male and female fish, resp. at 1 µg/l and 10 µg/L in absence of mortality. Plasma testosterone (T) statistically significantly decreased in males exposed to 10 and 100 µg/l in a dose-dependent manner, whereas in females a dose-dependent non-statistacally significant increase at ≥10 µg/L was observed. This confirms the alteration of sex hormones seen in the 21 day reproduction study of Ji *et al*. (2013), where these alterations were accompanied by up-regulation of the aromatase (CYP19a in the gonads and CYP19b in the brain) genes and down-regulation of CYP17 and 17βhsd genes. Authors confirmed that results of the water and solvent control were the same.

Exposure to 100 µg/L of BPS induced a significant increase in the number of Hypothalamus-GnRH3 neurons (Qiu *et al.*, 2016 and 2021). GnRH neurons integrate internal and external cues to control sexual maturation and fertility (Zhao *et al.*, 2016). In the study of Qiu *et al*. (2016), BPS also significantly up-regulated the expression of the reproduction-related genes kiss1/kiss1r (upstream regulator of GnRH neurons, also in fish), gnrh3 and erα, lhβ, fshβ after 5 days exposure. BPS did not affect kiss2, kiss2r and sv2 gene expression levels. Study on the implication of ERs in the neuroendocrine system suggest that not erβ, but erα mediates the effects of BPS as only a statistically significant increase of erα was observed at 25 hpf. Fadrozole was the main effective inhibitory compound on BPS-induced effects suggesting that the effects are mainly aromatase-mediated.

An aromatase B-mediated mechanism (aromatase B, the key enzyme for local estradiol synthesis) was demonstrated by Kinch *et al*. (2015), in embryonic zebrafish on day 5 post fertilisation following exposure to 0.0068 µM BPS (= 1.7 µg/L BPS). Androgens have been shown to increase CYP19a1b expression in zebrafish. After exposure to BPS, increase in neuronal birth in the rostral hypothalamus and in locomotory bursting activity was observed. This effect was reduced by transient knockdown of aromatase B, but not in the presence of an ER antagonist (ICI) demonstrating an aromatase-mediated mode of action.

Cano-Nicolau *et al. (*2016), demonstrated the induction of aromatase expression and estrogenic activity in zebrafish embryos after exposure to 1 µM of BPS (250 µg/L). BPS caused a 41-fold induction of CYP19a1b expression and a 6-fold induction CYP19a1b promotor activity in the brain of a transgenic zebrafish line, although the latter was not statistically significant. Furthermore, brain sections of 7 dpf larva brains demonstrated identical CYP19a1b transcripts distribution patterns in the whole brain as to EE2 and BPA. EE2 and BPA showed over-expression in posterior telencephalon, preoptic area and caudal hypothalamus, including the nucleus recessus posterioris where the induction was the strongest.

Furthermore, Moreman *et al. (*2017), exposed transgenic zebrafish expressing estrogen-responsive element linked to GFP (strain Tg(ERE:Gal4ff)(UAS:GFP)) to 10, 20 and 50 mg/L BPS from 1 to 120 hpf. BPS induced GFP expression, mainly in the heart (up to 2.7- and 10.8-fold more than in control at 20 and 50 mg/L, resp.), but also in the liver and the tail. The GFP expression was totally inhibited after addition of ICI 182,780, an estrogen receptor antagonist.

Le Fol *et al. (*2017), report a 4-fold induction of GFP expression at 30 and 60 µM BPS (7500 and 15 000 µg/l resp.) in a zebrafish embryo assay (EASZY assay) based on the CYP19a1b-GFP

transgenic line expressing the GFP under the control of the ER-regulated CYP19a1b aromatase gene in the brain. However, co-exposure with ICI 182,780 1 µM did not block the effect of BPS, suggesting the involvement of an ER-independent pathway. It should be noted that co-exposure with ICI was not tested at the concentration reaching the highest activation level measured in the dose response curve (60 µM). Furthermore, the ratio BPS/ICI (30 µM BPS vs 1 µM ICI) was very high at the concentration tested (30 µM BPS). Therefore, it is not surprising that the inhibition (at very low concentration of ICI compared to BPS) was not sufficiently to inhibit the effect of BPS on aromatase.

In Qiu *et al. (*2018b), embryonic zebrafish were exposed to environmentally relevant concentrations of 1, 10 and 100 µg/L BPS. Exposure to 100 µg/L BPS for 120 h increased significantly mRNA levels of erα and nf-κb in zebrafish. And co-exposure with the ER antagonist ICI 182,780 and NF-κb antagonist pyrrolidine dithiocarbamate (PDTC) significantly attenuated the stimulatory actions of BPS on gene expression of the cytokines il-1β, il-6 and tnfα. Antagonists of ER, TR and aromatase blocked many of the effects of BPS on reproduction-related gene expression in zebrafish embryos, providing evidence that those three pathways (partly) mediate the actions of BPS on the reproductive neuroendocrine system (Qiu *et al.*, 2016). The study of Qiu *et al.* (2021) confirms that fadrozole attenuated the stimulatory actions of BPS on fshβ.

Zebrafish embryos were exposed to 2.5, 12.5 and 25 mg/L for 96 h to assess developmental toxicity (Mu *et al.*, 2018). Neither ERα protein nor ERs-coding gene (esr1, esr2a, esr2b or vtg1) were induced or affected after 96 h of exposure.

After exposure of adult zebrafish to 0, solvent control (0.1% DMSO), 8, 40, 200 µg/mL BPS for 21 days, no sign. effect was seen on hepatic ERα mRNA activation in males and females compared to the solvent control (Park *et al.*, 2022). However, ERβ activation decreased significantly in males, while no effect was seen in females. Whole body concentrations of progesterone and 17ß-estradiol (E2) levels were significantly and dose-dependently increased in females while testosterone and 11-ketotestosterone were significantly decreased in male fish. E2 increased significantly and dose-dependently in males. The ratio E2/T was significantly increased in male fish indicating aromatase activity.

After exposure of zebrafish embryos (2hpf) to 1 and 100 µg/l untill 120 hpf, a statistically significant increase in FSH and E2 was reported by Qiu *et al.* (2021) resp. at ≥ 1* µg/l and 100* µg/L. Also levels of LH and GH increased, although not statistically significantly. Furthermore, this study confirms the findings of their previous study (Qiu *et al.*, 2016) concerning kiss1, lhβ an erα. Gnrh3-expression, however, was increased, but not significantly. BPS also statistically significantly upregulated cyp 19a1 and cyp 19a2 genes at 100* µg/L. Unlike their previous study, kiss2 was statistically significantly increased at 100* µg/L. While the studies of Qin *et al.* (2016 and 2018b) suggest that not erβ, but erα mediates the effects of BPS as only a statistically significant increase of erα was observed, the study of 2021 demonstrated a statistically significant increase of erβ (erβ1 at 1* and 100* µg/l and erβ2 at 100* µg/L).

- Other fish than zebrafish

Hormone balance was also disturbed after exposure of *Oreochromis mossambicus* juvenile and adult fish to 100, 120 and 140 mg/L BPS and 100, 125 and 150 mg/L resp. (Anjali *et al.*, 2019). Estradiol was statistically significantly increased in juvenile males, while significantly decreased in adult males, female juveniles and female adults. Furthermore, they observed a significant decrease of testosterone in juvenile and adult males and a significant increase of E2/T ratio in male juveniles and adults.

The *in vivo* coherent findings in zebrafish show a **clear alteration**, after **BPS exposure**, in the **transcription of genes involved in steroidogenesis** resulting in a **change in steroid hormones synthesis.**

Vitellogenin and gene transcription

VTG promotes oocyte maturation after it is secreted from the yolk particles of the oocytes into the bloodstream (Qin *et al.*, 2021).

- Zebrafish

In the developmental toxicity study, similar to OECD TG 234 described above (Naderi *et al.*, 2014), plasma VTG synthesis was statistically significantly and dose-dependently induced in females and males, resp. at 10 and 100 µg/l after 75d exposure. This response could be triggered through exerting estrogenic effects. Increase of E2 can also lead to an induction of VTG.

During a 7d exposure of adult male zebrafish to 0.1, 1 and 10 µM of BPS (25, 250 and 2500 µg/L resp.), also a statistically significant increase of plasma VTG was observed at 0.1 and 1 µM BPS (Le Fol *et al.*, 2017). At the highest dose however high mortality was observed.

Qin *et al.* (2021), observed statistically significantly increased VTG levels in plasma in female zebrafish only at 1 µg/L and in ovary at 1 and 100 µg/L after exposure from 2 hpf until 240 dpf to 1 and 100 µg/L of BPS, but no changes in liver were observed.

However, in a ZEOGRT (Unpublished study report, 2020), performed according to a zebrafish adapted OECD TG 240, it was concluded that no significant effect was seen on VTG (measured in head/tail homogenates) in F1. In this ZEOGRT, zebrafish were exposed to 2, 10, 50, 250 and 1250 µg/L for a total of 150 days in the absence of a solvent. VTG was calculated per mg whole fish body. It is known that homogenate tend to show slightly lower VTG values than plasma (Holbech *et al.*, 2001; Nilsen *et al.*, 2004; Örn *et al.*, 2006).

Park *et al*. (2022), observed a statistically significant down-regulation of hepatic VTG gene expression in adult females at ≥ 40 µg/mL, while a significant increase was seen in males at 200 µg/mL compared to the solvent control after exposure of 21 days to 0, solvent control (0.1% DMSO), 8, 40 and 200 µg/mL under semi-static conditions.

- Other fish than zebrafish

Exposure to BPS induced a statistically significant increase in Vitellogenin (Wang *et al.*, 2017a) in adult male guppies (*Poecilla reticulata*) at all doses (1, 10 and 100 µg/L of BPS), the maximal being reached at 10 µg/L BPS in all tissues (fin, liver and whole animal).

Most of the data show that **BPS** exposure leads to an **increased VTG concentration in male and female fish**.

5.7.4 Lines of evidence – T[19] modality

Data on T-modality are available but were not assessed further in detail in this report as a plausible link is postulated between reproductive adverse effects and the estrogenic modality and steroidogenesis. Data on binding and activation of thyroid hormone receptor were not consistent across studies.

[19] T - thyroidal

5.7.5 Lines of Evidence – Other modalities

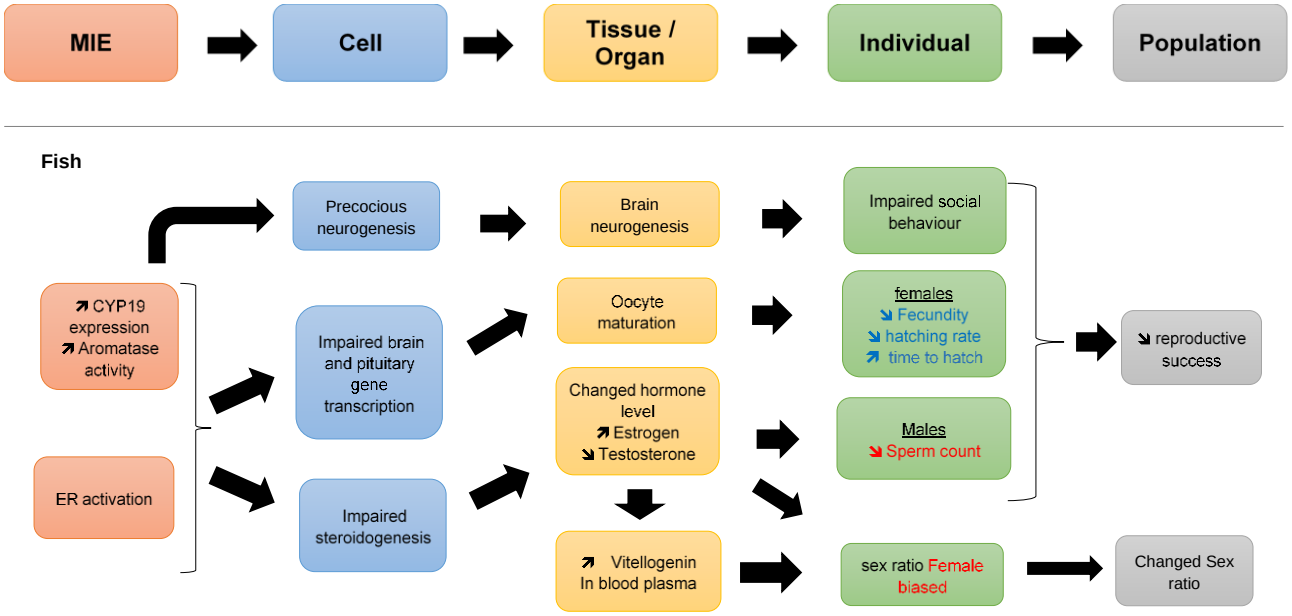
Several *in vitro* assays show that BPS can act on other modalities (AhR, CAR, GR, PPAR, …). These were not assessed further in detail as focus was put on Estrogen and Steroidogenesis. Some weak anti-androgenic activity has been observed *in vitro*, but the effect of BPS on this pathway is less obvious due to contradictory results.

5.7.6 Mode of Action (MoA) analysis

5.7.6.1 Postulation of MoA(s)

Postulated estrogenic and steroidogenic MoA affecting reproduction and development in fish is presented in Figure 5. The MoA presented here does not describe every detail of the biology, but instead focuses on describing critical steps, acknowledging that other activities could also influence each of the key events described.

Figure 5: Scheme showing the postulation of MoA(s)



EATS-mediated adverse effect
Sensitive but not diagnostic of EATS

Exposure to BPS leads to agonistic estrogenic activity

Several *in vitro* studies demonstrate a clear estrogenic activity of BPS via ER-binding and ER activation.

In vivo studies conducted with zebrafish show that **BPS impairs VTG levels and thus shows estrogenic mode of action**:

"VTG is normally produced by the liver of female oviparous vertebrates in response to circulating endogenous estrogen. It is a precursor of egg yolk proteins and, once produced in the liver, travels in the bloodstream to the ovary, where it is taken up and modified by developing eggs. The VTG synthesis is very limited, though detectable, in immature fish and adult male fish because they lack sufficient circulating estrogen. However, the liver is capable of synthesizing and secreting VTG in response to exogenous estrogen stimulation. […] The measurement of VTG serves for the detection of chemicals with estrogenic, anti-estrogenic, androgenic modes of

action and chemicals that interfere with steroidogenesis as for example aromatase inhibitors… […] The biological relevance of the VTG response following estrogenic/aromatase inhibition is established and has been broadly documented" (OECD TG 234).

BPS statistically significantly induced plasma VTG production at 100 µg/L in male zebrafish and at 10 and 100 µg/L in females (Naderi *et al.*, 2014) and even from 1µg/L in females [no measurement in males (by Qin *et al.*, 2021)] resp. after 75 days and 240 days. This induction is however not observed in F1 in a one-generation study (ZEOGRT, Unpublished study report, 2020).

Induction of vtg mRNA transcript expression level was also measured in male zebrafish (40-200* µg/mL) by Park *et al. (*2022) while, in females, a statistically significant enhanced transcription was measured at 8 µg/mL and, conversely a statistically significant decrease in vtg transcripts was assessed at 200 µg/mL. The estrogenic effect is also measured using transgenic zebrafish by measuring activation of an ERE-GFP system (Moreman *et al.*, 2017). BPS induced GFP expression, mainly in the heart (up to 2.7- and 10.8-fold more than in control at 20 and 50 mg/L, resp), but also in the liver and the tail. The GFP expression was totally inhibited after addition of ICI 182,780, an estrogen receptor antagonist.

BPS disrupts steroidogenesis (aromatase activity) in zebrafish by impairing gene transcription and consequently changing steroid hormone levels.

"Teleost fishes have two copies of the CYP*19a1 gene that encode two isoforms of aromatase:* CYP*19a1a encodes ovarian aromatase, while the* CYP*19a1b gene encodes brain aromatase (aromatase B). Aromatase B is strongly expressed in radial glial cells (RGC), that act as stem cells in mammals and fish and the* CYP*19a1b gene is very sensitive to estrogens, through a mechanism that involves a well conserved ERE" (Brion et al., 2012).*

A statistically significant increase in gonadal and brain aromatase transcript levels was measured in adult males (Ji *et al.*, 2013). This was accompanied by statistically significant changes in *CYP11a*, *3βhsd*, *CYP17* and *17βhsd* transcripts in testes and *hmgra* and *hmgrb* in ovaries. The significant upregulation of cyp19a1 (gonads) and cyp19a2 (brain) genes after embryonal exposure (1 and 100 *µg/L) BPS was confirmed by Qiu *et al.* (2021).

Furthermore, Cano-Nicolau *et al*. (2016), also demonstrated the induction of aromatase expression and estrogenic activity in the brain of CYP19a1b-GFP transgenic zebrafish larvae after exposure to 250 µg/l (1 µM, single concentration tested) of BPS (EASZY-assay). BPS caused a 41-fold induction of CYP19a1b expression and a 6-fold induction CYP19a1b promotor activity in the brain, although the latter was not statistically significant. Furthermore, brain sections of 7dpf larva brains demonstrated identical CYP19a1b transcripts distribution patterns in the whole brain as to EE2 and BPA. These findings were confirmed by Le Fol *et al*. (2017), who also reported an increased aromatase activity in the brain of transgenic aromatase GFP zebrafish embryos.

Exposure to BPS leads to altered steroid synthesis in zebrafish, characterised by increased E2 production in both sexes and decreased T production in males. Ji *et al*. (2013), measured a dose-dependent and statistically significant increase in E2 production (all doses in males: 0.5, 5 and 50 µg/L, up to 4-fold), and a statistically significant increase only at 50 µg/L for females (with 2.5-fold). Naderi *et al*. (2014), reported an increase of E2 production in a dose-dependent manner in both males and females and significantly from 1 to 100 µg/L and from 10 to 100 µg/L in males and females resp. Park *et al*. (2022), also reports a dose-dependent and statistically significant increase in whole body E2 in males (8*, 40*, 200* µg/mL BPS). All doses induced a significant (and dose-dependent) increase in whole body concentrations of E2 (8*, 40*, 200* µg/mL BPS) and Progesterone in females (40* and 200* µg/mL). Furthermore Qiu *et al.* (2021) observed a statistically significant increase of E2 after embryonal exposure (2hpf until 120hpf) at 1* and 100 * µg/L.

Furthermore, BPS disrupts endocrine signalling, neurogenesis in the brain and impacts behaviour.

"Fish receptor gene (fshr) is essential in folliculogenesis (Zhang et al., 2015b). When FShβ receptor genes are completely deleted in female zebrafish, the follicle activation completely fails. Ovarian follicles arrest at the primary growth -previtellogenic transition causing a delay of the onset of puberty, followed by complete reversion into fertile males. In fshr-deficient males, spermatogenesis was normal in adults, but the initiation of spermatogenesis in juveniles was retarded. Fshr can be activated by both FSH and LH in zebrafish. LH-deficient zebrafish showed normal gonadal growth, but females failed to spawn and were therefore infertile" (Zhang et al., 2015a).

"Zebrafish have four types of gonadotropin-releasing hormone receptors which are expressed in a variety of tissues including the brain, eye and gonads" (Tello et al., 2008). In all vertebrates, the brain hormone gonadotropin-releasing hormone (GnRH) activates a cascade of events leading to gametogenesis (Tello et al., 2008).

"Mature animals lacking (Preoptic area-hypo) GnRH3 neurons exhibited arrested oocyte development and reduced average oocyte diameter. Animals in which GnRH3 neurons were partially ablated exhibited normal oocyte development, however, their fecundity was significantly reduced" (Abraham et al., 2010).

A statistically significant increase in the transcript levels of gnrh3, gnrhR1 and 2, fsh, lhβ was measured in males, while the synthesis of gnrh3 and, fsh messengers were significantly decreased in females (Ji *et al.*, 2013). Low levels of BPS also up-regulated the expression of kiss1/kiss1r (upstream regulator of GnRH neurons), gnrh3 and erα in embryonic and early larval stages at 100 µg/L and showed a ER, TR and CYP19 mediated pathway (Qiu *et al.*, 2016). In this study, BPS significantly increased the number of Hypothalamus-GnRH3 neurons.

Exposure to BPS (6.8 nM=1.7µg/L) during the embryonic phase (0-5 dpf) resulted in an early 240% increase in neurogenesis in the rostral hypothalamus at 24 hpf and a significant increase (160%) in locomotor activity of the 5-dpf fish (Kinch *et al.*, 2015). These effects were absent when co-exposed in the presence of brain aromatase morpholinos, but still effective in co-treatment with 1 µM ICI 182,780. In zebrafish, the hypothalamus participates to the regulation of locomotor activity (McPherson *et al.*, 2016), suggesting that the increased hypothalamic neurogenesis was possibly responsible of the observed increased activity. In addition, the increased locomotor activity was related to changes in the hypothalamic production of neuroestrogens since transient knockdown of brain aromatase expressed specifically in hypothalamic progenitor cells counteracted these effects.

Exposure to BPS during this early period of neuronal development (2hpf-5 dpf) leads to delayed effects on behaviour, measured at 21 dpf (Naderi *et al.*, 2022). BPS altered social behaviour (statistically sign. decreased inter-individual distances at 0.25µg/L and 25 µg/L BPS and social preference (time spent near conspecifics) at 0.25 µg/L). These effects were accompanied by a statistically significant increase in anxiety (significant at 0.25 µg/L), measured by an increased thigmotaxis (tendency of an animal to remain close to the walls of an arena), and by changes in object recognition memory (a decrease in the time spent exploring a new object), sign at 25 µg/L. Co-exposure to the aromatase inhibitor fadrozole reversed the anxiogenic effects of BPS, excepted for object recognition memory, which was the only effect reversed by co-administration with the ER inhibitor ICI 182,780.Impaired social behaviour was also observed after exposure of adult zebrafish (9 months old) for 120 days (Naderi *et al.*, 2020). Fish groups exposed to 10 and 30 µg/L BPS spent less time studying new congener fish (decreased exploration ratio) compared to the control group in a dose-dependent manner (significant from 10 µg/L), indicating impaired memory or social recognition behaviour.

Shorter exposure (75 days) of adult fish (9 months old) to BPS (30µg/L) statistically significantly increased inter-individual distances in the zebrafish shoal (Salahinejad *et al.*, 2020). All doses tested (1, 10 and 30 µg/L) increased the distance from conspecifics (group preference test), indicating an impairment of social interactions. No effect on locomotor activity was noted.

Measurements on male offspring (6 months old) from F0 females exposed for 60 days (1, 10 and 30 µg/L) resulted in more ambiguous effects (Salahinejad *et al.*, 2022). A decrease in social recognition (fish group preference) was measured at the 1 µg/L dose only. Exposure to 10 µg/L

BPS (no effect at other doses) significantly decreased inter-individual distances, i.e. increased shoal cohesion in opposition to what was described by Naderi *et al.*, 2022. It should be noted that in the study of Salahinejad *et al.* (2022) the presence of BPS could not be confirmed in the eggs of exposed females.

Taken together, these data imply that BPS influences hypothalamic development and may act mainly through aromatase, but estrogenic mechanism cannot be excluded as well.

Exposure to BPS disrupts endocrine signalling and leads consequently to an impairment of gametogenesis.

Sperm production

For male zebrafish, histological analysis indicates that sperm production is affected in a dose-dependent manner (-5 to -60%) and significantly at 10 and 100 µg/L after embryos were exposed for 75 days (Naderi *et al.*, 2014). This effect is not observed by Park *et al*. (2022), when adult zebrafish are exposed, but it should be noted that exposure period in this study was much shorter (21 days) and exposure occurs at a different window.

No data on sperm morphology and sperm motility are available for zebrafish which weakens the evidence.

However, the result on sperm count in zebrafish is supported by the effects of BPS on male reproduction in rodents (see section 4.10.5.5): BPS exposure inconsistently affected the male reproductive organs among all studies in rodents. Some TG studies reported a reduced weight of testes and epididymis (OECD TG 408, Unpublished study report, 2014), a reduced seminal vesicles weight (Dose Range Finding Study, Unpublished study report, 2017a and OECD TG 421, Unpublished Study Report, 2000), or a reduced prostate weight (OECD TG 443, Unpublished study report, 2019), the other male reproductive organs not being affected. The other non-TG studies confirm this inconsistency.

However, histopathological analyses of rats exposed to BPS revealed that BPS modifies the structure of the testis (Ullah *et al.,* 2016, 2018b, 2019 and 2021).

Furthermore, several studies reported that BPS exposure affects the production of sperm already at low dose, resulting in significantly reduced sperm count and sperm motility in mice (Shi *et al*., 2017 and 2018) and rats (Ullah *et al.*, 2018b, 2019 and 2021). All these studies also showed that the spermatogenesis is disturbed after exposure to BPS in both mice and rats. Finally, Shi *et al.* (2019c), reported that exposing mice during the pregnancy could affect the third generation, demonstrating that these effects could even be transgenerational.

Among the TG studies, sperm count and sperm motility were assessed only in the EOGRTS (OECD TG 443, Unpublished study report, 2019), in which no reduction of sperm count has been observed in neither parental nor F1A cohorts. The sperm motility was slightly, but statistically significant reduced in F0 (88, 84*, 85* and 86*%, resp. at 0, 20, 60 and 180 mg/kg bw/d), although this was not observed in the F1A. The effect on sperm motility observed has to be considered as borderline, as the lowest value observed in F0 corresponds to the control value in F1A.

These effects of pre-, perinatal or adult BPS exposure on sperm count and sperm motility are remarkably consistent at low doses but were not observed at the high doses used for the EOGRTS (OECD TG 443, Unpublished study report, 2019).Therefore, even if there are some inconsistencies, these results show that BPS through estrogenic activity and/or steroidogenic disrupting properties affects the male reproductive system in rodents.

Using a weight of evidence approach, results in fish and rodents show that **BPS affects male reproductive system through estrogenic activity and/or steroidogenic disrupting properties.**

Oocyte maturation

"The gonadotropins, follicle-stimulating hormone and luteinizing hormone, have been shown to stimulate the production of 17α,20β-DHP either in vivo or in vitro" (Planas and Swanson, 1995). "17α,20β-Dihydroxy-4-pregnen-3-one (17α,20β-DHP) is an endogenous, maturation-inducing steroid that stimulates oocyte maturation in females of several teleost species" (Milla et al., 2008).

The histological analysis in the ZEOGRT (Unpublished study report, 2020), showed a dose-dependent decrease in the number of females with mature oocytes at the end of the experiment (170 days) for all concentrations (calculated by the dossier submitter using Cochrane-Armitage trend analysis) and was significantly affected at 1250 µg/L (calculated by the dossier submitter using Fisher exact test). The ovaries of females exposed to 40 and 200 µg/mL showed an excess of atretic oocytes after 21 days of exposure (Park *et al.*, 2022). Conversely, Qin *et al*. (2021), reported an increase in mature stages after 240 days of exposure. It should be noted that results in this study are expressed as a percentage of certain developmental stages not including atretic oocytes. Nevertheless, the authors noted the presence of atretic follicles and fibrosis observed at both concentrations studied (1 and 100 µg/L), showing altered quality of produced eggs in the exposed groups.

Furthermore, oocyte maturation in rodents is affected by BPS exposure. All studies reported a significant decrease of secondary follicles (Shi *et al.,* 2019a; Zhang *et al.*, 2020a and Liu *et al.*, 2021), preantral follicles (Nevoral *et al.*, 2018) and antral follicles (Ahsan *et al*., 2018; Nevoral *et al.*, 2018; Zhang *et al.*, 2020a and Liu *et al.*, 2021).

In parallel, Ahsan *et al*. (2018), and Ijaz *et al*. (2020), demonstrated a significant increase of atretic follicles, indicating a disrupted ovarian development and a decreased oocyte quality after BPS exposure.

As a consequence of changed hormone levels, disturbed HPG signalling and increased VTG production, zebrafish fecundity is impaired by BPS leading to impaired reproductive success on population level.

Ji *et al*. (2013), reported a statistically significant dose-dependent decrease at all doses in the number of eggs produced per female (number of eggs divided by two at 50 µg/L). In the study of Naderi *et al.* (2014), egg production (measured over 7 consecutive days) is halved at 10 and 100 µg/L. The ZEOGRT study (Unpublished study report, 2020) reports **non-monotonic effects of BPS with impaired fecundity at the low doses tested and no effect at higher** (environmentally unrealistic) concentrations above the mg/L. Potential non-monotonic dose response should be reproducible, which is the case here at low concentration as the findings are in accordance with those of the literature study and the range-finding study of the ZEOGRT. Exposure to BPS, resulted in the preliminary study (range-finding test) in a decrease in fecundity for concentrations of 3.2, 10 and 32 µg/L with reductions of 36, 70 and 50% resp. and was statistically significant at 10 µg/L (recalculated using the Wilcoxon test). The main F1 study showed a decrease in fecundity for all concentrations (by 43, 21, 36, 21 and 7% at 2, 10, 50, 250, and 1250 µg/L resp.) which was statistically significant at 2 and 50 µg/L. In contrast to the above studies, Qin *et al*. (2021), observed a statistically significant increase in the number of eggs/breeding tank/day after chronic exposure (2 hpf - 240 dpf) to 1 and 100 µg/L of BPS. However, this result is doubtful (number of eggs/female per day is unclear).

As a consequence of changed hormone levels and increased VTG production, zebrafish fertility is impaired by BPS leading to impaired reproductive success at population level.

Ji *et al*. (2013), reported a statistically significant dose-dependent decrease at all doses when eggs and parents were exposed; the decrease was statistically significant only at 5 and 50 µg/L if only parents were exposed. In the main study of the ZEOGRT no effect on fertility in F1 was observed. While in the F2-generation, although minor, a statistically significant difference was determined on the hatching success (0-4 days) at 10 (94*%), 250 (95*%) and 1250 µg/L (94*%). A decrease in fertility from 3.2 to 320 µg/L (9, 38, 36, 22 and 22%, resp. at µg/L; not significant) was measured in the preliminary study.

As a consequence of changed hormone levels, the sex ratio of zebrafish is altered by BPS.

In the study of Naderi *et al*. (2014), the proportion of females in the 10 and 100 µg/L treatment groups was 58.8% and 66.7% resp, compared to 46.3% in the control group (X2-test, X2= 12.14, p = 0.016). The ZEOGRT study (Unpublished study report, 2020) shows a non-significant decrease in the sex ratio for all concentrations except 250 µg/L. However, it is notable that, similarly to the range-finding study, the percentage of males in the control group in the main study was very low (41%) impacting the sensitivity of the observations. Despite this, the sex ratio at the 10 µg/L concentration fell below natural variation with only 29% of males.

As a consequence of impaired oocyte maturation, reproductive success (hatching) is impaired by BPS.

Hatching time and hatching rate are impaired when parents or embryos are exposed to BPS. Hatching rate was statistically significantly prolonged at all doses (0.5, 5 and 50 µg/l) when eggs and parents were exposed, and statistically significantly prolonged when only parents were exposed to 50 µg/L BPS (Ji *et al.*, 2013). Also hatchability was statistically significantly and dose-dependently reduced at all doses (0.5, 5 and 50 µg/L) when eggs and parents were exposed but only statistically significantly reduced at 5 and 50 µg/L if only parents were exposed (Ji *et al.*, 2013). No significant effect is observed in the F1 generation of the ZEOGRT study. However, embryo survival in F2 was impaired at all concentrations and significant at 10, 250 and 1250 µg/L (not sign. at 2 and 50 µg/L). Wei *et al*. (2018), reported also a statistically significant reduction in hatching at all concentrations in F1 (1, 10 and 100 µg/Lof BPS) after 60 and 72hpf. Naderi *et al*. (2014), reported a statistically significantly decreased hatching rate (~-40%) at 10 and 100 µg/L and an increased hatching time (~+10%). This is supported by the findings of Zhang *et al*. (2017) and Qin *et al*. (2021) where hatchability decreased statistically significantly after embryonal exposure resp. at 30 µg/L and 1 and 100 µg/L of BPS. Mu *et al*. (2018) (at high concentrations of BPS: (3, 6, 12.5, 25 and 50 mg/L) did not see any significant effect on hatching rate. Qiu *et al*. (2018b and 2021) measured an increase in hatching rate, an effect that they did not observe in their 2016 publication (Qiu *et al.*, 2016).

Supportive evidence :

Alterations in gonadal development are sometimes observed.

"The Gonadosomatic index may provide additional information about the gonad maturation and spawning readiness" (EFSA/ECHA ED guidance, 2018)

"A lesser GSI value accompanied by an inhibition of egg production has been reported in fish exposed to estrogenic compounds" (Van den Belt et al., 2001). "Reduction of the GSI in male fish is regarded as a sensitive parameter in reproductive studies with estrogenic substances" (EFS/ECHA ED guidance, 2018).

Qin *et al*. (2021), note a statistically significant increase in GSI at 100 µg/L without giving values. Conversely, Naderi *et al*. (2014), observed that the GSI of males and females was affected in a dose-dependent manner (statistically significant decrease at 10 and 100 µg/L in males and only at 100 µg/L in females) which was accompanied by a statistically significant decrease in egg production. Finally, Park *et al*. (2022), found that exposure to BPS had no effect on the GSI of males, but statistically significantly increased in females exposed to 40 µg/mL (16.06%) and decreased it at 200 µg/mL (5.17%).

