Substance Name: Dicyclohexyl phthalate

EC Number: 201-545-9

CAS Number: 84-61-7

SUPPORT DOCUMENT

TO THE OPINION OF THE MEMBER STATE COMMITTEE ON THE IDENTIFICATION FOR

DICYCLOHEXYL PHTHALATE

AS A SUBSTANCE OF VERY HIGH CONCERN BECAUSE OF ITS TOXIC FOR REPRODUCTION (ARTICLE 57C) AND ENDOCRINE DISRUPTING PROPERTIES WHICH CAUSE PROBABLE SERIOUS EFFECTS TO HUMAN HEALTH WHICH GIVE RISE TO AN EQUIVALENT LEVEL OF CONCERN TO THOSE OF CMR[1] AND PBT/VPVB[2] SUBSTANCES

Adopted on 9 June 2016

[1] CMR means carcinogenic, mutagenic or toxic for reproduction
[2] PBT means persistent, bioaccumulative and toxic; vPvB means very persistent and very bioaccumulative

CONTENT

TABLES

ABBREVIATIONS

AGD	Anogenital distance
AMH	Anti-Muellerian Hormone
AMH/MIS	Anti-Muellerian Hormone/Mullerian Inhibiting Substance
AR	Androgen Receptor
ATP	Adaptation to Technical Progress
BBP	butylbenzyl phthalate
DBP	di-n-butyl phthalate
DCHP	dicyclohexyl phthalate
DEP	diethyl phthalate
DEHP	di(2-ethylhexyl) phthalate
DHP	di-n-hexyl phthalate
DMP	dimethyl phthalate
DOP	di-n-octyl phthalate
DPeP	di-n-pentyl phthalate
DPrP	di-n-propyl phthalate
ED	endocrine disruptor
FSH	Follicle Stimulating Hormone
3β-HSD	3β-hydroxysteroid dehydrogenase
17β-HSD3	3β-hydroxysteroid dehydrogenase type 3
GD	Gestation day
LOAEL	Lowest observed adverse effect level
MCHP	moncyclohexyl phthalate
MIS	Mullerian Inhibiting Substance
MoA/HRF	Mode of Action/Human Relevance Framework
NOAEL	No Observed Adverse Effect Level
PCNA	Proliferating Cell Nuclear Antigen
PND	Postnatal day
RAC	Risk Assessment Committee
REACH	Regulation (EC) No 1907/2006 of the European Parliament and of the Council concerning Registration, Evaluation, Authorisation and Restriction of Chemicals
WHO/IPCS	World Health Organisation/International Programme on Chemical Safety

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

Substance Name: Dicyclohexyl phthalate (DCHP)

EC Number: 201-545-9

CAS number: 84-61-7

- DCHP should be identified as a substance meeting the criteria of Article 57 (c) of Regulation (EC) No 1907/2006 (REACH) as, according to the adopted opinion[3] of the Committee for Risk Assessment (RAC), the substance meets the criteria for classification in the hazard class reproductive toxicity category 1B, H360D (May damage the unborn child).

- DCHP 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 the REACH Regulation, owing to its endocrine disrupting properties in humans.

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

DCHP should be identified as a substance of very high concern in accordance with Article 57 (c) of Regulation (EC) 1907/2006 (REACH) because it meets the classification criteria for reproductive toxicity, category 1B.

In accordance with Article 37(4) of the Regulation (EC) No 1272/2008, the Committee for Risk Assessment (RAC) has adopted an opinion for harmonised classification and labelling of dicyclohexyl phthalate. At RAC-31 (December 2014) the RAC adopted the opinion that dicyclohexyl phthalate meets the criteria for classification as toxic for reproduction Repr. 1B, H360D ("May damage the unborn child."). DCHP is included in the 9th ATP to CLP, as agreed at the 44th meeting of the REACH Committee 3-4 February 2016.

DCHP 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 which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of REACH.

DCHP has been shown to adversely affect the endocrine system of mammals primarily through *in vivo* findings on reduced fetal testosterone production. These findings are further substantiated by mechanistic findings of inhibitory effects on enzymes in the steroidogenic biosynthesis pathway. The spectrum of effects observed in rats include increased areola mammae retention, decreased anogenital distance, prolonged preputial separation, genital malformations associated with small testis, signs of reduced sperm quality, atrophic tubules in prostate, prostatic intraepithelial neoplasia and testicular changes including tubular atrophy of which almost all can be considered adverse.

In conclusion, DCHP is considered an endocrine disruptor for human health as it fulfils the World Health Organisation/International Programme on Chemical Safety (WHO/IPCS)

[3] http://www.echa.europa.eu/web/guest/opinions-of-the-committee-for-risk-assessment-on-proposals-for-harmonised-classification-and-labelling/-/substance-rev/8702/term

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.

The endocrine disrupting properties of DCHP give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of REACH because scientific evidence shows that exposure during sensitive time windows of development are likely to cause irreversible developmental programming effects leading to severe effects on development and reproduction. This is regarded as particularly serious in relation to human health also because these adverse effects may first manifest themselves in later life stages because of exposure during early life stages and it may be difficult to establish a toxicological threshold for such effects with sufficient certainty. A reduced ability to reproduce considerably reduces the quality of life for the individuals affected, and it has a negative impact on society as it contributes to an increased financial burden.

Thus, DCHP should be identified as a substance of very high concern in accordance with Article 57 (c) and 57 (f) of Regulation (EC) 1907/2006 (REACH).

Registration dossiers submitted for the substance: Yes

Justification

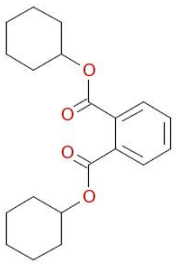
1. Identity of the substance and physical and chemical properties

1.1. Name and other identifiers of the substance

Table 1: Substance identity

EC number:	201-545-9
EC name:	dicyclohexyl phthalate
CAS number (in the EC inventory):	84-61-7
CAS number: **Deleted CAS numbers:**	- 55819-02-8 and 169741-16-6
CAS name:	1,2-benzenedicarboxylic acid, 1,2-dicyclohexyl ester; 84-61-7
IUPAC name:	dicyclohexyl phthalate
Index number in Annex VI of the CLP Regulation	607-719-00-4 (this index number is included in the 9th ATP)
Molecular formula:	$C_{20}H_{26}O_4$
Molecular weight range:	330.418
Synonyms:	1,2-Benzenedicarboxylic acid Dicyclohexylester DCHP

Structural formula:



1.2. Composition of the substance

Name: Dicyclohexyl phthalate

Description: 80 – 100 %

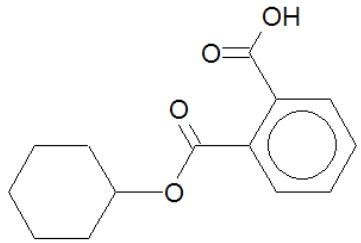
Substance type: mono-constituent

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

Table 2: Degradation (transformation) product/metabolite

EC number:	N.A.
EC name:	N.A.
SMILES:	OC(=O)c1ccccc1C(=O)OC1CCCCC1
CAS number (in the EC inventory):	N.A.
CAS number:	7517-36-4
CAS name:	1,2-Benzenedicarboxylic acid, 1-cyclohexyl ester
IUPAC name:	N.A.
Index number in Annex VI of the CLP Regulation	N.A.
Molecular formula:	$C_{14}H_{16}O_4$
Molecular weight range:	248.3
Synonyms:	*Monocyclohexyl phthalate* *MCHP*

Structural formula:



Indication of the process, organism and/or organ in which the formation takes place:

It is known from studies on other phthalates that the metabolic pathway follows at least two steps: a phase I hydrolysis followed by phase II conjugation. In the first step, the diester phthalate is hydrolysed into the primary metabolite monoester phthalate, which has been linked with adverse effects both *in vitro* and *in vivo* (Frederiksen et al. 2007). Data indicates that the metabolism of DCHP to the monoester (MCHP) would take place primarily at the intestine. The intestinal metabolism of DCHP to MCHP by both rats and human intestine (Lake et al 1977) is further confirmed by a study with 16 hr incubation of 1% DCHP *in vitro* (IUCLID Dataset, ECB).

