Annex XV report

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

Substance Name: isobutyl 4-hydroxybenzoate (isobutylparaben)

EC Number: 224-208-8

CAS Number: 4247-02-3

Submitted by: The Danish Environmental Protection Agency (DK-EPA)

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ABBREVIATIONS

A	Androgen
ADME	Absorption, distribution, metabolism and excretion
AGD(i)	Anogenital distance (index)
AO	Adverse outcome
AR	Androgen receptor
AUC	Area under curve
BP	Butylparaben
BW	Body weight
CaBP-9k	Vitamin-D-dependent calcium-binding protein
CAR	Constitutive androstane receptor
CHO	Chinese Hamster Ovary
CLP	Classification, Labelling and Packaging
C_{max}	Maximum concentration
COC	Cumulus oocyte complex
CYP	Cytochrome P450
DES	Diethylstilbestrol
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
E	Estrogen
E2	17-beta-estradiol
EAS	Estrogen/Androgen/ Steroidogenesis
EATS	Estrogen/Androgen/Thyroid /Steroidogenesis
EC	Effective concentration
EC_{50}	50 % effective concentration
ECHA	European Chemicals Agency
ED	Endocrine disruptor
ED_{50}	50 % effective dose
EE	Ethinyl estradiol
EFSA	European Food Safety Authority
ELISA	Enzyme linked
EP	Ethylparaben
ER	Estrogen receptor
ERC	Environmental release category
ERE	Estrogen response element
ESR1	Estrogen receptor 1
FSH(r)	Follicle stimulating hormone (receptor)
GD	Gestational day
GH3 cells	Rat pituitary tumor cell line
GREB1	Growth regulating estrogen receptor binding 1
hAR	Human androgen receptor
hER	Human estrogen receptor
HPLC-MS	High-performance liquid chromatography mass spectrometry
IBP	Isobutylparaben
IC50	50 % inhibitory concentration
ICI 182,780	High affinity estrogen receptor antagonist
INSL3	Insulin like peptide 3
IPCS	International Programme on Chemical Safety
IPP	Isopropylparaben
KE	Key event
KER	Key event relationship
LH (r)	Luteinising hormone (receptor)
LNCaP	Lymph Node Carcinoma of the Prostate cell line
LO(A)EL	Lowest observed (adverse) effect level
LoD	Limit of detection
LoE	Line of Evidence
LOEC	Lowest Observed Effect concentration
LOEL	Lowest Observed Effect level
µM	Micromolar
MCF-7	Breast cancer cell line. Michigan Cancer Foundation-7
MDA cells	MDA MB 231, breast cancer cell line derived at M.D. Anderson
MP	Methylparaben
MIE	Molecular initiating event
mg/kg	Milligram per kilograms
MoA	Mode of Action
nM	Nanomolar
NOEC	No-observed effect concentration
NO(A)EL	No observed (adverse) effect level
NTP	National toxicology program
OECD	Organisation for Economic Co-operation and Development
Pa	Pascal
PC	Product category
PC50	50 % positive control response
PD	Pup day
PHBA	Parahydroxybenzoic acid
pKa	Dissociation constant
PND	Postnatal day

PP	Propylparaben
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone Receptor
PROC	Process category
PXR	Pregnane X receptor
QSAR	Quantitative structure-activity relationship
RAAF	Read Across Assessment Framework
RACB	Reproductive Assessment by Continuous Breeding
REC/RIC20	Response equal to 20 % agonistic/inhibitory activity
ROS	Reactive oxygen species
s.c.	Subcutaneous
SCCS	Scientific Committee on Consumer Safety
SHBG	Sex hormone binding globulin
STAR	Steroidogenic acute regulatory protein
STOT SE	Specific target organ toxicity – single exposure
SU	Sector of end use
SVHC	Substance of very high concern
T3	Triiodothyronine
T4	Thyroxine
T47D cells	Human breast cancer cell line
T	Thyroid
TEB	Terminal end bud in mammary glands
TG	Test guideline
TR	Thyroid Receptor
TRHR	Thyrotropin-releasing hormone receptor
TSH	Thyroid stimulating hormone
TSHR	Thyroid stimulating hormone receptor
VO	Vaginal opening
WHO	World Health Organisation
WoE	Weight of evidence
ZR-75-1 cells	Human breast cancer cell line
4tOP	4-tert-octylphenol

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

Substance name: isobutyl 4-hydroxybenzoate (isobutylparaben, IBP)

EC number: 224-208-8

CAS number: 4247-02-3

- It is proposed to identify the substance 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

Isobutyl 4-hydroxybenzoate is proposed to be identified as a substance of very high concern (SVHC) 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 human health which gives 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.

Endocrine disrupting (ED) properties of IBP relevant for human health:

Estrogenic activity
There is strong evidence that IBP affects estrogen receptor (ER) binding and transactivation and estrogen dependent signalling in target cells *in vitro*. *In vivo*, there is moderate-strong evidence of estrogenic activity as evidenced in uterotrophic assays, showing increased uterine weight and altered expression of estrogen-regulated genes and proteins.

Adverse effects
There is low-moderate evidence of adverse effects on ovary and uterus histopathology after pubertal IBP exposure, due to lack of studies and limited study reliability. There are no reliable studies for IBP investigating adverse effects on sperm quality in perinatally exposed rats.

Therefore, a read across approach is proposed from the source substance butylparaben (BP) to the target substance IBP. BP has already been identified as a SVHC because of its endocrine disrupting properties to human health. The read-across is supported by the structural similarity of the substances and by similar estrogenic activity and potency observed *in vitro* and *in vivo*.

A number of rodent studies using oral gavage or subcutaneous exposure show moderate-strong evidence for adverse effects of BP on sperm count and quality, after perinatal exposure. No effect on endocrine related endpoints (sperm parameters and anogenital distance) are seen in a recent developmental dietary exposure study, using continuous

breeding protocol. However, the adverse findings observed in other studies should not be neglected. These inconsistencies can be considered to reflect differences in bioavailability using different study designs such as exposure routes and periods. Hence, after consideration of all available *in vivo* results for BP, there is still moderate-strong evidence that, under specific conditions, exposure to BP, and consequently to IBP, can cause adverse effects on sperm count and quality.

Plausible link between adverse effects and endocrine activity
The mode of action (MoA) analysis leads to the conclusion that IBP acts via an estrogenic MoA. Since limited information was available for IBP on adverse effects, information on BP was included in the MoA analysis (perinatal exposure). The molecular initiating event is activation of the ER(s). In developing males, increased ER signaling results in altered testicular development and subsequently altered testicular function in adulthood. In turn, reduced sperm count and quality are observed. The analysis led to the conclusion that it is biologically plausible that ER activation during development leads to the observed adverse effects on the male reproductive system following perinatal exposure to IBP.

Summary of the ED assessment
There is scientific evidence to conclude that IBP is an endocrine disruptor via the E (estrogen) modality, according to a MoA analysis including an evaluation of biological plausibility.

Equivalent level of concern
The adverse effects on BP are reduced sperm count and quality as observed in rodent studies using perinatal exposure. Effects are irreversible and are shown to occur later in life after exposure in the perinatal period only. These effects are considered severe as similar effects in humans could cause sub- and infertility. Sub- and infertility is not only detrimental to the propagation of the species, but also has a major impact on quality of life. Fertility treatment and counselling carries high societal costs.

No safe concentration/level can be derived from the available data on adverse reproductive effects via an endocrine MoA. Two of the available studies show reduced sperm count or quality in perinatally exposed rats at the lowest tested dose and therefore no no-observed-effect-level can be determined for this endpoint. The difficulty to establish a safe level with sufficient certainty raises concern particularly on the capacity to manage safe use of the substances for sensitive populations. Moreover, mixture effects, where substances act additively or with synergistic effects, cannot be excluded and this might impact the threshold of toxicity.

Altogether, IBP exposure gives rise to an equivalent level of concern to substances listed in Article 57 points (a) to (e) due to its endocrine disrupting properties for human health. Notably, the conclusion is reached using a read-across approach with BP as a source substance – a substance already identified as a SVHC because of its endocrine disrupting properties to human health.

Conclusion
Overall, it is concluded that the substance isobutyl 4-hydroxybenzoate (referred to as isobutylparaben, IBP) meets the criteria of 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 human health which gives rise to an equivalent level of concern to those substances listed in points (a) to (e) of Article 57 of the REACH Regulation.

Registration dossiers submitted for the substance: Yes

PART I

Justification

1. Identity of the substance and physical and chemical properties

Name and other identifiers of the substance

Table 1: Substance identity of isobutyl 4-hydroxybenzoate (referred to as IBP)

EC number:	224-208-8
EC name:	isobutyl 4-hydroxybenzoate
CAS number (in the EC inventory):	4247-02-3
IUPAC name:	2-methylpropyl 4-hydroxybenzoate isobutyl 4-hydroxybenzoate
Index number in Annex VI of the CLP Regulation	-
Molecular formula:	C11H1403
Molecular weight range:	194.23
Synonyms:	isobutylparaben

Structural formula:

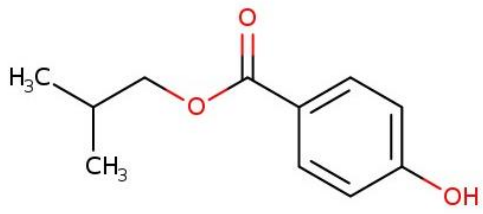

Figure 1: Structure of IBP

1.2 Composition of the substance

Name: isobutyl 4-hydroxybenzoate

Description: Organic, white solid

Substance type: Mono-constituent

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

See section 4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)for knowledge on ADME (absorption, distribution, metabolism and elimination) of parabens in general. Metabolites relevant for the SVHC assessment are also described.

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

Table 2: Substance identity of butyl-4-hydroxybenzate (referred to as BP)[1]

EC number:	202-318-7
EC name:	butyl 4-hydroxybenzoate
SMILES:	CCCCOC(=O)C1=CC=C(C=C1)O
CAS number (in the EC inventory):	94-26-8
CAS number:	94-26-8
IUPAC name:	butyl 4-hydroxybenzoate butyl *p*-hydroxybenzoate
Index number in Annex VI of the CLP Regulation	Not applicable
Molecular formula:	C11H14O3
Molecular weight range:	194.23
Synonyms:	butylparaben

Substance type: Mono-constituent

[1] Registration dossier of butyl-4-hydroxybenzoate https://echa.europa.eu/da/registration-dossier/-/registered-dossier/25335 (March 2022)

Structurally related substance(s) formula

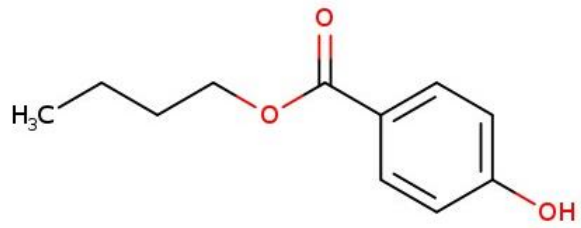

Figure 2: Structure of BP

The substances BP and IBP share close structural similarity, the only difference being that IBP has an isopropyl group at the end of the alkyl chain while BP has a butyl group. BP has already been identified as a substance of very high concern (SVHC) because of its endocrine disrupting properties to human health and a read across is proposed based on structural similarity and similar endocrine activity. Further information about structurally related substances can be found in 4.10.B. Read-across justification summary and Annex I - Additional information on read across approach.

1.5 Physicochemical properties

Table 3: Overview of physicochemical properties of IBP (based on the registration information)[2]

Property	Description of key information	Value [Unit]	Reference/source of information
Physical state at 20°C and 101.3 kPa	White solid		ECHA dissemination site
Melting/freezing point		70-74 °C.	ECHA dissemination site
Boiling point		323-332 °C	ECHA dissemination site
Vapour pressure		0.0005 Pascal (Pa) at 20 °C (OECD 104) 0.001 Pa at 20 °C (chemical safety assessment)	ECHA dissemination site
Density		1.24 g/cm³ at 20 °C	ECHA dissemination site
Water solubility		212 mg/L at 20 °C	ECHA dissemination site
Partition coefficient n-octanol/water (log value)		log Kow 3.43 at 23 °C	ECHA dissemination site

[2] Registration dossier of isobutyl-4-hydroxybenzoate https://echa.europa.eu/da/registration-dossier/-/registered-dossier/17752 (March 2022)

2. Harmonised classification and labelling

IBP does not have any harmonised classifications according to the CLP Regulation. According to the classifications provided in the REACH registration dossier, IBP may cause an allergic skin reaction (Skin Sens. 1B, H317), causes skin irritation (Skin Irrit. 2, H315), serious eye damage (Eye Dam. 1, H318) and serious eye irritation (Eye Irrit. 2, H319). IBP is very toxic to aquatic life (Aquatic Acute 1, H400) and toxic to aquatic life with long lasting effects (Aquatic Chronic 2, H411). IBP may cause respiratory irritation (STOT SE 3, H335)[3].

3. Environmental fate properties

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

4. Human health hazard assessment

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

No information available on the ECHA dissemination site[2] for IBP.

This assessment does not include a full review of ADME.

The route of exposure accounts for differences in bioavailability of parabens. For instance, Aubert *et al.* (2012) showed that parabens were well absorbed after oral and subcutaneous (s.c.) exposure, and partially absorbed after dermal administration. Oral absorption has shown to be dependent upon length of the paraben ester chain, in a study comparing the pharmacokinetics of methylparaben (MP), propylparaben (PP) and BP in rats (Aubert *et al.* 2012).

Upon oral, s.c. or dermal uptake, parabens are commonly metabolised (hydrolysed) by esterases with formation of a common metabolite parahydroxybenzoic acid (PHBA). A large portion of PHBA is excreted as p-hydroxyhippuric acid, the glycine conjugate of PHBA (Aubert 2009; Aubert *et al.;* 2012; Boberg *et al.* 2016). In humans, the presence of other metabolites in the urine has also been described (Moos *et al.* 2016).

Studies investigating the endocrine activity of the major metabolite PHBA show inconsistent results (Pugazhendhi *et al.* 2005; SCCS 2010; Watanabe *et al.* 2013; Ouedraogo *et al.* 2022). With regards to metabolism of parabens in developing animals, limited capacity to metabolise BP has been found in offspring relative to dams during lactational interval. This suggests higher internal exposure levels of the parent molecule during the developmental phase (Roberts *et al.* 2016).

Obringer *et al.* (2021) and Lester *et al.* (2021) describe how parabens are metabolised differently in the skin and liver depending on e.g., the alkyl chain length, isomeric form, alcohol group identity and plasma protein binding.

[3] C&L Inventory database: https://echa.europa.eu/da/information-on-chemicals/cl-inventory-database (June 2022)

4.2 Acute toxicity

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

4.3 Irritation

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

4.4 Corrosivity

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

4.5 Sensitisation

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

4.6 Repeated dose toxicity

No information on repeated dose toxicity of IBP is available on the ECHA dissemination site[2]. Carcinogenicity and reproductive toxicity studies may provide useful information on repeated dose toxicity, but no such studies were available either.

Two published studies investigating some aspects of repeated dose toxicity were identified: a pubertal study in female rats investigated effects on female reproductive endpoints after 19 days of oral exposure (Vo *et al*. 2010) and a dermal 28-day study investigated the effects on a wide range of endpoints in male and female rats (Kim *et al.* 2015).

Detailed study descriptions are provided in Annex II. Based on limited available data, it is not possible to determine whether IBP exposure causes repeated dose toxicity.

The identified developmental toxicity studies from the open literature are presented in section 4.9 Toxicity for reproduction and Annex III – Detailed study information on IBP and BP.

4.7 Mutagenicity

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

4.8 Carcinogenicity

No information was available on the ECHA dissemination site[2], which could inform on endocrine related carcinogenicity of IBP.

4.9 Toxicity for reproduction

No information was available on the ECHA dissemination site[2], which could inform on reproductive toxicity of IBP.

In the open literature, two developmental toxicity studies with IBP were identified. The results have been reported in the following four publications: Yang *et al.* 2016, Kawaguchi *et al.* 2009a, Kawaguchi *et al.* 2009b, Kawaguchi *et al.* 2010. Study descriptions for these studies are provided in Annex II, but due to many important shortcomings, these studies were deemed unreliable (Klimisch score 3). This score was given because the studies used low statistical power, improperly performed statistical analyses, poorly described and non-standardised experimental methods and poor control of experimental variables.

The only relevant studies for assessing the reproductive toxicity of IBP, were three uterotrophic studies (Darbre *et al.* 2002; Koda *et al.* 2005; Vo & Jeung 2009), a pubertal study in females (Vo *et al.* 2010) and a dermal 28-day study in male and female rats (Kim *et al*. 2015). Study summaries for these are provided in Annex II, and the studies are discussed in more detail in section 4.10 on endocrine disruption.

In summary, all three uterotrophic studies showed increased uterine weight after IBP exposure, indicating an estrogenic mechanism *in vivo*. The pubertal study in females had some shortcomings but did report adverse histopathological effects on uteri and ovaries, which indicates that female reproductive development may be adversely affected by IBP exposure. The dermal 28-day study found no effects on male or female reproductive organs or circulating steroid hormone concentrations.

Based on the very limited number of acceptable studies, it is not possible to assess whether exposure to IBP causes adverse effects on 'fertility and sexual function' or on 'development'.

4.10 Endocrine disruption (Human Health)

4.10.A. General approach

Overall strategy

This section presents an evaluation of the endocrine disrupting effects of IBP.

There are structural similarities between IBP and BP and the mechanistic data show similar endocrine activity between IBP and BP. Considering the very limited number of studies that have investigated endocrine-mediated adversity of IBP, a read-across from BP was applied when constructing the lines of evidence (LoE) for adverse effects for IBP. BP has already been identified as a SVHC because of its endocrine disrupting properties to human health. The mode of action (MoA) postulated for BP is estrogen receptor activation leading to decrease sperm count and quality after perinatal exposure (ECHA 2020).

Methodology

The evaluation of endocrine disrupting properties was carried out in accordance with the Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009 (ECHA/EFSA 2018), which is based on the WHO/IPCS definition of an endocrine disruptor (WHO/IPCS 2002).

The majority of the information has been reported in the following annexes:

- *Annex I – Additional information on read-across approach*
- *Annex II – Detailed study information (in vitro & in vivo* for IBP, *in vivo* for BP*)*
- *Annex III – LoE for endocrine activity and adverse effects*
- *Annex IV – MoA analysis*
- *Annex V – Human epidemiology studies*

A summary of the **read across** justification is presented below in section 4.10.B. while detailed tables are provided in Annex I.

An overview of the performed **literature search** is presented in section 4.10.C. This is followed by a brief summary of the studies on IBP and BP that are relevant for the ED assessment (section 4.10.D and 4.10.E). Detailed study summaries of all *in vitro* and *in vivo* studies of IBP and of relevant *in vivo* studies on BP are presented in Annex II.

The **LoE** for a) endocrine activity of IBP and b) endocrine mediated adverse effects of IBP and source substance BP (related to estrogenic, androgenic and steroidogenic (EAS) modalities) are summarised in section 4.10.1 and further presented in Annex III. The few data available for T modality are summarised in section 4.10.2.

A **MoA** analysis evaluating the biological plausibility of a causal relation between endocrine activity and adverse effect (for EAS modalities) is summarised in section 4.10.4 and further presented in Annex IV.

Annex V presents the human studies for IBP and BP.

Finally, section 4.10.5 presents an **overall conclusion** on endocrine disruption with regard to human health.

4.10.B. Read-across justification summary

A read-across approach can be applied using substances with physicochemical and/or toxicological properties that are likely to be similar to the substance in question or follow a regular pattern as a result of structural similarity.

All of these conditions are met in the proposed read-across from the source substance BP to the target substance IBP. The read-across is supported by the structural similarity of the substances BP and IBP (Annex I, Table 9), similarity in physico-chemical properties (Annex I, Table 10) and by the same estrogenic activity and potency observed *in vitro* and *in vivo* (Annex I, Table 11 and **Table 12**).

From these overview tables, showing the endocrine activity of a range of parabens *in vitro* and *in vivo*, the following pattern emerges:

- ***In vitro* estrogenicity:** Several types of *in vitro* assays investigating estrogenicity have been conducted for parabens of various chain length: ER binding, ER mediated proliferation, ER mediated gene expression as well as ER transactivational assays. All show a pattern of increasing potency of the paraben with growing alkyl R-group. The response of IBP is similar to, or higher than, that of BP in several of the studies conducted.
- ***In vitro* androgenicity/anti-androgenicity:** In androgen/anti-androgenic test assays, all of the parabens show minimal or no effects. However, when an effect (androgen receptor (AR) antagonism) is present, the potency seems to increase with chain length and be higher in isopropylparaben (IPP), propylparaben (PP), IBP and BP compared to methylparaben (MP) and ethylparaben (EP).
- **Other *in vitro* assays:** Results from *in vitro* assays testing other endocrine related endpoints (PXR, CAR, PPARα transactivation) show a higher potency of IBP and BP

than shorter chain parabens. Additionally, IBP seems to be more potent than BP in some assays.

- ***In vivo* estrogenicity:** Three uterotrophic assays with IBP revealed increased uterine weights. In general, the potency appeared to increase with growing alkyl R-group (i.e., lower lowest-observed-effect-levels (LOELs) with growing alkyl R-group).

In summary, the data overview tables in Annex I indicate that the chemical structure as well as *in vitro* and *in vivo* mechanistic effects of IBP are more closely related to PP and BP than the shorter chain parabens. Specifically, assays investigating estrogenic responses *in vitro* and *in vivo* point to a high potency of IBP and BP compared to shorter chain parabens. *In vitro* studies including both IBP and BP in many cases show a higher potency of IBP than BP. Using BP as a read-across substance can therefore be considered as realistic or it may even underestimate the toxicity of IBP.

Comparable conclusions related to the similar properties of different parabens have been reached by the European Union Scientific Committee on Consumer Safety (SCCS), who have on several occasions reviewed the (at the time available) data on estrogenic effects of parabens: *"In vitro studies show the potential of endocrine modifying effects of parabens, with estrogenic activity as a function of chain length"* (SCCS 2010). In addition, "*The in vivo estrogenic activities of parabens have been tested in uterotrophic assays employing female rodents, either immature or adult ovariectomised, after oral, subcutaneous or dermal administration. Butylparaben appeared to be more potent than Propyl-, Ethyl- and Methylparaben, and again the values remained several magnitudes of order below the potency of 17β-estradiol.*"

SCCS also considered that the uterotrophic study on BP, IBP and IPP showed similar potency of these compounds (SCCS 2010 with reference to Vo and Jeung 2009). Overall, SCCS 2010 concluded that the estrogenic activity of parabens appears to increase with increasing chain length, and this conclusion was carried forward to their later opinions (SCCS 2011) and (SCCS 2013). In addition, one uterotrophic study including both IBP and BP showed a similar effect size at the same dose level, indicating that there are no kinetic differences that would speak against using BP as a read-across substance.

A common precursor or breakdown products is an important aspect in the read-across assessment (ECHA 2016). Upon uptake the parabens are hydrolysed to the same common metabolite PHBA, by which a large portion is excreted as p-hydroxyhippuric acid, the glycine conjugate of PHBA (Aubert 2009, Aubert *et al.,* 2012; Boberg *et al.* 2016). Excretion of other metabolites in the urine has been found in a human study (Moos *et al.* 2016). Studies investigating the endocrine activity of this major metabolite PHBA show inconsistent results (Pugazhendhi *et al.* 2005; SCCS 2010; Watanabe *et al.* 2013; Ouedraogo *et al.* 2022).

Many factors appear to impact metabolism and bioavailability of parabens e.g., the route of exposure, alkyl chain length, isomeric form, alcohol group identity and plasma protein binding (Aubert 2009, Aubert *et al.,* 2012; Obringer *et al.* 2021; Lester *et al.* 2021), but overall data support the proposed read-across from the source substance BP to the target substance IBP.

4.10.C. Literature search

As presented in Sections 4.6, 4.8 and 4.9 on repeated dose toxicity, carcinogenicity and reproductive toxicity, no information on these endpoints is presented on **IBP** in the REACH registration dossier[2]. To secure inclusion of all relevant literature a single concept search strategy was used as suggested in ECHA/EFSA guidance document (ECHA/EFSA 2018). This type of search was conducted on October 6th 2020 in PubMed, PubChem and Web of

Science and search strings and number of hits are presented in the table below. Much of the literature review work was performed in 2021, but before finalisation of the SVHC support document, an additional literature search was performed in Pubmed on March 25th 2022, to ensure inclusion of any relevant new studies on IBP published prior to this final cut-off date. However, none of the additional 15 identified studies published between October 2020 and March 2022 were found relevant to include in this ED assessment of IBP on human health.

Table 4: Date, database, search string and number of articles.

Date of search	Database	Search string	Number of articles	Comment
6 October 2020	Pubmed	((((Isobutylparaben) OR (Isobutyl 4-hydroxybenzoate)) OR ("4247-02-3")) OR (isobutyl paraben)) OR ((isobutyl) AND (paraben))	88	
6 October 2020	PubChem	4247-02-3	44	No. of unique articles compared to pubmed: 18
6 October 2020	Web of Science	((((Isobutylparaben) OR (Isobutyl 4-hydroxybenzoate)) OR ("4247-02-3")) OR (isobutyl paraben)) OR ((isobutyl) AND (paraben))	77	No. of unique articles compared to pubmed: 16
25 March 2022	Pubmed	((((Isobutylparaben) OR (Isobutyl 4-hydroxybenzoate)) OR ("4247-02-3")) OR (isobutyl paraben)) OR ((isobutyl) AND (paraben)) limited to 2020-2022	20	Unique articles compared to Pubmed search performed on October 6th 2020: 15

After removal of duplicates, the articles were screened in three refinement steps based on title, abstract and full text according to the procedure described in ECHA/EFSA guidance document (ECHA/EFSA 2018). Articles were considered relevant when they contained primary data (i.e., not reviews) related to endocrine activity *via* EATS modalities (estrogenic, androgenic, thyroid, steroidogenesis), effects on outcomes related to reproduction or other modalities. Examples of articles not considered relevant included human exposure data without health parameters, methodological articles concerning detection and measurements of chemicals in samples (e.g., HPLC-MS methods), and articles not including studies on IBP.

- **1st screen based on title**: 56 articles considered relevant, 21 articles possibly relevant, and 54 articles considered irrelevant.
- **2nd screen based on abstract**: 34 articles were considered relevant. These were categorised into 12 *in vivo* studies, 17 *in vitro* studies and 5 epidemiological studies. 11 articles were considered possibly relevant and 20 articles considered irrelevant.
- **3rd screen based on full text**: 9 *in vivo*, 18 *in vitro* (one of them being from the 'possibly relevant list' and 2 being *in vivo* studies also), and one epidemiological study. 10 studies were considered irrelevant.

The 3rd screen resulted in the final dataset. Each *in vitro* and *in vivo* study is described in Annex II, while Annex V is used for reporting of the epidemiology study on IBP.

Additionally, a search in **Toxcast/CompTox Chemicals Dashboard**[4] was conducted on 10 June 2022 using the search term "isobutyl paraben". Results for assays dealing with the EATS modalities are presented in Annex III, following the *in vitro* results. *In silico* QSAR (Quantitative structure-activity relationship) results from the **Danish QSAR database**[5] are likewise presented in Annex III, following the *in vitro* results.

Epidemiological studies were evaluated according to EFSA recommendations (EFSA 2017), while assessment of study reliability for all other studies was conducted using Klimisch score (Klimisch *et al.* 1997). Four *in vivo* publications were assigned a Klimisch score 3 (unreliable). This score was given due to low statistical power, improperly performed statistical analyses, poorly described and non-standardised experimental methods and poor control of experimental variables. These studies are summarised in Annex II in the same manner as all other *in vivo* studies, but only results from studies with a klimisch score of 1 or 2 were included in the LoE.

The assessment of the toxicity information on the source substance **BP**, was primarily based on the previously performed literature review, as described in the SVHC support document (ECHA 2020), and in a publication by Boberg *et al.* (2020). However, since that literature review was performed in 2020, for the present report an updated literature search was performed in PubMed in October 2021, and again on March 25th 2022. The aim of this additional search was not to include all newly published literature on BP, but to investigate if any additional publications with specific relevance to the SVHC identification for endocrine disrupting properties had been published after the SVHC identification of BP. The Pubmed literature search was therefore limited to articles published from 2020- 2022, and the screening of titles and abstracts was based on very "strict" criteria, in that only *in vivo* studies in rodents, using BP as single test compound (not as part of a mixture) were included for further assessment. This selection process resulted in four additional *in vivo* publications (Oliveira *et al.* 2020, Hubbard *et al.* 2020; Bae *et al.* 2021, Ara *et al.* 2021). Results from these studies are summarised in section 4.10.E. For two of the studies that used perinatal exposure (Oliveira *et al.* 2020, Hubbard *et al.* 2020), results have also been added to the LoE for BP that were originally prepared for the SVHC support document (ECHA, 2020) and assessed for study reliability using the Klimisch score (Klimisch *et al.* 1997). In the other two studies (Bae *et al.* 2021, Ara *et al.* 2021) investigations of endocrine-relevant endpoints were done after pubertal or adult exposure animals and they were therefore not relevant for inclusion in the LoE.