No effect on gonad maturity index was reported in the ZEOGRT (Unpublished study report,

2020).

No conclusion can be drawn on GSI due to diverging results observed after exposure to BPS. It should however be noted that histological analysis of the gonads is the most accurate method for maturity staging compared to the gonadosomatic index which is a very general parameter (see oocyte maturation above).

5.7.6.2 Assessment of biological plausibility of the link between endocrine activity and adverse effect(s)

Several studies provide evidence of altered gametogenesis, fecundity, sex ratio and social behaviour, as well as an impact on endocrine modalities (estrogen and steroidogenesis). Adverse effects on development and reproduction in zebrafish (*Danio rerio*) were observed after exposure to BPS in the µg/L range. Reduced sexual functioning and fertility were characterised by reduced fecundity and impaired gametogenesis (reduced sperm count, decreased oocyte maturation). Furthermore, BPS altered hatching rate and caused a skewing of phenotypic sex ratio towards females. Notwithstanding that in a ZEOGRT (Unpublished study report, 2020), the findings on sex ratio were not significant, the same trend towards feminisation was observed as in the literature study (Naderi *et al.*, 2014) with the number of males close to, or even below, natural variation at low concentrations.

Bisphenols are known to target many different endocrine pathways. Available *in vitro* and *in vivo* studies show that BPS can interfere with the estrogen, androgen, steroidogenesis, thyroid, PPARγ, and other pathways. Clear evidence is available for the estrogen and steroidogenic modalities. *In vitro* ER binding assays demonstrate that BPS is capable to bind to the estrogen receptor, with IC50 ranging from 5.8 to 105 µM depending on the cell line used (rat and human). Several literature studies showed increased estrogen activity, although weak (ER reporter gene assays, proliferative assays and ER-regulated gene expression assays). Vitellogenin, a biomarker for estrogen activity was induced in male embryonic and adult zebrafish. Literature data also demonstrate a change in steroidal hormone balance with decreased testosterone and increased estradiol levels and an increased E2/T ratio in zebrafish. With regard to the steroidogenic pathway, several *in vitro* literature studies show a disturbance after exposure to BPS. Clear evidence was given in *in vivo* studies in zebrafish, where the impact on the synthesis of steroid hormones (decrease of testosterone and increase of estrogen) was accompanied by an increased expression of genes involved in steroidogenesis and specifically aromatase (CYP19a, CYP19b in testis and brain resp.).

Therefore, a mode of action analysis was performed on the estrogen and steroidogenesis pathways with focus on reproductive success and sex ratio.

Table 22: Analysis of mode of action

	Event	Supporting evidence
MIEa	ER activation	Strong evidence: • *In vitro* studies show binding of BPS to ER in mammalian cells (Blair *et al.*, 2000; Yamasaki *et al.*, 2004; Laws *et al.*, 2006; Akahori *et al.*, 2008; Zhang *et al.*, 2018; Liu *et al.*, 2019b). 1/1 study with Human U251 glia cells transfected with Zebrafish ERα, ERβ1 and Erβ2 showing very weak binding affinity (Cano-Nicolau *et al.*, 2016). • Several *in vitro* studies show an agonist activation of ER (among others Grignard *et al.*, 2012; Kang *et al.*, 2014. Dvorakova *et al.*, 2016; Le Fol *et al.*, 2017; Rosenmai *et al.*, 2014; Mesnage *et al.*, 2017; Kojima *et al.* 2018; Pelch *et al.*, 2019).

		• 2/2 in vitro studies with Zebrafish cells or human cells transfected with zfER (zfERα, zfERβ1 and zfERβ2) show agonistic activation of ER (resp. : Le Fol *et al*., 2017; Cano-Nicolau *et al*., 2016). • 1/1 *in vivo* study (transgenic zebrafish) showed activation of ERE (Moreman *et al.*, 2017).
MIEb	Aromatase activation/ ↗ CYP19 expression	Strong evidence: Aromatase activation *In vitro* studies show increased aromatase activation (Williams and Darbre, 2019).
KE1a	Impaired transcription of genes of HPG	Strong evidence: 2/2 Changed expression of GnrH *in vivo* in zebrafish Stat. sign. decrease of GnrH and fsh in adult females and stat. sign. increase in adult males, stat. sign. increase of GnrH and no effect on fsh in embryonic zebrafish (Ji *et al.*, 2013; Qiu *et al*., 2016). Increased transcription of gnrh3 but not stat. sign. (Qiu *et al*., 2021).
KE1b	Impaired transcription of genes involved in steroidogenesis	Strong evidence: Steroidogenesis • Steroidogenesis gene expression (other than CYP19a) affected *in vitro* in mammalian cells (Sidorkiewicz *et al.*, 2018; Feng *et al*., 2016). • 2/2 Increased CYP19a gene transcription in zebrafish (Ji *et al*., 2013; Qiu *et al*., 2021). • 5/5 Increased CYP19b gene transcription in zebrafish (Ji *et al*., 2013, Kinch *et al*., 2015 ; Cano-Nicolau *et al*., 2016; Le Fol *et al*., 2017; Qiu *et al*., 2021). • Support from mammalian data : enhanced aromatase expression in testes (Shi *et al*., 2018; Shi *et al*., 2019c).
KE1c	Precocious neurogenesis	Strong evidence: • Induction of precocious hypothalamic neurogenesis in embryonic zebrafish (Kinch *et al*., 2015)
KE2a	Impairment of Oocyte maturation	Strong evidence: • In zebrafish, the ZEOGRT reported decreased number of females with mature oocyte (Unpublished study report, 2020 - OECD TG 240 amended for zebrafish) but increase in another literature study is unclear (Qin *et al*., 2021). • 3/3 Excess of atretic oocytes in fish (Unpublished study report, 2020-TG 240 amended for zebrafish; Qin *et al*., 2021; Park *et al*., 2022).

		• Support from mammalian data, where several studies (except the EOGRTS) show an affected oocytes maturation *in vivo* in mammals (Shi et al., 2019a; Ahsan et al., 2018; Liu et al., 2021; Nevoral et al., 2018; Zhang et al., 2020a), showing an increase of primordial and atretic follicles, and a decrease of primary, secondary and antral follicles.
KE2b	Impaired steroid synthesis: ↗ Estrogen ↙ Testosterone	Strong evidence: • Several studies show impaired steroid synthesis in vitro in mammalian cells (Rosenmai *et al*., 2014; Goldinger *et al*., 2015, Feng *et al*., 2016; Eladak *et al*., 2015; Campen *et al*., 2018a; Téteau *et al*., 2020; Williams *et al*., 2019). • 4/4 Changed hormone level in *In vivo* in zebrafish (Ji *et al*., 2013; Naderi *et al*., 2014; Park *et al*., 2022; Qiu *et al*., 2021).
KE2c (← KE1c)	Brain neurogenesis	Strong evidence: • Increase of the Hypothalamic Gnrh3 neurons in embryonic zebrafish (Qiu *et al*., 2016; Qiu *et al*., 2021)
KE3	Vitellogenin induction	Strong evidence: • 3/3 increased VTG in blood plasma zebrafish (Naderi *et al*., 2014; Qin *et al*., 2021). • No effect observed in F1 in ZEOGRT (Unpublished study report, 2020 - OECD TG 240 amended for zebrafish). • 1/1 Vtg gene expression increased in males and decreased in females (Park *et al*., 2022).
AO1	Females ↙ Fecundity and hatching	Strong evidence: • *Fecundity* Reduced fecundity reported in 3/4 fish studies, including in ZEOGRT at low concentrations comparable to those tested in literature studies (Ji *et al*., 2013; Naderi *et al*., 2014; Unpublished study report, 2020 - TG 240 amended for zebrafish). However, increased fecundity in one study but results are not clear (Qin *et al*., 2021). • *Hatching* Reduced hatching rate in 6/10 fish studies (Ji *et al*., 2013; Naderi *et al*., 2014; ; Moreman *et al*., 2017; Wei *et al*., 2018; Qin *et al*., 2021), also sign. decreased in F2 (Unpublished study report, 2020 - TG 240 amended for zebrafish). 2 studies (Qiu *et al*., 2018b and 2021) reported an increase but results are in contradiction to the results of their previous study of 2016 (Qiu *et al*., 2016). Increased time to hatch in 3/4 fish studies (Ji *et al*., 2013; Naderi *et al*., 2014; Moreman *et al*., 2017).

AO2	↙ fertility Males	Medium evidence: • Decreased sperm count in 1/1 fish study (Naderi *et al*., 2014). • Support from mammalian data, in which sperm count is affected in several studies (Shi *et al*., 2017 and 2018; Ullah *et al*., 2018b, 2019 and 2021; but not in EOGRTS (Unpublished study report, 2019 – OECD TG 443).
AO3	Sex ratio (female biaised)	Strong evidence: Feminisation observed in 3/3 fish studies with sign. increase of female ratio to 58.8% (10 µg/l) and 66.7% (100 µg/l compared to control (46.3%) (Naderi *et al*., 2014) and a non-significant increase of female ratio reaching 71% at 10 µg/l (Unpublished study report, 2020 - TG 240 amended for zebrafish).
A04	Altered social behaviour	Strong evidence: • effects on social behaviour in 4/4 fish studies (Naderi *et al*., 2020 and 2022; Salahinejad *et al*., 2020 and 2022).

The hypothesised mode of action and the resulting AOs in vertebrate wildlife species are considered population relevant (see section 5.7.6.3). Evidence of endocrine disruptive properties of BPS on mammalian vertebrate species and especially the disruption of the estrogenic pathway in this case, provides further support for similar properties in mammalian vertebrates. This is due to the large commonalities between non-mammalian and mammalian vertebrate species concerning hormones, enzymes and receptors involved in the EATS' modalities.

5.7.6.2.1 Background on HPG axis and relation to sex change

See Annex III.

5.7.6.2.2 Plausible link between endocrine activity and adverse effects

- Altered reproductive success

Altered male fertility

As mentioned above, teleost fish have two copies of the CYP19 gene that encode two isoforms of aromatase: CYP19a (gonadal aromatase) and CYP19b (brain aromatase) *(Brion et al*., 2012). Furthermore, it is known that the CYP19b gene contains an estrogen response element (ERE) on the promotor region which is very sensitive to estrogen activity (Kallivretaki *et al.*, 2006). CYP19 is the enzyme in the steroidogenic pathway that converts testosterone into estradiol.

Androgens (testosterone and 11-ketotestosterone) play a pivotal role in spermatogenesis, development of secondary characteristics and in reproductive behaviour in teleost fish (Tang *et al*., 2018). Furthermore, it was demonstrated that a surplus of steroidal estrogens may alter sexual behaviour, development and reduce fertility in male fish (Tang *et al.*, 2017).

Sperm numbers were decreased after exposure to BPS. Furthermore, exposure to BPS leads to altered steroid synthesis in zebrafish, characterised by increased E2 production in both sexes and decreased T production in males. The change in hormone balance was accompanied by an upregulated CYP19 gene transcription.

However, no data are available on sperm morphology and sperm motility. Despite these uncertainties and the fact that also an effect was seen in rodents it is shown that BPS is capable of affecting the male reproductive system.

As a consequence of the disrupted aromatase activity resulting in a changed steroid hormone balance in male zebrafish, spermatogenesis is impacted after exposure to BPS leading to reduced sperm count.

Altered female reproductive success (oocyte maturation, fecundity and hatching)

"Estradiol treatment causes a significant increase in both kiss1 and kiss2 mRNA expression in the brain of juvenile zebrafish (Servili *et al.*, 2011).

Kisspeptins are ligands of the G-protein coupled receptor in the hypothalamus. G-protein coupled receptor signalling initiates the secretion of gonadotropin hormones which at their turn stimulate the release of follicle-stimulating hormone and luteinising hormone which have been shown to stimulate the production of 17α,20β-DHP*. "17α,20β-Dihydroxy-4-pregnen-3-one (17α,20β-DHP) is an endogenous, maturation-inducing steroid that stimulates oocyte maturation in females of several teleost species" (*Milla *et al.*, 2008).

BPS induced a statistically significant increase in the number of Hypothalamus-GnRH3 neurons at 100 µg/L during embryonic exposure (Qiu *et al.*, 2016 and 2021). GnRH neurons integrate internal and external cues to control sexual maturation and fertility (Zhao *et al.*, 2016). Additionally, in the study of Qiu *et al.* (2016) BPS also statistically significantly up-regulated the expression of the reproduction-related genes kiss1/kiss1r, gnrh3 and erα, lhβ, fshβ after 5 days exposure. The findings concerning kiss1, lhβ an erα were confirmed in the study of 2021 (Qiu *et al*., 2021). Gnrh3-expression, however, was non-significantly increased, but was seen together with a statistically significantly higher level of FSH. Unlike their previous study of 2016, kiss2 and erβ (erβ1 and erβ2) were statistically significantly increased. The observed effective inhibition by a known aromatase inhibitor (fadrozole) and ER-antagonist (ICI 182,780) suggests that these effects are mediated partially by ERα and aromatase. The disturbance on the HPG-axis was not only observed at embryonic stage, but also in adult fish where an up-regulation of fshβ, lhβ, fshr and lhr genes in adult males, and a decrease of fshβ in adult females support the fact that BPS can indirectly affect gonadotropin hormones (Ji *et al.*, 2013).

Transgenerational BPS exposure caused a decrease of the number of females with mature oocytes. Furthermore, an excess of atretic oocytes was observed in the ovaries of females impacting egg quality.

Furthermore, cumulative fecundity in adult populations is also adversely affected by ER agonists *(AOP 29, under development: https://aopwiki.org/aops/29).*

BPS caused a decrease in fecundity and reduced the hatching success due to a decreased quality of the eggs.

Although it should be noted that there is some degree of uncertainty due to the absence of measurements on all neuropeptides and hormones in the regulation of HPG axis in zebrafish, clear effects were demonstrated on the regulation of the transcription of the genes involved.

It is known that estrogen receptor agonism leads to reproductive dysfunction (AOP 29) and skewed sex ratio (AOP 52). Furthermore, as a consequence of the increased aromatase activity (increased estradiol), BPS exposure impacts steroid hormone balance in male zebrafish and spermatogenesis leading to reduced sperm count, while the alteration of gene expression of the HPG-axis in females ultimately leads to an alteration of the oocyte maturation and thus impacting the reproductive success (fecundity and hatchability).

As a consequence of the increased aromatase activity (increased estradiol), BPS

caused an alteration of gene expression of the HPG-axis in females ultimately leading to an alteration of the oocyte maturation and thus impacting the reproductive success (fecundity and hatchability).

Overall, BPS exposure can reduce reproductive performance arising as a result of alterations at several levels of HPG axis.

- skewed sex ratio

The HPG-axis is suggested to be the major signalling pathway regulating gonadal sex change. (EFSA/ECHA ED Guidance, 2018). Zebrafish are gonochoristic teleost, meaning that adult fish are female or male. However they are transient hermaphroditism during their juvenile stage.

As explained above, juvenile gonad development is regulated by expression of CYP19a and estrogens. Also maintenance of the sex in teleost fish is regulated by the inhibition of CYP19a gene (male sex) and oestrogen production (female sex). Furthermore, it was shown that GnRH knock out in zebrafish during sex determination leads to male sex-biased population (Rajendiran *et al.*, 2021).

Although still under development, AOPs 29 and 52 can be used in the assessment of biological plausibility of the link between endocrine activity (agonistic estrogen receptor) and adverse effect (skewed sex ratio):

- **AOP29:** Estrogen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/29), describing the linkage between ER agonism and impact on reproductive functioning:
  "*In terms of teleost fish, exposure to ER agonists leads to a suite of adverse outcomes depending upon whether exposures occur during or beyond the larval, juvenile and adult life-stages. For example, aquatic exposure to potent ER agonists during the larval and juvenile life-stages may lead to gonadal and renal pathology and skewed-sex ratios in adult fish (potentially 100% females). Larval, juvenile and adult male fish exposed to the same ER agonists display abnormal plasma or whole body levels of vitellogenin (VTG). Cumulative fecundity in adult populations is also adversely affected by ER agonists and this is an important endpoint in the OECD Test Guideline 229 Fish Short Term Reproduction Assay.*" (AOP 29, under development: https://aopwiki.org/aops/29)

Table 23: Mapping of AOP29 (Estrogen receptor agonism leading to reproductive dysfunction) for BPS

Sequence	Type	Defined in AOP	Evidence
1	MIE	Agonism, Estrogen receptor	*In vitro*: clear evidence in many studies *In vivo*: induction of VTG in male and female zebrafish (Naderi *et al.*, 2014) accompagnied with upregulated vtg gene transcription in males (Park *et al.*, 2022)
2	KE	Reduction, Cumulative fecundity and spawning	BPS caused a decrease in fecundity (Ji *et al.*, 2013; Naderi *et al.*, 2014; Unpublished study report 2020-OECD TG 240 amended for zebrafish)
3	KE	Increase, Plasma vitellogenin concentrations	Increased plasma VTG concentration in zebrafish (Naderi *et al.*, 2014; Le Fol *et al.*, 2017; Qin *et al.,* 2021; Park *et al.,* 2022)
4	KE	Increase, Vitellogenin synthesis in liver	No information
5	KE	Increase, Renal pathology due to VTG deposition	No information

6	AOP	Decrease, Population trajectory	No information
7	AOP	Altered, Reproductive behaviour	No information
8	AOP	Altered, Larval development	Time to hatch increased (Ji *et al.*, 2013; Naderi *et al.*, 2014; Moreman *et al.*, 2017)

- **AOP52:** Skewed sex ratio (https://aopwiki.org/aops/52)

Table 24: Mapping AOP52 (Skewed sex ratio) for BPS

Type	Defined in AOP	Evidence
MIE	Agonism, Estrogen receptor	*In vitro*: clear evidence in many studies *In vivo*: induction of VTG in male and female zebrafish (Naderi *et al.*, 2014) accompagnied with upregulated vtg gene transcription in males (Park *et al.*, 2022)
AOP	Skewed sex ratio	Sex ratio towards females (Naderi *et al.*, 2014), trend towards feminisation (Unpublished study report, 2020-OECD TG 240 amended for zebrafish)

Low levels of BPS up-regulated the expression of gnrh3 in embryonic brain tissue and early larval stages. BPS also impacted the transcript levels of gnrh3 in adult zebrafish with an increase in males and a significant decrease in females.

BPS leads to disturbed steroid synthesis in zebrafish exposed to BPS, characterised by increased E2 production in both sexes and decreased T production in males. Furthermore, increase in gonadal and brain aromatase transcript levels was measured in adult males after BPS exposure.

Additionally, the estrogen agonist mechanism seen *in vitro* is confirmed *in vivo* by measurement of VTG, an estrogen biomarker. Embryonic exposure to BPS induced plasma VTG production in male and female zebrafish. In adult males also an induction of the vtg transcript expression level was measured.

There is no information available on renal pathology, a KE in AOP29 (see Table 23).

Exposure to BPS (6.8 nM=1.7 µg/L) during the embryonic phase (0-5 dpf) results in an early 240% increase in neurogenesis in the rostral hypothalamus at 24 hpf and a significant increase (160%) in locomotor activity of the 5-dpf fish (Kinch *et al.*, 2015). These effects were absent when co-exposed in the presence of brain aromatase morpholinos but still effective in co-treatment with 1 µM ICI 182,780. Exposure during neurogenesis, adult exposure and maternal exposure (F1) lead also to impaired social behaviour (inter-individual distances, social preference (time spent near conspecifics), anxiety, object recognition memory (resp. Naderi *et al.*, 2022; Salahinejad *et al.*, 2020 and Naderi *et al.*, 2020; Salahinejad *et al.*, 2022). Effects on anxiety were sign. increased by the co-administration with ICI 182,780. No effect on locomotor activity was noted. However this is not part of AOP52 as it was demonstrated that aromatase modality is concerned in these studies.

A change in sex ratio towards feminisation was observed in zebrafish after exposure to BPS.

Due to estrogen agonist activity as well as the impact on the signalling pathway of the HPG axis (aromatase activity), exposure to BPS can lead to skewed sex ratio in fish.

5.7.6.3 Conclusion on the Mode of Action analysis

Based on the weight of evidence approach and considering the results of all available studies there is evidence that the adverse effects of BPS on sperm count and sex ratio in zebrafish are due to the estrogenic activity and to disrupted steroidogenesis. All those findings were observed at concentrations without mortality.

Skewed sex ratio is recognised as EATS-mediated effect. Altered gametogenesis as reduced sperm counts has been also observed. In fish, sperm count (mature sperm cells) is generally not assessed in the current standardised OECD TGs and is therefore not listed as EATS-mediated effect in the guidance document (EFSA/ECHA ED Guidance, 2018). However, based on the existing knowledge in mammals and the similarities with fish gametogenesis, this effect could be considered as EATS-mediated also in fish. The estrogenic activity of BPS is demonstrated in mammals and is further evidenced by vitellogenin induction in fish. Altered steroidogenesis may lead to the observed decreased sperm counts and altered oocyte maturation which, in turn, may lead to impaired hatchability of the eggs. Increased aromatase activity is consistently observed and is clearly responsible for effects on fish brain and behaviour. Impaired social behaviour may also result in reduced reproduction.

All mammalian data were considered in a weight of evidence approach. Knowing that there is a large degree of conservation of the endocrine system. This implies large commonalities between non-mammalian and mammalian vertebrate species in regard to hormones, enzymes and receptors involved in the EATS' modalities (OECD GD 150, 2018). Evidence of endocrine disruptive properties of BPS on mammalian vertebrate species therefore provides further support for similar properties in non-mammalian vertebrates, in particular with regard to disruption of estrogenic pathways.

Considering all relevant and reliable information in a weight of evidence approach, it is concluded that BPS is an endocrine disruptor according to the WHO/IPCS definition (WHO/IPCS, 2002) with regard to environment.

Table 25: Key event relationship

	Key event relationships (KERs)			
	MIE to KE1	**KE1 to KE2**	**KE2 to KE3**	**KE3 to AE**
Biological plausibility for the KERs	STRONG ER binding→ Estrogen signalling CYP19a activation→aromatase activation	STRONG Estrogen signalling→increase E2, decrease T secretion	Medium Increase E2, Decrease T→ reduced sperm	Medium Altered gametogenesis→altered reproductive success
	STRONG CYP19b activation→ GNRH activation	MEDIUM GNRH activation→ gonotropin hormone modulation	STRONG gonotropin hormone modulation→altered gamete maturation (oocytes)	STRONG altered gamete maturation→ altered reproductive success
	STRONG ER binding→ Estrogen signalling/ CYP19a activation→aromatase activation	STRONG Estrogen signalling/aromatase activation→increase E2, decrease T secretion		STRONG increase E2, decrease T→changed sex ratio (feminisation)
Empirical support for the KERs	Consistent *in vitro* and *in vivo* results for ER transactivation/ consistent increase in CYP19 upregulation	Consistent change in plasma steroids in 1 study	Reduced oocyte maturation/sperm counts	Reduced reproductive success (fecundity, fertility and hatchability)/ impaired sex-ratio Sex ratio is considered EATS-mediated

	MIE	**KE1**	**KE2**	**KE3**	**AE**
Essentiality of KEs					

Consistency	The KEs have been observed consistently in several studies examining embryonic and adult development and reproduction
Analogy	Other bisphenols and estrogenic compounds altering sex ratio[20]
Specificity	Effects have been observed in absence of general toxicity.

Identified uncertainties	**Comments**
Uncertainty 1 few results for gonadotropin release	It is not possible to determine exactly the mechanism behind it in absence of analysis of all neuropeptides and hormones involved in the regulation of the HPG axis.
Uncertainty 2 sperm counts	No data available on sperm morphology and sperm motility for zebrafish. However data are available for rodents
Uncertainty 3	
Uncertainty	

[20] European Chemicals Agency (ECHA) (2017b) : Support document for identification of 4,4'-isopropylidenediphenol (Bisphenol A, BPA) as substance of very high concern
European Chemicals Agency (ECHA) (2021) : Support document for identification of 4,4'-(1-methylpropylidene)bisphenol; (bisphenol B) as substance of very high concern

Overall conclusion on the postulated MoA
The overall biological plausibility is strong and substantiated by a strong empirical support for the majority of postulated KEs.

5.7.7 Overall conclusion on endocrine disruption with regards to environment

Based on all available scientific evidence (environmental data, supported by human health data) it can be concluded that BPS fulfils the WHO/IPCS (2002) definition of an endocrine disruptor with regard to the environment :

- It shows clear adverse effects in zebrafish by a change in reproduction and development, supported by clear adverse effects on reproduction in rodents. The reproductive endocrine system is highly conserved not only between mammals, but also between mammals and vertebrates like fish.
- It has endocrine modes of action: clear estrogenic mode of action and disruption of steroidogenesis.
- The adverse effects are considered EAS-mediated effects and are thus a consequence of the endocrine mode of action.

5.8 Other non-ED adverse effects

Not applicable.

5.9 Summary and discussion of the environmental hazard assessment

The substance 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) according to Article 57(f) of REACH Regulation.

6. Conclusions on the SVHC Properties

6.1 CMR assessment

BPS is covered by index number 604-098-00-1 of Regulation (EC) No 1272/2008 in Annex VI, part 3, Table 3 (the list of harmonised classification and labelling of hazardous substances) and it is classified in the hazard class toxic for reproduction category 1B (H360FD).

Therefore, this classification of the substance in Regulation (EC) No 1272/2008 shows that it meets the criteria for classification in the hazard class:

- toxic for reproduction category 1B in accordance with Article 57 (c) of REACH.

6.2 PBT and vPvB assessment

This section is not relevant for the identification of BPS as SVHC in accordance with Article 57 (c) and (f) of the REACH Regulation.

6.3 Assessment under Article 57(f)

6.3.1 *Summary of the data on the intrinsic/hazardous properties (providing scientific evidence of probable serious effects to HH and/or ENV)*

Data summarised below illustrate that BPS meets the WHO/IPCS definition of an endocrine disruptor:

- for humans due in particular and as specifically demonstrated in this dossier to its ED-mediated reprotoxic effects (fertility) AND,

- for the environment in particular and as specifically demonstrated in this dossier due to its ED-mediated effects on sexual development (feminisation) and reproduction (fertility).

Human Health:

Endocrine activity

In vitro ER binding assays demonstrate that BPS is capable to bind to the estrogen receptor, with IC50 ranging from 5.8 to 105 µM depending on the cell line used (rat and human). Several literature studies showed increased estrogen activity, although weak (ER reporter gene assays, proliferative assays and ER-regulated gene expression assays). *In vivo*, the increase in uterus weight (as seen in all three uterotrophic assays) is identified as a diagnostic parameter for oestrogenicity.

BPS is shown to have estrogenic activity.

As supportive information, BPS has also been shown to disrupt steroidogenesis in a range of *in vitro* assays. Despite differences depending on the experimental conditions, a clear trend towards decreased testosterone was observed. Furthermore, increased aromatase activity in the testes was found in several studies following exposure to BPS.

Some weak anti-androgenic activity has been observed *in vitro*, but the effect of BPS on this pathway is less obvious due to contradictory results. Similarly, binding and activation of thyroid hormone receptors resulted in contradictory findings.

Relevant adverse effects

The most relevant effects have been observed on female reproduction. Indeed, several adverse effects have been observed in rodent experimental studies conducted according to OECD TG studies and numerous literature publications:

- BPS consistently affects the estrous cyclicity in female rodents after different windows of exposure and in particular during adult exposure, as shown in OECD TGs 443 (F0 and F1B), 421 and 422 and other literature studies (e.g. Shi *et al*., 2019a) due to longer diestrus phases.

- Rodents, when exposed to BPS during different windows of exposure, display reduced number of implantation sites, as shown significantly in OECD TG 422, and as trend in OECD TGs 421 and 443 (Unpublished study report, 2000, 2017b and 2019)

- As a consequence, the number of born pups was significantly reduced in some studies, including OECD TGs 443, 422 and 421, and fertility index was affected, among others in OECD TGs 421 and 422. Fertility index was not modified in OECD TG 443, however the highest tested dose was only 180 mg/kg bw/d (compared to 300 mg/kg bw/d in OECD TGs 421 and 422).

Considering the results of all available studies, there is strong evidence that the adverse effects

on fertility and development which led to harmonised classification of BPS as Repr. 1B[21], particularly in females, are due to estrogenic activity of BPS.

Some other adverse effects have been observed on male reproductive system, but not consistently:

- BPS modifies the serum hormone levels in males, showing an increase of estradiol level and a decrease of testosterone in a coherent manner in all the available studies (Shi *et al*., 2018 and 2019c; Ullah *et al*., 2016, 2018a, 2018b and 2019, also supportive study Shi *et al*., 2017).

- BPS disrupts the spermatogenesis in rodents at low doses (Shi *et al*., 2018 and 2019c; Ullah *et al.*, 2016, 2018a, 2018b and 2019, also supportive study Shi *et al*., 2017).

- BPS affects the sperm quality at low doses (Shi *et al.*, 2018 and 2019c; Ullah *et al.,* 2019, also supportive screening study Shi *et al*., 2017). However, these effects on sperm were not confirmed in the OECD TG 443, in which animals exposed at higher doses only displayed a slightly reduced sperm motility and in P0 only.

Finally, BPS exposure resulted in a higher incidence of mammary gland atrophy in males in all OECD TG studies (OECD TGs 443, 422 and 408). Furthermore, a literature study reported low-dose BPS effects (2 to 200 µg/kg/d) on male mammary gland development (Kolla *et al.*, 2019). Knowing that the mammary gland is a very sensitive marker of endocrine activity, this remarkable consistency should be considered, even if the outcome and adversity of this effect is unknown.

Plausible link between adverse effects and endocrine activity

Considering the results of all available studies, there is strong evidence that the adverse effects on fertility in females are due to the oestrogenic activity of BPS. The increase in uterus weight (as seen in all three uterotrophic assays) is recognised as a diagnostic parameter for oestrogenicity. Furthermore, prolongation of the estrous cycle was consistently observed in the majority of studies. Finally, the number of implantation sites was decreased in three reproductive studies, resulting in decreased fertility and number of pups. All these parameters are considered as either EATS-mediated or sensitive to EATS modalities (OECD GD 150, 2018). The different effects of BPS, in particular on the female reproductive system leading to adversity on apical fertility endpoints and litter size, can be plausibly linked to the oestrogenic activity of the substance.

BPS, as other bisphenols, acts via different MoAs and leads to different adverse effects, such as effects on the male reproductive system or the male mammary gland most probably through decreased circulating testosterone levels. However, the data on these MoAs were less consistent, therefore they are considered as supportive, as they give further indications of the endocrine activity of BPS.

Human relevance:

Human relevance of MoA is assumed by default unless there is indication that this may not be the case. Hence, only if there is such indication, assessment of relevance for humans of the postulated MoA(s) needs to be carried out here (see Sections 3.5.4.4 & 3.3.1.3 of ED guidance, EFSA/ECHA ED Guidance, 2018).

Estrogen signalling plays a key role in mammalian reproduction. There is no reason to assume that the observed adverse effects on fertility by disruption of estrogen signalling in rats have no human relevance.

[21] RAC opinion of 10 December 2020

Conclusion human health:

Considering all relevant and reliable information in a Weight of Evidence Approach, it is concluded that BPS can be identified as an endocrine disruptor for human health in accordance with WHO/IPCS definition (WHO/IPCS, 2002).

Environment:

Endocrine activity:

See in Endocrine Mode of Action for Human Health above.

Vitellogenin, a biomarker for estrogen activity was induced in male embryonic and adult zebrafish.

BPS is shown to have estrogenic activity

Additional to the effects on estrogenic activity, *in vivo* studies in zebrafish show also a coherent picture of the impact of BPS exposure on the synthesis of steroid hormones (decrease of testosterone and increase in estradiol). This finding was accompanied by an alteration in the transcription of genes involved in steroidogenesis (including CYP19).

BPS is shown to affect steroidogenesis.

Relevant adverse effects:

- Reduced reproductive success

- Fecundity:

  BPS exposure leads to a significant decrease in egg production which was seen both in literature studies (Naderi *et al*., 2014) as well as in an ZEOGRT (adapted OECD TG 240). In the latter BPS impaired fecundity at low concentrations, similar to those for which an effect was seen in literature studies. In one study a significant increase in the number of eggs/breeding tank/day was observed (Qin *et al*., 2021). However, this result is unclear (number of eggs produced per female per reproduction day unclear).