1.4. Physicochemical properties

Table 3: Overview of physicochemical properties

Property	Description of key information	Value	Reference/source of information
Physical state at 20°C and 101.3 kPa	*Evidence due to substance observation and handling.*	*Crystalline powder with slightly aromatic odour*	*ECHA dissemination site (2015)*
Melting point	*Measured, ASTM E537-07*	*ca. 65.6 °C* *at 1 atm*	*ECHA dissemination site (2015)*
Boiling point	*Measured, ASTM E537-07*	*ca. 322.03 °C at 1 atm*	*ECHA dissemination site (2015)*
Vapour pressure	*Measured, Dew-Point and Tensimeter method*	*8.7×10^{-7} mm Hg at 25 °C*	*Werner, 1952 – from ECHA dissemination site (2015)*
Density	*Measured, USP 34-NF29 <616>*	*Density 0.787 g/ml*	*ECHA dissemination site (2015)*
Water solubility	*Measured, OECD 105/1995*	*1.015 mg/L (20°C and pH 7)*	*ECHA dissemination site (2015)*
Partition coefficient n-octanol/water (log value)	*Estimated value obtained by extrapolation from the calibration curve, OECD 117*	*Log Kow= 4.82 (25oC)* *Log Kow = 6.2 (estimated)*	*ECHA dissemination site (2015)* *KowWin (v1.68)*

2. Harmonised classification and labelling

In accordance with Article 37(4) of the Regulation (EC) No 1272/2008, the Committee for Risk Assessment (RAC) has at RAC-31 adopted an opinion for harmonised classification and labelling of dicyclohexyl phthalate stating that dicyclohexyl phthalate meets the criteria for classification as toxic for reproduction Repr. 1B, H360D ("May damage the unborn child.").

Therefore, dicyclohexyl phthalate meets the criteria of Article 57(c) of Regulation (EC) 1907/2006 (REACH Regulation). DCHP is included in the 9th ATP to CLP, which was agreed at the 44th meeting of the REACH Committee 3-4 February 2016.

Table 4: Classification according to Annex VI, Table 3.1 (list of harmonised classification and labelling of hazardous substances) of Regulation (EC) No 1272/2008

Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Spec. Conc. Limits, M-factors	Notes
				Hazard Class and Category Code(s)	Hazard statement code(s)	Pictogram, Signal Word Code(s)	Hazard statement code(s)	Suppl. Hazard statement code(s)		
607-719-00-4	Dicyclohexyl phthalate	201-545-9	84-61-7	Repr. 1B Skin Sens. 1	H360D H317	GSH07 GSH08 Dgr	H360D H317			

3. Environmental fate properties

Not relevant for this dossier.

4. Human health hazard assessment

Introduction

The human health hazard assessment is not relevant for the identification of the substance as SVHC in accordance with Article 57 (c) REACH, since this is based on the opinion adopted by RAC that DCHP meets the criteria for classification as a reproductive toxicant in hazard class reproductive toxicity category 1B. However, for the identification of the substance in accordance with Article 57 (f) data should be presented. Therefore, relevant data in relation to the endocrine disrupting properties have been collected and is presented in the following sections, including toxicity for reproduction.

General approach

Criteria on how to assess whether or not a biocide or pesticide substance has endocrine disrupting properties and/or is an endocrine disruptor are currently being developed in the European Union.

The basis for the criteria is envisaged to be the widely accepted definition of an endocrine disruptor by the WHO/IPCS (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).

It is assumed in this report that a substance should fulfil the recommendations from the European Commission's Endocrine Disrupters Expert Advisory group outlined above in order to be identified as an endocrine disruptor, and available information has accordingly been assessed based on:

- Adverse health effects
- Endocrine mode of action

- Plausible link between adverse effects and endocrine mode of action
- Human and/or environmental relevance

Furthermore, relating to REACH and the ongoing SVHC Roadmap work, the available data on DCHP has been presented and discussed in the Endocrine Disruptor Expert Group at ECHA. A broad majority considered the available data sufficient to conclude that the substance meets the WHO/IPCS definition of an endocrine disrupting substance and that the data are relevant for humans.

The most marked adverse effects of DCHP have been described for the male reproductive system and most work performed to elucidate the mode of action of DCHP has been carried out in experimental tests studying developing male rats. The following discussion therefore focuses on endocrine disrupting effects on male reproduction. DCHP may also have other endocrine disrupting modes of action, but these will only be discussed briefly.

4.1. Toxicokinetics (absorption, metabolism, distribution and elimination)

There is limited toxicokinetic data available for DCHP. Lake et al. (1977) showed that DCHP is hydrolysed to its monoester and to an alcohol moiety (cyclohexanol) *in vitro* by rat, ferret and primate (baboon) liver and intestinal preparations as well as by human intestinal preparations. Similar results were obtained for other phthalates that also were examined in the study; dimethyl phthalate (DMP), diethyl phthalate (DEP), di-n-butyl phthalate (DBP), di-n-octyl phthalate (DOP) and di(2-ethylhexyl) phthalate (DEHP). For all the compounds examined, the hepatic hydrolase activity generally decreased in the order baboon > rat > ferret (Lake et al., 1977). There are no data on the formation of oxygenated metabolites of DCHP.

Saito et al. (2010) showed that eight phthalates (diethyl phthalate (DEP), di-n-propyl phthalate (DPrP), di-n-butyl phthalate (DBP), di-n-pentyl phthalate (DPeP), di-n-hexyl phthalate (DHP), DEHP, n-butyl benzyl phthalate (BBP), and dicyclohexyl phthalate (DCHP)) were all hydrolyzed to their corresponding monoesters by both porcine and bovine pancreatic cholesterol esterases. The hydrolysis experiment with bovine pancreatic cholesterol esterases showed complete hydrolysis of each phthalate (5 pmole), except for BBP and DCHP, within 15 min; BBP and DCHP were hydrolyzed within 30 min and 6 h, respectively. The authors concluded that the rates of phthalate hydrolysis could be affected by the bulkiness of the alkyl side chains in the phthalate ester.

No data are available on absorption or elimination kinetics of DCHP. However, Fredriksen et al. (2007) present a review of metabolism of phthalates in humans, where human data are compared to data from rat studies. It is known that the metabolic pathway follows at least two steps: a phase I hydrolysis followed by phase II conjugation. In the first step, the diester phthalate is hydrolysed into the primary metabolite monoester phthalate, which has been linked with adverse effects both *in vitro* and *in vivo*. Humans excrete both primary and secondary metabolites of phthalates as do the rat, however the proportions of primary and secondary metabolites can vary between species. This review also shows that there may be differences in metabolism at different ages and that the pattern of metabolites can differ in different tissues, blood and urine. The review does however describe that phthalates are absorbed and excreted by humans and that phthalates can reach the fetus *in utero* and the nursing baby through the breast milk.

4.2. Toxicity for reproduction

This section does not aim to analyse in detail the reproductive toxicity information that has already been assessed in the CLH process[4]. The section summarises the relevant *in*

[4] http://www.echa.europa.eu/web/guest/opinions-of-the-committee-for-risk-assessment-on-proposals-for-

vivo studies (developmental toxicity and fertility studies) for DCHP (the robust study summaries are given in Annex I). The available information is further used in the hazard identification for endocrine properties described in Annex II.

The data used to evaluate the adverse effects of DCHP for the CLH-dossier is from one 2-generation study (OECD TG 416, 1983 protocol, key study, Hoshino et al. 2005) and three supporting experimental studies with exposure for a limited period during gestation (Aydogan and Barlas 2013, and Saillenfait et al 2009) and during gestation up to postnatal day (PND) 20 (Yamasaki 2009). An additional study (Aydogan and Barlas 2015) with exposure for a limited period during gestation has been published after the CLH process. The main effects observed are on male reproductive organs. The effects were organ weight and histopathological findings in prostate and testes, signs of reduced sperm quality, reduced anogenital distance (AGD, absolute as well as relative to the cubic root of the fetal weight) and retained areola mammae. This indicates that male reproductive organs are the target organs and that the effect is likely to be mediated through an anti-androgenic or estrogenic mode of action. The effect on areola mammae also appears to be more pronounced in the F2-generation, 63% ($p<0.01$) of the F2 litters were affected as compared to 16.1% ($p<0.01$) of the F1 litters.