An update on epidemiology studies for BP was performed for the period 2020-2022, using a cut-off date of March 25th 2022. Screening of abstracts and titles was performed in order to identify studies investigating how prenatal exposure to BP was associated to possible adverse effects in newborns, while disregarding all biomonitoring studies and all epidemiology studies correlating effect outcomes in adult individuals after adult exposure. 66 abstracts on BP were reviewed and one new relevant study identified, which investigated how anogenital distance (AGD) and reproductive hormone levels at three months of age (during mini-puberty) correlated to prenatal exposure of six parabens, including BP (Jensen *et al.* 2021). All of the other recently published epidemiology studies using birth cohorts, focused on parameters like birth weight, head circumference, % body fat, thyroid function, allergy and neurological development (Thürmann *et al.* 2021; Højsager *et al.* 2021; Hajizadeh *et al.* 2021; Freire *et al.* 2020; Li *et al.* 2020; Jamal *et al.* 2020; Leppert *et al.* 2020) but did not investigate effects on male reproductive development, i.e. the adverse effects in the postulated MoA for identifying BP as a SVHC based on its endocrine disrupting properties for human health (ECHA 2020).

[4] CompTox Chemicals Dashboard / TOXCAST (https://comptox.epa.gov/dashboard/)
[5] Danish (Q)SAR Database, http://qsar.food.dtu.dk, assessed 10 June 2022

4.10.D Study summaries for IBP

In vitro (incl. comptox and QSAR)
Study summaries for *in vitro* studies of IBP are provided in Annex II.

In vitro studies, described in Annex II, Table 13, showed that IBP binds to the estrogen receptor (ER) and increases ER transactivation and estrogen dependent signalling in target cells *in vitro* (Okubo *et al.* 2001; Darbre *et al.* 2002; Terasaki *et al.* 2009; Vo *et al.* 2010; Yang *et al.* 2011; Kim *et al.* 2011; Kim *et al.* 2012a; Kim *et al.* 2012b; *Vo et al.* 2012; Watanabe *et al.* 2013; Gonzalez *et al.* 2018; Comptox 2022; QSAR 2022). IBP has weak or no effects on the androgen receptor (Satoh *et al.* 2005; Kim *et al.* 2010; Kjaerstad *et al.* 2010; Watanabe *et al.* 2013; Comptox 2022; QSAR 2022).

In vivo
Study summaries for *in vivo* studies of IBP are provided in Annex II.

A relatively limited number of *in vivo* studies for IBP was identified, and they were all from the open literature as no studies relevant for assessment of ED properties were included in the registration dossier. Three uterotrophic studies, investigating endocrine activity *in vivo* (via E modality), found increased uterine weights after IBP exposure. The weight increases were seen at s.c. doses of 250 mg/kg and higher in both immature rats (Vo & Jeung, 2009) and ovariectomised rats (Koda *et al.* 2005), while a s.c. dose of approximately 90 mg/kg bw/day was high enough to increase uterine weights in immature mice.

A pubertal study in female rats (Vo *et al.* 2010) found adverse effects on uterus and ovaries, at oral doses of 62 mg/kg bw/day and above. The effects were seen as myometrial hyperplasia, increased number of cystic follicles and thinning of follicular epithelium. However, due to substantial limitations in the reporting of the histopathological assessments in the Vo *et al.* (2010) publication, the study only provided a weak to moderate degree of evidence of adverse effects on female reproduction.

A dermal 28-day study in male and female rats (Kim *et al.* 2015) found no mechanistic or adverse effects related to EATS modalities, using doses up to 600 mg/kg bw/day. It is possible that the dermal application of IBP may have changed the ADME properties of the test compound compared to s.c. or oral exposure. Furthermore, the study used a relatively low group size (n=5/sex), and even fewer animals for their histopathological examinations (n=3/sex). This study was therefore not sufficient to conclude that 28-day dermal IBP-exposure does not lead to adverse reproductive or endocrine effects.

The identified developmental toxicity studies (Kawaguchi *et al.* 2009a; Kawaguchi *et al.* 2009b; Kawaguchi *et al.* 2010; Yang *et al.* 2016) had severe limitations related to both methodology and reporting, and the results were deemed unreliable (Klimisch score 3). A description of findings as well as identified shortcomings is available in Annex II, Table 14.

Only one relevant human study on IBP was identified (Jurewicz *et al.* 2017). As described in Annex V, this study showed no association between IBP exposure and sperm chromosome disomy. However, the study was assessed to be of limited usefulness, as the timing of the exposure measurement was not considered relevant to the endpoint assessed.

4.10.E Study summaries for BP

BP has been identified as a SVHC, based on its endocrine disrupting properties for human health (ECHA 2020). As further explained in section 4.10.B and Annex I, a read-across approach from the source substance BP is suggested for the identification of IBP as an endocrine disrupting substance.

As summarised above, studies investigating adverse effects of IBP are very scarce. It is therefore here argued that *in vivo* studies for BP, showing adverse effects on male reproduction after developmental exposure, can be used to justify that IBP is an endocrine disrupting substance, using a read-across approach. Hence, a description of *in vivo* studies of BP was included in the present assessment of IBP.

In the SVHC support document for BP (ECHA 2020), study descriptions of all identified, relevant *in vivo* studies on BP were provided. These same study descriptions have also been included in the present assessment of IBP (Annex II, Table 15 supplemented with descriptions of additional perinatal *in vivo* studies, which were not yet published when BP was identified as a SVHC (Oliveira *et al.* 2020, Hubbard *et al.* 2020)). Two other new studies (Bae *et al.* 2021; Ara *et al.* 2021) also investigated endocrine endpoints but did not use perinatal exposure. These studies have been summarised below, but were not included in Table 15 of Annex II, or in the updated LoE for BP.

In short, Oliveira *et al.* (2020) investigated how BP exposure throughout the gestation period in rat dams, at s.c. doses of 100 and 200 mg/kg bw/day affected testicular development in the offspring. They found that relative testis weights were reduced on postnatal day (PND) 56 without change in body weight, and that the weight changes were associated with changes in the mitochondrial bioenergetics and antioxidant capacity of testis.

Hubbard *et al.* (2020) performed a large developmental continuous breeding study (reproductive assessment by continues breeding - RACB). BP exposure was dosed via the feed, at three doses, going as high as 3000 mg/kg bw/day. In the perinatally exposed offspring, no indications of endocrine disruption were seen, including lack of effects on AGD or on sperm quality. These results conflict with all other developmental toxicity studies with BP investigating sperm quality at high exposure doses, and the considerations on these new findings are discussed in section 4.10.1.1.

Studies by Ara *et al.* (2021) and Bae *et al.* (2021) also investigated how BP affected endocrine endpoints in rodents but did so in animals that were not exposed during perinatal development, but only during puberty and adulthood. As the LoE for BP focuses on perinatal exposure, study summaries for these studies have therefore not been included in Annex II, Table 15, or in the LoE for BP (section 4.10.1. and Annex III), but are instead briefly summarised below, and the results have been included in the overall WoE for BP, as supporting information.

Ara *et al.* 2021 dosed pubertal and adult mice with BP for 30 days (day 35-65), using an oral gavage dose of 150 µg/g body weight /day (which equals 150 mg/kg bw/day). The group size was 10/sex and the investigated endpoints included serum hormone levels, weights and histopathology of testes and ovaries (including analysis of sperm quality and follicle counts). In the males, BP exposure caused significant decreases in serum testosterone concentrations, altered testes weights, atrophy of the seminiferous tubules, aspermia, reduced sperm counts and increases in abnormal sperm. In the females, BP exposure led to increases in LH (luteinising hormone) and FSH (follicle stimulating hormone), decreased estradiol concentrations, increased ovary weights and differences in follicular count in the ovaries, showing markedly fewer primary follicles and a large increase in empty follicles, compared to controls. All of these effects were seen in the absence of systemic toxicity. While these findings indicate a rather consistent pattern of endocrine disrupting effects in the orally BP-exposed mice during (males) or just after puberty (females), the study only tested one dose of BP and it did have some clear limitations, especially related to the reporting, thus limiting the robustness of the conclusions that can be drawn from it.

Bae *et al.* (2021) performed a thirteen-week repeated dose toxicity study, in adult Sprague-Dawley rats, testing subcutaneous doses of 2, 10 and 50 mg/kg bw/day. Thus, in comparison with the Ara *et al.* 2021 study, this study used much lower BP doses, a different exposure route and adult animals rather than animals close to puberty. The group size was 10/sex and the assessed endpoints were also related to reproductive toxicity and endocrine disruption, as they tested estrous cyclicity, sperm quality and weight and histopathology of the following reproductive organs: testes, epididymis, prostate, ovary and uterus. The authors concluded that none of these endpoints were significantly affected by the s.c. BP exposure, but unfortunately most of the results could not be reviewed because they were not shown in the publication. The only endpoints where results were shown was the estrous cyclicity data, but here all exposure groups were said to have a mean of 100% regularity. The criteria for defining regularity were however not defined in the publication, so this data was difficult to evaluate. Overall, the study seemed scientifically sound, but it had some important limitations in its reporting, and the tested doses were quite low, thus any adverse endocrine effects would not necessarily be expected at these dose levels after adult exposure.

Like for the epidemiology study with IBP, human studies for the source substance BP, are also presented in Annex V. In the human studies available at the time of the SVHC support document (ECHA, 2020), no epidemiological studies examined the relationship between BP exposure in utero and effects on male reproductive parameters (hormone levels, sperm parameters) later in life. One study (Fernández *et al.* 2016) observed no association between placental BP levels and congenital malformations of the male genitalia (cryptorchidism and hypospadias). Residues of BP were, however, more frequently detected in cases versus controls. This was viewed as supporting human evidence for adverse effects of BP exposure during pregnancy. A few studies reported on relationships between BP exposure and maternal hormone levels and thus provided supporting evidence for endocrine disrupting activity of BP during pregnancy. One study showed a negative association between maternal urinary BP levels and maternal serum levels of estradiol and the estradiol/progesterone ratio (Aker *et al.* 2019). A larger study (Aker *et al.* 2016) showed a borderline trend of lower maternal testosterone levels with higher maternal BP levels and a significant negative association between maternal BP and sex hormone binding globulin (SHBG).

The literature update for the period 2020-22 identified one study (Jensen *et al.* 2021) that showed a trend of shorter AGD in boys and longer AGD in girls with higher maternal paraben exposure, although not significant for BP. In addition, FSH and LH concentrations were affected in girls with high prenatal paraben exposure, although not significant for BP. This provided supporting human evidence for adverse effects of paraben exposure during pregnancy, but generally the available epidemiology studies only played a minor role in the identification of BP as an endocrine disrupting substance, and this new study does not change that.

4.10.1 Lines of evidence - EAS modalities

The available information is structured into LoE tables, which are presented in Annex III:

Table 16 presents information on *in vitro* studies incl. comptox and QSAR on endocrine activity.
Table 17 presents information on *in vivo* studies on endocrine activity.
Table 18 presents information on adverse effects of IBP.
Table 19 presents additional LoE for specific endpoints of *in vivo* adversity of BP and IBP. This is based on the read-across approach presented above and in Annex I.

The integrated LoE on ***in vitro*** studies on **endocrine activity** (Table 16) led to a conclusion that there is strong evidence that IBP affects ER binding and increases ER

transactivation and estrogen dependent signalling in target cells *in vitro* (Okubo *et al.* 2001; Darbre *et al.* 2002; Terasaki *et al.* 2009; Vo *et al.* 2010; Vo *et al.* 2011; Yang *et al.* 2011; Kim *et al.* 2011; Kim *et al.* 2012a; Kim *et al.* 2012b; Vo *et al.* 2012; Watanabe *et al.* 2013; Gonzalez *et al.* 2018; Comptox 2022; QSAR 2022). IBP has weak or no effects on the androgen receptor (Satoh *et al.* 2005; Kim *et al.* 2010; Kjaerstad *et al.* 2010; Watanabe *et al.* 2013; Comptox 2022; QSAR 2022).

The integrated LoE on ***in vivo*** studies on **endocrine activity** of IBP (Table 17) led to the conclusion that there is moderate-strong evidence of estrogenic activity *in vivo* demonstrated by significant increases in uterus weight in three uterotrophic assays performed in immature or ovariectomised rats and mice (Darbre *et al.* 2002; Koda *et al.* 2005; Vo and Jeung 2009) and moderate evidence of altered regulation of estrogen-responsive genes in uterine tissue of immature rats (Vo and Jeung 2009). No effects on serum estradiol, testosterone, prolactin, FSH, LH and inhibin were reported, in the two studies investigating these endpoints, a pubertal study in female rats and a dermal 28-day study in adult male and female rats (Vo *et al.* 2010; Kim *et al.* 2015).

These two studies (Vo *et al.* 2010; Kim *et al.* 2015) were also the only ones that could inform on adverse effects *in vivo* of IBP, as the identified developmental studies on IBP, were assessed to be unreliable.
All *in vivo* study descriptions, including specification of study- and reporting limitations, are presented in Annex II, **Table 14***.*

As can be seen in the LoE table for **adverse effects of IBP** (**Table 18**), most endpoints were only assessed in a single study, complicating the overall assessment of each LoE. The only identified adverse effects were seen in the pubertal study in female rats. Here all three tested doses of IBP caused significant effects on ovary and uterus histopathology – seen as an increase in number of cystic follicles and decrease in corpora lutea, as well as a significant increase in the thickness of the uterus (Vo *et al.* 2010). The altered ovary and uterus histopathology however only provided weak evidence of adverse effects, as the study had marked limitations (as described in Annex II, **Table 14**). Other endpoints investigated in this study included vaginal opening (VO), estrous cyclicity and weight of ovaries and uterus. None of these were significantly affected. The 28-day study with dermal exposure also found no effect on male or female reproductive organs, or any sign of systemic toxicity at doses up to 600 mg/kg bw/day. It is possible that the dermal exposure may have caused different results than those seen in studies with oral or s.c. exposure. Overall, the integrated LoE on IBP showed insufficient data to conclude on adverse effects *in vivo*.

For **BP**, the integrated LoE showed sufficient data to conclude on **adverse effects *in vivo***, as presented in the SVHC support document for BP (ECHA 2020).

In Annex III, (Table 19) of the present report, the same LoE on adverse effect of BP are presented, with addition of data from two newer developmental toxicity studies (Oliveira *et al.* 2020 and Hubbard *et al.* 2020). These studies were not yet available at the time of the identification of BP as an SVHC.

Especially, the Hubbard *et al.* (2020) study is important to take into account, as it found no effects on AGD, sperm numbers or sperm motility in perinatally exposed offspring exposed to high doses of BP in the feed in a RACB study design. The implications of these new finding are discussed below.

4.10.1.1 Assembling and integration of LoE for endocrine Activity and Adversity - EAS modalities

The conclusions from the integrated LoE can be summarised as follows:

Weight of evidence (WoE) for endocrine activity of IBP – EAS modalities:
Strong evidence that IBP affects ER binding and transactivation and estrogen dependent signalling in target cells *in vitro*.
Moderate-strong evidence that IBP has estrogenic activity *in vivo* as evidenced in uterotrophic assays, showing increased uterine weight and altered expression of estrogen-regulated genes and proteins.

WoE for adverse effect of IBP and BP – EAS modalities:
Low-moderate evidence of adverse effects on ovary and uterus histopathology after pubertal **IBP** exposure, due to limited study reliability and lack of more studies.

No reliable studies for IBP investigating adverse effects on sperm quality in perinatally exposed rats.

For the source substance **BP**, a number of perinatal studies using oral gavage or s.c. exposure show moderate-strong evidence for adverse effects on sperm count, motility and number of normal sperm, after perinatal exposure (Guerra *et al.* 2017, Kang *et al.* 2002, Maske *et al.* 2020, Zhang *et al.* 2016, Boberg *et al*. 2016). On the other hand, no effects on sperm count or quality were seen with the recent dietary exposure National Toxicology Program (NTP) study, using continuous breeding protocol (Hubbard *et al.* 2020). Below the factors that might describe these discrepancies are discussed:

Some factors that might help explain these discrepancies are that the Hubbard *et al.* (2020) study used a different exposure scenario than the previous studies, by testing the effects of continuous BP exposure through the feed from F0 through to the F2 generation. This was different from e.g., the Boberg *et al.* (2016) study, where oral dosing was performed only during fetal and neonatal development of the F1 generation. Also, the exposure route differed between the studies which might account for some of the observed differences, as differences in bioavailability and metabolism may occur when using different exposure regimens. For instance, Aubert *et al.* (2012) showed that plasma C_{max} (maximum concentration) and area under the curve (AUC) values after oral or s.c. BP administration were 4-10 times higher than after dermal administration. If the same pattern holds true for IBP, this might explain why the dermal study by Kim *et al.* 2015 found no adverse effects at a dose of 600 mg/kg bw/day. Such differences could likely lead to differences in toxicological effects. Whether differences in ADME characteristics, and corresponding differences in C_{max} and AUC also exist for different oral exposures, i.e., gavage versus feed dosing, remains to be elucidated.

Apart from the dosing regimen, the studies by Hubbard *et al.* (2020) and Boberg *et al.* (2016), have relatively similar study designs. The dietary NTP study by Hubbard *et al.* used 22 litters per dose group and continuous dietary exposure, whereas the Boberg *et al*. (2016) study used 18 litters per dose group and exposure by gavage from gestational day (GD) 7 to PND 21 only. For the sperm analysis the Hubbard *et al.* (2020) study assessed 12-19 litters per dose group in control, low and mid doses, and 5-7 litters in the highest exposure group. In the Boberg *et al.* (2016) study rats from 13-17 litters per group were assessed for sperm count. Other studies showing altered sperm were smaller and used gavage or s.c. exposure in periods corresponding to the Boberg study (Zhang *et al.* 2016; Maske *et al.* 2020; Kang *et al.* 2002; Guerra *et al.* 2017). In the present evaluation of adverse effects, the NTP study results alone cannot negate the findings from all of the studies showing adverse effects on sperm quality using other exposure routes and periods. As discussed above, there may be differences in bioavailability using different study designs leading to the observed differences, but this remains to be elucidated.

Hence, after inclusion of all available *in vivo* results for BP on the endpoints in question in the ED assessment, there is still moderate-strong evidence that BP exposure under specific

exposure conditions can cause adverse effects on sperm count, sperm motility and reduced number of normal sperm cells (Guerra *et al.* 2017, Kang *et al.* 2002, Maske *et al.* 2020, Boberg *et al.* 2020).

In addition, there is some evidence of altered hormone levels, decreased AGD, and altered testicular histopathology in perinatally exposed rats (Boberg *et al.* 2016, Guerra *et al.* 2017, Kang *et al.* 2002, Maske *et al.* 2020, Taxvig *et al.* 2008, Zhang *et al.* 2014, Zhang *et al.* 2016), while other studies did not find these effects (Guerra *et al.* 2017, Hubbard *et al*. 2020). Effects on fertility were investigated in two studies showing no effect (Guerra *et al.* 2017, Hubbard *et al.* 2020) and one study showing a reduced number of implantation sites (Maske *et al.* 2020).

4.10.2 Lines of evidence - T modality

There is very little available knowledge on the effects of IBP on the T modality. The results are summarised below:

- Comptox Chemical Dashboard reported 0 out of 3 *in vitro* assays for thyroid interaction positive, i.e., no activity reported for thyroid receptor (TR), Thyroid stimulating hormone receptor (TSHR), and Thyrotropin-releasing hormone receptor (TRHR) (Comptox 2022) (**Table 16**).
- No effects on circulating T4 were seen after pubertal IBP exposure in female rats (Vo *et al.* 2010) and no effects on circulating T3 and TSH concentrations were seen in male and female rats in a dermal 28-day study (Kim *et al.* 2015) (**Table 17**).
- No effects on thyroid weights were seen after pubertal IBP exposure (Vo *et al.* 2010) (Table 18).

No other thyroid endpoints *in vitro* or *in vivo* have been assessed for IBP.

WoE for endocrine activity and adverse effects of IBP – T modality:

There were no indications of endocrine activity or of adverse effects of IBP suggesting thyroid hormone system disruption, but there was insufficient data to conclude with certainty on the T modality.

4.10.3 Lines of Evidence - Other modalities

For IBP, there was only little relevant evidence for other modalities than EATS identified. IBP appears to activate Pregnane X receptor (PXR), CAR (constitutive androstane receptor) and PPARα (Kamata *et al.* 2018; Fujino *et al.* 2019), though more studies are needed to substantiate these findings.

4.10.4 Mode of Action (MoA) analysis

4.10.4.1 Postulation of MoA(s)

To evaluate the plausible link between adverse effects and endocrine activity, a MoA analysis was carried out in accordance with ECHA/EFSA guidance (ECHA/EFSA 2018). ECHA/EFSA guidance highlights that both biological plausibility and empirical support are weighted, however biological plausibility is the most influential consideration.

This MoA analysis is presented in Annex IV, Table 20. Here, key events (KE) in the MoA and the degree of supporting evidence for each KE or adverse outcome (AO) is presented.

In the proposed MoA, the molecular initiating event (MIE) is "ER activation", which through a series of KEs leads to the adverse outcomes of "reduced sperm quality" and consequently "impaired fertility". It is noted that the MoA analysis was carried out for perinatal exposure only. As there are no reliable studies using perinatal IBP exposure, studies on the source substance BP showing reduced sperm count and sperm motility have been used.

The hypothesised MoA for the effects of IBP on male fertility is presented in Figure 3 (and in Annex IV, Table 20). Read-across from BP is used for identification of adverse effects. The use of BP as a read-across substance builds on a number of studies showing a similar or higher potency of IBP than BP regarding estrogenicity *in vitro* and *in vivo (*see read-across argumentation in section 4.10.B. and Annex I).

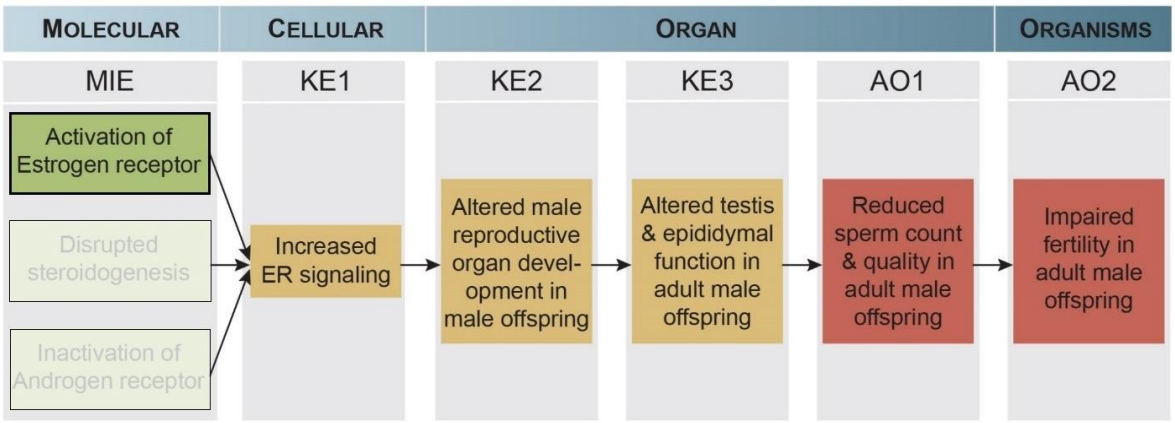

Figure 3: Hypothesised MoA for effects of BP and IBP on perinatally exposed male offspring (modified from Boberg et al. 2020). For BP three possible MIEs could lead to the observed AO, but experimental data on IBP only support the MIE of "ER activation" (as shown in Table 20 in Annex IV).

The hypothesised molecular initiating event (MIE) is activation of the ER(s). Other MIEs (androgen receptor antagonism and steroid synthesis inhibition) may lead to the same series of KE and ultimately the same AOs, but for IBP there is no data suggesting that these other MIEs are involved.

In developing males, increased ER signalling results in altered testicular development and subsequently altered testicular function in adulthood. In turn, reduced sperm count and quality are observed.

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

The biological plausibility of key event relationships (KERs) was analysed and presented in Table 21, Annex IV. There was a high degree of evidence for all the presented KERs, except the step "Increased ER signalling to altered reproductive development of offspring", for which the evidence was considered "Moderate to high". The evidence for the analyses was not limited to IBP or BP but, was strengthened by evidence from other models and studies on other endocrine disrupters affecting sperm count and quality. Similar argumentation was used in the analysis of biological plausibility of KERs for BP (ECHA 2020).

4.10.4.3 Human relevance of MoA

Human relevance is assumed, as there is no data indicating that these endocrine MoA are not relevant to humans.

4.10.4.4 Conclusion on the MoA analysis

The conclusions of the MoA analysis are presented in Annex IV, Table 22. The analysis was carried out for effects of perinatal exposure and included the following steps: an overview of KEs, an analysis of biological plausibility of KERs, considerations on dose and temporal concordance, human relevance and uncertainties.

The analysis led to the conclusion that it is biologically plausible that ER activation during development leads to adverse effects on male reproductive function following perinatal exposure. There was sufficient dose- and temporal concordance between KEs and the effects are assumed to be relevant to humans.

4.10.5 Overall conclusion on endocrine disruption with regards to human health

LoEs for IBP show clear evidence of endocrine activity *in vitro* and *in vivo*. Specifically, effects on estrogenic activity are considered strong. There are no reliable *in vivo* studies of IBP using perinatal exposure and very limited overall knowledge on the potential adverse effects of IBP exposure *in vivo*. Based on similarities in chemical structure, as well as *in vitro* and *in vivo* mechanistic effects and potency, IBP is considered closely related to BP. Specifically, assays investigating estrogenic responses *in vitro* and *in vivo,* point to a high potency of IBP and BP compared to shorter chain parabens. *In vitro* studies including both IBP and BP in many cases show a higher potency of IBP than BP. Using BP as a read-across substance can therefore be considered as realistic or it may even underestimate the toxicity of IBP.

For the source substance BP, a number of studies using oral gavage or s.c. exposure show moderate to strong evidence for adverse effects on sperm count, motility and number of normal sperm, after perinatal exposure. On the other hand, no effects on sperm count or quality were seen with the recent dietary exposure study, using continuous breeding protocol, performed by the NTP (Hubbard *et al.* 2020). Differences in ADME characteristics, and corresponding differences in C_{max} and AUC for different oral exposures, i.e., gavage versus feed dosing, remains to be elucidated. Based on a weight of evidence approach, this present report concludes that the NTP study results alone cannot negate the findings from all of the studies showing adverse effects on sperm quality after perinatal BP exposure. See 4.10.1.1 Assembling and integration of LoE for endocrine Activity and Adversity - EAS modalitiesfor the discussion of new studies on BP.

After inclusion of all available *in vivo* results for BP on the endpoints in question here, there is still moderate-strong evidence that BP exposure under specific developmental exposure conditions can cause adverse effects on sperm count, sperm motility and reduced number of normal sperm cells.

The MoA analysis of IBP is based on "EAS-mediated adversity", and the substance is considered to be an endocrine disrupter for E modality. No alternative non-endocrine MoA has been demonstrated.

In conclusion, based on a weight of evidence approach and read across to BP, IBP is assessed to meet the WHO/IPCS definition of an endocrine disruptor (WHO/IPCS 2002) as interpreted by the JRC Endocrine Advisory Group (2013), with estrogenic disrupting MoA, leading to adverse effect on the male reproductive system following perinatal exposure.

4.11 Other effects

None were identified.

4.12 Summary and discussion of human health hazard assessment

No additional human health hazards than those identified for endocrine disruption have been identified. Repetition of the arguments provided for the ED assessment was deemed unnecessary.

5. Environmental hazard assessment

Environmental data for IBP was not reviewed and has therefore not been included in this report.

6. Conclusions on the SVHC Properties

6.3 Assessment under Article 57(f)

6.3.1 *Summary of the data on the intrinsic/hazardous properties*

A short summary of the data on human health hazard assessment on EAS modalities as well as the read across justification are provided below:

Endocrine disruption

Endocrine activity

The integrated LoE on *in vitro* studies on endocrine activity of IBP led to a conclusion that there is strong evidence that IBP affects ER binding and transactivation and estrogen dependent signalling in target cells *in vitro.* IBP has weak or no effects on the androgen receptor but appears to activate PXR, CAR and PPARα.

The integrated LoE on *in vivo* studies on endocrine activity of IBP led to the conclusion that there is moderate-strong evidence of estrogenic activity *in vivo* as evidenced in uterotrophic assays, showing increased uterine weight and altered expression of estrogen-regulated genes and proteins. No effects on serum estradiol, testosterone, prolactin, FSH, LH, inhibin, TSH, T4 or T3 were reported.

Adverse effects

There was only low-moderate evidence of adverse effect on ovary and uterus histopathology after pubertal IBP exposure, due to lack of studies and limited study reliability. There were no reliable studies for IBP investigating adverse effects on sperm quality in perinatally exposed rats.

Therefore, a read across approach was proposed from the source substance BP to the target substance IBP. BP has already been identified as a SVHC because of its endocrine disrupting properties to human health. The read-across is supported by the structural similarity of the substances and by similar estrogenic activity and potency observed *in vitro* and *in vivo*.

For the source substance BP, a number of rodent studies using oral gavage or s.c. exposure show moderate-strong evidence for adverse effects on sperm count and quality, after perinatal exposure. These effects are considered severe as similar effects in humans could cause sub- and infertility, as described in 6.3.2. In addition, there is supporting evidence of adverse effects on AGD, sperm motility, hormone levels and testicular histopathology in perinatally exposed male rats. However, no effect on sperm parameters and AGD were seen in a recent developmental dietary exposure NTP study, using continuous breeding protocol. These inconsistencies can be considered to reflect differences in bioavailability using different study designs, such as exposure routes and period. However, in the present evaluation of adverse effects, the NTP study results alone cannot negate the findings from all of the other studies. Hence, after inclusion of all available *in vivo* results for BP on the endpoints in question here, there is still moderate-strong evidence that, under specific conditions, exposure to BP, and consequently to IBP, can cause adverse effects on sperm count and quality.

Plausible link between adverse effects and endocrine activity

The MoA analysis leads to the conclusion that IBP acts via an estrogenic MoA. Since limited information was available for IBP on adverse effects, information on BP was included in

the MoA analysis (perinatal exposure). The MIE is activation of the ER(s). In developing males, increased ER signaling results in altered testicular development and subsequently altered testicular function in adulthood. In turn, reduced sperm count and quality are observed.

