Substance Name: Dibutyl phthalate (DBP)

EC Number: 201-557-4

CAS Number: 84-74-2

SUPPORT DOCUMENT TO THE OPINION OF THE MEMBER STATE COMMITTEE FOR IDENTIFICATION OF

DIBUTYL PHTHALATE (DBP)

AS A SUBSTANCE OF VERY HIGH CONCERN BECAUSE OF ITS ENDOCRINE DISRUPTING PROPERTIES WHICH CAUSE PROBABLE SERIOUS EFFECTS TO HUMAN HEALTH AND THE ENVIRONMENT WHICH GIVE RISE TO AN EQUIVALENT LEVEL OF CONCERN TO THOSE OF CMR[1] AND PBT/vPvB[2] SUBSTANCES

Adopted on 11 December 2014

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

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

CONTENTS

TABLES

Substance Name(s): Dibutyl phthalate (DBP)

EC Number(s): 201-557-4

CAS Number(s): 84-74-2

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

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

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

DBP has been shown to adversely affect the endocrine system of mammals primarily through *in vivo* findings on reduced fetal testosterone. These findings are further substantiated by mechanistic findings, also *in vivo*, of down-regulation of genes in the steroidogenic biosynthesis pathway. The spectrum of adverse effects observed in rats include increased nipple retention, decreased anogenital distance, genital malformations, reduced number of spermatocytes and testicular changes including multinucleated gonocytes, tubular atrophy and Leydig cell hyperplasia.

In relation to the environment, adverse effects concerning development and reproduction are generally regarded as endpoints of particular relevance because such effects are likely to manifest themselves at the population level. The effects observed in rats are of particular concern for 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.

Adverse effects caused by exposure to DBP have also been identified in non-mammalian wildlife where the sex ratio (sex reversal of male fish to female fish) was affected in fish. The plausible connection to the endocrine system was also confirmed in fish where the anti-androgenic MoA could be verified in an anti-androgenic specific assay in stickleback. Hence the current data indicates also in fish that DBP has endocrine disruptive properties leading to adverse effects related to sexual development and reproduction.

In conclusion, when available information from toxicological and ecotoxicological studies are combined, DBP can be considered an endocrine disruptor for both the environment and for human health 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.

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 development may cause irreversible developmental programming effects leading to severe effects on development and reproduction, regarded as particularly serious in relation to human health and wildlife species, also because these adverse effects may first manifest themselves in later life stages as a consequence of exposure during early life stages. 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 both for human health and for environmental species.

Registration dossiers submitted for the substance: Yes

JUSTIFICATION

1 IDENTITY OF THE SUBSTANCE AND PHYSICAL AND CHEMICAL PROPERTIES

1.1 Name and other identifiers of the substance

Table 1: Substance identity

EC number:	201-557-4
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 (typical concentration of DBP 80-100%w/w)

1.3 Physico-chemical properties

Table 2: Overview of physicochemical properties

Property	Value	IUCLID section	REACH ref Annex, §
Physical state at 20°C and 101.3 kPa	*Oily liquid*	3.1	VII, 7.1
Melting/freezing point	*-69C*	3.2	VII, 7.2
Boiling point	*340C at 10,013 hPa*	3.3	VII, 7.3
Vapour pressure	*9.7±3.3 x 10-5 hPa at 25C*	3.6	VII, 7.5
Water solubility	*10 mg/L at 20C*	3.8	VII, 7.7
Partition coefficient n-octanol/water (log value)	*log Kow 4.57 at 20C*	3.7	VII, 7.8
Dissociation constant		3.21	XI, 7.16

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

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	

Classification and labelling of DBP according to Annex VI, Table 3.2 of Regulation (EC) No 1272/2008 (The list of harmonized classification and labelling of hazardous substances from Annex I to Directive 67/548/EEC)

Index No	International Chemical Identification	EC No	CAS No	Classification	Labelling	Concentration limits
607-318-00-4	dibutyl phthalate; DBP	201-557-4	84-74-2	Repr. Cat. 2; R61 Repr. Cat. 3; R62 N; R50	T; N R: 61-50-62 S: 53-45-61	

3 ENVIRONMENTAL FATE PROPERTIES

Infomation 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).

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 contributes 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.m3/mol indicates that DBP will only slowly volatilize 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

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

The toxicokinetics as described in the EU RAR for human health is cited below (EU RAR 2004):

"Dibutylphthalate is rapidly absorbed and excreted after oral administration as was demonstrated in studies in laboratory animals. Up to more than 90% of oral doses given to rats or hamsters was excreted in urine within 24-48 hours. Fecal excretion is low (1.0-8.2%). Also in man oral absorption of DBP takes place. After dermal exposure of rats to DBP ca. 60% of the dose was excreted in urine within 7 days. In feces ca. 12% of the dose was found. An *in vitro* study revealed slower absorption of DBP by the human skin (2.40 µg/cm2/hr) than by the rat skin (93.35 µg/cm2/hr). Data on absorption after exposure by inhalation are not available. A substantial fraction of DBP is initially excreted in the bile and subsequently enters the enterohepatic circulation.

No significant accumulation in tissues was observed in laboratory animals after oral as well as dermal exposure; limited inhalation data revealed an indication for some accumulation in tissues. The major part of DBP is hydrolysed to mono-n-butyl phthalate (MBP) and the corresponding alcohol prior to absorption by the small intestines, but hydrolysis can also occur in liver and kidneys. The metabolites that occur in urine are MBP, MBP-glucuronide, various ω- and ω-1- oxidation products of MBP (more polar ketones, carboxylates) and a small amount of free phthalic acid. Species differences in the excretion of MBP and its glucuronide were observed; rats excreted a larger proportion unconjugated MBP in urine than hamsters.

There are no data on biotransformation after dermal exposure and exposure by inhalation. Transplacental transfer of DBP and its metabolites was demonstrated in an oral study with 14C-labelled DBP in rats. Radioactivity in embryonic tissues contained less than 0.12-0.15% of the administered dose. MBP accounted for most of the radioactivity in maternal plasma, placenta and embryo. Unchanged DBP was found in only small amounts. No accumulation of radioactivity was seen in maternal or embryonic tissues."

4.2 Other effects: Endocrine disruption

4.2.1 General approach – Human Health

Criteria on how to assess whether or not a substance has endocrine disrupting properties and/or is an endocrine disruptor are currently being developed in the European Union. The timeline for the finalization of the process is not currently known.

The basis for the criteria is envisaged to be the widely accepted definition of an endocrine disruptor by the WHO/IPCS (WHO/International Programme on Chemical Safety 2002):

An endocrine disruptor is an exogenous substance or mixture that

1) alters function(s) of the endocrine system and

2) consequently causes

3) adverse health effects in an intact organism, or its progeny, or (sub)populations.

The European Commission's Endocrine Disrupters Expert Advisory group agreed in 2013 "that the elements for identification of an endocrine disrupter were demonstration of an adverse

effect for which there was convincing evidence of a biologically plausible causal link to an endocrine disrupting mode of action and for which disruption of the endocrine system was not a secondary consequence of other non-endocrine-mediated systemic toxicity. Relevance of the data to humans should be assumed in the absence of appropriate data demonstrating non-relevance." (JRC 2013)

As it is assumed in this report that a substance should fulfil these definitions in order to be identified as an endocrine disruptor, available information is assessed based on the following topics:

1) Adverse health effects
2) Endocrine mode of action
3) Plausible link between adverse effects and endocrine mode of action
4) Human relevance

The most marked adverse effects of DBP have been described for male reproductive system and most work performed to elucidate the mode of action of DBP has been carried out in experimental tests studying developing male rats. The following discussion therefore focuses on adverse effects on male reproduction induced by inhibition of steroid synthesis in fetal testis. DBP may also have other endocrine disrupting modes of action. Although data on these modes of action are sparse, data on estrogenic action will be discussed briefly here to give a complete overview of the possible endocrine disrupting modes of action of DBP.

4.2.2 Adverse health effects – Analysis of available information from rodent studies

a) Background

DBP is classified as a substance toxic to reproduction (Repr. 1B, H360Df) based on evidence of adverse effects on the reproductive organs in developing rodents. The spectrum of adverse effects observed in rats include increased nipple retention, decreased anogenital distance, genital malformations, reduced number of spermatocytes and testicular changes including multinucleated gonocytes, tubular atrophy and Leydig cell hyperplasia of which almost all are considered adverse (OECD 2008). The evidence of reproductive toxicity indicative of an anti-androgenic endocrine disrupting mode of action was described as follows in the EU risk assessment report from 2004 (EU RAR, 2004):

"*In several recent developmental studies in rats delayed preputial separation and a markedly disturbed development of the male reproductive tract (internal and external) of rat offspring exposed via their mothers during gestation or during gestation and lactation, was observed at oral doses ≥ 250 mg/kg bw. Maternal toxicity was seen at oral doses ≥500 mg/kg bw. In female offspring sporadic cases of reproductive tract malformations were observed at doses ≥ 250 mg/kg bw. Age at vaginal opening and estrus cyclicity were not affected. At the lowest oral dose level of 100 mg DBP/kg bw, studied in developmental studies in rats, still delayed preputial separation in male progeny was seen. The results of these studies indicate that DBP does not possess estrogenic activity but rather shows antiandrogenic activity.*" (EU RAR, 2004).

Here, "antiandrogenic activity" is used to describe a reduced activation of the androgen receptor. For some chemicals, this is achieved by antagonism of the androgen receptor, but for DBP and other phthalates the reduced activation of the androgen receptor is caused by interference with steroid hormone synthesis, as will be discussed below. In addition to the study by Ema et al. 1998,

No detailed evaluation of endocrine mode of action was included in the EU risk assessment report (EU RAR, 2004) except for the conclusion: "*In some special in vitro assays DBP showed weak estrogenic activity. The estrogenic effects were not confirmed in in vivo studies.*

Therefore the relevance of the effects observed in vitro for the in vivo estrogenic activity of DBP is questionable." (EU RAR, 2004, p. 120). One study included in the EU risk assessment report (EU RAR, 2004) found decreased ano-genital distance in male pups (Ema et al. 1998). Decreased ano-genital distance in male pups is generally known to be an androgen dependent endpoint and decreased anogenital distance is associated with an anti-androgenic mode of action (Bowman et al. 2003), and the findings by Ema et al. (1998) thus strengthens the hypothesis of DBP as an endocrine disruptor.