In the key study the most sensitive endpoints were lowered prostate weight, reduced AGD and retained areola mammae in rats (LOAEL 80-107 mg/kg bw/day, NOAEL 16-21 mg/kg bw/day) (Hoshino et al, 2005)[5].

Aydogan and Barlas (2015) reported that DCHP exposure (0, 20, 100 and 500 mg/kg bw/day) *in utero* (GD 6-19) resulted in an increased number of litters with resorptions (3% (control group), 33%, 31% and 26 %, respectively), decrease in the male fetal pups AGD (related to the cubic root of the body weight) and increased Inhibin B levels in all dose groups at GD 20. Testosterone and anti-Mullerian hormone (AMH/MIS), as well as the follicle stimulating hormone (FSH) to Inhibin B ratio decreased in the mid- and high dose groups. Histopathological effects in the testes were also evident in a dose dependent manner showing atrophic and small seminiferous chords, decreased germ cells in chords, Sertoli cell chords only, chords with cells detached from wall and presence of multinucleated germ cells. Immunohistochemical staining of testis tissue showed decreased presence of 3β-hydroxysteroid dehydrogenase (3β-HSD), AMH/MIS, and androgen receptor (AR) in all dose groups and in the highest dose group also decreased proliferating cell nuclear antigen (PCNA).

In the study by Aydogan and Barlas (2013)[6], histopathological changes were observed in testes, epididymis and prostate at all dose levels with a LOAEL of 20 mg/kg bw/day (tubular atrophy, germinal cell debris, apoptotic cells, and Sertoli cell vacuolisation in the testis; presence of spermatogenic cells in lumen of the epididymidis; and increase in atrophic tubules and intraepithelial neoplasia in the prostate). For testes and epididymis in prepubertal animals (PND20), the incidences of these findings increased in a dose dependent manner. For the prostate, the effects were dose dependent for pubertal (PND 32) and adult animals (PND90). Incidences of effects on the testes (including tubular atrophy) were statistically significantly increased in prepubertal and pubertal animals but not in adults. On the other hand, dose-dependently increased Sertoli cell vacuolisation was reported only in adult animals. The percentage of abnormal epididymal sperm was significantly increased at all doses (10.9% (control group), 27.6%, 23% and 27.4% at 0, 20, 100 and 500 mg/kg/day, respectively) in the adult animals.

There are no effects on fertility, mating and gestation and birth index in the key study (Hoshino et al 2005). In one of the supporting studies (Yamasaki et al. 2009)[7] the

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[5] Two-generation (OECD TG 416, 1983), with extra parameters for detecting endocrine disrupting activity (Yamasaki et al. 2005), for more details see Annex I.

[6] GD 6-19 *in utero* exposure with 0, 20, 100 and 500 mg/kg bw/day and evaluation of endpoints PND 20 (prepubertal),32 (pubertal) and 90 (adult).

[7] Exposure GD6 – PND20, oral gavage 0, 20, 100 or 500 mg/kg, 10/group

viability index at PND4 is slightly but statistically significantly lowered (-2.2%) in the high dose group. Delayed preputial separation and two cases of hypospadias[8], and a decreased relative weight of the levator ani/bulbocavernousus muscle is also reported in the high dose group.

The 2-generation key study (Hoshino et al 2005) reports an increased absolute and relative thyroid weight and signs of increased thyroid activity (hypertrophy of follicular cells) in the high dose group. This was not seen in the study by Yamasaki et al (2009), however the period for dosing in the latter was only GD6–PND20.

In some of the studies effects on body weight and weight gain are reported (Hoshino et al 2005, Saillenfait et al 2009). The effects on maternal body weight gain are, however, considered not to be of the magnitude that could explain the effects on male reproductive organs, thus the effects on male reproductive organs are relevant.

4.2.1. Summary and discussion of reproductive toxicity

In the RAC opinion (RAC-31, December 2014) for DCHP, the reproductive toxicity data are summarised as follows "*The experimental animal data for DCHP effects on development indicated a reduced AGD and an increased incidence of areola mammae in male pups. These effects were reported in three independent studies in the absence of marked maternal toxicity. In addition, prolonged preputial separation and hypospadias associated with small testis was described in one of the studies. The adverse effects observed in the Aydogan (2013) study in male reproductive organs, including testicular tubular atrophy and atrophic tubules in the prostate, occurred after in utero exposure and were considered as supportive evidence for developmental effects. Taken together, all these effects, which were observed following parental exposure in the absence of marked maternal toxicity, provide clear evidence of an adverse effect on development in the absence of other toxic effects. These effects have also been shown to occur following exposure to various transitional phthalates and are consistent with an anti-androgenic action of DCHP, which is considered relevant to humans.*"

The Aydogan and Barlas (2015) study (published after the decision by RAC) contributed further evidence on reproductive effects on the male reproductive system.

For the purpose of identification of DCHP as SVHC on the basis of an endocrine mode of action, the reproductive toxicity information together with additional mechanistic data have been analysed and are presented in section 4.3.2 below (and in more detail in Annex II).

4.3. Endocrine disrupting properties

4.3.1. Non-human information

4.3.1.1. *In vitro* mechanistic data

Estrogenic/Anti-estrogenic activity

DCHP gave mixed results in estrogenic *in vitro* assays. It induced MCF7 cell proliferation (Hong et al. 2005, Okubo et al. 2003) whereas its metabolite mono-cyclohexyl phthalate (MCHP) inhibited the 17β-estradiol induced MCF7 cell proliferation (Okubo et al., 2003). In a study by Nakai et al. (1999) it showed a characteristic biphasic binding curve with different affinities for the high and low binding sites on the estrogen receptor. Nishihara et al. (2000) found DCHP to be negative in a yeast two-hybrid assay with ERα, whereas in another assay utilising transcriptional activities via human receptors it was agonistic to ERα and antagonistic to ERβ (Takeuchi et al. 2005). DCHP gave negative estrogenic results in a couple of *in vivo* studies where it had no effect on CaBP-9k mRNA and protein levels in the uterus (Hong et al. 2005) and was negative (did not increase

[8] 10 litters with an average of 13.5 pups/litter, 45% of them being male=approx. 3% of the pups affected

uterine weight) in a uterotrophic assay (Yamasaki et al. 2002), thus no estrogenic effects were detected. In summary, studies with DCHP have shown different results for estrogenic/anti-estrogenic activity *in vitro*. The available *in vivo* tests in rats do not show estrogenic/anti-estrogenic activity for DCHP.

Androgenic/anti-androgenic

In a reporter gene assay, DCHP, as well as other C3 to C6 dialkyl phthalates (except diphenyl phthalate) inhibited human AR-mediated transcriptional activity (Takeuchi et al. 2005).

Steroidogenesis

DCHP inhibits the enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD3) *in vitro*, both of which are involved in biosynthesis of androgens in testis (Yuan et al. 2012). Furthermore, in a screening assay assessing fetal testosterone production *ex vivo* after *in utero* exposure DCHP significantly reduced testosterone production in the fetal testis (Furr et al. 2014).

Other hormones

Other *in vitro* assays indicate potential activity relating to effects on adipogenesis, thyroid receptor transcription and membrane signalling via the nicotinic acetylcholine receptor (nAChR) (Sargis et al 2010, Sugiyama et al 2005, Liu and Lin 2002, Lu et al 2004). Data showing adverse effects related to these hormonal effects are, however, not available, even though a change of thyroid weight and histopathological hyperthrophy of thyroid follicular cells were reported in the 2-generation study (Hoshini et al. 2005).