- Gametogenesis:

  BPS exposure impairs gametogenesis by decreasing sperm production (Ji *et al.*, 2013) and oocyte maturation (ZEOGRT OECD TG 240, Unpublished study report, 2020), which was also observed in rodents (Nevoral *et al.*, 2018; Ahsan *et al.*, 2018; Shi *et al.*, 2019a; Zhang *et al.*, 2020; Ijaz *et al.*, 2020; EOGRTS OECD TG 443, Unpublished study report 2019). Additionally, an excess of atretic oocytes in fish was found (Qin *et al.*, 2021; Park *et al.*, 2022). Potentially, as a consequence of this bad egg quality, BPS impacted hatchability by increasing the time to hatch and decreasing the hatching success (Ji *et al.*, 2013; Naderi *et al.*, 2014; Moreman *et al*., 2017; Wei *et al*., 2018; Qin *et al.*, 2019; ZEOGRT, Unpublished study report 2020).

- Behaviour:

  Exposure to BPS (6.8 nM=1.7 µg/L) during the embryonic phase (0-5 dpf) results in an early 240% increase in neurogenesis in the rostral hypothalamus at 24 hpf and a significant increase (160%) in locomotor activity of the 5-dpf fish (Kinch *et al*., 2015). These effects were absent when co-exposed in the presence of brain aromatase

morpholinos but still effective in co-treatment with 1 µM ICI 182,780. Exposure during neurogenesis lead also to a sign. decreased inter-individual distance (sign. at 0.25µg/L and 25 µg/L BPS) and social preference (time spent near conspecifics) at 0.25 µg/L at 21 dpf (Naderi *et al*., 2022). These effects are accompanied by a significant increase in anxiety (significant at 25 µg/L), measured by an increased thigmotaxis (tendency of an animal to remain close to the walls of an arena), and by changes in object recognition memory (a decrease in the time spent exploring a new object, significant at 25 µg/L). Effects on exploration ratio were not reversed by co-exposure to fadrozole, while effects on anxiety were sign. increased by the co-administration with ICI 182,780. However, this is not part of AOP52 as aromatase modality is concerned in these studies.

Social recognition behaviour and memory were also impaired after exposure of adult zebrafish (9 months old) for 120 days. Fish groups exposed to 10 and 30 µg/L BPS spent less time studying new congener fish compared to the control group in a dose-dependent manner (significant from 10 µg/L) (Naderi *et al*., 2020). Shorter exposure (75 days) of adult fish (9 months old) to BPS (30µg/L) significantly increased inter-individual distances in the zebrafish shoal (Salahinejad *et al*., 2020). All concentrations of BPS (1, 10 and 30 µg/L) increased the distance from conspecifics (group preference test), indicating an impairment of social interactions. No effect on locomotor activity was noted.

Measurements on male offspring (6 months old) from F0 females exposed for 60 days (1, 10 and 30 µg/L) resulted in more ambiguous effects (Salahinejad *et al*., 2022). A decrease in social recognition (fish group preference) was measured at the 1 µg/L dose only. Exposure to 10 µg/L BPS (no effect at other doses) significantly decreased inter-individual distances (i.e. increased shoal cohesion) in opposition to what was described by Naderi *et al*., 2022. It should be noted that in the study of Salahinejad *et al.*, 2022 the presence of BPS could not be confirmed in the eggs of exposed females.

Taken together, these data imply that BPS influences hypothalamic development and may act mainly through aromatase, but estrogenic mechanism cannot be excluded as well.

- Altered sex ratio:

BPS alters the sex ratio leading to feminisation. This was clearly demonstrated in a literature study (Naderi *et al.*, 2014). Also a trend towards feminisation was observed in a ZEOGRT (Unpublished study report, 2020), where the sex ratio at low concentrations (comparable to literature concentrations) was close to or even fell below natural variation.

Effects on reproductive success and sex ratio are considered EATS-mediated effects and thus providing evidence for both endocrine activity and adverse effect.

Weight of Evidence Approach:

There is a clear consistency in results from studies in a given species (especially in zebrafish) and from different vertebrate species (rats and fish) showing an alteration of the reproductive endocrine system which is a very well conserved mechanism across vertebrate species. A large degree of conservation of the primary amino acid sequences in proteins implies large commonalities between non-mammalian and mammalian vertebrate species in regard to hormones, enzymes and receptors involved in the EATS modalities (OECD GD 150, 2018). Evidence of endocrine disruptive properties of BPS on mammalian vertebrate species therefore provides further support for similar properties in non-mammalian vertebrates, in particular with regard to disruption of estrogenic pathways and steroidogenesis.

Population relevance:

For ecotoxicological tests, effects on apical endpoints, such as fecundity, altered sex ratio and growth, are generally considered adverse because they are population relevant (OECD guidance n°150, 2018). Studies in zebrafish showed that BPS can lead to skewing of the sex ratio towards females, as well as reducing fecundity. These adverse effects clearly demonstrate the impact on the population stability.

Biological plausibility between ED modality and ED adverse effects

Considering the results of all available studies, there is strong evidence that the adverse effects on reproductive success, sex ratio and behaviour in zebrafish are due to the estrogenic activity and disrupted steroidogenesis after exposure to BPS.

Several studies provide evidence of altered gametogenesis, fecundity, sex ratio and social behaviour, as well as an impact on endocrine modalities (estrogen activity and steroidogenesis). Adverse effects on development and reproduction in zebrafish (Danio rerio) were observed after exposure to BPS in the µg/L range. Reduced sexual functioning and fertility were characterised by reduced fecundity and impaired gametogenesis (reduced sperm count, decreased oocyte maturation). Furthermore, BPS altered hatching rate and caused a skewing of phenotypic sex ratio towards females. Notwithstanding that in a ZEOGRT (OECD TG 204 adapted for zebrafish), the findings on sex ratio were not significant, the same trend towards feminisation was observed as in the literature study (Naderi *et al.*, 2014) with the number of males close to or even below natural variation at low concentrations.

Bisphenols are known to target many different endocrine pathways. Available *in vitro* and *in vivo* studies show that BPS can interfere with the estrogen, androgen, steroidogenesis, thyroid, PPARγ, etc. pathways. Clear evidence is available for the estrogen and steroidogenic modalities. *In vitro* ER binding assays demonstrate that BPS is capable to bind to the estrogen receptor, with IC50 ranging from 5.8 to 105 µM depending on the cell line used (rat and human). Several literature studies showed increased estrogen activity, although weak (ER reporter gene assays, proliferative assays and ER-regulated gene expression assays). Vitellogenin, a biomarker for estrogen activity was induced in male embryonic and adult zebrafish. Literature data also demonstrate a change in steroidal hormone balance with decreased testosterone and increased estradiol levels and an increased E2/T ratio in zebrafish. Considering steroidogenic pathway, several *in vitro* literature studies show a disturbance after exposure to BPS. Clear evidence was given in *in vivo* studies in zebrafish, where the impact on the synthesis of steroid hormones (decrease of testosterone and increase of estrogen) was accompanied by an increased expression of genes involved in steroidogenesis and specifically aromatase (CYP19a, CYP19b in testis and brain resp.).

It is known that estrogen receptor agonism leads to reproductive dysfunction (AOP 29) and skewed sex ratio (AOP 52). Furthermore, as a consequence of the increased aromatase activity (increased estradiol), BPS exposure impacts steroid hormone balance in male zebrafish and spermatogenesis leading to reduced sperm count, while the alteration of gene expression of the HPG-axis in females ultimately leads to an alteration of the oocyte maturation and thus impacting the reproductive success (fecundity and hatchability).

Reproductive dysfunction is supported by rodent data: an increase in uterus weight and a prolongation in the estrous cycle. Uterotrophic assays in rodents are a strong highly diagnostic parameter for estrogenicity. Moreover, the number of implantation sites was decreased in three reproductive studies, resulting in decreased fertility and number of pups at birth. All these parameters are considered as either EATS-mediated or sensitive to EATS modalities (OECD GD 150, 2018). The different effects of BPS, in particular on the female reproductive system can be plausibly linked to the estrogenic activity of the substance and could explain the adverse impacts seen on apical fertility endpoints and litter size (see Table 15). These adverse effects have been observed at doses showing neither maternal toxicity nor general toxicity.

In conclusion BPS exposure can decline the reproductive success and change the sex ratio in fish due to an impact on estrogen activity and steroid synthesis leading to alterations (at several levels) of HPG axis.

Conclusion environment:

Considering all relevant and reliable information in a Weight of Evidence Approach, it is concluded that there is scientific evidence that BPS can be identified as an endocrine disruptor for the environment according to the WHO/IPCS (2002) definition of an endocrine disruptor.

Moreover, the use of mammalian data is widely accepted as part of the available evidence for endocrine activity and endocrine adversity for the environment, as described in EFSA/ECHA ED guidance, 2018: "*Mammalian data are always relevant for ED assessment on non-target organisms. Furthermore, there may be information on non-target organisms that could be relevant also for the ED assessment for humans."* […] "*Furthermore, because of the high level of conservation of the endocrine system across taxonomic groups, the mammalian data may also be relevant for other vertebrates (OECD, 2018b). Therefore, data on mammals and other taxa are considered together in a holistic approach as part of the available evidence…*".

6.3.2 Equivalent level of concern assessment

According to the REACH regulation, substances identified as SVHCs under article 57(f) shall give rise to an equivalent level of concern (ELoC) to those of other substances listed in points (a) to (e), on a case-by-case basis. BPS is an endocrine disruptor relevant for human health and environment as summarised above. In order to assess ELoC the following factors should be discussed in this document (discussion paper by ECHA (2012) with a specific focus on sensitisers):

- Characteristics of the effects:
  - Type of possible effects and severity
  - Irreversibility and delay of effects
- Other factors:
  - Quality of life affected (for human health effects)
  - Societal concern
  - derivation of 'safe concentration'

6.3.2.1 Human health

- **Type of possible effects and severity:**

BPS affects reproduction and development. The observed severe effects after exposure to BPS have led to the harmonised classification Repr. 1B (Committee for Risk Assessment, 2020)– a reproductive classification to which Art. 57 (c) of REACH Regulation directly refers. Through its ED Modes of Action (estrogenic, anti-androgenic and steroidogenesis), BPS affects estrous cycle, sperm quality, mammary gland, and therefore fulfils the WHO/IPCS (2002) definition of endocrine disruptor with regard to human health. Moreover, it is known that bisphenols target other possible endocrine MoA such as thyroid, PPARγ, etc.

- **Irreversible and delayed effects**

Exposure to BPS resulted in some of the ED-related effects, which are also observed after developmental exposure, with consequences that are observed later in life (disturbed estrous cycle) without a direct exposure. Such latency effects are indeed considered as permanent and irreversible.

- **Quality of life**

In the available studies it has been found that BPS prolonges the estrous cyclicity or leads to irregular cycle in rodents (see Section 4.10.2.1). Any disruption of ovarian cycles may trigger various outcomes in women, such as extension or shortening of the menstrual cycle, erratic period cycles, irregularity of menstruation flows, etc. These disruptions may have various impacts on everyday life such as abnormal bleeding (menstruation flow), disruption of fertility (due e.g. to fewer ovarian cycles and thus a lower probability of getting pregnant in the case of elongation of cycles), disruption of sexuality, discomfort and inconvenience, and generally a lower quality of life which can occur from puberty to menopause. The severity of possible effects can vary from a slight extension of ovarian cycles to complete amenorrhoea. Potential infertility is associated with a major source of worries and psychological distress for the individuals affected, leading to significant additional medical health care and costs for the affected individuals as for their partners and families.

- **Societal concern**

BPS was detected in different types of human biological fluids such as urine and blood, but also in ovarian follicles, placenta and amniotic fluids (see section 3.2.3.2.3).

Exposure to endocrine disruptors is of serious concern for the health of current and future generations (Demeneix and Slama, 2019). A reduced ability to reproduce negatively affects society as it contributes to an increased financial burden e.g. on the health care sector, both providing assisted fertilisation treatments and clinical treatment for individuals with adverse reproductive effects. The annual costs estimated in the above mentioned study are around €163 billion. Therefore, reduced fertility due to ED exposure is of general concern in the EU countries.

- **Derivation of safe level**

Endocrine regulation, which is set up during critical life stages in vertebrates, is a very complex feedback process. Any disturbance of this regulation during transient but vulnerable life stages can lead to irreversible effects during the entire lifetime or even in the following generations. Moreover, it is difficult to assess the latency of the effects based on the available ED specific test guidelines. Therefore, it is not possible to predict potential future effects and thus safe exposure levels for the human health.

Adverse effects caused by a chemical after the exposure during the sensitive time windows in the course of development vary dependently on the organism group and also on the individuals from the same group. Therefore, effects may be overlooked, not expressed or equivocal.

- **Avoid regrettable substitution as soon as possible**

BPS is the most widely used alternative to BPA in thermal paper (ECHA, 2020). Next to that, the following uses are known: monomer in production of PESU, monomer in a production of synthetic tanning agent, leather tanning, manufacture and recycling of paper, monomer or resin in the production of food contact material and adhesives, etc. It cannot be excluded that the industry may invest in substitution of BPA with BPS in other applications as well. Moreover, detection of BPS in different types of environmental compartments (see section 3.2.3.2.4) and human biological fluids such as urine and blood, but also in ovarian follicles, placenta and amniotic fluids (see section 3.2.3.2.2) reinforces the need to avoid further regrettable substitution and to protect human health and wildlife.

The same conclusions as listed above have been already indicated and agreed upon in the identification of BPA and BPB as endocrine disruptors for environment (ECHA, 2017).

6.3.2.2 Environment.

- **Severity: BPS affects reproduction and development**

It is known that bisphenols display many endocrine Modes of Action. This is also reflected in the available *in vitro* tests and *in vivo* studies in which different endocrine disrupting (ED) modes of action for BPS (estrogenic, anti-androgenic, affected steroidogenesis, thyroid, PPARγ, etc.) are reported.

For the environment, several studies show estrogen- and steroidogenesis-mediated adverse effects on development and reproduction in zebrafish (*Danio rerio*) after exposure to BPS in the µg/L range. The following was observed: reduced sexual functioning and fertility characterised by reduced fecundity, sperm count and oocyte maturation. Furthermore, exposure to BPS leads to altered steroid hormone balance (decreased testosterone and increased estrogen), altered hatching rate, reduced body length and weight in males, caused increased malformation rates in exposed F1 embryos and caused a skewing of phenotypic sex ratio towards females. In the available guideline study (ZEOGRT) only a trend towards feminisation was observed with the number of males close to or below natural variation (30%/70%) at low concentrations. Although this result was not significant, it can be considered as comparable to the findings in the literature study as it was observed at the similar concentrations (see Section 5.1.1.2 for more details).

Moreover, BPS is capable of inducing precocious hypothalamic neurogenesis in embryonic zebrafish which was associated to subsequent hyperactive behaviours in zebrafish larvae. Furthermore, embryonic exposure and maternal exposure to BPS can alter social behaviour and anxiety response (see section 5.7.3.1.2 for more details). Such adverse effect (group preferences, shoaling behaviour and social approaches) can affect the reproductive performance (e.g. critical for mating) and the development. Hence, alteration of these behaviours may lead to decreased fitness and may cause ultimately reduced survival of individuals and populations (Salahinejad *et al*., 2020).

Furthermore, in rodents clear endocrine-mediated effects were seen on reproduction (lower sperm count and motility, prolonged estrous cycle in females). 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 modalities (OECD GD 150, 2018). Evidence of endocrine disruptive properties of BPS on mammalian vertebrate species therefore provides further support for similar properties in non-mammalian vertebrates, in particular with regard to disruption of estrogenic pathways.

Apical effects of BPS are associated with developmental and reproductive disturbances and malformations at the organism level. These effects can severely affect population stability and recruitment and they are considered as serious effects for the species living in the environment, mammalians and non-mammalians. Furthermore, BPS can impair sex steroid hormones in fish.

- **Irreversible and delayed effects**

Effects on the next generation and population development were examined in a ZEOGRT (OECD TG 240 adapted for zebrafish, Unpublished study report, 2020). A trend towards feminisation was observed in F1 in this study. The findings were close to or even fell below natural variation at low concentrations and were comparable to the significant findings at concentrations tested in literature.

In the ZEOGRT study (Unpublished study report, 2020), non-monotonic effects of BPS were reported in F1 impairing fecundity at the low doses tested and with no effect at higher (environmentally unrealistic) concentrations above the mg/L. This reduction in egg production was confirmed by Naderi *et al.* (2014). Moreover, parental exposure to BPS caused alteration of the hatching rate (decrease) and time to hatch (delayed) of embryos, even when they were hatched in clean water (Ji *et al.*, 2013).

It is recognised that due to its MoA, a short time exposure may be sufficient to provoke long-term effects even if exposure ceases. *Moreover, effects potentially related to EATS modalities may be only observable during specific windows of exposure like specific life stage (e.g. larvae, juvenile, adult) and/or during specific stages of the reproductive cycle (e.g. gonadal development and differentiation, recrudescence, oocyte growth, final maturation). Therefore, whether or not endocrine-mediated effects are observable highly depends on the life stage tested (EFSA/ECHA ED Guidance, 2018).* Disturbance of this set up may result in effects during the entire life-time including sensitive life stages and even in the next generation with long-term consequences at the (sub)-population level (WHO/IPCS, 2002).

BPS can cause intergenerational developmental and reproductive effects.

- **Affecting a large variety of species in different ecosystems**

The ED Guidance (EFSA/ECHA ED guidance, 2018) states that: *(…) Furthermore, because of the high level of conservation of the endocrine system across taxonomic groups, the mammalian data may also be relevant for other vertebrates (OECD, 2018b). Therefore, data on mammals and other taxa are considered together in a holistic approach as part of the available evidence, but also for identifying potential data gaps when assembling lines of evidence for endocrine activity and/or endocrine-related adversity. This means, for example, that information on endocrine effects in fish/amphibians, could be used to investigate the mammalian data set with heightened scrutiny for similar effects and to target potential requests for the generation of further mammalian information, or vice versa. (…)*. In the identification of PDDP[22] and BPB[23] as endocrine disruptor for human health and environment, the argument of rats being an appropriate animal model for other mammalian wildlife species due to the general conservation of hormone systems between different mammalian species has been accepted (ECHA, 2021a, ECHA, 2021b).

As explained above, the reproductive endocrine system is highly conserved not only between mammals, but also between mammals and vertebrates like fish. Hence, adverse effects on reproduction as observed for rats are expected to occur in other mammals, and in addition, it is not excluded that exposure to BPS also leads to adverse effects (such as reproductive dysfunction and skewed sex ratios) in oviparous vertebrates.

Moreover, BPS is found in the Arctic (Kongsfjorden, Svalbard) in seabirds eggs of black-legged kittiwake and glaucous gull and in artic char muscle as explained in Section 3.3. Because BPS is assumed to act through different modes of action and target sites, a higher number of organisms may be affected. As this assessment considers only a small proportion of the existing species and thus all different organism groups were not studied, potential effects on other organisms remain unknown. Therefore, it can not be excluded that a number of other species may be very sensitive to an exposure of BPS. Adverse effects are thus not restricted to certain taxonomic groups or species.

It is generally known that effects are not restricted to certain environments. Other species, than freshwater fish, living in other compartments such as marine waters, sediments, and terrestrial environments are also affected. Monitoring studies (see section 3.2.3.2.3) show that BPS reaches diverse environmental compartments and biota, even at remote areas. This indicates that BPS is not restricted to certain environmental compartments, local sites or specific time points. It is recognised that due to its MoA, a short time exposure may be sufficient to provoke long-term effects even if exposure ceases. Effects may become more pronounced later, especially for migratory species which are more or less continuously exposed to low concentrations.

For BPS, the observed reproductive effects are considered adverse and serious with population level relevance for both human health and environment. ECHA ED Guidance states (EFSA/ECHA

[22] Phenol, alkylation products (mainly in para position) with C12-rich branched alkyl chains from oligomerisation, covering any individual isomers and/ or combinations

[23] European Chemicals Agency (ECHA) (2021) : Support document for identification of 4,4'-(1-methylpropylidene)bisphenol; (bisphenol B) as substance of very high concern

ED guidance, 2018): *Effects on growth, development, reproduction in single species are generally regarded relevant for the maintenance of the wild population (European Commission, 2011). Therefore, the relevance of such effects at the population level should be assumed when determining the adversity in the absence of appropriate scientific data demonstrating non-relevance. Behavioural changes and impaired ability to cope with additional stress are factors implicitly covered by the WHO definition of adversity, since they would affect the reproductive performance and the development. Therefore, behavioural changes or impaired ability to cope with additional stressors which have the potential to impact the population stability of non-target organisms would be considered in the definition of adversity. It is acknowledged however, that current standard tests are not specifically designed to specifically capture all behavioural effects (European Commission, 2018).*

Furthermore, reproductive effects caused by BPS are of particular concern for mammalian wildlife including top predator species and endangered species. Especially in case of endangered species their potentially low reproductive rate may lead to serious consequences for their population.

BPS may affect a large variety of species in different ecosystems and the effects seen are population relevant for both human health and environment.

- **Concern related to co-exposure and combined effects**

BPS and other bisphenols occurring in the environment can act jointly, so that exposures at comparatively low concentrations may lead to effects. BPS is part of the group of bisphenols, some of which share common MoA and may have additive effects. Common MoA and effects are demonstrated for BPA but some evidence also exists for other bisphenols i.e. bisphenol B, bisphenol F (Rosenmai *et al*., 2014; Rochester and Bolden, 2015; Le Fol *et al*., 2017; Pelch *et al*., 2019; Faheem and Bhandari, 2021).

Typical examples are sewage plant effluents (e.g. from paper mills) or water used in the paper recycling process, where BPS can occur jointly with other chemicals. These may be known endocrine disruptors with similar MoA but also chemicals with different MoA that act additively or even synergistically. It has been already recognised that bisphenols can act jointly (f.i.: share same MoA resulting in additive effects) with other chemicals occurring in the environment having the same bisphenol structure and displaying the same effect (ECHA, 2021b). Moreover, as it has been shown in Section 3.2.3.2, BPS is detected in the environment, in environmental species as well as in human fluids together with BPA and other bisphenols.

Because of the occurrence of BPS in different environmental compartments co-exposure and combined effects with other substances cannot be excluded.

- **Derivation of safe level:**

Endocrine regulation, which is set up during critical life stages in vertebrates, is a very complex feedback process. Any disturbance of this regulation during transient but vulnerable life stages can lead to irreversible effects during the entire life-time or even in the following generations. Moreover, it is difficult to assess the latency of the effects based on the available ED specific test guidelines. Therefore, it is not possible to predict potential future effects and thus safe exposure levels for the environment.

Adverse effects caused by a chemical after the exposure during the sensitive time windows in the course of development vary dependently on the organism group and also on the individuals from the same group. Therefore, effects may be overlooked, not expressed or equivocal. For BPS, for example, no data is available for all trophic levels, what makes it difficult to derive a safe exposure level in the environment, although it may exist.

Another reason may be that low effect concentrations are difficult to determine definitely as effects may only be observed in certain life stages or time windows.

Additionally, seasonal effects may lead to difficulty in predicting the impact on the development of different groups of organisms or individuals from the same group.

Besides, concentration response-relationships are often not monotonic. In case of BPS, a non-monotonic change in fecundity and hatching success was observed in fish. The support documents for identification of BPB and BPA as endocrine disruptors for human health and/or environment have shown that this non-monotonicity indeed appears. These effects can be explained by the fact that the hormonal receptors are sensitive to certain trigger concentrations, or that different modes of action are triggered.

It was shown that BPS, similarly to BPA and BPB, elicits effects via estrogenic agonism. Similarly as it has been observed for other bisphenols also other MoA are concomitant. For example, for BPS most of the data and effects observed are related to estrogen and steroidogenic pathways but BPS may also potentially interfere with anti-androgen, thyroid and PPARɣ, etc., while anti-androgenicity was also indicated for BPB and thyroid effect and anti-ecdysteroid action in arthropods for BPA.

The support document for identification of BPB as endocrine disruptor indicates that '*These multiple effects on various receptors and endpoints explain why a great variety of organisms may possibly be affected. Moreover, it has been demonstrated for BPA that certain species (e.g. fish, amphibians and snails) are sensitive at low concentrations (even below or around 1 µg/L), levels which are indeed measured in the environment'.*

Thus, as endangered species such as amphibian species are affected by BPA it may be the case for BPB. A limitation to take into account is that literature always provide data on species used in standard tests and commonly found in specific environments. But it was demonstrated for a large set of substances, that non-standard test species and non-traditional endpoints may be much more sensitive than endpoints usually considered in OECD standard test protocols (ECHA, 2017b). A great variety of taxonomic groups essential for the well-functioning of ecosystems were shown to be affected by BPA, making it probable that BPB will also affect the environment and other species living in it. Although the endocrine system with its hormones and functioning is conservative among vertebrate species, the specific hormones affected, binding affinities and modes of action differ between taxa. 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 entire environment.' (ECHA, 2021b) The arguments provided in the cited document are also relevant for BPS.

- **Avoid regrettable substitution as soon as possible**

BPS is the most widely used as alternative for BPA in thermal paper (ECHA, 2020). Next to that following uses are known: monomer in production of PESU, monomer in a production of synthetic tanning agent, leather tanning, manufacture and recycling of paper, monomer or resin in the production of food contact material and adhesives,… . It cannot be excluded that the industry may invest in substitution of BPA by BPS in other applications as well. Moreover detection of BPS in different type of environmental compartments (see section 3.2.3.2.4) and human biological fluids such as urine and blood but also in ovarian follicles, placenta and amniotic fluids (see section 3.2.3.2.2) reinforces the need to avoid further regrettable substitution and to protect human health and wildlife.

The same conclusions as listed above have been already indicated and agreed upon in the identification of BPA and BPB as endocrine disruptors for environment (ECHA, 2021b and ECHA, 2017).

6.3.2.3 Summary of the ELoC assessment

See Annex II.

6.3.3 Conclusion on the Article 57(f) assessment

BPS is identified as a substance of very high concern in accordance with Article 57 (c) and (f) of Regulation (EC) 1907/2006 (REACH):

- The substance is identified as a substance meeting the criteria of Article 57(c) of Regulation (EC) No 1907/2006 (REACH) owing to its classification in the hazard class toxic for reproduction category 1B[24], H360FD.

- The substance 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) according to Article 57(f) of REACH Regulation.

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

- BPS is covered by index number 604-098-00-1 of Regulation (EC) No 1272/2008 in Annex VI, part 3, Table 3 (the list of harmonised classification and labelling of hazardous substances)[25] and it is classified in the hazard class toxic for reproduction category 1B (H360FD).

  Therefore, this classification of the substance in Regulation (EC) No 1272/2008 allows its identification as substance of very high concern in accordance with Article 57(c) of REACH.

- In addition, BPS is identified as a substance of very high concern in accordance with Article 57(f) of Regulation (EC) 1907/2006 (REACH) because of its endocrine disrupting properties for which there is scientific evidence of probable serious effects to the environment and human health which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of the REACH Regulation.

Adverse effects

Human health:
BPS consistently affects the estrous cyclicity in female rodents, at different windows of exposure. All the available studies show irregular cycles, linked in most of them to a prolongation of the diestrus phase. The disturbance of estrous cycle is considered as EAS (estrogenic, androgenic, and steroidogenic)-mediated.

In addition, effects that are sensitive to, but not diagnostic of, EATS (as potentially linked to other Modes of Action) were also reported regarding rodent female reproduction. A statistically significant decrease of the number of embryo implantation sites was observed in reproductive toxicity studies, resulting in decreased fertility and number of pups.

Other developmental and male reproductive adverse effects were observed in the available rodent studies supporting the endocrine disrupting properties of BPS. These include EAS-mediated effects such as reduced sperm count and motility at low doses and a high incidence of male rodent mammary gland multifocal atrophy. Additionally, adverse effects sensitive to, but not diagnostic of, EATS were observed including dose-dependent increased post-implantation

[24] Classification in accordance with section 3.7 of Annex I to Regulation (EC) No 1272/2008.
[25] COMMISSION DELEGATED REGULATION (EU) 2022/692 of 16 February 2022 amending, for the purposes of its adaptation to technical and scientific progress, Regulation (EC) No 1272/2008 of the European Parliament and of the Council on classification, labelling and packaging of substances and mixtures (18th ATP)

loss in reproductive toxicity studies and higher adrenal glands weight, in particular in males, in several independent studies.

These adverse effects have been observed at doses showing neither maternal toxicity nor severe general toxicity. Moreover, since estrogen signalling is critical to reproductive success in all vertebrates including mammals, it is assumed that the observed adverse effects on fertility through disruption of estrogen signalling in rodents are relevant to humans.

The complexity of the effects sensitive to, but not diagnostic of, EATS observed following exposure to BPS suggests the interaction of multiple MoAs to produce the observed effects, increasing the concern for human health. For example, the consistent effects on the mammary gland in males in two rodent species provides an indication of hormonal disturbance and may have influence on e.g. human breast tumor development.

Environment:

There is evidence in literature that BPS affects sperm count and sex ratio in zebrafish (*Danio rerio*) after exposure in the µg/L range. In a ZEOGRT (OECD TG 240 adapted for zebrafish), the findings on sex ratio were not significant. However a similar trend towards feminisation was observed with the number of males close to or even below natural variation at low concentrations. These EAS-mediated effects were observed at concentrations below general toxicity.

In addition, effects that are sensitive to, but not diagnostic of, EATS (as potentially linked to other Modes of Action) were also reported regarding reproductive effects: reduced fecundity, reduced hatching rate and altered oocyte maturation in fish.

Other important adverse effects on brain neurogenesis and behaviour were identified in fish. Experimental data on zebrafish demonstrated that these effects depend on BPS-induced changes in aromatase activity.

Effects on apical endpoints such as fecundity and altered sex ratio are considered to impair population stability and recruitment. Therefore, these effects are to be considered population relevant for the environment.

BPS induces adverse effects on development and reproduction in rodents and fish.

Endocrine activity

Bisphenols are known to target many endocrine pathways. Consistent *in vivo* and *in vitro* evidence is available on steroidogenesis and in particular on estrogenic activity.

- *Estrogenic activity*

In vitro ER binding assays demonstrate that BPS is capable of binding to the estrogen receptor, with IC50 ranging from 5.8 to 105 µM depending on the cell line used (rat and human). Several *in vitro* literature studies using different cell cultures showed a weak increase in the estrogenic activity (ER reporter gene assays, proliferative assays and ER-regulated gene expression assays). *In vivo*, the increase in uterine weight, observed in all rodent uterotrophic assays, is a parameter diagnostic of estrogenicity.

Vitellogenin, a biomarker of estrogenic activity in fish, was induced in embryonic and adult male zebrafish. Literature data also reported a change in steroidal hormone balance with decreased testosterone and increased estradiol levels and an increased E2/T ratio in zebrafish.

BPS exhibits estrogenic activity.

- *Steroidogenesis*

In a range of *in vitro* assays investigating steroidogenesis following exposure with BPS, a clear trend towards decreased testosterone was observed. Furthermore, an increase in testis aromatase expression was observed in several studies following exposure to BPS. Several, but not all, *in vivo* studies, showed decrease in serum testosterone level in rodents.

Moreover, the impact on the synthesis of steroid hormones (decrease of testosterone and increase of estrogen) was clearly shown in *in vivo* studies with zebrafish. These findings were accompanied by an increased expression of genes involved in steroidogenesis and specifically in aromatase (CYP19a, CYP19b in testis and brain resp.).

BPS is shown to affect steroidogenesis.

Plausible link between adverse effects and endocrine activity

Human health:
Considering the results of all available experimental studies, there is strong evidence that the adverse effects on fertility in female rodents are due to the estrogenic activity of BPS. The increase in uterus weight (as seen in the available uterotrophic assays) is a strong diagnostic parameter for estrogenicity. Furthermore, the prolongation of the estrous cycle was consistently observed in the majority of the studies. In addition, the number of implantation sites was decreased in three reproductive studies, resulting in a decrease of both fertility and number of pups. All of these parameters are considered as either EATS-mediated or sensitive to, but not diagnostic of, EATS modalities. The different effects of BPS, in particular on the female reproductive system, can be plausibly linked to the estrogenic activity of the substance and could therefore explain the adverse impacts seen on fertility endpoints.

Other modes of action than those involving estrogenic activity and/or signalling pathways are likely. For example, altered testosterone production is probably linked to adverse effects on the male reproductive system (reduced sperm count and motility) or the male mammary gland. Despite the fact that these data give further indications of the endocrine activity of BPS, they are considered as supportive adverse human health effects.

In conclusion, the effects on the female reproductive organs and functional parameters are consistent with an estrogenic mode of action of BPS. The adverse effects on the estrous cycle are EATS-mediated, therefore, in the absence of information proving the contrary, the biologically plausible link is already pre-established based on existing scientific knowledge. There is strong evidence that the **adverse effects on fertility and sexual function are plausibly linked to the estrogenic activity of the substance. BPS is therefore an endocrine disruptor according to the WHO/IPCS definition with regard to human health.**

Environment:

Based on the weight of evidence approach and considering the results of all available studies there is evidence that the adverse effects of BPS on sperm count and sex ratio in zebrafish are due to the estrogenic activity and to disrupted steroidogenesis.

