Substance Name: Dibutyl phthalate

EC Number: 201-557-4

CAS Number: 84-74-2

MEMBER STATE COMMITTEE SUPPORT DOCUMENT FOR IDENTIFICATION OF

DIBUTYL PHTHALATE

AS A SUBSTANCE OF VERY HIGH CONCERN BECAUSE OF ITS ENDOCRINE DISRUPTING PROPERTIES (ARTICLE 57(F) - ENVIRONMENT) PROPERTIES

Adopted on 29 November 2023

CONTENTS

TABLES

ABBREVIATIONS

AKR1C14 : aldo-keto reductase family 1, member C14
AMA : Amphibian Metamorphosis Assay
AR : androgen receptor
AOX : acyl-CoA oxidase
BSI : brain-somatic index
BTC : betacellulin
BW : body weight
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 : 17β-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
fhERα : full-length recombinant estrogen receptors from fathead minnows
GSI : gonadosomatic index
hERa : human estrogen receptor α
17β-HSD : 17β-hydroxy-steroid dehydrogenase
H295R : human adreno-carcinoma cell line (NCI-H295R cells)
HLL : hind limb length
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
RBC : red blood cell
RSCABS : Rao-Scott Cochran Armitage by Slices
rtER : rainbow trout estrogen receptor
SRD5A1 : smooth endoplasmic reticulum steroid 5α-reductase 1
StAR : Steroidogenic Acute Regulatory Protein
SVL : snout-vent length

T : Testosterone
T3 : 3,3´,5-L-triiodothyronine
T4 : L-thyroxine
TRb : thyroid hormone receptor-beta
TSHa : alpha subunit of thyroid-stimulating hormone
TSHb : beta subunit of thyroid-stimulating hormone
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

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 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 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 has 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. Mammalian studies showed a decrease in mating, fertility, and pregnancy rates. Overall, developmental and reproductive toxicity tests in rodents provide evidence that the adverse effects elicited by DBP are population relevant and can be used, in parallel to the non-mammalian data, to conclude 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 steroidogenic MoA. DBP can disturb testicular differentiation and impaired spermatogenesis of amphibians which may be plausibly linked to the estrogenic or anti-androgenic activity. There is also evidence that DBP affects the thyroid axis leading to an accelerated metamorphic development in amphibians.

In conclusion, the link between endocrine EATS activity and the observed adverse effects on sexual development, reproductive function and on offspring in fish, amphibians and mammals as well as on testicular differentiation and impaired spermatogenesis of amphibians and on metamorphosis in amphibians 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, on mating, fertility, and pregnancy rates in rodents and on testicular differentiation and impaired spermatogenesis of amphibians and on metamorphosis in amphibians.

- It has EATS activity as clearly shown both *in vitro* and *in vivo*.

- The substance has an EATS mode of action, i.e., the adverse effects, including the recognised EATS mediated effects on reproduction in rodents, sex ratio in fish and on testicular differentiation and impaired spermatogenesis of amphibians and on metamorphosis in amphibians and also effects considered to be sensitive to, but not diagnostic of, EATS 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

1. Identity of the substance and physical and chemical properties

1.1 Name and other identifiers of the substance

Table 1: Substance identity

EC number:	201-557-4
EC name:	Dibutyl phthalate
CAS number (in the EC inventory):	84-74-2
CAS number:	84-74-2
CAS name:	1,2-Benzenedicarboxylic acid dibutyl ester
IUPAC name:	Dibutyl phthalate
Index number in Annex VI of the CLP Regulation	607-318-00-4
Molecular formula:	$C_{16}H_{22}O_4$
Molecular weight range:	278.34 g/mol
Synonyms:	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[1]

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^{-3} 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

Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling		Specific Conc. Limits, M-factors
				Hazard Class and Category Code(s)	Hazard Statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	
607-318-00-4	dibutyl phthalate; DBP	201-557-4	84-74-2	Repr. 1B Aquatic Acute 1	H360Df H400	GHS08 GHS09 Dgr	H360Df 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).

[1] 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 ready 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^3/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 focused 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 8th, 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. In addition, a targeted literature search was performed on available *in vivo* amphibian studies to find further evidence for the potential adverse effects of DBP on the hypothalamic-pituitary thyroid axis. The assessment of *in vivo* data focuses on the question whether adverse effects on sexual development and reproduction in fish and metamorphosis in amphibians can be inferred to originate from the presumed EATS 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

DBP has been extensively studied for EAS activity *in vitro*, which are present in the public literature. These literature studies are summarised as follows. 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 hERα (YES). Cells were incubated with serially diluted DBP concentrations and positive control (17-β estradiol) for 48 h at 32 °C in the presence of a substrate for β-galactosidase synthesis. DBP showed no estrogenic activity till the highest test concentration of 50 µM. However, DBP exhibited concentration-dependent anti-estrogenic activity at the concentrations between 0.5 and 50 µM, with an IC50 of 19.2 µM.

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^{-7} M to 10^{-3}M. Dose dependent estrogenic activity was detected in the yeast screen assay at concentrations between 10^{-5} M and 10^{-3} M. DBP at the concentration of 10^{-5} 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 17ß-estradiol to the fish estrogen receptor in an assay with cytosolic liver extracts of rainbow trout at concentrations of approximately 10^{-7} to 10^{-5} M. DBP also stimulated transcriptional activity of the estrogen receptor at concentrations of 10^{-6} and 10^{-5} 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^{-5} 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 (fhERα) and those from humans (hERa). 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 fhERα (IC50s of 1.98 x 10^{-5} M), while only partial displacement of E2 by DBP was achieved for hERa 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 fhERα than for hERα.