4.3.2. Mode of Action Framework

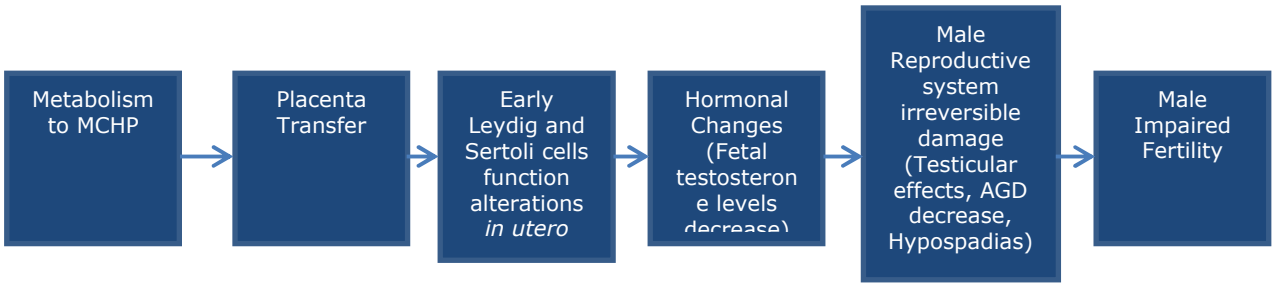
For the purpose of identification of DCHP as an endocrine disrupter, the WHO/IPCS Mode of Action/ Human Relevance Framework (MoA/HRF)[9] has been used.

The assessment of the reliability of the available *in vivo* DCHP studies is presented in Annex III. All available information has been used in a weight of evidence approach using the Bradford Hill considerations.

The MoA/HRF framework focused on:

- the weight of evidence analysis
- the establishment of the MoA endocrine mediated irreversible effects observed with DCHP in experimental species
- the establishment of human relevance

The MoA/HRF analysis is based on the following hypothesised mode of action:



The detailed MoA analysis is presented in Annex II of this document including the elements of male reproductive system adverse effects, endocrine mediated mode of action, establishment of a plausible causal relationship between adverse effects and the

[9] Available at: http://echa.europa.eu/support/guidance-on-reach-and-clp-implementation/formats

endocrine mode of action, and human relevance.

The summary of the species concordance analysis (human relevance) as developed with the use of the WHO/IPCS MoA/HRF are presented below.

Qualitative Concordance				***Quantitative Concordance***	
Key Event (name)	***(Evidence in Experimental Species)***	***(Evidence in Humans)***	***Confidence***	***(Evidence in Experimental Species)***	***Confidence***
Metabolism of DCHP to MCHP	Evidence based	Plausible and some evidence based	High		
Placenta Transfer	Likely, sufficient evidence available	Plausible	Medium		
Early Leydig and Sertoli cells function alterations *in utero*	Evidence based	Plausible	Medium	Evidence based (see section 5a in Annex II)	Medium
Hormonal changes (Fetal testosterone levels decrease)	Evidence based	Plausible	High	Evidence based (see section 5a in Annex II)	High
Male Reproduction system irreversible damage Testicular effects (Leydig cells, Sertoli cells, Epididymis)	Evidence based	Plausible	High	Evidence based (see section 5a in Annex II)	High
Male Impaired Fertility	Likely, not chemical specific (DCHP) based	Plausible	Medium		

4.3.3. Summary and discussion of endocrine disrupting properties

As outlined in section 4 (general approach), it is assumed in this report 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 available information has accordingly been assessed based on:

Adverse effects:

DCHP caused adverse effects on the male reproductive system in more than one whole-animal toxicity study (Hoshino et al. 2005, Yamasaki et al. 2009, Saillenfait et al. 2009, Aydogan and Barlas 2013 and 2015) of acceptable quality (one OECD test guideline compliant standard 2-generation study and four non-standard studies) with relevant routes of exposure (oral via diet/gavage). The spectrum of effects observed in rats include increased areola mammae retention, decreased anogenital distance, prolonged

preputial separation, genital malformations associated with small testis, signs of reduced sperm quality, atrophic tubules in prostate, prostatic intraepithelial neoplasia and testicular changes including tubular atrophy of which almost all can be considered adverse (OECD, 2008).

Endocrine mode of action:

The overall weight of evidence analysis shows that the adverse effects on the male reproductive organs observed following *in utero* exposure to DCHP are mediated via an endocrine (antiandrogenic) mode of action that involves irreversible effects induced by interference with steroidogenesis resulting in decreased testosterone levels during fetal development.

Plausible link between adverse effects and endocrine mode of action:
The MoA framework analysis establishes a plausible relationship between the distinct key events identified for the hypothesised endocrine mediated mode of action. There is a plausible mechanistic link between the toxic effects of concern and endocrine disruption (an anti-androgenic mode of action) as the cause of the adverse effects in the male reproductive system, observed in the studies described above. This takes into account dose-response relationships and temporal association.

Human relevance:

The effects observed in experimental animals are judged to be relevant to human health on the basis of biological plausibility, taking into account existing knowledge on established pathways for male reproductive system development across species, as well as the absence of contradicting data to exclude human relevance.

5. Environmental hazard assessment

Not relevant for this dossier.

6. Conclusions on the SVHC Properties

6.1. CMR assessment

According to the RAC-opinion (RAC-31, December 2014) DCHP meets the criteria for classification as Repro 1B (H360D, "May damage the unborn child").

Therefore, dicyclohexyl phthalate meets the criteria of Article 57(c) of Regulation (EC) 1907/2006 (REACH Regulation). DCHP is included in the 9th ATP to CLP, which was agreed at the 44th meeting of the REACH Committee 3-4 February 2016.

6.2. Equivalent level of concern assessment

6.2.1. Conclusion on fulfilment of WHO/IPCS definition of endocrine disruptor

A summary of the findings in chapters 4 and 5 are compared with the definition of an endocrine disruptor 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 disruptor.

According to the widely accepted definition of an endocrine disruptor by the WHO/IPCS (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 and/or environmental relevance

Re 1) The spectrum of effects on the male reproductive system observed in rats include increased areola mammae retention, decreased anogenital distance, prolonged preputial separation, genital malformations associated with small testis, signs of reduced sperm quality, atrophic tubules in prostate, prostatic intraepithelial neoplasia and testicular changes including tubular atrophy, of which almost all can be considered adverse (OECD, 2008). DCHP causes adverse – and serious – reproductive toxicity effects in rodents and a harmonised classification Repr. 1 B has been concluded.

Re 2) The overall weight of evidence analysis shows that the male reproductive effects observed following *in utero* exposure to DCHP are mediated via an endocrine (antiandrogenic) mode of action that involves irreversible effects induced by interference with steroidogenesis during fetal development.

Re 3) The MoA framework analysis establishes a plausible relationship between the distinct key events identified for the hypothesised endocrine mediated mode of action. There is a plausible mechanistic link between the toxic effects of concern and endocrine disruption (an anti-androgenic mode of action) as the cause of the adverse effects in the male reproductive system, this takes into account dose-response relationships and temporal association. It is considered biologically highly plausible that the observed adverse effects in rats are linked to the endocrine disrupting mode of action of DCHP and its metabolite MCHP.

Re 4) The effects observed in experimental animals are judged to be relevant to human health on the basis of biological plausibility, taking into account existing knowledge on established pathways for male reproductive system development across species, as well as the absence of contradicting data to exclude human relevance. DCHP causes serious adverse reproductive toxicity effects in rodents and a harmonized classification Repr. 1B has been concluded. Hence it can be concluded that human relevance has been agreed for the adverse effects.

In conclusion, when available information is combined DCHP is considered an endocrine disruptor 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.

6.2.2. 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 DCHP, in addition to fulfilling the definition of an endocrine disruptor from WHO/IPCS and further elaborated by the European Commission's Endocrine Disrupters Expert Advisory Group (JRC 2013), also fulfils Article 57(f), the following are considered.

Human health

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. 57(f) directly refers). In addition, the seriousness of the reproductive effects concerned can be characterized in the following way:

- *Potential severity*: DCHP adversely affects the normal development and the reproductive ability.
- *Irreversibility:* The adverse effects concerned such as reduced ability to produce semen 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 affect reproductive ability, such as reduced number of spermatocytes, testicular changes, tubular atrophy and organ malformations or misfunction, will not manifest themselves fully until reproductive age.
- *A toxicological threshold* for the endocrine mediated reproductive toxic effects may be difficult to establish for DCHP.
- *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 society as it contributes to an increased financial burden e.g. on the health care sector, both providing assisted fertilisation treatments and clinical treatment for individuals with adverse reproductive effects postnatally.