The analysis led to the conclusion that it is biologically plausible that ER activation during development leads to the observed adverse effects on the male reproductive system following perinatal exposure of IBP. There was sufficient dose- and temporal concordance between KEs and the effects are assumed to be relevant to humans.

No alternative non-endocrine MoA was demonstrated.

Conclusion

Based on a weight of evidence approach and read across to BP, it is concluded that IBP meets the WHO/IPCS definition of an endocrine disruptor (WHO/IPCS 2002) as interpreted by the JRC Endocrine Advisory Group (2013), with estrogenic disrupting MoA, leading to adverse effect on the male reproductive system following perinatal exposure.

6.3.2 Equivalent level of concern assessment

6.3.2.1 Human health

The adverse effects on BP are reduced sperm count and quality as observed in rodent studies using perinatal exposure. Effects are irreversible and are shown to occur later in life after exposure in the perinatal period only and will not manifest fully until reproductive age. These adverse effects on male reproductive system can have potentially serious effects on humans as similar effects in humans could cause sub- and infertility.

For humans, sub- and infertility is not only detrimental to the propagation of the species, but also has a major impact on quality of life. A reduced ability to reproduce negatively 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.

Based on the available studies, no safe concentration/level can be derived from the available data on adverse effects via endocrine MoA. Two of the available studies show reduced sperm count or quality in perinatally exposed rats at the lowest tested dose and thus, no NOAEL can be determined for this endpoint. This raises concern particularly on the capacity to manage safe use of the substance for sensitive populations. Moreover, mixture effects, where substances act additively or with synergistic effects, cannot be excluded and this might impact the threshold of toxicity.

Overall, it is concluded that the substance isobutyl 4-hydroxybenzoate (referred to as isobutylparaben, IBP) meet the criteria of 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 human health which gives rise to an equivalent level of concern to those substances listed in points (a) to (e) of Article 57 of the REACH Regulation. Studies on adverse effects of IBP are missing but read-across from BP provides sufficient evidence for adverse effects on male reproductive system following perinatal exposure. BP is already identified as a SVHC because of its endocrine disrupting properties to human health and a read-across justification of IBP and BP clearly shows structural similarity as well as similar effect patterns on estrogenic activity and potency *in vitro* and *in vivo*.

6.3.2.3 Summary of the ELoC assessment

IBP exposure gives rise to an equivalent level of concern to substances listed in Article 57 points (a) to (e) due to its endocrine disrupting properties for human health. Notably, the conclusion is reached using a read-across approach with BP as a source substance.

The adverse effects observed in rodent studies on BP are reduced sperm count and quality. These effects are considered severe as similar effects in humans could cause sub- and infertility. Effects are irreversible and are shown to occur later in life after exposure in the perinatal period only. For humans, sub- and infertility has a major impact on quality of life. Fertility treatment and counselling carries high societal costs. No safe concentration/level could be derived, which raises a particular concern for safe use of the substance for sensitive populations.

Altogether, this gives rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 REACH.

6.3.3 Conclusion on the Article 57(f) assessment

Isobutyl 4-hydroxybenzoate is proposed to be identified as a substance of very high concern (SVHC) 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 human health which gives 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.

Endocrine disrupting (ED) properties of IBP relevant for human health:

Estrogenic activity
There is strong evidence that IBP affects estrogen receptor (ER) binding and transactivation and estrogen dependent signalling in target cells *in vitro*. *In vivo,* there is moderate-strong evidence of estrogenic activity as evidenced in uterotrophic assays, showing increased uterine weight and altered expression of estrogen-regulated genes and proteins.

Adverse effects
There is low-moderate evidence of adverse effects on ovary and uterus histopathology after pubertal IBP exposure, due to lack of studies and limited study reliability. There are no reliable studies for IBP investigating adverse effects on sperm quality in perinatally exposed rats.

Therefore, a read across approach is proposed from the source substance butylparaben (BP) to the target substance IBP. BP has already been identified as a SVHC because of its endocrine disrupting properties to human health. The read-across is supported by the structural similarity of the substances and by similar estrogenic activity and potency observed *in vitro* and *in vivo*.

A number of rodent studies using oral gavage or subcutaneous exposure show moderate-strong evidence for adverse effects of BP on sperm count and quality, after perinatal exposure. No effect on endocrine related endpoints (sperm parameters and anogenital distance) are seen in a recent developmental dietary exposure study, using continuous breeding protocol. However, the adverse findings observed in other studies should not be neglected. These inconsistencies can be considered to reflect differences in bioavailability using different study designs such as exposure routes and periods. Hence, after consideration of all available *in vivo* results for BP, there is still moderate-strong evidence

that, under specific conditions, exposure to BP, and consequently to IBP, can cause adverse effects on sperm count and quality.

Plausible link between adverse effects and endocrine activity
The mode of action (MoA) analysis leads to the conclusion that IBP acts via an estrogenic MoA. Since limited information was available for IBP on adverse effects, information on BP was included in the MoA analysis (perinatal exposure). The molecular initiating event is activation of the ER(s). In developing males, increased ER signaling results in altered testicular development and subsequently altered testicular function in adulthood. In turn, reduced sperm count and quality are observed. The analysis led to the conclusion that it is biologically plausible that ER activation during development leads to the observed adverse effects on the male reproductive system following perinatal exposure to IBP.

Summary of the ED assessment
There is scientific evidence to conclude that IBP is an endocrine disruptor via the E (estrogen) modality, according to a MoA analysis including an evaluation of biological plausibility.

Equivalent level of concern
The adverse effects on BP are reduced sperm count and quality as observed in rodent studies using perinatal exposure. Effects are irreversible and are shown to occur later in life after exposure in the perinatal period only. These effects are considered severe as similar effects in humans could cause sub- and infertility. Sub- and infertility is not only detrimental to the propagation of the species, but also has a major impact on quality of life. Fertility treatment and counselling carries high societal costs.

No safe concentration/level can be derived from the available data on adverse reproductive effects via an endocrine MoA. Two of the available studies show reduced sperm count or quality in perinatally exposed rats at the lowest tested dose and therefore no no-observed-effect-level can be determined for this endpoint. The difficulty to establish a safe level with sufficient certainty raises concern particularly on the capacity to manage safe use of the substances for sensitive populations. Moreover mixture effects, where substances act additively or with synergistic effects, cannot be excluded and this might impact the threshold of toxicity.

Altogether, IBP exposure gives rise to an equivalent level of concern to substances listed in Article 57 points (a) to (e) due to its endocrine disrupting properties for human health. Notably, the conclusion is reached using a read-across approach with BP as a source substance – a substance already identified as a SVHC because of its endocrine disrupting properties to human health.

Conclusion
Overall, it is concluded that the substance isobutyl 4-hydroxybenzoate (referred to as isobutylparaben, IBP) meets the criteria of 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 human health which gives rise to an equivalent level of concern to those substances listed in points (a) to (e) of Article 57 of the REACH Regulation.

PART II

7. Registration and C&L notification status

7.1 Registration status

Table 5: Registration status

From the ECHA dissemination site[6]	
Registrations	☒ Full registration(s) (Art. 10) ☐ Intermediate registration(s) (Art. 17 and/or 18)

7.2 CLP notification status

Table 6: CLP notifications

	CLP Notifications[7]
Number of aggregated notifications	11
Total number of notifiers	532

No harmonised classifications apply to the substance. According to the classification and labelling data submitted to ECHA in the registration under REACH and the data notified by manufacturers or importers under CLP, IBP may cause an allergic skin reaction (Skin Sens. 1B, H317) and causes skin irritation (Skin Irrit. 2, H315). It causes serious eye damage (Eye Dam. 1, H318) and serious eye irritation (Eye Irrit. 2, H319). IBP is very toxic to aquatic life (Aquatic Acute 1, H400) and toxic to aquatic life with long lasting effects (Aquatic Chronic 2, H411). IBP may cause respiratory irritation (STOT SE 3, H335).

8. Total tonnage of the substance

Table 7: Tonnage status

Total tonnage band for the registered substance (excluding the volume registered under Art 17 or Art 18)[8]	1-10 t/pa
Tonnage information from public sources other than registration dossiers (if available)	N/A

[6] Registered substance dissemination site, https://echa.europa.eu/da/registration-dossier/-/registered-dossier/17752 (accessed 4 July 2022)
[7] C&L Inventory database, https://echa.europa.eu/da/information-on-chemicals/cl-inventory-database/-/discli/details/54124 (accessed 4 July 2022)
[8] Registered substance dissemination site, https://echa.europa.eu/da/registration-dossier/-/registered-dossier/17752 (accessed 4 July 2022)

9. Information on uses of the substance

Table 8: Uses

	Use(s)	**Registered use** *(If not, specify the source of the information)*	**Use likely to be in the scope of Authorisation**
Uses as intermediate	N/A		
Formulation or repacking	Coating products, fillers, putties, plasters, modelling clay and inks and toners. **Substance preparation:** Environmental release category (ERC): ERC2: Formulation into mixture Process category (PROC): PROC 1: Chemical production or refinery in closed process without likelihood of exposure or processes with equivalent containment conditions PROC 2: Chemical production or refinery in closed continuous process with occasional controlled exposure or processes with equivalent containment conditions PROC 3: Manufacture or formulation in the chemical industry in closed batch processes with occasional controlled exposure or processes with equivalent containment conditions PROC 4: Chemical production where opportunity for exposure arises PROC 5: Mixing or blending in batch processes PROC 7: Industrial spraying PROC 8a: Transfer of substance or mixture (charging and discharging) at non-dedicated facilities PROC 8b: Transfer of substance or mixture (charging and discharging) at dedicated facilities	Yes	Yes

	PROC 9: Transfer of substance or mixture into small containers (dedicated filling line, including weighing) PROC 15: Use as laboratory reagent <u>Product category formulated (PC):</u> PC 9a: Coatings and paints, thinners, paint removes PC 9b: Fillers, putties, plasters, modelling clay PC 18: Ink and toners **Manufacture of blends:** <u>Environmental release category (ERC):</u> ERC2: Formulation into mixture <u>Process category (PROC):</u> PROC 1: Chemical production or refinery in closed process without likelihood of exposure or processes with equivalent containment conditions PROC 2: Chemical production or refinery in closed continuous process with occasional controlled exposure or processes with equivalent containment conditions PROC 3: Manufacture or formulation in the chemical industry in closed batch processes with occasional controlled exposure or processes with equivalent containment conditions PROC 5: Mixing or blending in batch processes PROC 8a: Transfer of substance or mixture (charging and discharging) at non-dedicated facilities PROC 8b: Transfer of substance or mixture (charging and discharging) at dedicated facilities PROC 9: Transfer of substance or mixture into small containers (dedicated filling line, including weighing) PROC 14: Tabletting, compression, extrusion, pelletisation, granulation		

	PROC 15: Use as laboratory reagent Product category formulated (PC): PC 0: Other: Blends		
Uses at industrial sites	Laboratory chemicals. This substance has an industrial use resulting in manufacture of another substance (use of intermediates). This substance is used for the manufacture of: chemicals. Environmental release category (ERC): ERC6a: Use of intermediate Process categories (PROC): PROC 1: Chemical production or refinery in closed process without likelihood of exposure or processes with equivalent containment conditions PROC 2: Chemical production or refinery in closed continuous process with occasional controlled exposure or processes with equivalent containment conditions PROC 3: Manufacture or formulation in the chemical industry in closed batch processes with occasional controlled exposure or processes with equivalent containment conditions PROC 4: Chemical production where opportunity for exposure arises PROC 5: Mixing or blending in batch processes PROC 7: Industrial spraying PROC 8a: Transfer of substance or mixture (charging and discharging) at non-dedicated facilities PROC 8b: Transfer of substance or mixture (charging and discharging) at dedicated facilities PROC 9: Transfer of substance or mixture into small containers (dedicated filling line, including weighing) PROC 10: Roller application or brushing	Yes	No

	PROC 13: Treatment of articles by dipping and pouring PROC 14: Tabletting, compression, extrusion, pelletisation, granulation PROC 15: Use as laboratory reagent Product category used (PC): PC 19: Intermediate PC 21: Laboratory chemicals Sector of end use (SU): SU 9: Manufacture of fine chemicals		
Uses by professional workers	N/A		
Consumer uses	N/A		
Article service life	N/A		

10. Information on structure of the supply chain

There is one active registrant under REACH of this substance. The substance was first registered in 2016.

11. Additional information

11.1 Substances with similar hazard and use profiles on the Candidate List

The substance BP has already been included in the Candidate list due to its endocrine disrupting properties for human health (ECHA 2020). The substances BP and IBP share close structural similarity the only difference being that IBP has an isopropyl group at the end of the alkyl chain while BP has a butyl group. The available evidence suggest that BP and IBP share similar MoA and the data presented in this dossier suggests that they also share similar hazard properties.

BP is used by consumers, professional workers and in formulation or re-packing in cosmetics and personal care products and pharmaceuticals.

11.2 Alternatives

Other parabens could be used as alternatives to IBP as they share similar properties. Available evidence suggests that they all act via the same MoA. BP has already been included in the Candidate List (ECHA 2020) due to its endocrine disrupting properties and

other parabens are currently under assessment for potential endocrine disrupting properties. Substitution of IBP with other parabens with similar hazard profile should be avoided (regrettable substitution).

11.3 Existing EU legislation

IBP is included in Annex II to the Cosmetic Products Regulation (Regulation (EC) No 1223/2009), which lists the substances prohibited in cosmetic products (Ref. No. 1375). Therefore, IBP is banned from use in any cosmetic products marketed for sale or use in the European Union.

11.4 Previous assessments by other authorities/ongoing regulatory activities

An RMOA on IBP was prepared by Denmark in 2022 (ECHA, 2022). The RMOA concluded that there is sufficient evidence available to conclude that the substance is an endocrine disruptor to human health according to the WHO/IPCS criteria and that further risk management measures are needed. The RMOA assessed different risk management measures and concluded that the substance should be included on the Candidate List as a SVHC due to endocrine disrupting properties.

REFERENCES

References for Part I

Ahn H-J, An B-S, Jung E-M, et al (2012) Parabens inhibit the early phase of folliculogenesis and steroidogenesis in the ovaries of neonatal rats. Mol Reprod Dev 79:626–636. doi: 10.1002/mrd.22070

Aker AM, Ferguson KK, Rosario ZY, et al (2019) A repeated measures study of phenol, paraben and Triclocarban urinary biomarkers and circulating maternal hormones during gestation in the Puerto Rico PROTECT cohort. Environ Heal 18:28. doi: 10.1186/s12940-019-0459-5

Aker AM, Watkins DJ, Johns LE, et al (2016). Phenols and parabens in relation to reproductive and thyroid hormones in pregnant women. Environ Res 151:30–37. doi: 10.1016/j.envres.2016.07.002

Ara C, Asmatullah, Butt N, Ali S, Batool F, Shakir HA, Arshad A. (2021). Abnormal steroidogenesis, oxidative stress and reprotoxicity following prepubertal exposure to butylpataben in mice and protective effec of Curcuma longa. Env Sci Poll Res 28:6111-6121

Aubert N (2009) Blood Plasma Pharmacokinetics and Mass Balance of Total Radioactivity in Sprague-Dawley Rats Following Single Administration of Three Different Parabens (Methyl-, Butyl-, Propyl-) by Three Different Routes of Administration (Oral, Dermal, Sub-Cutaneous). CIT, Centre International de Toxicologie, Evreux, France. Study No. 34851 PAR, 26 November. doi.org: 10.1016/j.fct.2011.12.045.

Aubert N, Ameller T, Legrand JJ (2012) Systemic exposure to parabens: pharmacokinetics, tissue distribution, excretion balance and plasma metabolites of [14C]-methyl-, propyl- and butylparaben in rats after oral, topical or subcutaneous administration. Food Chem Toxicol 50:445–454

Bae J, Lee JDL, Song SW, Shin HC, Choi YK, Shin CY, Lee BM, Kim KB (2021) Thirteen-week subcutaneous repeated dose toxicit study of butylparaben and its toxicokinetics in rats. Arch Toxicol, 95:2037-50

Boberg J, Axelstad M, Svingen T, et al (2016) Multiple Endocrine Disrupting Effects in Rats Perinatally Exposed to Butylparaben. Toxicol Sci 152:244–256. doi: 10.1093/toxsci/kfw079

Boberg J, Taxvig C, Christiansen S, Hass U (2010) Possible endocrine disrupting effects of parabens and their metabolites. Reprod Toxicol 30:301–312. doi: 10.1016/j.reprotox.2010.03.011

Boberg J, Johansson HKL, Axelstad M, Olsen GPM, Johansen M, Holmboe SA, Andersson AM, Svingen T (2020) Environ Int. 2020 Nov;144:105996. doi: 10.1016/j.envint.2020.105996

CIR (2008) Final Amended Report on the Safety Assessment of Methylparaben, Ethylparaben, Propylparaben, Isopropylparaben, Butylparaben, Isobutylparaben, and Benzylparaben as used in Cosmetic Products. Int J Toxicol 27:1–82. doi: 10.1080/10915810802548359

Comptox, Comptox Chemical dashboard/Toxcast (2022) U.S. Tox21 Program, Tox21 Data https://comptox.epa.gov/dashboard/dsstoxdb/results?search=DTXSID4020749#invitrodb-bioassays-toxcast-tox21. Accessed 7 Oct 2020 and 10 June 2022

Cosmetic Products Regulation (2009) Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A02009R1223-20220731

Darbre PD, Byford JR, Shaw LE, et al (2002) Oestrogenic activity of isobutylparaben in vitro and in vivo. J Appl Toxicol 22:219–226. doi: 10.1002/jat.860

Delbès G, Levacher C, Duquenne C, et al (2005) Endogenous Estrogens Inhibit Mouse Fetal Leydig Cell Development via Estrogen Receptor α. Endocrinology 146:2454–2461. doi: 10.1210/en.2004-1540

Delbès G, Levacher C, Habert R (2006) Estrogen effects on fetal and neonatal testicular development. Reproduction 132:527–538. doi: 10.1530/rep.1.01231

Delbès G, Levacher C, Pairault C, et al (2004) Estrogen Receptor β-Mediated Inhibition of Male Germ Cell Line Development in Mice by Endogenous Estrogens during Perinatal Life. Endocrinology 145:3395–3403. doi: 10.1210/en.2003-1479

ECHA, European Chemicals Agency (2016) Practical Guide: How to use alternatives to animal testing Version 2.0. 19 July 2016. doi: 10.2823/194297

ECHA, European Chemicals Agency (2017) Read Across Assessment Framework (RAAF). doi: 10.2823/619212

ECHA, European Chemicals Agency (2020) Member State Committee support document for identification as a substance of very high concern because of its endocrine disrupting properties (article 57(f) – human health). Adopted on 28 May 2021.

ECHA/EFSA (2018) ECHA (European Chemicals Agency) and EFSA (European Food Safety Authority) with the technical support of the Joint Research Centre (JRC). Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 11. EFSA J 2018 16:5311, 135 pp. doi: https://doi.org/10.2903/j.efsa.2018.5311. ECHA-18-G-01-EN

EFSA, European Food Safety Authority (2017) Scientific opinion of the PPR panel on "Literature review of epidemiological studies linking exposure to pesticides and health effects. EFSA J 15(10):5007

Fernández MF, Arrebola JP, Jiménez-Díaz I, et al (2016) Bisphenol A and other phenols in human placenta from children with cryptorchidism or hypospadias. Reprod Toxicol 59:89–95. doi: 10.1016/j.reprotox.2015.11.002

Fisher JS, Turner KJ, Brown D, Sharpe RM (1999) Effect of neonatal exposure to estrogenic compounds on development of the excurrent ducts of the rat testis through puberty to adulthood. Environ Health Perspect 107:397–405. doi: 10.1289/ehp.99107397

Freire C, Vela-Soria F, Beneito A, Lopez-Espinosa MJ, Ibarluzea J, Barreto FB, Casas M, Vrijheid M, Fernandez-Tardon G, Riaño-Galan I, Fernandez MF (2020) INMA Project. Association of placental concentrations of phenolic endocrine disrupting chemicals with cognitive functioning in preschool children from the Environment and Childhood (INMA) Project. Int J Hyg Environ Health. 2020 Sep;230:113597. doi: 10.1016/j.ijheh.2020.113597

Fujino C, Watanabe Y, Sanoh S, et al (2019) Comparative study of the effect of 17 parabens on PXR-, CAR- and PPARα-mediated transcriptional activation. Food Chem Toxicol 133:110792. doi: 10.1016/j.fct.2019.110792

Golden R, Gandy J, Vollmer G (2005) A Review of the Endocrine Activity of Parabens and Implications for Potential Risks to Human Health. Crit Rev Toxicol 35:435–458. doi: 10.1080/10408440490920104

Gonzalez TL, Moos RK, Gersch CL, et al (2018) Metabolites of n-Butylparaben and iso-Butylparaben Exhibit Estrogenic Properties in MCF-7 and T47D Human Breast Cancer Cell Lines. Toxicol Sci 164:50–59. doi: 10.1093/toxsci/kfy063

Guerra MT, Sanabria M, Leite GAA, et al (2017) Maternal exposure to butyl paraben impairs testicular structure and sperm quality on male rats. Environ Toxicol 32:1273–1289. doi: 10.1002/tox.22323

Hajizadeh Y, Moradnia M, Kiani Feizabadi G, Rafiei N, Tahmasbizadeh M, Darvishmotevalli M, Fadaei S, Karimi H. (2021) The sex-specific association between maternal urinary paraben levels and offspring size at birth. Environ Sci Pollut Res Int. 2021 Jul;28(27):36029-36038. doi: 10.1007/s11356-021-13175-3.

Hossaini A, Larsen J-J, Larsen JC (2000) Lack of oestrogenic effects of food preservatives (parabens) in uterotrophic assays. Food Chem Toxicol 38:319–323. doi: 10.1016/S0278-6915(99)00160-X

Hubbard TD, Brix A, Blystone CR, McIntyre BS, Shockley K, Cunny H, Waidyanatha S, Turner KJ, McBride S, Roberts GK. (2020). Butylparaben multigenerational reproductive assessment by continuous breeding in Hsd:Sprague Dawley SD rats following dietary exposure. Reprod Toxicol. 2020 Sep; 96:258-272. doi: 10.1016/j.reprotox.2020.07.006.

Højsager FD, Kyhl HB, Frederiksen H, Juul A, Andersson AM, Andersen MS, Grøntved A,

Jensen TK. (2021) Prenatal Exposure to Butyl Paraben Is Associated With Fat Percentage in 7-Year-Old Boys.J Clin Endocrinol Metab. Jun 16;106(7):e2633-e2638. doi: 10.1210/clinem/dgab167.

Jamal A, Rastkari N, Dehghaniathar R, Nodehi RN, Nasseri S, Kashani H, Shamsipour M, Yunesian M. (2020) Prenatal urinary concentrations of environmental phenols and birth outcomes in the mother-infant pairs of Tehran Environment and Neurodevelopmental Disorders (TEND) cohort study. Environ Res. May;184:109331. doi: 10.1016/j.envres.2020.109331

Jefferson WN, Couse JF, Banks EP, Korach KS, Newbold RR. (2000) Expression of estrogen receptor beta is developmentally regulated in reproductive tissues of male and female mice. Biol Reprod 2000; 62:310-317

Jensen TK, Andersson AM, Main KM, Johannsen TH, Andersen MS, Kyhl HB, Juul A, Frederiksen H. (2021) Prenatal paraben exposure and anogenital distance and reproductive hormones during mini-puberty: A study from the Odense Child Cohort. Sci Total Environ. 2021 May 15;769:145119. doi: 10.1016/j.scitotenv.2021.145119.

JRC, Joint Research Centre (2013) Key Scientific issues relevant to the identification and characterisation of endocrine disrupting substances – Report of the Endocrine Disruptors Expert Advisory Group (ED EAG). Eds. Munn S. and Gourmenou M. Pp 32. Available at: https://ec.europa.eu/jrc/en/publication/eur-scientific-and-technical-research-reports/key-scientific-issues-relevant-identification-and-characterisation-endocrine-disrupting

Joseph A, Shur BD, Hess RA (2011) Estrogen, Efferent Ductules, and the Epididymis. Biol Reprod 84:207–217. doi: 10.1095/biolreprod.110.087353

Jurewicz J, Radwan M, Wielgomas B, et al (2017) Environmental exposure to parabens and sperm chromosome disomy. Int J Environ Health Res 27:332–343. doi: 10.1080/09603123.2017.1339784

Kamata R, Nakajima D, Shiraishi F (2018) Agonistic effects of diverse xenobiotics on the constitutive androstane receptor as detected in a recombinant yeast-cell assay. Toxicol Vitr 46:335–349. doi: 10.1016/j.tiv.2017.09.014

Kang K-S, Che J-H, Ryu D-Y, et al (2002) Decreased Sperm Number and Motile Activity on the F1 Offspring Maternally Exposed to Butyl p-Hydroxybenzoic Acid (Butyl Paraben). J Vet Med Sci 64:227–235. doi: 10.1292/jvms.64.227

Kawaguchi M, Irie K, Morohoshi K, et al (2009a) Maternal isobutyl-paraben exposure alters anxiety and passive avoidance test performance in adult male rats. Neurosci Res 65:136–140. doi: 10.1016/j.neures.2009.06.011

Kawaguchi M, Morohoshi K, Imai H, et al (2010) Maternal Exposure to Isobutyl-Paraben Impairs Social Recognition in Adult Female Rats. Exp Anim 59:631–635. doi: 10.1538/expanim.59.631

Kawaguchi M, Morohoshi K, Masuda J, et al (2009b) Maternal Isobutyl-Paraben Exposure decreases the Plasma Corticosterone Level in Dams and Sensitivity to Estrogen in Female Offspring Rats. J Vet Med Sci 71:1027–1033. doi: 10.1292/jvms.71.1027

Kim MJ, Kwack SJ, Lim SK, et al (2015) Toxicological evaluation of isopropylparaben and isobutylparaben mixture in Sprague-Dawley rats following 28 days of dermal exposure. Regul Toxicol Pharmacol 73:544–551. doi: 10.1016/j.yrtph.2015.08.005

Kim SM, Jung EM, An BS, et al (2012a) Additional effects of bisphenol A and paraben on the induction of calbindin-D9K and progesterone receptor via an estrogen receptor pathway in rat pituitary GH3 cells. J Physiol Pharmacol 63:445–455

Kim T-S, Kim C-Y, Lee H-K, et al (2011) Estrogenic Activity of Persistent Organic Pollutants and Parabens Based on the Stably Transfected Human Estrogen Receptor-α Transcriptional Activation Assay (OECD TG 455). Toxicol Res 27:181–184. doi: 10.5487/TR.2011.27.3.181

Kim TS, Yoon CY, Jung KK, et al (2010) In vitro study of Organization for Economic Co-operation and Development (OECD) endocrine disruptor screening and testing methods- establishment of a recombinant rat androgen receptor (rrAR) binding assay. J Toxicol Sci 35:239–243. doi: 10.2131/jts.35.239

Kim YR, Jung E-M, Choi K-C, Jeung E-B (2012b) Synergistic effects of octylphenol and isobutyl paraben on the expression of calbindin-D9k in GH3 rat pituitary cells. Int J

Mol Med 29:294–302. doi: 10.3892/ijmm.2011.823
Kjaerstad MB, Taxvig C, Andersen HR, Nellemann C (2010) Mixture effects of endocrine disrupting compounds in vitro. Int J Androl 33:425–433. doi: 10.1111/j.1365-2605.2009.01034.x
Klimisch H-J, Andreae M, Tillmann U (1997) A Systematic Approach for Evaluating the Quality of Experimental Toxicological and Ecotoxicological Data. Regul Toxicol Pharmacol 25:1–5. doi: 10.1006/rtph.1996.1076
Koda T, Umezu T, Kamata R, et al (2005) Uterotrophic effects of benzophenone derivatives and a p-hydroxybenzoate used in ultraviolet screens. Environ Res 98:40–45. doi: 10.1016/j.envres.2004.05.015
Lassurguere J, Livera G, Habert R, Jégou B (2003) Time- and Dose-Related Effects of Estradiol and Diethylstilbestrol on the Morphology and Function of the Fetal Rat Testis in Culture. Toxicol Sci 73:160–169. doi: 10.1093/toxsci/kfg065
Lehraiki A, Chamaillard C, Krust A, et al (2011) Genistein impairs early testosterone production in fetal mouse testis via estrogen receptor alpha. Toxicol Vitr 25:1542–1547. doi: 10.1016/j.tiv.2011.05.017
Lemini C, Hernández A, Jaimez R, et al (2004) Morphometric analysis of mice uteri treated with the preservatives methyl, ethyl, propyl, and butylparaben. Toxicol Ind Health 20:123–132. doi: 10.1191/0748233704th202oa
Lemini C, Jaimez R, Ávila ME, et al (2003) In vivo and in vitro estrogen bioactivities of alkyl parabens. Toxicol Ind Health 19:69–79. doi: 10.1191/0748233703th177oa
Leppert B, Strunz S, Seiwert B, Schlittenbauer L, Schlichting R, Pfeiffer C, Röder S, Bauer M, Borte M, Stangl GI, Schöneberg T, Schulz A, Karkossa I, Rolle-Kampczyk UE, Thürmann L, von Bergen M, Escher BI, Junge KM, Reemtsma T, Lehmann I, Polte T. (2020) Maternal paraben exposure triggers childhood overweight development. Nat Commun. Feb 11;11(1):561. doi: 10.1038/s41467-019-14202-1.
Lester C, Hewitt NJ, Müller-Vieira U, Mayer M, Ellison C, Duplan H, Genies C, Jacques-Jamin C, Fabian E, Sorrell I, Lange D, Schepky A, Grégoire S. (2021) Metabolism and plasma protein binding of 16 straight- and branched-chain parabens in in vitro liver and skin models. Toxicol In Vitro. 2021 Apr;72:105051. doi: 10.1016/j.tiv.2020.105051. PMID: 33188879.
Li W, Guo J, Wu C, Zhang J, Zhang L, Lv S, Lu D, Qi X, Feng C, Liang W, Chang X, Zhang Y, Xu H, Cao Y, Wang G, Zhou Z. (2020) Effects of prenatal exposure to five parabens on neonatal thyroid function and birth weight: Evidence from SMBCS study. Environ Res. Sep;188:109710. doi: 10.1016/j.envres.2020.109710
Martin O V., Shialis T, Lester JN, et al (2008) Testicular Dysgenesis Syndrome and the Estrogen Hypothesis: A Quantitative Meta-Analysis. Environ Health Perspect 116:149–157. doi: 10.1289/ehp.10545
Maske P, Dighe V, Mote C, Vanage G (2020) n-Butylparaben exposure through gestation and lactation impairs spermatogenesis and steroidogenesis causing reduced fertility in the F1 generation male rats. Environ Pollut 256:112957. doi: 10.1016/j.envpol.2019.112957
McLachlan JA (2001) Environmental Signaling: What Embryos and Evolution Teach Us About Endocrine Disrupting Chemicals. Endocr Rev 22:319–341. doi: 10.1210/edrv.22.3.0432
Meng F, Jiao X, Chen F, et al (2020) Isobutylparaben Negatively Affects Porcine Oocyte Maturation Through Increasing Oxidative Stress and Cytoskeletal Abnormalities. Environ Mol Mutagen 61:433–444. doi: 10.1002/em.22356
Moos RK, Angerer J, Dierkes G, Brüning T, and Koch HM. (2016) Metabolism and elimination of methyl, iso- and n-butyl paraben in human urine after single oral dosage. Arch Toxicol 2016;90:2699–2709. doi: 10.1007/s00204-015-1636-0
Nielsen M, Bjornsdottir S, Hoyer P, Byskov A (2000) Ontogeny of oestrogen receptor alpha in gonads and sex ducts of fetal and newborn mice. J Reprod Fertil 118:195–204. doi: 10.1530/jrf.0.1180195
Obringer C, Wu S, Troutman J, Karb M, Lester C. (2021) Effect of chain length and branching on the in vitro metabolism of a series of parabens in human liver S9, human skin S9, and human plasma. Regul Toxicol Pharmacol. 2021 Jun;122:104918. doi:

10.1016/j.yrtph.2021.104918. PMID: 33741472.