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 ERα expression vector rat ERα/pCI were exposed to E2 at concentrations of 1.0×10^{-12}–1.0×10^{-8}M, DMSO control, DBP and MBP for 24 h. MBP showed no agonistic activity, whereas DBP appeared weakly estrogenic at the concentration of 1.0×10^{-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 5α- dihydrotestosterone for 48 h at 32 °C in the presence of a substrate for β-galactosidase synthesis. DBP showed no androgenic activity till the highest test concentration of 50 µM. However, DBP exhibited concentration dependent anti-androgenic activity between 1 and 50 µM, with an IC50 of 12.2 µM.

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 5α-DHT at concentrations of 1.0×10^{-12}–1.0×10^{-7}M, DMSO control, DBP and MBP for 24 h. The results showed that DBP and MBP not only exhibited potent antiandrogenic activity, with IC50 value of 1.05×10^{-6}, and 1.22×10^{-7}M, respectively, but also showed the androgenic activity with EC50 value of 6.17×10^{-6} and 1.13×10^{-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 β-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 µM in DMSO) and MBP (0.1, 10, 1000 µM in DMSO) for 24 h. The MTT assay showed that DBP at concentrations 1000 and 2000 µM and MBP at the concentration of 2000 µM 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 µM did not show a decrease in the MTT assay. The mRNA levels of the side-chain cleavage enzyme (P450scc), cytochrome p450c17 (P450c17) and 3β-hydroxy-steroid dehydrogenase (3 β 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.

5.2.2.1 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=1.98x10^{-5}M	Weak binder to fER	Rider *et al.*, 2009

ER transactivation	hER	MCF 7	transactivation at 1X 10^{-6} M and higher	ER transactivation	Jobling *et al.*, 1995
ER transactivation	hER	yeast	transactivation at 10^{-5} M and higher	ER transactivation	Harris *et al.*, 1997
ER transactivation	rER	CV-1	EC50=1X10^{-4}M	ER transactivation	Shen *et al.*, 2009
ER transactivation	hER	yeast	IC50=1.92x10^{-5}M	Anti-estrogenic activity	Czernych *et al.*, 2013
AR transactivation	hAR	CV-1	IC50=1.05x10^{-6}M	Anti-antrogenic activity	Shen *et al.*, 2009
AR transactivation	hAR	CV-1	EC50=6.17x10^{-6}M	androgenic activity	Shen *et al.*, 2009
AR transactivation	hAR	yeast	IC50=1.22x10^{-5}M	Anti-antrogenic activity	Czernych *et al.*, 2013
Steroidogenesis	Murine MLTC-1	MLTC-1	biphasic effects on testosterone production	Alternation of steroidogenisis	Chen *et al.*, 2013
Steroidogenesis	Rat Leydig cells	Rat leydig cells	Inhibition of androgen production	inhibiton of steroidogenisis	Li *et al.*, 2013
Steroidogenesis	Human mural granulosa cell	Human mural granulosa cell	Inhibition of steroidogenesis at 100 mg/L	inhibiton of steroidogenisis	Adir *et al.*, 2017
Steroidogenesis	H295R	H295R cells	Reduced testosterone; increased cortisol	inhibiton of steroidogenisis	Källsten *et al.*, 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 ERα compared to human ERα (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 for EAS activity and adversity

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.

Aoki *et al.* (2011): In the present study, adult male three-spined sticklebacks (*Gasterosteus aculetaus*; 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. he 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±1.3, 64.9±2.5, 113.8±0.7 ng g^{-1} 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^{-1} feed) and five trial groups with 0.125, 0.25, 0.5, 1, and 2 g DBP·kg^{-1} 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^{-1} 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^{-1} 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^{-1} 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^{-1} 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 35dpf 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 500µg/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 100µg/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.

5.2.3.1 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 ERα and ERβ, 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
Sticklebacks	Adult male, 22d, 15 and 35 µg/L	T,↑;11-KT, ↔; spiggin, ↓; at 35 µg/L	Antiandrogenic activity	Comparable to TG229/230	Aoki *et al*., 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 *et al.*, 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 *et al.*, 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 & *et al.*, 2006
zebrafish	Adult male, 30d, 11, 113, 1133 µg/L	E2, ↔; T, ↑; testis histology changes, changes in genes of signalling	Effects on testis histology due to disruption of signalling		Chen *et al.*, 2020, 2021

		pathways	pathways		
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 *et al.*, 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 *et al.*, 2021
zebrafish	Adult, 45d; 100, 500 µg/L	VTG, ↓; testis histology change; fecundity↔	Reproductive toxicity indicative of PPAR-ER cross talk		Xu *et al.*, 2014
zebrafish	embryos, 35d, 25, 100 µg/L	CYP19a2, ↓ at 100 µg/L; VTG, ↑;, no histology change	EAS activity was reported		Ortiz-Zarragoitia & *et al.*, 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 *et al.*, 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 *et al.*, 2013
murray rainbowfish	Juvenile fish at 30 dph, 90d, 5, 15, 50 µg/L	Feminiszation; E2/11-KT, ↑; at 5 µg/L	estrogenic effects on sex ratio	Comparable to TG234	Bhatia *et al.*, 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	DBP induced reproductive toxicity, leading to a decreased fecundity in F0 and a reduced hatching,	Full life study	Hu *et al.*, 2020

		VTG and CYP19a and b, ↓; at top two groups in females Hatching, ↓; survival, ↓;growth, ↓; in F1	survival rate, and growth in the F1 generation via EAS pathways.		