The reproductive toxicity of DBP was thus evaluated to be likely induced via an endocrine disrupting mode of action. This conclusion is further substantiated by studies carried out after the publication of the EU risk assessment report for DBP (see below).

An overview of the key studies on effects of DBP on reproduction and development were given in the EU risk assessment report for DBP (EU RAR, 2004) and are presented in the table in Annex 1 to this report. These studies are considered reliable (i.e. in most cases with a Klimisch score 1 or 2). Detailed study summaries can be found in the EU risk assessment report.

b) Adverse effects indicative of endocrine disruption

Several studies on reproductive and endocrine effects of DBP *in vivo* have been published since data was collected for the EU risk assessment report. Key studies showing adverse effects and/or showing an in vivo endocrine mode of action of DBP are summarized in table 1 below.

All studies included in table 3 are generally evaluated as reliable (Klimisch score 1 or 2). The reliability of a few of these studies are evaluated as somewhat limited, because they use a rather low number of animals and only one dose level (Barlow et al. 2003a and b; Kleymenova et al. 2005; Kwack et al., 2009; Wilson et al. 2004), but these studies have anyway been included in the overview table because the findings of these studies in general are accordance with the more comprehensive studies shown in the table and hence can be used as supportive evidence. Overall, the dataset is evaluated as very reliable due to the consistency of the findings with regards to both the adverse effects and the mode of action.

Table 3 Summary of studies *in vivo* showing adverse effects and/or showing an *in vivo* endocrine mode of action.

Study design	**Effects**	**Reference**
Pregnant rats, dietary exposure GD 15 to PND 21. According to the authors and the EFSA opinion, 20 mg/kg feed corresponds to 1.5 to 3.0 mg/kg bw/day.	Details of a study by Lee et al. (2004) were found in the EFSA opinion on DBP from 2005 (citation in *italics*): *In a recent developmental toxicity study (Lee et al., 2004) with exposure during the period from late gestation (Gestational day 15) to the end of lactation on postnatal day 21 (PND 21), maternal rats were given DBP at dietary concentrations of 0, 20, 200, 2000 and 10000 mg/kg. Major results of this study are summarised below. At PND 2, anogenital distance was significantly reduced in 10000 mg/kg male offspring. At PND 14, the incidence of retained nipples/areolae was increased in all treated male offspring compared with controls but the increase was only significant at 10000 mg/kg. At PND 21, in males, reduction of spermatocyte development as manifested by a decreased number of spermatocytes was observed from 20 mg/kg with dose-dependent increased incidence or/and severity. A significant increase in scattered foci of aggregated Leydig cells was observed at 2000 mg/kg and 10000 mg/kg. In the epididymis, significantly decreased ductular cross sections, indicating reduced coiling, were observed at 2000 and 10000 mg/kg. In the mammary glands, dilatation of alveolar buds and/or ducts was seen in male offspring from 20 mg/kg with low incidence but not achieving statistical significance in any group. In female offspring, hypoplasia of the alveolar buds of the mammary glands was observed in animals from 20 mg/kg with a statistically significant increase at 20, 200, 2000 and 10000 mg/kg (P<0.05). At postnatal week 11 (PNW 11), in males, loss of germ cell development was significant at 2000 mg/kg and above. This lesion differed markedly in severity between animals.*	Lee et al., 2004

Study design	Effects	Reference
	Significant increases in vacuolar degeneration in the mammary glands of males was present from 20 mg/kg but with similar incidence and qualitative gradation of change across the dose groups (End of citation from EFSA 2005).	
Pregnant rats, gavage GD 12 to 21	This study examined the pathogenesis of testicular and epididymal effects of prenatal DBP exposure of rats from fetal life to adulthood at a dose of 500 mg/kg bw/day. Male offspring was examined at GD 16 to 21 and on PND 3, 7, 16, 21, 45 and 70. In the fetal testes, large aggregates of Leydig cells, multinucleated gonocytes, and increased numbers of gonocytes were detected on GD 17 to 21. These lesions resolved during the early postnatal period, while decreased numbers of spermatocytes were noted on PND 16 and 21. The testicular effects of DBP observed in late gestation (multinucleated gonocytes, increased numbers of gonocytes and clustering of Leydig cells) thus resolved, while a different set of morphological alterations appeared later on (focal dysgenesis in the seminiferous epithelium in some testes and general degeneration in others). Fetal epididymides of DBP-exposed animals exhibited decreased coiling of ducts - an effect, which progressed to malformation and degeneration in adulthood. The severity of testicular degeneration progressed with age and was suggested to result from the progressing epididymal lesions causing obstruction of flow from the testes.	Barlow et al., 2003
Pregnant rats, gavage GD 12 to 19	Pregnant Sprague-Dawley rats were gavaged with vehicle or 500 mg/kg bw/day of DBP from GD 12 to 19, and testicular expression of genes related to steroid synthesis and androgen signaling were examined. DBP reduced the expression of several genes in the androgen synthesis pathway including SRB1, StAR, P450scc, 3beta-HSD, P450c17, and c-kit, while expression of TRPM-2 was upregulated. In Leydig cells, reduced presence of lipid vacuoles in testes of DBP exposed fetuses, and immunohistochemical staining for SRB1 and StAR proteins supported the finding of downregulation of the genes for these proteins. Immunohistochemical staining also showed reduced staining for c-kit in gonocytes and increased staining for TRPM-2 in Sertoli cells. The authors concluded that this study confirmed the gene expression changes found by Shultz et al. (2001) and found that the data correlated with observations of decreased testosterone synthesis by fetal Feydig cells following DBP exposure.	Barlow et al., 2003
Pregnant rats, gavage from PND 24, through mating and pregnancy until gestation day 13	24-day old rats were exposed to 0, 250, 500 or 1000 mg/kg bw/day of DBP, i.e. from weaning through puberty, mating and gestation. At doses of 500 and 1000 mg/kg bw/day female fertility and ovarian function was impaired.	Gray et al., 2006
Pregnant rats, gavage GD 8 to 18	DBP decreased fetal testosterone production in rats at doses from 300 mg/kg bw/day (NOAEL 100 mg/kg bw/day). In this study, pregnant Sprague-Dawley rats were exposed to 33, 50, 100, 300, or 600 mg/kg bw/day of DBP from GD 8 to 18 by gavage in corn oil (n=3 to 4 dams per group). Testicular testosterone production ex vivo was assessed by incubation of testes of 18 day old fetuses for 3 hours and testosterone measurement in the media. Dose-related decreases in testosterone production was seen for BBP and the other tested phthalates (DIBP, BBP, and DEHP) from 300 mg/kg bw/day and above, and for DPP (dipentyl phthalate) from 100 mg/kg bw/day.	Howdeshell et al., 2008
Male rats, 4 weeks gavage	Groups of 6 SD rats were exposed to 500 mg/kg bw/day of DBP (or several other phthalates) daily for 4 weeks. Control rats received corn oil. Body weight gain was reduced and relative liver weights were increased with DBP (and other phthalates), but no changes in other organ weights were seen for DBP (testis weight decreased by DEHP and	Kwack et al., 2009

Study design	Effects	Reference
	MEHP). Sperm counts were lowered to 50% by DBP, whereas e.g. DEHP decreased sperm counts to 34%. Sperm motility (%) was significantly reduced by DBP and other phthalates. Other measures of sperm motility (types of velocity and straightness) were affected by DBP and other examined phthalates. Overall, DBP appears to affect sperm quality in a similar manner as e.g. DEHP and BBP.	
Pregnant rats, gavage GD 12 to GD16-20	Fetal exposure to 500 mg/kg bw/day of DBP reduced testosterone levels and increased the prevalence of Leydig cell aggregates and multinucleated gonocytes in fetal testis. Altered Sertoli cell structure (retracted apical processes, altered organization of the vimentin cytoskeleton and abnormal cell-cell contacts with gonocytes) was seen in DBP exposed animals. These effects were reversed after birth	Kleymenova et al. 2005
Pregnant rats, gavage GD 12 to 19	Decreased fetal testosterone concentration was seen in rats exposed to DBP at doses from 50 mg/kg bw/day with a NOAEL of 10 mg/kg bw/day. In that study, pregnant Sprague-Dawley rats were exposed to 0, 0.1, 1, 10, 50, 100, or 500 mg/kg bw/day of DBP from GD 12 to 19 by gavage in corn oil (n=1 to 4 litters per group, analysis of testosterone in 3-4 males per litter). Testosterone concentration was measured in testes of 19 day old fetuses	Lehmann et al., 2004
Pregnant rats, gavage GD 12 to 21	Dffects DBP on steroid hormone production and expression of steroidogenesis related genes were examined in fetal rats exposed orally to 500 mg/kg bw/day from gestation day 12 to 21. Male fetal testes were examined on GD 16, 19 and 21. Reduced testosterone concentration was seen in DBP exposed fetuses at GD 19 and 21; reduced testicular expression of steroidogenesis related genes P450scc and P450c17 was seen at GD 19, and reduced testicular expression of StAR and SR-B1 were seen at all examined ages.	Shultz et al., 2001
Pregnant rats, gavage GD 14 to 18	Effects of DEHP, DBP and BBP on steroid hormone production and insl3 gene expression were examined in fetal rats exposed orally from gestation day 14 to 18. Reduced testosterone production and insl3 gene expression was seen in males at GD 18.	Wilson et al., 2004

In summary, several rodent studies have demonstrated adverse reproductive effects of DBP (Ema et al., 1998; Gray et al., 1999; Kwack et al., 2009; Lee et al., 2004; Mylchreest et al., 1998, 1999; Zhang et al., 2004). Most well-described are the effects on the male reproductive system, e.g. increased nipple retention, decreased anogenital distance, reduced number of spermatocytes, changes in epididymis and testicular changes, including multinucleated gonocytes and aggregated Leydig cells (Table 1 and Annex 1). It is well-known that this type of effects can be induced via endocrine disrupting modes of action. Chemicals acting as androgen receptor antagonists can induce comparable effects (Wolf et al., 1999), but in the case of phthalates it is highly plausible that interference with steroid hormone synthesis in fetal testis in responsible for the anti-androgenic effects.

