

## **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:** Dibutyl phthalate (DBP)

**EC Number:** 201-557-4

**CAS Number:** 84-74-2

**Submitted by:** The Netherlands

**Date:** August 2023

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## ABBREVIATIONS

AKR1C14: aldo-keto reductase family 1, member C14  
AR : androgen receptor  
AOX : acyl-CoA oxidase  
BSI : brain-somatic index  
BTC : betacellulin  
CV-1 : green monkey kidney fibroblast  
CYP11A1 : cytochrome 11A1  
CYP19a : cytochrome 19a  
CYP19b : cytochrome 19b  
DBP : Dibutyl phthalate  
DMSO : dimethylsulfoxide  
dpf: days post fertilisation  
E2 : 17 $\beta$ -estradiol  
EAS : estrogenic, androgenic and steroidogenic  
EC ED EAG : European Commission Endocrine Disruptor Expert Advisory Group  
EC50 : effect concentration at 50 percent effect  
ED : endocrine disruption  
EGF : epidermal growth factor  
ER : estrogen receptor  
EREG : epiregulin  
fhER $\alpha$  : full-length recombinant estrogen receptors from fathead minnows  
GSI : gonadosomatic index  
hER $\alpha$  : human estrogen receptor  $\alpha$   
17 $\beta$ -HSD : 17 $\beta$ -hydroxy-steroid dehydrogenase  
H295R : human adreno-carcinoma cell line (NCI-H295R cells)  
HPG : hypothalamic-pituitary-gonad  
HSI : hepatic-somatic index  
HTS : high throughput screening  
IC50 : concentration with 50% inhibition  
INSL3 : insulin-like hormone 3  
KEGG : Kyoto Encyclopedia of Genes and Genomes  
11-KT : 11-keto testosterone  
MCF-7 : Michigan Cancer Foundation-7, human breast cancer cell line  
MDA-kb2 : human breast cancer cell line  
MLTC-1 : murine Leydig tumor cell  
MMTV : murine mammalian tumor virus  
MoA : Mode of Action  
MBP : monobutyl phthalate  
pERE-TATA-Luc : Plasmid Luciferase reporter containing vitellogenin Estrogen Response Element  
PVC : polyvinyl chloride  
qPCR : quantitative polymerase chain reaction  
rtER : rainbow trout estrogen receptor  
SRD5A1 : smooth endoplasmic reticulum steroid 5 $\alpha$ -reductase 1  
StAR : Steroidogenic Acute Regulatory Protein  
T : Testosterone  
VEGF-A : Vascular endothelial growth factor A  
VTG : vitellogenin  
WHO/IPCS : World Health Organisation / International Programme on Chemical Safety  
WoE : weight of evidence  
YES/YAS : Yeast estrogen screen (YES) and Yeast androgen screen (YAS)

ZR-75 : human breast cancer cell line

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

**Substance name:** Dibutyl phthalate (DBP)

**EC number:** 201-557-4

**CAS number:** 84-74-2

- Dibutyl phthalate (DBP) is proposed to be identified as a substance of equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of Regulation (EC) No 1907/2006 (REACH) according to Article 57(f) of REACH Regulation.

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

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

DBP has been classified as reproductive toxicant (Cat.1B) and been identified as a substance of very high concern in accordance with Article 57(f) of Regulation (EC) 1907/2006 (REACH) due to its endocrine disrupting (ED) properties for human health (ECHA, 2017). The spectrum of adverse effects observed in rats include impaired fertility, increased nipple retention, decreased anogenital distance, genital malformations, reduced number of spermatocytes and testicular changes including multinucleated gonocytes, tubular atrophy and Leydig cell hyperplasia. Overall, developmental and reproductive toxicity tests in rodents provide supporting evidence that the adverse effects elicited by DBP are population relevant and can be used to strengthen the conclusion that DBP is an ED for the environment.

DBP induces female biased sex ratio in murray rainbowfish and pikeperch via an estrogenic or anti-androgenic pathway. DBP also influences reproductive toxicity as shown in changes in gonad histology and a decrease in fecundity as well as adverse effects in their offspring like increased malformation and reduced hatching, survival rate, and growth in F1 as reported in murray rainbowfish and in zebrafish. These effects are related to an anti-estrogenic MoA or steroidogenesis MoA.

In conclusion, the link between endocrine EAS activity and the observed adverse effects on sexual development, reproductive function and on offspring is highly plausible. Therefore, there is scientific evidence that DBP fulfils the WHO/IPCS definition of an endocrine disruptor relevant for the environment. DBP can be considered an endocrine disruptor for the environment.

The effects of DBP due to its endocrine disrupting properties are considered to be of equivalent level of concern to CMR Cat. 1, PBT or vPvB substances as listed in Article 57 points (a) to (e) of the REACH Regulation. DBP is considered as a substance giving rise to an equivalent level of concern because scientific evidence shows that exposure during sensitive time windows of sexual development may cause irreversible developmental programming effects leading to severe effects on development and reproduction, regarded as particularly serious in relation to wildlife species, also because these adverse effects may first manifest themselves in later life stages or offspring. It is difficult to determine the most sensitive species and thus to quantify a safe level of exposure with regard to the endocrine mediated effects. Adverse effects on development and

reproduction are in addition generally regarded as endpoints of concern, and as such frequently used for regulatory hazard and risk assessment for environmental species.

Overall, based on all available scientific evidence, it can be concluded that DBP fulfils the WHO/IPCS definition of an endocrine disruptor:

- It shows clear population relevant adverse effects on sexual development, reproduction and offspring survival and growth in fish.
- It has EAS activity as clearly shown both in vitro and in vivo.
- The substance has an EAS mode of action, i.e., the adverse effects, including the recognised EAS-mediated effects on sex ratio and also effects considered to be sensitive to, but not diagnostic of, EAS modalities are biologically plausibly linked to the adverse effects

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

**Registration dossiers submitted for the substance:** Yes

## PART I

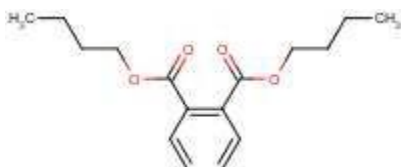
### 1. Identity of the substance and physical and chemical properties

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

**Table 1: Substance identity**

<b>EC number:</b>	201-557-4
<b>EC name:</b>	Dibutyl phthalate
<b>CAS number (in the EC inventory):</b>	84-74-2
<b>CAS number:</b>	84-74-2
<b>CAS name:</b>	1,2-Benzenedicarboxylic acid dibutyl ester
<b>IUPAC name:</b>	Dibutyl phthalate
<b>Index number in Annex VI of the CLP Regulation</b>	607-318-00-4
<b>Molecular formula:</b>	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>
<b>Molecular weight range:</b>	278.34 g/mol
<b>Synonyms:</b>	DBP

Structural formula:



#### 1.2 Composition of the substance

**Name:** DBP

**Description:** Mono constituent substance



### 1.3 Physicochemical properties

**Table 2: Overview of physicochemical properties<sup>1</sup>**

Property	Value
Physical state at 20 °C and 101.3 kPa	Oily liquid
Melting/freezing point	-67.2 °C at 101.3 kPa
Boiling point	340 °C at 101.3 kPa
Vapour pressure	9.7 10 <sup>-3</sup> Pa at 25 °C
Water solubility	11.4 mg/L at 25 °C
Partition coefficient n- octanol/water (log value)	log Kow 4.46 at 30 °C

## 2. Harmonised classification and labelling

DBP is listed in Regulation (EC) No 1272/2008 as follows:

Classification and labelling of DBP according to Annex VI, Table 3.1 of Regulation (EC) No 1272/2008

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

I n d e x N o	Internat ional Chemic al Identific ation	E C N o	C A S N o	Classification		Labelling		Spec ific Conc · Limit s, M- facto rs
				Hazard Class and Category Code(s)	Hazar d State ment Code( s)	Pictogra m, Signal Word Code(s)	Hazar d state ment Code( s)	
6 0 7 - 3 1 8 - 0 0 - 4	dibutyl  phthala te; DBP	2 0 1 - 5 5 7 - - 4	8 4 - 7 4 - - 2	Repr. 1B  Aquatic Acute 1	H360 Df  H400	GHS08  GHS09  Dgr	H360 Df  H400	

## 3. Environmental fate properties

Information on environmental fate properties, in particular persistency and bioaccumulation is included as background information. Fate related properties are not required for identification of SVHCs with endocrine disruptive properties according to Article 57(f).

<sup>1</sup> The information on physicochemical properties corresponds to the information reported in the registration dossiers.

### 3.1 Environmental fate

The environmental fate of DBP as concluded in the EU RAR for degradation, distribution and bioaccumulation is cited in the sections below (EU RAR, 2004).

DBP may be released into the environment during its production and subsequent life cycle stages, including disposal. Emissions to water and air are expected to be the most important entry routes of DBP (EU RAR, 2004). General characteristics of DBP which are relevant for the exposure assessment are given below.

### 3.2 Degradation

*"The contribution of hydrolysis to the overall environmental degradation of phthalate esters, including DBP, is expected to be low. Photo-oxidation by OH radicals contribute to the elimination of DBP from the atmosphere. An atmospheric half-life of about 1.8 days has been estimated for the photo-oxidation reaction. The metabolic pathway of aerobic and anaerobic biodegradation of phthalates can be summarised as follows. First the di-ester is hydrolysed into the mono-ester by esterases with low substrate specificity. Subsequently the mono-ester is converted into phthalic acid. There is ample evidence that DBP is readily biodegradable under aerobic conditions. The same literature sources indicate that biodegradation of DBP is much slower in the anaerobic environment, e.g., sediments or deeper soil or groundwater layers."* Citation from EU RAR (2004).

### 3.3 Distribution

*"The Henry's law constant of 0.27 Pa.m<sup>3</sup>/mol indicates that DBP will only slowly volatilise from surface waters, i.e., virtually all of the DBP will remain in the water phase at equilibrium. The octanol/water partition coefficient (Kow) of DBP is high and consequently the equilibrium between water and organic carbon in soil or sediment will be very much in favour of the soil or sediment. A Koc of 6,340 l/kg can be calculated using the log Kow of 4.57. Despite its low volatility, DBP has been reported as particulate and as a vapour in the atmosphere. In the air DBP is transported and removed by both wet and dry deposition."* Citation from EU RAR 2004.