5.2.4 Amphibian toxicity tests for EAS activity and adversity

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

The following studies were already included in the previous proposal made in 2014 (ECHA, 2014):

Ohtani et al. (2000): To examine the effects of dibutyl phthalate (DBP) on gonadal sex differentiation, genetically male tadpoles of *Rana rugosa* were exposed to dilute solutions of DBP at nominal concentrations of 0.1, 1, or 10 µM (27.8, 278 and 2780 µg/l respectively) (N=50) during days 19-23 after fertilization, which is the critical period of gonadal sex differentiation in *R. rugosa*. Tadpoles were necropsied on day 40. The genetically male tadpoles were produced from crossings between males (ZZ) of one local population, in which females are the heterogametic sex, and females (XX) of another local population, in which males are the heterogametic sex. As positive control groups, tadpoles were exposed to dilute solutions of 17β-estradiol (E2) at concentrations of 0.01, 0.1, or 1 µM during the same period.

The internal structure of the gonads was histologically examined in a total of 30 control tadpoles, 86 E2-treated tadpoles, and 90 DBP-treated tadpoles. The gonads of the control tadpoles all showed the typical structure of testes. In contrast, 0.01, 0.1, and 1 pM E2 treatments caused the undifferentiated gonads of 18, 63, and 100% of the tadpoles, respectively, to develop into gonads of complete or partial ovarian structure. After 0.1, 1, and 10 µM DBP treatment, 0, 7, and 17% of tadpoles, respectively, were similarly affected. These findings suggest that DBP was about 1,000-fold less potent than E2. Nevertheless, DBP disrupts the pathways of testicular differentiation in genetically male animals.

Lee et al. (2005a): Evaluated the effects of low concentrations of DBP on spermatogenesis in *Xenopus laevis*, the African clawed frog. Xenopus tadpoles were exposed to 0, 0.1, 0.5, 1.0, 5.0, or 10.0 ppm DBP, beginning at sexual differentiation (Nieuwkoop and Faber stage 52; 3 weeks of age) and continuing until 100% of controls metamorphosed (stage 66; 8 weeks of age). Upon necropsy at 33 weeks, 4–6% of DBP treated frogs had only one testis, and 2–4% had retained oviducts. In all DBP treatment groups, seminiferous tubule diameter and the average number of germ cell nests per tubule were lower, and the number of tubules with no germ cells was significantly higher ($p < 0.05$). The percent of secondary spermatogonial cell nests significantly decreased ($p < 0.05$) in 1.0, 5.0, and 10.0 ppm groups. Several lesions occurred in DBP-exposed testes including denudation of germ cells, vacuolization of Sertoli cell cytoplasm, thickening of lamina propria of seminiferous tubules, and focal lymphocytic infiltration. Entire sections of testes containing almost exclusively mature spermatozoa were found in 1.0, 5.0, and 10.0 ppm DBP-exposed testes, indicating impairment of spermiation. Testicular hypoplasia and seminiferous tubular dysgenesis were also evident in DBP-treated frogs. Thus, subchronic exposure to low concentrations of DBP impairs spermatogenesis in frogs.

5.2.5 Rodent toxicity tests for EAS activity and adversity

Several rodent studies, included in the supporting document to the opinion of MSC for identification of DBP as ED for human health, have demonstrated an endocrine mode of action *in vivo*, which is substantiated by mechanistic data from *in vitro* studies. These studies showed decreased testosterone levels, indicating an anti-androgenic mode of action of DBP due to effects on steroidogenesis. It is biologically highly plausible that the suggested anti-androgenic mode of action gives rise to the adverse reproductive effects of DBP, such as a decrease in mating, fertility, and pregnancy rates (ECHA, 2014). This is further substantiated by a study of Mahood *et al*. (2007).

Mahood et al. (2007) evaluated fetal and adult end points in Wistar rats after in utero DBP exposure of 0, 4, 20, 100, or 500 mg/kg from gestation day 13.5 to 20.5 or 21.5 through maternal oral gavage. A dose dependent decrease in the fertility of the male offspring was noted starting at the 20 mg/kg dose when offspring were housed for one week with proven fertile females. At 500 mg/kg, infertility was statistically significant. Ninety percent of the animals exposed to 500 mg/kg DBP showed cryptorchidism, the absence of one or both testes from the scrotum, and a significant decrease in testicular weight at gestation day 21.5 and adulthood. Testicular testosterone levels were significantly decreased in gestation day 21.5 animals with 100 and 500 mg/kg exposures. In gestation day 21.5 testis sections, the authors noted an increase in occurrence of multinucleated gonocytes starting at 20 mg/kg, with significance achieved at 100 mg/kg, a decrease in Leydig cell number, and an increase in Leydig cell size at the 100 and 500 mg/kg doses. These fetal endpoints suggest abnormal development of the testis. Focal dysgenesis in adult rats was statistically significant at 500 mg/kg dose, although focal dysgenesis was also noted with the 100 mg/kg dose. Focal dysgenesis was defined as malformed seminiferous tubules with intratubular Leydig cells and immature sertoli cells in testis with no other malformations.