The female reproductive system and the thyroid hormone system may also be affected by DBP. Endocrine disruption is related to adverse effects in males as well as females and increasing attention to effects on the female reproductive system as well as improved methods for detection of effects in females has led to a still growing number of findings of adverse effects of phthalates in females. For DBP, few studies describing effects on female reproduction can be found in the literature. In a study on DBP exposure from weaning through puberty, mating and gestation, female fertility and ovarian function was affected by DBP, and the authors concluded that "these results should be considered when evaluating mechanisms of reproductive toxicity for the PE [phthalate esters, ed.] because it is likely that these reproductive alterations in the female rat arise via a mode of action similar to that operative in male rats" (Gray et al., 2006). Moreover, DBP has been found to interact with the thyroid hormone receptor *in vitro* (Ghisari and Bonefeld-Jorgensen 2009). However, the possible thyroid disrupting potential of DBP has not been assessed in this report.

In conclusion, several rodent studies have demonstrated adverse effects in intact organisms, especially on male reproductive development and adult male reproductive organs.

4.2.3 Endocrine mode of action

The studies in table 3 show adverse effects of DBP and/or an endocrine mode of action *in vivo*. Mode of action defined as effects on organ/tissue/organism/physiological level. The *in vivo* mode of action data show effects on steroidogenesis, e.g. decreased testosterone production, further substantiated by mechanistic *in vivo* data showing reduced expression of genes involved in steroidogenesis pathways. Mechanism of action defined as effects at the cellular/sub-cellular/organelle/biochemical level (genes, receptors, enzymes etc). Several studies on testosterone production and steroidogenesis in fetal male rats show an endocrine disrupting mode of action of DBP *in vivo* (Barlow et al. 2003, Howdeshell et al. 2008, Kleymenova et al. 2005, Lehmann et al. 2004, Shultz et al. 2001, Wilson et al. 2004). It is important to note that the initial events at the molecular level for DEHP and related phthalates are not known, but that there is strong weight of evidence for an anti-androgen mode of action related to decreased fetal testosterone production. Several target genes involved in the development and function of fetal Leydig and Sertoli cells have been identified and several studies have shown reduced expression of genes in the steroid biosynthesis pathway (Howdeshell et al., 2008; Wilson et al., 2004).

It should be noted that phthalates are absorbed as monoesters and/or rapidly metabolized to monoesters. Monoesters are transported across the placenta and reach the fetus (David 2006). Thus, it is the metabolites of phthalate diesters that are endocrine disrupting and mainly effects of metabolites are relevant.

Decreased fetal testosterone production is considered a key event in a cascade leading to adverse effects in the male reproductive system, as visualized by NRC, 2008. The reduction in testosterone production will decrease the activity of the androgen receptor in target tissues and interfere with androgen-mediated development. This will in turn lead to reproductive tract malformations including effects on anogenital distance, nipple retention, reproductive tract malformations and semen quality (Fig. 1).

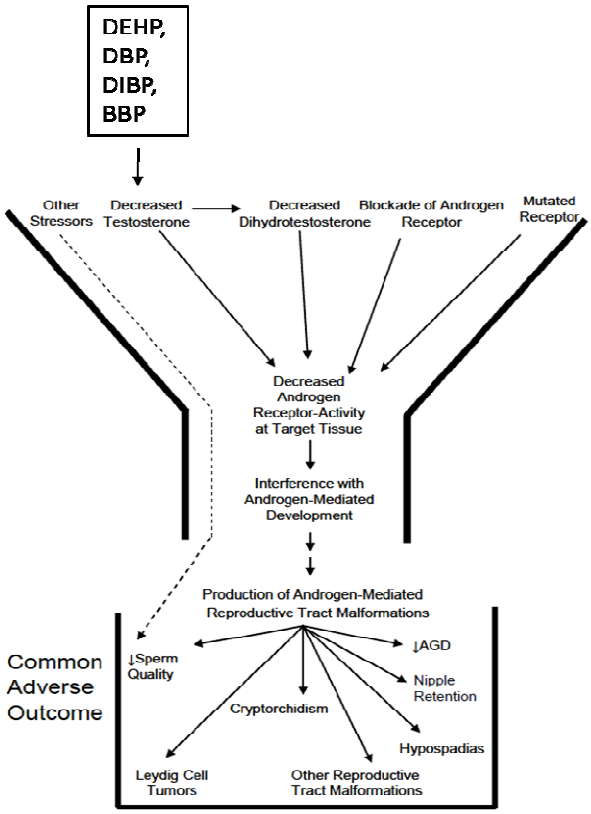

Fig. 1. Modified from NRC, 2008.

DBP has been found to interact with estrogen receptors *in vitro* in some assays (Ghisari and Bonefeld-Jorgensen 2009, Zacharewski et al., 1998), but adverse effects on estrogen related endpoints *in vivo* have not been detected (Zacharewski et al., 1998, Ahmad et al., 2013). *In vitro* studies have shown no interaction of DBP with androgen receptors (Kim et al., 2010, Krüger et al., 2008). Although phthalate diesters may interact with estrogen or androgen receptors, no interactions with monoesters have been found, as reviewed by David 2006. Thus, the effects of the phthalates are considered to be primarily related to changes in steroidogenesis.

In conclusion, several rodent studies have demonstrated an endocrine mode of action *in vivo,* which is substantiated by mechanistic data from *in vivo* 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 give rise to the adverse reproductive effects of DBP reported in the previous section.

4.2.4 Plausible link between adverse effects and endocrine mode of action

Altered steroidogenesis is related to adverse effects in males as well as females. The adverse effects of DBP on male reproductive system can be attributed to decreased testosterone levels, i.e. an anti-androgenic mode of action (EU RAR 2004). Investigation of toxicological effects of DBP in rat studies have provided convincing evidence that exposure can cause changes in the developing endocrine system as well as irreversible adverse reproductive effects. Anogenital distance and nipple retention in male pups are some of the adverse effects observed and are generally known to be androgen dependant, and decreases in anogenital distance and increases in nipple retention in males is associated with an anti-androgenic mode of action (Bowman et al. 2003; Wolf et al., 1999; Imperato-McGinley et al. 1985; Imperato-McGinley et al. 1986). Targeted studies on phthalate mode of action showed changes in steroidogenesis, including reduced testosterone production and down-regulation of genes involved in steroid

synthesis (Howdeshell et al., 2008; Wilson et al., 2004). Reduced testosterone production can in turn impair androgen signalling in androgen sensitive target organs during sensitive periods of development. Based on these findings it is highly biologically plausible that the observed adverse effects are linked to the endocrine disrupting mode of action of DBP.

4.2.5 Further work substantiating the plausible link between adverse effects and endocrine mode of action

In addition to the above studies showing an endocrine disrupting mode of action of DBP, a review paper by David, 2006, describes alternative cascades of events that could lead to the adverse health effects observed for DBP.

Path A describes how altered gene expression for cholesterol transport and steroidogenesis in Leydig cells (Lehmann et al., 2004, Schultz et al., 2001, Barlow et al., 2003, Lee et al., 2004; Liu et al., 2005) can lead to decreased cholesterol transport (Schultz et al., 2001, Gazouli et al., 2002, Barlow et al., 2003) and subsequent decreased T synthesis (Bell et al., 1978, Foster et al., 1983, Parks et al., 2000, Akingbemi et al., 2001; Zhu et al., 2005). In turn, this can lead to the adverse health effects of hypospadias and underdeveloped secondary sex organs (Wine et al., 1997, Mylchreest et al., 1998, 1999, 2000, Gray et al., 1999, 2000, Parks et al., 2000).

Path B describes how altered gene expression of insl3 protein in Leydig cells (Lehmann et al., 2004; Liu et al., 2005) can lead to decreased levels of insl3 (Wilson et al., 2004; Liu et al., 2005) and failure of gubernacular ligament to develop (Nef and Parada, 1999). In turn, this can lead to the adverse health effect of cryptorchidism (Gray et al., 1999, 2000, Parks et al., 2000).

Path C describes effects on Sertoli cells and gonocytes including presence of multinucleated gonocytes in the seminiferous tubules. Influences on Sertoli cells are not clear but include decreased expression of cyclin D2 in neonatal Sertoli cells, decreased gene expression for cell junctions, decrease in Sertoli cell proliferation, interference with cytoskeleton, decreased intercellular communication, and inhibition of gap junctional intercellular communication (Liu et al., 2005, Li and Kim, 2003, Li et al., 1998, 2000, Kleymenova et al. 2005, Yu et al., 2005, Kang et al 2002). Additionally, decreased T production in Leydig cells may lead to inhibition of Sertoli cell numbers (Atanassova et al., 2005). Gonocyte effects may be related to Sertoli cell changes, but this has not been clarified.

Overall, it is highly biologically plausible that the described adverse effects on the male reproductive system are induced through an endocrine disrupting mode of action mainly related to altered steroidogenesis following exposure to DBP.

4.2.6 Human relevance

Human relevance of the experimental data will be addressed also using read across to other phthalates when relevant, as data on human relevance of the effects of DBP are sparse. This approach is considered justified, as many similarities have been found between phthalate esters containing a straight-chain backbone of approximately 4-6 carbons. DEHP is branched with straight C6 backbones. DBP is linear with straight C4 backbones. BBP has one benzyl side chain and a straight C4 backbone. DBP and BBP share the same metabolite, mono-butyl phthalate. DIBP is the branched isoform of DBP with straight C3 backbones. For these phthalates many similarities also have been found between (1) adverse effects in endocrine related organs, (2) in *in vivo* endocrine modes of action and (3) a plausible link between the adverse effects and the endocrine modes of action.

For example, several studies have shown similar adverse effects and endocrine mode of action for phthalates containing a straight chain backbone of 3-7 carbons. Adverse effects on

reproductive organs, genital development and nipple retention were observed in males exposed to DEHP, BBP, DINP or DBP (Gray et al. 1999; Gray et al. 2000). Moreover, DEHP, DBP, BBP, DPP, DIBP and DINP reduced testosterone production, indicating an anti-androgenic mode of action of these phthalates (Borch et al. 2004; Howdeshell et al. 2008; Liu et al. 2005). As the adverse effects of the phthalates plausibly are linked to their anti-androgenic mode of action, a read-across between phthalates is considered relevant, for example when evaluating human relevance.

Due to recent studies showing differences in male reproductive effects of these phthalates between different species (rats, mice and marmosets), the issue of human relevance has been debated. Current knowledge indicates that phthalate induced effects on fetal testosterone production are not consistently found in mice, marmoset or human testis (ex vivo), but that changes in germ cell development can be induced by phthalates in different species.