Skewed sex ratio is recognised as an EAS-mediated effect. Altered gametogenesis as reduced sperm counts has been also observed. Based on the existing knowledge in mammals and the similarities with fish gametogenesis, reduced sperm count is considered as EAS-mediated also in fish. The estrogenic activity of BPS is demonstrated in mammals and is further evidenced by vitellogenin induction in fish. Altered steroidogenesis may lead to the observed decreased sperm counts and altered oocyte maturation which, in turn, may lead to impaired hatchability of the eggs. Increased aromatase activity is consistently observed and is clearly responsible for effects on fish brain and behaviour. Impaired social behaviour may also result in reduced reproduction.

There is a large degree of conservation of the endocrine system, implying large commonalities between non-mammalian and mammalian vertebrate species in regard to hormones, enzymes and receptors involved in the EATS modalities. All mammalian data provide substantial evidence that BPS can disrupt particularly estrogenic pathways. Therefore, those data were also considered in the Weight of Evidence approach for the assessment of the ED properties in the environment and thus wildlife species.

Considering all relevant and reliable information in a weight of evidence approach, it is concluded that BPS is an endocrine disruptor according to the WHO/IPCS definition with regard to environment.

Equivalent level of concern:

The effects of BPS due to its endocrine disrupting properties are considered to be of equivalent level of concern to CMR Cat. 1, PBT or vPvB substances as listed in Article 57 points (a) to (e) of the REACH Regulation.

Based on the scientific evidence, the effects on organisms and populations are considered to be severe and irreversible as effects on estrous cycle, sex ratio, etc. are observed following developmental exposure. Such effects are considered to impair population stability and recruitment. Moreover, a wide range of taxa in different ecosystems may be adversely affected due to conservation of the endocrine system. However, the difference between taxa concerning specific hormones affected, binding affinities and modes of action makes it difficult to determine the most sensitive species and thus to quantify a safe level of exposure with regard to the endocrine mediated effects.

Bisphenols are widely used and can be found together in the environment. It has been already recognised that bisphenols can act jointly in the environment by sharing the same mode of action resulting in additive effects. Bisphenols can also act together with chemicals other than bisphenols (sharing the same and/or a different MoA) occurring in the environment, at comparatively low concentrations, displaying the same and/or additional effects. This supports equivalent level of concern as endocrine disruptors with similar MoA but also chemicals with different MoA can act additively or even synergistically.

In conclusion:

Based on all available scientific evidence, it can be concluded that BPS fulfils the WHO/IPCS (2002)[26] definition of an endocrine disruptor:

- It shows clear reproductive adverse effect in rodents and fish. The reproductive endocrine system is highly conserved not only between mammals, but also between mammals and other vertebrates like fish.

- It has endocrine modes of action: clear estrogenic mode of action and alteration of steroidogenesis.

- The adverse effects, including the recognised EAS-mediated effects (e.g. on estrous cycle and sex ratio) and effects sensitive, but not diagnostic of EAS (e.g. fecundity, fertility, implantation sites and number of pups), are a consequence of the endocrine modes of action.

The assessment performed demonstrates that there is scientific evidence of **probable serious effects of BPS to the environment and human health due to its endocrine disrupting properties, which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of the REACH Regulation.**

[26] An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, its progeny or (sub)populations.

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Annex I - Lines of evidence for adverse effects and endocrine activity

As outlined in the ED guidance (EFSA/ECHA ED Guidance, 2018), if there are indications that a substance may act via multiple MoAs, the assessment should start with the MoA for which the most convincing evidence is available. BPS may have multiple modes of action that interact or superimpose and are difficult to distinguish from each other.

The most convincing evidence for endocrine disruption caused after exposure to BPS is available for estrogenic activity. In this respect only the lines of evidence supporting the postulated MoA are presented underneath.

The reliability of the studies has been assessed with ToxRTool. This assessment tool was developed by the European Commission's Joint Research Center in 2009 (Segal *et al*., 2015). It builds on Klimisch categories by providing additional criteria and guidance for assessing the reliability of (eco)toxicological studies. ToxRTool is applicable to various types of experimental data, endpoints and studies (study reports, peer-reviewed publications). The most informative studies were given a reliability score of 1 (reliable without restriction) or 2 (reliable with restriction). Due to the very high number of literature studies on BPS, it has been decided to perform the assessment only of:

- *in vitro* studies based on TG or TG-like protocols,
- *in vivo* studies in rodents demonstrating an adverse effect resulting in impaired female reproduction or considered as supportive adverse effect,
- *in vivo* studies showing adverse effects in zebrafish resulting in impaired reproduction and development.

Table X1: Lines of evidence for endocrine activity in silico by BPS

It was decided not to perform a thorough scientific literature search for *in silico* data because sufficient amount of *in vitro* data on estrogen modality are available.

Table X2: Lines of evidence for endocrine activity in vitro by BPS

A literature search was performed until 30.08.2021 (except for Park *et al*., 2022; Salahinejad *et al*., 2022 and Naderi *et al*., 2022). Studies were listed according to cell lines.

1. ESTROGEN MODALITY

Group-ing	*Line of evidence*	*Reference*	*Study relia-bility (ToxR Tool)*	*Guideline / test type*	*Species / cell lines*	*Test duration*	*Duration unit*	*Conc. or conc. range*	*Conc. unit*	*Observed effects (positive/negative)*	*Assessment of each line of evidence*	*Assessment of the integrated line of evidence*
In vitro mechanistic	**ER binding**	Blair *et al.*, 2000	1	OPPTS 890.1250	Rat uterine cytosol	20	h	10^{-11} to 10^{-3}	M	IC50 = 1.05 x10^{-4} M RBA (compared to E2) = 0.0009%	Very weak affinity for ER	Overall positive evidence for endocrine activity
		Laws *et al.*, 2006	-	Estrogen receptor competitive binding assay	Rat uterine cytosol	18	h	10^{-7} to 10^{-4}	M	IC50=64.0 µM Ki=82.4 µM 50-74% displacement of 17β-estradiol	Weak estrogen receptor binding activity	
		Hashimoto and Nakamura, 2000	-	Fluorescence polarisation	Human Erα	1	h	10^{-7} to 10^{-3}	M	~70% displacement of the fluorescence ligand ES1 from the hERα-ES1 complex at 10^{-3} M	Estrogen binding activity	
		Hashimoto *et al.*, 2001	-	Fluorescence polarisation	Human Erα	1	h	10^{-7} to 10^{-3}	M	~70% displacement of the fluorescence ligand ES1 from the hERα-ES1 complex at 10^{-3} M	Estrogen binding activity	
		METI, 2002	-	Estrogen receptor competitive binding assays	Human ERα					RBA=0.0055% Weak relative activity (RA) of BPS regarding E2. The RA is the ratio between the concentration giving 50% of the maximum transcriptional activity of E2 1 nM (PC50) of BPS divided by the PC50 of E2.	Weak estrogen receptor binding affinity	

Group-ing	Line of evidence	Reference	Study relia-bility (ToxR Tool)	Guideline / test type	Species / cell lines	Test duration	Duration unit	Conc. or conc. range	Conc. unit	Observed effects (positive/negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence
										RA = 0.000254		
		Yamasaki *et al.*, 2004	-	Estrogen receptor competitive binding assays	Human recombinant ERα	1	h	10^{-11} to 10^{-4}	M	RBA=0.0055%	Weak estrogen receptor binding activity	
		Akahori *et al.*, 2008	-	Estrogen receptor binding assay	hERα-LBD fused with glutathione S-transferase and expressed in *E. coli*	1	h	10^{-11} to 10^{-4}	M	Log RBA = -2.26 (RBA=0.00549%)	Weak estrogen receptor binding activity	
		Molina-Molina *et al.*, 2013	-	Luciferase reporter gene assay	HELN-hERα and –hERβ	3	h	0.01–10	µM	in HELN-hERα: IC50=6560 nM RBA=0.001% in HELN-hERβ IC50= 3452 nM RBA=0.006%		
		Rajasärkkä *et al.*, 2014	-	Yeast reporter assay	based on BPA-targeted receptor (BPA-R)	3	h	10^{-7} to 10^{-2}	M/L	2.5 orders of magnitude less potent than BPA	Weak affinity for ER	
		Rajasärkkä *et al.*, 2014	-	Yeast reporter assay	hERα	3	h	10^{-7} to 10^{-2}	M/L	2 orders of magnitude less potent than BPA	Weak affinity for ER	
		Stossi *et al.*, 2014	-	Fragment complementation assay (PCA)	ERα-LBD and Erβ-LBD stably expressed by fusion protein constructs in HEK93T cells	8	h	10^{-10}-10^{-4}	M	Erα and Erβ: low activity (log EC50=-4)	Low ER binding activity	

Group-ing	Line of evidence	Reference	Study relia-bility (ToxR Tool)	Guideline / test type	Species / cell lines	Test duration	Duration unit	Conc. or conc. range	Conc. unit	Observed effects (positive/negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence
		Stossi *et al.*, 2014	-	PRL assay	ERα-LBD and Erβ-LBD stably expressed by fusion protein constructs in HEK93T cells	30	min	10^{-10}-10^{-4}	M	Erα: no activity ERβ: EC50: 10^{-5} M	No Erα binding activity Low Erβ binding activity	
		Zhang *et al.*, 2018	-	Fluorescence polarisation assay	hERα-LBD	2	h	1-10	µM	IC50=5.78 µM	Estrogen binding activity	
		Eilebrecht *et al.*, 2019	-	Estrogen Receptor Transactivation assay	HEKT 293 (ERα and ER)β	2	h	0.01, 0.1, 1.0, 10 and 100	µM	ERα: 26.8% inhibition IC50: nd ERβ: 25.75% inhibition IC50= 7.8 µM, RBA: 0.012%	Estrogen binding activity	
		Keminer *et al.*, 2020	-	Fluorescence polarisation (Polar screen E-TM wcompetitor assay)	ERα and ERβ	2	h	10	µM	No inhibition of binding to the fluorescent compound (ERα nor ERβ)	No effect	
		Liu *et al.*, 2019b	-	Competitive binding assay	ERα and ERβ	1	h	10	µM	ERα: IC50>10 µM Erβ: IC50>10 µM	Extremely weakly active	
		Cano-Nicolau *et al.*, 2016	-	Competitive binding assay	Human U251 glia cells transfected with Zebrafish ERα, ERβ1 and Erβ2	24	h	10^{-10} to 10^{-5}	M	Almost no binding affinity to move zfERα, zfERβ 1 and zfERβ 2 (probably due to the absence of binding to ERα)	Very weak estrogen receptor binding	
	ER activity (agonist)	Tox Cast Pathway model (ER)	-	high-throughput ER binding model						Agonist (AUC) : 0.263 agonist (above the threshold of 0.1) and 0 antagonist. Cytotoxicity limit 13.64 µM	Estrogenic activity	Overall positive evidence for endocrine

Group-ing	Line of evidence	Reference	Study relia-bility (ToxR Tool)	Guideline / test type	Species / cell lines	Test duration	Duration unit	Conc. or conc. range	Conc. unit	Observed effects (positive/negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence
		Nishihara *et al.*, 2000	-	Yeast two-hybrid assay	Yeast cells Saccharomyces cerevisia (hERα + coactivator TF2)	4	H			No estrogenic activity up to 1 x10^{-3} M (i.e. 10% of the agonistic activity of 10^{-7} M E2 was not reached)	No estrogenic activity	activity
		Hashimoto Y. and Nakamura M., 2000	-	Yeast two-hybrid system (ERα)	Yeast cells (hERα)	4	H	10^{-7} to 10^{-3}	M	No estrogen activity between 10^{-7} and 10^{-3} M (i.e. the relative β-galactosidase activity (rate of β-galactosidase activity divided by that of 10^{-7} M E2) was below 0.1)	No estrogenic activity	
		Hashimoto *et al*., 2001	-	Yeast two-hybrid assay	Yeast cells	4	H	10^{-7} to 10^{-3}	M	significant ↗ of β-galactosidase activity at concentrations of 10^{-3} M in the presence of metabolic activation No induction in the absence of S9mix	Estrogenic activity after metabolism	
		Chen *et al.*, 2002	-	Yeast two – hybrid assay	Yeast cells (ERα)	4	h	0.001-1000	mg/L	Weak estrogen activity: only 130 units at 200 mg/L compared to optimum concentration of 17β-estradiol (1230 units)	Weak estrogenic activity	
		Skledar *et al.*, 2016	-	Yeast assay (Xenometrix)	Yeast cells (hERα)	48	h	30 nM to 300 mM		Agonist activity: EC50 = 8.4 x10^{-5} M	Estrogen agonist activity	
		Dvorakova *et al.*, 2016	2	YES assay	Yeast cells (hERα)	4	h	10^{-8} to 10^{-4}	mg/L	Estrogenic agonist activity observed		
		Conroy-Ben *et al.*, 2018	-	YES assay	Yeast cells (hER)	4	h	10^{-10}-10^{-3}	M	Weak estrogenic activity EC50 = 5.88 x10^{-4} M	Weak estrogenic activity	

Group-ing	Line of evidence	Reference	Study relia-bility (ToxR Tool)	Guideline / test type	Species / cell lines	Test duration	Duration unit	Conc. or conc. range	Conc. unit	Observed effects (positive/negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence
										Compared to 17β-estradiol (EE=1), EE of BPS= 1.07 $x10^{-6}$		
		Ruan *et al.*, 2015	-	Bioluminescent Yeast reporter assay (BLYES)	Yeast cells expressing estrogen-response elements	12	h	10-100000	nM	2 orders of magnitude less potent than BPA EC50 = 4.13 $x10^{5}$ nM EEF = 1.05 $x10^{-6}$	Weak estrogenic activity	
		Grignard *et al.*, 2012	1	Transactivation assays (comparable to OECD TG 455)	MELN cells	24	h	10^{-15}- to 10^{-4}	M	EC50= 4.24 $x10^{-6}$ M	Weak estrogenic activity	
		Molina-Molina *et al.*, 2013	-	Luciferase reporter gene assay	MELN cells	16	h	10^{-2} to 10^{-5}	M	EC50= 12.10 µM	Estrogenic activity	
		Grignard *et al.*, 2012	-	Transactivation assays (comparable to OECD TG 455)	BG1Luc4E2 cells	24	h	10^{-15}- to 10^{-4}	M	EC50= 4.93 $x10^{-6}$ M	Weak estrogenic activity	
		Simon *et al.*, 2016	-	Reporter gene assay	BG1Luc4E2	24 or 48	h	10^{-6}, 10^{-5}, 10^{-4} and 0.001 Dissolved in 1% DMSO	M	Agonistic effect: relative response >50%	Estrogenic activity	
		Dvořáková *et al.*, 2016	2	ER transactivation assay (OECD TG 455/457)	VM7Luc4E2 (formerly BG1Luc4E2)	24	h	10^{-7} to 10^{-2}	mg/L	Estrogenic agonist activity observed		

Group-ing	Line of evidence	Reference	Study relia-bility (ToxR Tool)	Guideline / test type	Species / cell lines	Test duration	Duration unit	Conc. or conc. range	Conc. unit	Observed effects (positive/negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence
		Rosenmai *et al.*, 2014	-	Reporter gene assay	BG1Luc cells	22	h	0.0001-100	µM	ER activity ↗ Emax= 222% EC50= 1.17 µM	Estrogenic activity	
		Grignard *et al.*, 2012	1	Transactivation assays	BG1Luc4E2 cells	24	h	10^{-15}- to 10^{-4}	M	Weak estrogenic activity EC50= 4.93 x10^{-6} M	Weak estrogenic activity	
		Molina-Molina *et al.*, 2013	-	Luciferase reporter gene assay	HELN-hERα and –hERβ	3	h	0.01– 10	µM	in HELN-hERα: EC50= 3.96 µM IC50= 6560 nM RBA= 0.001% in HELN-hERβ EC50= 1.72 µM IC50= 3452 nM RBA= 0.006%		
		Grimaldi *et al.*, 2019	-	Reporter gene assay	HELN ERα HELN ERβ	16	h	0.001–10	µM	ERα : EC50= 4.47 +/-0.59 µM ERβ : EC50= 1.72 +/-0.11 µM	Estrogen agonistic activity	
		Hashimoto and Nakamura, 2000	-	E-screen	MCF-7	144	h	10^{-7} to 10^{-3}	M	Proliferation at 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M	Estrogenic activity	
		Hashimoto *et al.*, 2001	-	E-screen	MCF-7	144	h	10^{-9} to 10^{-4}	M	↗ of MCF7 cell growth at 10^{-6} and 10^{-5} M (1.5-fold)	Estrogenic activity	
		Kitamura *et al.*, 2005	-	ERE (Estrogen Response Element)-luciferase reporter assay	MCF-7	24	h	10^{-9} to 10^{-4}	M	Significant estrogen agonist activity EC50= 1.1 µM		
		Kuruto-Niwa *et al.*, 2005	-	Cell proliferation assay	MCF-7	3	d	10^{-9} to 10^{-4}	M	Weak estrogen potency EEF of 5.54 x10^{-6}		

Group-ing	Line of evidence	Reference	Study relia-bility (ToxR Tool)	Guideline / test type	Species / cell lines	Test duration	Duration unit	Conc. or conc. range	Conc. unit	Observed effects (positive/negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence
		Molina-Molina *et al.*, 2013	-	E-screen	MCF-7	6	d	0.01-10	µM	↗ of cell proliferation (3.7-fold) at 10 µM		
		Stossi *et al.*, 2014	-	Cell proliferation assay	MCF-7	6	d	10	µM	↗ proliferation RPE= 97.54% RPP= 0.000105%	Estrogenic activity	
		Simon *et al.*, 2016	-	Reporter gene assay	MCF-7 (MVV-Luc)	24 or 48	h	10^{-6}, 10^{-5}, 10^{-4} and 0.001	M	Agonistic effect: relative response >50%	Estrogenic activity	
		Kim *et al.*, 2017	-	Cell proliferation assay	MCF-7	48	h	10^{-9}-10^{-7}	M	↗ proliferation of MCF-7 cells (10^{-6} to 10^{-5} M)		
		Mesnage *et al.*, 2017	-	E-screen	MCF-7	6	d	10^{-11}-10^{-4}	M	↗ proliferation AC50= 1.33 µM	Weak estrogenic activity	
		Li *et al.*, 2018	-	Luciferase Reporter gene assay	MCF-7		h	1-1000	nM	ERα specific binding: ERE-mediated activation at 1000 nM (3-fold)	Estrogenic activity	
		Molina-Molina *et al.*, 2019	-	E-screen	MCF-7	6	d	0.01-10	µM	No positive correlation between BPS concentrations in thermal paper and estrogenic activity	No effect	
		Williams and Darbre, 2019	-	Proliferation assay	MCF-7	7	d	10^{-11}-10^{-4}	M	Significant ↗ proliferation of ERα-dependent MCF-7 after exposure to 10^{-8} M	Estrogenic activity	
		Atlas and Dimitrova , 2019	-	Proliferation assay	MCF-7	7	d	0.00001-10	µM	↗ proliferation of MCF-7 (expressing ERα and ERβ) at 1 µM	Estrogenic activity	
		Park *et al.*, 2020	-	Protein isolation and western blotting	MCF-7	24	h	0.01-10	µM	Significant ↘ of ERα by 0.53-fold after exposure to 10 µM		
		Atlas and Dimitrova , 2019	-	Growth in 3D	MCF-10A	30	d	1	µM	No induction of proliferation of MCF-10A (expressing ERβ but not ERα) at 1 µM	No effect	

Group-ing	Line of evidence	Reference	Study relia-bility (ToxR Tool)	Guideline / test type	Species / cell lines	Test duration	Duration unit	Conc. or conc. range	Conc. unit	Observed effects (positive/negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence
		Atlas and Dimitrova , 2019	-	Luciferase reporter gene assay	MCF-12	7	d	0.00001-10	µM	↗ Activation of ERα at 1 µM (2.5-fold) and at 10 µM (5-fold), ERβ followed a similar trend	Estrogenic activity	
		Williams and Darbre, 2019	-	Proliferation assay	ZR-75-1	7	d	10^{-11}-10^{-4}	M	Significant ↗ proliferation of ERα-dependent ZR-75-1 after exposure to 10^{-8} M	Estrogenic activity	
		Williams and Darbre , 2019	-	Proliferation assay	HMF3A	7	d	10^{-11}-10^{-4}	M	No proliferation of ERα and Erβ-dependent HMF3A	No effect	
		Mesnage *et al.*, 2017	-	E-screen	T47-D	6	d	10^{-11}-10^{-4}	M	Proliferation ↗ Same trend as seen in MCF-7 but at a lesser extend	Weak estrogenic activity	
		Conley *et al.,* 2016	-	Estrogen receptor transcription al activation assay	T47D-KBluc cells (ERα and Erβ)	24	h	10^{-10}-10^{-5}	M	ERα: EC50 = 6.43 x10^{-7} M RPF = 1.55 x10^{-6}		
		Mesnage *et al.*, 2017	-	ERE-mediated Luciferase reporter assay	T47D-KB-luc cells	24	h	10^{-11}-10^{-4}	M	AC50 = 1.5 µM addition of ICI 182,780 (100 nM) antagonised the effect of BPS	Weak estrogenic activity	
		Mesnage *et al.*, 2017	-	E-screen	MDA-MB-231 cells					No cell proliferation	No effect	
		Kang *et al.*, 2014	1	OECD TG 455	Stable transfected Human MVLN cells	72	h	0.0001-5	µM	EC50= 6.97 x10^{5} µM REP50= 4.09 x10^{-9} at 0.5 µM of BPS (REP = relative potency to E2) metabolites formed by S9 fraction showed increasing estrogenic activity	Estrogenic activity	
		Boucher *et al.*, 2016	-	Estrogen-response element (ERE-	Human preadipocytes	2 and 4	d	0.1 nM to 25 µM		↗ Activation of ERE-luciferase reporter up to 3-fold		

Group-ing	Line of evidence	Reference	Study relia-bility (ToxR Tool)	Guideline / test type	Species / cell lines	Test duration	Duration unit	Conc. or conc. range	Conc. unit	Observed effects (positive/negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence
				luciferase) transcription assay								
		Li *et al.*, 2018	-	ERE-mediated Luciferase reporter assay	Human endometrial adenocarcinoma, Ishikawa/vec	18	h	1-1000	nM	Ishikawa/Vec ERE-mediated activation is weak No Erβ-mediated activation	Weak estrogenic activity: Response is cell-type specific	
		Li *et al.*, 2018	-	ERE-mediated Luciferase reporter assay	Human endometrial adenocarcinoma ,Ishikawa/ERα,	18	h	1-1000	nM	Ishikawa/ERα ERE-mediated activation at 1000 nM (3-fold)		
		Li *et al.*, 2018	-	Luciferase reporter assay	HepG2 (ERα and ERβ)	18	h	1-1000	nM	BPS only weakly activated the ERα ERE at 100 nM (8-fold), at 1000 nM (30-fold) No ERβ ERE mediated activation		
		Pelch *et al.*, 2019	-	Luciferase reporter assay	human Transiently transfected HepG2, ERα and ERβ,	18	h	3 nM to 10 µM		ERα Rela EC50= 1.3 $x10^{-6}$ ERβ Rela EC50= 2.1 $x10^{-6}$	Estrogenic activity (Erα and Erβ)	
		Park *et al.*, 2020	1	Stable transfected transcriptional activation (OECD TG 455)	HeLa9903 (hERα)	20-24	h			EC50 of 5.98 $x10^{-6}$ M PC50= 0.43 µM (response that is 50% of the maximal positive control response)	Estrogenic activity	
		Li *et al.*, 2018	-	Luciferase reporter gene assay	BG-1FR cells		h	1-1000	nM	ERE-mediated activation at 1000 nM (± 5-fold)		

Group-ing	Line of evidence	Reference	Study relia-bility (ToxR Tool)	Guideline / test type	Species / cell lines	Test duration	Duration unit	Conc. or conc. range	Conc. unit	Observed effects (positive/negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence
		Kojima *et al.*, 2018	-	Luciferase reporter gene assay	CHO-K1 cells	24	h	10^{-9}-10^{-5}	M	ERα: REC50 = 5.4 x10^{-7} M ERβ: REC50 = 5.4 x10^{-7} M	Estrogenic activity	
		Teng *et al.*, 2013	-	Transient transfection luciferase assays	Monkey kidney CV1 cells	24	h	10^{-8}–10^{-4}	M	EC50 = 2.20 x10^{-6} M	Weak estrogen agonist activity	
		Atlas and Dimitrova, 2019	-	Reporter gene assay	COS-7 cells (from monkey kidney) transfected with VP16-ERα or VP-16-ERβ	24	h	0.01, 0.0, 0.1, 1 and 10	µM	ERα: Sign. ↗ at 1.0 (2.5-fold) and 10 µM (5-fold) ERβ: sign. ↗ 10 µM: similar trend as ERα	Estrogenic activity	
		Keminer *et al.*, 2020	-	Fluorescence polarisation (Polar screen E-TM wcompetitor assay)	ERα and ERβ	2	h	>10	µM	No nuclear receptor binding		
		Le Fol *et al.*, 2017	-	Transactivation assay	Zebrafish hepatic reporter cells (zfERα, zfERβ1 and zfERβ2)					EC50 of 4.058 µM in zfERα, 1.016 µM in zfERβ1 and 2.468 µM in zfERβ2 (REPE2) to each receptor was 3.7 x10^{-5}, 3.0 x10^{-5} and 2.4 x10^{-5} resp. => slightly more potent towards zfERβs than zfERα	Estrogenic activity	
		Qiu *et al.*, 2018a	-	Macrophage exposure experiment	Fish primary macrophages of *Cyprinus carpio*	6	h	0.1 1,10, 100 and 1000	µg/L	Significantly ↗ of ERα and ERβ expression at conc ≥ 1 µg/L	Estrogenic activity	
		Cano-Nicolau *et al.*, 2016	-	Fluorescence in situ hybridisation	Human U251 glia cells transfected					No stimulation of transcriptional activity of zebrafish nuclear receptors (ERα, ERβ1,and ERβ2)		

Group-ing	Line of evidence	Reference	Study relia-bility (ToxR Tool)	Guideline / test type	Species / cell lines	Test duration	Duration unit	Conc. or conc. range	Conc. unit	Observed effects (positive/negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence
					with zebrafish ERα, ERβ1 and ERβ2							
		Campen *et al.*, 2018	-		Bovine granulosa and theca cells					Estrogenic agonist: ↗ production of estradiol in ganulosa cells, no effect in presence of LH	Supportive	
		Viñas and Watson, 2013	-	Estrogen-induced (via membrane ERα) non-genomic signalling	Rat GH3/B6/F10 pituitary cell line	24	h	10^{-15} to 10^{-7}	M	ERK-activation similar to E2 No significant JNK activation BPS can alter the timing of response to E2 at very low concentrations.	Supportive	
		Ma *et al.*, 2015	-	probe-based RT-PCR assay	Chicken embryonic hepatocytes			1-300	µM	Significant upregulation of apoII (by 30.7-fold) and Vtg (by 10.3-fold) after exposure to 300 µM BPS		
		Zalmanova *et al.*, 2017	-		Pig oocytes	72	h	3 nM, 300 nM or 30 µM		Impact on maturation rates, spindle morphology and cumulus expansion	Supportive	
	ER Activity (antagonist)	Tox Cast Pathway model (ER)	-	high-throughput ER binding model						Antagonist (AUC) :0 Cytotoxicity limit 13.64 µM	No anti-estrogenic activity	
		Kitamura *et al.*, 2005	-	ERE (Estrogen Response Element)-luciferase reporter assay	MCF-7 cells	24	h	10^{-10}	M	No significant antagonistic activity	No effect	Overall negative evidence
		Kuruto-Niwa *et al.*, 2005	-	Cell proliferation assay	MCF-7	3	d	10^{-9}-10^{-4}	M	No antagonistic activity	No effect	
		Teng *et al.*, 2013	-	Transient transfection luciferase assays	Monkey kidney CV1 cells	24	h	3 x10^{-8} to 1 x10^{-13}	M	No antagonistic activity	No effect	

Group-ing	***Line of evidence***	***Reference***	***Study relia-bility (ToxR Tool)***	***Guideline / test type***	***Species / cell lines***	***Test duration***	***Duration unit***	***Conc. or conc. range***	***Conc. unit***	***Observed effects (positive/negative)***	***Assessment of each line of evidence***	***Assessment of the integrated line of evidence***
		Dvořáková *et al.*, 2016	2		VM7Luc4E 2					No antagonistic estrogen activity		
		Simon *et al.*, 2016	-	Reporter gene assay	T47-D (TARM-Luc, TM-Luc and TGRM-Luc)	24 or 48	h	10 mM, 1mM and 100 µM		No agonistic nor antagonistic activity	No effect	
		Mesnage *et al.*, 2017	-	ERE-mediated Luciferase reporter assay	T47D-KB-luc cells	24	h	10^{-11}-10^{-4}	M	Antagonised activation with addition of ICI 182,780 (100 nm)	Weak antagonistic estrogen activity	
		Okazaki *et al.*, 2017	-		MCF-7 and SK-BR-3 cells					Reduced transcriptional activity (antagonist), but not statistically significant		
		Pelch *et al.*, 2019	-	Luciferase reporter assay	human Transiently transfected HepG2, ERα and ERβ	18	h	3 nM to 10 µM		ERα Rela EC50= - ERβ Rela EC50= -	No antagonistic estrogen activity	
		Kojima *et al.*, 2018	-	Luciferase reporter gene assay	CHO-K1 cells	24	h	10^{-12}-10^{-5}	M	No agonistic activity	No effect	

2. STEROIDOGENESIS

Group-ing	Line of evidence	Reference	Study relia-bility (ToxR Tool)	Guideline / test type	Species / cell lines	Test duration	Duration unit	Conc. or conc. range	Conc. unit	Observed effects (positive/negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence
In vitro mechanistic	**Aromatase**	Williams and Darbre, 2019	-	Aromatase activity assay	MCF-7	8	h	10^{-8}	M	Sign. ↗ of aromatase activity after exposure to 10^{-8} M BPS	Impact on steroidogenesis: Increased aromatase activity	Overall increase of aromatase activity
		Williams and Darbre, 2019	-	Aromatase activity assay	ZR-75-1	8	h	10^{-8}	M	Sign. ↗ of aromatase activity after exposure to 10^{-8} M BPS		
		Williams and Darbre, 2019	-	Aromatase activity assay	HMF3A	8	h	10^{-8}	M	Sign. ↗ of aromatase activity after exposure to 10^{-8} M BPS		
	Steroid hormone production	ToxCast	-	CEETOX H295R	human	48	h			Active but above cytotoxicity limit of 13.64 µM • 11-deoxycoticosterone: AC50= 21.9 µM • Androstenedione: AC50= 32.6 µM • Cortisol: AC50= 33.4 µM • Estradiol: AC50= 16.2 µM • Testosterone: AC50 =30.7 µM Active below cytotoxicity limit: • 11-Deoxycortisol: AC50= 4.46 µM • Progesterone: AC50= 1.25 µM • 17-alpha-hydroxypregnelone: AC50= 1.08 µM	Effect on steroidogenesis	Overall impact on steroidogenesis with decreasing testosterone and increasing estradiol levels

Group-ing	Line of evidence	Reference	Study relia-bility (ToxR Tool)	Guideline / test type	Species / cell lines	Test duration	Duration unit	Conc. or conc. range	Conc. unit	Observed effects (positive/negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence
		Rosenmai *et al*., 2014	1	H295R steroidogenesis assay	human adrenal cortico-carcinoma cells	48	h	0.8-50	µM	No change in 17β-estradiol or estrone. Sign. ↘ in male hormones: dehydroandrosterone: 70%, androstenedione: 57% and testosterone: 62% ↘ of cortisol: 74% and 70% corticosterone ↗ of progesterone (744%) and 17-α-OH progesterone (1676%)	Changes in steroid hormone levels	
		Goldinger *et al.*, 2015	1	H295R steroidogenesis assay (OECD 456)	human adrenal cortico-carcinoma cells	48	h	0.1, 0.3, 1, 3, 10, 30 and 100	µM	Inhibition of free testosterone at 30 µM No sign. effect on estradiol level	Changes in hormone levels	
		Feng *et al.*, 2016	-	H295R cell line	human adrenal cortico-carcinoma cells	48	h	0, 0.1, 1, 10, 30, 50, and 70	µM	↗ of progesterone production: 50.3% at 1 µM, 91.0% at 10 µM ↘ in production: - aldosterone: 20.5%, 20.9%, 50.3%, 62.7%, 74.5%, and 75.2% of the control at 0.1, 1, 10, 30, 50, and 70 mM BPS, resp - cortisol: 55.9%, 71.7%, and 79.0%, resp at 30, 50 and 70 µM - Testosterone: 34.0%, 69.1%, 82.4%, and 86.8%, resp between 10-70 µM	Change in hormone levels	
		Roelofs *et al.*, 2015	-	Steroid hormone profile (14 hormones)	MA-10 Leydig cells	48	h	10	µM	↗ of pregnenolone (P5, 0.77 ng/mL ± -0.17) and progesterone (P4, 7.14 ng/mL ± 1.36) compared to DMSO control 0.37 ± 0.18 and 1.77 ± 0.70 resp	Change in hormone levels	

Group-ing	Line of evidence	Reference	Study relia-bility (ToxR Tool)	Guideline / test type	Species / cell lines	Test duration	Duration unit	Conc. or conc. range	Conc. unit	Observed effects (positive/negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence
		Eladak *et al.*, 2015	-	Fetal testis assay	Mouse fetal testis explants	3	d	10-10 000	nM	Significant ↘ of basal testosterone secretion from 100 nM	Change in hormone levels	
		Eladak *et al.*, 2015	-	Fetal testis assay	human fetal testis explants	3	d	10-10 000	nM	Significant ↘ of basal testosterone secretion from 10 nM		
		Campen *et al.*, 2018	-	Hormone production (estradiol, androstenedione, progesterone)	Bovine granulosa cells	6	d	1-100	µM	↗ estradiol production at 100 µM, no effect when cells were stimulated with FSH No effect on progesterone production, nor in the presence of FSH	Change in hormone level	
		Campen *et al.*, 2018	-	Hormone production (androstenedione and progesterone)	Bovine thecal cells	6	d	1-100	µM	No effect on androstenedione or progesterone production		
		Téteau *et al.*, 2020	-	Progesterone/estradiol assay	Ovine granulosa cells	48	h	0, 10, 50 and 100	µM	↘ progesterone production (by 22% after exposure to 20 µM) ↗ estradiol production (by 2-fold after exposure to 10 µM). Changed expression of PR and ER	Progesterone: plays important role in oocytes maturation and development. Low ovulation levels in women were associated with low serum progesterone levels. Suggested that reduction of progesterone by BPS has an harmful effect on oocyte quality and consequently on fertility.	