6.2.3. Conclusion on whether the substance gives rise to an equivalent level of concern

As stated above, DCHP should be identified as a substance of very high concern in accordance with Article 57 (c) of Regulation (EC) 1907/2006 (REACH) because it meets the classification criteria for reproductive toxicity, category 1B.

In accordance with Article 37(4) of the Regulation (EC) No 1272/20084, the Committee for Risk Assessment (RAC) has adopted an opinion for harmonised classification and labelling of dicyclohexyl phthalate. At RAC-31 (December 2014) the RAC adopted the opinion that dicyclohexyl phthalate meets the criteria for classification as toxic for reproduction Repr. 1B, H360D ("May damage the unborn child.").

In addition, DCHP 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 which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of REACH.

DCHP has been shown to adversely affect the endocrine system of mammals primarily through *in vivo* findings on reduced fetal testosterone production. These findings are further substantiated by mechanistic findings of inhibitory effects on enzymes in the steroidogenic biosynthesis pathway. The spectrum of effects observed in rats include increased areola mammae retention, decreased anogenital distance, prolonged preputial separation, genital malformations associated with small testis, signs of reduced sperm quality, atrophic tubules in prostate, prostatic intraepithelial neoplasia and testicular changes including tubular atrophy, of which almost all can be considered adverse.

In conclusion, DCHP is considered an endocrine disruptor 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.

The endocrine disrupting properties of DCHP give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of REACH because scientific evidence shows that exposure during sensitive time windows of development are likely to cause irreversible developmental programming effects leading to severe effects on development and reproduction. This is regarded as particularly serious in relation to human health also because these adverse effects may first manifest themselves in later life stages because of exposure during early life stages and it may be difficult to establish a toxicological threshold for such effects with sufficient certainty. A reduced ability to reproduce considerably reduces the quality of life for the individuals affected, and it has a negative impact on society as it contributes to an increased financial burden.

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Annex I - Robust study summaries of toxicological *in vivo* studies on DCHP

The study summaries below are copied from the CLH-report, except for Aydogan and Barlas (2015), which was published after the harmonised classification of the substance was adopted by RAC. The studies included are considered relevant and reliable, using the Scirap tool[10] and reliability criteria (for more information on this see Annex III). In addition, the level in the OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters[11] (2012) to which the study belongs is indicated.

Hoshino et al. 2005 – Key study

Reference	Hoshino N., Iwai M., Okazaki Y. A Two-Generation Reproductive Study of Dicyclohexyl Phthalate in Rats. The Journal of Toxicological Sciences, Volume 30, Special Issue, 79-96, 2005 Additional information in: Yamasaki K., Takahashi M, Yasuda M. 2005. Two-Generation Reproductive Toxicity Studies in rats with extra parameters for detecting endocrine disrupting activity: Introductory overview of results from nine chemicals. The Journal of Toxicological Sciences, Volume 30, Special Issue, 1-4, 2005
Reliability	R1
OECD conceptual framework	Level 5
Method	• Two-generation study (dietary) in accordance with OECD TG 416 of 1983, protocol enhanced with parameters to detect endocrine disrupting activity. • 24 animals /sex/dose • Rats (Crj:CD(SD)IGS • F0: 5 week of age at start of dosing
Test substance & Dose	DCHP (CAS No. 84-61-7, 99.9% purity) 0, 240, 1200, or 6000 ppm (corresponding to for F0 males : 0, 16, 80 and 402; F0 female: 0, 21, 105 and 511; F1 males: 0, 18, 90 and 457; F1 females: 0, 21, 107 and 534 mg/kg bw/day, respectively, when taking mean daily intake during the entire dosing period into account) F0 males: dosed at least 10 weeks before mating and during mating F0 females: dosed at least 10 weeks before start of mating continuing until weaning of F1 offspring (PND 21). F1: from PND21 continuing to end of mating for males (mating at ~14 – 15 weeks of age), and females being dosed until lactation day 21.
Results	<u>Effects on body weights, necropsy and clinical observation</u> • F0 males: no significant effects on body weights. No clinical signs. • F0 females: slightly decreased body weights ($p<0.01$ from 2 weeks of dosing continuing until end of lactation for high dose group (~ 10-12 % lower body weight, as compared to controls, from premating until PND 21 as judged from the graphical presentation of this data in the paper) and for intermediate group on occasional days (mostly $p<0.05$) up until end of pregnancy and more frequently during the period of lactation ($p<0.05$ /0.01). At end of study the intermediate dose group weighed ~5% less than the controls. No clinical signs. • F1 males: A very slightly decreased weight from birth and onwards (but statistically significant $p<0.01$) in high dose animals. The effects on body weight got more pronounced as treatment continued over time and after ~10 weeks of dosing decreased body weights ($p<0.01$) was also

[10] www.scirap.org
[11] Available at: http://www.oecd.org/env/ehs/testing/OECD%20Conceptual%20Framework%20for%20Testing%20and%20Assessment%20of%20Endocrine%20Disrupters%20for%20the%20public%20website.pdf

	observed in the intermediate dose group (4% less in the intermediate and 9% less in the high dose group as compared to the controls as judged from the graphical presentation of this data). No clinical signs. • F1 high dose females showed a somewhat lower weight at birth until weaning ($p<0.01$) and then also during the entire period of gestation and lactation ($p<0.05$/0.0l, being maximum 8-9 % less as compared to controls as judge from the graphical presentation of the data). No clinical signs. Organ weights and histopathology • Increased absolute (+21%) and relative (+24%) liver weight of males and females (+9% and +19%, respectively) in the high dose groups of the F0 generation. An increased relative liver weight in the F1 generation (+14% M and +16% F), animals at the high dose level. At the intermediate dose level, an increased relative weight (+6%) in F0 females and a decreased absolute weight (-12%) in F1 male were recorded. • At histopathological examination, an increased incidence of diffuse hypertrophy (severity score slight) of hepatocytes was observed at the high dose level (both genders of F0 and F1 generation) and at a lower incidence in F0 males and females at the intermediate dose level. • Increased thyroid weight was seen at the high dose level in the F0 generation (males: ~+30% both in absolute and relative terms but only seen in left gland; females: +15-24% in only relative weight of both glands). No effects in F1 generation. Increased incidence of thyroid follicular cell hypertrophy (severity slight) in high dose animals (F0 and F1 animals) and intermediate F0 males. • Increased hyaline droplets in the renal proximal tubular epithelium were observed in both F0 and F1 males including controls without a dose response for the slight severity grade. For the moderate severity grade a high incidence (F0: 15; F1: 8), as compared to the controls (1 in both) was recorded in the high dose males. • Statistically significant decrease in absolute (19%, 16% and 28% less as compared to controls in low, intermediate and high dose groups, respectively) and relative (statistically significant only at the high dose level, -19%) weight of the prostate in F1 (no effects on prostate weight in the F0). Diffuse atrophy of the seminiferous tubules (severe grade) was seen in 3 high dose males of the F1 generation and a lack of sperm in the epididymal tubules was also observed in these animals. Focal atrophy (slight severity) was seen in 1, 0, 2, 6 males in the control, low, intermediate and high dose groups, respectively, in the F1 generation. Effects on fertility and hormone levels No statistically significant effect on mating or fertility indices or on the number of days between start of mating until day of confirmed copulation, or on gestation length or gestation index for the F0 and F1 generations. The values for the mating and fertility indices showed slight tendencies for decrease in the F1 high dose group (90.5 and 89.5 as compared to 95 and 100%, respectively). The authors considered that this was associated with the testicular changes (soft and/or small size) recognized in three males at necropsy. In the other F1 high dose males copulation and resultant pregnancies were normal. Dose dependent decrease in number of testicular homogenization resistant spermatids in the intermediate and high dose (15 and 24 % less as compared to controls) of the F1 generation (no effect observed in F0 and F2 was not examined). In the F1 male parents of the high dose group, soft and small sized testes were observed in one animal, and examination of this rat revealed no sperm. There were no effects on epididymal sperm motility, number or morphology in either F0 or F1 generation (endpoint not examined in F2). Minimal (+5% longer) but statistically significant increase of the estrous cycle length was recorded for the F0 high dose group (no effect recorded in F1) but no females displayed abnormal cycles. The effect was thought to be secondary to the suppression of body weight gain by the authors. There were no dose-dependent effects on testosterone/estradiol, FSH and LH levels in F0 or F1 animals. Developmental effects • F1 and F2: No effects on sex ratio, litter size, viability index or on survival. No effects on physical development as revealed by effects on pinna unfolding or on time point for incisor eruption or eye opening. • Slightly (4-6%, but statistically significant), decreased birth weight in high dose F1 males and females. The effects on bodyweight were observed throughout lactation and at weaning pups (males and females) weighed 11 - 12% less than the controls. F2 males and females weighed about the same as the controls at birth and up until postnatal day 21 when a slight (8-9%, $p<0.01$) reduced body weight was observed at the high dose level. • Time point for pre-putial separation was delayed (not statistically significant) and coincided with a statistically significantly decreased body weight at day of preputial separation in F1 high dose males. No effects on day of vaginal opening in F1 females. • Male pups showed a decreased absolute (F1: -7%, $p<0.01$; F2: -9% $p<0.01$) and relative (F1: -8%, $p<0.01$; F2: -9%, $p<0.01$) anogenital distance at the high dose level and this effect was also seen at the