Several studies are indicative of species differences in the reproductive effects of phthalates. In a study by Tomonari et al. (2006), no reproductive effects were seen in male marmosets (n=5-6 per dose group) exposed to DEHP by oral gavage at 100, 500 and 2500 mg/kg bw/day from 3 months of age until sexual maturity (18 months). Similarly, no reproductive effects were seen in a study by Kurata et al., 1998, in which male marmosets (n=4 per dose group) were dosed with 100, 500 and 2500 mg/kg bw/day of DEHP during 12-15 months of age. However, in another study on 4-day-old marmosets (5 co-twins and 4 non-twins, total n=14) treated for 14 days with 500 mg/kg bw/day of MBP, an increased Leydig cell volume was observed (Hallmark et al., 2007). A second study from the same authors revealed suppressed blood testosterone levels in male marmosets (n = 9) exposed at 2-7 days of age to a single dose of 500 mg/kg bw/day of MBP (measurement 5h after dose). In 4 day old co-twin marmosets (5 co-twins, n=10) were exposed to MBP neonatally during 14 days, and no effects on germ cell number or differentiation were apparent (McKinnell et al., 2009). It has been argued that the critical programming window for reproductive effects in marmosets is exposure during week 7 to 15 of gestation, but MBP did not alter the male reproductive system in the one study using this exposure period (McKinnell et al., 2009). In that study, no effects on testicular morphology, reproductive tract, testosterone levels at birth, germ cell number nor germ cell proliferation were observed in male offspring (n=6) of pregnant marmosets exposed to 500 mg/kg bw/day MBP from GD 49-105 (McKinnell et al., 2009). However, unusual clusters of undifferentiated germ cells were found in two of six males examined at birth, and the biological significance of this observation is unclear. Overall, data from marmoset studies are weakened by a low number of animals, and results appear to depend on the timing of exposure.

In mice it has proved difficult to find comparable effects of phthalates on testosterone production to those seen in rats. A study in fetal mice exposed to DBP did reveal changes in several immediate genes, but no decreases were observed in testosterone levels or in genes related to cholesterol homeostasis or steroidogenesis as would be expected for rats (Gaido et al., 2007). The study in fetal DBP-exposed mice showing no influence on steroidogenesis did reveal comparable changes in germ cells to those seen in fetal rats, i.e. increased seminiferous cord diameter, and increased numbers of multinucleated gonocytes (Gaido et al., 2007). *In vitro* studies on cultured rat, but not human, fetal testes have shown the ability of phthalates to reduce testosterone production, indicating species differences in sensitivity to the testosterone suppressing effect of phthalates (Hallmark et al., 2007; Lambrot et al., 2009, Chauvigné et al., 2009). In these *in vitro* studies human testis samples were from first or second trimester fetuses, but it is not clear whether these ages correspond to the sensitive window for phthalate exposure in rats (Lambrot et al., 2009, Hallmark et al., 2007). Data from *in vitro* studies are not consistent, as an vitro study on adult human testes has shown that exposure to DEHP and MEHP impaired testosterone production, and that the measured concentrations of phthalate metabolites in the incubated testes were as low as the phthalate metabolite levels measured in humans (Desdoits-Lethimonier et al., 2012).

In contrast to the possible differences seen between species regarding phthalate-induced changes in testosterone production, there appears to be similarities between rats, mice,

marmosets and humans regarding influence of phthalate exposure on germ cell proliferation and differentiation. *In vitro* studies on phthalate exposure of fetal testis tissue have been able to show comparable changes in germ cells whether using testes from rats, mice or humans (Lambrot et al., 2009, Lehraiki et al., 2009, Chauvigné et al., 2009, Habert et al., 2009). This clearly supports the possibility that reproductive effects of phthalates are relevant to humans.

Another experimental model has been applied for species comparisons, i.e. transplantation of testicular tissue from fetal rats or humans to a (transgenic) castrated mouse. A study using this model was able to demonstrate a testosterone inhibiting effect of DBP when using rat fetal testis explants, but not when using human fetal testis explants (Mitchell et al., 2012). However, there were several differences in study design between the fetal rat testis graft and the fetal human testis graft study, including duration of grafting before exposure and timing of exposure and age of the testis explant at the time of exposure. In the fetal human graft study, mice were supplied with hCG to promote testosterone production, whereas no LH (luteinizing hormone) stimulation was necessary for the rat graft to produce testosterone, and absolute testosterone levels therefore greatly differed in the two experimental setups (Mitchell et al., 2012). The differences in study design between the fetal rat testis graft study and the fetal human testis graft study thus complicate conclusions, and no firm conclusions regarding human relevance can be made on the basis of this study.

Another recent study comparing phthalate effects on rat, mouse and human testis in xenotransplant studies revealed similar effects as those described by Mitchell et al 2012 (Heger et al., 2012). Fetal testis xenotransplant studies revealed that effects on steroidogenic gene expression and ex vivo testosterone production were only seen with fetal rat testis, whereas multinuclear gonocytes were seen with rat, mouse and human fetal testis tissue (Heger et al 2012). Another study on fetal human testis xenografts showed that DBP did not affect testosterone levels or weights of androgen-sensitive host organs, whereas a CYP17A1 inhibitor, abiraterone acetate, did (Spade et al., 2013). DBP increased the number of multinucleated germ cells and altered the expression of oxidative stress response genes and actin cytoskeleton genes (Spade et al., 2013). These gene expression changes may reflect possible mechanistic targets that are suggested as subjects for further studies. Changes in the seminiferous chords may be important to germ cell development and may be related to persistent effects on testes as seen in the testicular dysgenesis syndrome (Toppari et al., 2010).

Human epidemiological studies are difficult to interpret due to the effects being delayed relative to the time of exposure. Interestingly, a study comparing phthalate exposure in mother's milk and testosterone levels in their infant sons revealed correlations between exposure to certain phthalate monoesters and the ratio of LH to testosterone (Main et al., 2006). This is in good agreement with the marmoset study showing that neonatal phthalate exposure impaired testosterone production and induced testicular effects characteristic for high LH levels (Hallmark et al., 2007)., and may indicate that the neonatal period may be a sensitive window of exposure for humans/primates. As described by Welsh et al., 2008, testosterone levels peak in late gestation in rats, but earlier (week 14-18) in humans, and this coincides with important periods of differentiation of reproductive organs. However, reproductive development continues postnatally in humans and may also be sensitive to exposure to endocrine disrupting compounds during early development (den Hond and Schoeters, 2006, Jacobson-Dickman and Lee, 2009).

In a recent review, data on phthalate toxicity to the fetal rat testis were compared with data from studies using mice or human testicular tissue (Johnson et al., 2012). The overall conclusions were that species-specific differences in testicular response following in utero phthalate exposure between mice and rats were observed, and that the response of human fetal testis to phthalate exposure may be more comparable to the response of a mouse than a rat. This review recognized two different pathways of phthalate effect on the fetal testes, namely a) suppression of steroidogenic gene expression and suppressed testosterone secretion and b) increase in multinucleated gonocyte number. It is suggested that understanding of molecular mechanisms responsible for the differences in sensitivity or resistance to

developmental phthalate exposure and more insight into the molecular pathways controlling steroidogenesis in the human fetal testis is warranted. In relation to risk assessment Johnson et al. (2012) conclude that "molecular mechanistic understanding will be needed for risk assessment to progress beyond the default protective assumption that humans respond similarly to the most sensitive species".

A recent publication provides a critical assessment of in vivo and in vitro studies exploring phthalate effects in humans (Albert and Jegou 2014). This paper highlights the variation among species in the window of susceptibility to the effects of phthalates and variation among species in timing of the development of the testis. Another conclusion of this literature study is that the indications of species differences found in e.g xenografting studies have methodological limitations and that "Caution before concluding that phthalates are innocuous in the human feta testis should be kept until these issues have been addressed" (Albert and Jegou 2014).

In their assessment of this Background document to the Opinion on the Annex XV dossier proposing restrictions on four phthalates (ECHA 2012), RAC concluded regarding human relevance of reproductive effects of these four phthalates: "For marmosets, however, limited data are available for in utero, peri- and neonatal exposure. There is no study with exposure during the entire life cycle such as the multigeneration studies in rats. In fact, there is only one developmental toxicity study (using a single high dose of MBP) with a period of exposure that covers the sensitive window for the programming of the male reproductive system, demonstrating some effects on the testes of neonatal marmosets of which the toxicological significance is unclear. This, combined with the relatively low number of (non-inbred) animals tested in the marmoset studies, makes it difficult to compare the results with those found in (inbred) rats. All in all, RAC concluded that there is too much uncertainty in the data available to allow a conclusion on humans being less, equally or more sensitive than rats, and thus suggested not to deviate from the default interspecies factor of 10."

Overall, there are clear indications of species differences in metabolism (Kurata 2012a, 2012b) and possibly in effects on fetal steroidogenesis, but there are also important differences in timing and duration of exposure in the experimental studies showing these species differences. Thus, the current knowledge on species differences is not sufficient to disregard the human relevance of phthalate effects. There are clear indications that changes in germ cell development can be induced by phthalates in several species including rats, mice, marmosets and xenotransplanted human fetal testis tissue. The implications or importance of these germ cell changes on long term effects on male reproduction are not fully elucidated, but it is evident from the current knowledge on the human testicular dysgenesis syndrome that early changes in the seminiferous chords may be important to germ cell development and related to persistent effects on testes (Toppari et al., 2010).

For possible female reproductive effects related to steroidogenesis interference and for possible thyroid disrupting effects of DBP the issue of human relevance has not been addressed. It is, however plausible that the endocrine disrupting effects of DBP may be of relevance to humans whether related to steroidogenesis interference (in males or females) or to thyroid disruption. It is therefore assumed that these effects may also be relevant to humans, as no data demonstrate non-relevance.

4.2.7 Summary – Human health

Based on the definition of endocrine disrupters by WHO/IPCS in 2002 (WHO/International Programme on Chemical Safety 2002) and the recommendation from the European Commission's Endocrine Disrupter Expert Advisory Group in 2013, the following four topics are covered to clarify how DBP fulfills the definition of being an endocrine disrupter:

1) Adverse health effects
2) Mode of action

3) Causality / plausible link between adverse effects and mode of action
4) Human relevance of experimental data

The EU risk assessment report on DBP from 2004 (EU RAR, 2004) acknowledges that "The results of these studies indicate that DBP does not possess estrogenic activity but rather shows antiandrogenic activity" and that based on the studies available when the EU risk assessment report on DEHP from 2008 (EU RAR 2008) was published "The results of recently performed *in vivo* studies on rats exposed to DEHP or DBP support the hypothesis that exposure to phthalates may be provoked by an antiandrogen mechanism". More recent studies published after the data was collected for the EU risk assessment report confirm this hypothesis.