5.2.6 *In vitro* information indicative of thyroid activity

The following studies were already included in the previous proposal made in 2014 (ECHA, 2014):

Sugiyama et al. (2005): The authors developed a thyroid hormone (TH) inducible primary screening assay for the identification and assessment of chemicals that interfere with the TH signalling pathway within target cells. The assay was developed in a *Xenopus laevis* cell line that was transduced with a self-inactivating (SIN) lentivirus vector (LV) containing a luciferase gene. The luciferase activation in this cell line was TH-specific: 3,3´,5-L-triiodothyronine (T3) > 3,3´5-L-triiodothyroacetic acid (Triac) > 3,3´,5-D-triiodothyronine (D-T3), > L-thyroxine (T4) > 3,3´,5´-L-triiodothyronine (rT3). The application of the ligand-dependent luciferase assay for screening for thyroid system-disrupting chemicals revealed that three phthalates (dicyclohexyl phthalate, n-butylbenzyl phthalate, and di-n-butyl phthalate), two herbicides (ioxynil and pentachlorophenol) and a miticide (dicofol) had 3,3´,5-L-triiodothyronine- T3- antagonist activity at concentrations ranging from 10-6 to 10-5 M. These chemicals also inhibited the expression of the endogenous primary T3-response TH nuclear receptor b (TRb) gene. The inhibitory characteristics of these chemicals were similar for both assays performed, although the assay for T3-dependent activation of TRb gene was more sensitive than the luciferase assay. These results indicate that the luciferase assay was a rapid method with a small intra-assay variation for the primary screening of thyroid system-disrupting chemicals. Of the six chemicals, only n-butylbenzyl phthalate and pentachlorophenol exhibited T3- antagonist activity in an *in vivo* metamorphosis-based assay after 5 days exposure (T3- dependent activation of TRb gene in T3- induced metamorphosing tadpoles). It should be noted that chemicals elicited thyroid system-disrupting activity in the luciferase assay did not always interfere with the thyroid system *in vivo*.

Shimada & Yamauchi. (2004): The authors characterized the 3,5,3´-L-triiodothyronine (T3)-uptake system on the plasma membrane of *Rana catesbeiana* tadpole red blood cells (RBCs) in the presence of a variety of inhibitors and potentially competing amino acids. To

investigate the effect of endocrine-disrupting chemicals (EDCs) on [125I]T3 uptake, RBCs were incubated with [125I]T3 in the presence of each chemical. Among the test chemicals, di-n-butyl phthalate, n-butylbenzyl phthalate and the miticide, dicofol, were the most powerful inhibitors of [125I]T3 uptake, with an IC50 of 2•2 μM, which was one order of magnitude greater than that for T3 (IC50, 0•14 μM). The results raise the possibility that the T3-uptake system on the plasma membrane of the tadpole RBCs could be a candidate target site for some EDCs and can modulate cellular T3 response.

Shen et al. (2011). In agreen monkey kidney fibroblast (CV-1) cell line transiently transfected with SMRT and hTRβ, DBP and MBP enhanced the interactions between co-repressor SMRT (silencing mediator for retinoid and thyroid hormone receptors) and TRβ in a dose-dependent manner.

5.2.7 Amphibian toxicity tests for thyroid activity and adversity

The following first two studies were already included in the previous proposal made in 2014 (ECHA, 2014):

Shen et al. (2011). Nieuwkoop and Faber stage 51 *Xenopus laevis* (N=20) were exposed to DBP and MBP (2, 10 or 15 mg/L nominal, DMSO as solvent) separately for 21 days. The two test chemicals decelerated spontaneous metamorphosis in X. laevis at concentrations of 10 and 15 mg/L. Moreover, MBP seemed to possess stronger activity. The effects of DBP and MBP on inducing changes of expression of selected thyroid hormone response genes: thyroid hormone receptor-beta (TRb), retinoid X receptor gamma (RXRc), alpha and beta subunits of thyroid-stimulating hormone (TSHa and TSHb) were detected by qPCR at all concentratios of the compounds.

Lee et al. (2005b): this study was undertaken to investigate the effects of environmentally relevant concentrations of DBP on development in Xenopus laevis African clawed frogs. Developmental effects of DBP on Xenopus embryos were determined using the 96-h frog embryo teratogenesis assay–Xenopus (FETAX). Embryos (n = 300/group) were exposed from gastrulation (stage 8–11) through primary organogenesis (stage 46) to 0.1, 0.5, 1, 5, 10, or 15 ppm DBP (nominal concentrations) dissolved in 0.01% dimethyl sulfoxide (DMSO), vehicle alone (0.01% DMSO; solvent control), or FETAX culture medium only (control; n = 600). At 96 h, mortalities for control, solvent control, and 0.1, 0.5, 1, 5, 10, and 15 ppm DBP were 5, 4, 6, 5, 5, 9, 18, and 52%, respectively; the incidence of developmental malformations in the surviving tadpoles was 7, 9, 15, 37, 51, 53, 90, and 100%. The average length of embryos was significantly lower in all DBP treatment groups. Thus, DBP significantly affected development of Xenopus embryos at low, environmentally relevant concentrations.