Group-ing	Line of evidence	Reference	Study relia-bility (ToxR Tool)	Guideline / test type	Species / cell lines	Test duration	Duration unit	Conc. or conc. range	Conc. unit	Observed effects (positive/negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence
		Ullah *et al.*, 2016	-	Testosterone production	Adult Sprague Dawley rat testis explants	2	h	0, 0.5, 1, 10 and 100	ng/mL	↘ testosterone concentration but not sign. compared to control 56.06, 53.17, 53.68, 54.56, 52.93 ng/g tissue after exposure to 0, 0.5, 1, 10 and 100 ng/mL, resp		
		Ullah *et al.*, 2018a	-		Adult Sprague Dawley rat explants	2	h	0, 1, 10 and 100 ng/ml		↘ testosterone, but not sign. compared to control: 54.27, 53.15, 51.65 and 52.00 ng/g tissue after exposure to 0, 1, 10 and 100 ng/mL, resp		
		Desdoits-Lethimonier *et al.*, 2017	-	Testosterone production	Adult human testis explants	24 or 48	h	10^{-9} to 10^{-5}	M	At 24h: sign. ↗ of testosterone at 10^{-6} M but slight ↘ at 10^{-5} M At 48h: sign. ↘ of testosterone at 10^{-8} M, slightly ↗ at higher doses Production of insulin-like factor 3: ↗ at all conc. and time but only sign. at 10^{-9} and 10^{-8} at 48h		
		Jambor *et al.*, 2019	-	Testosterone production	Mice TM3 leydig cells	24	h	0, 0.04, 0.2, 1, 2.5, 5, 10, 25, 50	µg/mL	↘ in production of testosterone at 10 µg/ml (91.2%), 25 µg/ml (93.1%) and 50 µg/ml (80.6% but not sign. different from control		
	Gene expression	Sidorkiewicz *et al.*, 2018	-	Gene expression	mouse spermatocyte GC-2 cells	24, 48 and 72	h	10^{-10} or 10^{-8}	M	↗ gene expression of erα and erβ ↗ expression of genes involved in steroidogenesis: StAr, CYP11a1, Hsd17b3, CYP17a1 and CYP19a1		
		Roelofs *et al.*, 2015	-	Gene expression	MA-10 Leydig cells	6	h	10	µM	No upregulation of StAR gene expression 8.4-fold ↗ of 5αRed1 gene expression		

Group-ing	Line of evidence	Reference	Study relia-bility (ToxR Tool)	Guideline / test type	Species / cell lines	Test duration	Duration unit	Conc. or conc. range	Conc. unit	Observed effects (positive/negative)	Assessment of each line of evidence	Assessment of the integrated line of evidence
		Feng *et al.*, 2016	-	Gene expression	human adrenocortical carcinoma cell line (H295R)	48	h	0, 0.1, 1, 10, 30, 50, and 70	µM	No alteration of StAR, CYP11A1, HSD3B2, CYP11B2, 17b-HSD and CYP19A1 ↘CYP11B1 at 10, 30, 50 and 70 µM CYP17A1 from 30 µM		
		Téteau *et al.*, 2020	-	Gene expression: hormonal receptor genes AR, PR, ESR1 and ESR2 and steroidgenic enzyme genes: CYP19A1, CYP11A1 and HSD3B1 and cholesterol: StAR	Ovine granulosa cells	48	h	0, 10, 50 and 100	µM	Sign. ↗ of ESR1 (at 10, 50 and 100 µM) and ESR2 (at 50 and 100 µM) Expression of AR, PR, CYP19A1, CYP11A1, HSD3B1 and StAR not affected		

Table X3: Lines of evidence for endocrine activity in vivo and adversity by BPS

Group-ing	*Line of evidence*	*Species / cell lines*	*Route of exposure*	*Exposure duration*	*Concentrations tested*	*Solvent*	*Observed effects (positive/negative/trend)*	*Reference*	*Study reliability (ToxR Tool)*	*Assess-ment of each line of evidence*	*Mod-ality*
Human health											
In vivo mechanistic	Uterotrophic assay	Rat (SD) Immature female rat	Subcutaneous injection	3 days	20, 100 and 500 mg/kg bw/d	Olive oil	Sign. increase in absolute and relative uterine blotted and wet weight at 20 mg/kg bw/day ($p<0.05$) and at 500 mg/kg bw/day ($p<0.01$).	Yamasaki *et al.*, 2004	1	Overall positive evidence of estrogenic activity	***E***
		Rat (SD) Immature female rat	Subcutaneous injection	3 days	79.4 µM/kg bw/d and 1995 µM/kg bw/d		Weak estrogenic effect (LogLED estrogenic 1.9 µmol/kg bw/d).	Akahori Y. *et al.*, 2008	3		
		Ovariectomised Sprague Dawley rats (60 day old)	Gavage	4 days	50, 100, 200, 400 and 800 mg/kg bw/d		Sign. increase in uterine blotted and wet weight from 50 mg/kg bw/d ($p<0.05$) with an uterus wet and blotted weight at of 156.7±13.4 and 121.4±7.6 respectively at 800 mg/kg bw/d versus 31.6±2.9 and 25.4±3.0 in control group	Conley *et al.*, 2016	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
Parameters EATS-mediated	Estrous cyclicity	Rat (SD)	Oral Drinking water	P0: from 10w before mating to PND 21 F1A: for 13w F1B: to PND 21 F2A: for 11w F2B: for 3w F3: for 8w	0, 20, 60 and 180 mg/kg bw/d	0.5% CMC	Prolonged estrous cycle in - P0 (3.9, 3.9, 3.9 and 4.1* days (St. dev. : 0.10, 0.26, 0.14 and 0.22)) and in - F1B (3.9, 4.0, 4.0 and 4.5 days (St. dev. : 0.29, 0.16, 0.13 and 1.51)) No effect on F1A (4.1 days in all groups) ↗ mean nb of days in diestrus stage in P0 (6.3, 74, 7.7 and 9.0 days) and F1B (6.8 ± 2.4, 8.4* ± 4.5, 9.2** ± 3.1 and 11.8** ± 6.1 days). Slight effect in F1A (4.5, 4.6, 4.8 and 5.4 days)	Unpublished study report, 2019 OECD TG 443	1	Overall positive evidence. Irregular estrous cycle, with prolonged diestrus phases.	***E***
		Rat (SD)	Oral	Total of 40 to 46d for females (from 14d premating to PND3)	0, 10, 60 and 300 mg/kg bw/d	0.5 % aqueous sodium CMC solution with 0.1 % Tween 80	Prolonged estrous cycle (4.08, 4.01, 4.14 and 5,57** days) ↗ nb of animals with longer diestrus (0, 0, 1 and 5*/12 females)	Unpublished study report, 2000 OECD TG 421	1		
		Rat (SD)	Oral	Males: 10w Females: From pre-mating until PND21	0, 30, 100 and 300 mg/kg bw/d	CMC	Prolonged estrous cycle (4.2, 3.97, 4.01 and 5.16** days)	Unpublished study report, 2017b OECD TG 422	1		
		Mice (CD1)	Oral (gavage)	GD 10.5 to 17.5	0, 0.05, 0.5 and 5 mg/kg bw/d		No significative difference on the number of estrous cycles	Tucker *et al.*, 2018	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Mice (CD1)	Oral (pipette)	GD 7 to PND 0	0, 0.5 and 50 µg/kg bw/d		In F3 : Irregular estrous cycle with several days in estrus, metestrus or diestrus Percentage of days in proestrus: No clear dose-response	Shi *et al.*, 2019b	2		
		Mice (CD1)	Oral	GD 11 to birth	0, 0.5, 20 and 50 µg/kg bw/d		Irregular estrous cycles with longer estrus and diestrus phases at all doses	Shi *et al.*, 2019a	1		
		Rat (strain not specified)	Sub-cutaneous	PND 1 to 10	0, 0.5, 5 and 50 mg/kg bw/d		Altered estrous cycle reported (irregular pattern)	Ahsan *et al.*, 2018	2		
	Ovaries weight	Rat (Wistar)	Oral	90d	0, 100, 300 and 1000 mg/kg bw/d (for males, the highest dose changed to 600 mg/kg bw/d onwards 70d)	CMC	Sign. ↗ rela ovaries weight at highest dose (0.047, 0.048, 0.052 and 0.061* % (St. dev. : 0.008, 0.005, 0.01 and 0.012)) Abs weight ↗ at the highest dose (104.7, 104.0, 106.9 and 126.9 mg (St. dev. : 20.205, 11.963, 19.284 and 30.33)	Unpublished study report, 2014 OECD TG 408	1		
		Rat (SD)	Oral	Total of 40 to 46d for females (from 14d premating to PND3)	0, 10, 60 and 300 mg/kg bw/d	0.5 % aqueous sodium CMC solution with 0.1 % Tween 80	Ovaries weight did not show modification (110.35, 116.02, 114.86 and 105.63 mg (St. dev. : 11.28, 15.45, 9.53 and 10.57))	Unpublished study report, 2000 OECD TG 421	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Rat (SD)	Oral	28d	0, 40, 200 and 1000 mg/kg bw/d for main groups 0, 200 and 1000 mg/kg bw/d for recovery groups	0.5 % CMC aqueous solution	Ovaries weight did not show sign. modification - in main groups : abs : 86.1, 92.0, 85.1 and 76.5 mg (St. dev. : 12.9, 19.1, 15.3 and 21.6); rela : 36.7, 38.9, 38.1 and 34.9 % (St. dev. : 4.1, 6.6, 6.3 and 7.4) - in recovery groups : abs : 77.0, 75.4 and 70.4 mg (St. dev. : 8.6, 15.2 and 13.1); rela : 29.1, 29.4 and 28.7 % (St. dev. : 3.1, 5.9 and 4.7)	Unpublished study report, 1999 OECD TG 407	1		
		Rat (SD)	Oral	Males: 10w Females: From pre-mating until PND21	0, 30, 100 and 300 mg/kg bw/d	CMC	Ovaries weight did not show modification (0.035, 0.035, 0.037 and 0.034 g)	Unpublished study report, 2017b OECD TG 422	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Rat (SD)	Oral Drinking water	P0: from 10w before mating to PND 21 F1A: for 13w F1B: to PND 21 F2A: for 11w F2B: for 3w F3: for 8w	0, 20, 60 and 180 mg/kg bw/d	0.5% CMC	Ovaries weight unaffected : P0 : abs : 102.7, 111.1, 106.0 and 104.0 mg (St. dev. : 16.844, 20.06, 12.452 and 13.437); rela : 0.038, 0.04, 0.039 and 0.038 % (St. dev. : 0.006, 0.007, 0.004 and 0.004) F1A : abs : 82.2, 82.9, 88.2 and 86.222 g (St. dev. : 12.593, 13.242, 14.972 and 14.437); rela : 0.034, 0.034, 0.036 and 0.034 % (St. dev. : 0.005, 0.004, 0.006 and 0.006) F1B : abs : 109.542, 109.5, 113.217 and 106.833 g (St. dev. : 14.289, 13.587, 13.501 and 12.651); rela : 0.038, 0.039, 0.037 and 0.035 % (St. dev. : 0.005 in all groups)	Unpublished study report, 2019 OECD TG 443	1		
		Rat (strain not specified)	Sub-cutaneous	PND 1 to PND 10	0, 0.5, 5, and 50 mg/kg bw/d		Sign. ↘ abs paired ovarian weight (129.8, 122.2, 119.6* and 115.6** mg (SEM : 1.50, 4.54, 1.08 and 1.08))	Ahsan *et al.*, 2018	2		
		Mice (ICR) (adult)	Drinking water	4w	0, 0.001, 0.1, 10 and 100 ng/g bw/d		Sign. dose-dependent ↘ of the ovarian volume and rela weight	Nevoral *et al.*, 2018	2		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Rat (SD)	Intra-peritoneal	28d	0, 0.05, 0.5, 5 and 50 mg/kg bw/d		Ovaries weight sign. ↘ (0.184, 0.174, 0.164, 0.145 and 0.122* g)	Ijaz *et al.*, 2020	-		
	Uterus histopathology	Rat (Wistar)	Oral	90d	0, 100, 300 and 1000 mg/kg bw/d (for males, the highest dose changed to 600 mg/kg bw/d onwards 70 days)	CMC	↗ incidence of uterus squamous metaplasia (0, 2, 2 and 5)	Unpublished study report, 2014 OECD TG 408	1		
	Uterus weight	Rat (SD)	Oral Drinking water	P0: from 10w before mating to PND 21 F1A: for 13w F1B: to PND 21 F2A: for 11w F2B: for 3w F3: for 8w	0, 20, 60 and 180 mg/kg bw/d	0.5% CMC	Unaffected in P0 (abs : 0.728, 0.709, 0.75 and 0.737 g (St. dev. : 0.361, 0.216, 0.195 and 0.228); rela : 0.268, 0.255, 0.274 and 0.273 % (0.133, 0.074, 0.075 and 0.094)) F1A : trend ↗ uterus weight (abs : 0.707, 0.709, 0.716 and 0.823 g (St. dev. : 0.26, 0.218, 0.281 and 0.251); rela : 0.294, 0.299, 0.288 and 0.328 % (St. dev. : 0.109, 0.108, 0.108 and 0.103)) In F1B, uterus weight was ↗ at the mid dose (abs : 0.747, 0.669, 1.666 and 0.778 g (St. dev. : 0.284, 0.25, 4.707 and 0.29); rela : 0.255, 0.235, 0.538 and 0.255 % (St. dev. : 0.09, 0.086, 1.495 and 0.099))	Unpublished study report, 2019 OECD TG 443	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Rat (SD)	Oral	Males: 10w Females: From pre-mating until PND21	0, 30, 100 and 300 mg/kg bw/d	CMC	Trend ↗ (0.197, 0.224, 0.224 and 0.307 g)	Unpublished study report, 2017b OECD TG 422	1		
		Rat (SD)	Oral	total of 40 to 46d for females (from 14d premating to PND3)	0, 10, 60 and 300 mg/kg bw/d	0.5 % aqueous sodium CMC solution with 0.1 % Tween 80	Uterus weight did not show modification (0.691, 0.683, 0.713 and 0.700 g (St. dev. : 0.076, 0.143, 0.100 and 0.131))	Unpublished study report, 2000 OECD TG 421	1		
		Rat (Wistar)	Oral	90d	0, 100, 300 and 1000 mg/kg bw/d (for males, the highest dose changed to 600 mg/kg bw/d onwards 70d)	CMC	Uterus weight showed variation (abs : 0.724, 0.864, 1.284 and 0.648 g (St. dev. : 0.263, 0.396, 0.795 and 0.145) ; rela : 0.332, 0.41, 0.615 and 0.315 % (St. dev. : 0.135, 0.205, 0.36 and 0.069))	Unpublished study report, 2014 OECD TG 408	1		
		Rat (strain not specified)	Sub-cutaneous	PND 1 to PND 10	0, 0.5, 5, and 50 mg/kg bw/d		Sign. ↘ rela weight (1.63, 1.55, 1.55 and 1.35*** (SEM : 0.02, 0.05, 0.01 and 0.03)) Sign. ↘ abs weight (259.2, 250.2, 252.2 and 234.0 mg (SEM : 0.37, 2.60, 0.73 and 2.34))	Ahsan *et al.*, 2018	2		
		Mice (CD-1)	Sub-cutaneous	From birth to PND 60	0, 50 µg or 10 mg/kg bw		No sign. differences	Shi *et al.*, 2017	3		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Rat (SD)	Intra-peritoneal	28d	0, 0.05, 0.5, 5 and 50 mg/kg bw/d		Uterine weights sign. ↘ (abs: 256.4, 244.2*, 240.6*, 236.0* and 215.0* mg; rela : 1.40, 1.59, 1.63, 1.63* and 1.40* mg/g)	Ijaz *et al.*, 2020	-		
	Sperm quality	Rat (SD)	Oral Drinking water	P0: from 10w before mating to PND 21 F1A: for 13w F1B: to PND 21 F2A: for 11w F2B: for 3w F3: for 8w	0, 20, 60 and 180 mg/kg bw/d	0.5% CMC	P0 : ↘ sperm motility (88, 84*, 85* and 86* % (St. dev. : 5, 7, 8 and 4)) Other sperm parameters not affected F1B : No effect on sperm motility (84, 83, 84 and 83% (St. dev. : 10, 8, 7 and 8)) Other sperm parameters not affected	Unpublished study report, 2019 OECD TG 443	1	Overall positive evidence. Reduced sperm count and motility at low-dose, but not at higher dose. Considered as supportive adverse effect.	***ES***
		Mice (CD-1)	Sub-cutaneous	From birth to PND 60 (exposure every 3d)	0, 50 µg or 10 mg/kg bw		↘ sperm count (6.4, 2.5** and 3.8** x10^6/ml (SEM : 0.2, 0.2 and 0.3)) ↘ sperm motility (76.8, 67.2* and 63.1**% (SEM : 1.2, 1.7 and 2.1))	Shi *et al.*, 2017	3		
		Mice (CD-1)	Oral	GD 11 to birth	0, 0.5, 20 and 50 µg/kg bw/d		↘ sperm count (66** and 55*** % resp. at 0.5 and 20 µg/kg bw/d, but no effect at high dose) ↘ sperm motility at 0.5 µg/kg bw/d, but no effect at higher doses	Shi *et al.*, 2018	2		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Mice (CD-1)	Oral	GD 7 to birth in F1 (effects in F3 males)	0, 0.5 and 50 µg/kg bw/d		Transgenerational effects: ↘ sperm count (40%*** and 48%*** of reduction) Sign. ↘ sperm motility at 0.5 µg/kg bw/d, but not at higher dose	Shi *et al.*, 2019c	-		
		Rat (SD)	Oral	28d	0, 1, 5, 25 and 50 µg/kg bw/d		Trend ↘ nb spermatocytes (74.1, 73.6, 70.5, 71.1 and 69.6 (SEM : 1.37, 1.43, 1.21, 1.28 and 1.40)) and spermatid (248.3, 246.0, 247.4, 244.3 and 241.0 (SEM : 1.98, 2.54, 2.69, 2.06 and 1.44))	Ullah *et al.*, 2016 Similar to OECD TG 407	-		
		Rat (SD)	Drinking water	From PND 23 and for 48w	0, 5, 25 and 50 µg/kg bw/d		↘ sperm motility (79.6, 78.1, 75.3 and 74.3** % (SEM : 0.54, 0.51, 1.10 and 0.74)) ↘ DSP (53.3, 52.2, 50.3 and 48.2** $x10^6$ (SEM : 0.6, 0.5, 0.8 and 0.5)) Nb of different cell types in testis : ↘ spermatogonia (65.7, 63.4, 63.6 and 61.6* (Sem : 0.62, 1.05, 1.15 and 0.87)) spermatocytes (77.1, 74.7, 73.8 and 72.1* (SEM : 1.06, 1.30, 1.23 and 1.24)) and spermatids (257.3, 250.0, 248.3 and 244.0** (SEM : 1.79, 2.77, 2.52 and 2.01))	Ullah *et al.*, 2018b	-		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Rat (SD)	Drinking water	GD 1 to 21	0, 5, 25 and 50 µg/kg bw/d		↘ sperm motility (79.6, 78.1, 75.3* and 74.3**% (SEM : 0.54, 0.51, 1.10 and 0.74)) ↘ DSP (73.4, 63.3, 62.3 and 61.3* x10^6 (SEM : 0.6, 1.5, 0.2 and 0.6)) Nb of different cell types in testis : ↘ spermatogonia (64.7, 62.4, 62.6 and 60.6* (SEM : 0.61, 1.04, 1.14 and 0.85)), spermatocytes (76.1, 73.7, 72,8 and 71.1* (SEM : 1.05, 1.31, 1.22 and 1.23)) and spermatids (256.3, 251.1, 247.3 and 243.0* (SEM : 1.77, 2.75, 2.51 and 2.03))	Ullah *et al.*, 2019a	-		
		Rat (SD)		28 days	0, 5, 25 and 50 mg/kg bw/day BPS		↘ daily sperm production (sign. at high dose) ↘ sperm motility trend (87.8, 85.1, 84.9 and 83.7%)	Ullah *et al.*, 2019b Similar to OECD TG 407	-		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Rat (SD)	Drinking water	From PND 23 and for 48w	0, 0.5, 5 and 50 µg/l BPS		↘ sperm motility (77.9, 78.2, 73.9 and 72.9* % (SEM : 1.28, 1.77, 2.13 and 1.06)) ↘ daily sperm production (52.0, 50.6, 49.7 and 47.3** x10^6 (SEM : 0.78, 0.89, 0.93 and 0.36)) ↗ spermatogonia (36.4, 61.8, 61.3 and 59.4* (SEM : 1.12, 0.92, 1.51, 1.22)) ↘ spermatocytes (75.5, 75.2, 70.1** and 68.3*** (SEM : 1.30, 1.35, 1.14 and 1.07)) and spermatids (258.5, 258.2, 251.4* and 246.3** (SEM : 1.92, 2.68, 1.54 and 1.87))	Ullah *et al.*, 2021	-		
	Epididymide weight	Rat (Wistar)	Oral	90 days	0, 100, 300 and 1000 mg/kg bw/d (for males, the highest dose changed to 600 mg/kg bw/d onwards 70d)	CMC	Abs weight sign. ↘ (1.209, 1.16, 1.126 and 1.072** g (St. dev. : 0.102, 0.096, 0.088 and 0.082)) Rela weight sign. ↗ (0.308, 0.31, 0.316 and 0.346** % (St. dev. : 0.029, 0.033, 0.027 and 0.027))	Unpublished study report, 2014 OECD TG 408	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Rat (SD)	Oral	total of 40 to 46d for females (from 14d premating to PND3)	0, 10, 60 and 300 mg/kg bw/d	0.5 % aqueous sodium CMC solution with 0.1 % Tween 80	Epididymis weight did not show modification (1.355, 1.292, 1.328 and 1.292 g (St. dev. : 0.090, 0.099, 0.105 and 0.067))	Unpublished study report, 2000 OECD TG 421	1		
		Rat (SD)	Oral Drinking water	P0: from 10w before mating to PND 21 F1A: for 13w F1B: to PND 21 F2A: for 11w F2B: for 3w F3: for 8w	0, 20, 60 and 180 mg/kg bw/d	0.5% CMC	Epididymis weight unaffected P0 : abs : 1.304, 1.328, 1.3 and 1.3 g (St. dev. : 0.081, 0.099, 0.122 and 0.112) F1A : abs : 1.167, 1.159, 1.14 and 1.151 g (St. dev. : 0.097, 0.093, 0.147 and 0.089) F1B : abs : 1.324, 1.347, 1.319 and 1.308 g (St. dev. : 0.098, 0.098, 0.108 and 0.097)	Unpublished study report, 2019 OECD TG 443	1		
		Rat (SD)	Drinking water	From PND 23 and for 48w	0, 5, 25 and 50 µg/kg bw/d		Abs paired epididymis weight dose dependently ↘ (1.44, 1.43, 1.42 and 1.41 g (SEM : 0.03, 0.05, 0.04 and 0.02)) Rela epididymis weight : 2.65, 2.63, 2.60 and 2.56** mg/g (SEM : 0.03, 0.03, 0.02 and 0.02)	Ullah *et al.*, 2018b	-		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Rat (SD)	Drinking water	From PND 23 and for 48w	0, 0.5, 5 and 50 µg/l BPS		Abs paired epididymis weight : 1.42, 1.41, 1.39 and 1.36 g (SEM : 0.01, 0.01, 0.02 and 0.01) Rela epididymis weight : 2.64, 2.62, 2.60 and 2.50*** (SEM : 0.02, 0.02, 0.03 and 0.02)	Ullah *et al.*, 2021	-		
	Prostate weight	Rat (SD)	Oral Drinking water	P0: from 10w before mating to PND 21 F1A: for 13w F1B: to PND 21 F2A: for 11w F2B: for 3w F3: for 8w	0, 20, 60 and 180 mg/kg bw/d	0.5% CMC	↘ prostate abs. weight : Sign in F1A (1.163, 1.118, 1.053* and 1.046** g (St. dev. : 0.16, 0.147, 0.181 and 0.205) Trend at F0 (1.478, 1.423, 1.454 and 1.335 g (St. dev. : 0.226, 0.237, 0.193 and 0.2)) and F1B (1.557, 1.489, 1.47 and 1.398 g (St. dev. : 0.268, 0.267, 0.213 and 0.232))	Unpublished study report, 2019 OECD TG 443	1		
		Rat (SD)	Drinking water Gavage	28d	0, 100, 300 and 600 mg/kg bw/d		Rela prostate weight ↘ at the highest dose (- 15 %)	Unpublished study report, 2017a RF for OECD TG 443	1		
		Rat (SD)	Oral	total of 40 to 46d for females (from 14d premating to PND3)	0, 10, 60 and 300 mg/kg bw/d	0.5 % aqueous sodium CMC solution with 0.1 % Tween 80	Prostate weight did not show modification (0.723, 0.746, 0.777 and 0.708 g (St. dev. : 0.082, 0.106, 0.137 and 0.210))	Unpublished study report, 2000 OECD TG 421	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Rat (SD)	Oral	Males: 10w Females: From pre-mating until PND21	0, 30, 100 and 300 mg/kg bw/d	CMC	Prostate weight did not show modification (0.302, 0.303, 0.278 and 0.297 g)	Unpublished study report, 2017b OECD TG 422	1		
		Rat (SD)	Drinking water	From PND 23 and for 48w	0, 5, 25 and 50 µg/kg bw/d		Relative prostate weight slightly ↘ (2.71, 2.67, 2.68 and 2.64 mg/g (SEM : 0.05, 0.03, 0.04 and 0.03))	Ullah *et al.*, 2018b	-		
		Rat (SD)	Drinking water	GD 1 to 21	0, 5, 25 and 50 µg/kg bw/d		Not sign. modified (0.53, 0.54, 0.47 and 0.48 g (SEM : 0.05, 0.02, 0.04 and 0.03))	Ullah *et al.*, 2019a	-		
	Seminal weight	Rat (SD)	Oral	Total of 40 to 46d for females (from 14d premating to PND3)	0, 10, 60 and 300 mg/kg bw/d	0.5 % aqueous sodium CMC solution with 0.1 % Tween 80	↘ abs seminal vesicle weight (sign. at the highest dose : 2.825, 2.718, 2.860 and 2.428** g (St. dev. : 0.314, 0.249, 0.295 and 0.376)) Rela weight : 0.552, 0.531, 0.546 and 0.498 % (St. dev. : 0.073, 0.069, 0.062 and 0.055))	Unpublished study report, 2000 OECD TG 421	1		
		Rat (SD)	Oral	Males: 10w Females: From pre-mating until PND21	0, 30, 100 and 300 mg/kg bw/d	CMC	Sem. Ves. Weight did not showed modification (0.357, 0.366, 0.336 and 0.348 g)	Unpublished study report, 2017b OECD TG 422	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Rat (SD)	Oral Drinking water	P0: from 10w before mating to PND 21 F1A: for 13w F1B: to PND 21 F2A: for 11w F2B: for 3w F3: for 8w	0, 20, 60 and 180 mg/kg bw/d	0.5% CMC	Seminal weight unaffected P0 : abs : 1.924, 1.788, 1.847 and 1.834 g (St. dev. : 0.306, 0.261, 0.204 and 0.202) ; rela : 0.37, 0.352, 0.355 and 0.365 % (St. dev. : 0.055, 0.055, 0.041 and 0.055) F1A : abs : 1.353, 1.254, 1.285 and 1.258 g (St. dev. : 0.205, 0.193, 0.205 and 0.186) ; rela : 0.3, 0.282, 0.284 and 0.291 % (St. dev. : 0.056, 0.047, 0.043 and 0.039) F1B : abs : 1.808, 1.725, 1.813 and 1.74 g (St. dev. : 0.201, 0.241, 0.272 and 0.23); rela : 0.34, 0.327, 0.332 and 0.342 % (St. dev. : 0.052, 0.052, 0.052 and 0.04)	Unpublished study report, 2019 OECD TG 443	1		
		Rat (SD)	Drinking water Gavage	28d	0, 100, 300 and 600 mg/kg bw/d		Rel seminal weight ↘ at the highest dose (- 16 %)	Unpublished study report, 2017a RF for OECD TG 443	1		
		Rat (SD)	Drinking water	From PND 23 and for 48w	0, 5, 25 and 50 µg/kg bw/d		Abs seminal weight sign. ↘ (1.9, 1.87, 1.83* and 1.79** g (SEM : 0.04, 0.02, 0.03 and 0.03))	Ullah *et al.*, 2018b	-		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Rat (SD)	Drinking water	GD 1 to 21	0, 5, 25 and 50 µg/kg bw/d		Sign. ↘ at the highest dose (1.17, 1.16, 1.14 and 1.13* g (SEM : 0.01, 0.01, 0.02 and 0.06))	Ullah *et al.*, 2019a	-		
		Rat (SD)	Drinking water	From PND 23 and for 48w	0, 0.5, 5 and 50 µg/l BPS		Abs sem. Ves. Weight : 1.89, 1.87, 1.81* and 1.79** (SEM : 0.02, 0.02, 0.01 and 0.02)	Ullah *et al.*, 2021	-		
	Testes weight	Rat (Wistar)	Oral	90d	0, 100, 300 and 1000 mg/kg bw/d (for males, the highest dose changed to 600 mg/kg bw/d onwards 70d)	CMC	Abs weight sign. ↘ (3.914, 3.862, 3.636* and 3.592* g (St. dev. : 0.325, 0.305, 0.202 a,d 0.226)) Rela weight sign. ↗ (0.999, 1.035, 1.021 and 1.162** % (St. dev. : 0.126, 0.123, 0.063 and 0.115))	Unpublished study report, 2014 OECD TG 408	1		
		Rat (SD)	Oral	Total of 40 to 46d for females (from 14d premating to PND3)	0, 10, 60 and 300 mg/kg bw/d	0.5 % aqueous sodium CMC solution with 0.1 % Tween 80	Testes weight did not show modification (3.559, 3.480, 3.554 and 3.503 g (St. dev. : 0.325, 0.192, 0.241 and 0.150))	Unpublished study report, 2000 OECD TG 421	1		
		Rat (SD)	Oral	Males: 10w Females: From pre-mating until PND21	0, 30, 100 and 300 mg/kg bw/d	CMC	Testes weight was very slightly ↗ at the highest dose (0.685, 0.663, 0.663 and 0.734 g)	Unpublished study report, 2017b OECD TG 422	1		

Group-ing	*Line of evidence*	*Species / cell lines*	*Route of exposure*	*Exposure duration*	*Concentrations tested*	*Solvent*	*Observed effects (positive/negative/trend)*	*Reference*	*Study reliability (ToxR Tool)*	*Assess-ment of each line of evidence*	*Mod-ality*
		Rat (SD)	Oral	28d	0, 40, 200 and 1000 mg/kg bw/d for main groups 0, 200 and 1000 mg/kg bw/d for recovery groups	0.5 % CMC aqueous solution	Rela weight sign. ↗ in main groups (0.79, 0.81, 0.84 and 0.96**% (St. dev. : 0.04, 0.09, 0.07 and 0.05)) Abs weight unaffected in main groups (3.07, 2.94, 3.06 and 2.96 g (0.14, 0.12, 0.23 and 0.24)) Recovery groups (abs and rela weight unaffected)	Unpublished study report, 1999 OECD TG 407	1		