### 3.4 Bioaccumulation

*"The high Kow of DBP indicates that the substance has a potential for bioaccumulation. However, the actual degree of bioaccumulation in vivo will be determined by the metabolisation and the elimination rate of the substance. The available BCF data demonstrate a relatively low bioconcentration, but also indicate that higher BCF values are obtained when the BCF is calculated for the total amount of metabolites using 14C-labelled material. The experimental BCF of 1.8 l/kg for DBP from the recent study is used in the further risk assessment for secondary poisoning (aquatic route). In the risk characterisation attention will be paid to the possible consequences of using a higher value. No experimental BCF data are available for terrestrial species. EUSES calculates a BCF worm of 13 kg/kg."* Citation from EU RAR (2004).

## 4. Human health hazard assessment

Dibutyl phthalate (DBP) has been identified as substance having reproductive toxicity (Repro. 1B) and endocrine disrupting properties whose effects to human health give rise to an equivalent level of concern according to Article 57(f) of REACH (ECHA, 2008, 2014). The following was copied from the summary of the 2014-ED assessment report for human health (ECHA, 2014).

*"Rodent studies have demonstrated adverse reproductive effects, especially in male reproductive organs, such as testicular changes, decreased number of spermatocytes and decreased*

*anogenital distance and nipple retention, and it is considered as highly plausible that these effects are induced by an endocrine mode of action of DBP. Further, studies on DBP also showed decreased levels of testosterone and other effects on steroidogenesis such as e.g., reduced expression of genes in the steroid biosynthesis pathway, confirming an endocrine disrupting mode of action of DBP. There is convincing evidence of a plausible link between the adverse effects observed in males and the anti-androgenic mode of action of DBP.*

*The anti-androgenic related effects of DBP that are suspected to be relevant in humans are congenital malformations of the male reproductive organs, reduced semen quality, reduced male reproductive hormone levels, and changes in pubertal timing including changes in breast development. It has been hypothesised that these disorders may comprise a testicular dysgenesis syndrome with a common origin in fetal life. Testicular cancer may also be part of this syndrome.*

*Effects on female reproduction have also been reported as well as effects on the thyroid system. An estrogenic and a thyroid mode of action of DBP cannot be excluded.*

*In conclusion, DBP is classified as toxic to reproduction based on evidence of adverse effects on the reproductive organs in developing male rodents, and these adverse effects are attributed to the anti-androgenic mode of action of DBP. Thus, DBP is considered as an endocrine disrupter that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism and its progeny.”*

## **5. Environmental hazard assessment**

The environment hazard assessment is focuses on the endocrine disrupting properties of DBP found in several fish species. To put this information in perspective only the general toxicity data for fish will be described as presented in the registration dossier, without a thorough evaluation of the original fish toxicity studies.

### **5.1 Aquatic toxicity to fish**

Four acute toxicity studies were conducted on 4 species of fish: Fathead minnow, Bluegill sunfish, Sheepshead minnow or Rainbow trout. The lowest 96 h LC50 value of 0.48 mg/L based on measured test concentration was found for bluegill sunfish (*Lepomis macrochirus*).

The general long-term effects were investigated in an early life toxicity test in rainbow trout for 99 days (60 days post hatch). The total length and wet weight of rainbow trout after 99 days of exposure (the most sensitive exposure parameter) were significantly reduced in comparison to control at 0.19 mg/L DBP. The lowest observed effect concentration (LOEC) was 0.19 mg/L, and the no observed effect concentration (NOEC) was 0.10 mg/L DBP.

### **5.2 Assessment of Endocrine Disruption Potential**

#### **5.2.1 General approach for the assessment of endocrine disruption properties**

The following targeted assessment focuses on tests and endpoints that can be conclusive for endocrine disrupting properties in the environment. To evaluate whether or not DBP fulfils the WHO/IPCS definition (WHO/IPCS, 2002) of an endocrine disruptor as interpreted by the EC ED EAG (JRC, 2013), both *in vitro* data and *in vivo* data were taken into account, in order to demonstrate:

- Population relevant adverse effects
- Endocrine activity
- Plausible link between adverse effects and endocrine activity, i.e. endocrine mode

of action (MoA)

This targeted assessment builds upon a previous proposal made in 2014 (ECHA, 2014) supplemented with reliable and relevant *in vitro* tests and fish toxicity tests relevant to EAS mediated activity and adversity published after 2014. For this a search in the PubMed was conducted by using key words “dibutyl phthalate and estrogen receptor”, “dibutyl phthalate and androgen receptor”, “dibutyl phthalate and steroidogenesis” and “dibutyl phthalate and fish” revealing 61, 62, 52 and 126 results, respectively (accessed June 8<sup>th</sup>, 2023). From these results a selection was made considering whether DBP has been directly tested in an EAS assay, and whether ED related effects on sexual development and reproduction life stages have been studied. The assessment of *in vivo* data focuses on the question whether adverse effects on sexual development and reproduction can be inferred to originate from the presumed EAS modes of action or to be a consequence of general systemic toxicity. The studies were considered on the basis of their relevance, reliability and adequacy for the analysis and were qualitatively weighted based on expert judgement to produce a conclusion on the selected adverse effects and their ED MoA in order to conclude if DBP fulfils the WHO/IPCS definition.

## 5.2 2 In vitro information indicative of EAS activity

### 5.2.2.1 In vitro EAS studies

DBP has been tested in the ToxCast™ HTS assays for the EAS activity. A search in the US EPA CompTox DB (<https://comptox.epa.gov/dashboard/>) was performed for DBP by using its CAS number of 84-74-2 (accessed June 8th, 2023). Bioactive data for DBP was available for seven ER assays, four AR assays and six steroidogenesis assays. These results provide evidence that DBP interferes with the EAS modalities.

#### Estrogen modality

**Czernych et al. (2013):** Estrogenic activity of DBP was tested in the XenoScreen YES assay using yeast cells *Saccharomyces cerevisiae* which were stably transfected with a gene encoding the human estrogen receptor hER $\alpha$  (YES). Cells were incubated with serially diluted DBP concentrations and positive control (17- $\beta$  estradiol) for 48 h at 32 °C in the presence of a substrate for  $\beta$ -galactosidase synthesis. DBP showed no estrogenic activity till the highest test concentration of 50  $\mu$ M. However, DBP exhibited concentration-dependent anti-estrogenic activity at the concentrations between 0.5 and 50  $\mu$ M, with an IC<sub>50</sub> of 19.2  $\mu$ M.

**Harris et al. (1997):** This study was included in the EU RAR. A brief description is provided here. The estrogenic activity of DBP was investigated in a recombinant yeast screen assay and in the proliferation test of two estrogen-responsive breast cancer cell lines. MCF-7 and ZR-75 cells The test DBP concentrations ranged from 5 X 10<sup>-7</sup> M to 10<sup>-3</sup>M. Dose dependent estrogenic activity was detected in the yeast screen assay at concentrations between 10<sup>-5</sup> M and 10<sup>-3</sup> M. DBP at the concentration of 10<sup>-5</sup> M stimulated proliferation of MCF-7 and ZR-75 cells, which further confirmed the estrogenic responses observed in the yeast assay.

**Jobling et al. (1995):** This study was included in the EU RAR. A brief description is provided here. The estrogenic activity of DBP including ER binding effects in fish, transactivation in MCF-7 cells and proliferative effects in estrogen-responsive human breast cancer cell line, ZR-75 cells, were studied. DBP reduced the binding of 17 $\beta$ -estradiol to the fish estrogen receptor in an assay with cytosolic liver extracts of rainbow trout at concentrations of approximately 10<sup>-7</sup> to 10<sup>-5</sup> M. DBP also stimulated transcriptional activity of the estrogen receptor at concentrations of 10<sup>-6</sup> and 10<sup>-5</sup> M in an assay with transiently transfected MCF-7 cells. DBP showed mitogenic effects on cell growth of ZR-75 cells at the concentration of 10<sup>-5</sup> M, indicating weak estrogenicity.

**Rider et al. (2009):** The objectives of the present study were to evaluate the performance of two different in vitro assay systems, a whole cell and a cell-free competitive binding assay, in assessing whether binding of chemicals differs significantly between full-length recombinant

estrogen receptors from fathead minnows (fhER $\alpha$ ) and those from humans (hER $\alpha$ ). DBP was one of the test chemicals. Results showed that both assays were effective in identifying strong binders, weak binders, and nonbinders to the two receptors. DBP displayed a complete binding curve with fhER $\alpha$  (IC<sub>50</sub>s of  $1.98 \times 10^{-5}$  M), while only partial displacement of E2 by DBP was achieved for hER $\alpha$  at the same concentration. Higher concentrations could not be tested due to visual evidence of precipitation of DBP in the media at the highest concentration tested. Overall, DBP is a weak binder to both receptors, with a higher affinity for fhER $\alpha$  than for hER $\alpha$ .

**Shen et al. (2009):** Estrogenic activity of DBP and MBP was studied in a luciferase reporter gene assay. Green monkey kidney fibroblast (CV-1) cells transfected with luciferase reporter plasmid pERE-TATA-Luc+ and rat ER $\alpha$  expression vector rat ER $\alpha$ /pCI were exposed to E2 at concentrations of  $1.0 \times 10^{-12}$ – $1.0 \times 10^{-8}$ M, DMSO control, DBP and MBP for 24 h. MBP showed no agonistic activity, whereas DBP appeared weakly estrogenic at the concentration of  $1.0 \times 10^{-4}$  M.

#### Androgen modality

**Czernych et al. (2013):** Androgenic activity of DBP was tested in the XenoScreen YAS assay, which uses yeast cells *Saccharomyces cerevisiae* stably transfected with a gene encoding the human androgen receptor hAR. Cells were incubated with serially diluted DBP concentrations and positive control 5 $\alpha$ - dihydrotestosterone for 48 h at 32 °C in the presence of a substrate for  $\beta$ -galactosidase synthesis. DBP showed no androgenic activity till the highest test concentration of 50  $\mu$ M. However, DBP exhibited concentration dependent anti-androgenic activity between 1 and 50  $\mu$ M, with an IC<sub>50</sub> of 12.2  $\mu$ M.