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 hypothesized 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

5.1 Other effects: Endocrine disruption

5.1.1 General approach -Environment

To clarify how DBP fulfills the definition of being an endocrine disrupter, the topics described in chapter 4 will be covered in relation to the environment. It is important to emphasize that the results from chapter 4 are also relevant for mammalian wildlife, especially to wildlife species with low reproductive output including top predators and other larger mammals (including endangered species), because any negative effect on development or reproduction has a high likelihood of leading to serious effects at the population level for such species. As described for human health, in this report it is assumed that a substance should fulfil the recommendations from the European Commission's Endocrine Disrupters Expert Advisory group in order to be identified as an endocrine disruptor, and the available information is assessed based on the following topics:

1) Adverse effects

2) Endocrine mode of action

3) Plausible link between adverse effects and endocrine mode of action

For considering endocrine disrupting effects in the environment, data from both terrestrial and aquatic species should be analyzed. This is in conformity with the agreement of the European Commission's Endocrine Disrupters Expert Advisory group that "In relation to ecotoxicology, data on all species, including mammalian data generated to assess human toxicity, are generally considered relevant for the assessment of effects on ecosystems. In addition, since ecotoxicological assessment relates to impact at the population level rather than the individual level, relevance is applied in the context of identified adverse effects being relevant for the population" (JRC 2013).

Hence the fourth issue that should be considered as regards endocrine disrupters in relation to the environment is – not as for human health, human relevance – but rather environmental relevance, i.e. whether the adverse effects observed are also likely to cause effects at the population level.

Generally in regulatory ecotoxicology effects on survival, growth, but in particular development and reproduction are considered relevant endpoints for effects on populations and as such these endpoints are used to derive regulatory hazard and risk assessment decisions. It is noted that effects after longer time exposure relating to development and reproduction are generally preferred types of data for such decision.

Hence, the reproductive effects of DBP on mammals are of regulatory relevance for the environment.

5.1.2 Effects in the aquatic compartment (including sediment)

Overviews of the key studies on effects of DBP on wildlife were given in the EU risk assessment report for DBP (2004) and the WHO/UNEP/IPCS DBP Environmental Health Criteria 189 (WHO (1997)). In vivo studies from the report which include endpoints relevant for the assessment of endocrine disrupting effects are presented in the table below. Detailed study summaries can be found in the EU risk assessment report and WHO (1997).

Table 4. key studies on effects of DBP on wildlife including endpoints relevant for the assessment of endocrine disrupting effects as given in the EU risk assessment report for DBP (EU RAR 2004)

Species	**Vehicle**	**Exp. period**	**Endpoint**	**Effect conc. (mg/l)**		**Comment**	**Reference and estimated reliability score (Klimisch)**
Rainbow trout (Oncorhynchus mykiss) ELS	Flow through	99 days	Length and weight	NOEC LOEC	0.10 0.19	Length and weight. This NOEC was used in the EU RAR, 2004	Ward & Boerie (1991) (Score 4)[3]
Cyprinodontiform fish (Rivulus	Semi-static (3 weekly	147 days (DPB exposure	fecundity, viability of embryos, and	NOEC LOEC	1 2	Embryo viability affected	Davis (1988) (Score 2)

[3] GLP guideline study but only limited data available (secondary source)

marmoratus)	renewals)	21 weeks. Depuration period 9 weeks)	skeletal anomalies				

Beside the studies described above some long term tests on fish and invertebrates were presented in the WHO/UNEP/IPCS DBP Environmental Health Criteria 189 report (WHO 1997) but none of these studies had endpoints that could be related to endocrine disruption. The study by Ward & Boerie (1991) is included here because the growth effects could be anti-thyroid related and because the NOEC is used in the EU RAR, 2004 for risk assessment of DBP. Davis (1988) observed a significant decrease in the post-exposure mean number of fertile eggs and in the exposure period mean embryonic viability.

As described in chapter 4 Studies on estrogenic activity of DBP were presented in the EU Risk assessment report for DBP and the conclusion was: *In some special in vitro assays DBP showed weak estrogenic activity. The estrogenic effects were not confirmed in in vivo studies. Therefore the relevance of the effects observed in vitro for the in vivo estrogenic activity of DBP is questionable.* (EU RAR 2004). Two *in vivo* studies on juvenile rainbow trout where 500 and 1000 mg/kg DBP were intraperitoneally injected could also not confirm estrogenicity (Christiansen et al. 1998, 2000).

Several studies have been performed with endocrine relevant endpoints included since the EU 2004 RAR.

5.1.2.1 Studies conducted after the EU RAR (2004) – Fish

5.1.2.1.1 Short-term toxicity to fish

No endocrine relevant endpoints are included in the short-term toxicity tests to fish and therefore these studies are not discussed in this part of the dossier.

5.1.2.1.2 Long-term toxicity to fish

At the end of each study summary an estimated reliability score (Klimisch 1997) is given. In this respect is should be noted that significant effects from studies with only nominal exposure concentrations are also seen as reliable because the conclusions on DEHP SVHC properties are hazard based:

Tollefsen et al 2002: DBP were found to bind competitively to the Atlantic salmon (*Salmo salar*) Sex steroid Binding Protein (SBP) and potentially disrupt the endocrine function of these proteins. Furthermore, both weakly acting (DBP) and potent estrogen mimics (ethynylestradiol), were able to induce a substantial up-regulation of circulating levels of SBP in vivo. Modulation of SBP-levels was found to be a more sensitive endpoint than chemically induced interference with classical ER-mediated mechanisms for weakly acting estrogen mimics like di-(n-butyl) phthalate. Modulation of the high affinity estrogen-binding proteins in plasma of juvenile Atlantic salmon exposed to DBP for 14 days (N=6-8) occurred from 19 µg/l with a NOEC of 9 µg/l. (Klimisch Score 2 (non-guideline study)).

Jarmołowicz et al. (2013): The aim of the study was to describe the impact of di-n-butyl phthalate on the development of the reproductive system of European pikeperch (*Sander lucioperca*) during the sex differentiation period (age 61–96 days post hatch). A total of 240 fish were divided into 6 groups (40 fish per circulation tank). Treatments consisted of a control group (0 g di-n-butyl phthalate·kg^{-1} feed) and five trial groups with 0.125, 0.25, 0.5, 1, and 2 g di-nbutyl phthalate·kg^{-1} feed, respectively. The feed was delivered by automatic band

feeders 18 h per day. Histological changes of the fish gonads, sex ratio, survival and growth of fish were evaluated. Di-n-butyl phthalate seriously disturbed sex differentiation process of pikeperch. Histopathological analyses revealed that the administration of 2 g di-n-butyl phthalate·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 feminization process (intersex gonads) at concentrations of 1 g and 2 g di-n-butyl phthalate·kg^{-1} were observed. All analyzed concentrations delayed testicular development. Phthalate did not have a significant impact on the survival or growth rates of the pikeperch. This is the first report of disruption sex differentiation processes in fish by di-n-butyl phthalate- (Klimisch Score 2 (non-guideline study)).

Mankidy et al. (2013): The study investigated cytotoxicity, endocrine disruptive effects mediated via AhR, lipid peroxidation and effects on expression of enzymes of xenobiotic metabolism caused by di-(2-ethy hexyl) phthalate (DEHP), diethyl phthalate (DEP), dibutyl phthalate (DBP) and benzyl butyl phthalate (BBP) in developing fish embryos (*Pimephales promelas*). Oxidative stress was identified as the critical mechanism of toxicity (CMTA) in the case of DEHP and DEP, while the efficient removal of DBP and BBP by phase 1 enzymes resulted in lesser toxicity. DEHP and DEP did not mimic estradiol (E2) in transactivation studies, but at concentrations of 10 mg/L synthesis of sex steroid hormones was affected. Exposure to 10 mg BBP/L resulted in weak transactivation of the estrogen receptor (ER). All phthalates exhibited weak potency as agonists of the aryl hydrocarbon receptor (AhR). The order of potency of the 4 phthalates studied was; DEHP > DEP > BBP » DBP. A concentration of 1 mg/l DBP caused an 8 fold induction of CYP2K19 gene. In summary exposure to 10 mg DBP/L resulted in weak transactivation of the estrogen receptor (ER) in a MVLN transactivation reporter assay. Exposure of fertilized fathead minnow eggs to 1 mg/l BBP for 96 h increased the androgen receptor mRNA significantly. (Klimisch Score 2 (non-guideline study)).

Aoki et al. (2011): In the present study, adult male three-spined sticklebacks (*Gasterosteus aculetaus*; n=8) were exposed to di-n-butyl phthalate 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. This deviation from nominal values is expected because DBP is metabolized to mono-n butyl phthalate in fish: e.g. 75% within 4 hours in channel catfish (Hogan et al 1975). Plasma testosterone concentrations were significantly higher in males from the 35 µg DBP/L group compared with the solvent control, whereas plasma 11-ketotestosterone concentrations were not significantly affected. Expression of steroid acute regulatory protein and 3b-hydroxysteroid dehydrogenase remained unchanged. Spiggin concentrations were significantly lower in the males exposed to 35 µg DBP/L. It should be noted that changes in male spiggin concentration has not yet been directly connected to adverse effect at the population level. However, such changes in reproductive behavior in fish seems to be both ecotoxicological relevant and of concern. It was reported that five of thirty-six samples had DEHP contamination. This occurrence of DEHP cannot explain the dose dependent decline in spiggin observed. Nest building appeared to be slower in some males exposed to DBP, but this was not statistically significant. These results suggest that DBP has anti-androgenic effects in fish. (Klimisch Score 2 (non-guideline study)).

Bhatia et al. (2013): The present study investigated the changes in ovarian histology and serum vitellogenin concentrations in adult Murray rainbowfish (*Melanotaenia fluviatilis*) after exposure to nominal 125 µg/L, 250 µg/L, 500 µg/L, and 1000 µg/L DnBP for 7 d (two replicates of 4 fish per concentration). Measured concentrations were 10-30% of nominal after 24 h. Treatment at 125 µg/L to 1000 µg/L DnBP 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 DnBP. The circulating levels of plasma vitellogenin were significantly lower in the fish exposed to 500 µg/L and 1000 µg/L DnBP ($p<0.05$). These data show that a continuous exposure to subacute concentrations of DnBP for 7 d can cause anti-estrogenicity in female adult Murray rainbowfish. (Klimisch Score 2 (non-guideline study)).