Okubo T, Yokoyama Y, Kano K, Kano I (2001) ER-dependent estrogenic activity of parabens assessed by proliferation of human breast cancer MCF-7 cells and expression of ERα and PR. Food Chem Toxicol 39:1225–1232. doi: 10.1016/S0278-6915(01)00073-4

Oliviera MM, Martins F, Silva MG, Correia E, Videira R, Peixoto F (2020). Use of Parabens (Methyl and Butyl) during the Gestation Period: Mitochondrial Bioenergetics of the Testes and Antioxidant Capacity Alterations in Testes and Other Vital Organs of the F1 Generation. Antioxidants (Basel). 2020 Dec 18;9(12):1302. doi: 10.3390/antiox9121302.

Pugazhendhi D, Pope GS, Darbre PD. (2005) Oestrogenic activity of p-hydroxybenzoic acid (common metabolite of paraben esters) and methylparaben in human breast cancer cell lines. J Appl Toxicol. Jul-Aug;25(4):301-9. doi: 10.1002/jat.1066. PMID: 16021681.

Ouedraogo G, Alexander-White C, Bury D, Clewell HJ 3rd, Cronin M, Cull T, Dent M, Desprez B, Detroyer A, Ellison C, Giammanco S, Hack E, Hewitt NJ, Kenna G, Klaric M, Kreiling R, Lester C, Mahony C, Mombelli E, Naciff J, O'Brien J, Schepky A, Tozer S, van der Burg B, van Vugt-Lussenburg B, Stuard S, Cosmetics Europe. (2022) Read-across and new approach methodologies applied in a 10-step framework for cosmetics safety assessment - A case study with parabens. Regul Toxicol Pharmacol. 2022 Jul;132:105161. doi: 10.1016/j.yrtph.2022.105161. PMID: 35508214.

QSAR, Danish (Q)SAR Database (2022), http://qsar.food.dtu.dk, assessed 10. June, 2022

Riad MA, Abd-Rabo MM, Abd El Aziz SA, et al (2018) Reproductive toxic impact of subchronic treatment with combined butylparaben and triclosan in weanling male rats. J Biochem Mol Toxicol 32:e22037. doi: 10.1002/jbt.22037

Roberts GK, Waidyanatha S, Kissling GE, Fletcher BL, Silinski MAR, Fennell TR, Cunny HC, Robinson VG, Blystone CR. (2016) Exposure to butyl paraben during gestation and lactation in Hsd:Sprague dawley SD rats via dosed feed. Toxicol Rep. 2016 Sep 11;3:774-783. doi: 10.1016/j.toxrep.2016.09.004. PMID: 28959604; PMCID: PMC5616079.

Routledge EJ, Parker J, Odum J, et al (1998) Some Alkyl Hydroxy Benzoate Preservatives (Parabens) Are Estrogenic. Toxicol Appl Pharmacol 153:12–19. doi: 10.1006/taap.1998.8544

Sathyanarayana S, Swan SH, Farin FM, et al (2012) A pilot study of the association between genetic polymorphisms involved in estrogen signaling and infant male genital phenotypes. Asian J Androl 14:766–772. doi: 10.1038/aja.2012.27

Satoh K, Nonaka R, Ohyama K, Nagai F (2005) Androgenic and Antiandrogenic Effects of Alkylphenols and Parabens Assessed Using the Reporter Gene Assay with Stably Transfected CHO-K1 Cells (AR-EcoScreen System). J Heal Sci 51:557–568. doi: 10.1248/jhs.51.557

SCCS Scientific Committee on Consumer Safety (2010) SCCS/1348/10 Opinion on parabens. 14 December 2010, revision of 22 March 2011. http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_041.pdf

SCCS Scientific Committee on Consumer Safety (2011) Clarification on Opinion SCCS/1348/10 in the light of the Danish clause of safeguard banning the use of parabens in cosmetic products intended for children under three years of age. http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_069.pdf

SCCS Scientific Committee on Consumer Safety (2013) Opinion on parabens, 3 May 2013. Updated request for a scientific opinion on propyl- and butylparaben.

Schwartz CL, Christiansen S, Vinggaard AM, et al (2019) Anogenital distance as a toxicological or clinical marker for fetal androgen action and risk for reproductive disorders. Arch Toxicol 93:253–272. doi: 10.1007/s00204-018-2350-5

Shaw J, DeCatanzaro D (2009) Estrogenicity of parabens revisited: Impact of parabens on early pregnancy and an uterotrophic assay in mice. Reprod Toxicol 28:26–31. doi: 10.1016/j.reprotox.2009.03.003

Sivaraman L, Pouliot L, Wang B, et al (2018) Safety assessment of propylparaben in juvenile rats. Regul Toxicol Pharmacol 92:370–381. doi: 10.1016/j.yrtph.2017.12.009

Stewart MK, Mattiske DM, Pask AJ (2018) In utero exposure to both high- and low-dose diethylstilbestrol disrupts mouse genital tubercle development†. Biol Reprod 99:1184–1193. doi: 10.1093/biolre/ioy142

Taxvig C, Vinggaard AM, Hass U, et al (2008) Do Parabens Have the Ability to Interfere with Steroidogenesis? Toxicol Sci 106:206–213. doi: 10.1093/toxsci/kfn148

Terasaki M, Kamata R, Shiraishi F, Makino M (2009) Evaluation of estrogenic activity of parabens and their chlorinated derivatives by using the yeast two-hybrid assay and the enzyme-linked immunosorbent assay. Environ Toxicol Chem 28:204–208. doi: 10.1897/08-225.1

Thürmann L, Herberth G, Seiwert B, Schlittenbauer L, Rolle-Kampczyk U, Röder S, Sack U, Borte M, von Bergen M, Trump S, Reemtsma T, Lehmann I. (2021) Prenatal paraben exposure and atopic dermatitis-related outcomes among children. Allergy. Oct;76(10):3122-3132. doi: 10.1111/all.14890.

Vo TTB, AN B-S, YANG H, et al (2012) Calbindin-D9k as a sensitive molecular biomarker for evaluating the synergistic impact of estrogenic chemicals on GH3 rat pituitary cells. Int J Mol Med 30:1233–1240. doi: 10.3892/ijmm.2012.1122

Vo TTB, Jeung E (2009) An Evaluation of Estrogenic Activity of Parabens Using Uterine Calbindin-D9k Gene in an Immature Rat Model. Toxicol Sci 112:68–77. doi: 10.1093/toxsci/kfp176

Vo TTB, Jung E-M, Choi K-C, et al (2011) Estrogen receptor α is involved in the induction of Calbindin-D9k and progesterone receptor by parabens in GH3 cells: A biomarker gene for screening xenoestrogens. Steroids 76:675–681. doi: 10.1016/j.steroids.2011.03.006

Vo TTB, Yoo Y-M, Choi K-C, Jeung E-B (2010) Potential estrogenic effect(s) of parabens at the prepubertal stage of a postnatal female rat model. Reprod Toxicol 29:306–316. doi: 10.1016/j.reprotox.2010.01.013

Watanabe Y, Kojima H, Takeuchi S, et al (2013) Comparative study on transcriptional activity of 17 parabens mediated by estrogen receptor α and β and androgen receptor. Food Chem Toxicol 57:227–234. doi: 10.1016/j.fct.2013.03.036

WHO/IPCS (2002) World Health Organization, International Programme on Chemical Safety. Global assessment of the state-of-the-science of endocrine disruptors. WHO/PCS/EDC/02.2

Yang H, Nguyen T-T, An B-S, et al (2011) Synergistic effects of parabens on the induction of calbindin-D 9k gene expression act via a progesterone receptor-mediated pathway in GH3 cells. Hum Exp Toxicol 31:134–144. doi: 10.1177/0960327111422402

Yang Y-J, Hong Y-P, Chae SA (2016) Reduction in semen quality after mixed exposure to bisphenol A and isobutylparaben in utero and during lactation periods. Hum Exp Toxicol 35:902–911. doi: 10.1177/0960327115608927

Zhang L, Ding S, Qiao P, et al (2016) n- butylparaben induces male reproductive disorders via regulation of estradiol and estrogen receptors. J Appl Toxicol 36:1223–1234. doi: 10.1002/jat.3291

Zhang L, Dong L, Ding S, et al (2014) Effects of n-butylparaben on steroidogenesis and spermatogenesis through changed E2 levels in male rat offspring. Environ Toxicol Pharmacol 37:705–717. doi: 10.1016/j.etap.2014.01.016

References for Part II

ECHA, European Chemicals Agency (2020) Member State Committee support document for identification of butyl 4-hydroxybenzoate as a substance of very high concern because of its endocrine disrupting properties (article 57(f) – human health). Adopted on 28 May 2020. European Chemical Agency

ECHA, European Chemicals Agency (2022) Isobutyl 4-hydroxybenzoate, information on regularity risk management action published on ECHA's website, https://echa.europa.eu/da/assessment-regulatory-needs/-/dislist/details/0b0236e185d60683 (accessed 19 August 2022)

Annex I - Additional information on read across approach

A read-across approach can be applied using substances with physicochemical and/or toxicological properties that are likely to be similar to the substance in question or, follow a regular pattern as a result of structural similarity. Below it is specified why all of these conditions are met in the proposed read-across from source substance BP to target substance IBP, with the overall aim of filling a data gap for adverse effects of IBP.

In general, the read-across approach can be applied in the assessment of a property of a target substance when the source substance(s) in relation to the endpoint under assessment are likely to be similar or follow a regular pattern as a result of structural similarity. According to ECHA's practical guide "How to use alternatives to animal testing: Section 4.4. Read-across and categories" (ECHA 2016) similarities may be due to a common functional group, common precursor or breakdown products, constant pattern in changing potency or common constituents or chemical class.

All of these conditions are met in the proposed read-across from source substance BP to target substance IBP. The read-across is supported by the structural similarity of the substances BP and IBP (**Table 9**) and similar physico-chemical properties (**Table 10**), estrogenic activity and potency observed *in vitro* and *in vivo* (**Table 11** and **Table 12**). The read-across approach follows the principles of the Read Across Assessment Framework (RAAF) (ECHA, 2017).

Structural similarities
The group of parabens consists of closely related substances with very similar structures and properties as shown in Table 10. The generic structure of parabens is presented in Figure 4 where R indicates an alkyl group with varying numbers of carbon (**Table 9**).

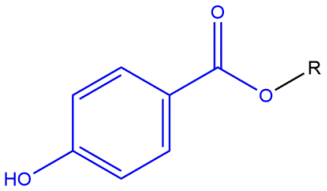

Figure 4: Paraben phenolic acids. A generic structure wherein R is an alkyl group with 1 to 4 carbons.

Table 9: Structural information and octanol/water partition coefficient for six parabens. Data from (CIR 2008) with reference to (Golden et al. 2005).

	MP	**EP**	**IPP**	**PP**	**IBP**	**BP**
Carbons in alkyl chain	1	2	3 (branched)	3	4 (branched)	4
Alkyl chain backbone length	1	2	2	3	3	4
Molecular weight (g/mol)	152.16	166.18	180.21	180.22	194.25	194.23
Octanol/water partition coefficient	1.66	2.19	2.71	2.91	3.4	3.24

MP = methylparaben, EP = ethylparaben, IPP = isopropylparaben, PP = propylparaben, IBP = isobutylparaben, BP = butylparaben.

Chemical and physical properties
Additional physical-chemical properties of six parabens are presented in Table 10. Based on the data provided in **Table 9** and Table 10, it can clearly be seen that the chemical and physical properties of IBP and BP are quite similar in many respects.

Table 10: Chemical and physical properties of six parabens.
Data modified from (CIR 2017) and (Cherian *et al*. 2020)

	MP	**EP**	**IPP**	**PP**	**IBP**	**BP**
Physical Form	Powder Liquid	Crystals or powder		Crystal or powder	Solid, powder	Crystals or powder
Colour	White or colourless	Colourless or white		Colourless or white	White	White
Odour	Characteristic			Odourless or faint		Odourless
Molecular Weight g/mol	152.16	166.18	180.22	180.21	194.25	194.23
Density g/cm3 at 137.2oC at 20˚C	1.1208 1.209±0.06	1.291		1.0630 1.28	1.105±0.06	
Vapor pressure mmHg at 25˚C	$2.37x10^{-4}$	$9.29x10^{-5}$		5.55x10-4	0.000381	1.86x10-4
Melting Point ˚C	131 125-128	116-118 115-118	96-97	96.2-98 95-98	72.95	68-69 68-72
Boiling Point ˚C	270-280 265 140-141	297-298	294	294 271	302.3 ± 15.0	309.2 ± 15.0
Water Solubility g/L at 25˚C	$2.50x10^{3}$ Slightly soluble	0.885		0.0500 Insoluble	2.24	0.0027x102 Insoluble
Other Solubility	Very soluble Slightly soluble Very soluble Slightly soluble (Alcohol Benzene Ether Glycerin)	Very soluble Very soluble Slightly soluble (Alcohol, Ether, Glycerin)		Soluble Soluble (Alcohol, Ether)		Soluble Soluble Slightly soluble (Alcohol, Ether, Glycerin)
log Kow	1.93	2.47 2.27		2.34 2.81	3.04e (log Pow)	
Disassociation constants (pKa) at 25˚C	8.17 8.31±0.13			8.35		8.37 8.47

MP = methylparaben, EP = ethylparaben, IPP = isopropylparaben, PP = propylparaben, IBP = isobutylparaben, BP = butylparaben.

Toxicologic profile
Below, the read-across justification shows that IBP has similar toxicological effects as BP in *in vitro* studies on endocrine activity and in mechanistic *in vivo* screening studies.

A comparison of endocrine activity *in vitro* and *in vivo* of parabens with an alkyl group of one to four carbons is presented in Table 11 and **Table 12**. This overview points to a similar profile of endocrine activity *in vitro* and *in vivo* of BP and IBP.

Overall, data on metabolism support the proposed read-across from source substance BP to target substance IBP. As mentioned previously, a common precursor or breakdown products is an important aspect in the read-across assessment (ECHA 2016). Upon uptake the parabens are hydrolysed to the same common metabolite PHBA, by which a large portion is excreted as p-hydroxyhippuric acid, the glycine conjugate of PHBA (Aubert 2009, Aubert *et al.,* 2012; Boberg *et al.* 2016). See 4.10.B. Read-across justification summary and 4.1 Toxicokinetics for more information.

Table 11: Overview of in vitro endocrine activity of six parabens.
Potency of the compounds in the different *in vitro* assays increases with colour change from white (no effect) through light grey to dark grey (5 levels of effects in total). Thus, the colour code indicates the strength of effect.

		MP	**EP**	**IPP**	**PP**	**IBP**	**BP**
Darbre *et al.* 2002	**ERα** competition binding (% [3H]oestradiol binding inhibition) at 100 000; 500 000; 1 000 000-fold molar excess					81%, 86%, 87%	60%, 80%, 86%
Vo *et al.* 2010	**ERα** relative binding affinities (50 % inhibitory concentration (IC50) of 17b - estradiol/IC50 of parabens)×100, IC50 = conc. that inhibits binding of Fluormone ES2 to ER by 50%)		0.006	0.019	0.016	0.144	0.06
	ERβ relative binding affinities (IC50 of 17b - estradiol/IC50 of parabens)×100, IC50 = conc. that inhibits binding of Fluormone ES2 to ER by 50%)		0.005	0.017	0.018	0.11	0.057
Okubo *et al.* 2001	Relative **proliferative potency** in MCF7 cells (is the ratio of C_{max} of the test compound to that of 17b-estradiol. So the larger the number, the more potent the substance)	1.5x10^-7	1.5x10^-6	6x10^-5	1.5x10^-6	6x10^-5	1.5x10^-6
	ERα Relative binding affinity (IC50 of DES/IC50 of competitor)x100, DES=100)		0.011	0.04	0.033	0.11	0.053
	ERβ Relative binding affinity (IC50 of DES/IC50 of competitor)x100, DES=100)		0.011	0.054	0.044	0.093	0.123
Kim *et al.* 2011	**ERα** (Stably Transfected Human ER-α transcriptional activation assay), **-Log Relative transcriptional activation** (100 × (PC50) of 17-beta-estradiol (E2)/(PC50) of test compound, PC50 = the concentration of chemical estimated to cause 50% of activity of the positive control response on a plate by plate basis.) **17b-estradiol = 2**		−2.64016	−2.73993	−2.84164	−2.34008	−1.63752

Vo *et al.* 2011	Relative CaBP-9k gene expression, Rat GH3 cells lowest observed effect concentration (LOEC) values (M)	Low increase, LOEC=10^{-4}	Low increase, LOEC=10^{-4}	High increase, LOEC=10^{-5}	Low increase, LOEC=10^{-5}	High increase, LOEC=10^{-5}	High increase, LOEC=10^{-5}
	Relative CaBP-9k protein expression, Rat GH3 cells. LOEC values (M)	Low increase, LOEC=10^{-5}	Low increase, LOEC=10^{-7}	Medium increase, LOEC=10^{-6}	Medium increase, LOEC=10^{-5}	High increase, LOEC=10^{-7}	Medium increase, LOEC=10^{-6}
	Relative PR (progesterone receptor) gene expression LOEC values (M)	Low increase, LOEC=10^{-5}	Low increase, LOEC=10^{-5}	High increase, LOEC=10^{-5}	Low increase, LOEC=10^{-5}	High increase, LOEC=10^{-7}	High increase, LOEC=10^{-7}
	Relative PR protein expression LOEC values (M)	No effect	No effect	Medium expression, LOEC=10^{-6}	Medium expression, LOEC=10^{-5}	High expression, LOEC=10^{-5}	Medium increase, LOEC=10^{-4}
	Relative ERα gene expression	No effect	No effect	No effect	No effect	No effect	No effect
	Relative ERα protein expression	N effect	No effect	No effect	No effect	No effect	No effect
	Estrogen response element (ERE) activity in GH3 cells			Medium activity	Medium activity	High activity	Medium activity
Yang *et al.* 2011	**Relative CaBP-9k gene expression**, Rat GH3 cells	small increase	small increase	small increase	small increase	large increase	large increase
	Relative CaBP-9k protein expression, Rat GH3 cells	small increase	small increase	large increase	large increase	large increase	large increase
	Relative PR (Progesterone Receptor)-B gene expression, Rat GH3 cells	small increase	small increase	medium increase	small increase	large increase	large increase
	Relative PR-B protein expression, Rat GH3 cells	no effect	no effect	no effect	no effect	no effect	no effect
Gonzalez *et al.* 2018	**Proliferation,** EC50 (μM): in breast cancer cell lines (MCF-7 cells)					0.3	1.2
	Proliferation mediated by ERα, IC50 (nM)**:** Blocking of proliferation with ER agonist at identified EC50s for proliferation (a higher number means that more of the inhibitor was needed to induce inhibition - hence the paraben is more potent)					1.4	0.8
	Estrogenic response. Induction of estrogen regulated gene GREB1 in ER positive MCF7 cells					~ 36 fold induction	~ 30 fold induction
Terasaki *et al*. 2009	**ERα agonism** (yeast two-hybrid assay). Data reported as EC x 10 = effective concentration test solution producing a chemiluminescent signal 10-fold that of the blank control. The smaller the value the more potent chemical.	No effect	No effect	2200 ± 350	4100 ± 270	680 ± 160	2300 ± 340

	ERα competitive binding. Enzyme linked immunosorbent assay (ELISA). Data reported as IC50 (nM)	No effect	No effect	4600 ± 1000	31000 ± 10000	3600 ± 850	20000 ± 2600
Watanabe *et al.* 2013	**ERα reporter gene assay** (human): Tested 10^-8 to 10-5 M. Data reported as 20% relative effect concentration (REC20): The concentration of the test compound showing agonistic activity equivalent to 20% of that of 10^-9 M E2.	No effect	4.3 x 10^-6	3.0 x 10^-7	6.1 x 10^-7	1.2 x 10^-7	2.9 x 10^-7
	ERβ reporter gene assay (human): Tested 10^-8 to 10-5 M. Data reported as 20% relative effect concentration (REC20)	No effect	1.2 x 10^-6	1.6 x 10^-7	1.7 x 10^-7	4.3 x 10^-8	1.5 x 10^-7
Kjaerstad *et al.* 2010	**AR agonism**, AR-transfected Chinese Hamster Ovary (CHO) cells	no effect	no effect	no effect	no effect	no effect	no effect
	AR antagonism, AR-transfected CHO cells	no effect	no effect		no effect	weak effect	no effect
Satoh *et al.* 2005	**AR inhibition**, IC50: Concentration of test chemical for 50% reduction in dihydrotestosterone (DHT) induced luciferase activity. AR agonism: No effect of any chemicals at doses tested	>1x10^-4	>1x10^-4	4.2x10^-5	8.6x10^-5	7.6x10^-5	6.8x10^-5
	AR-binding, IC50: Concentration of test chemical for 50% reduction in testosterone binding. They only observed 40% at 1.9x10^-4 and did not test higher concentrations	No effect	No effect	>1.9x10^-4	>1.9x10^-4	>1.9x10^-4	>1.9x10^-4
Kim *et al.* 2010	**AR (rat recombinant) relative binding affinity** (DHT=100)				0.0019	0.0058	0.0029
Fujino *et al.* 2019	**PXR reporter gene assay** (human): Tested 0.3-30 µM, LOEC reported (µM). Positive control (rifampicin) LOEC = 1µM)	No effect	30	30	No effect	10	10
	PXR reporter gene assay (rat): Tested 0.03-30 µM, LOEC reported (µM). Positive control (PCN) LOEC = 0.1 µM	No effect	30	30	30	10	10
	CAR reporter gene assay (rat): Tested 1-30 µM, LOEC reported (µM). Positive control (artemisinin) LOEC = 3 µM	No effect	30	30	No effect	10	30
	PPARα reporter gene assay (rat): Tested 1-30 µM, LOEC reported (µM). Positive control (bezafibrate) LOEC = 10 µM	1	No effect	30	3	10	30

Watanabe *et al.* 2013	**CAR reporter gene assay** (yeast two hybrid assay). Data reported as EC x 10 = effective concentration test solution producing a chemiluminsecent signal 10-fold that of the blank control. The smaller the value the more potent chemical.				7400 ± 830	3300 ± 330	

MP = methylparaben, EP = ethylparaben, IPP = isopropylparaben, PP = propylparaben, IBP = isobutylparaben, BP = butylparaben.

Table 12: Overview of *in vivo* endocrine activity and adverse effects of six parabens. Estrogenic potency of the compounds increases with colour change from light grey to dark grey (5 levels of effects in total), thus the colour code indicates the strength of effect. Most data are adapted from (Boberg *et al.* 2010).

		MP	EP	IPP	PP	IBP	BP
***In vivo* estrogenic.** Doses in mg/kg bw/day, **s.c.** exposure unless otherwise stated.							
Vo and Jeung 2009	Immature rats, uterotrophic			NOEL 250 LOEL 1000		NOEL 250 LOEL 1000	NOEL 250 LOEL 1000
Darbre *et al.* 2002	Immature mice, uterotrophic					LOEL 72	
Koda *et al.* 2005	Ovx rats uterotrophic					NOEL 100 LOEL 250	
Lemini *et al.* 2003	Immature rats uterotrophic	NOEL 16.5 LOEL 55	NOEL 60 LOEL 180		NOEL 20 LOEL 65		NOEL 20 LOEL 70
Lemini *et al.* 2003	Immature mice uterotrophic	NOEL 5.5 LOEL 16.5	NOEL 18 LOEL 60		NOEL 6.5 LOEL 20		NOEL 0.7 LOEL 7
Lemini *et al.* 2003	Ovx mice uterotrophic	NOEL 55 LOEL 165	NOEL 6 LOEL 18		NOEL 6.5 LOEL 20		NOEL 7 LOEL 21
Lemini *et al.* 2004	Ovx mice uterotrophic	LOEL 55	LOEL 60		LOEL 65		LOEL 70
Routledge *et al.* 1998	Immature rat uterotrophic	No effect up to 80 (s.c.) or 800 (oral)					NOEL 200 LOEL 400
Hossaini *et al.* 2000	Immature mice uterotrophic	No effect at 100 (s.c.) or at 1, 10, 100 (oral)	No effect at 100 (s.c.) or 1000 (oral)	No effect at 100 (s.c.) or at 1, 10, 100 (oral)			No effect at 100 (s.c.)
	Immature rats, uterotrophic						LOEL 100 in immature rats (s.c.)
Shaw and DeCatanzaro 2009	Ovx mice uterotrophic						No effect up to 1000 (23% increase, non-significant)
Ahn *et al.* 2012	Rat PND 1-7, uterus weight PND8	No effect at 62.5, 250, 1000			No effect at 62.5, 250, 1000		NOEL 250 LOEL 1000
Sivaraman *et al.* 2018	Immature rats uterotrophic				No effect (oral) (29% increase, non-significant)		
Pubertal assay or related. Doses in mg/kg bw/day unless otherwise stated.							
Vo *et al.* 2010	Female pubertal assay in rats, s.c.	No change in VO or uterine epithelium thickness at 62.5, 250 or 1000	No change in VO or uterine epithelium thickness at 62.5, 250 or 1000	Delay in VO at 250 and 1000, increased uterine epithelium thickness at 1000		No change in VO, increased uterine epithelium thickness at 62.5, 250 and 1000	No change in VO, increased uterine epithelium thickness at 62.5, 250 and 1000
Sivaraman *et al*. 2018	Rats PND 4-90, oral gavage, n=10-25				Early VO, slight delay PPS at 1000 (NOEL 100), no change in estrous cycle or fertility		

MP = methylparaben, EP = ethylparaben, IPP = isopropylparaben, PP = propylparaben, IBP = isobutylparaben, BP = butylparaben.

Annex II – Detailed study information on IBP and BP

In Annex II, all relevant information on *in vitro* studies (Table 13) and *in vivo* studies (**Table 14**) on IBP is presented. This is followed by a table presenting selected relevant *in vivo* results from source substance BP (**Table 15**)

In vitro studies on IBP

Table 13: Study overview, *in vitro* studies on IBP incl. Comptox and QSAR. Effects described are statistically significant unless otherwise indicated. Reliability has been assessed using Klimisch score.