Group-ing	*Line of evidence*	*Species / cell lines*	*Route of exposure*	*Exposure duration*	*Concentrations tested*	*Solvent*	*Observed effects (positive/negative/trend)*	*Reference*	*Study reliability (ToxR Tool)*	*Assess-ment of each line of evidence*	*Mod-ality*
		Rat (SD)	Oral Drinking water	P0: from 10w before mating to PND 21 F1A: for 13w F1B: to PND 21 F2A: for 11w F2B: for 3w F3: for 8w	0, 20, 60 and 180 mg/kg bw/d	0.5% CMC	Testes weight unaffected P0 : abs : 3.642, 3.739, 3.53 and 3.659 g (St. dev. : 0.279, 0.25, 0.337 and 0.323); rela : 0.703, 0.739, 0.679 and 0.726 % (St. dev. : 0.072, 0.104, 0.066 and 0.07) F1A : abs : 3.655, 3.56, 3.69 and 3.63 g (St. dev. : 0.315, 0.318, 0.325 and 0.296); rela : 0.807, 0.801, 0.818 and 0.842 % (St. dev. : 0.095, 0.095, 0.091 and 0.076) F1B : abs : 3.853, 3.954, 3.874 and 3.857 g (St. dev. : 0.317, 0.314, 0.28 and 0.304): rela : 0.723, 0.75, 0.712 and 0.764 % (St. dev. : 0.076, 0.087, 0.074 and 0.11)	Unpublished study report, 2019 OECD TG 443	1		
		Mice (CD-1)	Sub-cutaneous	From birth to PND 60	0, 50 µg or 10 mg/kg bw		Testes weight (abs or rela) unaffected	Shi *et al.*, 2017	3		
		Rat (SD)	Drinking water	From PND 23 and for 48w	0, 5, 25 and 50 µg/kg bw/d		Testis weight dose dependently ↘ (3.68, 3.55, 3.51 and 3.50 g (SEM : 0.08, 0.04, 0.05 and 0.03))	Ullah *et al.*, 2018b	-		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Rat (SD)	Oral	28d	0, 1, 5, 25 and 50 µg/kg bw/d		Right testis weight : 1.292, 1.24, 1.146, 1.55 and 1.112 g (SEM : 0.04, 0.07, 0.04, 0.03 and 0.16) Left testis weight : 1.37, 1.31, 1.22, 1.30 and 1.12 g (SEM : 0.05, 0.07, 0.03, 0.01 and 0.07)	Ullah *et al.*, 2016 Similar to OECD TG 407	-		
		Rat (SD)	Drinking water	GD 1 to 21	0, 5, 25 and 50 µg/kg bw/d		Abs right testis weight : 1.16, 1.15, 1.16 and 1.16 (SEM : 0.05, 0.06, 0.07 and 0.08) Abs left testis weight : 1.14, 1.13, 1.12 and 1.14 (SEM : 0.01, 0.09, 0.09 and 0.01)	Ullah *et al.*, 2019a	-		
		Rat (SD)	Drinking water	From PND 23 and for 48w	0, 0.5, 5 and 50 µg/l BPS		Abs paired testis : 3.66, 3.59, 3.52 and 3.50 (SEM : 0.08, 0.06, 0.03 and 0.02)	Ullah *et al.*, 2021	-		
	Testes histopathology	Mice (CD-1)	Sub-cutaneous	From birth to PND 60	0, 50 µg or 10 mg/kg bw		↗ the proportion of tubules in stage VII compared to control testes ↘ of the percentage of tubules in stage VIII At stage IX to XIII, no sign differences in tubules distribution	Shi *et al.*, 2017	3		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Rat (SD)	Drinking water	GD 1 to 21	0, 5, 25 and 50 µg/kg bw/d		Testicular cells strongly modified Area of seminiferous tubule : 88.12, 86.37, 84.74** and 83.886***% (SEM : 0.4, 0.2, 0.5 and 0.5) Area of interstitial space : 14.17, 13.17, 12.95 and 12.15*% (SEM : 0.5, 0.3, 0.5 and 0.2) Area of lumen : 15.65, 15.67, 15.37 and 15.04***% (SEM : 0.1, 0.1, 0.3 and 0.1) Seminiferous tubule diameter : 226.32, 226.24, 224.17 and 222.32* µm (SEM : 2.8, 0.3, 0.4 and 0.5 Seminiferous tubule epithelial height : 59.03, 58.43, 58.09 and 61.14** µm (SEM : 0.2, 0.1, 0.6 and 0.3)	Ullah *et al.*, 2019a	-		
		Rat (SD)	Oral	28d	0, 1, 5, 25 and 50 µg/kg bw/d		↘ epithelial height of seminiferous tubules (70.6, 69.7, 65.2, 59.7* and 59.2* µm (SEM : 2.77, 3.22, 2.67, 3.31 and 2.95))	Ullah *et al.*, 2016 Similar to OECD TG 407	-		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Rat (SD)	Drinking water	From PND 23 and for 48w	0, 5, 25 and 50 µg/kg bw/d		↘ epithelial height (71.2, 66.3, 64.4 and 62.0* µM (SEM : 1.90, 2.65, 1.87 and 2.72))	Ullah *et al.*, 2018b	-		
		Rat (SD)	Drinking water	From PND 23 and for 48w	0, 0.5, 5 and 50 µg/l BPS		↘ epithelial height (73.1, 73.6, 68.1* and 68.11* µM (SEM : 1.55, 1.72, 0.97 and 0.85))	Ullah *et al.*, 2021	-		
	Male mammary gland histopathology	Rat (SD)	Oral Drinking water	P0: from 10w before mating to PND 21 F1A: for 13w F1B: to PND 21 F2A: for 11w F2B: for 3w F3: for 8w	0, 20, 60 and 180 mg/kg bw/d	0.5% CMC	Atrophy of male mammary gland in F1A (0, 0, 2 and 7 animals affected of 20 animals per group) No effect in P0 nor F1B	Unpublished study report, 2019 OECD TG 443	1	Overall positive evidence. Affected morphology of male mammary gland (atrophy at high dose, enlargement at low dose). Considered as supportive adverse effect.	***EAS***
		Rat (SD)	Drinking water Gavage	28d	0, 100, 300 and 600 mg/kg bw/d		↗ incidence of diffuse atrophy (3 at mid dose and 4 at highest dose)	Unpublished study report, 2017a RF for OECD TG 443	1		
		Rat (SD)	Oral	Males: 10w Females: From pre-mating until PND21	0, 30, 100 and 300 mg/kg bw/d	CMC	Atrophy of male mammary gland (0, 0, 0 and 10 animals affected of 10 animals per group)	Unpublished study report, 2017b OECD TG 422	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Rat (Wistar)	Oral	90d	0, 100, 300 and 1000 mg/kg bw/d (for males, the highest dose changed to 600 mg/kg bw/d onwards 70 days)	CMC	Atrophy of male mammary gland (0, 0, 7 and 10 animals affected of 10 animals per group) Furthermore, severity increased (at mid dose, 7 of grade 1 while at the highest dose 4 of grade 2, 2 of grade 3, 3 of grade 4 and 1 of grade 5)	Unpublished study report, 2014 OECD TG 408	1		
		Mice (CD-1)	Oral	GD 9 to PND 2 or 20	0, 2 and 200 µg/kg bw/d		Changed morphology of the mammary gland in adult males (larger epithelial trees)	Kolla *et al.*, 2019	-		
Sensitive to, but not diagnostic of EATS	Post-implantation loss	Rat (SD)	Oral Drinking water	P0: from 10w before mating to PND 21 F1A: for 13w F1B: to PND 21 F2A: for 11w F2B: for 3w F3: for 8w	0, 20, 60 and 180 mg/kg bw/d	0.5% CMC	P0 : ↗ mean nr of post-implantation loss (0.5, 0.8, 1.3* and 1.5**, corresponding to 3.1, 5.9, 9.4* and 10.5** %) F1B : ↗ mean nr of post-implantation loss (0.9, 0.8, 1.1 and 3.3**, corresponding to 6.4, 5.3, 11.1 and 24.6** %).	Unpublished study report, 2019 OECD TG 443	1	Overall positive evidence. Increase of post-implantation loss. Considered as supportive adverse effect.	**ES**
		Rat (SD)	Oral	Males: 10w Females: From pre-mating until PND21	0, 30, 100 and 300 mg/kg bw/d	CMC	↗ % of post-implantation loss (3.6, 5.2, 6.5 and 34.6** %)	Unpublished study report, 2017b OECD TG 422	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Rat (Wistar)	Oral	GD 6 - 19	0, 30, 100 and 300 mg/kg bw/d		Very slightly ↗ at the highest dose (4.7, 3.9, 3.9 and 6.3 %)	Unpublished study report, 2014 OECD TG 414	1		
	Implantation index	Rat (SD)	Oral	Total of 40 to 46d for females (from 14d premating to PND3)	0, 10, 60 and 300 mg/kg bw/d	0.5 % aqueous sodium CMC solution with 0.1 % Tween 80	Sign. ↘ at the highest dose (95.8, 80.84, 86.15 and 64.89** %)	Unpublished study report, 2000 OECD TG 421	1	Overall positive evidence. Decrease of implantation sites.	***E***
		Rat (SD)	Oral	Males: 10w Females: From pre-mating until PND21	0, 30, 100 and 300 mg/kg bw/d	CMC	Mean nb of implantation sites sign. ↘ at the highest dose (15.8, 15.0, 15.5 and 10.4 **)	Unpublished study report, 2017 OECD TG 422	1		
		Rat (SD)	Oral Drinking water	P0: from 10w before mating to PND 21 F1A: for 13w F1B: to PND 21 F2A: for 11w F2B: for 3w F3: for 8w	0, 20, 60 and 180 mg/kg bw/d	0.5% CMC	Mean number of implantation sites was moderately modified in the cohort 1B at the highest dose (15.2, 14.6, 15.4 and 13.7 (St. dev. : 2.55, 2.12, 3.65 and 3.83)). Mean number of implantation sites in P0 : 15.3, 14.8, 14.9 and 14.3 (St. dev. : 1.90, 3.56, 2.68 and 3.14)	Unpublished study report, 2019 OECD TG 443	1		

Group-ing	***Line of evidence***	***Species / cell lines***	***Route of exposure***	***Exposure duration***	***Concentrations tested***	***Solvent***	***Observed effects (positive/negative/trend)***	***Reference***	***Study reliability (ToxR Tool)***	***Assess-ment of each line of evidence***	***Mod-ality***
	Fertility	Rat (SD)	Oral Drinking water	P0: from 10w before mating to PND 21 F1A: for 13w F1B: to PND 21 F2A: for 11w F2B: for 3w F3: for 8w	0, 20, 60 and 180 mg/kg bw/d	0.5% CMC	P0 Fertility index not affected (96, 91, 100 and 96 %) F1B Fertility index not affected (100, 100, 96, 96 %)	Unpublished study report, 2019 OECD TG 443	1	Overall positive evidence. Reduced fertility.	***ES***
		Rat (SD)	Oral	Total of 40 to 46d for females (from 14d premating to PND3)	0, 10, 60 and 300 mg/kg bw/d	0.5 % aqueous sodium CMC solution with 0.1 % Tween 80	↘ Fertility index (91.7, 91.7, 100.0 and 58.3%) (Nb of pregnant females/nb of copulated females : 11/12, 11/12, 12/12 and 7/12)	Unpublished study report, 2000 OECD TG 421	1		
		Rat (SD)	Oral	Males: 10w Females: From pre-mating until PND21	0, 30, 100 and 300 mg/kg bw/d	CMC	↘ Fertility index (100, 90, 100 and 60%)	Unpublished study report, 2017 OECD TG 422	1		
		Mice (CD-1)	Sub-cutaneous	From birth to PND 60	0, 50 µg or 10 mg/kg bw		No reduced fertility, but both treated males and females need more time to successfully mate, suggesting subfertility (some of the animals took over 10 days and up to 16 days to become pregnant)	Shi *et al.*, 2017	2		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Rat (strain not specified)	Sub-cutaneous	PND 1 to PND 10	0, 0.5, 5, and 50 mg/kg bw/d		Sign. ↘ fertility at highest doses (60** % vs 100 % in all other groups)	Ahsan *et al.,* 2018	2		
		Mice (ICR) (adult)	Drinking water	4w	0, 0.001, 0.1, 10 and 100 µg/kg bw/d		Sign. ↘ fertility at 10 µg/kg bw/d, but enhanced at 100 µg/kg bw/d	Nevoral *et al.*, 2018	2		
		Mice (CD-1)	Oral	GD 11 to birth	0, 0.5, 20 and 50 µg/kg bw/d		No impact on fertility in young mice But fertility ↘ in 9 months old mice (100, 66.7, 40 and 40 %)	Shi *et al.*, 2019a	1		
	Number of pups delivered	Rat (SD)	Oral Drinking water	P0: from 10w before mating to PND 21 F1A: for 13w F1B: to PND 21 F2A: for 11w F2B: for 3w F3: for 8w	0, 20, 60 and 180 mg/kg bw/d	0.5% CMC	P0 : ↘ Mean nr of pups delivered (14.9, 14.0, 13.5 and 12.7 (St. dev. : 1.94, 3.66, 2.86 and 3.33)) F1B : ↘ Mean nr of pups delivered (14.3, 13.8, 14.9 and 11.4** (St. dev. : 2.82, 2.35, 1.73 and 4.49))	Unpublished study report, 2019 OECD TG 443	1	Overall positive evidence. Reduced number of pups.	***ES***
		Rat (SD)	Oral	Total of 40 to 46d for females (from 14d premating to PND3)	0, 10, 60 and 300 mg/kg bw/d	0.5 % aqueous sodium CMC solution with 0.1 % Tween 80	↘ Mean nr of pups delivered (14.2, 12.5, 13.5 and 9.1)	Unpublished study report, 2000 OECD TG 421	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		Rat (SD)	Oral	Males: 10w Females: From pre-mating until PND21	0, 30, 100 and 300 mg/kg bw/d	CMC	↘ Mean nr of pups delivered (15.2, 14.1, 14.5 and 10.8**)	Unpublished study report, 2017 OECD TG 422	1		
		Rat (Wistar)	Gavage	GD 6 – 19	0, 30, 100 and 300 mg/kg bw/d		Slightly ↘ mean nb of live foetuses (10.1 at the highest dose vs 10.6 in all other groups (St. dev. : 1.61, 1.83, 1.71 and 1.80))	Unpublished study report, 2014 OECD TG 414	1		
		Mice (CD1)	Oral	GD 11 to birth	0, 0.5, 20 and 50 µg/kg bw/d		Trend decrease in 3 months old mice : 12.0, 12.8 and 11.0 in 6 months old mice : 7.8, 7.0 and 12.3 in 9 months old mice : 2.0, 1.3* and 2.0	Shi *et al.*, 2019a	1		
		Mice (CD1)	Oral (pipette)	GD 7 to PND 0	0, 0.5 and 50 µg/kg bw/d		In F4 : No sign differences observed at any ages	Shi *et al.*, 2019b	2		
		Rat (strain not specified)	Sub-cutaneous	PND 1 to 10	0, 0.5, 5 and 50 mg/kg bw/d		Sign. ↘ nb of pups born per female mated with untreated males (8.80, 8.80, 8.60 and 5.33 (SEM : 0.58, 0.58, 0.75 and 0.67))	Ahsan *et al.*, 2018	2		

Environment

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent*	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assesss-ment of each line of evidence	Mod-ality

Solvent* : if not indicated otherwise, results were compared to solvent control

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
In vivo mechanistic	Hormonal changes	*Danio rerio*	Water	3-4 months old: 21d	**0**, SC, 0.5, 5 and 50 µg/L Results compared to blank	0.1% Methanol (v/v) No sign. difference between water and solvent control	E2: stat. sign. ↗ in male at ≥0.5µg/L (0.5*, 5* and 50* µg/L) and female adult fish at 50* µg/L T: stat. sign. ↘ in males at 50 *µg/L, no effect in females E2/T ratio: stat. sign. ↗ at 0.5*, 5* and 50* µg/L in males and at 50* µg/L in females	Ji *et al.*, 2013 following OECD guideline 229 with modifications	1	Overall positive evidence of hormonal changes: ↗ E2, ↙ T	**S**
		Danio rerio	Water	2-75 dpf	0, **SC**, 0.1, 1, 10 and 100 µg/L Results compared to SC	0.01 % acetone (v/v) Authors confirmed that results of water and solvent control were the same	E2: stat. sign. ↗ in males (at 1*, 10* and 100* µg/L) and females (at 10* and 100* µg/L) T: stat. sign. ↘ in males at 10* and 100* µg/L, no sign. effect in females but dose-dependent ↗ from 10 µg/L ↘ T3 and T4 in males (10* and 100* µg/L) and females 100* µg/L	Naderi *et al.*, 2014 OECD TG 234	1		**S** T
		Danio rerio	Water	2 hpf – 168 hpf	SC, 1, 3, 10, and 30 µg/L	0.01% DMSO (v/v)	Stat. sign. ↘ of whole-body T4 at 10** and 40** µg/L and T3 at 30* µg/L Stat. sign. ↗ whole-body TSH at 10* and 30** µg/L	Zhang *et al.*, 2017	1		**T**
		Danio rerio	Water	2 hpf – 120 dpf	SC, 1, 10 and 100 µg/L	0.002% DMSO (v/v)	F0 females: stat. sign. ↘ T4 and stat. sign. ↗ T3 at all conc.: T4: 8.09 ± 1.08, 5.74 ± 0.87*, 5.46 ± 0.39* and 6.33 ± 0.57* T3: 1.01 ± 0.12, 1.51 ±	Wei *et al.*, 2018	1		**T**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							0.14*, 1.56 ± 0.26* and 1.64 ± 0.25* F0 males: sign. ↗ in T3 at 1 and 40 µg/L: T4: 5.76 ± 0.63, 5.77 ± 0.76, 6.29 ± 1.02 and 5.65 ± 0.35 T3: 1.35 ± 0.23, 1.67 ± 0.17*, 1.79 ± 0.20* and 1.24 ± 0.23 F1 eggs: sign. ↘ T4 and sign. ↗ T3 at all conc T4: 6.96 ± 0.84, 5.10 ± 0.68*, 5.24 ± 1.12* and 4.90 ± 0.92* T3: 0.43 ± 0.07, 0.73 ± 0.13*, 0.68 ± 0.11* and 0.66 ± 0.14*				
		Danio rerio	Water	Embryos (2 hpf): until 120 hpf (5 dpf)	**0**, SC, 1 and 100 µg/L Results compared to blank	0.005% DMSO (no sign. differenc e with water control)	LH: ↗, but not stat. sign. FSH: stat. sign. ↗ at 100* µg/L E2: stat. sign. ↗ at 1* and 100* µg/L GH : slightly ↗, but not stat; sign. At 100 µg/L BPS (single conc. used): LH: BPS + ICI: ↙ at 100* µg/L compared to control, ↙ but not sig. compared to BPS LH: BPS + FAD: ↙, but not sign. FSH: BPS + ICI: ↗ at 100* µg/L compared to control, ↗ but not sig. compared to BPS FSH: BPS + FAD: ↗ at 100*	Qiu *et al.*, 2021	1		**EAS**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							µg/L compared to control, no stat. difference with BPS				
		Danio rerio	Water	Adults (3-4 month): 21 d	0, SC, 8, 40 and 200 µg/mL	0.1% DMSO (no sign. difference with water control in reproductive parameters)	Females: E2: stat sign. ↗ at ≥ 8 µg/L (0.398 ± 0.183, 0.965* ± 0.204, 1.326 ± 0.321*,1.162 ± 0.203* ng/g) T: no effect (0.007 ± 0.002, 0.006 ± 0.003, 0.007 ± 0.003, 0.006 ± 0.003 ng/g) Progesterone: stat. sign. ↗ at 40 µg/L (6.90 ± 4.3, 11.36 ± 1.69, 12.30 ± 2.79*, 13.54 ± 2.19* ng/g) Cortisol: no effect (0.042 ± 0.011, 0.280 ± 0.352, 0.129 ± 0.108, 0.073 ± 0.041 ng/g) 11-KT: no effect (0.142 ± 0.047, 0.160 ± 0.228, 0.106 ± 0.026, 0.114 ± 0.040 ng/g) Males: E2: stat. sign. ↗ at all concentrations (0.201 ± 0.058, 0.380 ± 0.091*, 0.572 ± 0.212*, 0.663 ± 0.297* ng/g) T: Stat. sign. ↘ of T at all concentrations (0.011 ± 0.004, 0.004 ± 0.002*, 0.003 ± 0.002*, 0.003 ± 0.001* ng/g) Progesterone: no effect (4.48 ± 2.05, 5.08 ± 2.07, 5.40 ± 3.59, 4.48 ± 2.48 Cortisol: no effect (0.064 ± 0.073, 0.104 ± 0.119, 0.085 ± 0.046, 0.106 ± 0.092	Park *et al.*, 2022	1		**S**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							ng/g) 11-KT: stat. sign. at 40 and 200 µg/L (0.237 ± 0.108, 0.273 ± 0.117, 0.132 ± 0.117*, 0.114 ± 0.075* ng/g) Stat sign. ↗ T/E2 ratio at all conc. =>Aromatase activity Sign. ↗ of thyroid hormones T3: F: 5.80 ± 1.16, 10.00 ± 0.71*, 12.40 ± 0.87*, 16.20 ± 1.77* pg/g M: 5.00 ± 0.95, 10.40 ± 1.54*, 14.00 ± 1.48*, 14.60 ± 2.56* pg/g T4: F: 21.80 ± 2.92, 32.20 ± 3.61*, 44.40 ± 2.27*, 58.80 ± 3.04* pg/g M: 18.40 ± 1.81, 24.20 ± 3.25*, 32.80 ± 1.74*, 36.40 ± 2.89* pg/g				T
		Salmo trutta	Cholesterol pellets	Juvenile: 2 and 8 weeks	Pellets containing 2 mg/kg fish and 20 mg/kg fish of BPS	5% of cocoa	No effect on T3 and T4 in plasma after 2 and 4 weeks of exposure No effect on T3/T4 ratio in plasma	Ribera JM, 2015	/		**T**
		Salmo trutta	Cholesterol implant (injection in abdominal cavity)	Juvenile (+/-1yr old): 2 and 8 weeks	Pellets containing 2 mg/kg fish and 20 mg/kg fish of BPS	/	Stat. sign. ↗ of T3 at 20* mg/kg after 2 weeks but no longer at 8 weeks. No effect on T4 No effect on T3/T4 ratio	Frenzilli *et al.*, 2021	/		**T**
		Oreochromis mossambicus	Water	Juveniles (bw: 45 +/- 2 g): 4,8 and 12	100, 120 and 140 mg/L Adults: 100, 125 and 150 mg/L	/	E2 levels Juvenile males: time dependent stat. sign. ↗ of E2 (100*, 120* and 140*	Anjali *et al.*, 2019	/	Supportive evidence for hormonal changes (↗	**S**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
				d			mg/L), Juvenile females: time dependent stat. stign. ↘ of E2 (100*, 120* and 140* mg/L) Adult males: time dependent stat.sign. ↘ (100*, 125* and 150* mg/L), Adult females: time-dependent stat.sign. ↗ (at 100*, 125* and 150* mg/L) <u>T and E2/T ratio</u>: Juveniles: stat. sign. ↘ of T (100*, 120* and 140* mg/l) and ↗ of E2/T ratio (100*, 120* and 140 mg/L Adults: Stat. sign. ↘ of T (100*, 125* and 150 mg/L) and ↗ of E2/T ratio (100*, 125* and 150 mg/L) <u>T3 and T4 serum level</u>: Juveniles: T3: stat. sign.↘ T3 at 100* mg/L, ↗ at 120 mg/L after 3 days and stat. sign. ↗ after 6 and 9 days exposure, T4: stat. sign. ↘ at all conc. (100*, 120*, and 140* mg/L) Adults: time dependent stat. sign. ↗ or T3 (100*, 125* and 150* mg/L) and T4 (100 *, 125* and 150* mg/L <u>Cortisol serum level</u>: Juveniles:			E, ↙ T) in other species than zebrafish	**T**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							stat. sign. ↙ at 100* mg/L after 3, 6 and 9 days exposure Stat.sign.. ↗ at 120* mg/L after 9 days exposure and at 140* mg/L after 6 and 9 days of exposureCortisol serum level ↙ Adults: stat. sign. ↙ at 100*, 125* after 4, 8 and 12 days and 150* mg/L after 8 and 12 days of exposure				
		Chicken	Injection in air cell of fertilised eggs	Fertilised eggs: 20-22d	**SC**, 0.27, 0.91, 10.6, 52.8 and 207 µg/g egg	~ 1µg/L DMSO	No effect on plasma T4	Crump *et al.*, 2016	/		**T**
	Vitellogenin	*Danio rerio*	Water	2-75 dpf	0, **SC**, 0.1; 1; 10 and 100 µg/L Results compared to SC	0.01 % acetone (v/v) Authors confirmed that results of water and solvent control were the same	Stat. sign. ↗ plasma VTG in females (10* and 100* µg/L) and males at 100* µg/l resp., dose-dependently	Naderi *et al.*, 2014	1	Overall positive evidence: ↗ VTG in males and females	**E**
		Danio rerio	Water	6 month old: 7 d	0, **SC**, 0.1, 1 and 10 µM (25, 250 and 2500 µg/L) Results compared to SC	0.01% DMSO (v/v)	Stat. sign. ↗ in males at 0.1*** and 1*** µM (high mortality at 10 µM)	Le Fol *et al.*, 2017	1		**E**
		Danio rerio	Water	F0 (+/-30 wks old): 49-50d, F1: 125-128d F2: 96h	**0**, 2, 10, 50, 250 and 1250 µg/l	/	Not measured in F0 No sign. effect in F1 Males: 14.2 ± 133, 9.4 ± 64, 9.8 ± 10, 7.9 ± 41, 7.3 ± 12 and 7.7 ± 41% ng/mg	Unpublished study report, 2020 (ZEOGRT (OECD TG	1		**E**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							(median ± %CV) Females: 33475 ± 24, 43586 ± 31, 30561 ± 56, 30732 ± 7, 32071 ± 23 and 27927 ± 31 ng/mg (median ± %CV)	240 adapted for zebrafish))			
		Danio rerio	Water	2 hpf – 240 dpf	**SC**, 1 and 100 µg/L	0.002% DMSO	Females: Liver VTG: non-sign. ↙ compared to SC Plasma VTG: stat. sign. ↗ at 1** µg/L, non-sign. ↗ at 100 µg/L Ovary VTG: stat. sign ↗ at 1**and 100** µg/L	Qin *et al.*, 2021	2	Results questionable	**E**
		Danio rerio	Water	Adults (3-4 month): 21 d	0, SC, 8, 40 and 200 µg/mL	0.1% DMSO (no sign. difference with water control in reproductive parameters)	Females: Statistically significant ↙ at ≥ 40 µg/mL (hepatic VTG) Males: statistically significant ↗ at 200 µg/mL	Park *et al.*, 2022	1	Overall positive evidence: ↗ VTG in males	**E**
		Salmo trutta	Cholesterol pellets	Juvenile: 2 and 8 weeks	Pellets containing 2 mg/kg fish and 20 mg/kg fish of BPS	5% of cocoa	Slight ↗ of VTG in juveniles at 20 mg/kg but not stat. sign. after 2 weeks and 8 weeks exposure	Ribera J.M., 2015	/	Increasing trend in VTG although adminis-tration route is not environmentally relevant	**E**
		Salmo trutta	Cholesterol implant (injection in abdominal cavity)	Juvenile: 2 and 8 weeks	Pellets containing 2 mg/kg fish and 20 mg/kg fish of BPS	/	↗ in VTG in juveniles, but not sign. at 2 and 20 mg/kg	Frenzilli *et al.*, 2021	/		**E**
		Poecilia reticulata (guppies)	Water	Adult: 21d	**SC**, 1, 10 and 100 µg/L	Solvent not specified	Stat. sign. ↗ in males at all conc., Brain (0, 50**, 100** and 200** ng/L), eyes (0, 50**, 100** and 200** ng/L), Gonad (0, 50**, 100** and 200** ng/L),	Wang *et al.*, 2017a	/	Supporting evidence for increased VGT (in males) in other species than zebrafish	**E**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							skin (0, 50**, 100** and 200** ng/L), fin (0, 50**, 100** and 200** ng/L), whole animal (0, 50**, 100** and 200** ng/L) and liver (0, 50**, 100** and 200** ng/L) max. reached at 10 µg/l in fin, liver and whole animal in males				
	Estrogen binding	*Danio rerio* ERE-transgenic zebrafish	Water	1–120 hpf	**SC**, 1, 20 and 50 mg/L	0.01% ethanol	Induced GFP expression of estrogen responsive element (2.7 and 10.8 fold ↗ at 20** and 50** mg/l BPS resp.); totally inhibited by ICI	Moreman *et al.*, 2017	1	Estrogen activity	**E**
EATS-mediated parameters	Sperm count	*Danio rerio*	Water	2-75 dpf	0, **SC**, 0.1, 1, 10 and 100 µg/L Results compared to SC	0.01 % acetone (v/v) Authors confirmed that results of water and solvent control were the same	Sign. ↘ in sperm count at 10* and 100* µg/L, dose dependently	Naderi *et al.*, 2014	1	WOE approach: overall evidence: no data on sperm morphology and sperm motility measured in zebrafish but effects on on sperm count and sperm motility are consistently observed at low doses in rodents	**EAS**
	Specific gonad histopathology	*Danio rerio*	Water	F0 (+/-30 wks old): 49-50d, F1: 125-128d F2: 96h	0, 2, 10, 50, 250 and 1250 µg/l	/	No significant effects on gonad maturation in F1 males and females ↘ in mature females: 14,89%, 7,41%, 3.64%, 9.26%, 4.88% and 1,82%	Unpublished study report, 2020 (ZEOGRT (OECD TG	1	Overall positive evidence of reduced oocyte maturation	**EAS**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							resp. at 0, 2, 10, 50, 250 and 1250 µg/l	240 adapted for zebrafish))		(except for Qin *et al.*, 2021)	
		Danio rerio	Water	2 hpf – 240 dpf	**SC**, 1 and 100 µg/L	0.002% DMSO	Stat. sign. ↘ in primary oocytes, ↗ of full grown stage oocytes, sign. ↗ of follicle- and vitellogenic-stage oocytes But % atretic follicles were not included	Qin *et al.*, 2021	2	Supported by effects on oocyte maturation in rodents	**E**: Lipid metabolism: BPS promoted the excessive use of lipids as an energy source for oocyte maturation
		Danio rerio	Water	Adults (3-4 month): 21 d	0, SC, 8, 40 and 200 µg/mL Results compared to SC	0.1% DMSO (no sign. difference with water control in reproductive parameters)	Females: affected oocyte maturation at 40 and 200 µg/L (regressed oocytes characterised by excess of oocyte atresia) Males: no effect on testicular development	Park *et al.*, 2022	1		**EAS**
		Danio rerio	Water	Adult zebrafish (6 months old) - 14 d	F0**:** 0, **SC**, 1, 10, 100 µg/L	0.002% DMSO	Females: The proportions (%) of perinucleolar oocytes, cortical alveolar oocytes and late vitellogenic and spawning oocytes in the 0, 1, 10 and 100 µg/L BPS groups were 46.72, 68.96, 55.56 and 59.14; 22.41, 17.55, 23.31 and 15.60; and 28.12, 11.46, 17.89 and	Wang *et al.*, 2020b	1		**EAS**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							13.68, respectively Males: Spermatogenic cysts in the testes of males in the BPS treatments were loose, and the sperm were spilled in comparison to those in the control group				
	Sex ratio	*Danio rerio*	Water	2 dpf: 75 d	0, **SC**, 0,1, 1, 10 and 100 µg/l Results compared to SC	0.01 % acetone (v/v) Authors confirmed that results of water and solvent control were the same	Feminisation in F0: 58.8 and 66.7% females at 10 and 100 µg/L resp. compared to control (46.3%) (X2-test, X2= 12.14, p = 0.016)	Naderi *et al.*, 2014	1	Overall positive evidence of feminisation	**EAS**
		Danio rerio	Water	<4 hpf-148 dpf	0, 0.0032, 0.010, 0.032, 0.1, 0.320, 1.0, 3.2 and 10.0 mg/L	/	Non-sign. effect on sex ratio (resp. 32%, 30%, 41%, 33%, 37%, 37%, 40%, 50% and 39% M at 0, 0.0032, 0.010, 0.032, 0.1, 0.320, 1.0, 3.2 and 10.0 mg/L) But very low % of males in control (32%)	Unpublished study report, 2020 (Range finding study: OECD TG 210 + OECD TG 234 + OECD TG 229)	1		**EAS**
		Danio rerio	Water	F0 (+/-30 wks old): 49-50 d, F1: 125-128 d F2: 96 h	0, 2, 10, 50, 250 and 1250 µg/l	/	Non-sign. ↙ of males (resp. 41/59, 31/68, 29/71, 33/67, 49/51, 30/70 M/F at 0, 2, 10, 50, 250 and 1250 µg/l) Although low % males in control group (41%), males decreased at 10 µg/l to 29%	Unpublished study report, 2020 (ZEOGRT (OECD TG 240 adapted	1		**EAS**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							(outside natural variation)	for zebrafish))			
	Transcriptional activity of Cyp19	*Danio rerio*	Water	3-4 months: 21 d	**0**, SC, 0.5, 5 and 50 µg/L Results compared to blank	0.1% Methanol (v/v) No sign. difference between water and solvent control	Males: stat sign. ↗ of Cyp19a and 19b gene expression compared to blank: expressed by 2.65 fold (β=0.837, p<0.05) and 2.81-fold (β=0.750, p<0.05) resp. at 50 µg/l compared to control (Cyp 19a: 1.00 ± 0.35, 1.00 ± 0.19, 1.54 ± 0.79, 1.65 ± 0.39 and 2.81 ± 0.69* Cyp 19b: 1.00 ± 0.16, 0.96 ± 0.13, 1.04 ± 0.42, 2.23 ± 0.52 and 2.65 ± 0.24*) Females: no stat. sign. difference with blank (Cyp 19a: 1.00 ± 0.22, 1.11 ± 0.14, 1.44 ± 0.52, 0.96 ± 0.45 and 1.07 ± 0.09 Cyp 19b: 1.00 ± 0.13, 1.28 ± 0.31, 0.90 ± 0.18, 0.85 ± 0.44 and 0.61 ± 0.28)	Ji *et al.*, 2013	1	Overall evidence on upregulated transcription of Cyp19 gene	**S**
		Danio rerio	Water	1-7 dpf	SC and 1 µM (250 µg/L)	0.1% DMSO (v/v)	41-fold ↗ of CYP19a1b expression at 250* µg/L	Cano-Nicolau *et al.*, 2016	1		**S**
		Danio rerio	Water	0-4 dpf	0, **SC**, 0.25, 0.5, 1, 2.5, 5, 10, 20, 30 and 60 µM Results compared to SC	0.01% DMSO (v/v)	4-fold ↗ at 30*** and 60*** µM (resp. 7500 and 15 000 µg/L), but not inhibited by ICI	Le Fol *et al.*, 2017	1		**S**
		Danio rerio	Water	1d-4dpf	SC and 1 µM (205 µg/L) EASZY assay	0.1% DMSO (v/v)	6-fold ↗ of CYP19a1b in transgenic CYP19a1b-GFP zebrafish at 250* µg/L CYP19a1b transcripts	Cano-Nicolau *et al.*, 2016	1		**S**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							distribution patterns in the whole brain were identical to EE2: over-expression in posterior telencephalon, preoptic area and caudal hypothalamus, including the nucleus recessus posterioris were the induction was the strongest				
		Danio rerio	water	Embryos (2hpf): until 120 hpf (5dpf)	**0**, SC, 1 and 100 µg/L Results compared to blank	0.005% DMSO (no sign. differenc e with water control)	Stat. sign. upregulation of CYP19a1 (gonad) and CYP19a2 (brain) at 100* µg/L	Qiu *et al.*, 2021	1		**S**
	Gene transcriptio n in gonads (excl. Cyp19a)	*Danio rerio*	Water	3-4 months: 21d	**0**, SC, 0.5, 5 and 50 µg/L Results compared to blank	0.1% Methanol (v/v) No sign. difference between water and solvent control	Males: stat. sign. ↗ at 50* µg/L of fshr, lhr, hmgra, hmgrb, CYP11a and 3βhsd mRNA transcription in testis *fshr*: 1.00 ± 0.13, 1.07 ± 0.43, 1.01 ± 0.45, 1.39 ± 0.73 and 2.65 ± 0.41* *lhr*: 1.00 ± 0.09, 1.04 ± 0.32, 2.59 ± 0.90, 2.89 ± 0.99 and 3.18 ± 1.00* *hmgra*: 1.00 ± 0.46, 1.21 ± 0.28, 1.24 ± 0.43, 2.19 ± 0.35 and 2.29 ± 0.29* *hmgrb*: 1.00 ± 0.15, 1.20 ± 0.45, 1.33 ± 0.17, 1.75 ± 0.50 and 1.96 ± 0.78* *star*: 1.00 ± 0.20, 0.91 ± 0.26, 0.81 ± 0.22, 0.67 ± 0.35 and 0.53 ± 0.19 *cyp11a*: 1.00 ± 0.31, 1.14 ± 0.45, 1.17 ± 0.07, 1.60 ± 0.38 and 2.37 ± 0.92* *3βhsd*: 1.00 ± 0.30, 1.16 ± 0.22, 1.60 ± 0.54, 1.65 ± 0.62 and 2.07 ± 0.77*	Ji *et al.*, 2013	1	Overall evidence of changes in gene transcription in gonads (excl. Cyp19a)	**EAS**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							Cyp17: 1.00 ± 0.05, 1.05 ± 0.21, 1.37 ± 0.25, 1.14 ± 0.48 and 0.47 ± 0.10* *17βhsd*: 1.00 ± 0.16, 1.10 ± 0.23, 0.54 ± 0.18, 0.51 ± 0.18 and 0.39 ± 0.13* Females: Stat. sign. ↘ of hmgra and hmgrb mRNA expression at 50* µg/L fshr: 1.00 ± 0.26, 1.15 ± 0.28, 1.24 ± 0.52, 1.16 ± 0.51 and 1.40 ± 0.39 lhr: 1.00 ± 0.26, 1.01 ± 0.36, 1.10 ± 0.27, 1.19 ± 0.78 and 1.34 ± 0.49 hmgra: 1.00 ± 0.14, 1.02 ± 0.30, 0.46 ± 0.36, 0.38 ± 0.12 and 0.28 ± 0.21* hmgrb: 1.00 ± 0.31, 1.30 ± 0.37, 1.43 ± 0.30, 0.53 ± 0.21 and 0.44 ± 0.22* star: 1.00 ± 0.26, 1.06 ± 0.21, 1.29 ± 0.46, 1.09 ± 0.30 and 0.65 ± 0.16 cyp11a: 1.00 ± 0.12, 1.08 ± 0.24, 1.22 ± 0.16, 0.72 ± 0.43 and 0.86 ± 0.35 3βhsd: 1.00 ± 0.27, 1.18 ± 0.16, 1.17 ± 0..04, 1.06 ± 0.33 and 1.20 ± 0.30 Cyp17: 1.00 ± 0.40, 1.09 ± 0.51, 1.14 ± 0.26, 0.93 ± 0.16 and 0.61 ± 0.11 17βhsd: 1.00 ± 0.11, 1.13 ± 0.27, 1.17 ± 0.25, 0.83 ± 0.43 and 0.99 ± 0.37				
	Gene trans-cription in	*Danio rerio*	Water	3-4 months: 21d	**0**, SC, 0.5, 5 and 50 µg/L	0.1% Methanol (v/v)	Males: sign. ↗ of gnrh3, gnrhr1, gnrhr2, fshβ, lhβ at 50* µg/L	Ji *et al.*, 2013	1	Overall evidence of changes in	**EAS**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
	brain (excl. Cyp 19b)				Results compared to blank	No sign. difference between water and solvent control	*gnrh2*: 1.00 ± 0.28, 1.03 ± 0.24, 0.92 ± 0.19, 1.23 ± 0.32 and 1.32 ± 0.22 *gnrh3*: 1.00 ± 0.30, 0.93 ± 0.12, 1.07 ± 0.27, 1.53 ± 0.07 and 2.22 ± 0.81* *gnrhr1*: 1.00 ± 0.36, 1.17 ± 0.51, 1.46 ± 0.46, 1.36 ± 0.26 and 1.80 ± 0.18* *gnrhr2*: 1.00 ± 0.14, 0.91 ± 0.31, 0.91 ± 0.36, 1.72 ± 0.34 and 2.19 ± 0.60* *gnrhr4*: 1.00 ± 0.23, 0.99 ± 0.21, 0.86 ± 0.34, 0.94 ± 0.36 and 0.97 ± 0.42 *fshβ*: 1.00 ± 0.08, 1.01 ± 0.35, 1.35 ± 0.38, 1.24 ± 0.51 and 2.09 ± 0.69* *lhβ*: 1.00 ± 0.10, 1.14 ± 0.25, 1.56 ± 0.45, 2.01 ± 0.71 and 2.32 ± 0.42* *era*: 1.00 ± 0.36, 1.08 ± 0.31, 0.83 ± 0.14, 0.66 ± 0.15 and 0.53 ± 0.22 *er2β*: 1.00 ± 0.07, 0.98 ± 0.29, 0.90 ± 0.60, 0.82 ± 0.20 and 0.73 ± 0.33 *ar*: 1.00 ± 0.13, 0.88 ± 0.18, 0.98 ± 0.30, 1.09 ± 0.35 and 0.95 ± 0.41 Females: sign. ↘regulation of Gnrh3 and fshβ at 50* µg/L *gnrh2*: 1.00 ± 0.16, 1.10 ± 0.52, 1.14 ± 0.49, 0.93 ± 0.51 and 0.53 ± 0.22 *gnrh3*: 1.00 ± 0.29, 1.05 ± 0.25, 1.29 ± 0.16, 0.80 ± 0.35 and 0.49 ± 0.18* *gnrhr1*: 1.00 ± 0.25, 0.98 ±			gene transcription in brain (excl Cyp19b	