Ortiz-Zarragoitia & Cajaraville (2005). The purpose of the work was to investigate if xenoestrogens are able to cause proliferation of liver peroxisomes using zebrafish (*Danio rerio*) as a model. Adult male zebrafish (2 replicates of 30 fish per exposure group) were exposed for 15 days (semi-static, daily water renewal, nominal concentrations) to 17b-estradiol (E2) and the xenoestrogens dibutylphthalate (DBP), methoxychlor (MXC), 4-tert-octylphenol (OP) and 17a-ethynylestradiol (EE2). All five tested compounds caused significant proliferation of liver peroxisomes ($p <0.05$) as indicated by increased peroxisomal surface and numerical densities and elevated activities of the peroxisomal b-oxidation enzyme acyl-CoA oxidase (AOX). In the case of DBP, MXC and E2, positive significant correlations between peroxisomal density parameters and AOX were found. The treatments did not produce gross alterations in testis histology, but spermatogenic cell proliferation was disturbed in E2 and EE2-treated groups and vitellogenin levels increased significantly in fish exposed to MXC, OP, EE2 and E2 with respect to controls. Furthermore, a significant correlation between vitellogenin levels and AOX activity was found for MXC, OP and EE2 treatments, suggesting that for the latter xenoestrogens early estrogenic effects are associated with liver peroxisome proliferation. No such association occurred with typical peroxisome proliferators such as DBP. No effect on vitellogenin after 15 days exposure to 500 µg/l. (Klimisch Score 2 (non-guideline study)).

Ortiz-Zarragoitia & Cajaraville (2006): The aim of the work was to study the effects of the peroxisome proliferator dibutylphthalate (DBP) and the xenoestrogen 17β-ethynylestradiol (EE2) on liver peroxisomes, reproduction, and development of zebrafish (*Danio rerio*). In experiment 1, newly fertilized zebrafish eggs (250 eggs, in two replicates for each experimental group with static water renewal) were exposed for five weeks, covering the entire period of sexual determination, to nominal concentrations of 25 and 100 µg/L of DBP and 5 µg/L of EE2. In experiment 2, adult female zebrafish (N=10) were exposed for 15 d to 100 and 500 µg/L of DBP and 5 µg/L of EE2, and afterward, they were paired with untreated males to study the effects in the resultant offspring. Ethynylestradiol provoked marked mortality (~50%) and delayed development of larvae as well as sterility of adult females, possibly related to alterations in aromatase gene expression. Ethynylestradiol up-regulated vitellogenin expression in the early life stages and increased vitellogenin synthesis and accumulation in adult females. Ethynylestradiol caused liver peroxisome proliferation in early life stages but not in adult females. Dibutylphthalate caused teratogenic effects in early life stages and mortality of the larvae obtained from exposed females. The number of eggs obtained from females exposed to both phthalate concentrations did not differ from that of controls (an average of 50 eggs per female and day in the group receiving 500 µg/L of DBP, 41eggs per female and day in the group receiving 100 µg/L of DBP, and 48 eggs per female and day in the control group). However, mortality of fry was increased significantly in a concentration-dependent manner in the DBP-exposed groups, reaching 70% mortality after 25 d postfertilization in fry from the females exposed to 500 µg/L of DBP. Marked mortality was observed during the period covering the transition from endogenous food intake (vitelline sac) to exogenous food intake at 9 to 14 d postfertilization. Dibutylphthalate provoked liver peroxisome proliferation and up-regulation of cytochrome P450A1 in early life stages at the end of the exposure and in adult females. Dibutylphthalate also upregulated the expression of aromatase genes. In conclusion, the xenoestrogen EE2 caused liver peroxisome proliferation in early life stages of zebrafish, but the peroxisome proliferator DBP did not behave as a typical xenoestrogen. Overall, changes in gene expression were more marked during early life stages than in adult female zebrafish. Even though DBP up-regulated CYP19A2 expression in adult female zebrafish (presumably via the PPAR-response element or by an indirect mechanism), it did not induce other estrogen receptor– target genes, such as VTG. (Klimisch Score 2 (non-guideline study)).

5.1.2.2 Studies conducted after the EU RAR (2004) - Amphibians

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. (Klimisch Score 2 (non-guideline study)).

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. (Klimisch Score 2 (non-guideline study)).

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. (Klimisch Score 2 (non-guideline study)).

Shen et al. (2011). Nieuwkoop and Faber stage 51 *Xenopus laevis* (N=20)vwere 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 concentrations of the compounds. Using mammalian two-hybrid assay *in vitro*, we found that DBP and MBP enhanced the interactions between co-repressor SMRT (silencing mediator for retinoid and thyroid hormone receptors) and TR in a dose-dependent manner, and MBP displayed more markedly. In addition, MBP at low concentrations (2 and 10 mg/L) caused aberrant methylation of TRb in head tissue. (Klimisch Score 2 (non-guideline study)[4].

Lee et al. (2005a): Evaluated the effects of low concentrations of DBP on spermatogenesis in *Xenopus laevis*, the African clawed frog The study consisted of three definitive 96-h frog embryo teratogenesis assay–Xenopus (FETAX) tests, each represented by one clutch of embryos from a single breeding pair, and each test was conducted in duplicate. Embryos (n = 300/group) were randomly assigned to the following treatment groups: 0.1, 0.5, 1, 5, 10, or 15 ppm DBP (nominal concentrations) dissolved in 0.01% DMSO, vehicle alone (0.01% DMSO; solvent control), or FETAX solution only (control; n = 600) 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 *Xenopus laevis* frogs. (Klimisch Score 2 (non-guideline study)[5].

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. (Klimisch Score 2 (non-guideline study)[6].

[4] The high dose of DBP of 15 mg/l is above the maximum water solubility of approximately 10 mg/l.

[5] The high dose of DBP of 15 mg/l is above the maximum water solubility of approximately 10 mg/l

[6] The high dose of DBP of 15 mg/l is above the maximum water solubility of approximately 10 mg/l

5.1.2.3 Adverse effects related to endocrine disruption

Below, the endocrine disruptive effects in non-mammalian aquatic (vertebrate) species are summarized from the above mentioned studies.

One of the reported fish studies describes an endocrine specific adverse effect: The significantly affected sex ratio in the pikeperch (Jarmołowicz et al., 2013), where the proportion of phenotypic males declined in a dose dependent manner is adverse according to the OECD ED GD (OECD 2012). The effect could be caused by either estrogenic or anti-androgenic MoA of DBP. In conclusion DBP is anti-androgenic in fish and anti-thyroidal/anti-androgenic in amphibians. Adversity is confirmed in fish but no adversity can be confirmed in amphibians.

Further, as described in chapter 4, several rodent studies have demonstrated adverse reproductive effects. These studies are relevant to some mammalian wildlife as top predator species where the described reproductive effects are expected to cause effects at population level because of a natural low reproductive output.

5.1.2.4 Endocrine mode of action

Below, the influence of DBP on the endocrine system in non-mammalian aquatic (vertebrate) species are summarized from the above mentioned studies.

Fish - effects on vitellogenin (Vtg) concentration in fish after waterborne and/or injection exposure to DBP has been investigated in a few studies. The only study showing effect is Bahtia et al. (2013) where the circulating levels of plasma vitellogenin were significantly lower in the female Murray rainbowfish (*Melanotaenia fluviatilis*) exposed to 500 µg/L and 1000 µg/L DBP ($p<0.05$) which could be a sign on anti-estrogenicity – but also a systemic toxic effect. In conclusion, there are signs of anti-estrogenicity in one study, otherwise no effect.

Fish - effects on spiggin: The concentration of the androgen dependent protein spiggin, declined significantly in adult male three-spined sticklebacks (*Gasterosteus aculetaus*) after exposure to 35 µg DBP/L for 22 d (Aoki et al., 2011). This effect is regarded as being anti-androgenic. In conclusion, DBP is anti-androgenic due to decline of spiggin.

Fish - effects on steroidogenesis: DBP was found to bind competitively to the Atlantic salmon (*Salmo salar*) Sex steroid Binding Protein (SBP) and potentially disrupt the endocrine function of these proteins. Furthermore, DBP was able to induce a substantial up-regulation of circulating levels of SBP *in vivo* Modulation of the high affinity estrogen-binding proteins in plasma of juvenile Atlantic salmon exposed to DBP occurred from 19 µg/l with a NOEC of 9 µg/l sex steroid-binding protein (Tollefsen et al. (2002). Mankidy et al. (2013) investigated several biomarkers for steroidogenesis and concluded that DBP was the only phthalate that showed no effects with respect to any of the endocrine disrupting end points in the study. In another study (Ortiz-Zarragoitia & Cajaraville (2006)) DBP up-regulated CYP19A2 expression in adult female zebrafish (presumably via the PPAR-response element or by an indirect mechanism) but it did not induce other estrogen receptor– target genes, such as VTG. In conclusion, no specific MoA can explain the observed effects on steroidogenesis.

Fish - phenotypic sex: Jarmołowicz et al. (2013) observed that DBP seriously disturbed sex differentiation process of pikeperch (*Sander lucioperca*) after exposure during the sex differentiation period (age 61–96 days post hatch). Histopathological analyses revealed that the administration of 1 and 2 g DBP/kg via food significantly affected the sex ratio. The feminization process (intersex gonads) at concentrations of 1 g and 2 g di-n-butyl phthalate/kg were observed. All analyzed concentrations delayed testicular development. The results support an anti-androgenic MoA.

Fish - thyroidal effects: Not tested in fish. It is not possible to conclude if the reduced growth at 190 µg/l in juvenile rainbow trout (Ward & Boerie (1991)) is thyroid mediated.

Fish - reproduction: No effect on fecundity was seen at 500 µg/l DBP in zebrafish in Ortiz-Zarragoitia & Cajaraville (2006). Davis (1988) observed a significant decrease in the number of fertile eggs after 21 weeks semi-static exposure to 2 mg/l DBP in *Rivulus marmoratus* and significant decline in embryonic viability during a 9 week depuration period. In conclusion, a decrease in egg fertility and embryonic viability is observed at high concentrations – MoA is not indicated.

In vitro: See chapter 4.

Amphibians - developmental/thyroidal effects: Two *in vitro* studies revealed anti-thyroidal (T3-antagonist) activity of DBP in amphibians (Shimada & Yamauchi. (2004); Sugiyama et al. (2005)). A follow up *in vivo* study by Sugiyama et al. (2005) could not confirm DBP T3-antagonism. Shen et al. (2011) observed decelerated spontaneous metamorphosis in *X. laevis* at concentrations of 10 and 15 mg/L. Moreover, The effects of DBP 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 concentrations of the compound. Lee et al. (2005b) investigated developmental effects of DBP on *Xenopus* embryos using the 96-h frog embryo teratogenesis assay–Xenopus (FETAX). 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. In conclusion there are signs of anti-thyroid effects – as decelerated metamorphosis and decreased growth.