The following study was found in the additional literature search:

Kamel et al (2022) investigated the potential effects of DBP on the hypothalamic-pituitary thyroid axis of *Xenopus laevis* using a 21-day Amphibian Metamorphosis Assay (AMA). *Xenopus laevis* larvae at NF stage 51 were exposed at mean measured concentrations of 4.76, 13.4, 46.0, and 143 μg/L. Endpoints included mortality, developmental stage, hind limb length (HLL), snout-vent length (SVL), body weight (BW), and thyroid histopathology. An increase in developmental stage (i.e., accelerated metamorphic development) frequency distribution was observed at 143 μg/L. HLLs were significantly greater than the control on day 7 for the 46 and 143 μg/L treatments and day 21 for the 13.4, 46, and 143 μg/L treatments. Normalized HLL in the 146 μg/L DBP treatment was significantly greater than the control on day 21. Exposure to DBP was associated statistically with increased frequencies of mild follicular cell hypertrophy in the 13.4, 46.0, and 143 μg/L treatment groups, and an increased frequency of moderate follicular cell hypertrophy in the 143 μg/L group (RSCABS $p < 0.05$). Conversely, the prevalence and severity of follicular cell hyperplasia were generally comparable between control and DBP-exposed frogs. SVLs were significantly greater than the control in the 13.4, 46.0, and 143 μg/L DBP treatments on day

7, on day 21 for NF stage ≤60 larvae, and on day 21 for NF stage >60. BWs measured in the 13.4, 46.0 and 143 µg/L DBP treatments on day 7 were significantly greater than the control. BWs for NF stage ≤60 specimens in the 13.4, 46.0 and 143 µg/L DBP treatment on day 21 were significantly greater than the control. BWs for NF stage >60 specimens in the 46.0 and 143 µg/L DBP treatment at day 21 were significantly greater than the control. The authors argued that this finding is suggestive of inhibited thyroid hormone feedback on the hypothalamus, which would lead to increased release of TSH. Elevated TSH release in turn causes the thyroid follicular epithelium to proliferate and become more metabolically active, resulting in hyperplasia and/or hypertrophy. Based on this study the authors concluded that DBP showed potential effects on amphibian metamorphosis and thyroid activity.

The authors argued that because Shen et al (2011) exposed the animals at much higher concentrations, there is a greater chance of inducing systemic toxicity, which could induce developmental delay. While Shen et al. (2011) did not specifically investigate systemic toxicity, it is supported at the highest concentration (15 mg/L) where there was a substantially significant decrease in whole body length compared to controls. They also mentioned that in a preliminary 4-day range finding experiment, performed for this study 100% mortality in NF stage 51 larvae was observed at both 10 and 1 mg/L.

5.2.8 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 ERα and ERβ, 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).

Several rodent studies have demonstrated an endocrine mode of action *in vivo*, which is substantiated by mechanistic data from *in vitro* studies. These studies showed decreased testosterone levels, indicating an anti-androgenic mode of action of DBP due to effects on steroidogenesis. It is biologically highly plausible that the suggested anti-androgenic mode of action gives rise to the adverse reproductive effects of DBP, such as a decrease in mating, fertility, and pregnancy rates (ECHA, 2014). This is further substantiated by a study of Mahood *et al*. (2007), who found after in utero exposure a dose dependent decrease in fertility of male offspring rats. Similarly, DBP can disturb testicular differentiation and impaired spermatogenesis of amphibians which may be plausibly linked to the estrogenic or anti-androgenic activity.

Several studies provided evidence that DBP also has thyroid related activity in amphibians of which Kamel *et al*. (2022) provided evidence that DBP affects the thyroid axis leading to an accelerated metamorphic development.

5.2.9 Conclusion regarding ED properties relevant to the environment

5.2.9.1 Adverse effects relevant to the ED identification

Twelve fish 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.

Mammalian studies showed a decrease in mating, fertility, and pregnancy rates (ECHA, 2014). These adverse effects are generally regarded as endpoints of particular relevance because such effects are likely to manifest themselves at the population level. The effects observed in rats are of particular concern for wildlife species with a natural low reproductive output, including top predators and other mammals (including endangered species) as negative effects on reproduction has an even higher potential for causing long term negative effect at the population level for such taxa.

DBP can also disturb testicular differentiation and impaired spermatogenesis and affect metamorphosis of amphibians.

5.2.9.2 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; Jarmołowicz *et al.*, 2013; Bhatia *et al.*, 2014b; Hu *et al.*, 2020).

Several studies provided evidence that DBP also has estrogenic or antiandrogenic activity as well as thyroid related activity in amphibians.

5.2.10.3. 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. Changes in testicular differentiation and spermatogenesis may be plausibly linked to the estrogenic or anti-androgenic activity of DBP.

Several rodent studies have demonstrated an endocrine mode of action *in vivo*, which is substantiated by mechanistic data from *in vitro* studies. Several of the studies showed decreased testosterone levels, indicating an anti-androgenic mode of action of DBP due to effects on steroidogenesis. It is biologically highly plausible that the suggested anti-androgenic mode of action gives rise to the adverse reproductive effects of DBP, such as a decrease in mating, fertility, and pregnancy rates (ECHA, 2014).

There is evidence that DBP affects the thyroid axis leading to an accelerated metamorphic development.