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							0.31, 1.10 ± 0.54, 1.30 ± 0.43 and 0.85 ± 0.68 *gnrhr2*: 1.00 ± 0.36, 1.09 ± 0.40, 1.29 ± 0.17, 1.06 ± 0.05 and 0.62 ± 0.21 *gnrhr4*: 1.00 ± 0.40, 1.10 ± 0.44, 1.22 ± 0.33, 1.28 ± 0.31 and 0.58 ± 0.19 *fshβ*: 1.00 ± 0.10, 1.26 ± 0.19, 0.96 ± 0.33, 0.68 ± 0.26 and 0.46 ± 0.22* *lhβ*: 1.00 ± 0.25, 1.10 ± 0.16, 0.96 ± 0.30, 0.86 ± 0.27and 0.73 ± 0.36 *era*: 1.00 ± 0.38, 1.05 ± 0.79, 0.99 ± 0.41, 0.76 ± 0.20 and 0.44 ± 0.17 *er2β*: 1.00 ± 0.47, 1.00 ± 0.70, 1.19 ± 0.50, 1.25 ± 0.23 and 0.98 ± 0.52 *ar*: 1.00 ± 0.14, 1.13 ± 0.62, 1.15 ± 0.21, 1.27 ± 0.28 and 0.99 ± 0.52				
		Danio rerio	Water	4 hpf – 25 hpf 4 hpf - 120 hpf	0, **SC**, 100 µg/L Results compared to SC	0.005% DMSO No sign. difference between blank and solvent control	kiss1: stat; sign. ↗ at 100* µg/l after 25 hpf and at 100* and 1000* µg/L at 120 hpf kiss1r: stat. sign. ↗ at 10* and 100* µg/l after 25 hpf and 10*, 100* and 1000* after 120 hpf gnrh3: stat. sign. ↗ at 100* µg/L after 25hpf and 10*, 100* and 1000* µg/L after 120 hpf lhβ: stat. sign. ↗ at 10*, and 100* µg/L after 25hpf and 10* and 1000* µg/L after 120 hpf fshβ: stat. sign. ↗ at 1000*	Qiu *et al.*, 2016	1		**EATS**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							µg/L after 120 hpf sv2: stat. sign. ↗ at 10*, 100* and 1000* µg/L after 120 hpf only measured at 100 µg/l BPS and 25 hpf: kiss1: stat; sign. ↗ at 100* µg/l kiss2: ↗, but no stat. sign. effect kiss1r: ↗, but no stat. sign. effect kiss2r: ↗, but no stat. sign. effect gnrh3: stat; sign. ↗ at 100* µg/l lhβ: ↗, but no stat. sign. effect fshβ: ↗, but no stat. sign. effect sv2: ↙, but no stat. sign. effect erα: stat; sign. ↗ at 100* µg/l erβ: ↙, but no stat. sign. effect At 120 hpf and 100 µg/L BPS: -BPS+ICI (ER antagonist, 1 µM): no stat. sign. inhibition of stimulatory actions of BPS on kiss1r, lhβ and erα, stat. sign. inhibition of stimulatory actions of gnrh3 and fshβ -BPS+FAD (Aromatase inhibitor, 1 µM): stat. sign. ↙ expression of kiss1r, gnrh3, lhβ and erα				

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							*BPA+AMIO (THR antagonist, 1 µM): stat. stat. sign. ↙ expression of kiss1r and lhβ				
		Danio rerio	water	Embryos (2hpf): until 120 hpf (5dpf)	**0**, SC, 1 and 100 µg/L Results compared to blank	0.005% DMSO (no sign. difference with water control)	Kiss 1: dose dependent ↗, stat. sign at 100* µg/L Kiss2: dose dependent ↗, stat. sign at 100* µg/L Gnrh3: ↗, but not stat.sign. Fshβ: ↗, but not stat.sign. Lhβ: stat. sign. ↗ at 1* and 100 µg/L Anp: stat. sign. ↗ at 1* and 100 µg/L Ren: dose dependent ↗, stat. sign at 100* µg/L Pth1: dose dependent ↗, stat. sign at 100* µg/L Gh: dose dependent ↗, stat. sign at 100* µg/L Pr1: ↗, stat. sign. at 1* µg/l At 100 µg/L BPS(single concentration used): lhβ: BPS + ICI: non-sign.↙ lhβ : BPS+ FAD: ↙ at 100* µg/L compared to control, ↙ but not stat. different from BPS fshβ: BPS + ICI: ↙ at 100* µg/L compared to control, ↙ but not stat. different from BPS fshβ: BPS + FAD: ↙ at 100* µg/L compared to control and BPS	Qiu *et al.*, 2021	1		**EATS**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
	Hepatic gene trans-cription	*Danio rerio*	Water	Adults (3-4 month): 21 d	0, SC, 8, 40 and 200 µg/mL	0.1% DMSO (no sign. difference with water control in reproductive parameters)	Females: ↘ of VTGmRNA expression at 40 and 200* µg/L, while ↗ at 8* µg/L no effect on Erα and ERβ gene expression Males: ↗ of VTG mRNA expression at 40 and 200* µg/L, while ERβ mRNA stat sign. ↘ at 40* and 200* µg/L No effect on Erα mRNA activation	Park *et al.*, 2022	1	Disturbed oocyte maturation linked to disturbed vitello-genesis	**E**
	Homo-genates: Gene trans-cription	*Danio rerio*	Water	2hpf - 168 hpf	**SC**, 1,3,10 and 30µg/L	0.01% (v/v) DMSO	mRNA expression: crh: stat.sign. ↗ at 10** and 30** µg/L pax8: stat. sign. ↗ at 30** µg/L slc5a5: stat. sign. ↗ at 30** µg/L tg: stat.sign. ↗ at 10** and 30** µg/L ttr: stat.sgin. ↙ at 3**, 10** and 30** µg/L trα: no effect trβ: no effect dio1: stat. sign. ↗ at 10* and 30** µg/L dio2: stat.sign. ↗ at 30** µg/L dio3: no effect ugt1ab: stat. sign. ↗ 10** and 30** µg/L	Zhang *et al.*, 2017	1		**T**
		Danio rerio	Water	4 - 120 hpf	0, **SC,** 0.1, 1, 10, 100 and 1000 µg/L Results compared to SC	0.005% DMSO No sign. difference between blank and solvent	Stat. sign. ↗ expression of Erα (100* and 1000* µg/L) and nf-kb genes (100* and 1000* µg/L)	Qiu *et al.*, 2018b	2		**E**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
						control					
		Danio rerio	Water	1 hpf - 96 hpf	0, **SC**, 3, 6, 12.5, 25 and 50 mg/L Results compared to SC	0.01% acetone, except for 12.5 mg/L (0.125 mg/L acetone) and 25 mg/L (0.25 mg/L acetone) No sign. difference between blank and solvent control	No induction of ERα protein (very slight increase at 5 and 25 mg/L) nor ERs-coding gene (esr1, esr2a, esr2b or vtg1- >25 mg/l BPS effective concentration needed to disrupt ER transcription) associated with metabolism of estrogen	Mu *et al.*, 2018	1		**E**
		Chironomus riparius	Water	Early phase of fourth instar larvae: 24 h, 24h BPS+24h in fresh culture medium without BPS	0.5, 5, 50 and 500 µg/L	0.05% DMSO	Stat. sign. change of EcR (↗ at 5*, 50* and 500* mg/L afterer 24h and ↙ at 0.5*, 5*, 50* and 500* µg/L after 24h+24h exposure), ERR (↗ at 0.5, 5*, 50* and 500* mg/L at 24h and ↙ at 5*, 50* and 500* µg/L after 24h+24h, CYP18a1 (↗ at 0.5*, 5*, 50* and 500 µg/l at 24h, ↙ at 500* µg/L at 24h+24h), hsp70 (↗ at 0.5*, 5* and 50 µg/L at 24h, ↙ non sign. after 24h+24h) and hsp40 (↗ at 5*, 50* and 500* µg/l at 24h, ↙ at 0.5*, 5*, 50* and 500* µg/l after 24h+24h), CYP4g (↗ at 5*, 50* and 500* µg/l at 24h, ↙ non sign. at 24h+24h), GPx (↗	Herrero *et al.*, 2018	/	Supportive evidence on changes in gene transciption	**EAS**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							at 0.5, 5*, 50* and 500 µg/L at 24h, ↙ non sign. at 24h+24h, and GSTd3 (↗ at 5, 50* and 500* µg/L at 24h, ↙ non sign. at 24h+24h)↗ of E74 (non sign. at 24h, ↙ at 0.5*, 5, 50 and 500 µg/L at 48h),vtg (↗ non. sign. at 50 and 500 µg/L after 24h, ↗ non sign. at all conc. after 48h) and rpL13; ↘ of its2 (0.5*, 5, 50 and 500 µg/L at 24h, ↙ at 0.5, 5, 50 and 500* µg/l at 24+24h), rpL4 (↙ at 0.5* and ↗ at 5, 50 µg/l at 24h, ↙ non sign. at 24h+24h), rpl13 (↙ at 0.5 and 5 µg/l and ↗ at 50 and 500 µg/l at 24h, ↗ at 5, 50 and 500 µg/L), GAPDH: no changes				
		Chicken	Injection in air cell of fertilised eggs	Fertilised eggs: 20-22 d	**SC**, 0.27, 0.91, 10.6, 52.8 and 207 µg/g egg	~ 1µg/L DMSO	Thyroid: Dio1: ↗ (52.8* µg/g (3.09-fold), 207 µg/g) Xenobiotic metabolism: Ugt1a9 and sult1b1: stat. ↗ (52.8* and 207 µg/g) Lipid metabolism/homeostasis: Ascl5 and Cyp7b1: ↗ (52.8* and 207 µg/g) Heat-shock: Hsp90ab1: ↗ (52.8* and 207 µg/g)	Crump *et al.*, 2016	/	Thyroid, xenobiotic metabolism, lipid metabolism/ homeostasis, heat shock	**T**
Sensitive to, but not diagnos	Behaviour	*Danio rerio*	Water	Age within 3 hpf: 0-5 dpf	**SC** and 0.0068 µM=1.7 µg/L	0.08% MeOH (v/v)	240% ↗ in neuronal birth in the rostral hypothalamus at 24 hpf and a significant (160%) ↗ in locomotor	Kinch *et al.*, 2015	1	Overall positive evidence on changed	**S**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							bursting activity which was reduced by transient knockdown of AroB but not by ICI			social behavior	
		Danio rerio	Water	2 – 120 hpf	**SC**, 1, 10 and 100 µg/L	0.002% DMSO (v/v)	↘ spontaneous movement inside chorion of 48hpf ebmryosof F1: stat sign. 1*, 10*, 100* µg/L	Wei *et al.*, 2018	1		
		Danio rerio	Water	1 hpf - 6 dpf	**SC**, 0.03, 0.3 and 3 mg/L	0.01% DMSO (v/v)	↘ locomotory activity and altered retinal structure partially by increasing oxidative stress and suppressing of levels of neurodevelopment genes (α1-tubulin, elavl3, gap43, mbp, syn2a and gfap) Total distance travelled and average speed : ↙ dose-dependently and sign. at ≥0.3 ***µg/L.	Gu *et al.*, 2019	1		**EAS**
		Danio rerio	Water	Adult zebrafish (9 month old) – 75 d exposure	**SC**, 1, 10 and 30 µg/L 1 µg/L E2	0.01% DMSO (v/v)	* shoaling - inter-individual distance: ↗ dose-dependently, 30* µg/L and 1µg/l E2 - Excursion from shoal: non-sign., non-monotonic ↗ * social preference: dose - depentenly ↗, sign at 1µg/L*, 10µg/l*, 30 µg/L* and 1 µg/L E2* * locomotory activity: ↙, no sign. effect	Salahinejad *et al.*, 2020	1		**EAS**
		Danio rerio	Water	Adult zebrafish: maternal exposure (9 months old): 60 d Offspring:	**SC**, 1, 10 and 30 µg/L 1 µg/L E2	0.01% DMSO (v/v)	* shoaling: - inter-individual distance: non-monotonic, but only sign. ↙ at 10 µg/L* * group preference (form groups with conspecifics): non-dose-depent ↙, only sign. at 1 µg/L BPS*	Salahinejad *et al.*, 2022	1		**EAS**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
				6 months old			* anxiety response: ↙ (inverted u-shape), sign. ↙ at 1* and 30 µg/L* BPS and 1 µg/l E2*				
		Danio rerio	Water	Adult zebrafish (9 months old): 120 d exposure	**SC**, 1,10 and 30 µg/L 1 µg/L E2	0.01% DMSO (v/v)	* object recognition: Dose-dependent ↙ in time spent with novel object, sign. at 10* and 30* µg/L * object placement: Time spent with moved object: ↗ at 1* and 10 µg/L, ↙ at 30* µg/L * social recognition: Dose-dependent ↙ in time spent exploring unfamiliar conspecific, sign. at 10* and 30* µg/L	Naderi *et al.*, 2020	1		**EAS**
		Danio rerio	Water	21 dpf old larvae (exposed from 2 hpf - 5 dpf)	**SC**, 0.25µg/l (0.001µM), 2.5 µg/l (0.01µM) and 25 µg/L (0.1µM) 25µg/L BPS+FAD 25µg/L BPS+ICI	~0.001% DMSO (v/v)	* anxiety (thigmotaxis): ↗, but only sign at 0.25µg/L* BPS+FAD:=ctrl BPS+ICI: ↗* * social behaviour: - social preference (time spent near conspecifics): only ↙ at 25 µg/L* BPS+FAD:=ctrl BPS+ICI: ↗* - interfish distance: inverted u-shape, sign. at 0.25* and 25* µg/L BPS+FAD: ↙ BPS+ICI: ↙* * object recognition: dose-dependent ↙ from 2.5 µg/L, sign. at 25 µg/L* BPS+FAD: ↙* BPS+ICI: ↙(slight)	Naderi *et al.*, 2022	1		**EAS**
		Danio rerio	Water	Adult zebrafish (6 months	F0**:** 0, **SC**, 1, 10, 100 µg/L	0.002% DMSO	Females: *shoaling - Time ratio spent in the	Wang *et al.*, 2020b	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
				old) - 14 d			area close to other fish: sign. ↙ at 100* µg/L - Time ratio in far area: sign. ↗ at 100* µg/L *anxiety: - Time spent at the bottom: sign. ↙ at 10* µg/L - Freezing frequency at the bottom: decreasing trend but not sign. Males: *shoaling - Time ratio spent in the area close to other fish: sign. ↙ at 1**, 10* and 100* µg/L - Time ratio in far area: trend to increase but not sign. *anxiety: - Time spent at the bottom: trend to increase but not sign. - Freezing frequency at the bottom: sign. ↗ at 10* µg/L Number of attacks of F and M: No sign. effect but increased in males				
		Danio rerio	water	Embryos (2hpf): until 120 hpf (5dpf)	**0**, SC, 1 and 100 µg/L Results compared to blank	0.005% DMSO (no sign. difference with water control)	↙ in movement distances but not stat. sign.	Qiu *et al.*, 2021	1		
	Neuro-genesis	*Danio rerio*	Water	Age within 3 hpf: 0-5 dpf	**SC** and 0.0068 µM=1.7 µg/L	0.08% MeOH (v/v)	240% ↗ in neuronal birth in the rostral hypothalamus at 24 hpf and a significant	Kinch *et al.*, 2015	1	Overall evidence on effect on	**S**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							(160%) ↗ in locomotor bursting activity which was reduced by transient knockdown of AroB but not by ICI			neuro-genesis depending on aromatase activity	
		Danio rerio	Water	4 hpf – 25 hpf	0, **SC**, 100 µg/L Results compared to SC	0.005% DMSO No sign. difference between blank and solvent control	Stat. sign. ↗ of Hypothalamic GnrH3 neurons, no effect on Terminal Nerve GnrH3	Qiu *et al.*, 2016	1		**EAS**
		Danio rerio	Water	Embryos (2 hpf): until 120 hpf (5 dpf)	**0**, SC, 1 and 100 µg/L Results compared to blank	0.005% DMSO (no sign. differenc e with water control)	Stat. sign. ↗ of Hypothalamic GnrH3 neurons, no stat. sign. effect on Terminal Nerve GnrH3	Qiu *et al.*, 2021	1		**EAS**
	Growth	*Danio rerio*	Water	2-75 dpf	0, **SC**, 0.1, 1, 10 and 100 µg/L Results compared to SC	0.01 % acetone (v/v) Authors confirmed that results of water and solvent control were the same	Body weight and length sign. ↘ in males at 100* µg/L Males: Body weight: SC: 238.33 ± 9.09, 239,17 ± 7.39, 240 ± 7.56, 235,58 ± 6.36, 176.83 ±6.36* mg Length: SC: 24.28 ± 1.024, 22.68 ± 0.617, 23.48 ± 0.774, 23.45 ± 1.163, 19.41 ± 0.548* mm Females Body weight: SC: 271.33 ±7.41, 269.67 ± 7.13, 273.58 ± 6.16, 282 ±5.32, 276.33 ± 5.19	Naderi *et al.*, 2014	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							Length: SC: 28.84 ±0.565, 28.15 ± 0.706, 28.77 ± 0.64, 28.67 ± 0.409, 26.67 ± 0.578				
		Danio rerio	Water	2 hpf – 168 hpf	**SC**, 1, 3, 10, and 30 µg/L	0.01% DMSO (v/v)	No sign. effect on body weight and length at 168hpf Weight: 0.4 ± 0.02, 0.4 ± 0.04, 0.43 ± 0.05, 0.38 ± 0.01 and 0.37 ± 0.03 mg Length: 3.73 ± 0.15, 3.50 ± 0.23, 3.63 ± 0.27, 3.47 ± 0.38 and 3.80 ± 0.17 mm	Zhang *et al.*, 2017	1		
		Danio rerio	Water	4 - 120 hpf	0, **SC**, 0.1, 1, 10, 100 and 1000 µg/L Results compared to SC	0.005% DMSO No sign. difference between blank and solvent control	Concentration-dependent ↘ (stat. sign. at 100* and 1000* µg/L) at 120 hpf	Qiu *et al.*, 2018b	2		
		Danio rerio	Water	4 - 120 hpf	**SC**, 0.4, 2, 10 and 50 mg/L	< 0.1% DMSO (v/v)	No sign. effect (3.84 ± 0.18, 3.84 ± 0.18, 3.87 ± 0.14, 3.82 ± 0.14 and 4.09 ±0.14 mm)	Lee *et al.*, 2019	1		
		Danio rerio	Water	<4 hpf - 148 dpf	0, 0.0032, 0.010, 0.032, 0.1, 0.320, 1.0, 3.2 and 10.0 mg/L	/	No sign. effect at day 33/34 Length: 2.2, 2.2, 2.3, 2.2, 2.2, 2.2, 2.1, 2.4, 2.4 cm Weight: 95.4, 94.6, 105.6, 99.5, 90.3, 95.8, 86.4, 117.6, 114.9 mg Sign. effect on length at day 65 Length: 3.4, 3.4*, 3.2, 3.3, 3.2, 3.0*, 3.1, 3.3, 3.5 cm Weight: 407, 332, 372, 392, 340, 288, 325, 330, 406cmg Sign. effect on length and weight (males + females) at	Unpublished study report, 2020 (Range finding study: OECD TG 210 + OECD TG 234 + OECD TG 229)	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							all conc. at day 105/106 Length: F: 3.9, 3.6**, 3.5**, 3.7**, 3.5**, 3.6**, 3.6**, 3.7**, 3.9** cm M: 3.9, 3.4, 3.5, 3.4, 3.4, 3.4, 3.2*, 3.6, 3.5 cm Weight: F: 652, 451**, 420**, 482**, 429**, 524**, 443**, 554**, 581** mg M: 519, 301*, 394, 345, 315*, 353*, 249*, 453*, 386* mg; Slightly reduced length in females at 320 µg/l and males at 1000 µg/L at day 148 Length: F: 4, 3.9, 3.9, 3.9, 3.8, 3.8*, 3.8, 4.1, 4 cm M: 3.8, 3.8, 3.7, 3.7, 3.8, 3.7, 3.6*, 3.9, 3.9 cm Weight: F: 700, 687, 655, 674, 627, 580, 672, 830, 747 mg M: 520, 532, 463, 483, 529, 499, 449, 449, 591, 583 mg				
		Danio rerio	Water	F0 (+/-30 wks old): 49-50 d, F1: 125 - 128 d F2: 96 h	0, 2, 10, 50, 250 and 1250 µg/l	/	Length: F0: no effect at sacrifice (day 50) - M: 3.7, 3.8, 3.7, 3.8, 3.7 and 3.8 cm - F: 3.5, 3.5, 3.5, 3.5, 3.5 and 3.5 cm F1: stat sign. affected after day 35 (1.7, 1.7, 1.6*, 1.6*, 4.6* and 1.7* cm), the effect was transient and no longer observed at day 65 (2.7, 2.8, 2.7, 2.7, 2.7 and	Unpublished study report, 2020 (ZEOGRT (OECD TG 240 adapted for zebrafish))	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							2.8 cm) At day 125-128: - M: 3.7, 3.6, 3.6, 3.5, 3.6 and 3.5* cm - F: 3.6, 3.6, 3.6, 3.6, 3.6 and 3.6 cm Body weight: F0: at sacrifice (day 50) - M: 443, 474, 463, 465, 452,and 456 mg - F: 415, 389, 396, 410, 415 and 368 mg F1: at sacrifice day 125-128 - M:403, 408, 374, 387, 391 and 367 mg - F: 449, 453, 437, 437, 450 and 435 mg				
		Danio rerio	Water	2 hpf – 240 dpf	**SC**, 1 and 100 µg/L	0.002% DMSO	Females: Length: no effect Weight: stat. sign. ↗ at 1** and 100** µg/L at day 240	Qin *et al.*, 2021	2		
		Danio rerio	Water	Embryos (2 hpf): until 120 hpf (5 dpf)	**0**, SC, 1 and 100 µg/L Results compared to blank	0.005% DMSO (no sign. differenc e with water control)	Dose dependent ↙ of body length, stat. sign. at 100* µg/L at 120 hpf	Qiu *et al.*, 2021	1		
		Danio rerio	Water	Adult zebrafish (6 months old) - 14 d	F0**:** 0, **SC**, 1, 10, 100 µg/L	0.002% DMSO	No effect on body weight and length in males and females	Wang *et al.*, 2020b	1		
		Chicken	Injection in air cell of fertilised eggs	Fertilised eggs: 20-22 d	**SC**, 0.27, 0.91, 10.6, 52.8 and 207 µg/g egg	~ 1µg/L DMSO	Stat. sign. decrease in embryonic weight and tarsus length at 207* µg/g	Crump *et al.*, 2016	/	Complement -ary information	
	Morpho-logical abnormal-	*Danio rerio*	Water	3-4 months: 21 d	**0**, SC, 0.5, 5 and 50 µg/L	0.1% Methanol (v/v)	↗ malformations in continuous exposed F1: Cardiac edema, shortened	Ji *et al.*, 2013	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
	ities				Results compared to blank	No sign. difference between water and solvent control	tails and severe spinal kyphosis After 16d exposure: no sign. effect After 21d exposure: stat. sign. ↗ of % malformations at 5* and 50* µg/L				
		Danio rerio	Water	2 hpf – 168 hpf	SC, 1, 3, 10, and 30 µg/L	0.01% DMSO (v/v)	No sign. effect at 168 hpf (3.35 ± 0.88, 3.02 ± 1.53, 3.34 ± 1.45, 3.67 ± 0.88 and 3.96 ± 1.20 %)	Zhang *et al.*, 2017	1		
		Danio rerio	Water	1 hpf- 96 hpf	SC, 10, 20, 50, 100, 200 and 300 mg/L	0.01% ethanol	cardiac edema, craniofacial abnormalities and spinal deformity at ≥ 200 mg/L	Moreman *et al.*, 2017	1		
		Danio rerio	Water	1hpf - 96 hpf	0, **SC**, 3, 6, 12.5, 25 and 50 mg/L Results compared to SC	0.01% acetone, except for 12.5 mg/L (0.125 mg/L acetone) and 25 mg/L (0.25 mg/L acetone) No sign. difference between blank and solvent control	No effect on abnormality incidence (yolk sac edema, spine deformation, pigmentation inhibition and pericardial edema)	Mu *et al.*, 2018	1		
		Danio rerio	Water	4 - 120 hpf	**SC**, 0.4, 2, 10 and 50 mg/L	< 0.1% DMSO (v/v)	No sign. abnormalities (0.0 ± 0.0, 20.0 ± 23.1, 5.0 ± 10.0, 4.2 ± 8.3 and 10.1 ± 12.5 %)	Lee *et al.*, 2019	1		
		Danio rerio	Water	2 hpf – 240 dpf	**SC**, 1 and 100 µg/L	0.002% DMSO	Stat. sign. ↗ at 100** µg/L in F1	Qin *et al.*, 2021	2		
		Danio rerio	Water	Adult zebrafish	F0**:** 0, **SC**, 1, 10, 100 µg/L	0.002% DMSO	Pigmentation Females: sign. ↙ of melanin	Wang *et al.*, 2020b	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
				(6 months old) - 14 d			at 1*, 10** and 100** µg/L Males: decrease of melanin but not sign. Sign. ↙ of yellow pigment at 1**, 10** and 100** µg/L				
	Gonado-somatic index	*Danio rerio*		3-4 months: 21d	**0**, SC, 0.5, 5 and 50 µg/L Results compared to blank	0.1% Methanol (v/v) No sign. difference between water and solvent control	Males: stat. sign. ↘ at 50 µg/l (resp. 1.13 ± 0.26, 1.09 ± 0.24, 0.89 ± 0.14, 0.90 ± 0.11 and 0.83 ± 0.16* at at 0, solvent control, 0.5, 5 and 50 µg/L) Females: stat sign. ↘ at ≥ 0.5 µg/L (resp. 14.00 ± 1.62, 14.01 ±0.77, 11.41 ± 2.18*, 9.74 ± 1.05* and 8.94 ± 2.00* at 0, solvent control, 0.5, 5 and 50 µg/L; p <0.05)	Ji *et al.*, 2013	1	Additional information but not conclusive	
		Danio rerio	Water	2-75 dpf	0, **SC**, 0.1, 1, 10 and 100 µg/L Results compared to SC	0.01 % acetone (v/v) Authors confirmed that results of water and solvent control were the same	Males : stat. sign. ↘ at ≥ 10 µg/L (1.08 +/- 0.024, 1.07 ± 0.028, 1.02 ± 0.033, 0.82 ± 0.022*, 0.74 ± 0.02* at solvent control, 0.1, 1, 10 and 100 µg/L) Females: stat. sign. ↘ at 100 µg/L (11.49 ±0.257, 10.89 ± 0.258, 10.87 ± 0.282, 10.82 ± 0.26 and 9.02 ± 0.138* at solvent control, 0.1, 1, 10 and 100 µg/L)	Naderi *et al.*, 2014	1		
		Danio rerio	Water	2 hpf – 240 dpf	**SC**, 1 and 100 µg/L	0.002% DMSO	Stat. sign. ↗ 100 µg/L (0.01< p < 0.05)	Qin *et al.*, 2021	2		
		Danio rerio	Water	Adults (3-4 months): 21 d	0, **SC**, 8, 40 and 200 µg/L Results compared to SC	0.1% DMSO (no sign. difference with water	Females: stat. sign. ↗ of GSI at 40 g/L, while a sign. ↘ at 200 µg/L (8.17 +/- 1.18, 11.00 +/- 1.76, 16.06 +/- 3.26 and 5.17 +/- 1.86	Park. *et al.*, 2022	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
						control in reproductive parameters)	% at control, 8, 40 and 200 µg/L resp. Males: no effect on GSI (1.09 +/- 0.10, 1.04 +/- 0.13, 1.11 +/- 0.16, 1.12 +/- 0.26 % at control, 8, 40 and 200 µg/L resp.				
	Reproduction (fecundity, fertility)	*Danio rerio*	Water	3-4 months: 21d	**0**, SC, 0.5, 5 and 50 µg/L Results compared to blank	0.1% Methanol (v/v) No sign. difference between water and solvent control	Stat. sign. and dose-dependent ↘ fecundity at ≥ 0.5 µg/L in F0 (0.5*, 5*, 50* µg/L)	Ji *et al.*, 2013	1	Overall positive evidence of decreased fecundity	**EAS**
		Danio rerio	Water	2-75 dpf	0, **SC**, 0.1, 1, 10 and 100 µg/L Results compared to SC	0.01 % acetone (v/v) Authors confirmed that results of water and solvent control were the same	Stat. sign. and dose-dependent ↘ fecundity at 10* and 100* µg/L, dose-dependently	Naderi *et al.*, 2014	1		**EAS**
		Danio rerio	Water	<4 hpf - 148 dpf	0, 0.0032, 0.010, 0.032, 0.1, 0.320, 1.0, 3.2 and 10.0 mg/L	/	Using Jonckeere-Terpstra test: non-significant ↘ fecundity at 3.2, 1.0 and 32 µg/L (22, 14, 7, 11, 23, 17, 28, 33 and 37 eggs/female reproduction day) No sign. effect on fertility (45%, 41%, 28%, 29%, 35%, 35%, 45%, 65% and 66%, but fertility in controls (45%) did not meet the validity criteria of > 80%	Unpublished study report, 2020 (Range finding study: OECD TG 210 + OECD TG 234 + OECD TG 229)	1		**EAS**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							Recalculation of fecundity by DS (Wilcoxon test): stat. sign. ↘ at 10* µg/L (70%), non-sign.↘ at 3.2 (36%) and 32 µg/L (50%)				
		Danio rerio	Water	F0 (+/- 30 wks old): 49-50 d, F1: 125-128 d F2: 96 h	0, 2, 10, 50, 250 and 1250 µg/l	/	F0: No effect on fecundity (21, 19, 17, 21, 21 and 19 eggs/female reproduction day) F1: ↘ fecundity at all concentrations (14, 8*, 11, 9*, 11 and 13 eggs/female reproduction day) No effect on fertility in F0 (92.2, 92.2, 91.6, 90.0, 92.4 and 91.3%) and F1 (91.4, 90.5, 88.7, 90.8, 94.1 and 93.5%)	Unpublished study report, 2020 (ZEOGRT (OECD TG 240 adapted for zebrafish))	1		**EAS**
		Danio rerio	Water	2 hpf – 240 dpf	**SC**, 1 and 100 µg/L	0.002% DMSO	Stat. sign. ↗ in fecundity at 1 and 100 µg/L but number of eggs/female is unclear (0.01 <p<0.05)	Qin *et al.*, 2021	2		**EAS**
		Caenorhabditis elegans (nematode)	Concentration dissolved in solvent and put on plates	First larval L1 stage - 4d (24h post L4-stage)	**SC**, 125, 250 and 500 µM (internal dose resp. <0.1 µg/g, 0.21 µg/g and 0.39 µg/g	0.1% ethanol	Slight non-sign. ↗ in egg number, stat. sign. ↙ in brood size at 125* and 500 µM	Chen *et al.,* 2016	/	Complement-ary information	
	Survival	*Danio rerio*	Water	3-4 months: 21 d	**0**, SC, 0.5, 5 and 50 µg/L Results compared to blank	0.1% Methanol (v/v) No sign. difference between water and solvent control	No mortality	Ji *et al.*, 2013	1		
		Danio rerio	Water	2-75 dpf	0, **SC**, 0.1, 1, 10 and 100 µg/L	0.01 % acetone (v/v)	Sign. mortality at 100* µg/L in all zebrafish (+/-70% survival compared to +/-	Naderi *et al.*, 2014	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
					Results compared to SC	Authors confirmed that results of water and solvent control were the same	95% in control) No distinction made between males and females				
		Danio rerio	Water	4 hpf - 120 hpf	0, **SC**, 100 µg/L Results compared to SC	0.005% DMSO No sign. difference between blank and solvent control	No sign. effect on survival of embryos after 120hpf exposure to 100 µg/L BPS	Qiu *et al.,* 2016	1		
		Danio rerio	water	2 hpf - 168 hpf	SC, 1, 3, 10, and 30 µg/L Results compared to SC	0.01% DMSO (v/v)	No sign.mortality (86.4; 88.1; 85.7; 83.6 and 85.5%)	Zhang *et al.*, 2017	1		
		Danio rerio	Water	1 hpf - 96 hpf	**SC**, 10, 20, 50, 100, 200 and 300 mg/L	0.01% ethanol	96 hpf LC50 = 199 mg/L	Moreman *et al.*, 2017	1		
		Danio rerio	Water	6 months old: 7 d	0, **SC**, 0.1, 1 and 10 µM Results compared to SC	0.01% DMSO (v/v)	High mortality at 10 µM (2500 µg/L)	Le Fol *et al.*, 2017	1		
		Danio rerio	Water	1-96 hpf	0, **SC**, 3, 6, 12.5, 25 and 50 mg/L Results compared to SC	0.01% acetone, except for 12.5 mg/L (0.125 mg/L acetone) and 25 mg/L (0.25 mg/L	No sign. mortality (no 96h LC50 could be determined)	Mu *et al.*, 2018	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
						acetone) No sign. difference between blank and solvent control					
		Danio rerio	Water	4 - 120 hpf	0, SC, 0.1, 1, 10, 100 and 1000 µg/L Results compared to SC	0.005% DMSO No sign. difference between blank and solvent control	No effect on survival at 120 hpf	Qiu *et al.*, 2018b	2		
		Danio rerio	Water	4 - 120 hpf	**SC**, 0.4, 2, 10 and 50 mg/L	< 0.1% DMSO (v/v)	No sign. mortality (87.5 ± 16.0, 83.3 ± 0.0, 79.2 ± 16.0, 95.8 ±83, 87.5 ± 16.0 %)	Lee *et al.*, 2019	1		
		Danio rerio	Water	2 hpf – 240 dpf	**SC**, 1 and 100 µg/L	0.002% DMSO	Survival ↙ (87, 75 and 82%) after 24hpf	Qin *et al.*, 2021	2		
		Danio rerio	Water	<4 hpf - 148 dpf	0, 0, 0.0032, 0.010, 0.032, 0.1, 0.320, 1.0, 3.2 and 10.0 mg/L	/	No sign. effect on survival: At hatch: 98, 98, 98, 98, 100, 98, 98, 93 and 98% At day 33/34: 98, 98, 98, 98, 100, 98, 93, 93, 98% At day 65: 95, 98, 95, 98, 100, 98, 95, 93, 95% At day 105/106: 95, 97, 95, 98, 100, 98, 95, 90 and 94%	Unpublished study report, 2020 (Range finding study: OECD TG 210 + OECD TG 234 + OECD TG 229)	1		
		Danio rerio	Water	F0 (+/- 30 wks old): 49-50 d, F1: 125-128 d F2: 96 h	0, 0, 2, 10, 50, 250 and 1250 µg/l	/	No effects on survival in F0 (day 49-50: 100, 100, 100, 100 100 and 100%), F1 (day 35-125/128: 99, 99, 99, 100 100 and 98%) F2: stat sign. ↘ at 10, 250	Unpublished study report, 2020 (ZEOGRT (OECD TG 240	1		