Reference	Study design	Quick overview results	Results description	Study quality and assessment
Gonzalez *et al.* 2018	MCF-7 cells T47D cells LNCaP cells Proliferation: plated and allowed to attach overnight GREB1: MCF cells were treated for 2, 4 and 6h. ER transcription assay: 18 h exposure Proliferation: 10^{-7} - $10^{-4.5}$ M Androgenic properties: 10^{-11} - 10^{-5} M MDA-MB-231: 10^{-8} - 10^{-5} M (IBP), 10^{-7} - $10^{-4.5}$ M (2OH) ICI 182, 780: 10^{-12} - 10^{-6} M GREB1: 10 µM IBP or 2OH ER-transcription assay: 10, 20 µM (E2 positive control 100 nM) IBP: ≥ 98% 2OH: ≥ 95%	IC50 =0.3 µM Proliferation MCF-7 and T47D cells: EC50: 0.3 µM but unclear if based on MCF-7 or T47D results. Androgenic properties (LNCaP) (suppl. mat) No effect Proliferation in cells lacking ERα (MDA-MB-231) (suppl. mat) No proliferation registered Treatment with Fulvestrant IBP: IC50 1.4 nM GREB1 expression E2: 29 fold induction IBP: 36 fold induction. ICI exposure reversed these effects ER transcription assay IBP: induction (data not shown) E2: induction (100 nM)	In MCF-7 cells proliferation was induced in a concentration dependent manner (EC50: 0.3 µM) after IBP exposure. IBP also showed effect in T47D cells. It is unclear if the given EC-value stems from MCF7 or T47D cells, but IBP showed proliferation in both cell lines. Non-ERα expressing cells (MDA-MB-231) showed no effect on cell proliferation after exposure to IBP, indicating requirement of ERα for signalling (supplementary material). To confirm that proliferation was induced via ERα, MCF-7 cells were treated with the identified EC50 values of IBP and increasing concentrations of the anti-estrogen Fulvestrant (ICI 182, 780). The proliferation of IBP was inhibited at 1.4 nM. The gene expression level of *GREB1*, a critical downstream target of ERα signalling, was investigated in MCF-7 cells after exposure. E2 was used as positive control (induced a 29 fold expression of *GREB1*) and IBP induced GREB1 expression at 36-fold, compared to vehicle control. Co-treatment with Fulvestrant blocked the effects. Estrogenic activity was also confirmed in an ER-dependent transcription assay using an ERE-luciferase reporter construct. IBP induced transcription (data not shown). E2 used as positive control, high induction at 100 nM (approximately 5 fold). No cytotoxicity was seen.	*Reliability 2.* - Acceptable, generally well-documented study, relevant controls included. Not too high vehicle (ethanol or DMSO (Dimethyl sulfoxide) concentrations (less than 0.1% (v/v)) Shortcomings: 6 technical replicates and no independent experiments and experiment with only one concentration of IBP. Inconsistent in reporting, but all information is available, however some without referral. Despite shortcomings, the authors examined the estrogenic activity with different methods. Proliferation was observed in estrogen dependent cells (two cell lines) and not in estrogen independent cells. IBP induced ER-mediated gene expression and ER reporter assay, effects which were reversed by ICI exposure.

		Cytotoxicity No effect		
Meng *et al.* 2020	Cumulus oocyte complexes (COCs). Porcine. 27 or 44 h 0, 100, 200, 300, 400, 600 µM IBP: > 97%	LOEC = 400 µM Blocked cumulus cell expansion and affected first polar body extrusion (400, 600 µM) Further studies at 400 µM: ↑ ratio of abnormal spindles ↑ misaligned chromosomes Disrupted F-actin cytoskeleton ↑ oxidative stress (fluorescence intensity) ↑apoptosis (fluorescence intensity) ↑ disrupted histone modification (fluorescence intensity)	The effect of IBP on cultured COCs from porcine ovaries was investigated. Morphology of COCs was affected by exposure - 400 and 600 µM IBP blocked cumulus cell expansion. First polar body extrusion was also affected at 400 and 600 µM. This indicates IBP could interfere with meiotic maturation. Further studies were conducted in COCs exposed to 400 µM IBP. In these samples, spindle morphology was disrupted and the ratio of abnormal spindles was significantly increased in exposed samples. Occurrence of misaligned chromosomes was also significantly increased in the IBP exposed group. The F-actin cytoskeleton also seemed to be disrupted in porcine oocytes and oxidative stress seemed significantly higher in IBP exposed group (fluorescence intensity of ROS). Significantly increased apoptosis was also seen (fluorescence intensity).	*Reliability 3.* - Acceptable vehicle (DMSO) concentration (less than 0.1% in medium). - Shortcomings: Several of the endpoints are quantified by fluorescence intensity. This can be a problematic measurement and requires the exact same exposure time for all samples. This is not described in the article. Also, the oxidative stress and apoptosis could be due to direct toxicity of the compound at the concentrations used. It is not possible to determine if this is the case based on the data.
Fujino *et al.* 2019	Human PXR (hPXR), rat PXR (rPXR), rat CAR (rCAR) and rat PPARα (rPPARα) reporter gene assays. Not reported in this publication, refers to a previous. PXR: 0, 0.3, 1, 3, 10, 30 µM rPXR: 0, 0.03, 0.1, 0.3, 1, 3, 10, 30 µM rCAR: 0, 1, 3, 10, 30 µM IBP: > 99% 4-HBA (same as PHBA; general metabolite from parabens): > 95%	LOEC = 10 µM hPXR: ↑ activity at 10 and 30 µM IBP No effect of 4-HBA (Positive control, rifampicin, from 1 µM) rPXR: ↑ activity at 10 and 30 µM IBP No effect of 4-HBA (Positive control, PCN, from 0.1 µM) rCAR: ↑ activity at 10 and 30 µM IBP No effect of 4-HBA (Positive control, artemisinin, from 3 µM) rPPARα: ↑ activity at 10 and 30 µM IBP No effect of 4-HBA (Positive control bezafibrate, from 10 µM) No cytotoxicity seen for IBP or	In this study, the authors investigated if IBP altered the hormone-metabolising activities via PXR, CAR and PPARα. The studies were conducted in hPXR as well as rPXR, rCAR and rPPARα reporter gene assays. For hPXR, IBP significantly increased relative activity of hPXR at 10 and 30 µM. For rPXR, IBP significantly increased relative activity at 10 and 30 µM. For rCAR, IBP significantly increased relative activity at 10 and 30 µM. For rPPARα bezafibrate induced relative activity from 10 µM. IBP significantly increased relative activity at 10 and 30 µM. No effects were seen in any of the assays after exposure to 4-HBA. No cytotoxicity seen for IBP and 4-HBA.	*Reliability 2.* - Acceptable. Repeated in three independent experiments. Inclusion of positive controls for all assays, and assessment of cell viability. - Shortcomings: DMSO used as vehicle, in controls the levels are appropriate at 0.1%, but concentration in IBP groups not clearly stated. Method description is very short, authors refer to other articles.

		4-HBA		
Terasaki *et al.* 2009	Reporter gene assay (yeast cells transfected with hERα) and hERα competitive binding assay Two-hybrid assay: 4 h at 30 °C Binding assay: 1 + 1 h + 20 min Two-hybrid: IBP: 16-1000 nM Binding assay: 3.8-38000 nM IBP: analytical grade or HPLC grade	Relative activity = 9.2 x 10^{-5} Relative activity (to E2) in yeast two hybrid assay IBP: 2.0 x 10^{-4} Relative activity (to DES) in competitive binding assay IBP: 2.8 x 10^{-3} Cytotoxicity evaluation not described in M&M or results.	In this study, the estrogenic activity of IBP was investigated in a yeast two-hybrid assay with human ERα and in a human ERα competitive binding assay. In the yeast two-hybrid assay, E2 was used as positive control. The activity of IBP, relative to E2, was 2.0 x 10^{-4}. IBP was the most potent paraben among several different parabens tested. In the competitive binding assay, diethylstilbestrol (DES) was used as positive control. The activity of IBP, relative to DES, was 2.8x10^{-3}. Description of testing cytotoxicity was not reported.	*Reliability 2.* - Acceptable. Two hybrid assay repeated in three independent experiments and hERa competitive assay in two independent experiments. This is acceptable. - Shortcomings: Vehicle concentration in medium not reported, cytotoxicity does not appear tested. This is not needed for the receptor binding assay. Cytotoxicity (not reported) could potentially affect results on receptor activity, however a complimentary study on receptor binding was performed, collectively supporting the notion that interference with estrogen receptor/signalling can be induced by IBP.
Watanabe *et al.* 2013	Reporter gene assay (CHO cells transiently transfected with hERα, hERβ or hAR) 24 hours 10^{-8} - 10^{-5} M > 99%	REC20 = 4.3 x 10-8 M <u>REC20</u> ERα: 1.2 x 10-7 M ERβ: 4.3 x 10-8 M AR: no agonistic or antagonistic activity E2 on ERα: 2.5 x 10-12 M E2 on ERβ: 5.3 x 10-12 M Hydroxy flutamide on AR: 1.8 x 10-8 M	In this study the activity of IBP on hERα, hERβ or hAR was investigated. CHO cells were transfected with plasmids for hERα, hERβ or hAR. In ER cells, E2 was used as positive control and in AR cells DHT was used as positive control and hydroxy flutamide as inhibitor. The results were reported as the concentration where test substances reached 20% of control compound activity (REC20 or RIC20). For ERα this was at 1.2 x 10-7 M, ERβ 4.3 x 10-8 M and for AR no activity was seen with IsoBP treatment. The activity of estradiol on ERα was 2.5 x 10-12 M, ERβ 5.3 x 10-12 M and for hydroxyflutamide on AR 1.8 x 10-8 M. The data showed that IBP is an agonist on both ERα and ERβ with a higher affinity towards ERβ. No cytotoxicity was seen at the tested concentrations.	*Reliability 2* - Acceptable - Generally well reported Vehicle (DMSO) concentrations acceptable (less than 0.1%). Cytotoxicity examined. Relevant positive controls included. Three independent experiments in duplicates.
Kamata *et al.* 2018	Reporter gene assay (yeast cells transfected with CAR) 4 hours at 30 °C 156 nM- 10 µM (minimum 7 different conc.) ≥ 90 %	EC x 10 = 3300 ± 330 nM <u>Positive control 4tOP:</u> ECx10 of 13 ± 4.0 nM Luminescence intensity at 10 nM of 7.9 ± 1.3 times that of the blank control <u>IBP:</u> EC x 10 of 3300 ± 330 nM Relative activity (compared to	In this study, 549 compounds, among these IBP, were screened for activity in a CAR reporter gene assay. Each compound was tested in minimum seven concentrations (156 nM- 10 µM). CAR has no known potent agonist, but based on a previous study 4-tert-octylphenol (4tOP) was used. 4tOP yielded and EC10 of 13 ± 4.0 nM and luminescence intensity at 10 nM of 7.9 ± 1.3 times that of the blank control. The EC10 for IBP was 3300 ± 330 nM and the relative activity (compared to 4tOP) was 0.0042 ± 0.0017.	*Reliability 2.* - Acceptable. Seven concentrations tested in duplicates. Positives from this initial screening were tested in two additional independent experiments. DMSO at concentration of 1%.

		4tOP) was 0.0042 ± 0.0017.		
Darbre *et al.* 2002	MCF7 & ZR-75-1 & MDA-MB-231 human breast cancer cells Gene expression: 24 hours, 7 days MCF7 and ZR-75-1 proliferation, MCF7 and ZR-75-1 IBP effect on estrogen-induced proliferation: 14 days MDA-MB-231 proliferation: 8 days Competition: binding of [3H]oestradiol at 16 × 10^−10 M and 1–1.000.000-fold molar excess of unlabeled compounds. MCF7 proliferation: 10^-11 to 10^-4 M Gene expression, ZR-75-1 proliferation, MDA-MB-231 proliferation, and MCF7 and ZR-75-1 IBP effect on estrogen-induced proliferation: 10^-9 to 10^-4	LOEL: 10^-6 M Estrogen regulated genes↑ estrogen dependent growth of cell lines ↑ Binding of oestradiol to ERα.↓ Effects could be inhibited by addition of ER antagonist.	Estradiol ERα binding was inhibited by IBP. [3H]oestradiol binding could be inhibited by IBP by 81% at 100 000-fold molar excess, by 86% at 500 000-fold molar excess and by 87% at 1 000 000-fold molar excess. Stably transfected estrogen sensitive reporter gene ERE-CAT in MCF7 human breast cancer cells. CAT gene expression could be increased by IBP at concentrations of 10^−6 M, 10^−5 M and 10^−4 M. The magnitude of the effect of IBP at 10^−5 M was the same as for 17β-oestradiol at 10^−8 M. The same was found after 7 days of exposure (data not shown). pS2 gene (estrogen regulated) expression was increased by IBP: 10^−5 M could increase levels of pS2 mRNA after both 24 h and 8 days. Dose–response studies showed that IBP could increase pS2 levels weakly at 10^−6 M and strongly at 10^−5 M. The increase in pS2 mRNA by 10^−5 M IBP could be inhibited by the anti-estrogen ICI 182 780. IBP stimulated MCF7 and ZR-75-1 proliferation at 10^−6 M, 10^−5 M and 10^−4 M. The proliferation effect of 10^−5 M IBP could be inhibited by the pure anti-estrogen ICI 182 780. at 10−7 M There was no proliferation in MDA-MB-231 cells that lack ERα. IBP was assayed for its ability to antagonise the growth-promoting action of 10−10 M 17β-oestradiol. No significant antagonism of 10−10 M 17β-oestradiol action on cell growth was found, even when using 10^−4 M IBP in either MCF7 cells or ZR-75-1 cells. The in vitro studies investigated the estrogenic activity of isobutyl paraben at several levels, including at receptor, at gene and at a cellular physiologic response level. The in vitro part of the study provides strong evidence of an estrogenic mode of action of IBP.	*Reliability 2.* - Acceptable, generally well-documented study, relevant controls included. Not too high vehicle (ethanol) concentrations (1:10000 v/v). Several assays demonstrating a similar mechanism. - Shortcomings: CAS number and purity of IBP are not reported. IBP was a gift from Nipa Laboratories (Mid-Glamorgan, Wales). No reporting of cell viability with IBP exposure. Apparent low independent experiment number (1-2) with triplicates within same experiment Despite the low number of independent experiments, the authors examine the same MoA in several ways: ER alpha binding, ER mediated reporter expression and endogenous pS2 expression, increased proliferation in two estrogen dependent cell lines. ICI reversed the observed effects in several of the assays
Vo *et al.* 2010	ER competitive binding assay using a fluorescent estrogen, Fluormone ES2. Purity IBP unknown Test concentrations not reported	ERα IC50: 2.07×10^−6 M ERβ IC50: 2.75x10^−6 M competitive ligand binding assay ERα binding ↑ ERβ binding ↑	The binding affinity of IBP to ERα and ERβ was investigated. Isobutyl paraben showed affinity to both receptors and no preference to any of the receptors could be determined. The study provides weak evidence of an estrogenic mode of action of IBP.	*Reliability 2.* - The in vitro study is described very briefly and insufficiently as described below. However, the study reports results for E2, as a positive control, suggesting that the assay works. Shortcomings: - concentrations of E2 and parabens

				tested are not reported - The source and concentrations of ER alpha and beta added in the binding affinity experiment are not reported - exposure period is not reported - Purity of IBP is not reported. - Vehicle (ethanol) concentration is not reported
Okubo *et al.* 2001	MCF-7 cells Ligand Screening - ER (α) and (β) - System (Toyobo Co., Osaka, Japan) MCF-7 proliferation: 6 days Gene expression: 48 hrs. MCF-7 proliferation: 10^-7 to 10^-4 M Gene expression: 10^-5 M	EC50 MCF-7 proliferation: 6.8x10^-7 M LOEL gene expression and protein expression: 10^-5 M IC50 ERα competition binding: 2.7x10^-5 IC50 ERβ competition binding: 2.8x10^-5 MCF-7 proliferation: ↑ Addition of ER antagonist suppressed effect ERα gene expression, 48h ↓ PR expression, 24h and 48h ↑ ERα protein expression, 48h ↓ ERα binding ↑ ERβ binding ↑	Maximal MCF-7 proliferation (C_{max}) at 5x10^-6 M. EC50 MCF-7 proliferation: 6.8x10^-7 M. EC50 for 17b-estradiol: 1.6x10^-12 M. When various concentrations of ER antagonist ICI 182,780 were added in the presence of parabens, cell proliferative effects were also suppressed dose-dependently. IBP no effect on ERa expression at 24h, decrease at 48h. Gradual increase of PR expression, which reached about four to five times the control level at 24 and 48 h. IBP has much smaller effects than 17b-estradiol on gene expression of receptors, but they nevertheless similarly lower the level of ERa and raise that of PR. A small decrease in ERa protein level relative to control with parabens were observed. ERα and β binding by IBP had about 1000-fold lower affinity than DES. Based on the data on estrogenic activity of IBP at the gene, protein and cellular proliferation levels together with the study on affinity to human ERs, the study provides strong evidence of an estrogenic mode of action of isobutyl paraben.	*Reliability 2.* - Acceptable, generally well-documented study, relevant controls included. Not too high vehicle (ethanol) concentrations, the final concentration of ethanol in the culture medium did not exceed 0.1%, more than three independent experiments - It is not mentioned whether they assessed cytotoxicity, however since the IBP concentrations used induce proliferation, this implies limited cytotoxicity. There are indications of compromised viability with ICI at or above 10^-8 M as proliferation is reduced compared to vehicle control at these concentrations. - Shortcomings: CAS number and purity of isobutyl paraben is not reported.
Satoh *et al.* 2005	AR-Eco Screen, CHO-K1 cells AR competitive binding assay 24 hours, concentrations not stated but estimated to 1-100 µM based on graphs/99%	IC_{50}: $7.6x10^{-5}$ M for AR antagonism AR agonism: no effect AR binding: partial ↑	No cytotoxicity was observed at the tested concentrations. No AR agonistic activity was observed. IBP however, showed AR antagonistic activity with an IC_{50} of 7.6x10-5 M. IBP partially inhibited testosterone binding to the AR, about 40% at $1.9x10^{-4}$ M	*Reliability 2.* - Acceptable, generally well-documented study, relevant controls included and vehicle (DMSO) concentration at 0.1%. Cytotoxicity was monitored. Shortcomings: - Concentration range not reported, but based on graphs could be assumed to be 1-100 µM.
Kim *et al.* 2010	Recombinant rat AR binding assay ~24 hours ("over night")	IC_{50}: $3.1x10^{-4}$ M AR binding: ↑	Isobutyl paraben showed higher competitive affinity to AR than other parabens tested. The binding affinity relative to DHT was almost 17000 times lower for IBP.	*Reliability 2* - The protocol for the in vitro study is described in detail, triplicates were performed and purity of the

	Unknown but estimated around 10^{-4} to 10^{-3} M / 99%			compound is noted, however - concentrations tested are not reported, figures are of very low quality, hard to assess. - vehicle (likely DMSO) and concentration of vehicle is not described
Kim *et al.* 2011	hERα-HeLa-9903 cells transfected with human ERα and luciferase reporter. 20-24 hours 10^{-10} to 10^{-5} M	relative to positive control (E2) EC_{50}: $6.3x10^{-7}$ M ERα agonism: ↑	The study showed (22,000 fold) lower estrogenic activity of isobutyl paraben compared to 17β-estradiol. Compared to other parabens tested, IBP and BP were the two parabens with highest estrogenic activity.	*Reliability 2.* - Acceptable, generally well-documented study, relevant controls included giving responses comparable to OECD TG. Short comings: - vehicle (DMSO) concentration not reported. - Chemical purity not reported. - Cytotoxicity was not monitored. - number of replicates is not described. Despite shortcomings they test several concentrations and establish concentration-response relationship for IBP.
Vo *et al.* 2011	GH3 rat pituitary cells 24 hours 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} M / 99%	LOEL: 10^{-7} M CaBP-9k protein expression: ↑ (from 10^-7 M) CaBP-9k gene expression: ↑ (from 10^-5 M) ERα gene and protein expression: no statistically sign. Difference PR gene expression: ↑ (from 10^-7 M) PR protein expression: ↑ (from 10^-5 M) ERE activity: ↑ (at only dose tested 10^-4 M)	Expression of CaBP-9k gene was increased from 10^-5 M and protein was upregulated at all tested doses. An effect that was blocked by pre-incubation with ER antagonist ICI 182,780. ERα gene and protein expression showed no statistically significant difference after IBP exposure at all concentrations tested. The PR gene expression was upregulated in all the tested doses of IBP but only from 10^-5 M. This effect was also blocked with ICI. An ERE-luciferase construct showed that ER activity increased with 10^-4M IBP exposure and that this effect was partially blocked by ICI pre-treatment.	*Reliability 2.* - Final DMSO conc. 0.1% Shortcomings: - Generally errors in figures. 1, 2, 3 and 4 showing mRNA in both A and B sections instead of protein in A section. - Generally figures with errors in captions. - They discuss mRNA and protein together as one even though there are differences in the results - No cytotoxicity was examined Despite shortcomings, they report results similar to Kim et al 2012a and b which shows consistency in results.
Kim *et al.* 2012b	GH3 rat pituitary cells 24 hours 0.1, 1, 10 µM / 99%	LOEL: 0.1 µM CaBP-9k gene expression: ↑ (from 0.1 µM)	Expression of the ER reporter gene and expression of CaBP-9k gene (used as marker for estrogenic activity) and protein was upregulated at all tested doses of isobutyl paraben. An effect	*Reliability 2.* - Acceptable, generally well-documented study, relevant controls

		CaBP-9k protein expression: ↑ (from 0.1 µM) Progesterone receptor (PR) gene expression: ↑ (from 0.1 µM) PR protein expression: ↑ (0.1 µM) ER reporter gene expression: ↑(from 1 µM) (Kim *et al.* 2012a and b are two independent experiments)	that was blocked by pre-incubation with ER antagonist ICI 182,780. The PR gene and protein expression was also upregulated in all the tested doses of IBP. PR changes were also reversed with ICI exposure	included and vehicle (DMSO) concentration at 0.1%. They performed triplicate reactions. Shortcomings: - Cytotoxicity was not monitored - Number of repeats is a little unclear. They state they performed all experiments in triplicates for gene and protein expression and experiments were repeated at least four times for reporter gene assay
Kim *et al*. 2012a	GH3 rat pituitary cells 24 hours 0.1, 1 and 10 µM / 99%	LOEL: 10^{-7} M ER activation (luciferase reporter): ↑ (from 10^{-7} M) CaBP-9k gene expression: ↑ (from 10^{-7} M) CaBP-9k protein expression: ↑ (from 10^{-7} M) Progesterone receptor (PR) gene expression: ↑ (from 10^{-7} M) PR protein expression: ↑ (from 10^{-5} M)	Dose-dependent increase in ER activity based on luciferase reporter signal. Increased CaBP-9k gene and protein expression in all IBP doses tested. This effect could be blocked by addition of ER antagonist ICI 182,780. The PR gene and protein expression was also upregulated with IBP exposure. Pre-incubation with ICI blocked the PR increase.	*Reliability 2.* - Acceptable, generally well-documented study, relevant controls included and vehicle (DMSO) concentration at 0.1%. . Short comings: - Cytotoxicity was not monitored - Number of repeats is a little unclear. They state they performed all experiments in triplicates for gene and protein expression and experiments were repeated at least four times for reporter gene assay
Vo *et al.* 2012	GH3 rat pituitary cells 24 hours 10^{-7}, 10^{-6} and 10^{-5} M IBP purity is not reported but it was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan)	LOEL: 10^{-7} M CaBP-9k gene expression: ↑ (from 10^{-7} M) CaBP-9k protein expression: ↑ (from 10^{-7} M) Effect on CaBP-9k and PR reversible by ERa antagonist co-treatment. Progesterone receptor (PR) gene expression: ↑ (from 10^{-7} M) PR protein expression: ↑ (from 10^{-7} M) ERα gene expression: no difference in fig 5, but a significant decrease in fig 6. ERα protein expression: no difference	Increased CaBP-9k gene and protein expression in all IBP doses tested. This effect was blocked by pre-treatment with ICI 182,780 (fulvestrant). The PR gene and protein expression was also upregulated in all the tested doses of IBP. Pre-incubation with ICI blocked the PR increase. No difference in ERα gene expression was observed in the 1st experiment at all doses tested but a significant decrease was observed in a 2nd experiment using 10^{-5} M IBP.	*Reliability 2.* - Experiments generally well described and relevant negative and positive controls were included. All experiment repeated three times. The estrogenicity was measured in many ways, both at different molecular levels and with antagonist ICI Shortcomings: - IBP purity is not reported - vehicle (DMSO) centration is not reported - discrepancy between ERα gene expression across experiments. No induction in 1st experiment, a significant decrease ↓ in 2nd experiment. - No cell viability measured

Yang *et al.* 2011	GH3 rat pituitary cells 25 hours 10^{-5} M IBP purity is not reported but it was obtained from Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan).)	LOEL: 10^{-5} M CaBP-9k gene expression: ↑ CaBP-9k protein expression: ↑ ERα gene and protein expression: no difference Progesterone receptor B (PR-B) gene expression: ↑ PR-B protein expression: no difference	Increased CaBP-9k gene and protein expression and increased PR-B gene but not protein expression. No effects were observed on ERa gene or protein expression after IBP treatment. Pre-treatment with ER antagonist ICI 182,780 for 1 hour before paraben addition blocked all effects observed on CaBP and PR, but increased ERα gene and protein expression when given with a combination of IBP and BP.	*Reliability 2* - generally well described with relevant positive and negative controls included. - vehicle (DMSO) concentration at 0.1%. - experiments performed in triplicates Shortcomings: -IBP purity is not reported - single concentration tested
Kjaerstad *et al.* 2010	AR-transfected Chinese Hamster Ovary (CHO) cells Exposure time not clearly stated.0.025–50 uM/ 98% pure	LOEL: 25 µM AR antagonism: ↑ (≥25µM) AR agonism: no effect	The AR antagonistic effect of isobutylparaben was statistically significant at concentrations of 25 µM and above.	*Reliability 2.* - Acceptable, generally well-documented study, relevant controls included and cytotoxicity monitored. Purity of IBP is reported. Three independent experiments in quadruplicates. Shortcomings: - exposure time is not reported
CompTox 2022	In vitro AR activity		Two assays were performed, the TOX21_AR_BLA and the TOX21_AR_LUC_MDAKB2. Both assays were performed in agonist and antagonist mode and viability was examined. In both assays IBP was active for AR antagonism at non-cytotoxic concentrations, however in the TOX21_AR_LUC_MDAKB2 assay antagonism was only observed with the addition of 0.5 nM R1881 and not 10 nM R1881. No agonism was observed in the two assays.	
CompTox 2022	In vitro ERα activity		Two assays were performed, the TOX21_ERa_BLA and the TOX21_ERa_LUC_VM7. Both assays were performed in agonist and antagonist mode and viability was examined. In both assays IBP exhibited agonistic effects on receptor activity, whereas antagonism was only observed in the TOX21_ERa_BLA assay. There were no hit calls for compromised cell viability in the two assays.	
CompTox 2022	In vitro ERβ activity		One assay was performed, the TOX21_ERb_BLA. The assay was performed in agonist and antagonist mode and viability was examined. A positive hit call for antagonism was observed. No other effects were reported.	
CompTox 2022	In vitro aromatase		Inactive in 1/1 assays with CYP (cytochrome P450) 19A1	

	inhibition		activity	
CompTox 2022	In vitro thyroid hormone system disruptors		Three assays were performed all dealing with receptor activity, the TOX21, TRHR_HEK293, TOX21_TR_LUC_GH3, and TOX21_TSHR_HTRF assays. No effects were observed in the three assays	
QSAR 2022	In vitro AR activity		IBP is predicted to be negative (in domain) for AR inhibition.	
QSAR 2022	In vitro ER activity		IBP is predicted to be positive (in domain) for ER binding (full and balanced training set) and ER activation in the QSAR Battery combining predictions from the CASE Ultra, the Leadscope and the SciQSAR models. In addition, the OECD QSAR Toolbox (v.4.2) profilers predicts the parent substance to be a moderate ER binder while metabolites are predicted to be weak ER binders.	

In vivo studies on IBP

Table 14: Study overview, *in vivo* studies on IBP. Effects described are statistically significant unless otherwise indicated. Reliability has been assessed using Klimisch Scores.

Reference	Study design	NO(A)EL/LO(A)EL Quick overview results	Results description	Study quality and assessment
Darbre *et al.* 2002	Uterotrophic assay in immature CD1 mice, treated by s.c. injection on PND 18-20 and killed on PND 21 n=7/group 0, 1.2, 12 mg/mouse. Doses were not reported relative to body weight, but since average mouse BW was ~13 grams, the doses corresponded to approximately 90 and 900 mg/kg bw/day	NOEL: not identified LOEL: ~90 mg/kg bw/day Uterus weight relative to body weight ↑ in both dose groups (by ~ 30% and 60% respectively). The % increase was based on figure and no calculations of statistical significance were performed in the paper.	This study investigated the estrogenic activity of IBP in an immature uterotrophic assay in mice. IBP exposure increased relative uterine weight at both tested doses (by approximately by 30 % and 60 %), but whether these increases were statistically significant was not reported. No other in vivo endpoints were reported, e.g. no information on body weights in the paraben treated mice. IBP was also assessed in a number of in vitro assays (see in vitro section)	*Reliability 2.* - *In vivo* description was acceptable, but with several shortcomings: - very little information on study design (no information on housing conditions, CAS number or purity of IBP) - only indirect information on doses relative to body weight - limited information on study results (no information on toxicity, body weight in paraben exposed mice and no absolute uterine weights). - No statistical analysis of in vivo results
Koda *et al.* 2005	Uterotrophic assay in ovariectomised adult Sprague Dawley rats (OVX one week after arrival, and recovery for 2 weeks) Exposure: 3 days, euthanised on day 4, n = 6 0, 100, 250, 625 mg/kg/day Ethinyl estradiol (EE) (daily s.c. injections) used as positive control 0.01, 0.03, 0.1, 0.3, 1, 3, 10 µg/kg/day	NOEL: 100 mg/kg/day LOEL: 250 mg/kg/day. Absolute and relative wet uterine weight ↑ in 250 & 625 mg/kg/day, by ~20% and 70% respectively. Absolute and relative blotted uterine weight ↑ in 250 & 625 mg/kg/day, by ~ 23% and 51% respectively Body weight (no significant effect) <u>Positive control EE</u> Blotted and wet uterine weight ↑ from 0.3 - 10 µg/kg/day, by ~100-500%	IBP increased wet and blotted uterine weight at 250 and 625 mg/kg/day. At these doses no significant effects on body weight were observed, and weight increases were seen on both absolute and relative uterine weights. Wet uterine weights increased after exposure to EE of 0.3 µg/kg/day and upwards Using the Hill equation, ED10 for IBP was calculated to 590 mg/kg bw/day and ED50 660 mg/kg bw/day for wet weight, and 230 and 550 mg/kg bw/day for blotted weight. The relative estrogenic potency of IBP compared to EE was estimated to approximately 1/4,000,000	*Reliability 1.* - Acceptable - Well performed and reported - all basic scientific principles met. - Not performed according to OECD test guideline, but very similar - No shortcomings identified, except lack of reporting of the purity of IBP
Vo and Jeung 2009	Uterotrophic study in immature female Sprague- Dawley rats	NOEL : 62.5 mg/kg bw /day LOEL: 250 mg/kg bw/day. ↑ in uterus	250 and 1000 mg/kg bw/day IBP significantly increased uterine wet weight relative to bw, approximately 3- and 5 fold compared to controls.	*Reliability 2.* - Acceptable, but with some shortcomings.