**Shen et al. (2009):** Androgenic activity of DBP and MBP was studied in a luciferase reporter gene assays. The MDA-kb2 cell line stably transformed with murine mammalian tumor virus (MMTV)-luciferase was exposed to 5 $\alpha$ -DHT at concentrations of  $1.0 \times 10^{-12}$ – $1.0 \times 10^{-7}$ M, DMSO control, DBP and MBP for 24 h. The results showed that DBP and MBP not only exhibited potent antiandrogenic activity, with IC<sub>50</sub> value of  $1.05 \times 10^{-6}$ , and  $1.22 \times 10^{-7}$ M, respectively, but also showed the androgenic activity with EC<sub>50</sub> value of  $6.17 \times 10^{-6}$  and  $1.13 \times 10^{-5}$ M.

#### Steroidogenesis modality

**Adir et al. (2017):** Effects of DBP exposure on mural granulosa cell function were investigated in primary cultures from women undergoing in vitro fertilisation. Cultured cells were treated with DBP at concentrations of 0.01, 0.1, 1, 10, 100 mg/L for 48 h. Treatment with 100 mg/L DBP resulted in significantly lower 17  $\beta$ -estradiol and progesterone production ( $p < 0.01$ ). It also resulted in altered mRNA expression of steroidogenic, angiogenic, and epidermal growth factor-like growth factor genes: CYP11A1 ( $p < 0.001$ ), CYP19A1 (aromatase) ( $p < 0.001$ ), VEGF-A ( $p < 0.02$ ), BTC ( $p = 0.009$ ), and EREG ( $p = 0.04$ ). StAR expression was impaired after exposure to both 10 and 100 mg/L ( $p < 0.03$  and  $p < 0.001$ , respectively). The authors concluded that DBP exposure led to altered steroidogenesis and dysregulation of angiogenesis and EGF-like factors in human mural granulosa cells.

**Chen et al. (2013):** The objective of this study was to evaluate the effects of DBP and its active metabolite monobutyl phthalate (MBP) on steroidogenesis in the murine Leydig tumour cell line MLTC-1 in vitro. MLTC-1 cells were incubated with various concentrations of DBP (0.01, 1 and 100  $\mu$ M in DMSO) and MBP (0.1, 10, 1000  $\mu$ M in DMSO) for 24 h. The MTT assay showed that DBP at concentrations 1000 and 2000  $\mu$ M and MBP at the concentration of 2000  $\mu$ M significantly reduced the cell viability compared to the control. Testosterone secretion was stimulated at the lowest doses and inhibited at higher treatment doses of DBP and MBP. This inhibition is not due to cytotoxicity because the highest test concentration of 100  $\mu$ M did not show a decrease in the MTT assay. The mRNA levels of the side-chain cleavage enzyme (P450scc), cytochrome p450c17 (P450c17) and 3 $\beta$ -hydroxy-steroid dehydrogenase (3  $\beta$  HSD) were significantly reduced in the DBP and MBP exposed groups, whereas the transcription and translation of insulin-like hormone 3 (INSL3) was affected by DBP and MBP. Alterations of the steroidogenic enzymes and INSL3 in MLTC-1 cells may be involved in the biphasic effects of DBP/MBP on androgen production.

**Källsten et al. (2022):** Human adrenocortical H295R cells were exposed to 1–500 µM of DBP or MBP for 48 h. Quantification of steroid hormones in the cell medium by liquid chromatography-mass spectrometry revealed that both DBP and MBP decreased testosterone, androstenedione, corticosterone, and progesterone levels, in particular after dibutyryl-cyclic-AMP stimulation of steroidogenesis. Western blot analysis of key steroidogenic proteins showed that DBP induced a dose-dependent decrease of CYP11A1 and HSD3β2 levels, while MBP only significantly decreased CYP17A1 levels, indicating that the substances affect early steps of the steroidogenesis differently. Both DBP and MBP exposure also lead to a dose-related decrease in HSD17β3, the enzyme which catalyzes the final step in the testosterone biosynthesis pathway, although these effects were not statistically significant. Interestingly, DBP increased the cortisol concentration, which may be due to the non-significant CYP11B1 increase in DBP-exposed cells. In contrast, MBP decreased cortisol concentration. Moreover, the analysis of superoxide generation and quantification of the protein oxidation marker nitrotyrosine demonstrated that DBP induced oxidative stress in H295R cells while MBP reduced protein nitrotyrosine levels. These findings confirm the anti-androgenic effects of DBP and MBP and reveal several differences in their toxicological mechanisms.

**Li et al. (2017):** Effects of DBP and its active metabolite monobutyl phthalate (MBP) on steroidogenesis were studied in immature Leydig cells isolated from rats. These cells were cultured with 0.05–50 µM DBP or MBP for 3 h in combination with testosterone synthesis regulator or intermediate. The concentrations of 5α-androstanediol and testosterone in the media were measured, and the mRNA levels of the androgen biosynthetic genes were detected by qPCR. The direct actions of DBP or MBP on *Cyp11a1*, *Cyp17a1*, *Srd5a1*, and *Akr1c14* activities were measured. MBP inhibited androgen production by the immature Leydig cell at as low as 50 nM, while 50 µM was required for DBP to suppress its androgen production. MBP mainly downregulated *Cyp11a1* and *Hsd3b1* expression levels at 50 nM. However, 50 µM DBP downregulated *Star*, *Hsd3b1*, and *Hsd17b3* expression levels and directly inhibited *Cyp11a1* and *Cyp17a1* activities. In conclusion, DBP is metabolised to the more potent inhibitor MBP which downregulated the expression levels of some androgen biosynthetic enzymes.

### 5.2.2.2 Summary of *in vitro* EAS endocrine activities

The following table provides an overview of the available *in vitro* tests with DBP for EAS activity.

**Table 4: Results of EAS related *in vitro* assays**

assay category	species	cell	results	conclusion	reference
ER binding	rtER	Liver extract from rt	Decreased the binding of E2 to rtER	Influence ER binding to E2	Jobling et al., 1995
ER binding	hERα	COS cells	Low binding	Weak binder to hERα	Rider et al., 2009
ER binding	fER	COS cells	IC50=1.98x 10 <sup>-5</sup> M	Weak binder to fER	Rider et al., 2009
ER transactivation	hER	MCF7	transactivation > 1X 10 <sup>-5</sup> M	ER transactivation	Jobling et al., 1995
ER transactivation	hER	yeast	transactivation at 10 <sup>-6</sup> - 10 <sup>-5</sup> M	ER transactivation	Harris et al., 1997
ER transactivation	rER	CV-1	EC50=1X10 <sup>-4</sup> M	ER transactivation	Shen et al., 2009
ER transactivation	hER	yeast	IC50=1.92x 10 <sup>-5</sup> M	Anti-estrogenic activity	Czernych et

n					<i>al.</i> , 2013
AR transactivation	hAR	CV-1	IC50=1.05x 10 <sup>-6</sup> M	Anti-androgenic activity	Shen <i>et al.</i> , 2009
AR transactivation	hAR	CV-1	EC50=6.17x 10 <sup>-6</sup> M	androgenic activity	Shen <i>et al.</i> , 2009
AR transactivation	hAR	yeast	IC50=1.22x 10 <sup>-5</sup> M	Anti-androgenic activity	Czernych <i>et al.</i> , 2013
Steroidogenesis	Murine MLTC-1	MLTC-1	biphasic effects on testosterone production	Alternation of steroidogenesis	Chen <i>et al.</i> , 2013
Steroidogenesis	Rat Leydig cells	Rat Leydig cells	Inhibition of androgen production	inhibitor of steroidogenesis	Li <i>et al.</i> , 2013
Steroidogenesis	Human mural granulosa cell	Human mural granulosa cell	Inhibition of steroidogenesis at 100 mg/L	inhibitor of steroidogenesis	Adir <i>et al.</i> , 2017
Steroidogenesis	H295R	H295R cells	Reduced testosterone ; increased cortisol	inhibitor of steroidogenesis	Källsten <i>et al.</i> , 2022

Note, AR, androgen receptor; ER, estrogen receptor; h, human; f, fathead minnow, r, rat, rt, rainbow trout; COS cells, African green monkey kidney fibroblast cells of origin containing simian virus 40 (COS) cells; CV-1, Green monkey kidney fibroblast (CV-1) cell line; IC50, 50% inhibition; EC50, 50% effect concentration.

As shown in the above table, seven *in vitro* experiments have been conducted to examine the binding and transactivation of DBP to ERs (Jobling *et al.*, 1995; Harris *et al.*, 1997; Rider *et al.*, 2009; Shen *et al.*, 2009; Czernych *et al.*, 2013). DBP exhibits weak binding to both human and fish ERs, with a higher affinity for fish ER $\alpha$  compared to human ER $\alpha$  (Rider *et al.*, 2009). Transactivation of ERs has been tested in four experiments involving human and rat ERs. These studies have observed both estrogenic and anti-estrogenic activity (Jobling *et al.*, 1995; Harris *et al.*, 1997; Shen *et al.*, 2009; Czernych *et al.*, 2013). Collectively, these studies demonstrate that DBP binds to and transactivates ERs in various vertebrate species. The transactivation of human ARs by DBP has also been investigated, revealing both androgenic activity and anti-androgenic activity (Shen *et al.*, 2009; Czernych *et al.*, 2013). Furthermore, steroidogenesis assays conducted with murine MLTC-1 cells, rat Leydig cells, H295R cells, and human mural granulosa cells have revealed that DBP interferes with steroidogenesis (Adir *et al.*, 2017; Chen *et al.*, 2013; Li *et al.*, 2013 and Källsten *et al.*, 2022). In summary, the *in vitro* assays indicate that DBP has EAS activity.

### 5.2.3 Fish toxicity tests

This section presents and discusses fish studies that have tested with DBP and are relevant to the ED identification. These fish studies are considered reliable as Klimisch Score 2, non-guideline studies, and summarised as follows.

### 5.2.3.1 Fish studies tested for EAS activity and adversity

**Aoki et al. (2011):** In the present study, adult male three-spined sticklebacks (*Gasterosteus aculeatus*; n=8) were exposed to DBP at nominal concentrations of 50 and 100 µg/L for 22 d and analyzed for changes in nesting behavior, plasma androgen concentrations, spiggin concentrations, and steroidogenic gene expression. The mean measured concentrations of the nominal 50 and 100 µg DBP/L tanks during the experiment were 15.23±6.28 and 35.20±8.03 µg DBP/L respectively. No significant differences in weight, length, or gonadosomatic index were noted. Plasma testosterone concentrations were significantly higher in males from the 35 µg DBP/L group compared with the methanol control, whereas plasma 11-ketotestosterone concentrations were not significantly affected. Expression of steroidogenic genes StAR and 3β-hydroxysteroid dehydrogenase remained unchanged. Spiggin concentrations were significantly lower in the males exposed to 35 µg DBP/L. Nest building appeared to be slower in some males exposed to 35 µg DBP/L, but this was not statistically significant. These results suggest that DBP has antiandrogenic effects in fish.