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							and 1250 µg/L (100, 95, 94*, 95, 95* and 94*%)	adapted for zebrafish))			
		Danio rerio	Water	Adult zebrafish (9 months old): 120d exposure	**SC**, 1,10 and 30 µg/L 1 µg/L E2	0.01% DMSO (v/v)	No mortality	Naderi *et al.*, 2020	1		
		Danio rerio	Water	Adult zebrafish (9 months old) – 75 d exposure	1, 10 and 30 µg/L 1 µg/L E2	0.01% DMSO (v/v)	No mortality	Salahinejad *et al.*, 2020	1		
		Danio rerio	Water	Adults (3-4 months): 21 d	0, SC, 8, 40 and 200 µg/mL	0.1% DMSO (no sign. difference with water control in reproductive parameters)	No mortality	Park *et al.*, 2022	1		
		Danio rerio	Water	Adult zebrafish: maternal exposure (9 months old): 60 d Offspring: 6 months old	1, 10 and 30 µg/L 1 µg/L E2	0.01% DMSO (v/v)	No mortality	Salahinejad *et al.*, 2022	1		
		Chironomus riparius	Water	Early phase of fourth instar larvae: 24 h, 24h BPS+24h	0.5, 5, 50 and 500 µg/L	0.05% DMSO	No significant effect on survival	Herrero *et al.*, 2018	/	Complementary information	

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
				in fresh culture medium without BPS							
		Chicken	Injection	4 d old (E4) – E19	210 nmol/g egg		57% mortality in ovo compared to control (24%)	Mentor *et al.*, 2020	/		
		Caenorhabditis elegans (nematode)	Concentration dissolved in solvent and put on plates	First larval L1 stage - 4d (24h post L4-stage)	125, 250 and 500 µM (internal dose resp. <0.1 µg/g, 0.21 µg/g and 0.39 µg/g	0.1% ethanol	Stat. sign. mortality at all doses, 5-fold increase at 500 µM (125*, 250** and 500** µM)	Chen *et al.*, 2016	/		
	Time to maturity (time to first spawn)	/									
	Hatching success	*Danio rerio*	Water	3-4 months: 21d	**0**, SC, 0.5, 5 and 50 µg/L Results compared to blank	0.1% Methanol (v/v) No sign. difference between water and solvent control	Dose-dependent ↘ hatching rate: After 16d exposure: stat. sign. at ≥ 5* µg/L (5*, 50 µg/L) After 21d exposure: Stat. sign. at ≥ 0.5* µg/l (0.5*, 5*, 50* µg/L)	Ji *et al.*, 2013	1	Overall positive evidence of reduced hatching success	**EATS**
		Danio rerio	Water	2 - 75 dpf	0, **SC,** 0.1, 1, 10 and 100 µg/L Results compared to SC	0.01 % acetone (v/v) Authors confirmed that results of water and solvent control were the same	Dose-dependent ↘ hatching rate, stat. sign. ≥ 10* µg/L	Naderi *et al.*, 2014	1		**EATS**
		Danio rerio	Water	2 hpf – 168 hpf	SC, 1, 3, 10, and 30 µg/L	0.01% DMSO (v/v)	Stat. sign. ↘ in hatching rate at 72 hpf, while restored at 96 hpf	Zhang *et al.*, 2017	1		**EATS**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
							At 72hpf: 81.2 ± 2.65, 79.5 ± 4.04, 73.3 ± 3.76, 74.1 ± 3.2 and 62.3 ± 5.55* % At 96hpf: 94.5 ± 1.2, 93.3 ± 1.0, 90.8 ± 1.4, 91.2 ± 1.7 and 90.8 ± 1.9 %				
		Danio rerio	Water	1 hpf - 96 hpf	100, 200 and 300 mg/L	0.01% ethanol	72 hpf hatching rate: EC50=155 mg/L ↙, not sign. but dose-dependent	Moreman *et al.*, 2017	1		**EATS**
		Danio rerio	Water	4 hpf – 54 hpf	0.1, 1, 10, 100 and 1000 µg/L Results compared to SC	0.005% DMSO	Stat. sign. ↗ hatching rate at 48 and 54h ≥1 µg/L (1*, 10* and 100* µg/l) but no effect at 1000 µg/L	Qiu *et al.*, 2018b	1		**EATS**
		Danio rerio	Water	1 - 96 hpf	0, **SC**, 2.5, 12.5 and 25 mg/L Results compared to SC	0.01% acetone, except for 12.5 mg/L (0.125 mg/L acetone) and 25 mg/L (0.25 mg/L acetone) No sign. difference between blank and SC	No effect on hatching (no or very slight difference with control after 48 hpf, 72 hpf and 96 hpf)	Mu *et al.*, 2018	1		**EATS**
		Danio rerio In compliance with OECD TG 210	Water	2 – 120 hpf	1, 10 and 100 µg/L	0.002% DMSO (v/v)	Sign. ↘ hatching rate in F1 at all concentrations 48 hpf: 1*, 10* and 100* µg/L 60 hpf: 1*, 10* and 100* µg/L	Wei *et al.*, 2018	1		**EATS**

Group-ing	Line of evidence	Species / cell lines	Route of exposure	Exposure duration	Concentrations tested	Solvent	Observed effects (positive/negative/trend)	Reference	Study reliability (ToxR Tool)	Assess-ment of each line of evidence	Mod-ality
		and 230					72 hpf: 1*, 10* and 100* µg/L				
		Danio rerio	Water	4 - 120 hpf	0.4, 2, 10 and 50 mg/L	< 0.1% DMSO (v/v)	No sign. effect on hatchability (87.5 ± 16.0, 83.3 ± 0.0, 79.2 ± 16.0, 95.8 ± 83 and 87.5 ± 16.0 %)	Lee *et al.*, 2019	1		
		Danio rerio	Water	F0 (+/- 30 wks old): 49-50d, F1: 125-128d F2: 96h	0, 2, 10, 50, 250 and 1250 µg/l	/	F1: No effect F2: 95.00 +/--5.000%, **93.750 +/--6.250%***, 95.000 +/- -5.000%, 95.000 +/- -5.000% and **93.750 +/- -6.250%*** vs control (100.000+/- 0.000%)	Unpublished study report, 2020 (ZEOGRT (OECD TG 240 adapted for zebrafish))	1		**EATS**
		Danio rerio	Water	2 hpf – 240 dpf	**SC**, 1 and 100 µg/L	0.002% DMSO	Sign. ↘ at 1 µg/L at 60 and 72 hpf and ↗ in 100 µg/L at 48 hpf	Qin *et al.*, 2021	2		**EATS**
		Danio rerio	Water	Embryos (2hpf): until 120 hpf (5dpf)	**0**, SC, 1 and 100 µg/L Results compared to blank	0.005% DMSO (no sign. difference with water control)	Dose dependent ↗, stat. sign. at 100A* µg/L at 48hpf	Qiu *et al.*, 2021	1		**EATS**
		Chicken	Injection in air cell of fertilised eggs	Fertilised eggs: 20 - 22 d	**SC**, 0.27, 0.91, 10.6, 52.8 and 207 µg/g egg	~ 1 µg/L DMSO	Sign. reduction in pipping success at 207 mg/L (58%) Resp. 93, 100, 100, 93, 89, 58%	Crump *et al.*, 2016	/	Complement-ary information	
	Embryo time to hatch	*Danio rerio*	Water	3-4 months: 21 d	**0**, SC 0.5, 5 and 50 µg/L Results compared to blank	0.1% Methanol (v/v) No sign. difference between water and	Dose-dependent ↗ time to hatch: stat. sign. at 50* µg/L after 16d of exposure and ≥ 0.5* µg/L after 21d of exposure (0.5*, 5*, 50* µg/L)	Ji *et al.*, 2013	1	Overall positive evidence of increased time to hatch	**EATS**

Group-ing	***Line of evidence***	***Species / cell lines***	***Route of exposure***	***Exposure duration***	***Concentrations tested***	***Solvent***	***Observed effects (positive/negative/trend)***	***Reference***	***Study reliability (ToxR Tool)***	***Assess-ment of each line of evidence***	***Mod-ality***
						solvent control					
		Danio rerio	Water	2-75 dpf	0, **SC**, 0.1, 1, 10 and 100 µg/L Results compared to SC	0.01 % acetone (v/v) Authors confirmed that results of water and solvent control were the same	Stat. sign. ↗ at ≥ 10* µg/L, dose-dependent	Naderi *et al.*, 2014	1		**EATS**
		Danio rerio	Water	1 hpf - 96 hpf	**SC**, 10, 20, 50, 100, 200 and 300 mg/L	0.01% ethanol	↗ hatching time at 72 hpf	Moreman *et al.*, 2017	1		**EATS**
		Danio rerio	Water	4 - 120 hpf	**SC**, 0.4, 2, 10 and 50 mg/L	< 0.1% DMSO (v/v)	↗ hatching (2.09 ± 0.11, 2.90 ± 0.12*, 2.51 ± 0.37, 2.38 ± 0.34, 2.51 ± 0.26 days)	Lee *et al.*, 2019	1		**EATS**
	Histopathology (liver, kidney)	/									

Annex II - ELoC summary reporting

Table Y1: Summary table regarding ELOC

	Alteration of reproductive function	Disruption of mammary gland development	Overall conclusion
Possible serious health effects?*	Harmonised classification as Repr. 1B for fertility illustrates possible serious dysfunction.	Associated with effects on mammary Gland (males) leading to breast cancer.	YES Pattern of ED-related Effects associated with serious dysfunction Or pathologies Clearly related to serious Illness
Delay of health effects?	Developmental exposure can induce/result in effects on the reproductive function and this even without systematic direct exposure (latency effect).	Developmen tal and/or post-natal exposures can induce/resultin effects on the mammary gland and this even without systematic direct exposure (latency effect).	YES Developmental exposure has been shown to have consequences in relation to ED-related effects later in life. Future generations not protected.
Irreversibility of health effects?	Developmental exposure can lead to latency effects suggesting that such effects are irreversible.	Developmental and/or post-natal exposures can lead to latency effects suggesting that such effects are irreversible.	YES All ED related effects may be induced after developmental exposure without direct exposure.
Quality of life impaired?	Impacting mental/psychological wellbeing, as well as normal physiology.	Impaired quality of life associated with breast cancer. Impacting mental/psychological wellbeing, as well as normal physiology.	YES Impaired quality of life associated with several ED-related effects of BPS

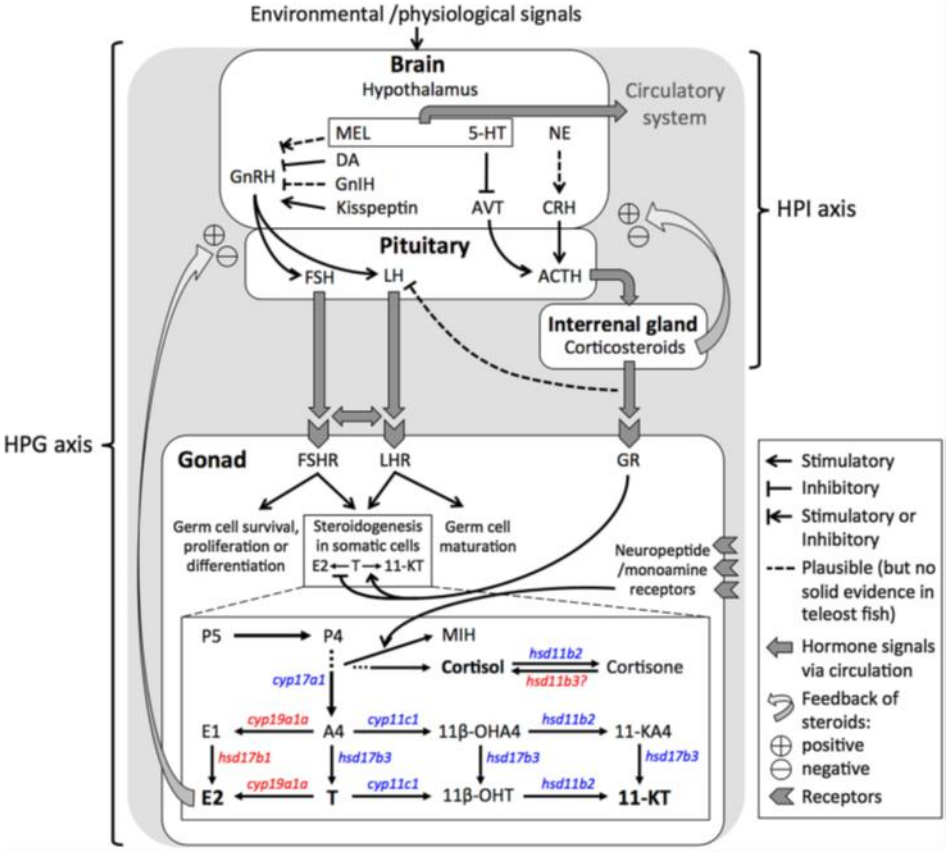
Societal concern?	Concern on fertility can lead worldwide to serious costs in terms of healthcare.	Concern on breast cancer can lead worldwide to serious costs in terms of healthcare.	YES Multiple ED related effects associated with major societal/ethical health concerns and socioeconomic burden for the society as a whole
Is derivation of a 'safe concentration' possible?	Impossible to predict potential future effects and safe exposure levels for the human health, due to the complexity of the endocrine system. Adverse effects caused by a chemical after the exposure during the sensitive time windows in the course of development vary dependently on the organism group and also on the individual from the same group. Therefore, effects may be overlooked, not expressed or equivocal.	Impossible to predict potential future effects and safe exposure levels for the human health, due to the complexity of the endocrine system. Adverse effects caused by a chemical after the exposure during the sensitive time windows in the course of development vary dependently on the organism group and also on the individual from the same group. Therefore, effects may be overlooked, not expressed or equivocal.	MOST PROBABLY NO Derivation of safe concentration associated with large uncertainties

**This factor is intended to discuss the severity of the effects and not their probability*

Annex III - Background on HPG axis and relation to sex change in fish

Due to its conserved role in mediating steroidogenesis and reproduction in vertebrates, the hypothalamic-pituitary-gonadal (HPG) axis is suggested to be the major signalling pathway regulating gonadal sex change (Liu *et al*., 2016). As shown in figure 6, the key elements in the HPG-axis of fish are the gonadotropin-releasing hormone (GnRH), the pituitary gonadotropins (follicle-stimulating hormone [FSH] and luteinising hormone [LH]) and steroid hormones.

Figure 6: Schematic of the HPG and HPI axes and neuroendocrine regulation of steroidogenesis in fish



Solid lines indicate interactions with support from fish models; dashed lines indicate interactions with support from non-teleost systems, but with no solid evidence in fish; question marks denote uncertain reactions. In the steroidogenic pathways, genes colored in red and blue showed female- and male-biased expression in the gonad of bluehead wrasses, respectively (Liu *et al*., 2015). 5-HT, serotonin; 11b-OHA4, 11b-hydroxyandrostenedione; 11b-OHT, 11b-hydroxytestosterone; 11-KA4, 11-ketoandrostenedione; 11-KT, 11-ketotestosterone; ACTH, adrenocorticotropic hormone; A4, androstenedione; AVT, arginine vasotocin; CRH, corticotropin-releasing hormone; DA, dopamine; E1, estrone; E2, 17b-estradiol; FSH, follicle-stimulating hormone; FSHR, folliclestimulating hormone receptor; GR, glucocorticoid receptor; GnIH, gonadotropin inhibitory hormone; GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; LHR, luteinizing hormone receptor; MEL, melatonin; MIH, maturation-inducing hormone; NE, norepinephrine; P4, progesterone; P5, pregnenolone; T, testosterone. (From Liu *et al.*, 2016).

- Brain

Kisspeptins are proteins encoded by the Kiss1 gene in the hypothalamus of teleost fish. Kiss1 neurons are major integrators of environmental, metabolic, and gonadal signals. As such, they play key roles in the central triggering of puberty, acting upstream from GnRH

(Servili *et al.*, 2011). GnRH, a decapeptide secreted from the hypothalamus has two GnRH forms (GnRH3 and GnRH2) in zebrafish. In adult zebrafish, GnRH3 is localised to the midbrain tegmentum while GnRH3 is localised in the olfactory bulbs terminal nerves as well as in the preoptic area of the hypothalamus. Furthermore, it was shown that levels of GnRH3 peptide in the adult zebrafish pituitary were 3 to 4-fold higher than those of GnRH2 (Rajendiran *et al.*, 2021). GnRH3 has taken over the role of GnRH1 present in fish having three forms of GnRH. Therefore, GnRH3 is considered to be the hypophysiotropic form. GnRH transcripts and GnRH receptors in fish are initiated prior to the setup of the HPG axis and starts at early stages of development. GnRH, released to the pituitary, stimulates gonadotropin hormone synthesis (including FSH and LSH) and secretion. FSH and LH released into the circulatory system then bind to their receptors in the gonads.

- Gonads

FSH and LSH mediate steroidogenesis in the gonads by stimulating the synthesis of androgen and oestrogen and are responsible for gonadal differentiation and function by regulating spermiation and ovulation (Rajendiran *et al.*, 2021). Both gonadotropins can have a positive or negative feedback on the HPG axis. On their turn, the released gonadal steroids can exert a feedback to the hypothalamus and control release of GnRH and gonadotropins (Liu *et al.*, 2016).

- Aromatase (CYP19) in gonad and brain

The CYP19 protein also called aromatase, catalysing the conversion of androgens (androstenedione and testosterone) into estrogens (estrone and 17β-estradiol), is encoded by CYP19 gene. Expression of these genes depends on the need of estrogen by the brain, retina, pituitary and ovary (Rajendiran *et al.*, 2021). In teleost fish it is expressed in the gonads (see Figure 6) and the brain (see Figure 7) resp. by CYP19a and CYP19b. CYP19a is also known as CYP19a1, CYP19a1a and ovarian aromatase; CYP19b as Cyp19a2, Cyp19a1b and brain aromatase.

The brain of teleost fish is characterised by an extremely high capacity to aromatise androgens into estrogens (Menuet *et al.*, 2005). This **brain aromatase** is strongly expressed in radial glial cells which are pluripotent cells and considered the unique cell type expressing aromatase in zebrafish brain. In contrast to rodents, brain aromatase activity and expression in zebrafish is low during embryonic development and starts to increase during sexual maturation, corresponding to a high circulation of steroid hormones like estradiol and testosterone. Furthermore, expression of aromatase in the brain of fish is also strongly stimulated by estrogens and some androgens (Coumailleau *et al.*, 2015). *Estrogen-dependent regulation of brain aromatase gene expression requires functional ERs and the binding of liganded-ER on ERE and half ERE located in the promoter region of the cyp19a1b gene* (Le Fol *et al.*, 2017). During early development, three estrogen receptors (ERα: esr1, ERβ1: esr2b and ERβ2: esr2a) are expressed and clearly increase between 24hp and 48hpf together with an increase of cyp19a1b mRNA. It is known that embryonic exposure to an estrogen compound can lead to a dramatic increase in aromatase expression already at low dose as cyp19a1b-gene is very sensitive to estrogens and as a consequence impact brain development and functioning (f.i. EE2 disrupts the GnRH neuronal network, by increasing the number of GnrH-immunoreactive neurons and fibers in the forebrain and by modifying the migration pattern of those nurons (Coumailleau *et al.*, 2015).

It is known that brain of fish keeps growing in adults in parallel with high aromatase expression. Furthermore, it is suggested that estrogens inhibit cell proliferation and cell migration in adults.

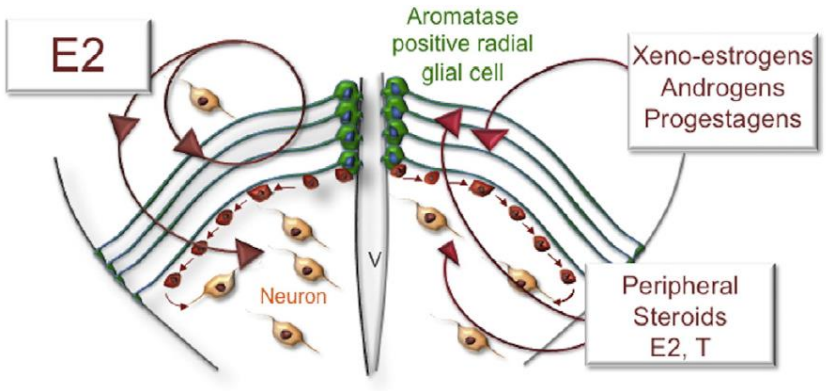


Figure 7: Schematic representation of aromatase positive-cells Radial glial cells (green cells) in the brain of zebrafish (from Coumailleau *et al.*, 2015)
Radial glial cells can proliferate and generate neuroblasts (red cells) that migrate along the radial processes and give birth to neurons (orange cells). RGCs can produce estrogens that can either act on neighboring neurons or on RGC themselves to modulate their neurogenic activity. RGCs are also target for peripheral steroids and xeno-hormones.

The CYP19a (**gonadal aromatase**) is primarily involved in the estrogen synthesis in the granulosa follicle cells in the ovary but also in the theca interstitial cells of previtellogenic ovaries and interstitial cells of the testis. Gonadal transition in sex-changing fish is accompanied by changes in plasma concentrations of gonadal steroids. 17b-estradiol (E2) and 11-ketotestosterone (11-KT) are resp. the major estrogen and androgen hormones controlling the gonad differentiation and maintaining the sexual phenotype in teleost fish (Liu *et al.*, 2016). A juvenile ovary disappears when there is low expression of CYP19a while feminisation is seen when zebrafish are exposed during development to xenoestrogens or higher concentration of estrogens (Rajendiran *et al.*, 2021). Male sex in teleost fish is maintained due to the inhibition of CYP19a gene, while female sex is maintained due to oestrogen production.