	intermediate dose level in F2 (-7% and -7% for absolute and relative distance, p<0.01). • The percentage of litters with male pups that had areola mammae was clearly increased at the high dose level (16.1% in F1 and 63.2% in F2, as compared to 0% in controls). The effect was also evident at the intermediate dose level but only in the F2 generation (18.4% as compared to 0% in the controls). However no nipples were recorded in the male pups of either generation. • NOAEL for effects on the parental animals, including the endocrine system was 240 ppm based on effects on liver and body weights. • NOAEL for reproductive adverse effects on parental animals is 240 ppm for males and 1200 ppm for females. NOAEL for offspring is 240 ppm for males and 1200 ppm for females.

The authors summarize the study in the abstract as follows:
"*The reproductive toxicity of dicyclohexyl phthalate (DCHP) was evaluated in a two generation test in which male and female Sprague-Dawley (SD) rats of parental (F0) and F1 generation were exposed to DCHP in the diet at concentrations of 0 (control), 240, 1200 or 6000 ppm. With regard to the effects on the F0 and F1 parental animals, changes included inhibition of body weight gain and food consumption, diffuse hypertrophy of hepatocytes, and hypertrophy of thyroidal follicular epithelial cells at the doses of 1200 ppm and 6000 ppm. The following changes were observed in the 6000 ppm group: increase weights of the liver and thyroid, increased hyaline droplets in the renal proximal tubular epithelium (F0 and F1 males), reduction of prostatic weight (F1 males), and diffuse and/or focal atrophy of testicular seminiferous tubules (F1 males). In addition, slight prolongation of the estrous cycle was noted in the F0 females of the 6000 ppm group, along with reduced spermatid head counts in the testes (homogenation-resistant spermatids) in F1 male receiving doses of 1200 ppm or 6000 ppm. It is thought that the prolonged estrous cycle was secondary to the suppression of body weight gain. There were no test substance related changes in clinical signs and reproductive capability (mating, fertility, gestation and birth index), or in data for the delivery and lactational periods, or serum hormone levels. With regard to effects on the offspring, inhibition of body weight gain was found in the F1 and F2 6000 ppm, and decrease of anogenital distance (AGD) and appearance of areola mammae were observed in the F1 male 6000 ppm and F2 male receiving doses of 1200 ppm or 6000 ppm. No effects of DCHP treatment on the offspring were observed on results of clinical signs, the number of the pups delivered, sex ratio, viability, physical development, reflex and response tests, external abnormalities, organ weights, or necropsy findings. From the present study of DCHP administered to rats over two-generations, the no observed effect level (NOEL) for effects on the parental animals including the endocrine system, is considered to be 240 ppm. With regard to the reproductive toxicological effects on the parental animals, the NOEL is 240 ppm for males and 1200 ppm for females. For offspring, the NOEL values are concluded to be 240 ppm for males and 1200 ppm for females.*"

Yamasaki 2009 – Supporting study

Reference	Yamasaki K., Okuda H., Takeuchi T., Minobe Y. Effects of *in utero* through lactational exposure to dicyclohexyl phthalate p,p'-DDE in Sprague-Dawley rats. Toxicolcogy Letters 189,2009,14-20.
Reliability	R2
OECD conceptual framework	Level 4
Method	• 40 mated Crl:CD(SD)IGS female rats (F0) (~12 weeks old) subdivided into 4 equally sized groups (10/group). • Culling at PND 4, to litter size of 8 aiming for 4 pups/sex when possible. • At weaning pups (F1) in each group were randomly subdivided into 2 sub-groups. A. Sacrificed at 10 weeks of age. Examined externally (nipples and effect on external sex organs), vaginal cytology from 8 weeks. Necropsied and examined internally for ectopic or atrophic testes; agenesis of the gubernaculums, epididymis and sex accessory glands; and epididymal granulomas. The following organs were weighed after necropsy: uterus, ovaries, testes, epididymis, ventral prostate, seminal vesicles with coagulation gland, levator ani /bulbocavernosus muscles, brain, liver, adrenals, kidneys, thyroids, and pituitary. B. 2 females and 2 males/dam were mated at 12 weeks to assess reproductive performance and possible effects on early embryonic development (cesarean sections performed on gestation day 13). Adult males and females necropsied and the same organs as in subgroup A were weighed.

	Non-GLP study
Test substance & Dose	• 0, 20, 100 or 500 mg/kg bw/day of DCHP (CAS No. 84-61-7, 99.9% purity) via oral gavage between gestation day (GD) 6 and postnatal day (PND) 20 • Vehicle: olive oil • Dose volume: 2 ml/kg
Results	Adult toxicity • F0: No effects on body weight. Dose-dependent increased liver weights (absolute and relative), being statistically significantly ($p<0.05$) higher at the intermediate and high dose level (+7 and +24 % as compared to controls). No information on weights of other organs. • F0: Dystocia in one high dose female that died on GD 23 before parturition was completed; otherwise no effect on reproductive performance. • F1 (at necropsy week 10) o Decreased ($p<0.05$) ventral prostate weight at the low and high dose (-16% and -28% as compared to controls), but no dose dependency since the mid dose was less affected (-10%) than the low dose. o Decreased ($p<0.05$) relative weight (-12% as compared to controls) of the levator ani/bulbocavernosus muscle and slight histological changes, including decreased testicular germ cells and degenerated renal proximal tubules (incidence data not shown) in the high dose group. o No statistically significant effects on body weight, relative weights of the brain, pituitary, thyroid, adrenal, kidney, liver, ovary and uterus. • No effect on reproductive performance of F1-generation at 12 weeks of age (Sub- group B). Developmental effects • F1:Minimal (-2.2%) but statistically significantly decreased viability index on PND 4 in the high dose group. No effect on live birth index, sex ratio at PND 0, number of live pups on PND 4 or PND 21 or on weaning index on PND 21. • F1: Significantly decreased male and female pup weight at PND 14 and/or PND 21 (detailed data not provided). • F1 high dose male: o Hypospadias (combined with small testes) in 2 male pups, one sacrificed at 7 weeks due to poor condition. o ~2 days delayed ($p<0.05$) preputial separation in high dose males. No information provided for lower dose levels. o PND 4: Statistically significantly ($p<0.05$) decreased anogenital distance (absolute,-15%, as well as relative to the cubic root of the bodyweight, -13%). No information provided for lower dose levels. o PND 13: An increase in the numbers of pups/litter with areolas/nipple retention (2.7 as compared to 0 in the controls; $p<0.05$) as well as in the litter incidence of areolas/nipples retention (67.6% as compared to 0 in controls; $p<0.05$). No data provided for the lower dose groups • No effects on vaginal opening (examined from day 21 and onwards) or estrous cycling was observed in F1 females.