Amphibians - spermatogenesis: Lee et al. (2005a) evaluated the effects of low concentrations of DBP on spermatogenesis in *Xenopus laevis*, and observed several lesions 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. In conclusion: DBP affects spermatogenesis- causing different structural changes of testis. MoA could be anti-androgenic but this is not confirmed

Amphibians - phenotypic sex: Othani et al. (2000) investigated the effect of DBP on genetically male tadpoles of *Rana rugosa* and found that gonads of the control tadpoles all showed the typical structure of testes. In contrast, after 0.1, 1, and 10 µM DBP (27.8, 278 and 2780 µg/l) treatment, 0, 7, and 17% of tadpoles, respectively, develop gonads of complete or partial ovarian structure. In conclusion, genetic male tadpoles developed intersex or reversal to gonads. This effect could be driven by either an estrogenic or an anti-androgenic MoA.

5.1.2.5 Plausible link between adverse effects and endocrine mode of action

As seen from the ecotoxicological studies described above, several endocrine pathways could be affected by DBP. Anti-thyroid effects were confirmed in amphibians and anti-androgenicity was confirmed in fish where both sex ratio (Jarmołowicz et al., 2013) and spiggin (Aoki et al., 2011) were affected.

5.1.3 Summary - Environment

Fish: One of the reported fish studies describes an endocrine specific adverse effect according to the OECD ED GD (OECD 2012): DBP significantly affected sex ratio in the pikeperch (*S. lucioperca*), where the proportion of phenotypic males declined in a dose dependent manner. The effect could be caused by either estrogenic or anti-androgenic MoA of DBP. The

concentration of the androgen dependent protein spiggin, declined significantly in adult male three-spined sticklebacks (*G. aculetaus*) after exposure to DBP. This effect is regarded as being anti-androgenic.

Amphibians: Decelerated metamorphosis and decreased growth in *Xenopus laevis.* These findings were substantiated by two *in vitro* studies showing anti-thyroidal activity of DBP as a T3-antagonist. Studies on *Xenopus* showed effects on spermatogenesis and in *Rana rugosa* genetic male tadpoles developed intersex or reversal to gonads. Effects on spermatogenesis could be through an anti-androgenic MoA and effects of tadpole sex could be either an estrogenic or anti-androgenic MoA. However, MoA for these endpoints could not be confirmed.

Mammals: The data on effects of DBP on rodents as presented in chapter 4 are directly transferable to hazard assessment of DBP in relation to some mammalian wildlife including top predator species and large mammals (including endangered species), where the described reproductive effects are expected to cause effects at population level because of a natural low reproductive output.

6 CONCLUSIONS ON THE SVHC PROPERTIES

6.1 Conclusion on fulfilment of WHO definition of endocrine disruptor

A summary of the findings in chapters 4 and 5 are compared with the definition of an endocrine disrupter as given by WHO/IPCS, and as further elaborated by the European Commission's Endocrine Disrupters Expert Advisory Group (JRC 2013) on elements for identification of an endocrine disrupter.

According to the widely accepted definition of an endocrine disruptor by the WHO/IPCS (WHO/International Programme on Chemical Safety 2002), an "*endocrine disruptor is an exogenous substance or mixture that*

1) alters function(s) of the endocrine system and 2) consequently causes 3) adverse health effects in an intact organism, or its progeny, or (sub)populations."

This has been further elaborated by the European Commission's Endocrine Disrupters Expert Advisory Group that has recommended that for a substance to be identified as an endocrine disruptor, available information should be assessed as regards the following topics:

1) Adverse effects
2) Endocrine mode of action
3) Plausible link between adverse effects and endocrine mode of action
4) Human relevance (for human health only)

In relation to effects on wildlife (the environment) the above mentioned topic 4) human relevance should be replaced with "environmental relevance" (see section "5.1.1 General approach – Environment").

Re 1) The spectrum of adverse effects observed in rats include increased nipple retention, decreased anogenital distance, genital malformations, reduced number of spermatocytes and testicular changes including multinucleated gonocytes, tubular atrophy and Leydig cell hyperplasia. DBP causes adverse –and serious – reproductive toxicity effects in rodents and a harmonized classification Rep. 1 B has been concluded.

Adverse effects caused by exposure to DBP have also been identified in non-mammalian wildlife where the sex ratio was affected in fish. Hence the current data indicates also in fish that DBP has endocrine disruptive properties leading to adverse effects related to sexual development and reproduction.

Re 2) DBP has been shown to adversely affect the endocrine system of mammals primarily through *in vivo* findings on reduced fetal testosterone. These findings are further substantiated by mechanistic findings, also *in vivo*, of down-regulation of genes in the steroidogenic biosynthesis pathway. The plausible connection to the endocrine system was also confirmed in fish where an anti-androgenic or estrogenic MoA is could cause the observed change in sex ratio. An anti-androgenic mode of action was further supported as a decline in the protein spiggin was observed in the anti-androgenic specific assay in three-spined stickleback.

Re 3) The link between the endocrine mode of action of DBP has been concluded in numerous investigations in rodents (mode of action on the steroidogenic biosynthesis pathway) and has also been shown in fish (anti-androgenic or estrogenic mode of action). It is considered biologically highly plausible that the observed adverse effects in rats and fish are linked to the endocrine disrupting mode of action of DBP.

Re 4) DBP causes serious adverse reproductive toxicity effects in rodents and based on an assessment of human relevance using also other available information, a harmonized classification Repr. 1B has been concluded. Hence it can be concluded that human relevance has been agreed for the adverse effects. In respect to the environment sex reversal is regarded as a serious population relevant adverse effect endpoint in fish. The ED MOA and reproductive toxicity data from laboratory rodents are highly relevant for at least some mammalian wildlife species due to the similarities between mammalian species and their hormone systems. The laboratory ED MOA and reproductive toxicity data on fish are relevant for at least some wildlife fish species. Hence both human and environmental relevance can be concluded.

In relation to the environment, adverse effects concerning development and reproduction are generally regarded as endpoints of particular relevance because such effects are likely to manifest themselves at the population level. The effects observed in rats are of particular concern for 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.

In conclusion, when available information from toxicological and ecotoxicological studies are combined, DBP can be considered an endocrine disruptor for both the environment and for human health as it fulfils the WHO/IPCS definition of an endocrine disruptor, the recommendations from the European Commission's Endocrine Disrupters Expert Advisory Group for a substance to be identified as an endocrine disruptor

6.2 Conclusion on fulfilment of Article 57(f)

Article 57(f) states that: "substances – such as those having endocrine disrupting properties or those having persistent, bioaccumulative and toxic properties or very persistent and very bioaccumulative properties, which do not fulfil the criteria of points (d) or (e) – for which there is scientific evidence of probable serious effects to human health or the environment which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) and which are identified on a case-by-case basis in accordance with the procedure set out in Article 59."

In order to conclude on whether DBP, in addition to fulfilling the definition of an endocrine disrupter as given by WHO/IPCS and further elaborated by the European Commission's Endocrine Disrupters Expert Advisory Group (JRC 2013), also fulfils Article 57(f), the following should be considered:

For substances with endocrine disrupting properties, the scientific interpretation of Article 57(f) for regulatory implementation can be linked to the WHO/IPCS definition. Thus,

(i) "endocrine disrupting properties" (REACH art. 57.f) is another way of formulating "altering the functions of the endocrine system" (WHO definition),

(ii) "scientific evidence of probable serious effects" (art. 57 f.) is another way to explain the cause-effect relationship (WHO definition)_between the endocrine activity and the adverse effects,

(iii) "equivalent level of concern" is another way to describe that the adverse effects (of endocrine disruptors, WHO definition) are serious at the same level as CMRs or PBT/vPvBs. (ELoC according to REACH art. 57 f)

The differences between REACH Art. 57f as regards substances with ED properties of ELoC and the WHO definition of endocrine disrupters are:

a) The requirement for proving a causal relationship between the ED MOA and adverse/ serious effects is seemingly a bit higher according to the WHO definition, because this definition (rather unreflectively) uses the term "causes" whereas REACH art. 57 f expresses that the linkage between ED properties and serious effects should be "probable" (realizing that making a definitive causal proof may in reality not be possible even though the evidence is strong and also realizing that "waiting for the ultimate proof" may not be appropriate as basis for regulation).

b) The seriousness of the adverse effects is expressed slightly differently in the WHO definition and in the art. 57 f (ELoC part). This is probably due to the fact that the scope for the WHO definition is more narrow focussing on only endocrine disrupters ("causing adverse health effects in an intact organism, or its progeny, or (sub)populations.") whereas the scope of art. 57 f concerns both endocrine disrupters and in addition other types of serious chemical properties raising ELoC as those of CMRs or vPvB/PBTs.

Human health

With regard to assessing whether DBP, which fulfils the WHO/IPCS definition of an endocrine disruptor for human health, also fulfils Article 57(f) the following elements are considered:

Re. endocrine disrupting properties

DBP has systematically been shown to adversely affect the endocrine system of mammals primarily through *in vivo* findings on reduced fetal testosterone. These findings are further substantiated by mechanistic findings, also *in vivo*, of down-regulation of genes in the steroidogenic biosynthesis pathway.