**Bhatia et al. (2013):** The present study investigated the changes in ovarian histology and serum vitellogenin concentrations in adult female Murray rainbowfish (*Melanotaenia fluviatilis*) after exposure to nominal 125 µg/L, 250 µg/L, 500 µg/L, and 1000 µg/L DBP for 7 d. Control water and 0.001% (v/v) methanol solvent controls were also set up. Two replicates containing 4 fish each were set up for each concentration and the exposure was semi-static. Measured concentrations were 10-30% of nominal after 24 h. Treatment at 125 µg/L to 1000 µg/L DBP for 7 d had no significant effect on the survival, condition factor, gonadosomatic index, hepatosomatic index, and developmental stage of the fish. Based on the histological investigation, the sizes of the previtellogenic oocytes in the fish treated at 250 µg/L to 1000 µg/L were found to be significantly higher than in the corresponding control fish (p<0.05). The early vitellogenic oocytes in the fish treated at 1000 µg/L were significantly smaller relative to those in the unexposed fish (p<0.05). Histological changes like chorion folding, shrunken ooplasm, impaired yolk production, granulomatous inflammation, and interstitial fibrosis were observed in the ovaries of the fish treated with DBP. The circulating levels of plasma vitellogenin were significantly lower in the fish exposed to 500 µg/L and 1000 µg/L DBP (p<0.05). These data show that a continuous exposure to subacute concentrations of DBP for 7 d can cause anti-estrogenicity in female adult Murray rainbowfish.

**Bhatia et al. (2014a):** The present study investigated the effects of 7-day exposure to nominal concentrations of 125 µg/L, 250 µg/L, 500 µg/L, and 1000 µg/L DBP on the reproductive health in adult male Murray rainbowfish (*Melanotaenia fluviatilis*). Control water and 0.001% (v/v) methanol solvent controls were also set up. Two replicates containing 4 fish each were set up for each concentration and the exposure was semi-static. Measured concentrations of DBP before the start of the test ranged between 38 and 56% of the nominal concentrations. After 24 h of exposure, the actual concentrations of DBP were 14 ± 1, 21 ± 7, 74 ± 26 and 112 ± 41 µg/L, for nominal additions of 125, 250, 500 and 1000 µg/L, respectively. Treatment at 125 µg/L to 1000 µg/L DBP for 7 d had no significant effect on the survival, condition factor, gonadosomatic index, and hepatosomatic index. Exposures to 125–1000 µg/L DBP for 7 d did not induce ova-testes condition in the male Murray rainbowfish. However, the testes of the fish exposed to the highest concentration of DBP showed degeneration signs like the presence of vacuolated cells, apoptotic cell bodies, interstitial fibrosis and asynchronous development. The sizes of spermatogonia, Type A and B spermatocytes, and spermatids were significantly smaller relative to the controls after treatment with 125 µg/L to 1000 µg/L DBP. This was accompanied by a significant increase in the proportion of spermatogonia in fish treated with 250–1000 µg/L of DBP in comparison to the unexposed fish. At the end of the exposure period, the expressions of the transcripts for the androgen receptors α and β were significantly elevated in the livers of the fish treated with 500 and 1000 µg/L of DBP. In addition, there was also an increase in the circulating concentrations of VTG in the plasma in the top two higher treatment groups. An induction in the activity of aromatase was noted in the brains of 1000 µg/L DBP-treated fish. This was accompanied by an increase in the hepatic expression of the genes encoding for the oestrogen receptors α and β and choriogenin L. Collectively, an increase in the proportion of



spermatogonia in the testes, the upregulation of the genes for the oestrogen receptors and choriogenin in the liver, an induction in the brain aromatase activity and the increase in the circulating levels of plasma VTG suggest that continuous exposures for 7 days to sub-acute concentrations of DBP can adversely affect the reproductive health of the male Murray rainbowfish by an estrogenic mode of action.

**Bhatia et al. (2014b):** The aim of the present study was to evaluate whether long-term exposures to environmentally relevant concentrations of DBP disrupt the reproduction-based endpoints in juvenile Murray rainbowfish (*Melanotaenia fluviatilis*). Sexually undifferentiated Murray rainbowfish (30 dph, approximately 10 mg) were exposed in 1 litre of water in glass beakers to 5, 15 and 50 µg/L DBP in a semi-static system for 30, 60 and 90 days. Water control and 0.0005% methanol solvent control beakers were also used. Four beakers containing four fish in each were used (16 fish per treatment per time interval with 240 total fish). After 30 days of exposure, the fish were transferred to beakers containing 2 litre of water to account for their growth. The effects on survival, body growth, whole-body concentrations of sex steroid hormones and gonadal staging were investigated. Survival was not affected by the DBP exposure. Body length and weight were decreased only after 60 and 90 days of exposure but were not decreased at 30 days at the concentration of 5 µg/L. The lowest observed effective concentration to affect the body weight and length after 90 days was 5 µg/L. Complete feminisation of the gonad was noted in fish exposed to 5 µg/L for 90 days and to 15 and 50 µg/L of DBP for 30 or 60 days. It is noted that feminisation is not always accompanied with a decrease in body weight and length at the sampling of 30, 60, and 90 days. These results suggest that changes in sex ratio is not related to general toxicity. After 90 days of exposure to DBP, the ovaries were regressed and immature as opposed to the control fish which were in early-vitellogenic stage. Testes, present only in fish exposed to 5 µg/L of DBP for 30 or 60 days, were immature in comparison to the control fish that contained testes in the mid-spermatogenic phase. The E2/11-KT ratio was significantly higher only after exposures to 5 µg/L DBP for 90 days and 50 µg/L DBP for 30 days. The data suggest that exposures to 5 µg/L DBP for 30 days did not have profound effects on body growth and gonadal differentiation of fish. However, treatment with 15 and 50 µg/L DBP could induce complete feminisation of the gonads with altered E2 and 11-KT levels in 30 days. Although the gonads were completely feminised, the oocyte-development was hindered in DBP-treated fish. Based on the changes in E2/11-KT and the effects on the gonadal differentiation and development with skewed proportions of sex, favouring the females, an estrogenic mode of action was suggested. This study covers the sexual development stages. The effects on sex ratio were time and concentration dependent.

**Chen et al. (2015):** Wild-type TU strain zebrafish at 20 dpf were exposed to DBP at concentrations of 100 and 500 µg/L. A solvent control containing 0.005% DMSO was also included. The exposure was performed semi-statically, and one-half of the solution in the tank was replaced every day. At 45, 90 and 115 dpf, fish from each treatment group were sampled for evaluation. Sex ratio, length, weight, and condition factor at 115 dpf were reported. The average measured concentrations during the toxicity test ranged from 80% to 116% of the nominal concentrations. At 45 dpf, the survival rate of zebrafish was comparable in all treatments. DBP did not induce VTG synthesis. At 90 dpf, there were no significant alterations in the liver of fish exposed to DBP. Gills were subject to concentration dependent damage including amalgamation of gill lamellae and clubbing at the tips of the secondary lamellae. At 115 dpf, well-developed testes were observed in fish exposed to the low concentration of DBP, whereas a reduced number of spermatozoa and increased number of spermatogonia and spermatocyte were found in fish exposed to the high concentration of DBP compared with those in the solvent control. A reduced number of vitellogenic oocytes was found in fish exposed to both concentrations of DBP. At 115 dpf, DBP exposure showed no effects on sex ratio. There were no statistical differences in the survival rates, body length, body weight, and condition factors between the solvent control and each treatment group at 115 dpf. Overall, this study showed that DBP induced histological alternation in gonads.

**Chen et al. (2020):** Effects of DBP on male reproductive toxicity and the underlying toxicological mechanisms were reported. Adult wild-type AB strain 4-month old male zebrafish

were semi-statically exposed to DBP at concentrations of 11, 113 and 1133 µg/L for 30 days. A control group and a solvent control group (0.05% (v/v) DMSO) were employed. Effects on plasma hormone secretion, testis histology and transcriptomics were examined. The measured concentrations of DBP after exposure solution renewal (T0) were 82.7%-99.1% of the nominal concentrations. After 24 h (before renewing, T24), the concentrations of DBP declined, leaving 57.1%-77.0% residual. Both the condition factor and the GSI in the exposure groups did not present significant changes when comparing to the control. Plasma E2 in male zebrafish showed a slight decrease with increasing DBP concentrations but not significant, while plasma T levels increased with increasing DBP concentrations and significant elevation appeared at the highest DBP concentration. The highest concentration of DBP exposure caused imbalance ratio of T/E2 and a delayed development of testis with an expansion of the intercellular space. These effects were consistent with the testis transcriptome analysis for which 2795 genes were differentially expressed in the highest DBP exposure, with 1404 up-regulated genes and 1391 down-regulated genes. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that DBP exposure could affect cytokine-cytokine receptor interaction. The authors concluded that DBP exposure may interfere with the reproductive-related signalling pathways to inhibit the steroid hormones and retinoic acid synthesis and promote cell adhesion and apoptosis, and thereby disrupt spermatogenesis and elicit male reproductive toxicity in zebrafish.

**Chen et al. (2021):** The aims of this study were to assess the effects of parental exposure to DBP on early development of zebrafish offspring, and to explore the potential molecular mechanisms involved. Adult wild-type AB strain 4-month old male and female zebrafish were semi-statically exposed to DBP at concentrations of 11, 113 and 1133 µg/L for 30 days. Early developmental toxicity in F1 embryos was studied for 5 days. A control group and a solvent control group (0.05% (v/v) DMSO) were employed. The early developmental indicators and transcriptomic profiles of F1 larvae were examined after parental exposure to DBP for 30 days. The measured concentrations of DBP after exposure solution renewal (T0) were 90.5%-99.9% of the nominal concentrations. DBP concentrations in the F1 embryos after parental exposure to 11, 113 and 1133 µg/L were  $12.7 \pm 1.3$ ,  $64.9 \pm 2.5$ ,  $113.8 \pm 0.7$  ng g<sup>-1</sup> wet weight, respectively. After exposure, there was no significant change in the gonadosomatic index (GSI) of male zebrafish, while the GSI of females was significantly reduced at the highest DBP concentration. Pathological changes in the gonads of females have been observed after the DBP exposure of the highest concentration. Compared with the control group, the average number of eggs spawned was decreased with increasing exposure concentrations of DBP. Parental exposure to the highest concentration of DBP induced significant reduction in spawning. No effects were observed for hatching rate and body length at 96 hpf. Parental exposure to DBP at all test concentrations resulted in increased heart rate at 96 hpf and increased the prevalence of malformations and mortality in F1 larvae. The transcriptomic analysis revealed that the molecular mechanisms involved unfolded protein binding, E-box binding and photoreceptor activity in F1 larvae. These findings provide initial insights in the potential mechanism of action of parental exposure to DBP. However, the DBP-induced EAS modalities were not investigated. Whether these effects on gonad histology, fecundity, and F1 larvae were endocrine-mediated effects cannot be determined based on this study.