	PND 14 -16 n=8 62.5, 250, and 1000 mg/kg bw/day s.c. injection EE (daily s.c. injections) used as positive control ; 1 mg/kg/day	weight relative to body weight at 250 and 1000 mg/kg bw/day, by ~ 180% and 380% (read from graph). Uterine relative CaBP-9k gene expression ↑ at 1000 mg/kg bw/day. Uterine relative CaBP-9k protein expression ↑ at 250 & 1000 mg/kg bw/day Uterine PR mRNA and protein were unaffected.	Absolute uterine weights were not provided, and neither were body weights. EE increased relative uterine weights 15 fold. However, this high EE dose may also have reduced female body weights, which adds some uncertainly to the provided results on relative uterine weight. CaBP-9k, is a cytosolic calcium-binding protein. The expression of uterine CaBP-9k was markedly upregulated by E2. IBP also significantly increased CaBP-9K. Gene expression was significantly increased in the 1000 mg /kg group, while both 250 and 1000 mg/kg/day increased protein expression of CaBP-9k. EE and 1000 mg of butylparaben and IPP significantly increased uterine ERα mRNA and protein. This was not assessed for IBP. IBP did not alter progesterone receptor gene transcription or protein levels.	- dosing was performed very early in the female development (PND 14-16). Typically, uterotrophic studies in immature females are performed from GD19 or GD20. How this early exposure affected the results is unclear. - No information on toxicity, body weights or absolute uterine weights was provided. - some information on study design was missing (bottles and cages were not described as phytoestrogen-free and purity information on IBP was lacking) - The dose of the positive control compound was very high (1 mg/kg bw/day, given s.c.). It is not reported whether this exposure led to any systemic toxicity.
Vo *et al.* 2010	Pubertal study in female Sprague-Dawley rats (n=10), dosed on PND 21-40 with 0, 62.5, 250, and 1000 mg/kg bw/day by oral gavage	NOEL: not identified LOEL = 62.5 mg/kg bw/day VO: 2 days earlier in low and mid dose groups (non-significant) but no effect in high dose group. Estrous cycles during exposure period: no difference Organ weights at PND41: no difference on: bodyweight, uterus, ovary, Kidney, pituitary, adrenal, thyroid Histopathology: Uterus - Thickness of morphometric measurement: ↑ (62.5, 250, 1000mg) Ovary - Incidence of "Decrease of corpora lutea, increase in the number of cystic follicles" ↑ (62.5, 250, 1000mg) Serum hormones, PND41: Estradiol: non-significant, not dose - related ↓ of 30-60% in all dose groups Prolactin: non-significant ↑ (1000mg)	At all doses of IBP myometrial hyperplasia was seen in uteri. It is less clear what changes were observed histologically in ovaries, but a decreased number of corpora lutea, increased number of cystic follicles and thinning of the follicular epithelium was reported. Due to substantial reporting- (and possibly methodological) limitations related to the histological assessments, the study only provides weak evidence of adverse effects in female reproduction. The role of IBP on thyroid hormones is unclear as the changes in T4 levels were likely chance findings. A ligand binding assay was performed to assess the estrogenic activity in vitro (see in vitro).	*Reliability 2.* - Acceptable study, but with clear shortcomings - Histopathological examinations were very poorly described, making it difficult to assess how well the analyses were performed. - The dose of the positive control compound was so high that the animals in this group had a 48% lower body weight that control animals - It is not consistently described whether organ weights were reported as absolute or relative to body weight. - Estrous cycle regularity was assessed from PND21 when the female rats are not yet sexually mature. Since the mean day of VO in some of the groups was above 36, this left less than 4 days to properly assess estrous

		T4: Significant ↓ (62.5), no difference (250, 1000mg)		cyclicity - Purity of IBP was not reported.
Kim *et al.* 2015	Dermal 28 day study in male and female Sprague- Dawley rats. 5 weeks old when purchased. Age at exposure beginning, unclear. Exposed 5 days per week for 28 days. Timing of sacrifice, relative to last dose-application is unclear. n = 5/sex/ group Histopathology n = 3 samples / group 0, 50, 100, 300, 600 mg/ kg bw 98% purity. Applied on shaved skin.	NOEL: not determined LOEL: 50 mg/kg bw (but only for biochemical effect and skin lesions). No ED-related endpoints were affected at any dose. <u>Male & female rats</u> - BW weight (no effect) - Food and water consumption (no effect) - Relative weight of brain, heart, liver, kidney, uterus, vagina, prostate, testis (no effect) - Haematological parameters (no effect) - Histopathology brain, liver, heart, kidney (no effect) <u>Male rats</u> - Na^+ (↑, 50, 100, 300, 600 mg/kg bw) – dose response) - Cl^- (↑, 300 and 600 mg/kg bw) - Other biochemical parameters (no effect) - serum T3, TSH, insulin (no effect) serum E2, FSH, T (no effect) - histopathological skin lesions (no effect) <u>Female rats</u> - Cl^- (↑, 600 mg/kg bw) - serum T3, TSH, insulin (no effect) - serum E2, T (no effect) - different types of histopathological skin lesions were found at all doses (↑, 50, 100, 300, 600 mg/kg bw)	No ED related effects were reported (organ weights, histopathology and serum hormone levels) after exposure. Also, no effect on BW, food and water consumption in either sex. In male and female rats, no effects were seen on relative weight of brain, heart, liver, kidney, uterus or vagina and no effects were seen on histopathology of brain, liver, heart, and kidney. In male and female rats, no effect on haematological parameters. In male rats, a significant increase in the biochemical parameter Na^+ was seen with increasing dose, starting at 50 mg/kg bw (dose-response). A significant increase in Cl^- was also seen at 300 and 600 mg/kg bw. No effects were seen on other biochemical parameters. No effects were seen on serum T3, TSH, insulin, E2, FSH, or testosterone. No effects were seen on male skin histopathology. In female rats, a significant increase in Cl^- was seen at 600 mg/kg bw. No effects were seen on other biochemical parameters. No effects were seen on serum T3, TSH, insulin, E2 or testosterone (based on the text (no results shown) FSH was not tested in the females). In the females, different types of skin lesions were seen in all exposure groups.	*Reliability 2.* - Acceptable, but with some shortcomings. The authors state that the study was performed according to OECD guideline no. 410, however assessing histopathology in only 3 animals per sex/group is not in accordance with this OECD TG which states that "All animals in the study should be subjected to a full gross necropsy" The dermal exposure seems to have been applied on week days only and not during weekends, but it is unclear when the terminal sacrifice was performed, relative to last dose-application. It is possible that the dermal exposure used in this study, may have resulted in other ADME characteristics of IBP than the oral or s.c. exposures used in other studies, and that this may explain the lack of effects on any ED-related endpoints. Such differences have been shown for BP (Aubert *et al*. 2012)
Yang *et al.* 2016	Reproductive toxicity study in Sprague-Dawley rats. Dams exposed from GD 6 - PND 21, and male offspring examined n = 3 pregnant dams / group	NOEL: not identified LOEL: 2,5 mg/kg bw/day (based on significant effect on sperm parameters)	Only one very low dose of 2.5 mg/kg bw/day was tested. In male offspring, adverse effects were seen at the age of 70 days on two sperm parameters. Sperm count and sperm motility were ↓ by approximately 50% and 30%, respectively (read of graph). A BPA dose of 0.05 mg/kg and a mixture dose of BPA and IBP resulted in similar decreases in both	*Reliability 3.* - Unreliable study, with many important shortcomings - Litter effects were not accounted for, which is unacceptable in a developmental toxicity study with dosing of the pregnant and lactating dams - Group size was reported to be

	0, 2.5 mg/kg bw/ day ≥ 99% purity Oral gavage		parameters. During prenatal and pubertal development no significant effects of IBP were seen on; body weight, AGD, nipple retention, testes descent or preputial separation. On PND 70 testes and epididymis weights and histopathology were unaffected. FSH, LH and testosterone levels were also unaffected, whereas E2 was significantly decreased (by 26%) *Note For NR, testis descent, day of PPS, BW, AGDI, testis and epididymis weight, serum hormones, histology of testis and epididymis, n was reported to be 8. This means that more than 1 pup from each litter was used in the statistical analysis, but litter effects were not taken into account. For sperm count and mobility n=5, which still means more than one pup per litter was used for analysis. Also, litter effects were not mentioned in data analysis section, suggesting that the authors did not take this very important aspect into account.	5-8, however, only 3 pregnant dams were included in the IBP group, so in reality a very small group size. - No consideration for litter when performing culling - Only one dose tested - Only very low dose tested - No discussion of whether the effects seen in all dose groups on sperm parameters could be explained by unusually high values in the control group. - The authors removed outliers based on studentised deviate test. One should always be careful with removing outliers, as these may indicate biological effects even though they might be mathematical outliers
Kawaguchi *et al.* 2009a	Developmental study in Sprague-Dawley rats. Dams were exposed from 3 weeks before mating until weaning at PND21. Dosed via implanted silastic capsule,	LOEL = 4.36 mg/L/day Dams at PND21: plasma corticosterone ↓, uterus weight↑ no effect on: LH, FSH, E2, T4, T3, pituitary weight and adrenal weight (n=7-8). Offspring AGD, PND7 (n=7-8): no difference VO and Bw (n=5-8): no difference Estrous cycle (n=5-8), 7 weeks old: no difference No effect at weaning PND21 on: LH, Corticosterone, E2, T, Inhibin, T4, T3, testis, pituitary and adrenal weight, uterus weight. No effects at 17 weeks old females, diestrus, on: LH, PRL, E2	No overt signs of toxicity were seen. There were no effects on litter size or ratio of male pups. Plasma corticosterone levels in dams were decreased after IBP exposure and uterus weight was increased. No effects were found in the offspring except: the weight of the uterus in estradiol-treated ovariectomised offspring at 12 weeks of age was decreased by IBP exposure during gestation and lactation. The authors speculate that there was a decrease in expression of ER and the rate of proliferation in the uterus.	*Reliability 3.* - Unreliable study with many important shortcomings All three publications report results from the same developmental toxicity study. The study design and reporting in all three publications have important shortcomings. Therefore, all three are deemed unreliable (score 3). The main drawbacks are: the group size used, lack of consideration of litter as the statistical unit in the data analysis, testing of only one dose was and poor description of this dose. None of the publications provide precise information as to how many litters were included in this
Kawaguchi *et al.* 2009b	Sprague-Dawley rats Developmental. Dams were exposed from 3 weeks before mating	LOEL = 4.36 mg/L/day Elevated plus maze, 6weeks old: males: time spent in closed arms↑ time in open arms↓, female no effect	No overt signs of toxicity was observed. Male offspring exposed to IBP perinatally spent shorter time in the open arms of the elevated plus maze and showed decreased performance in the	

	until weaning at PND21. Behavioural examination of offspring n=7-8 /sex	Open field, 5 weeks old: No difference Passive avoidance task, 7 weeks old: males: no transfer response↓, female: no difference. Morris water maze, 7 weeks old: no difference	passive avoidance test. No effects on were seen in the open field or the Morris water maze performance of the males or in any behavioural tests in the female offspring.	study and the litter effect was not considered in the statistical evaluations, making the results unreliable. For some endpoints the group size was reported to be 7-8, but in other places it is stated that there were 6 control litters and 5 IBP litters. This is much too small to provide reliable data on behavioural measurements, especially since the litter effect was not included in the statistical analysis of the data. Information on the tested dose is also very scarce, and purity of IBP was not reported. It was stated in all three publications that *in vitro* the used capsules secreted 4.36 mg/L/day. In order to compare this dose to other studies, we have calculated that this corresponds to 436 ppm and hence we approximate that the dam and offspring exposure was ~35 mg/kg bw/day
Kawaguchi *et al.* 2010	Sprague-Dawley rats Perinatal. Dams were exposed from 3 weeks before mating until end of experiment Social (recognition) behaviour of female offspring was investigated. n = 6 control litters, 5 IBP litters.	LOEL: 4.36 mg/l/day	Female offspring were gonadectomised and at 16 weeks of age, social recognition was tested. For three days females were individually placed in a square open-field apparatus for 10 minutes. On the fourth day an unfamiliar rat was placed in the field for 60 seconds and the amount of time and the frequency of investigation (sniffing) of the intruder-rat was recorded. They were tested four times with 10 minutes intervals. On the fifth trial a new intruder-rat was place in the field. IBP treated rats showed impaired social behaviour and did not have a change in frequency of interaction with the new rat in the fight trial, which was seen in the controls.	

In vivo adverse effects of BP

The table below presents *in vivo* studies on adverse effects of BP. The adverse effect evaluation is only presented for perinatal exposure, as this exposure window was considered most relevant in the evaluation of endocrine disrupting properties of BP in the SVHC document for BP (ECHA 2020).

Table 15: In vivo studies reporting ED-related adverse effects, seen after perinatal exposure to BP. Study summaries from the SVHC support document for BP (ECHA 2020) supplemented with study summaries for two new *in vivo* studies and addition of substance purity. Effects described are statistically significant unless otherwise indicated.

Reference	Method	Relevant Endpoints & Effects	Comments/Notes	Klimisch Score
Fisher *et al.* 1999	Wistar rats. Neonatal repeated, s.c injection (PND 2-18). Dose: 0, 2 mg/ kg bw /day, n= 6. Other substances tested were DES, EE, bisphenol A, genistein, octylphenol.	No observed effect level NOEL = 2 mg/kg bw /day (only dose tested) Testis weight - not affected Testis histopathology - not affected DES and EE caused dose-related changes in testis weight, distension of the rete testis and efferent ducts, epithelial cell height in the efferent ducts and expression of aquaporin-1. Minor effects were seen with the less potent estrogenic compounds.	Only one dose of BP (2 mg/kg bw/day) was tested with no detectable effects on any of the measured reproductive parameters (absolute testis weight and histological examination).	*Reliability 2.* - Acceptable study, which meets basic scientific principles - One shortcoming is that purity of BP is not reported. - Single dose tested
Kang *et al.* 2002	Sprague- Dawley rats. Development of male reproductive system, s.c. (GD6-PND20). Doses 0, 100, 200 mg/ kg bw/day, n = 5-7 for organ weight/histology, 5 form sperm parameters and 3 for gene expression	LOAEL = 100 mg/kg bw/day Pups: Live births ↓ Surviving to weaning ↓ AGD not affected Weight: Testis ↓↑ Prostate ↓ Seminal vesicle ↓ Sperm: Numbers ↓ Motility ↓ Morphology ↓ ERα and ERβ expression in testis ↓↑	At both dosage levels, the weights of testes, seminal vesicles and prostate glands were decreased, together with the sperm count and the sperm motile activity in the epididymis. Testicular expression of ERα and ERβ mRNA was significantly increased at the highest dosage level.	*Reliability 2.* - Acceptable, well-documented study - One shortcoming is that purity of BP is not reported
Taxvig *et al.* 2008	Wistar rats. Development of male reproductive system, s.c. (GD7-21). Dose: 0, 200, 400 mg/kg bw/day, n = 13-18. Purity 99%	AGD not affected: Testosterone not affected Progesterone not affected, Cortisol not affected Testis and adrenal histopathology not affected, Adrenal not affected	Hormones measured in dams GD 21: 17α-hydroxyprogesterone and progesterone, no effects of exposure. Female AGD also measured with no effects reported.	*Reliability 2.* - Acceptable, well-documented study, comparable to guideline standards

Zhang *et al.* 2014	Wistar rats. Development of male reproductive system, oral (gavage) (GD7-PND21). Doses: 0, 64, 160, 400, 1000 mg/ kg bw/day, n =7-8. Purity 99%	NOAEL 64 mg/kg bw/day, LOAEL 160 mg/kg bw/day Pups: Sex ratio ↓ Body weight ↓ AGD ↓ Puberty (delayed) Weight: Testis ↓ Epididymis ↓ Seminal vesicle ↓ Hormones: Testosterone ↓ Estradiol ↑ Progesterone ↑ LH ↓↑ FSH ↓↑ Sperm numbers and daily sperm production ↓ Histopathology testis (affected PND 21 and 90)	Dams: FSH and LH ↑. Offspring affected at several ages (for many endpoints PND 21, 35, 49, 90, 180). Male offspring: sex ratio affected (fewer males) Bw decreased from PND 0-49, but not affected PND 90-180. Weight of testis, epididymis and seminal vesicles decreased, however several overlaps with reduced BW; relative weights not reported. AGD shortened on PND1 and 21 (also coincides with reduced BW). Testis histopathology affected on PND 21 and 90 with reduced and loosely arranged germ cells, reduced layers of seminiferous tubules, reduced numbers of spermatocytes. No obvious effects on Leydig cells. Testosterone levels reduced at 400 mg/kg/day and 1000 mg/kg/day and estradiol levels increased at 1000 mg/kg/day.	*Reliability 2.* - Acceptable, well-documented study - Basic data given, comparable to guideline standards
Zhang *et al.* 2016	Wistar rats. Mechanisms of ED and reproductive disorders, oral (gavage) (GD7-PND21). Doses: 0, 64, 160, 400, 1000 mg/kg bw/day, n = 7-8. Purity 99%	NOAEL = 160 mg/kg bw/day (effects are seen at protein level at this dose) Body weight ↓ Weight: Testis not affected Epididymis ↓ Seminal vesicle not affected Hormones: Testosterone ↓ Estradiol ↑ Gene expression: Star, P450scc, Sult1e1 (affected) Gene and protein expression: ERα, ERβ, AR (affected) Methylation of ERα promoter ↓ Histopathology testis (affected)	Data is possibly based on the same animal study as Zhang *et al.* 2014.	*Reliability 2.* - Acceptable, well-documented study, which meets basic scientific principles - Concern: some data seem to already have been reported in Zhang *et al.* 2014 and are likely reported here again without reference to the previous study.
Boberg *et al.* 2016	Wistar rats. Development of male reproductive system, oral (gavage) (GD7-21 and PND1-22). Doses: 0, 10, 100, 500 mg/kg bw/day, n = 18. purity >99.0 %,	LOAEL = 10 mg/kg bw/day AGD and AGDi ↓ Nipple retention not affected Puberty not affected Weight: Testis not affected	AGD and AGDi shortened in both males and females. Number of sperm in cauda reduced in all dose groups. Genes (cell markers, receptors (AR, FSHr, LHr), steroidogenesis) were investigated in testis PND 16 and in adulthood. Down regulation of Cyp19a1 in all exposure groups was seen on PND16. No other effects seen on gene expression. Hormone levels (estradiol measured PND16 males and PND 22 females): no effect.	*Reliability 2.* - Acceptable, well-documented study, comparable to guideline standards

		Prostate ↓ Seminal vesicle ↓ Epididymis not affected LABC not affected Bulbourethral gland not affected Sperm numbers ↓ Histopathology: Epididymis not affected Testis not affected Prostate (affected) Gene expression Cyp19a1 ↓ Hormones - Estradiol not affected	Mammary gland was investigated in females. PND 22: higher number of TEBs in two highest dose groups (100, 500 mg/kg bw/day). Increased outgrowth towards the lymphnode in 100 mg/kg bw/day. Adult: no clear effects seen.	
Guerra *et al*. 2017	Wistar rats. Male reproductive development, s.c. (GD 12 - PND21). Doses: 0, 10, 100, 200 mg/kg bw/day, n = 8/group.	NOAEL = 10 mg/kg/day AGD not affected Nipple retention not affected Puberty not affected Weight: Pituitary not affected Testis not affected Epididymis not affected Prostate not affected Seminal vesicle not affected Vas deferens not affected Histopathology: Fetal testis not affected PND 110 testis ↑ Leydig cells Hormones: Testosterone ↑ FSH ↓ LH ↓ Sperm: Spermatogenesis kinetics ↑↓ Sperm counts not affected Motile sperm ↓ Non-motile sperm not affected Normal morphology ↓ Abnormal morphology ↑ Testis morphometry (no of cells) not affected ESR1 and AR protein in testis ↓ Sexual behaviour Fertility not affected	Effect on testosterone, LH and FSH levels measured at PND 110 in male offspring on, 200 mg/kg /day. No effect on AGD (PND 1, all doses). Reproductive organ weights not affected except from: Increase in number of Leydig cells in interstitium of adult testes at PND 110 at 100 and 200 mg/kg bw/day. Decreased intensity of IHC staining of ESR1 in spermatids and AR protein in sertoli cell nuclei in testis from adults at 200 mg/kg/day. Effect on sperm: Change in spermatogenesis kinetics (PND 110). % seminiferous tubules in stage I – VI decreased at 200 mg/kg bw/day and stages VII-VIII increased at 10 and 200 mg/kg bw/day. Decreased motile sperm with progressive trajectory (%) at PND 110, 10 mg/kg bw/day, while slight decreases at 100 and 200 mg/kg bw/day (not statistically significant). Motile sperm with non-progressive trajectory (%) at PND 110: increased at 10 mg/kg bw/day, while slight increases at 100 and 200 mg/kg bw/day (not statistically significant). No change in % non-motile sperm (PND 110). Decreased normal morphology (%) at PND 110 in all dose groups (10, 100, 200 mg/kg bw/day). Increased abnormal head (characteristic curvature missing) (%) at PND 110 in all dose groups (10, 100, 200 mg/kg bw/day).	*Reliability 2.* - Acceptable, well-documented study, comparable to guideline standards - One shortcoming is that purity of BP is not reported.

Maske *et al.* 2020	Holtzman rats. Male reproductive development, s.c. (GD6 – PND21). Dose: 0, 10, 100, 1000 mg/ kg bw/day, n = 6-10 / group.	LOAEL = 10 mg/kg bw/day Testicular descent (delayed) Puberty (delayed) Weight: Testis not affected Epididymis ↓ Seminal vesicles ↑ Prostate ↑ Hypothalamus ↓ Pituitary ↓↑ Hormones: Testosterone ↓↑ Estradiol ↓↑ LH ↑ Testis histopathology (affected) Sperm: Motility ↓ Sperm count ↓ Daily sperm prod. ↓ Sperm transit time ↓ Fertility (affected) Gene expression: AR ↑ ERα ↑ ERβ ↑ Insl3 ↑ Star ↓	Testicular descent was delayed in the two highest dose groups and balano-preputial separation (puberty) delayed in the 10 mg/kg bw group. Weight of reproductive organs was affected at several ages and doses, not necessarily dose-response. Hormone levels were also affected at several ages. Sperm related parameters were affected and pattern of reduced motility, count, production etc. was seen. Reduced fertility was seen in naïve females mated with the exposed males. Gene expression in testis was affected.	*Reliability 2.* - Acceptable, well-documented study - One shortcoming is that purity of BP is not reported
Oliveira *et al.* 2020	Wistar rats, GD 1-22 Direct (s.c.). Doses: 100 and 200 mg/kg bw/day. N = 7–10 females/group.	LOAEL: 100 mg/kg bw/day Testis weight: ↓ Mitochondrial bioenergetics (testis): altered Antioxidant capacity (testis): altered	Relative testis weight was reduced in male offspring on PND 56 without change in body weight at doses of 100 and 200 mg/kg bw/day BP. This was associated with changes in the mitochondrial bioenergetics and antioxidant capacity of testis.	Reliability 2. - Acceptable, generally well-documented study -One shortcoming is that purity of BP is not reported
Hubbard *et al.* 2020	Sprague-Dawley rats. Continuous breeding from F0 through F2 generation, feed. Doses: 0, 5000, 15000, or 40000 ppm N=22/sex/group. In the gestation period, these doses corresponded to ~340 mg/kg, ~ 1000 mg/kg bw/day and ~3000 mg/kg bw/day, while the	LOAEL = 15000 ppm (for reproductive endpoints, liver effects were observed at 5000 ppm) Body weight ↓ (15000, 40000 ppm) AGD: no effect Nipple retention: no effect Testis descent: no effect Balano-preputial separation: no effect Sperm numbers and motility: no effect Testis histopathology: no effect Testis weight: ↑ (relative at doses of	BP exposure did not affect fertility, fecundity, pubertal attainment, or reproductive parameters (except testis weights) in F0, F1, or F2 generations. Exposure-dependent increases in liver weights, and incidences of non-neoplastic liver lesions were observed at 5000 ppm. Lower bw across all groups	Reliability 1. - Reliable without restrictions

	doses during the lactation period were almost twice as high. Purity 99.7 %	15000 and 40000 ppm corresponding to around 1400 and 4800 mg/kg/day) Fertility: No effect (mated/pair, littered/pair, littered/mated, duration of precoital or gestational intervals) with three natural matings per generation (F0 and F1). A significant decreasing trend in total litter size occurred in F0&F1 pairings increasing with exposure and a significant decrease in total litter size in 40000 ppm group in F0 and F1.		

Annex III – Lines of Evidence (LoE) for endocrine activity and adverse effects

Table 16: Lines of evidence for endocrine activity *in vitro* by IBP, incl. Comptox and QSAR

Reference	Effect target	Model	Species	Concentration	NOEC/LOEC/ IC_{xx}/EC_{xx}	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence
Terasaki *et al.* 2009 Reliability 2	Estrogen receptor (ER) binding	hERα competitive binding assay	Human	3.8-38000 nM	Activity relative to DES = 2.8×10^{-3}	Change	Binding of IBP to hERα established.	**Strong evidence that IBP consistently affects the ER *in vitro.*** ERα binding was assessed in 3 studies and IBP had an effect in all studies. QSAR predicted IBP to bind and activate ER. ERβ interference was investigated in 1 study, and showed effect. ER transactivation was assessed in 5 studies and IBP increased ER activity in all studies. Comptox showed activity in 4 assays (2 agonism, 2 antagonism).
Darbre *et al.* 2002 Reliability 2	ER binding	hERα competitive binding assay	Human	1–1.000.000-fold molar excess	LOEL: 100 000-fold molar excess	Change	Estradiol ERα binding was inhibited by IBP.	
Okubo *et al.* 2001 Reliability 2	ER binding	Ligand Screening - ERα - System	Human	Not reported	IC50 = 2.7×10^{-5} M	Change	Competitive binding with E2 showed an IC50 = 2.7×10^{-5} M for IBP. DES was used as a positive control with IC50 = $3.0 \times 10^{-8.}$ M. IBP had approximately 1000- fold lower affinity than DES.	
Okubo *et al*. 2001 Reliability 2	ER binding	Ligand Screening - ERβ - System	Human	Not reported	IC50 = 2.8×10^{-5} M	Change	Competitive binding with E2 showed an IC50 = 2.8×10^{-5} M for IBP. DES was used as a positive control with IC50 = $2.6 \times 10^{-8.}$ M. IBP had approximately 1000- fold lower affinity than DES.	
QSAR 2022	ER binding	High throughput assays					IBP is predicted to be for ER binding and ER activation. In addition, the OECD QSAR Toolbox (v.4.2) profilers predicts the parent substance to be a moderate ER binder	
Watanabe *et al.* 2013 Reliability 2	ER transactivation	CHO cells, transiently transfected with hERα	Human	10^{-8} - 10^{-5} M	REC20 = 1.2×10^{-7} M	Increase	Increased activity. E2 was used as positive control. The results were reported as 'the concentration where IBP reached 20% of E2 activity' (REC20). No cytotoxicity at tested	

							concentrations.	
Watanabe *et al.* 2013 Reliability 2	ER transactivation	CHO cells, transiently transfected with hERβ	Human	10^{-8} - 10^{-5} M	REC20 = 4.3 x 10^{-8} M	Increase	Increased activity. E2 was used as positive control. The results were reported as 'the concentration where IBP reached 20% of E2 activity' (REC20). No cytotoxicity at tested concentrations.	
Gonzales *et al.* 2018 Reliability 2	ER transactivation	ERE-luciferase reporter assay co-transfected into MCF-7 cells	Human	10, 20 µM	Data not shown	Increase	Induction reported, but data not shown in article. E2 used as positive control. No cytotoxicity.	
Kim *et al.* 2012a Reliability 2	ER transactivation	GH3 cells, transient transfection with ERE plasmid	Rat	10^{-7}, 10^{-6} and 10^{-5} M	LOEC = 10^{-7} M	Increase	Dose-dependent increase in ER activity based on luciferase reporter signal. Cytotoxicity was not investigated.	
Kim *et al.* 2011 Reliability 2	ER transactivation	hERα-HeLa-9903 cells transfected with hERα and luciferase reporter.	Human	10^{-10} to 10^{-5} M	EC_{50}: 6.3x10^{-7} M (relative to positive control (E2))	Increase	ER transactivation was increased. The estrogenic activity was 22,000 fold lower than the positive control 17β-estradiol. Cytotoxicity was not investigated.	
Terasaki *et al.* 2009 Reliability 2	ER transactivation	Yeast cells (Saccharomyces cerevisiae Y190), transfected with hERα	Human	16-1000 nM	Activity relative to E2 = 2.0 x 10^{-4}	Increase	Increased activity, relative to the positive control E2. IBP was the most potent paraben among several different parabens tested. No information on cytotoxicity in the study.	
CompTox 2022	ER transactivation	High throughput assays					IBP exhibited agonistic activity in two assays (TOX21_ERa_BLA and TOX21_ERa_LUC_VM7). Antagonistic activity was observed in two assays (TOX21_ERa_BLA and TOX21_ERb_BLA). There were no hit calls for compromised cell viability.	