The authors summarize the study in the abstract as follows:

"Anti-androgenic chemicals alter sexual differentiation by a variety of mechanisms, and the mechanisms between phthalate esters and p,p'-DDE are considered to be different. We performed an in utero through lactational exposure assay using dicyclohexyl phthalate and p,p'-DDE to investigate the sexual differentiation of these chemicals. Pregnant CD (SD) IGS rats were given dicyclohexyl phthalate or p,p'-DDE orally from gestational day (GD) 6 to postnatal day (PND) 20, and the endocrine-mediated effects in dams and their offspring were examined. The reproductive performance of offspring was also examined. The doses of dicyclohexyl phthalate were 0, 20, 100, and 500 mg/kg/day, and those of p,p'-DDE were 5, 15, and 50mg/kg/day. Using the dicyclohexyl phthalate, a dam in the 500 mg/kg group showed dystocia and died. The viability index of offspring on PND 4 decreased in the 500 mg/kg group. Prolonged preputial separation, reduced ano-genital distance, increased areolas/nipple retention, hypospadia, decreased ventral prostate and levator ani/bulbocavernosus muscle weights and decreased testicular germ cells were observed in male offspring in the 500 mg/kg group. In the assay using p,p'-DDE, decreased viability index of offspring on PND 21, prolonged preputial separation in male offspring and early vaginal opening in female offspring were observed in the 50mg/kg group. The copulation and fertility indices decreased in the reproductive performance of offspring in the 50mg/kg group. The endocrine-mediated effects were detected in offspring of dams given 100mg/kg dicyclohexyl phthalate, and in offspring of dams given 20mg/kg p,p'-DDE. Our results suggest that the in utero

through lactational exposure assay is a useful method to detect endocrine-mediated effects and that further comparative study between this assay and two-generation reproductive test are necessary when this assay becomes one of the definitive tests."

Saillenfait et al 2009 – Supporting study

Reference	Saillenfait, A. M., Gallissot F., Sabata J. P., Differential developmental toxicities of di-nhexyl phthalate and dicyclohexyl phthalate administered orally to rats, Journal of Applied Toxicology, 2009, 29: 510-521.
Reliability	R1
OECD conceptual framework	Level 4
Method	• Oral (gavage), female SD rats • Main study o 24-25 females/dose level. Study protocol resembled that of a Prenatal developmental toxicity study (OECD TG 414). In addition, anogenital distance was measured on GD 21. • Satellite study o 6-9 animals/dose level, dosing interval as main study, for examination of liver effects (Clin Path, enzyme activity and liver weights) on GD 21. Non-GLP study. (No information on how the offspring was randomized into the 3 different survival groups)
Test substance & Dose	• 0, 250, 500 or 750 mg/kg bw/day of DCHP (CAS No. 84-61-7, 99% purity) from GD 6 until GD 20 • Vehicle: olive oil • Dose volume 10 ml/kg
Results	Main study <u>Maternal body weights & clinical signs</u> • There were no mortalities or adverse clinical findings. • Decreased body weight gain during the first 3 days of dosing (30 and 43% in the high and intermediate dose) and in the high dose animals also during late gestation (51% less during GD 18-21) as well as for the entire dosing period (22% less). High dose animals also had a decreased corrected body weight gain for the entire dosing period (50%) indicating clear (but not overt) maternal toxicity at the high dose level. <u>Developmental effects</u> • No effects on post-implantation loss or on number of dead fetuses or on sex ratio. • Fetal weights (male, females and combined) were decreased (~11%) at the high dose level • Decreased anogenital distance (absolute and relative to the cubic root of bodyweight) in male fetuses in all DCHP dose groups (absolute distance: -9, -12 and -17% in the low, intermediate and high dose groups, respectively, as compared to the controls; relative distance: -8 , -11, -14% in the low, intermediate and high dose groups, respectively). • Fetal pathology: Diaphragmatic hernia was seen in one control fetus. Three fetuses from three different litters were malformed at the high dose level. One fetus had omphalocele, another had diaphragmatic hernia and a third had a thoracic vertebra malformation. These findings were considered isolated and not related to DCHP treatment by the authors. Satellite study - liver weights and limited Clinical Pathology • Significantly increased relative liver weight (+17%; $p<0.01$) in intermediate and high dose (+28%; $p<0.01$) animals. • Dose dependent increased (+75, + 90, +108% as compared to the controls; $p<0.01$) activity of hepatic palmitoyl CoA oxidase (a peroxisomal enzyme marker) at all dose levels. Increase in ASAT, (+49%) and in ALAT (+116%; $p<0.01$) but no statistically significant effects on cholesterol or triglyceride levels, in the high dose group. • No adverse finding at the histopathological examination of the liver.

The authors summarize the study in the abstract as follows:
"*The objective of this study was to evaluate the developmental toxic potential of di-n-hexyl phthalate (DnHP) and dicyclohexyl phthalate (DCHP) in rats. Pregnant Sprague-Dawley rats were exposed to DnHP or DCHP at doses of 0 (olive oil), 250, 500 and 750 mg kg(-1) per day, by gavage, on gestational days (GD) 6-20. Maternal food consumption and body weight gain were significantly reduced at 750 mg kg(-1) per day of DnHP and at the two high doses of DCHP. Slight changes in liver weight associated with peroxisomal enzyme induction were seen in dams treated with DnHP or DCHP. DnHP caused dose-related developmental toxic effects, including marked*

embryo mortality at 750 mg kg(-1) per day, and presence of malformations (mainly cleft palate, eye defects and axial skeleton abnormalities) and significant decreases in fetal weight at 500 and 750 mg kg(-1) per day. Significant delay of ossification and increase in the incidence of skeletal variants (e.g. supernumerary lumbar ribs) also appeared at 250 mg kg(-1) per day. DCHP produced fetal growth retardation at 750 mg kg(-1) per day, as evidenced by significant reduction of fetal weight. DnHP and DCHP induced a significant and dose-related decrease in the anogenital distance of male fetuses at all doses, and there was a significant increase in the incidence of male fetuses with undescended testis at 500 and 750 mg kg(-1) per day of DnHP. In conclusion, DnHP showed clear embryolethality and teratogenicity, but not DCHP. There was evidence that both phthalates could alter the development of the male reproductive system after in utero exposure, DnHP being much more potent than DCHP."