5.2.9.4 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. The adverse effects on reproduction observed in rodent species are also highly plausible related to an endocrine disrupting mode of action. In addition, there is also evidence that DBP can affect the thyroid axis leading to an accelerated metamorphic development. 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
sex differentiation	***In vitro evidence***	Changes in sex ratio can be	**Environmental relevance:**

female biased sex ratio was observed in murray rainbowfish and pikeperch exposed to DBP. ***Reproduction*** 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 Disruption of testicular differentiation and impaired spermatogenesis in amphibians Decrease in mating, fertility, and pregnancy rates in rodents Accelerated metamorphic development in amphibians	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 steorogenesis activity were reported in three studies. ***In vivo evidence*** Changes in VTG, E2, testosterone, 11-KT, spiggin, sex ratioand steroidogenesis gene expression e.g. CYP19A in murray rainbowfish, pikeperch, sticklebacks and zebrafish *In vitro* and *in vivo* evidence for thyroid related activity in amphibians	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. Changes in testicular differentiation and spermatogenesis can be plausible linked to the estrogenic or anti-androgenic activity. Evidence for effect on the thyroid axis leading to an accelerated metamorphic development	**effects on populations and generations** Sex ratio and fecundity are considered as population relevant endpoints. Impact on sex ratio, reproduction and survival of F1 generation are considered environmental relevance. The effects observed in rodents are of particular concern for wildlife species with a natural low reproductive output, including top predators and other mammals (including endangered species) as negative effects on reproduction has an even higher potential for causing long term negative effect at the population level for such taxa

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. There is also evidence that DBP changes in testicular differentiation and spermatogenesis via an estrogenic or anti-androgenic activity.

Several rodent studies have demonstrated an endocrine mode of action *in vivo*, which is substantiated by mechanistic data from *in vitro* studies. Several of the studies showed decreased testosterone levels, indicating an anti-androgenic mode of action of DBP due to effects on steroidogenesis. It is biologically highly plausible that the suggested anti-androgenic mode of action gives rise to the adverse reproductive effects of DBP, such as a decrease in mating, fertility, and pregnancy rates (ECHA, 2014).

Several studies provided evidence that DBP also has thyroid related activity in amphibians of which Kamel et al (2022) provided evidence that DBP affects the thyroid axis leading to an accelerated metamorphic development.

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 Disrupters 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.

Mammalian studies showed a decrease in mating, fertility, and pregnancy rates (ECHA, 2014). These adverse effects are generally regarded as endpoints of particular relevance because such effects are likely to manifest themselves at the population level. The effects observed in rats are of particular concern for wildlife species with a natural low reproductive output, including top predators and other mammals (including endangered species) as negative effects on reproduction haves an even higher potential for causing long term negative effect at the population level for such taxa.

DBP can disturb testicular differentiation and impaired spermatogenesis of amphibians and as such affect their reproductive success.

There is also evidence that DBP affects the thyroid axis leading to an accelerated metamorphic development.

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. DBP also causes adverse reproductive effects in rodents by inducing a decrease in mating, fertility, and pregnancy rates. In addition, it interferes with the HPT axis and accelerates metamorphic development of amphibians. Such 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

Phthalates including DBP have been detected in environmental samples (NIVA, 2019; 2021). 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 and including testing of endocrine mediated adverse effects 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 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. Mammalian studies showed a decrease in mating, fertility, and pregnancy rates. Overall, developmental and reproductive toxicity tests in rodents provide evidence that the adverse effects elicited by DBP are population relevant and can be used, in parallel to the non-mammalian data, to conclude that DBP is an environmental ED.

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. DBP can disturb testicular differentiation and impaired spermatogenesis of amphibians which may be plausibly linked to the estrogenic or anti-androgenic activity. There is also evidence that DBP affects the thyroid axis leading to an accelerated metamorphic development

In conclusion, the link between endocrine EATS activity and the observed adverse effects on sexual development, reproductive function and on offspring in fish, amphibians and mammals as well as on metamorphosis in amphibians 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, on mating, fertility, and pregnancy rates in rodents and on testicular differentiation and impaired spermatogenesis of amphibians and on metamorphosis in amphibians.

- It has EATS activity as clearly shown both *in vitro* and *in vivo*.

- The substance has an EATS mode of action, i.e., the adverse effects, including the recognised EATS-mediated effects on reproduction in rodents, sex ratio in fish and testicular differentiation and impaired spermatogenesis of amphibians and on metamorphosis in amphibians 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.

References

Adir M, Combelles CMH, Mansur A, Ophir L, Hourvitz A, Orvieto R, Dor J, Machtinger R. 2017. Dibutyl phthalate impairs steroidogenesis and a subset of LH-dependent genes in cultured human mural granulosa cell in vitro Reprod Toxicol. 2017 Apr;69:13-18. doi: 10.1016/j.reprotox.2016.12.007.

Aoki KAA, Harris CA, Katsiadaki I, Sumpter JP. 2011. Evidence that DI-n-Butyl Phthalate has antiandrogenic effects in fish. Environmental Toxicology and Chemistry, 30, No. 6, 1338–1345.

Bhatia H, Kumar A, Du J, Chapman J, McLaughlin MJ. 2013. Di-n-Butyl Phthalate causes antiestrogenic effects in female Murray Rainbowfish (*Melanotaenia fluviatilis*). Environmental Toxicology and Chemistry, 32, No. 10, 2335–2344.

Bhatia H, Kumar A, Ogino Y, Gregg A, Chapman J, McLaughlin MJ, Iguchi T. 2014a. Di-n-butyl phthalate causes estrogenic effects in adult male Murray rainbowfish (*Melanotaenia fluviatilis*). Aquat Toxicol. 149:103-15.