**Hu et al. (2020):** The objective of the study was to investigate whether long-term exposure to low concentrations of DBP can affect fish reproduction. Wild-type AB strain zebrafish (*Danio rerio*) embryos (F0) were exposed to measured DBP concentrations of 4.9, 13.6 and 43.8 µg/L from 2 hours post-fertilisation (hpf) until sexual maturation for four months in a semi-static system. At approximately 100 dpf, the solvent control and DBP-exposed fish (12 males and 12 females) were paired in clean water (without DBP), and the eggs were collected for 21 days. After each of these spawns, the egg numbers were recorded and fecundity was reported as the cumulative average number of eggs produced per female per day. A control group and a solvent control group (0.01% (v/v) DMSO) were employed. Exposure of the F0 generation to DBP did not significantly affect hatching, malformation, growth or survival. With regard to the F1 generation, >80% of the control embryos hatched successfully at 4 dpf, whereas the hatching rate was significantly reduced in the F1 embryos from the 43.8 µg/L DBP treatment group. The hatching rate was restored to levels similar to control levels at 5 dpf (>90%). The malformation

was not significantly changed in the F1 derived from the exposed F0 fish. However, there was a significant decrease in survival rates and growth (body weight) in F1 embryos derived from parents exposed to 43.8 µg/L DBP. There were no effects on growth, BSI and HSI in females or males exposed to any concentration of DBP. The GSI in females was significantly increased in the groups exposed to 13.6 and 43.8 µg/L DBP. The percentage of vitellogenic oocytes was significantly increased by 38.1% and 54.8% in the adults exposed to 13.6 and 43.8 µg/L DBP, respectively. However, the percentage of preovulatory oocytes was significantly decreased by 24.6% and 29.5% in the 13.6 and 43.8 µg/L DBP group, respectively. In males, only exposure to 43.6 µg/L DBP significantly increased the GSI. There were no obvious histological alterations observed in the testes in the groups exposed to DBP exposure. Egg production was significantly reduced by 28.9% in the group exposed to 43.6 µg/L DBP when compared with the solvent control. The results demonstrate that chronic exposure to DBP (43.8 µg/L) impaired the reproductive function of zebrafish, as verified by reduced egg production and modifications to gonadal histology of the treated fish. Plasma 17β-estradiol levels in female zebrafish decreased significantly in a concentration-dependent manner, while testosterone levels in males increased significantly when fish were exposed to 43.8 µg/L DBP. Real-time polymerase chain reaction was performed to examine selected genes in the hypothalamic-pituitary-gonadal (HPG) axis and liver. Hepatic vitellogenin gene transcription was downregulated in both males and females, suggesting that DBP possesses anti-estrogenic activity. The disturbed steroid hormones were accompanied by the significant alterations in gene expression along the HPG axis including an increase in follicle-stimulating hormone-β (FSHβ), and a decrease in luteinising hormone-β (LHβ), and in CYP19b in the 13.6 and 43.8 µg/L DBP group in both sexes. Additionally, parental exposure to DBP caused reduced hatching and survival rate as well as decreased growth in the F1 generation. Taken together, this is a full life cycle toxicity test. The results demonstrate that long-term exposure to low concentrations of DBP in zebrafish could cause reproductive toxicity, implying that DBP could have significant adverse effects on fish populations, particularly in a highly DBP-contaminated aquatic environment.

**Hu et al. (2021):** This study investigated the developmental toxicity of paternal exposure to DBP on offspring in zebrafish. Adult male AB strain zebrafish with normal reproductive function were semi-statically exposed to nominal DBP concentrations of 200, 600, and 1800 µg/L for 30 days, and then mated with females. Final acetone concentrations were 0.1% in each group. Thirty embryos per group were randomly selected to be observed, and malformations were recorded and photographed and then raised until adulthood of 6 months old. The mating and observations were repeated three times, for a total of 90 embryos per group. The results showed that the percentage of malformations, such as edema and a bent trunk, was increased in the 600, and 1800 µg/L DBP exposure groups, the heart rate and spontaneous contraction decreased in the 600, and 1800 µg/L DBP exposure groups and migration of primordial germ cells was disrupted in some F1 embryos in all DBP exposure groups after paternal exposure. The axial skeleton was affected in some F1 adults in the 1800 µg/L DBP exposure group. The study demonstrated the developmental toxicity of paternal DBP exposure in zebrafish.

**Jarmołowicz et al. (2013):** The ten-week-long experiment was divided into two stages of five weeks each in a flow-through system. The aim of the study was to describe the impact of DBP on the development of the reproductive system of European pikeperch (*Sander lucioperca*) during the sex differentiation period (age 61–96 days post hatch, dph). A total of 240 fish were divided into 6 groups (40 fish per circulation tank). Treatments consisted of a control group (0 g DBP·kg<sup>-1</sup> feed) and five trial groups with 0.125, 0.25, 0.5, 1, and 2 g DBP·kg<sup>-1</sup> feed, respectively. The feed was delivered by automatic band feeders 18 h per day. In the second stage of the experiment (97–132 dph), the fish were fed only commercial feed. Fish mortality was monitored daily. At the end of both stages of the experiment, the fish were weighed and measured for length. At the beginning of the experiment (60 dph) and after the first and second stages, the gonads of 15 individuals from each group were collected for histological analyses. The degree of gonad development, histological changes, and sex ratio were evaluated. No effects on fish survival and growth were observed during the whole period of experiment. Prior to the beginning of the experiment, the pikeperch aged 60 dph exhibited no signs of differentiation. After 96 dph, the sex ratios in the control group and in the groups treated with

0.125 and 0.25 g DBP·kg<sup>-1</sup> were about 1:1. DBP disturbed the sex differentiation process of pikeperch, leading to more females in the group treated with 2 g DBP·kg<sup>-1</sup> feed. After 132 dph, effects of DBP on sex ratio remains comparable to those observed at 96 dph. Histopathological analyses revealed that the administration of 2 g DBP·kg<sup>-1</sup> significantly affected the sex ratio. Male sex declined from 53% in the control group to 27% in the two highest exposure groups. 6.7% intersex was observed in these groups also. Intersex did not appear in lower doses or the control group. The feminisation process (intersex gonads) at concentrations of 1 g and 2 g DBP·kg<sup>-1</sup> were observed. All analysed concentrations delayed testicular development. The authors suggested that the effects of DBP on sex differentiation might be via anti-androgen modality in fish.

**Ortiz-Zarragoitia et al. (2006):** The aim of the work was to study the effects of the peroxisome proliferator dibutylphthalate (DBP) on liver peroxisomes, reproduction, and development of zebrafish (*Danio rerio*). In experiment 1, newly fertilised zebrafish eggs (250 eggs, in two replicates for each experimental group with static water renewal) were exposed for five weeks to nominal concentrations of 25 and 100 µg/L of DBP. Survival did not differ significantly between DBP exposed animals and control animals. DBP showed reduced *CYP19A2* gene expression at 6 and 10 dpf in the 100 µg/L concentration, with no changes in the 25 µg/L concentration. Although not statistically significant, *CYP19A1* expression was elevated at 4 and 35 dpf in the 100 µg/L of DBP treatment when compared with controls. A reduction and an up-regulation in *VTG* gene expression was observed at 6 dpf and 21 dpf, respectively, after exposure to 100 µg/L of DBP. Down-regulation of *CYP1A* gene expression occurred at 4 dpf, but afterward, it was up-regulated at 10 dpf and 35 dpf in zebrafish exposed to 25 µg/L of DBP. Exposure to 100 µg/L of DBP down-regulated *CYP1A* expression compared with controls at 10 dpf, but at 21 dpf, this effect disappeared. The activity of AOX also was statistically significantly induced in zebrafish exposed to 100 µg/L of DBP compared to control animals at 35 dpf. Liver peroxisomal volume density was increased after 100 µg/L of DBP exposures at 35 dpf, similar to the observations for AOX activity. This increase was accompanied by a significantly higher number of peroxisomes in both experimental groups compared with control animals. At 21 dpf, animals were not sexually differentiated in any experimental group, and no gonad-like structures were discernible except for one possible male in the control group. At 35 dpf, all animals from control and the two DBP treated groups were sexually differentiated. Previtellogenic and early vitellogenic oocytes were observed in female animals from the control (six females) and 100 µg/L of DBP (two females) group. No females were observed in the 25 µg/L of DBP group, and all 10 animals from this group were males, showing testes with different cysts containing all spermatogenic cells, including spermatozoa. Well-developed testes also were observed in male animals from control (four males) and 100 µg/L of DBP (eight males) groups.

In experiment 2, adult female zebrafish (N=10) were exposed for 15 d to 100 and 500 µg/L of DBP, and afterward, they were paired with untreated males to study the effects in the resultant offspring. The number of eggs obtained from females exposed to both DBP concentrations did not differ from that of controls. However, DBP caused teratogenic effects in early life stages and mortality of the larvae obtained from exposed females. Mortality of fry was increased significantly in a concentration-dependent manner in the DBP-exposed groups, reaching 70% mortality after 25 d post-fertilisation in fry from the females exposed to 500 µg/L of DBP. The expression of *CYP19A2* in the brain was up-regulated in females treated with 100 µg/L of DBP. Ovarian *CYP19A1* expression was significantly upregulated with both DBP concentrations. The AOX protein levels were significantly increased in livers from females exposed to both DBP concentrations compared to control females. Liver *VTG* levels did not differ between control and DBP-exposed females. Similar to control females, DBP-exposed females had ovaries containing oocytes at different gametogenic stages. Despite significant changes in the expression of aromatases, DBP-exposed females did not show any histological alterations in their gonads and were able to spawn successfully. However, the viability of fry obtained from these females was severely affected, showing high mortalities. Maternal exposure to DBP could affect larval development and survival.