Kim *et al.* 2012a Reliability 2	Estrogenic response in target cells	GH3 cells (pituitary cell line)	Rat	10^{-7}, 10^{-6} and 10^{-5} M	LOEC = 10^{-7} M	Increase	Gene and protein expression of CaBP-9k (used as a marker for estrogenic activity) was upregulated at all concentrations tested. The effect was blocked by addition of ER antagonist ICI 182,780. PR gene and protein expression was also upregulated at all doses tested. Pre-incubation with ICI blocked PR increase. Cytotoxicity was not investigated.	**Strong evidence that IBP consistently affects estrogen-regulated genes and proteins and increases estrogen-induced cell proliferation.** IBP binds to hERa in two and ERb in one experiment. Further, estrogen mediated transactivation in reporter gene assays was reported in 5 studies. Increased proliferation in estrogen dependent cell lines was reported in 3 studies, two of these studies tested in two different estrogen dependent cell lines. Estrogen dependent gene and protein expression of CaBP was observed in four studies. Gene expression of estrogen dependent genes GREB and ps2 was induced in one study. Generally. PR gene and protein expression was upregulated with
Kim *et al.* 2012b Reliability 2	Estrogenic response in target cells	GH3 cells (pituitary cell line)	Rat	0.1, 1, 10 µM	LOEC = 0.1 µM	Increase	Gene and protein expression of CaBP-9k (used as marker for estrogenic activity) was upregulated at all concentrations tested. The effect was blocked by pre-incubation with ER antagonist ICI 182,780. PR gene and protein expression was also upregulated at all doses. Cytotoxicity was not investigated.	
Vo *et al.* 2012 Reliability 2	Estrogenic response in target cells	GH3 rat pituitary cells	Rat	10^-7, 10^-6 and 10^-5 M	LOEC: 10^−7 M	Increase	Increased CaBP-9k gene and protein expression in all IBP doses tested. This effect was blocked by pre-treatment with ICI 182,780 (fulvestrant, ER antagonist). The PR gene and protein expression was also upregulated in all the tested doses of IBP. Pre-incubation with ICI blocked the PR increase. Cytotoxicity was not reported	
Vo *et al.* 2012 Reliability 2	Estrogenic response in target cells	GH3 rat pituitary cells	Rat	10^-7, 10^-6 and 10^-5 M	No effect	No effect	No change in ERa protein expression was observed after 24 hours. ERa gene expression was not affected in one experiment, but showed a significant decrease in another	
Yang *et al.* 2011 Reliability 2	Estrogenic response in target cells	GH3 rat pituitary cells	Rat	10^{-5} M	LOEL: 10^-5 M	Increase	Increased CaBP-9k gene and protein expression and increased PR-B gene expression. Pre-treatment with ER antagonist ICI 182,780 for 1 hour blocked all effects observed	
Yang *et al.* 2011 Reliability 2	Estrogenic response in target cells	GH3 rat pituitary cells	Rat	10^{-5} M	No effect	No effect	ERα gene and protein expression and PR-B protein expression were not affected after 25 hours of exposure	

Gonzales *et al.* 2018 Reliability 2	Estrogenic response in target cells	MCF-7 and T47D cells	Human	10^{-7} - $10^{-4.5}$ M	EC50 = 0.3 µM. Unclear if based MCF-7 or T47D cells, but in both cell-lines proliferation was induced	Increase	Proliferation was induced in a concentration dependent manner. No cytotoxicity.	exposure to IBP, and ER gene expression was either not affected or decreased. ICI (an anti-estrogen) reversed the observed effects.
Gonzales *et al.* 2018 Reliability 2	Estrogenic response in target cells	MCF-7	Human	0.3 µM IBP; 10^{-12} - 10^{-6} M ICI 182, 780	IC50 = 1.4 nM	Decrease	To investigate if proliferation in MCF-7 cells is induced via ERα, MCF-7 cells were treated with 0.3 µM IBP (EC50 value for MCF-7 proliferation) and increasing concentrations of the anti-estrogen Fulvestrant (ICI 182, 780). The proliferation of IBP was inhibited. No cytotoxicity.	
Gonzales *et al*. 2018 Reliability 2	Estrogenic response in target cells	MCF-7	Human	10 µM	10 µM	Increase	The gene expression level of GREB1, a critical downstream target of ERα signaling, was investigated. E2 was used as positive control (induced a 29-fold expression of GREB1) and IBP induced GREB1 expression at 36 fold compared to vehicle control. Co-treatment with Fulvestrant (ICI 182, 780) blocked the effects. No cytotoxicity.	
Okubo *et al.* 2001 Reliability 2	Estrogenic response in target cells	MCF-7	Human	10^{-7} to 10^{-4} M	EC50 = $6.8x10^{-7}$ M	Increase	IBP increased proliferation of MCF cells. Simultaneous addition of the ER antagonist ICI 182,780 suppressed proliferation in a dose-dependent manner, showing ER mediated proliferation. E2 was used as positive control. No information on cytotoxicity in the study, however, test concentrations led to increased proliferation, implying no cytotoxicity, except for ICI at concentrations at or above 10^-8 M (increased proliferation seen).	
Okubo *et al*. 2001 Reliability 2	Estrogenic response in target cells	MCF-7	Human	10^{-5} M	LOEC = 10^{-5} M	Change	After 48 h exposure both ERα gene and protein expression was downregulated (not after 24 h). PR expression was increased. This was the same pattern as seen for the positive control E2. No information on cytotoxicity in the study, but the concentration is within the range	

							where increased proliferation was seen, implying no cytotoxicity, except for ICI at concentrations at or above 10^-8 M, as ICI tested alone led to an apparent decrease in cell numbers at these concentrations.	
Darbre *et al.* 2002 Reliability 2	Estrogenic response in target cells	MCF-7	Human	10^-11 to 10^-4 M	LOEC: 10^−6 M	Increase	Proliferation was induced. Cytotoxicity was not reported	
Darbre *et al.* 2002 Reliability 2	Estrogenic response in target cells	MCF-7	Human	10^-9 to 10^-4	LOEL: 10^−6 M	Increase	Estrogen sensitive reporter gene ERE-CAT and ps2 increased. Cytotoxicity was not reported	
Gonzales *et al.* 2018 Reliability 2	Estrogenic response in target cells	MDA-MB-231 (non ERα expressing cells)	Human	10^{-8} - 10^{-5} M	No effect	No effect	Non-ERα expressing cells showed no effect on cell proliferation after exposure.	
Darbre *et al.* 2002 Reliability 2	Estrogenic response in target cells	ZR-75-1	Human	10^-9 to 10^-4	LOEL: 10^−6 M	Increase	Proliferation was induced. Cytotoxicity was not reported	
Satoh *et al.* 2005 Reliability 2	Androgen receptor binding	AR competitive binding assay	Human	Not reported	40% partial inhibition at 1.9 x 10^{-4} M	Change	Competitive binding to AR was investigated by co-administration of testosterone. IBP partially inhibited testosterone binding to AR (cell free assay).	**Moderate evidence of weak or no effects on the AR.**
Kim *et al.* 2010 Reliability 2	Androgen receptor binding	AR competitive binding assay	Rat	Estimated from graph: 10^{-4} to 10^{-3} M	IC50: 3.1x10^{-4} M	Change	Competitive affinity to AR was higher than for other parabens tested. However, the binding affinity relative to DHT was almost 17 000 times lower for IBP (cell free assay).	3 studies investigating AR agonism showed no effect.
Satoh *et al.* 2005 Reliability 2	Androgen receptor transactivation	AR-Eco Screen (CHO-K1 cells stably transfected with AR)	Human	Estimated from graph: 1-100 µM	IC50 = 7.6x10^{-5} M	Decrease	No agonistic activity was seen. However, antagonism was seen after exposure. No cytotoxicity at tested concentrations.	3 studies investigating AR antagonism showed either no effect or a weak effect. 2/8 assays in Comptox reported antagonism but at high concentrations.
Kjaerstad *et al.* 2010 Reliability 2	Androgen receptor transactivation	CHO cells transfected with AR	Human	0.025–50 uM	LOEC = 25 µM	Decrease	AR antagonism was seen at concentration ≥25µM. No agonism was seen. No cytotoxicity at tested concentrations.	

Watanabe *et al.* 2013 Reliability 2	Androgen receptor transactivation	CHO cells, transiently transfected with hAR	Human	10^{-8} - 10^{-5} M	No effect	No effect	No agonistic or antagonistic activity, or cytotoxicity at tested concentrations.	2 studies investigating AR binding showed effects at high concentrations or only a partial inhibition of testosterone binding. QSAR was negative for AR inhibition
CompTox 2022	Androgen receptor transactivation	High throughput screens					IBP was active for AR antagonism at non-cytotoxic concentrations in two assay, however in one assay this activity was dependent on the agonist concentration added. No agonism was observed.	
QSAR 2022	Androgen receptor transactivation	High throughput screens					IBP is predicted to be negative (in domain) for AR inhibition.	
Fujino *et al.* 2019 Reliability 2	Other	COS-1, transfected with PXR	Human	0, 0.3, 1, 3, 10, 30 µM	LOEC = 10 µM	Increase	Increased PWR transactivation at 10 and 30 µM. Rifampicin used as positive control. No cytotoxicity.	**Moderate evidence that IBP activates PXR, CAR and PPARα.** The results on PXR and PPAR are acceptable, but further evidence from other studies is needed to confirm the effects**.** Two studies show ability of IBP to induce CAR activity suggesting moderate evidence for this mechanism.
Fujino *et al.* 2019 Reliability 2	Other	COS-1, transfected with PXR	Rat	0, 0.03, 0.1, 0.3, 1, 3, 10, 30 µM	LOEC = 10 µM	Increase	Increased PXR transactivation at 10 and 30 µM. PCN used as positive control. No cytotoxicity.	
Fujino *et al.* 2019 Reliability 2	Other	COS-1, transfected with CAR	Rat	0, 1, 3, 10, 30 µM	LOEC = 10 µM	Increase	Increased CAR transactivation at 10 and 30 µM. Artemisinin used as positive control. No cytotoxicity.	
Fujino *et al.* 2019 Reliability 2	Other	COS-1, transfected with PPARα	Rat	0, 1, 3, 10, 30 µM	LOEC = 10 µM	Increase	Increased PPARα transactivation at 10 and 30 µM. Bezafibrate used as positive control. No cytotoxicity.	
Kamata *et al.* 2018 Reliability 2	Other	Yeast cells (*Saccharomyces cerevisiae* Y190), transfected with CAR	Human	156 nM- 10 µM	EC x 10 = 3300 ± 330 nM	Increase	An increase was seen in CAR transactivation, however, smaller than that for the positive control (4tOP yielded and ECx10 of 13 ± 4.0 nM). EC x 10 is defined as the test solution concentration producing luminescence intensity ten times that of the blank control.	

CompTox 2022	CYP19	High throughput screens					Inactive in 1/1 assays with CYP19A1 activity	**Weak evidence of no effect on aromatase (CYP19a1) based on one screen in Comptox**
CompTox 2022	Thyroid	High throughput screens					No hit calls for receptor activity on TRHR, TR and TSHR.	**No effect on TRHR, TR and TSHR based on screens in Comptox**

Table 17: Lines of evidence for endocrine activity *in vivo* by IBP

Reference	Effect target	Species	Exposure	Route	Dose	LO(A)EL (mg/kg bw/day)	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence
Koda *et al.* 2005 Reliability 1	Uterine weight	Rat	10-11 weeks old ovarectomised, 3 days	s.c. injection	100, 250, 625 mg/kg bw/day	250	Increase	Significant increase in uterus wet weight after 250 and 625 mg/kg bw/day for 3 days	**Moderate-strong evidence of estrogenic effects in the uterotrophic assay.** IBP caused increased uterine weights in rats and mice in all performed studies
Darbre *et al*. 2002 Reliability 2	Uterine weight	Mouse	PND 18-20	s.c. injection	~90 , 900 mg/kg bw/day	90	Increase	IBP increased relative uterine weight (/g bw) in immature mice at 1.2 and 12 mg/mouse (uterine assay)	
Vo and Jeung 2009 Reliability 2	Uterine weight	Rat	PND 14-16	s.c. injection	62.5, 250, 1000 mg/kg bw/day	250	Increase	Significant increase in relative uterus weight after 250 and 1000 mg/kg bw/day for 3 days	
Vo and Jeung 2009 Reliability 2	Gene expression	Rat	PND 14-16	s.c. injection	62.5, 250, 1000 mg/kg bw/day	1000	Increase	Significant increase in uterine relative CaBP-9k gene expression at 1000 mg/kg bw/day for 3 days	**Moderate evidence of effects on estrogen regulated gene and protein expression.** IBP affected estrogen responsive target genes. Altered expression of uterine CaBP-9k and ERα but not progesterone gene and protein expression after a short postnatal exposure.
Vo and Jeung 2009 Reliability 2	Gene expression	Rat	PND 14-16	s.c. injection	62.5, 250, 1000 mg/kg bw/day	1000	Decrease	Significant decrease in uterine relative ERα gene expression after 1000 mg/kg bw/day for 3 days	
Vo and Jeung 2009 Reliability 2	Gene expression	Rat	PND 14-16	s.c. injection	62.5, 250, 1000 mg/kg bw/day		No effect	No effect in uterus relative progesterone receptor mRNA after 62.5, 250 and 1000 mg/kg bw/day for 3 days	
Vo and Jeung 2009 Reliability 2	Protein expression	Rat	PND 14-16	s.c. injection	62.5, 250, 1000 mg/kg bw/day	250	Increase	Significant increase in uterine relative CaBP-9k protein expression after 250 and 1000 mg/kg bw/day for 3 days	

Vo and Jeung 2009 Reliability 2	Protein expression	Rat	PND 14-16	s.c. injection	62.5, 250, 1000 mg/kg bw/day	1000	Decrease	Significant decrease in uterine relative ERα protein expression after 1000 mg/kg bw/day for 3 days	
Vo and Jeung 2009 Reliability 2	Protein expression	Rat	PND 14-16	s.c. injection	62.5, 250, 1000 mg/kg bw/day		No effect	No effect in uterus relative progesterone receptor protein after 62.5, 250 and 1000 mg/kg bw/day for 3 days	
Vo *et al.* 2010 Reliability 2	Estradiol level	Rat	Pubertal assay, PND 21-40	Oral	62.5, 250, 1000 mg/kg bw/day	>1000	No effect	No significant effect on estradiol levels in female rats, after pubertal exposure	**No effects on estradiol levels.**
Kim *et al.* 2015 Reliability 2	Estradiol level	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw	>600 mg/kg bw	No effect	No effect in estradiol levels in male and female rats after 28-day dermal exposure	
Vo *et al.* 2010 Reliability 2	Prolactin level	Rat	Pubertal assay, PND 21-40	Oral	62.5, 250, 1000 mg/kg bw/day	>1000	No effect	No significant effect on prolactin levels in female rats, after pubertal exposure	**No effects on prolactin levels.**
Kim *et al.* 2015 Reliability 2	Testosterone level	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw	>600 mg/kg bw	No effect	No effect (male and female)	**No effects on testosterone levels**
Kim *et al.* 2015 Reliability 2	FSH level	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw	>600 mg/kg bw	No effect	No effect (male)	**No effects on FSH levels**
Kim *et al.* 2015 Reliability 2	T3 level	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw	>600 mg/kg bw	No effect	No effect (male and female)	**No effects on T3 levels**
Kim *et al.* 2015 Reliability 2	TSH level	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw	>600 mg/kg bw	No effect	No effect (male and female)	**No effect on adult TSH levels**
Vo *et al.* 2010 Reliability 2	T4 level	Rat	Pubertal assay, PND 21-40	Oral	62.5, 250, 1000 mg/kg bw/day	(62.5)	Decrease	Significant decrease in serum T4 levels in the low dose group at PND 41 but no significant effect at 250 and 1000 mg/kg bw/day	**No consistent evidence of reduced serum T4 levels.**

Table 18: Lines of evidence for adversity *in vivo* by IBP

Reference	Effect classification	Effect target	Species	Exposure	Route	Dose	LO(A)EL (mg/kg bw/day)	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence
Vo *et al.* 2010 Reliability 2	EAS-mediated	Age at VO	Rat	Pubertal assay, PND 21-40	Oral	62.5, 250, 1000 mg/kg bw/day		No effect	No significant effect, however a nominal increase from control at day 31.6 to 1000mg dose group at day 33.6	No effect on age at VO
Vo *et al.* 2010 Reliability 2	EAS-mediated	Estrous cyclicity	Rat	Pubertal assay, PND 21-40	Oral	62.5, 250, 1000 mg/kg bw/day		No effect	No effect after 62.5, 250, and 1000 mg/kg bw/day	No effect on estrous cycling (but uncertainties regarding assessment).
Vo *et al.* 2010 Reliability 2	EAS-mediated	Ovary histopathology	Rat	Pubertal assay, PND 21-40	Oral	62.5, 250, 1000 mg/kg bw/day	62.5	increase	Significant increase at PND 41 in incidents of "Decrease of corpora lutea, increase in the number of cystic follicles"	Some evidence of altered ovary histopathology with pubertal exposure.
Vo *et al.* 2010 Reliability 2	EAS-mediated	Ovary weight	Rat	Pubertal assay, PND 21-40	Oral	62.5, 250, 1000 mg/kg bw/day		No effect	Weight relative to bw at PND 41. No effect after 62.5, 250, and 1000 mg/kg bw/day	No effect on ovary weight with pubertal exposure
Vo *et al.* 2010 Reliability 2	EAS-mediated	Uterus histopathology	Rat	Pubertal assay, PND 21-40	Oral	62.5, 250, 1000 mg/kg bw/day	62.5	increase	Significant increase at PND 41 in thickness of morphometric measurement after62.5, 250, 1000 mg/kg bw/day	Some evidence of altered uterus histopathology with pubertal exposure
Kim *et al.* 2015 Reliability 2	EAS-mediated	Uterus weight	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw		No effect	No effect on uterus weight, after dermal exposure	No effect on uterus weight
Vo *et al.* 2010 Reliability 2	EAS-mediated	Uterus weight	Rat	Pubertal assay, PND 21-40	Oral	62.5, 250, 1000 mg/kg bw/day		No effect	Weight relative to bw at PND 41. No effect after 62.5, 250, and 1000 mg/kg bw/day	

Kim *et al.* 2015 Reliability 2	EAS-mediated	Vagina weight	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw		No effect	No effect	No effect on vagina weight after dermal exposure
Kim *et al.* 2015 Reliability 2	EAS-mediated	Prostate weight	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw		No effect	No effect	No effect on prostate weight after dermal exposure
Kim *et al.* 2015 Reliability 2	EAS-mediated	Testis weight	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw		No effect	No effect	No effect on testis weight after dermal exposure
Vo *et al.* 2010 Reliability 2	T-mediated	Thyroid weight, relative	Rat	Pubertal assay, PND 21-40	Oral	62.5, 250, 1000 mg/kg bw/day		No effect	Weight relative to bw at PND 41. No effect after 62.5, 250, and 1000 mg/kg bw/day	No effect on thyroid weight
Vo *et al.* 2010 Reliability 2	Sensitive to, but not diagnostic of, EAST	Adrenal weight, relative	Rat	Pubertal assay, PND 21-40	Oral	62.5, 250, 1000 mg/kg bw/day		No effect	Weight relative to bw at PND 41. No effect after 62.5, 250, and 1000 mg/kg bw/day	No effect on adrenal weight
Vo *et al.* 2010 Reliability 2	Sensitive to, but not diagnostic of, EAST	Pituitary weight, relative	Rat	Pubertal assay, PND 21-40	Oral	62.5, 250, 1000 mg/kg bw/day		No effect	Weight relative to bw at PND 41. No effect after 62.5, 250, and 1000 mg/kg bw/day	No effects on pituitary weight
Vo *et al.* 2010 Reliability 2	Systemic toxicity	Body weight	Rat	Pubertal assay, PND 21-40	Oral	62.5, 250, 1000 mg/kg bw/day		No effect	No effect after 62.5, 250, and 1000 mg/kg bw/day	No effect on body weight up to 1000 mg/kg bw/day in rats
Kim *et al.* 2015 Reliability 2	Systemic toxicity	Body weight	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw		No effect	No effect on bw in the study	
Kim *et al.* 2015 Reliability 2	Systemic toxicity	Haema tology	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw		No effect	No effect on hematological parameters	No effect on haematological parameters

Kim *et al.* 2015 Reliability 2	Systemic toxicity	Food consumption	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw		No effect	No effect (no effect on food and water consumption in the study)	No effect on food consumption
Kim *et al.* 2015 Reliability 2	Target organ toxicity	Heart histopathology	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw		No effect	No effect	No effect on heart histopathology or weight up to 600 mg/kg bw/day in adult rats
Kim *et al.* 2015 Reliability 2	Target organ toxicity	Heart weight	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw		No effect	No effect	
Kim *et al.* 2015 Reliability 2	Target organ toxicity	Kidney histopathology	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw		No effect	No effect	No effect on kidney histopathology
Kim *et al*. 2015 Reliability 2	Target organ toxicity	Kidney weight	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw		No effect	No effect	No effect on kidney weight
Vo *et al.* 2010 Reliability 2	Target organ toxicity	Kidney weight, relative	Rat	Pubertal assay, PND 21-40	Oral	62.5, 250, 1000 mg/kg bw/day		No effect	No effect	
Kim *et al.* 2015 Reliability 2	Target organ toxicity	Liver histopathology	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw		No effect	No effect	No effect on liver histopathology and weight
Kim *et al.* 2015 Reliability 2	Target organ toxicity	Liver weight	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw		No effect	No effect	
Kim *et al.* 2015 Reliability 2	Target organ toxicity	Brain histopathology	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw		No effect	No effect	No effect on brain weight or histopathology
Kim *et al.* 2015 Reliability 2	Target organ toxicity	Brain weight	Rat	28-day study	Dermal	0, 50, 100, 300, 600 mg/kg bw		No effect	No effect	

Table 19: Lines of evidence for adversity *in vivo* by BP (from ECHA 2020) and IBP (based on read across) – with addition of data from two new studies; Oliveira *et al.* 2020 and Hubbard *et al.* 2020.

As in the SVHC support document for BP (ECHA 2020), only studies with perinatal exposure to BP, where male offspring were examined for effects on AGD or sperm quality have been included. Data on systemic toxicity and target organ toxicity has been added to table from ECHA 2020.

Lines of evidence for **adverse effect with perinatal exposure to BP**. EAS modalities.						
Reference	**Grouping**	**Line of evidence**	**Study design**	**Effect dose (mg/kg bw/d)**	**Observed effects**	**Assessment of each line of evidence**
Kang *et al.* 2002 Reliability 2	EAS mediated	Anogenital distance (AGD)	Rat, GD6-PND20 Direct (s.c.)	-	No effect at PND 1 at doses of 100 and 200 mg/kg bw/day	**Low to moderate evidence of effects on male AGD.** Inconsistency between the studies may be due to different exposure periods, dose levels and measuring sensitivity.
Taxvig *et al.* 2008 Reliability 2	EAS mediated	AGD	Rat, GD7-GD21 Direct (s.c.)	-	No effect at GD 21 at doses of 200 and 400 mg/kg bw/day	
Zhang *et al.* 2014 Reliability 2	EAS mediated	AGD	Rat, GD 7 – PND 21, Oral	400	Reduced at PND 1 and 21 at 400 and 1000 mg/kg bw/day and not at 64 or 160 mg/kg bw/day	
Boberg *et al.* 2016 Reliability 2	EAS mediated	AGD	Rat, GD 7 – PND 22, Oral	100	Reduced of AGD and AGDi at PND 1 at 100 and 500 mg/kg bw/day	
Guerra *et al.* 2017 Reliability 2	EAS mediated	AGD	Rat, GD 12 – PND 21, Direct (s.c.)	-	No effect at PND 1 at doses of 10, 100, 200 mg/kg bw/day.	
Hubbard *et al.* 2020 Reliability 1	EAS mediated	AGD	Rat, continuous from F0 through F2 generation, feed	-	No effect at PND 1 in F1 and F2 generations at doses of 5000, 15000, 40000 ppm corresponding to around 335, 990, 3170 mg/kg/day.	
Fisher *et al.* 1999 Reliability 2	EAS-mediated	Testis histopathology	Rat, PND 2-18 Direct (s.c.)	-	No effect at PND 18. Only one low dose (2 mg/kg bw/day) and evaluation of aquaporins and rete testis morphology only.	**Moderate evidence of adverse effect on testes histopathology.** No effects on fetal testis in two studies. Signs of histological effects on seminiferous tubules of prepubertal testes in one study. Change in adult testes in four out of five studies,
Kang *et al.* 2002 Reliability 2	EAS-mediated	Testis histopathology	Rat GD6-PND20 Direct (s.c.)	100	Decrease. The numbers of spermatogonia, spermatocytes, round spermatids and elongated spermatids were investigated in seminiferous tubules. Round and elongated spermatids were reduced in both exposure groups (100 and 200 mg/kg bw/day).	
Taxvig *et al.* 2008	EAS-mediated	Testis histopath	Rat, GD7-GD21	-	No effect on foetal testis histopathology at GD 21.	