Bhatia H, Kumar A, Chapman JC, McLaughlin MJ. 2014b. Long-term exposures to di-n-butyl phthalate inhibit body growth and impair gonad development in juvenile Murray rainbowfish (*Melanotaenia fluviatilis*). J Appl Toxicol. 35:806-16.

Chen X, Zhou QH, Leng L, Chen X, Sun ZR, Tang NJ. 2013. Effects of di(n-butyl) and monobutyl phthalate on steroidogenesis pathways in the murine Leydig tumor cell line MLTC-1 Environ Toxicol Pharmacol. 2013 Sep;36(2):332-338. doi: 10.1016/j.etap.2013.04.013.

Chen P, Li S, Liu L, Xu N. 2015. Long-term effects of binary mixtures of 17α-ethinyl estradiol and dibutyl phthalate in a partial life-cycle test with zebrafish (*Danio rerio*). Environ Toxicol Chem. 34(3):518-26.

Chen H, Chen K, Qiu X, Xu H, Mao G, Zhao T, Feng W, Okeke ES, Wu X, Yang L. 2020. The reproductive toxicity and potential mechanisms of combined exposure to dibutyl phthalate and diisobutyl phthalate in male zebrafish (*Danio rerio*), Chemosphere. 2020 Nov;258:127238. doi: 10.1016/j.chemosphere.2020.127238.

Chen H, Feng W, Chen K, Qiu X, Xu H, Mao G, Zhao T, Ding Y, Wu X. 2021. Transcriptomic analysis reveals potential mechanisms of toxicity in a combined exposure to dibutyl phthalate and diisobutyl phthalate in zebrafish (*Danio rerio*) ovary**.** Aquat Toxicol. 2019 Nov;216:105290. doi: 10.1016/j.aquatox.2019.105290.

Czernych R, Chraniuk M, Zagożdżon P, Wolska L. 2017. Characterization of estrogenic and androgenic activity of phthalates by the XenoScreen YES/YAS in vitro assay Environ Toxicol Pharmacol. 2017 Jul;53:95-104. doi: 10.1016/j.etap.2017.05.010.

Dang Z.C. Audinot V., Papapoulos S. Boutin J.A., and Löwik C. Peroxisome proliferator-activated receptor γ (PPARγ) as a molecular target for the soy phytoestrogen genistein. *Journal of Biological Chemistry.* 2003, 278, 962-967.

Dang Z.C. and Löwik C. Dose-dependent effects of phytoestrogens on bone: molecular mechanisms, review paper, *Trends in Endocrinology and Metabolism.* 2005, 16, 207-213.

Dang Z.C. Dose-dependent effects of soy phyto-oestrogen genistein on adipocytes: mechanisms of action. *Obesity Review.* 2009, 10, 342-349.

EU RAR. 2004. European Chemicals Bureau (2004). European Union Risk Assessment Report. Dibutyl phthalate.with addendum 2004. Ehttp://echa.europa.eu/documents/10162/ba7f7c39- dab6-4dca-bc8e-dfab7ac53e37

ECHA. 2008 Member State Committee support document for identification as a substance of very high concern. https://echa.europa.eu/fi/registry-of-svhc-intentions/-/dislist/details/0b0236e180e4831b

ECHA. 2014. Proposal for identification of a substance of very high concern on the basis of the criteria set out in reach article 57-- Dibutyl phthalate (DBP).

ECHA. 2014 Support document to the opinion of the member state committee for identification of dibutyl phthalate

ECHA. 2017. Inclusion of substances of very high concern in the Candidate List for eventual inclusion in Annex XIV.

Harris CA, Henttu P, Parker MG, Sumpter JP. 1997. The estrogenic activity of phthalate esters in vitro Environ Health Perspect. 1997 Aug;105(8):802-11. doi: 10.1289/ehp.97105802.

Hu JX, Jiang K, Tang X, Liu H, Zhang H, Yang X, Nie X, Luo H. 2020. Chronic exposure to di-n-butyl phthalate causes reproductive toxicity in zebrafish. J Appl Toxicol. 2020 Dec;40(12):1694-1703. doi: 10.1002/jat.4030.

Hu J, Xia M, Wang Y, Tian F, Sun B, Yang M, Yang W, Ding X, Xu H, Li W. 2021. Paternal exposure to di-n-butyl-phthalate induced developmental toxicity in zebrafish (*Danio rerio*)Birth Defects Res. 2021 Jan 1;113(1):14-21. doi: 10.1002/bdr2.1812.

Jarmołowicz S, Demska-Zakęś K, Zakęś Z. 2013. Impact of di-n-butyl phthalate on reproductive system development in European pikeperch (*Sander lucioperca*). Acta Vet. Brno 82: 197–201.

Jobling S, Reynolds T, White R, Parker MG, Sumpter JP. 1995. A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. Environ. Health Perspect. 103(6), 582-587.

JRC. 2013. Key scientific issues relevant to the identification and characterisation of endocrine disrupting substances. Report of the ED Expert Advisory Group (ED EAG). Reference Report by Munn S, Goumenou M, Joint Research Centre of the European Commission. European Union 2013.