**Xu et al. (2014):** Three-month-old wild type TU strain male zebrafish were exposed to 100 and 500 µg/L DBP for 45 days under semi-static conditions. After 21 days of exposure, three males from each treatment group were randomly sampled for the analysis of vitellogenin (VTG) induction, and acyl-CoA oxidase (AOX). A solvent control group (0.005% (v/v) DMSO) was included. Reproductive performance was assessed after 30d of depuration in freshwater following 45 d exposure. Two male fish, from each treatment which has gone through the recovery period, were mated with two 4-month-old unexposed female zebrafish with normal spawning capacity. The breeding trials were run for 3 weeks. After 21d exposure, no statistical difference was found among the weights, lengths and condition factors of different treatment groups. A decrease in VTG levels was observed in both DBP groups, with a significant difference in the 100 µg/L DBP group. The AOX levels at both DBP exposure groups were significantly higher than the control. After 45d exposure, delayed gametogenesis was observed for the DBP groups, indicated by fewer spermatozoa and more spermatocytes. The developmental delay of testis partially recovered after a 30d depuration in clean water. No difference was reported for fecundity, fertilisation rate, survival rates and hatching rate of the F1 generation. The authors concluded that the effects on VTG, AOX and reproductive toxicity, were indicative of PPAR-ER cross talk.

### 5.2.3.2 Summary of fish studies tested for EAS endocrine activities and adversity

The following table provides an overview of the available in vivo fish tests with DBP for EAS-mediated activities and adversity. DBP has been tested in four fish species including murray rainbowfish, pikeperch, stickleback and zebrafish. Changes in VTG, E2, testosterone, 11-KT, spiggin, gene expressions of ER $\alpha$  and ER $\beta$ , aromatase, CYP19a1 and 2, and sex ratio have been reported in different studies of all four fish species (details see following table). The studies stated that DBP induces estrogenic, anti-estrogenic, anti-androgenic, and steroidogenesis activity, i.e., EAS activity in four fish species.

Female biased sex ratio has been reported in murray rainbowfish and pikeperch exposed to DBP during the sensitive window of sexual differentiation (Jarmołowicz *et al.*, 2013; Bhatia *et al.*, 2014b). One study in zebrafish of the TU strain covered the sexual development stages and did not show a change in sex ratio induced by DBP (Chen *et al.*, 2015). In the same study, VTG was also not changed. These results suggest that zebrafish of the TU strain at the exposure stages may be less responsive to DBP. In contrast to this study, a decrease in VTG was reported in adult zebrafish of the TU strain exposed to the same concentrations. Adult murray rainbowfish exposed to DBP had changes in gonad histology (Bhatia *et al.*, 2013; 2014a). Similarly, gonad histopathological changes were reported in zebrafish when exposure to DBP started at the juvenile or adult stages (Chen *et al.*, 2015; 2020; 2021; Xu *et al.*, 2014). A decrease in fecundity in F0 and increased malformations in F1 have been reported in zebrafish (Ortiz-Zarragoitia *et al.*, 2006; Hu *et al.*, 2021; Hu *et al.*, 2020; Chen *et al.*, 2021). These results reveal that DBP targets reproductive organs, leading to decreased fecundity and increased malformation in F1. In a zebrafish full life cycle toxicity test, chronic exposure to DBP at the concentration of 43.8 µg/L impaired reproductive function, as verified by reduced egg production and modifications to gonadal histology. Parental exposure to DBP caused reduced hatching and survival rate as well as decreased growth in the F1 generation (Hu *et al.*, 2020). In summary, twelve studies were included in this ED assessment, with two studies comparable to TG234 (Jarmołowicz *et al.*, 2013; Bhatia *et al.*, 2014b); seven studies similar to TG229/230 (Aoki *et al.*, 2011; Bhatia *et al.*, 2013; 2014a; Chen *et al.*, 2020; 2021; Hu *et al.*, 2021; Ortiz-Zarragoitia *et al.*, 2006; Xu *et al.*, 2014); and one zebrafish partial life cycle toxicity test (Chen *et al.*, 2015) and one zebrafish full life toxicity test (Hu *et al.*, 2020). All these studies showed that DBP targeted sexual development and reproduction, leading to population relevant adverse effects on sex ratio; fecundity and survival, malformation and growth of F1.

**Table 5: Overview of results of EAS related in vivo assays**

Species	Exposure	Results	Conclusions	Remarks	Reference
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ANNEX XV – IDENTIFICATION OF DIBUTYL PHTHALATE AS SVHC

Sticklebacks	Adult male, 22d, 15 and 35 µg/L	T, ↑; 11-KT, ↔; spiggin, ↓; at 35 µg/L	Antiandrogenic activity	Comparable to TG229/230	Aoki <i>et al.</i> , 2011
Murray rainbowfish	Adult female, 7d, 125, 250, 500, 1000 µg/L	Ovarian histology changes at 250 µg/L and above	Anti-estrogenic effects on ovarian histology		Bhatia <i>et al.</i> , 2013
Murray rainbowfish	Adult male, 7d, 125, 250, 500, 1000 µg/L	testis histology changes at 125 µg/L and above;  VTG, ↑ at 500 and 1000 µg/L; ERα and ERβ, ↑; choriogenin, ↑, Aromatase, ↑	Estrogenic effects on testis histology		Bhatia <i>et al.</i> , 2014a
zebrafish	Adult female, 15d, 100, 500 µg/L	Fecundity, ↔; survival in F1, ↓; CYP19a1 and 2, ↑; VTG ↔;	EAS activity, high mortalities in F1		Ortiz-Zarragoitia & <i>et al.</i> , 2006
zebrafish	Adult male, 30d, 11, 113, 1133 µg/L	E2, ↔; T, ↑; testis histology changes, changes in genes of signalling pathways	Effects on testis histology due to disruption of signalling pathways		Chen <i>et al.</i> , 2020, 2021
zebrafish	Adult male, 30d; 200, 600, 1800 µg/L	Malformations, ↑; heart rate, ↓; axial skeleton was affected in F1	Exposure of DBP to F0 males induced developmental toxicity in F1.		Hu <i>et al.</i> , 2021
zebrafish	Adult males and females, 30d, 11, 113, 1133 µg/L	Histology changes in ovary, fecundity, ↓; at 1133 µg/L; hatching, ↔, malformation, ↑ at 11 µg/L and above in F1	Adverse effects on reproduction and on F1 development	Comparable to TG229	Chen <i>et al.</i> , 2021
zebrafish	Adult, 45d; 100, 500 µg/L	VTG, ↓; testis histology change; fecundity ↔	Reproductive toxicity indicative of PPAR-ER cross talk		Xu <i>et al.</i> , 2014
zebrafish	embryos, 35d, 25, 100 µg/L	CYP19a2, ↓ at 100 µg/L; VTG, ↑; no	EAS activity was reported		Ortiz-Zarragoitia & <i>et</i>

		histology change			<i>al.</i> , 2006
zebrafish	Fry at 20 dpf, 115 dpf, 100 and 500 µg/L	VTG, ↔  Sex ratio, ↔  Gonad histology changes at 500 µg/L	No EAS conclusion	Covering sex differentiation period	Chen <i>et al.</i> , 2015
pikeperch	Fry at 60 dph, 36d-exposure+36d recovery, oral, 0.125, 0.25, 0.5, 1, and 2 g/kg.feed.	Female biased sex ratio at 2g/kg	Female biased sex ratio may be due to anti-androgen modality	Comparable to TG234	Jarmołowicz <i>et al.</i> , 2013
murray rainbowfish	Juvenile fish at 30 dph, 90d, 5, 15, 50 µg/L	Feminization;  E2/11-KT, ↑; at 5 µg/L	estrogenic effects on sex ratio	Comparable to TG234	Bhatia <i>et al.</i> , 2014b
zebrafish	Embryos,  4 months; 4.9, 13.6, 43.8 µg/L	Fecundity, ↓; gonad histology changes; T, ↑ in males at 43.8 µg/L; E2↓; gene expression of VTG and CYP19a and b, ↓; at top two groups in females  Hatching, ↓; survival, ↓; growth, ↓; in F1	DBP induced reproductive toxicity, leading to a decreased fecundity in F0 and a reduced hatching, survival rate, and growth in the F1 generation via EAS pathways.	Full life study	Hu <i>et al.</i> , 2020

#### 5.2.4 Plausible link between adverse effects and endocrine mode of action

Changes in sex ratio were observed in two fish species, pikeperch and murray rainbowfish. These changes were time and concentration dependent and were not related to general toxicity (Jarmołowicz *et al.*, 2013; Bhatia *et al.*, 2014b). Female biased sex ratio in these two fish species suggests that such an effect is mediated by anti-androgenicity or estrogenicity (OECD, 2011). Several in vitro studies show that DBP is a weak ER binder and transactivates ERs (Jobling *et al.*, 1995; Harris *et al.*, 1997; Rider *et al.*, 2009; Shen *et al.*, 2009; Czernych *et al.*, 2013). In vivo fish studies in zebrafish and murray rainbowfish further support that DBP induces estrogenicity (Jarmołowicz *et al.*, 2013; Bhatia *et al.*, 2014a and 2014b; Ortiz-Zarragoitia *et al.*, 2006). These in vitro and in vivo results further support that changes in sex ratio may be mediated by ERs. Similarly, there are also in vitro results (Czernych *et al.*, 2013) and in vivo fish tests (Aoki *et al.*, 2011; Jarmołowicz *et al.*, 2013; Bhatia *et al.*, 2014b) supporting that DBP

induces anti-androgenicity. In summary, both in vitro and in vivo evidence demonstrates that DBP induced female biased sex ratio may be mediated by the EA modalities.