Reliability 2		ology	Direct (s.c.)			but different endpoints examined.
Zhang *et al.* 2014 Reliability 2	EAS-mediated	Testis histopathology	Rat GD 7 – PND 21 Oral	400	Change. PND 21: reduced and loosely arranged germ cells, reduced layers of seminiferous tubules in the two highest dose groups (400 and 1000 mg/kg bw/day). No obvious effects on Leydig cells. PND 90: Expanded lumens of the seminiferous tubules, reduced layer of seminiferous tubules, and reduced number of spermatocyte cells in the two highest dose groups (400 and 1000 mg/kg bw/day)	
Zhang *et al*. 2016 Reliability 2	EAS-mediated	Testis histopathology	Rat, GD 7 – PND 21 Oral	400	Change. As above, same study as Zhang et al. (2014).	
Boberg *et al.* 2016 Reliability 2	EAS-mediated	Testis histopathology	Rat, GD 7 – PND 22, Oral	-	No effect. Examined at PND 90, control and high dose, no morphometrical analyses.	
Guerra *et al.* 2017 Reliability 2	EAS-mediated	Testis histopathology	Rat GD 12 – PND 21 Direct (s.c.)	-	No effect at GD 20 on number of foetal Leydig cells, diameter of seminiferous cords, number of gonocytes/cord	
Guerra *et al.* 2017 Reliability 2	EAS-mediated	Testis histopathology	Rat GD 12 – PND 21 Direct (s.c.)	100	Increase in number of Leydig cells in interstitium of adult testes at PND 110 in two highest dose groups (100 and 200 mg/kg bw/day). No effect on testicular morphometry PND 110 (number of Sertoli cells, nuclear Leydig cell volume, nuclear Leydic cell area)	
Guerra *et al.* 2017 Reliability 2	EAS-mediated	Testis histopathology	Rat GD 12 – PND 21 Direct (s.c.)	200	Decrease in IHC staining intensity on estrogen receptor(ESR)1 and AR in adult: reduced ESR1 in elongated spermatids in stage I – VI and rounded spermatids on stage VII – VIII and reduced AR in Sertoli cell nuclei in stages VII – VIII in high dose group (200 mg/kg bw/day).	
Guerra *et al.* 2017 Reliability 2	EAS-mediated	Testis histopathology	Rat GD 12 – PND 21 Direct (s.c.)	10	Change. Spermatogenesis kinetics PND 110. Percentage of seminiferous tubules in stage I – VI decreased in high dose group (200 mg/kg bw/day) and stages VII-VIII increased in low and high dose (10 and 200 mg/kg bw/day).	
Maske *et al.* 2020 Reliability 2	EAS-mediated	Testis histopathology	Rat GD 6- PND 21 Direct (s.c.)	10	Change. Seminiferous tubules and germinal layers were evaluated on PND 30, 45 and 75. Effects were seen on tubules (degenerative) and the germ layer (arrangement) on PND 30 (10, 100 and 1000 mg/kg bw) and on PND 45 and 75 degenerative changes on tubules and reduced spermatogenesis was seen at 10, 100 and 1000 mg/kg bw	
Hubbard *et al.* 2020 Reliability 1	EAS-mediated	Testis histopathology	Rat, continuous from F0 through F2	-	No effect. Examined at 30-31 and 13-14 weeks of age.	

			generation, feed			
Fisher *et al.* 1999 Reliability 2	EAS-mediated	Testis weight	Rat, PND 2-18 Direct (s.c.)	-	No effect. Absolute weight, one low dose of 2 mg/kg bw/day.	**No clear pattern of effects on testis weight in prepuberty or adulthood following perinatal exposure.**
Kang *et al.* 2002 Reliability 2	EAS-mediated	Testis weight	Rat, GD6-PND20 Direct (s.c.)	100	Change. Investigated at PND 21, 49, 70 and 90. 100 mg/kg bw/day led to increase on PND 21 and decrease on PND 49. 200 mg/kg bw/day led to increase on PND 90.	
Zhang *et al.* 2014 Reliability 2	EAS-mediated	Testis weight	Rat GD 7 – PND 21 Oral	400	Decrease. Absolute weights measured PND 21, 35, 49, 90, 180. Reduced in the two highest dose groups (400 and 1000 mg/kg bw/day) on PND 21, 35, and 49. BW also affected at these ages. On PND 90 affected in the three highest dose groups (160, 400 and 1000 mg/kg bw/day), no effect on BW at this age. No effect on testis weight on PND 180.	
Zhang *et al.* 2016 Reliability 2	EAS-mediated	Testis weight	Rat GD 7 – PND 21 Oral	-	No effect. Relative weights PND 21 and 90. Same study as Zhang et al. (2014), but the study by Zhang *et al.* (2014) is possibly a subgroup of animals from Zhang *et al.* (2016), therefore different effects (BW at PND 90 affected in the study by Zhang *et al.* (2016) but not *Zhang et al.* (2014)).	
Boberg *et al.* 2016 Reliability 2	EAS-mediated	Testis weight	Rat, GD 7 – PND 22, Oral	-	No effect. Testis weighed in offspring on PND16, 22, and 80 – 90. Absolute weights analysed using body weight as covariate in ANOVA.	
Guerra *et al.* 2017 Reliability 2	EAS-mediated	Testis weight	Rat, GD 12 – PND 21, Direct (s.c.)	-	No effect. Absolute weight PND 110	
Maske *et al.* 2020 Reliability 2	EAS-mediated	Testis weight	Rat, GD 6-PND 21, Direct (s.c.)	-	No effect. Testis weighed in male offspring on PND 30, 45 and 75.	
Oliveira *et al.* 2020 Reliability 2	EAS-mediated	Testis weight	Rat, GD 1-22 Direct (s.c.)	100	Decrease. Relative testis weight reduced in male offspring on PND 56 without change in body weight at doses of 100 and 200 mg/kg bw/day. Associated with changes in the mitochondrial bioenergetics and antioxidant capacity of testis.	
Hubbard *et al.* 2020 Reliability 1	EAS-mediated	Testis weight	Rat, continuous from F0 through F2 generation, feed	~1400	Increase in relative left and right testis weight at 13-14 and 30-31 weeks of age at doses of 15000 and 40000 ppm (corresponding to around 1400 and 4800 mg/kg/day) concomitant with significantly lower body weights.	
Kang *et al.* 2002	EAS-mediated	Sperm numbers	Rat, GD6-PND20, Direct	100	Decrease. Sperm count in caudal epididymis. Decreased to 50% of control in both exposure groups (100 and 200 mg/kg bw/day).	**Moderate-strong evidence for adverse effect on**

Reliability 2			(s.c.)			**sperm count**. Two studies (Guerra *et al.* 2017 and Hubbard et al. 2020) did not show effect at doses up to around 5000 mg/kg bw/day (40,000 ppm) and another study (Zhang et al. 2014) showed effect only at the highest doses of 400 and 1000 mg/kg bw/day. In Maske *et al.* (2020) effect was statistically significant in the middle dose (100 mg/kg bw/day), but the high dose (1000 mg/kg bw/day) is likely affected as the average value is approximately 80% of control and the variance in the control group is rather big. Effects are considered serious and irreversible.
Zhang *et al.* 2014 Reliability 2	EAS-mediated	Sperm numbers	Rat, GD 7 – PND 21, Oral	400	Decrease. Numbers of sperm in cauda epididymis and daily sperm production on PND 90 reduced in two high dose groups (400 and 1000 mg/kg bw/day, no effect at 64 or 160 mg/kg bw/day).	
Boberg *et al.* 2016 Reliability 2	EAS-mediated	Sperm numbers	Rat, GD 7 – PND 22, Oral	10	Decrease. Number of sperm in cauda epididymis measured on PND 90. Reduced in all dose groups (10, 100, 500 mg/kg bw/day).	
Guerra *et al.* 2017 Reliability 2	EAS-mediated	Sperm numbers	Rat, GD 12 – PND 21, Direct (s.c.)	-	No effect. Sperm counts on PND 110 (testis and epididymis) at doses of 10, 100 or 200 mg/kg bw/day	
Maske *et al.* 2020 Reliability 2	EAS-mediated	Sperm numbers	Rat GD 6- PND 21 Direct (s.c.)	100	Decrease. Sperm count was investigated on PND 75 and a decrease was seen at 100 mg/kg bw/day (67% of control), but not at 1000 mg/kg bw/day (however, the average value in this group is approximately 80% of control and the variance in the control group is rather big)	
Hubbard *et al.* 2020 Reliability 2	EAS-mediated	Sperm numbers	Rat, continuous from F0 through F2 generation, feed	-	No effect. Examined at 30-31 and 13-14 weeks of age.	
Kang *et al.* 2002 Reliability 2	EAS-mediated	Sperm motility	Rat, GD6-PND20 Direct (s.c.)	100	Decrease. Sperm motile activity (%) was decreased in both exposure groups of 100 and 200 mg/kg bw/day.	**Moderate to strong evidence for adverse effect on sperm motility.** Reduced sperm motility in three studies and no effect in one but lack of dose-response and different methods applied.
Guerra *et al.* 2017 Reliability 2	EAS-mediated	Sperm motility	Rat GD 12 – PND 21 Direct (s.c.)	10	Decrease. Motile sperm with progressive trajectory (%) at PND 110: decreased in low dose only (10 mg/kg bw/day) while slight decreases at 100 and 200 mg/kg bw/day were not statistically significant. Motile sperm with non-progressive trajectory (%) at PND 110: increased in low dose group (10 mg/kg bw/day) while slight increases at 100 and 200 mg/kg bw/day were not statistically significant. No change in % non-motile sperm at PND 110.	
Maske *et al.* 2020 Reliability 2	EAS-mediated	Sperm motility	Rat, GD 6-PND 21 Direct (s.c.)	100	Decrease. Sperm motility was investigated on PND 75 and a decrease was seen at 100 mg/kg bw/day, but not in the the high dose group (1000 mg/kg bw/day)	
Hubbard *et al.* 2020 Reliability 2	EAS-mediated	Sperm motility	Rat, continuous from F0 through F2	-	No effect. Examined at 30-31 and 13-14 weeks of age.	

			generation, feed			
Guerra *et al.* 2017 Reliability 2	EAS-mediated	Sperm morphology	Rat GD 12 – PND 21 Direct (s.c.)	10	Decrease. Normal morphology (%) at PND 110: decrease in all dose groups (10, 100, 200 mg/kg bw/day). Abnormal head (characteristic curvature missing) (%) at PND 110: increase in all dose groups (10, 100, 200 mg/kg bw/day).	**Some evidence for adverse effect on sperm morphology**. Reduced percentage of normal sperm in one study; same effect size at all doses. Effects are considered to be serious and irreversible.
Guerra *et al.* 2017 Reliability 2	Sensitive to, but not diagnostic of, EAS	Fertility (mammals)	Rat, GD 12 – PND 21, Direct (s.c.)	-	No effect on fertility with natural mating (age not clear) or in utero artificial insemination (PND 110).	**No clear evidence of effect on fertility.** Potential effects on fertility in rats indicated by low number of implantation sites. The effect on pre-/post-implantation loss is not considered to be dose-related, as pregnant females were not exposed.
Maske *et al.* 2020 Reliability 2	Sensitive to, but not diagnostic of, EAS	Fertility	Rat GD 6- PND 21 Direct (s.c.)	100	Change: Naïve females were sired by exposed male offspring. Decrease in mean no. of implantation sites (100 mg/kg bw/day). Increase in % pre-implantation loss in 100 and 1000 mg/kg bw/day as well as increase in % post-implantation loss at 1000 mg/kg bw/day. No effect on copulation, time taken for copulation, no. of copulated females showing resorptions, or mean number of corpus luteum	
Hubbard *et al.* 2020 Reliability 2	Sensitive to, but not diagnostic of, EAS	Fertility	Rat, cont. from F0 through F2 generation, feed	-	No effect on fertility (mated/pair, littered/pair, littered/mated, duration of precoital or gestational intervals) with three natural matings per generation (F0 and F1). A significant decreasing trend in total litter size occurred in F0&F1 pairings increasing with exposure.	
Kang *et al.* 2002 Reliability 2	Systemic toxicity	Body weight	Rat, GD6-PND20 Direct (s.c.)		No effect on bw in the pregnant and lactating dams at any dose level. Decreased body weight in male F1 offspring at 100 mg/kg on PND49 but not in 200 mg/kg.	No or minimal effect (F1 males) on bw in most studies. In one study effect on bw was found in all groups.
Taxvig *et al.* 2008 Reliability 2	Systemic toxicity	Body weight	Rat, GD7-GD21 Direct (s.c.)		No effect on bw	
Zhang *et al.* 2014 Reliability 2	Systemic toxicity	Body weight	Rat, GD 7 – PND 21, Oral		There was no effect in dams. In male offspring, bw decreased from PND 0-49, but not affected PND 90-180.	
Zhang *et al*. 2016 Reliability 2	Systemic toxicity	Body weight	Rat, GD 7 – PND 21 Oral		Data is possibly based on the same animal study as Zhang et al. 2014.	
Boberg *et al.* 2016	Systemic toxicity	Body weight	Rat, GD 7 – PND 22, Oral		No effect on bw in dams or offspring.	

Reliability 2						
Guerra *et al.* 2017 Reliability 2	Systemic toxicity	Body weight	Rat GD 12 – PND 21 Direct (s.c.)		No marked effect on bw in dams or male offspring.	
Maske *et al.* 2020 Reliability 2	Systemic toxicity	Body weight	Rat GD 6- PND 21 Direct (s.c.)		Increased bw in F1 males from PND 4 to PND 75 (except at PND 45) exposed to 10 mg/kg BP. No effect in other treatment groups.	
Oliveira *et al.* 2020 Reliability 2	Systemic toxicity	Body weight	Rat, GD 1-22 Direct (s.c.)		No effect on bw	
Hubbard *et al.* 2020 Reliability 1	Systemic toxicity	Body weight	Rat, continuous from F0 through F2 generation, feed		Lower bw across all groups (F0 males and females, F0 dams, F1 and F2 pups), with highest magnitude of lower bw noted in perinatally exposed rats of F1 and F2 cohorts.	
Kang *et al.* 2002 Reliability 2	Systemic toxicity	Food consumption	Rat, GD6-PND20 Direct (s.c.)		No effect on food consumption at any dose level in the pregnant and lactating dams at any dose level	No effect on food consumption
Oliveira *et al.* 2020 Reliability 2	Systemic toxicity	Food consumption	Rat, GD 1-22 Direct (s.c.)		No remarkable changes	
Hubbard *et al.* 2020 Reliability 1	Target organ toxicity	Liver weight	Rat, continuous from F0 through F2 generation, feed		Evidence of hepatic toxicity (effect on liver weight, liver histology). Predominant in F1 cohort.	Liver identified as target organ of BP toxicity in one study but not in another.
Oliveira *et al.* 2020 Reliability 2	Target organ toxicity	Liver weight	Rat, GD 1-22 Direct (s.c.)		No change in relative mass	
Hubbard *et al.* 2020 Reliability 1	Target organ toxicity	Kidney weight	Rat, continuous from F0 through F2 generation, feed		Reduced absolute kidney weight, 40.000 ppm (F0 males + females + F1 males)	Effect on kidney weight only at high dose level.
Oliveira *et*	Target	Kidney	Rat, GD 1-22		No change in relative mass	

al. 2020 Reliability 2	organ toxicity	weight	Direct (s.c.)			
Hubbard *et al.* 2020 Reliability 1	Target organ toxicity	Spleen weight	Rat, continuous from F0 through F2 generation, feed		Decreased absolute spleen weight, 40.000 ppm (F0 males + F1 males (parental to F2)). Increased absolute spleen weight (F0 females)	Effect on spleen weight only at high dose level.
Oliveira *et al.* 2020 Reliability 2	Target organ toxicity	Heart weight	Rat, GD 1-22 Direct (s.c.)		No change in relative mass	No effect on heart

Annex IV – Mode of action (MoA) analysis

Table 20: Summary table on key events in MoA analysis for IBP. Since limited information is available for IBP on adverse effects, information on BP is included in the MoA analysis (based on the read-across argumentation presented in Annex I).

Title	**Activation of ER to Impaired fertility of male offspring**	
Hypothesi	The molecular initiating event is activation of the ER(s). In developing males, increased ER signalling results in altered testicular development in offspring and subsequently altered testicular function in adulthood. In turn, reduced sperm count and quality is observed in offspring.	
	Brief description of event	**Supporting evidence**
MIE	Molecular: Activation of ER	High. LoE show strong evidence for endocrine activity related to ER activation. Several studies show ER agonistic response similar to estrogen (Gonzales *et al.* 2018; Kim *et al.* 2011; Kim *et al.* 2012a; Terasaki *et al.* 2009; Watanabe *et al.* 2013).
KE1	Increased ER signalling	High. Several studies show effects of IBP on growth of estrogen sensitive cells (Darbre *et al.* 2002; Gonzales *et al.* 2018; Okubo *et al.* 2001) or tissues (uterotrophic assay *in vivo* (Darbre *et al.* 2002; Koda *et al.* 2005; Vo and Jeung 2009) or showed increased expression of estrogen-regulated genes and proteins in cell lines (Darbre *et al.* 2002; Gonzales *et al.* 2018; Kim *et al.* 2012a; Kim *et al.* 2012b; Kim *et al.* 2011; Okubo *et al.* 2001; Vo *et al.* 2012; Yang *et al.* 2011).
KE2	Organ: Altered reproductive development of male offspring	No reliable data for IBP, moderate for BP. For BP, reduced AGD was seen in some, but not all studies (Riad *et al.* 2018; Zhang *et al.* 2014; Guerra *et al.* 2017; Kang *et al.* 2002; Boberg *et al.* 2016; Taxvig *et al.* 2008; Hubbard *et al.* 2020). Changes in histology of prepubertal testes were seen in one study (Zhang *et al.* 2014).
KE3	Organ: Altered testicular and epididymal function of adult offspring	No reliable data for IBP, moderate for BP. For BP, altered serum hormone levels were seen in several studies (Zhang *et al.* 2014; Zhang *et al.* 2016; Guerra *et al.* 2017), and altered testicular histology and gene expression was seen in perinatally exposed adults (Guerra *et al.* 2017; Kang *et al.* 2002), whereas effects on testis weights were seen in some but not all studies.
AO 1	Organ: Reduced sperm count and quality of offspring's	No reliable data for IBP, moderate - high for BP. For BP, studies using s.c. or oral gavage showed reduced epididymal sperm count (50-75% of control; Kang *et al.* 2002; Boberg *et al.* 2016; Zhang *et al.* 2014) but no change in epididymal sperm count in another study (Guerra *et al.* 2017). Reduced sperm motility (60% of control; Kang *et al.* 2002) and reduced percentage of progressive motile sperm (low dose only, Guerra *et al.* 2017). Increased percentage of sperm with head abnormalities and reduced percentage of normal sperm (Guerra *et al.* 2017). A developmental dietary exposure study showed no effect on the investigated sperm parameters (Hubbard *et al.* 2020).
AO 2	Organism: Impaired fertility of male offspring	IBP and BP: Low evidence for effect in rodents, but high plausibility that impaired sperm count and quality in humans lead to impaired fertility (see Biological plausibility table below). For BP, no effect was seen on fertility assessed by natural mating or artificial insemination (Guerra *et al.* 2017).

As presented in the SVHC support document for BP (ECHA 2020) additional MIEs leading to the same key events and ultimately the same AO as outlines above, are altered steroidogenesis and AR antagonism causing increased estradiol levels. In developing rodent males, this would lead to increased ER signalling resulting in altered testicular development in offspring and subsequently altered testicular function in adulthood. In turn, reduced sperm count and sperm quality would be observed. However, in the case of IBP, the supporting evidence for these MoAs are low, as the lines of evidence show no evidence for endocrine activity related to altered steroidogenesis and weak or no effects on the AR.

Table 21: Analysis of biological plausibility of key event relationships.

Title	**Activation of ER to Impaired fertility of male offspring**	
	Brief description of key event relationship (KER)	Supporting evidence
MIE to KE1	ER activation to Increased transcription of ER regulated genes	High. ER activation leads to increased ERs signalling.
KE1 to KE2	Increased ER signalling to Altered reproductive development of offspring	Moderate to high. ERα is expressed in fetal Leydig cells (Nielsen *et al.* 2000) and has regulatory effects on steroidogenesis; endogenous estrogens inhibit testicular development and function in fetal/neonatal life (Delbès *et al.* 2005; Delbés *et al.* 2006). Exogenous 'estrogens' lead to decreased testosterone levels in rodents (Lassurguere *et al.* 2003; Delbès *et al.* 2004; Delbés *et al.*2005; Lehraiki *et al.* 2011). In turn, reduced testosterone levels in male fetus may cause masculinisation failure (Stewart *et al.*2018; Schwartz *et al.* 2019). Human evidence is scarce, but one study has shown association between polymorphism of ESR1 (coding for ERα) and short AGD in boys (Sathyanarayana *et al.* 2012) ERβ is expressed in late gestation gonocytes (Jefferson *et al.* 2000) and regulates apoptotic/mitotic rate during late gestation (Delbès *et al.* 2004), and ERβ activation by estrogenic compounds could cause altered gonocyte proliferation (Delbès *et al.* 2006). There are several examples of exogenous estrogens altering male reproductive development in rodents. Developmentally estrogenised male mice display retained or cryptorchid testes, decrease in sperm number, increase in abnormal sperm, retained Müllerian ducts, epididymal cysts, hypospadias, and prostatic disease. Such phenotype has been seen, in whole or in part, in mice, rats, hamsters, and humans exposed to estrogens *in utero* (McLachlan 2001). Biological pathways leading from ER activation to effects on testes are currently not well described and may include alterations of androgen-dependent processes such as suppression of testosterone production and downregulation of the expression of the androgen receptor protein in reproductive target tissues including the testes (Martin *et al.* 2008). The epididymis is highly responsive to androgens, but estrogen has a predominant role in efferent ductules and initial segment epididymis (Joseph *et al.* 2011). Interference with ER signalling can thus affect epididymal development both directly and secondary to altered AR signalling.
KE2 to KE3	Altered reproductive development of offspring to Altered testicular and epididymal function in adult offspring	High. Correct development of the reproductive system in early life is essential to achieve optimal reproductive function in adulthood. It is highly biologically plausible that impaired reproductive development is a cause of altered testicular and epididymal function in adulthood.
KE3 to AO1	Altered testicular and epididymal function in adult offspring to Reduced sperm count and quality in offspring	High. Correct function of testis and epididymis is necessary for an optimal sperm count and quality (motility, morphology)
AO1 to AO2	Reduced sperm count and quality in offspring to Impaired fertility of male offspring	High. There is clear evidence that impaired sperm count and quality in humans leads to impaired fertility. In rodents, reproductive function is less sensitive to reductions in sperm count and quality.

The biological plausibility of key event relationships was analysed as presented in Table 21. The evidence for this analysis is not limited to isobutyl- or BP, but is strengthened by evidence from other models and studies on other endocrine disrupters affecting sperm count and quality of offspring. This analysis of biological plausibility thus includes understanding of physiology, endocrinology and toxicology, and information from studies on other chemicals or knockout models.

Table 22: Conclusions on MoA analysis.

Mode of action analysis	There is strong evidence of ER activation. There is a lack of studies investigating adverse effects of IBP but read-across from studies on BP provide strong evidence for this.
Biological plausibility	It is biologically plausible that adverse effects are due to the endocrine activity of BP, and that the same will be seen for IBP exposure.
Dose	In most *in vivo* studies on source substance BP, indicators of key events related to endocrine activity (e.g., altered hormone levels and altered AGD) were affected at the same doses causing adverse effects on sperm parameters. Between studies, there are however some differences in effective doses, possibly depending on study design and sensitivity.
Temporal concordance	Regarding temporal concordance, it is noted that key events are observed in the hypothesised order. For source substance BP, *in vivo* indicators of key events related to endocrine activity are seen in developing animals, and adverse effects on sperm parameters are seen in adulthood, i.e., long after the end of exposure. This is in line with the conclusion that developmental changes in the male reproductive system are the cause of adult adverse effects.
Essentiality	For determining essentiality, it should be demonstrated whether or not downstream KEs and/or the adverse effect is prevented/decreased if an upstream event is experimentally blocked. It was not examined whether counteracting the endocrine related key events would prevent adverse effects (of source substance BP) with perinatal exposure.
Human relevance	There are no data indicating that these endocrine modes of action are not relevant to humans. Thus, human relevance is assumed by default. No epidemiological studies examined the relationship between IBP or BP exposure *in utero* and effect on male reproductive parameters (hormone levels, sperm parameters) later in life. A few studies showed supportive evidence for an endocrine disrupting activity of BP during pregnancy (see ECHA 2020).
Identified uncertainties	
No one- or two-generation studies have been performed	IBP has not been tested in any reliable developmental toxicity studies, and BP has not been tested in one- or two-generation studies. However, a recent RABC study with BP has been published, showing no adverse effects on reproductive endpoints, when using high dietary doses of BP (Hubbard *et al.* 2020). The results from all other perinatal studies using BP doses above 400 mg/kg bw/day show the adverse effects on sperm quality in adult rodents, and the studies indicate that the adverse effects originate from developmental changes in male reproductive development.
Different effect levels/no effect levels observed in different *in vivo* studies.	Adverse effects (reduced sperm count and quality) are seen at different dose levels in different studies on BP. Differences in study design may explain some differences in results between studies in patterns of late-life effects in male offspring. The two studies including doses of 400 mg/kg bw/day or above both showed reduced sperm counts at these doses (Zhang *et al.* 2014; Boberg *et al.* 2016), but at lower doses some studies showed effect and other studies did not. One study contrasts these findings, as high dietary doses of BP (up to 3000 mg/kg bw/day) did not affect sperm quality in a recent RABC study (Hubbard *et al.* 2020).
Lack of a clear description of biological pathways leading from ER activation or androgen receptor antagonism in utero to adverse effects on testis function in adulthood.	It is biologically plausible that the alteration in sperm count and quality observed for BP is due to endocrine disruption during development. The uncertainty in describing biological pathways also applies to several other estrogenic or anti-androgenic substances for which perinatal exposures lead to reduction in sperm count and/or quality. It is stated in EFSA/ECHA guidance (ECHA/EFSA 2018) that to conclude on the biological plausibility of the link (between MoA and adversity), it may not be necessary to have demonstrated the whole sequence of events leading to the

	adverse effect for the substance under evaluation. Existing knowledge from endocrinology or toxicology may be sufficient to assess the link and conclude on the biological plausibility between adverse effects and the endocrine activity. It is noted that in some cases, "*the MoA analysis could be very simple; when an adverse effect is 'EATS-mediated', the biologically plausible link is already pre-established in the absence of information proving the contrary (i.e., a fully developed non-ED MoA). This is because, in the case of 'EATS-mediated' parameters, where the pattern of effects is deemed adverse, the biological plausibility that the adverse effects are caused via an EATS-mediated MoA is high, based on existing knowledge and theory (i.e. coherence analysis), and as such, it may not be necessary to generate further empirical data on the substance under evaluation to substantiate the link between the observed adverse effect(s) and an endocrine-mediated MoA*." (ECHA/EFSA 2018, section 3.5.2)
Conclusion: IBP likely acts mainly via binding to the ER and it is biologically plausible that the endocrine activity of IBP leads to observed adverse effects on the male reproductive system, as is the case for source substance BP. For ER activation (directly or due to increased estradiol levels) the evidence for each key event relationship is considered "High", except the step "Increased ER signalling to altered reproductive development of offspring", for which the evidence is considered "Moderate to high". There is sufficient dose and temporal concordance between key events, and effects are assumed relevant to humans.	

Annex V – Human epidemiology studies

Study overview of human studies

Only one study on IBP was identified of relevance to ED evaluation (Jurewicz *et al.* 2017). As noted in Table 23, this study, which showed no association of exposure with sperm chromosome disomy, is of limited usefulness, as the timing of exposure measurement was not considered relevant to the endpoint assessed.

Note - to open the embedded Excel worksheets:

1. Open the Attachments panel on the left hand side.
2. In the Attachments panel, double-click the listed attachment.

In case the embedded excel sheet does not open, it is provided as a separate file on the consultation page.

Table 23: Human studies on IBP. Quality scores according to EFSA (2017).

Reference	Method	Relevant Endpoints & Effects (Effects Are Annotated With Arrows Or 'Affected')	Comments/Notes	Quality Score
Jurewicz *et al.* 2017 Study evaluation: Jurewicz et al 2017.xlsx	Cross-sectional study 2008-2011. Men aged 22-45 years, average 32 years. Normal sperm conc. 15-300 mill/ml and saliva and urine random spot sample.	No effect. Assessment of sperm chromosome disomy (XX, YY, XY, 1313, 1818, 2121). The urinary concentration of MP, PP, BP, and iBuP were not significantly associated with any of the examined sperm chromosome disomy. However, 85% of samples with IBP<limit of detection (LOD).	For effects on sperm disomy it might be more relevant to assess IBP exposure during spermatogenesis (2-3 months before sampling of sperm). LOD higher than in other studies resulting in low detection rate for IBP in the samples.	Low relevance of timing of exposure measurement in relation to endpoint studied. Moderate to high quality of study reporting.

For source substance BP, no epidemiological studies examined the relationship between BP exposure in utero and effects on male reproductive parameters (hormone levels, sperm parameters) later in life (ECHA 2020). One study (Fernández *et al.* 2016) observed no association between placental BP levels and congenital malformations of the male genitalia (cryptorchidism and hypospadias). Residues of BP were however, more frequently detected in cases versus controls. This is supporting evidence for adverse effects of BP exposure during pregnancy.

A few studies reported on relationships between BP exposure and maternal hormone levels and thus provided supporting evidence for endocrine disrupting activity of BP during pregnancy. One study showed a negative association between maternal urinary butyl paraben levels and maternal serum levels of estradiol and the estradiol/progesterone ratio (Aker *et al.* 2019). A larger study (Aker *et al.* 2016) showed a borderline trend of lower maternal testosterone levels with higher maternal BP levels and a significant negative association between maternal BP and SHBG.

An update on epidemiology studies for BP was performed for the period 2020-2022, using a cut-off date of March 25th 2022. Screening of abstracts and titles was performed in order to identify studies investigating how prenatal exposure to BP was associated to possible

adverse effects in newborns, while disregarding all biomonitoring studies and all epidemiology studies correlating effect outcomes in adult individuals after adult exposure. 66 abstracts were reviewed and one new relevant study identified, which investigated how anogenital distance (AGD) and reproductive hormone levels at three months of age (during mini-puberty) correlated to prenatal exposure of six parabens including BP (Jensen *et al.* 2021).

The literature update for the period 2020-22 identified one study (Jensen *et al.* 2021) that showed that higher maternal paraben exposure was associated with shorter AGD in boys and longer AGD in girls. In addition, FSH and LH concentrations were affected in girls with high prenatal paraben exposure. This also provided supporting human evidence for adverse effects of BP exposure during pregnancy.

Table 24: Human studies on BP. Quality scores according to EFSA (2017).

Reference	Method	Relevant Endpoints & Effects	Comments/Notes	Quality Score
Aker *et al.* 2016	Cross-sectional study in a prospective cohort of pregnant women aged 18-40 years, gestation week 16-20 and 24-28, 2010-2012. Parabens measured in urine spot samples and hormones measured in maternal serum.	Maternal serum hormone levels at time of sampling: estradiol, progesterone, SHBG, estradiol/progesterone ratio, TSH, fT3, fT4. Maternal urinary BP levels were significantly negatively associated with maternal serum levels of estradiol (-8.5% per IQR, p=0.05) and the estradiol/progesterone ratio (-9.3% per IQR, p=0.04 (no effect on progesterone)) and significantly positively associated with maternal serum levels of fT4 (5.6% per IQR, p=0.01)		Moderate
Aker *et al.* 2019	Cross-sectional study in a prospective cohort of pregnant women aged 18-40 years, gestation week 16-20 and 24-28, 2012-17. Parabens measured in urine spot samples and hormones measured in maternal serum.	Maternal serum hormone levels at time of sampling: estriol, progesterone, testosterone, SHBG, progesterone/estriol ratio, corticotropin-releasing hormone, TSH, T3, fT3, T4, fT4. BP was associated with a decrease in maternal SHBG (-5.3% change per BP IQR, p=0.01). Also associated with a tendency of lower testosterone (-6.8% change per BP IQR, p=0.06) and estriol (-5.2%, p=0.13).		Moderate
Fernández *et al.* 2016	Nested case-control study in a prospective cohort, 2000-2002. Newborn males, 51 controls and 28 cases of	No associations between levels of BP in placenta at term and congenital malformations (cryptorchidism/hypospadia). Residues of BP were, however, more frequently detected in cases than in controls (85.7% vs. 66.7%, respectively, P = 0.054).		Moderate

	cryptorchidism.			
Jensen *et al.* 2021 Study evaluation:  Jensen et al 2021.xlsx	Prospective study on pregnant Danish women 2010-2012 with follow up in children at 3 months of age. Spot urine from 536 pregnant women analysed for six parabens. AGD measured in 452 children and serum hormone levels in 198 children.	No significant associations between BP exposure and AGD or hormone levels. BP below level of detection in majority of samples. Higher maternal paraben exposure was associated with shorter AGD in male offspring and longer AGD in girls, although only significant for MeP in boys. In addition, FSH, LH, DHEAS, 17-OHP concentrations were lower in girls with high prenatal paraben exposure, whereas no consistent pattern was found in boys. Overall, no statistically significant associations between BP exposure and the included outcomes were seen, but an important factor to mention here is that only 32.6% of the samples had BP concentrations above the level of detection, which markedly reduces the probability of finding statistically significant associations between exposure and effect.		Moderate