Källsten L, Pierozan P, Martin JW, Karlsson O. 2022. Di-n-Butyl Phthalate and Its Monoester Metabolite Impairs Steroid Hormone Biosynthesis in Human Cells: Mechanistic In Vitro Studies Cells. 2022 Sep 27;11(19):3029. doi: 10.3390/cells11193029.

Kamel A, Matten SR, Lynn SG, Wolf JC, Fort DJ. 2022. Amphibian Metamorphosis Assay: Investigation of the potential effects of five chemicals on the hypothalamic-pituitary thyroid axis of *Xenopus laevis*, Regulatory Toxicology and Pharmacology, 134, 105241.

Lee SK, Veeramachaneni DNR. 2005a. Subchronic Exposure to Low Concentrations of Di-n-Butyl Phthalate Disrupts Spermatogenesis in *Xenopus laevis* Frogs. Toxicological Sciences 84, 394–407.

Lee SK, Owens GA, Veeramachaneni DNR. 2005b. Exposure to Low Concentrations of Din-butyl Phthalate during Embryogenesis Reduces survivability and Impairs Development of Xenopus Laevis Frogs. Journal of Toxicology and Environmental Health, Part A, 68:763–772.

Li L, Chen X, Hu G, Wang S, Xu R, Zhu Q, Li X, Wang M, Lian QQ, Ge RS. 2017. Comparison of the Effects of Dibutyl and Monobutyl Phthalates on the Steroidogenesis of Rat Immature Leydig Cells Biomed Res Int. 2016;2016:1376526. doi: 10.1155/2016/1376526.

Mahood IK, Scott HM, Brown R, Hallmark M, Walker M, Sharpe RM. 2007. In utero Exposure to di(n-butyl) phthalate and testicular dysgenesis: Comparison of fetal and adult end points and their dose sensitivity. Environmental Health Prospectives , 115 (1), 55-61.

NIVA, 2019. Screening programme 2019: https://www.miljodirektoratet.no/sharepoint/downloaditem/?id=01FM3LD2WQPCTJHRCVKBB2IE6NPKE47T5L.

NIVA, 2021. Environmental Contaminants in an Urban Fjord: https://www.miljodirektoratet.no/publikasjoner/2023/januar-2023/environmental-contaminants-in-an-urban-fjord-2021

OECD (Organisation for Economic Co-operation and Development). 2011. Test No. 234: Fish Sexual Development Test. In: OECD Guidelines for the Testing of Chemicals, Section 2 OECD Publishing, Paris pp. https://doi.org/10.1787/9789264122369-en

Ohtani H, Miura I, Ichikawa Y. 2000. Effects of Dibutyl Phthalate as an Environmental Endocrine Disruptor on Gonadal Sex Differentiation of Genetic Males of the Frog *Rana rugosa*. Environmental Health Perspectives, Vol. 108, No. 12. 1189-1193

Ortiz-Zarragoitia M, Trant JM, Cajaraville MP. 2006. Effects of Dibutylphthalate and Ethynylestradiol on liver peroxisomes, reproduction and development of zebrafish. Environmental Toxicology and Chemistry, Vol. 25, No. 9, 2394–2404.

Rider CV, Hartig PC, Cardon MC, Wilson VS. 2009. Comparison of chemical binding to recombinant fathead minnow and human estrogen receptors alpha in whole cell and cell-free binding assays Environ Toxicol Chem. 2009 Oct;28(10):2175-81.

Shen O, Du G, Sun H, Wu W, Jiang Y, Song L, Wang X. 2009. Comparison of in vitro hormone activities of selected phthalates using reporter gene assays. Toxicol Lett. 2009 Dec 1;191(1):9-14. doi: 10.1016/j.toxlet.2009.07.019.

Shen O, Wu W, Du G, Liu R, Yu L, Sun H, Han X, Jiang Y, Shi W, Hu W, Song L, Xia Y, Wang S, Wang X. 2011. PLoS ONE. April 2011 | Volume 6 | Issue 4 | e19159.

Shimada N & Yamauchi K. 2004. Characteristics of 3,5,3-triiodothyronine (T3)-uptake system of tadpole red blood cells: effect of endocrine-disrupting chemicals on cellular T3 response. Journal of Endocrinology 183, 627–637.

Sugiyama S-I, Shimada N, Miyoshi H, Yamauchi K. Detection of Thyroid System Disrupting Chemicals Using in Vitro and in Vivo Screening Assays in Xenopus laevis. Toxicological Sciences 88(2), 367–374 (2005)

Tuan Tran H, Lin C, Bui XT, Ky Nguyen M, Dan Thanh Cao N, Mukhtar H, Giang Hoang H, Varjani S, Hao Ngo H, Nghiem LD. 2022. Phthalates in the environment: characteristics, fate and transport, and advanced wastewater treatment technologies. Bioresour Technol. 2022 Jan;344(Pt B):126249. doi: 10.1016/j.biortech.2021.126249. Epub 2021 Oct 31. PMID: 34732372.

WHO/International Programme on Chemical Safety. Global assessment of the state-of-the- science of endocrine disruptors – 2002 (Damstra T, Barlow S, Bergman A, Kavlock R, Van Der Kraak G, eds.). http://www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en/

Xu N, Chen P, Liu L, Zeng Y, Zhou H, Li S. 2014. Effects of combined exposure to 17α-ethynylestradiol and dibutyl phthalate on the growth and reproduction of adult male zebrafish (*Danio rerio*). Ecotoxicol Environ Saf. 107:61-70.