A disruptive effect on the gonad histology has been observed in adult zebrafish and murray rainbowfish exposed to DBP for 7 days and longer (Bhatia *et al.*, 2013 and 2014a; Chen *et al.*, 2020; Xu *et al.*, 2014). When exposure to DBP started from the juvenile stage, such a disruptive effect on gonad histology was also evidenced in these two species of fish (Bhatia *et al.*, 2014b; Chen *et al.*, 2015 and 2021; Ortiz-Zarragoitia *et al.*, 2006). These histopathological changes were DBP specific and were not related to general toxicity. It has been further demonstrated that DBP decreased fecundity and caused an increase in malformation and a decrease in hatching, survival rate and growth in the F1 generation of zebrafish, which was considered to be induced by an anti-estrogenic activity (Chen *et al.*, 2021; Hu *et al.*, 2020; Hu *et al.*, 2021; Ortiz-Zarragoitia *et al.*, 2006; Xu *et al.*, 2014). Concurrently, changes in biomarkers including VTG, E2, testosterone, 11-KT, spiggin, sex ratio and gene expressions of ER $\alpha$  and ER $\beta$ , CYP19a1 and 2, etc. have been reported in different studies of four fish species. These changes in biomarkers indicate that DBP acts via EAS modalities. Overall, the available fish studies demonstrated EAS-mediated adverse effects on fecundity and survival, malformation and growth of F1.

It is important to note that the underlying MoAs, i.e., estrogenicity, anti-estrogenicity, and anti-androgenicity, and steroidogenesis for DBP-induced population relevant adverse effects on sex ratio, reproduction and offspring health were concluded on the basis of changes in biomarkers. For example, based on a decrease in VTG in female Murray rainbowfish, anti-estrogenicity of DBP was concluded by the authors (Bhatia *et al.*, 2013); similarly, DBP induced estrogenicity was also concluded on the basis of an increase in VTG in male Murray rainbowfish by the same authors (Bhatia *et al.* 2013). These conclusions on estrogenicity and anti-estrogenicity seem to be conflicting. However, these MoAs may reflect a balanced result of multiple MoAs (Dang and Lowik, 2005). In fact, multiple MoAs induced by the same substance have often been reported. For instance, phytoestrogens like genistein and daidzein induce both estrogenicity and anti-estrogenicity in a dose response way (Dang and Lowik, 2005). Estrogenicity and anti-estrogenicity is a balanced result of cross-talk between ERs and PPARs induced by phytoestrogens (Dang *et al.*, 2003; Dang, 2009). Similarly, DBP induces also multiple MoAs. Responses of biomarkers may be a balanced result of DBP-induced multiple MoAs. So far, no studies are available investigating how the different MoAs of DBP interplay with each other. The available evidence could support the plausibility of EAS mediated adversity but could not pinpoint the exact adverse outcome pathways (AOPs).

## **5.2.5 Conclusion regarding ED properties relevant to the environment**

### **5.2.5.1 Adverse effects relevant to the ED identification**

Twelve studies were included in this ED assessment, with two studies covering sexual development stages (Jarmołowicz *et al.*, 2013; Bhatia *et al.*, 2014b); seven studies similar to OECD TG 229/230 (Aoki *et al.*, 2011; Bhatia *et al.*, 2013 and 2014a; Chen *et al.*, 2020 and 2021; Hu *et al.*, 2021; Ortiz-Zarragoitia *et al.*, 2006; Xu *et al.*, 2014); and one zebrafish partial life cycle toxicity test (Chen *et al.*, 2015) and one zebrafish full life toxicity test (Hu *et al.*, 2020). All these studies showed that DBP targeted sexual development and reproduction, leading to population relevant adverse effects on sex ratio; fecundity and survival, malformation and growth of F1.

### **5.2.5.2 Endocrine activity**

The available in vitro data reveal that DBP binds to and transactivates ERs in various vertebrate species. Additionally, androgenic activity and/or anti-androgenic activity were reported in transactivation assays with human ARs (Shen *et al.*, 2009; Czernych *et al.*, 2013). Furthermore, steroidogenesis assays have demonstrated that DBP interferes with steroidogenesis (Chen *et al.*, 2013; Li *et al.*, 2013 and Källsten *et al.*, 2022). In summary, the in vitro assays indicate that DBP influences EAS modalities. Similarly, EAS activity has also been detected in four fish species



including murray rainbowfish, pikeperch, sticklebacks and zebrafish, indicated by a change in spiggin, VTG, E2, testosterone, 11-KT, and steroidogenesis gene expression e.g., CYP19A (Ortiz-Zarragoitia *et al.*, 2006; Aoki *et al.*, 2011; Jarmolowicz *et al.*, 2013; Bhatia *et al.*, 2014b; Hu *et al.*, 2020).

### 5.2.5.3. Plausible link between adverse effects and endocrine activity

Sex ratio is considered as an EATS-mediated endpoint. Female biased sex ratio in murray rainbowfish and pikeperch suggests that this adverse effect is mediated via an estrogenic or anti-androgenic pathway. This conclusion is further supported by the available in vitro and in vivo fish tests. DBP-induced reproductive toxicity as shown in changes in gonad histology and a decrease in fecundity as well as adverse effects in their offspring like increased malformation and reduced hatching, survival rate, and growth in F1 have been reported in murray rainbowfish and in zebrafish. These endpoints are considered as “sensitive to, but not diagnostic of EATS” endpoints and the underlying MoAs should be elucidated. In several studies, these reproductive changes are associated with changes in VTG, sex ratio, E2, testosterone, 11-KT and in gene expression of CYP19a and b, 17β-HSD etc. These results reveal that reproductive dysfunction resulted from DBP induced EAS activity. Overall, the link between endocrine EAS activity and the observed adverse effects on sexual development, reproductive function and on offspring is highly plausible.

### 5.2.5.4 Conclusion on ED properties

Overall, DBP has EAS activity and induces adverse effects on sex differentiation in two species of fish and on reproductive function and offspring that are plausibly mediated by this endocrine activity. Therefore, there is scientific evidence that DBP fulfils the definition of an endocrine disruptor relevant for the environment.

**Table 6: Summary of evidence showing that DBP fulfils the definition of an endocrine disruptor**

Adverse effects	Endocrine activity	Plausible link	Relevance
<p><b>sex differentiation</b> female biased sex ratio was observed in murray rainbowfish and pikeperch exposed to DBP.</p> <p><b>Reproduction</b> Histological changes in ovary and testis in murray rainbowfish and zebrafish; A decrease in fecundity in zebrafish; increased malformation and reduced hatching, survival rate, and growth in F1 in zebrafish</p>	<p><b>In vitro evidence</b> Positive EAS activity has been reported in the ToxCast assays. DBP binds to fish and human ERs and transactivates human and rate ERs. Androgenicity and anti-androgenicity were induced by DBP Changes in steroidogenesis activity were reported in three studies.</p> <p><b>In vivo evidence</b> Changes in VTG, E2, testosterone, 11-KT, spiggin,</p>	<p>Changes in sex ratio can be plausible linked to the estrogenic and anti-androgenic activity of DBP. Alteration of reproduction can be plausibly linked to an anti-estrogenic activity. Overall, the link between endocrine EAS activity and the observed adverse effects on sexual development, reproductive function and on offspring is highly plausible.</p>	<p><b>Environmental relevance: effects on populations and generations</b> Sex ratio and fecundity are considered as population relevant endpoints. Impact on sex ratio, reproduction and survival of F1 generation are considered environmental relevance.</p>

	sex ratio and steroidogenesis gene expression e.g. CYP19A in murray rainbowfish, pikeperch, sticklebacks and zebrafish		
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## 6. Conclusions on the SVHC Properties

### 6.1 CMR assessment

DBP is covered by index number 607-318-00-4 of Regulation (EC) No 1272/2008 in Annex VI, part 3, Table 3 (the list of harmonised classification and labelling of hazardous substances) and it is classified in the hazard class toxic for reproduction category 1B (H360FD). Based on this classification it has already been concluded that the substance meets the criteria for classification in the hazard class:

- toxic for reproduction category 1B in accordance with Article 57 (c) of REACH.

### 6.2 PBT and vPvB assessment

This section is not relevant for the identification of DBP as SVHC in accordance with Article 57 (c) and (f) of the REACH Regulation.

### 6.3 Assessment under Article 57(f)

#### 6.3.1 Summary of the data on the intrinsic/hazardous properties (providing scientific evidence of probable serious effects to the environment)

DBP has been classified as reproductive toxicant (Cat.1B) and been identified as a substance of very high concern in accordance with Article 57(f) of Regulation (EC) 1907/2006 (REACH) due to its endocrine disrupting (ED) properties for human health (ECHA, 2017). The spectrum of adverse effects observed in rats include impaired fertility, increased nipple retention, decreased anogenital distance, genital malformations, reduced number of spermatocytes and testicular changes including multinucleated gonocytes, tubular atrophy and Leydig cell hyperplasia. Overall, developmental and reproductive toxicity tests in rodents provide supporting evidence that is potentially population relevant and can be used to strengthen the conclusion that DBP as an environmental ED for mammals in the environment cannot be excluded. This document focused on non-mammalian studies for the ED properties for the environment. A targeted assessment by focusing on EAS activity and fish was performed.

Adverse effects concerning sexual development and reproduction are generally regarded as endpoints of particular relevance because such effects are likely to manifest themselves at the population level. Female biased sex ratio has been reported in murray rainbowfish and pikeperch exposed to DBP during the sensitive window of sexual differentiation. DBP-induced reproductive toxicity as shown in changes in gonad histology and a decrease in fecundity as well as adverse effects in their offspring like increased malformation and reduced hatching, survival rate, and growth in F1 have been reported in murray rainbowfish and in zebrafish. The plausible connection to the endocrine system was also confirmed in fish where changes in spiggin (anti- androgenic MoA) in stickleback as well as changes in sex ratio and VTG (estrogenic and anti-estrogenic MoA) in zebrafish. Impaired reproductive effects are accompanied with changes in VTG, E2, testosterone, 11-KT and in gene expression of CYP19a and b, 17β-HSD, indicating estrogenic, anti-estrogenic and steroidogenesis MoAs. In summary, studies show that DBP disrupted sexual differentiation, leading to a change to female biased sex ratio; DBP impaired reproductive organs and caused a decrease in fecundity and adverse effects in their offspring via EAS mediated

modalities. Hence the current data indicates in fish that DBP has endocrine disruptive properties leading to adverse effects related to sexual development and reproduction. It is important to note that the available studies provide strong evidence on EAS mediated effects based on changes in biomarkers in fish. These multiple MoAs may interact with each other, leading to different effects on biomarkers in fish. So far, no studies have further show DBP-induced effects of interaction of different receptors on biomarkers. This limitation, however, does not influence ED identification of DBP for the environment.

In conclusion, DBP can be considered an endocrine disruptor for the environment as it fulfils the WHO/IPCS definition of an endocrine disruptor and the recommendations from the European Commission's Endocrine Disruptors Expert Advisory Group for a substance to be identified as an endocrine disruptor.