Re. scientific evidence

Altered steroidogenesis is related to adverse effects in males as well as in females, and the adverse effects of DBP may be attributed to decreased testosterone levels, i.e. an anti-androgenic mode of action. Consistent findings in rats provide convincing evidence that DBP can cause irreversible adverse reproductive effects. It is biologically highly plausible that the observed adverse effects are linked to/caused by the endocrine disrupting mode of action of DBP

Re. probable serious effects

DBP systematically has shown a wide spectrum of adverse effects observed in rats which include increased nipple retention, decreased anogenital distance, genital malformations, reduced number of spermatocytes and testicular changes including multinucleated gonocytes, tubular atrophy and Leydig cell hyperplasia. These development/reproductive toxicity effects have led to the harmonized classification Repr. 1B

Re. equivalent level of concern

The observed serious developmental/reproductive toxic effects are of an equivalent level of concern to substances classified with CMR Cat 1 because they have led to the harmonized classification Repr. 1B (i.e a CMR classification to which Art. 57f directly refers). In addition, the seriousness of the reproductive effects concerned can be characterized in the following way:

- Potential severity of health effects: DBP adversely affects the normal development and the reproductive ability Irreversibility of health effects: the adverse effects concerned such as reduced ability to produce semen (Leydig cell hyperplasis) or a malformed reproductive system are irreversible / long lasting reproductive changes.
- Delay of effects: There is a long latency period between early impacts and occurrence of the adverse effects. Impacts during early development which adversely affects reproductive ability such as reduced number of spermatocytes, testicular changes, tubular atrophy and organ malformations or mis-function, will not manifest themselves fully until reproductive age.
- Quality of life: A reduced ability to reproduce considerably affects the quality of life negatively for the individuals affected as well as for their partners and families. Reduced fertility is of general concern in the EU countries.
- Negative impact on society: A reduced ability to reproduce negatively affects the society as it contributes to a significant increased financial burden on the health care sector, both providing assisted fertilisation treatments and clinical treatment for individuals with adverse reproductive effects post-natally. In addition, the fertility rate in many EU countries (including in Denmark) is decreasing.
- No toxicological threshold for the endocrine disruption caused reproductive toxic effects has yet been scientifically proposed, discussed and concluded and/or agreed for DBP.

Environment

When assessing whether DBP, which fulfils the WHO/IPCS definition of an endocrine disruptor for the environment, also fulfils Article 57(f), the following elements are considered:

Re. endocrine disrupting properties

In fish, DBP affected sex ratio in the pikeperch (S. lucioperca), which is an endocrine specific adverse effect, where the proportion of phenotypic males declined in a dose dependent manner. The effect could be caused by either estrogenic or anti-androgenic MoA of DBP. The concentration of the androgen dependent protein spiggin, declined significantly in adult male three-spined sticklebacks (G. aculetaus) after exposure to DBP.

In amphibians, findings of decelerated metamorphosis and decreased growth in Xenopus laevis were substantiated by two in vitro studies showing anti-thyroidal activity of DBP as a T3-antagonist. Studies on Xenopus showed effects on spermatogenesis and in Rana rugosa genetic male tadpoles developed intersex or reversal to gonads. Effects on spermatogenesis could be through an anti-androgenic MoA and effects of tadpole sex could be either an estrogenic or anti-androgenic MoA.

Ad (ii) *scientific evidence*

Change in sex ratio of fish is seen as both adverse and highly likely to be a marker of endocrine disruption. This is substantiated by a decline in the concentration of the androgen dependent protein spiggin in adult male three-spined sticklebacks (*G. aculetaus*) which is regarded as an anti-androgenic effect. Anti-androgenicity was confirmed in fish where both sex ratio and spiggin were affected. Anti-thyroid effects were confirmed in amphibians; however

the mode of action for effects on spermatogenesis in *Xenopus* and intersex/reversal to gonads in tadpoles of *Rana rugosa* could not be confirmed. Overall, several endocrine pathways could be affected by DBP.

Re. probable serious effects.

DBP has adverse effects on the phenotypic sex in the pikeperch (*S. lucioperca*) according to the OECD ED GD. In amphibians, DBP decelerated metamorphosis and decreased growth in *Xenopus laevis and* effects on spermatogenesis in *Xenopus* and intersex/reversal to gonads in tadpoles of *Rana rugosa* were observed but the mode of action for these effects could not be confirmed.

Re. equivalent level of concern

- *Potential severity of ecotoxicological effects* – DBP may adversely affect the reproductive ability of fish populations by changing male fish into female fish. Such reproductive effects are considered an adverse and serious effect with population level relevance. DBP also as mentioned above causes developmental and reproductive toxicity effects in laboratory rat, which due to the general conservation of hormone systems between different mammalian species is also an appropriate animal model for mammalian wildlife species.

- *Irreversibility of effects:* Endocrine modulation is a very complex feedback process that is set up during critical early life stages in fish and mammalian species. 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 is 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.

- *Broad environmental relevance*: Effects on reproductive ability via an estrogenic mode of action has a broad environmental relevance. Due to the conservatism of estrogen receptors it is very likely that a wide range of wildlife species with different function in the 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 (inclusive 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.

- Finally. no toxicological threshold for the endocrine disruption caused reproductive toxic effects has yet been scientifically proposed, discussed and concluded and/or agreed for DBP.

6.3 Conclusion

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

DBP has been shown to adversely affect the endocrine system of mammals primarily through *in vivo* findings on reduced fetal testosterone. These findings are further substantiated by mechanistic findings, also *in vivo*, of down-regulation of genes in the steroidogenic biosynthesis pathway. The spectrum of adverse effects observed in rats include increased nipple retention, decreased anogenital distance, genital malformations, reduced number of spermatocytes and testicular changes including multinucleated gonocytes, tubular atrophy and Leydig cell hyperplasia.

In relation to the environment, adverse effects concerning development and reproduction are generally regarded as endpoints of particular relevance because such effects are likely to manifest themselves at the population level. The effects observed in rats are of particular concern for 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.

Adverse effects caused by exposure to DBP have also been identified in non-mammalian wildlife where the sex ratio (sex reversal of male fish to female fish) was affected in fish. The plausible connection to the endocrine system was also confirmed in fish where the anti-androgenic MoA could be verified in an anti-androgenic specific assay in stickleback. Hence the current data indicates also in fish that DBP has endocrine disruptive properties leading to adverse effects related to sexual development and reproduction.

In conclusion, when available information from toxicological and ecotoxicological studies are combined, DBP can be considered an endocrine disruptor for both the environment and for human health 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.

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 development may cause irreversible developmental programming effects leading to severe effects on development and reproduction, regarded as particularly serious in relation to human health and wildlife species, also because these adverse effects may first manifest themselves in later life stages as a consequence of exposure during early life stages. 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 both for human health and for environmental species.

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ANNEX 1 - OVERVIEW OF KEY STUDIES FROM TABLES IN THE EU RISK ASSESSMENT REPORT FOR DBP (2004)

Species	Protocol	Results
mouse	continuous breeding protocol (one generation) 0, 0.03, 0.3 and 1.0% in diet (~0, 40, 420 and 1,410 mg/kg bw)	115 d (including 7 d premating and 98 d during cohabitation) NOAEL for embryotoxicity and parental toxicity is 0.3% in diet (~420 mg/kg bw (Lamb et al., 1987; Morissey et al., 1989)
rat	continuous breeding protocol (two generations) 0, 0.1, 0.5 and 1.0% in diet (~ 0, 52, 256 and 509 mg/kg bw for males and 0, 80, 385 and 794 mg/kg bw for females).	119 d (including 7 d premating and 112 d during cohabitation). 0.1% in diet (52 mg/kg bw for males; 80 mg/kg bw for females) is the LOAEL for embryotoxicity. The NOAEL for maternal toxicity is 0.5% in the diet (385 mg/kg bw) (NTP, 1995; Wine et al., 1997)
rat	other; 0, 120 and 600 mg/kg bw 3 mos exposure followed by a 7d mating period	NOAEL 600 mg/kg bw for maternal toxicity and embryotoxicity (Nikoronow et al., 1973)
rat	other; 0, 5, 50 and 500 mg/kg bw via the diet to male rats only, 60 days before mating up to weaning of F1 pups	NOAEL 500 mg/kg bw with respect to fertility of male rats and embryotoxicity (IRDC, 1984)
Rat	other; 0, 5, 50 and 500 mg/kg bw via the diet to female rats only, 14 days prior to mating up to weaning of F1 pups. F1 pups fed 7 weeks postweaning	NOAEL for maternal toxicity, female fertility and embryotoxicity is 50 mg/kg bw (IRDC, 1984)
Rat	other; 0, 250, 500 and 1,000 mg/kg bw exposure of P0 generation only; two generations were produced	LOAEL 250 mg/kg bw Effects: delayed puberty in males of P0 generation, urogenital abnormalities and decreased fertility of F1 males and females (Gray et al., 1999)
mouse	other: 0, 0.005, 0.05 or 0.5% in diet (based upon food intake 0.05 and 0.5% were calculated to be 100 and 400 mg/kg bw) day 1- 18 of gestation	NOAEL 0.05% in diet (100 mg/kg bw) for maternal as well as embryotoxicity and teratogenicity (Hamano et al., 1977)
mouse	other: 0, 0.05, 0.1, 0.2, 0.4, 1.0% in diet (~80, 180, 350, 660 and 2,100 mg/kg bw) day 1-18 of gestation	NOAEL for embryotoxicity is 0.2% (~350 mg/kg bw); NOAEL for maternal toxicity and teratogenicity is 0.4% (~660 mg/kg bw) (Shiota et al., 1980)
rat	other; 500, 630, 750, 1,000 mg/kg bw day 7-15 of gestation	NOAEL 500 mg/kg bw for teratogenicity. 500 mg/kg b.w is a LOAEL for maternal and embryotoxicity (Ema et al., 1993)
rat	other; 0, 0.5, 1.0 or 2.0% in the diet (~331, 555 and 661 mg/kg bw) from day 11-21 of gestation	NOAEL 0.5% in diet (~331 mg/kg bw). Critical effect: undescended testes, decreased anogenital distance in male progeny (Ema et al., 1998)
rat	other; 0, 120 and 600 mg/kg bw day 1-21 of gestation	NOAEL 120 mg/kg bw for embryotoxicity (Nikoronow et al., 1973)
rat	other; 0, 250, 500 and 750 mg/kg bw from day 3 of gestation throughout gestation and lactation. Pups were allowed to mature.	LOAEL 250 mg/kg bw Critical effect: disturbed development of male reproductive tract (Mylchreest et al., 1998)
rat	other; 0, 500, 1,000, 1,500 and 2,000 mg/kg bw on day 14 of gestation	NOAEL 500 mg/kg bw. At doses ≥1,000 mg/kg bw higher incidences of skeletal variations. At doses ≥1500 mg/kg bw increased no. of resorptions and reduced fetal body wts.

		(Saillenfait et al., 1998)
rat	other; 0, 100, 250 and 500 mg/kg bw from day 12-21 of gestation	LOAEL 100 mg/kg bw. Critical effect: delayed (2-days) preputial separation (one litter) (Mylchreest et al., 1999)
rat	other; 0, 250, 500 and 1,000 mg/kg bw exposure of P0 generation only; two generations were produced	LOAEL 250 mg/kg bw for delayed puberty in males of P0 generation, urogenital abnormalities and decreased fertility of F1 males and females (Gray et al., 1999)