### **6.3.2 Equivalent level of concern assessment**

In agreement with the REACH legal text, substances identified as SVHCs under article 57(f) shall give rise to an equivalent level of concern to those of other substances listed in points (a) to (e), on a case-by-case basis. A number of factors relevant to assess that an adverse health effect represents an equivalent level of concern (ELoC) is identified in a discussion paper by ECHA (2012) with a specific focus on sensitisers.:

#### ***Characteristics of the effects:***

- Type of possible effects
- Irreversibility of effects
- Delay of effects

#### ***Other factors:***

- Quality of life affected (for human health effects)
- Societal concern
- Is derivation of 'safe concentration' possible?

The following description includes the elements relevant to the analysis in an environmental context.

#### **Probable serious effects**

DBP influences both sexual development and reproduction. DBP has adverse effects on the phenotypic sex in murray rainbowfish and pikeperch. DBP-induced reproductive toxicity as shown in changes in gonad histology and a decrease in fecundity as well as adverse effects in their offspring like increased malformation and reduced hatching, survival rate, and growth in F1 have been reported in murray rainbowfish and in zebrafish. These endpoints on sexual development and reproduction are considered population relevant.

#### ***Potential severity of ecotoxicological effects***

DBP may adversely affect the reproductive ability of fish populations by changing male fish into female fish. DBP may also induce adverse effects on reproductive organs and fecundity, and influence offspring survival and growth. Such reproductive effects are considered an adverse and serious effect with population level relevance and can lead to serious effects on ecosystems.

#### ***Irreversible and delayed effects***

Change in sex ratio of fish populations is an irreversible effect with long term implications on both the population itself and populations of other species dependent on this population. If for example the sex ratio of a fish population becomes significantly skewed and male fish becomes too scarce the population will not be able to maintain its size or may go through "a genetic bottle neck" reducing its natural genetic variability and thereby potentially diminishing the adaptation

of the population to environmental changes. Exposure during juvenile period of fish may cause gonad histopathological changes, leading to delayed effects on survival and growth of offspring. There is evidence that a short time exposure may be sufficient to provoke long-term effects even if exposure ceases.

### ***Broad environmental relevance***

Effects on sexual development and reproductive ability via endocrine EAS MoA has a broad environmental relevance. Due to the conservatism of endocrine EAS modalities, it is very likely that a wide range of wildlife species with different function in ecosystems could be affected. Further, the severity of effects of DBP on rodents are of particular concern in relation to mammalian wildlife including top predator species and other mammals (including endangered species), where the described reproductive effects are expected to cause serious effects at population level because of a natural low reproductive output of such taxa.

### ***Concern related to co-exposure and combined effects***

DBP can act jointly with other chemicals occurring in the environment having the same chemical structure and displaying the same effect. DBP is part of the group of phthalates, some of which have already been identified as endocrine disruptors (i.e. diisobutyl phthalate (DIBP) bis (2-ethylhexyl)phthalate (DEHP), benzyl butyl phthalate (BBP) and dicyclohexyl phthalate (DCHP). These substances share common MoAs and may have additive effects. Environmental occurrence show that DBP is detected in the environment together with other phthalates (Tuan Tran *et al.*, 2022).

### ***Derivation of safe level***

Endocrine regulation is a very complex feedback process. Any disturbance of this regulation during transient but vulnerable life stages can lead to irreversible effects during the entire lifetime or even in the following generations. Moreover, it is difficult to assess the latency of the effects based on the available ED specific test guidelines. Therefore, it is not possible to predict potential future effects and thus safe exposure levels for the environment.

A considerable amount of fish toxicity studies covering the whole life cycle of fish are available for DBP. However, these studies have only tested four fish species and species differences in effects have been observed. Toxic effects of DBP on different species of fish and other taxa may be overlooked for the environment. *Moreover, whether or not endocrine-mediated effects are observable may highly vary with the life stages tested because some effects potentially related to EAS modalities may be only observable during specific windows of exposure.* Disturbance of these specific life stages may result in effects on the entire life-time and even in the next generation with long-term consequences at the population level.

### **6.3.3 Conclusion on the Article 57(f) assessment**

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

DBP has been classified as reproductive toxicant (Cat.1B) and been identified as a substance of very high concern in accordance with Article 57(f) of Regulation (EC) 1907/2006 (REACH) due to its endocrine disrupting (ED) properties for human health (ECHA, 2017). The spectrum of adverse effects observed in rats include impaired fertility, increased nipple retention, decreased anogenital distance, genital malformations, reduced number of spermatocytes and testicular changes including multinucleated gonocytes, tubular atrophy and Leydig cell hyperplasia. Overall, developmental and reproductive toxicity tests in rodents provide supporting evidence that the adverse effects elicited by DBP are population relevant and can be used to strengthen

the conclusion that DBP is an ED for the environment.

DBP induces female biased sex ratio in murray rainbowfish and pikeperch via an estrogenic or anti-androgenic pathway. DBP also influences reproductive toxicity as shown in changes in gonad histology and a decrease in fecundity as well as adverse effects in their offspring like increased malformation and reduced hatching, survival rate, and growth in F1 as reported in murray rainbowfish and in zebrafish. These effects are related to an anti-estrogenic MoA or steroidogenesis MoA.

In conclusion, the link between endocrine EAS activity and the observed adverse effects on sexual development, reproductive function and on offspring is highly plausible. Therefore, there is scientific evidence that DBP fulfils the WHO/IPCS definition of an endocrine disruptor relevant for the environment. DBP can be considered an endocrine disruptor for the environment.

The effects of DBP due to its endocrine disrupting properties are considered to be of equivalent level of concern to CMR Cat. 1, PBT or vPvB substances as listed in Article 57 points (a) to (e) of the REACH Regulation. DBP is considered as a substance giving rise to an equivalent level of concern because scientific evidence shows that exposure during sensitive time windows of sexual development may cause irreversible developmental programming effects leading to severe effects on development and reproduction, regarded as particularly serious in relation to wildlife species, also because these adverse effects may first manifest themselves in later life stages or offspring. It is difficult to determine the most sensitive species and thus to quantify a safe level of exposure with regard to the endocrine mediated effects. Adverse effects on development and reproduction are in addition generally regarded as endpoints of concern, and as such frequently used for regulatory hazard and risk assessment for environmental species.

Overall, based on all available scientific evidence, it can be concluded that DBP fulfils the WHO/IPCS definition of an endocrine disruptor:

- It shows clear population relevant adverse effects on sexual development, reproduction and offspring survival and growth in fish.
- It has EAS activity as clearly shown both in vitro and in vivo.
- The substance has an EAS mode of action, i.e., the adverse effects, including the recognised EAS-mediated effects on sex ratio and also effects considered to be sensitive to, but not diagnostic of, EAS modalities are biologically plausibly linked to the adverse effects

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

## Part II

### 7. Registration and C&L notification status

#### 7.1 Registration status

**Table 7: Registration status**

From the ECHA dissemination site <sup>2</sup>	
Registrations	<input checked="" type="checkbox"/> Full registration(s) (Art. 10)  <input type="checkbox"/> Intermediate registration(s) (Art. 17 and/or 18)

#### 7.2 CLP notification status

**Table 8: CLP notifications**

	CLP Notifications <sup>3</sup>
Number of aggregated notifications	35
Total number of notifiers	1269

The self-classification listed is:

- Repr. 1B (H360Df)
- Aq. Acute 1
- Car Cat 1A
- Aq Chron 1

### 8. Total tonnage of the substance

**Table 9: Tonnage status**

Total tonnage band for the registered substance (excluding the volume registered under Art 17 or Art 18) <sup>9</sup>	≥ 1000 to < 10000 tonnes per year
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<sup>2</sup> The link to the substance specific entry on the dissemination site ( accessed on 27 July 2023): [Registration Dossier - ECHA \(europa.eu\)](https://echa.europa.eu/registration-dossier)

<sup>3</sup> C&L Inventory database, (accessed on 27 July 2023) [Inventaris van indelingen en etiketteringen \(europa.eu\)](https://echa.europa.eu/inventaris-van-indelingen-en-etiketteringen)

## 9. Information on uses of the substance

Table 10: Uses

	Use(s)	Registered use	Use <u>likely</u> to be in the scope of Authorisation
<b>Uses as intermediate</b>		-	-
<b>Formulation or repacking</b>	<ul style="list-style-type: none"> <li>- Formulation in polymers</li> <li>- Formulation in propellants</li> </ul>	Yes	Yes
<b>Uses at industrial sites</b>	<ul style="list-style-type: none"> <li>- Use as an absorption solvent in a closed system in the manufacture of maleic anhydride (MA)</li> <li>- Use in Propellants</li> <li>- Industrial use of DBP for production of PVC articles</li> <li>- Laboratory use</li> <li>- Use in ceramics</li> </ul>	Yes	Yes
<b>Uses by professional workers</b>	Laboratory use	Yes	Yes
<b>Consumer uses</b>	-	No	No
<b>Article service life</b>	Contact with PVC products e.g.: handling of building materials, contact with flooring, professional clothing, etc.	Yes	Yes

## 10. Information on structure of the supply chain

DBP is commonly used as a plasticiser in various products. Some downstream users of DBP include manufacturers of:

- PVC products: DBP is often added to PVC to increase its flexibility and durability, making it suitable for products like vinyl flooring, cables, and coated fabrics.
- Adhesives: DBP can be used as a plasticiser in adhesives to improve their performance and adhesion properties.
- Inks: DBP is used in some types of inks to enhance their flow and printing characteristics.

## 11. Additional information

No additional information.

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

Substances that have a similar hazard profile to DBP are other phthalates, especially those with similar chemical structures and properties. Those that are already identified as endocrine disruptors are diisobutyl phthalate (DIBP) bis (2-ethylhexyl)phthalate (DEHP), benzyl butyl phthalate (BBP) and dicyclohexyl phthalate (DCHP).

### **11.2 Alternatives**

Several alternatives to dibutyl phthalate (DBP) have been developed and adopted in various industries. Some of them are longer chain phthalates, such as diisononyl phthalate, diisodecyl phthalate and dioctyl phthalate. There are however also non-phthalate plasticisers available such as dibutyl sebacate that can be used as adhesives and coatings, acetyl tributyl citrate that can be used as plasticiser in products like food packaging, medical devices, and children's toys, or epoxidised soybean oil that can be used as a DBP replacement in PVC products (Harmon and Otter, 2022; Vieira *et al.*, 2011).



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