Aydogan and Barlas 2013- Supporting study

Reference	Aydogan A. M., Barlas N. Developmental effects of prenatal di-n-hexyl phthalate and dicyclohexyl phthalate exposure on reproductive tract of male rats: Postnatal outcomes, Food and Chemical Toxicology, 2013, 51:123-136
Reliability	R1
OECD conceptual framework	Level 4
Method	• Pregnant Wistar rats • After delivery all pups were allowed to grow with their dam for 1 month and then male pups were separated and housed 4/cage until they were killed on PND 20 (pre-pubertal), PND 32 (pubertal) or PND 90 (adult). Group size per age and dose level was 8-10 animals. There is no information on how offspring were randomized into the 3 different survival groups. • At necropsy the F1 animals were weighed. Testis, epididymis, ventral prostate and seminal vesicle were weighed and processed for histopathological examination except for left caput epididymis of adult animals which was processed for analysis of sperm head count and sperm morphology. • In connection with sacrifice, blood was collected from the heart samples for analysis of serum concentration of testosterone, estradiol, FSH, LH, inhibin B and MIS/AMH. • Non-GLP study
Test substance & Dose	• DCHP (CAS No. 84-61-7, purity 99%) was administered via gavage at 0, 20, 100 or 500 mg/kg bw/day to separate groups of pregnant dams from GD6 until GD 19. • Vehicle: corn oil • Dosing volume 0.25 ml
Results	• No information on maternal clinical signs, food consumption or maternal body weights during gestation or during lactation. No information on effects on litter size at birth or on pup survival or on birth weight or weight gain during lactation. No information on clinical signs, food consumption or weights in offspring during the study. Only bodyweight of offspring at termination is reported. No information on effects on anogenital distance. <u>Body weights (F1) at termination of study</u> • ↓ body weight ($p<0.05$) only at the low dose of pre-pubertal stage rats. No effect at any dose levels at the pubertal or adult stages. <u>Weights of reproductive organ</u> • ↓ absolute testis weight ($p<0.05$) at the low and high dose group (no dose dependency), and ↑relative testis weight ($p<0.05$) in intermediate dose group at the pre-pubertal stage. ↓ (absolute and relative, $p<0.05$) testis weight at the high dose level, and a ↓ relative weight at the intermediate dose levels (no dose-dependency) at the pubertal stage. No effects on testis weights at the adult stage. • ↓Absolute weight of the epididymis in the low dose group and no effects on the combined seminal and prostate weights were recorded at the pre-pubertal stage. At the pubertal stage no effect was seen on the weight of the epididymis or on the seminal vesicle but a ↑ ($p<0.05$) relative prostate weight was noted at the high dose level. At the adult stage the only effects observed were a ↑ ($p<0.05$) of the absolute weights of the epididymis and of the prostate at the high dose level. Histopathological examination (no grading of severity was reported). • Testis: dose dependent ↑ ($p<0.05$) incidence of tubular atrophy (nos. of affected animals: 0/10, 6/10, 5/10, 8/10; 0/10, 3/10, 8/10, 10/10 at the different dose levels of pre-pubertal and pubertal rats respectively) and of germinal cell debris (nos. of affected animals: 0/10, 3/10, 6/10, 9/10; 0/10, 3/10, 10/10/ 10/10 at the different dose levels of pre-pubertal and pubertal animals, respectively). In adult animals a much lower and not statistically significant incidence of tubular atrophy was recorded (0/10, 2/10, 0/10, 2/10 at the different dose levels). A dose dependent ↑ ($p<0.05$) incidence of Sertoli cell

	vacuolization (0/10, 6/10, 4/10, 8/10 at the different dose levels) was recorded in adult animals. • Epididymis: dose dependent ↑incidence of presence of spermatogenic cells in lumen at all age stages (incidence in high dose group was 8/10, 10/10 and 8/10 at the pre-pubertal, pubertal and adult stage, respectively, as compared to no observations in control animals at any stage of development). • Prostate: ↑incidence of atrophic tubules (0/10, 7/10, 9/10, 5/10; 0/10, 5/10, 10/10,10/10; 0/10; 5/10, 8/10, 10/10 at the different dose levels of pre-pubertal, pubertal and adult rats, respectively) and of intraepithelial neoplasia (incidence: 0/10, 7/10, 9/10, 5/10; 0/10, 3/10, 10/10, 10/10; 0/10, 5/10, 8/10, 8/10 at the different dose levels of pre-pubertal, pubertal and adult rats, respectively). Sperm analysis (manual analysis). • No effects on epidiymal sperm counts. ↑ ($p<0.05$) percentage of abnormal sperms of approximately the same magnitude at all dose levels (10.9, 27.6, 23.0 and 27.4% in the control, low, intermediate and high dose group, respectively).

The authors summarise the study in the abstract as follows:
"*The present study is to investigate the effects of in utero di-n-hexyl phthalate (DHP) and dicyclohexyl phthalate exposure (DCHP) on the development of male reproductive tract at prepubertal, pubertal and adult stages. Pregnant rats were exposed to DHP and DCHP at doses of 0, 20, 100 and 500mg/kg/day, by gavage, on gestational days (GD) 6-19. Testosterone (T) levels of prepubertal rats diminished at high dose DHP and middle dose DCHP groups. MIS/AMH levels elevated in DHP and DCHP groups. T levels of pubertal rats decreased in low and high dose DHP and DCHP groups. Inhibin B levels of adult rats diminished in DCHP groups. Atrophic and amorphous tubules, spermatogenic cell debris, apoptotic cells, adherent tubules, Sertoli cell vacuolisation, prostatic atrophic tubules and prostatic intraepithelial neoplasia (PIN) were observed in the reproductive organs of treated animals at all developmental stages. There was an increase in immunoexpression of MIS/AMH in testes of treated rats. There were no changes in sperm head count but percentages of abnormal sperms increased. The diameters of seminiferous and epididymal tubules in treatment groups were significantly lower. This study shows that DHP and DCHP may have antiandrogenic effects on male reproductive development before and after birth.*"

Aydogan and Barlas 2015 – Supporting study

Reference	Aydogan A. M., Barlas N. Influence of in utero di-n-hexyl phthalate and dicyclohexyl phthalate on fetal testicular development in rats. Toxicology Letters, 2015, 233: 125-137
Reliability	R1
OECD conceptual framework	Level 4
Method	• Pregnant time-mated Wistar albino rats (2 months old, weighing 200-220g) • 10 pregnant females (litters) per group randomly assigned to control and treatment. • Maternal body weight, food consumption and clinical signs of toxicity of animals were recorded daily. At necropsy on GD20 in the morning, the dams were euthanized by cervical dislocation and the litters with resorptions, percentage resorption, length (from head to end of the tail) and weight of male pups were recorded. Anogenital distance (AGD) was measured on male pups at necropsy using digital calipers. • Fetal blood was collected for hormone analyses • Testes tissue was examined with histopathological analysis as well as immunostaining examinations. • Non-GLP study
Test substance & Dose	• DCHP (CAS No. 84-61-7, purity 99%) was administered via gavage at 0, 20, 100 or 500 mg/kg bw/day to separate groups of pregnant dams from GD6 until GD 19. • Vehicle: corn oil • Dosing volume 0.25 ml
Results	<u>Maternal and fetal outcomes</u> • There was no significant difference in body weight gain and food and water intakes of pregnant rats among groups. • There were no significant differences in the length of pregnancy, the numbers of implantation sites, live pups, male pups and male/female ratio in treatment groups compared to control group • The litters with resorption increased in all DCHP-treated groups (3, 33,31 and 26% resorption respectively) • The AGD per cube root of body weight ratio of male pups in all DCHP-treated groups decreased when compared to control groups.

	Hormone levels • Testosterone and MIS/AMH decreased in the 100 and 500 mg/kg bw/day treatment groups. • Inhibin B levels increased in all treatment groups. • FSH levels of male pups in the 20 and 100 mg/kg bw/day groups increased. • The FSH to Inhibin B ratio was reduced in all groups Histopathology Exposure to DCHP during gestation caused adverse histopathologic effects on the development of testis. • Atrophic and small seminiferous chords significantly increased in the testis of male pups in all treatment groups (for 0/10, 8/10, 10/10 and 10/10, respectively) • Reduced numbers of germ cells in chords (for 0/10, 5/10, 7/10 and 8/10, respectively) • Some chords had only Sertoli cells, significant in the 100 and 500 mg/kg/day DCHP treatment groups (0/10, 3/10, 5/10 and 7/10, respectively). • In addition, the cells in the chords were detached from chord wall (for 0/10, 6/10, 8/10 and 10/10, respectively), and there were picnotic cells in the chords of treatment groups in contrast to control group. • Moreover there were multinucleated giant cells in the chords especially in the high dose treatment groups (for 0/10, 2/10, 5/10 and 9/10, respectively). Immunohistochemical analysis of 3β-HSD, MIS/AMH, AR and PCNA in testes • Proliferating cell nuclear antigen (PCNA) staining decreased in the interstitial area of the testes and there were groups of cells that were not stained. The immunodensity decreased in the high dose group. • The 3β-HSD immunostaining of Leydig cells decreased in a dose dependent manner. • The Leydig cells and and peritubular myoid cells obtained almost no immunostaining for the androgen receptor (AR). • Immunodensities for MIS/AMH and AR decreased in all dose groups compared to controls. Leydig cell number and clusters • The number of Leydig cell clusters was reduced in all DCHP treatment groups as compared to controls. • The percentage of small Leydig cell clusters decreased, while the percentage of medium and large clusters of Leydig cells increased as compared to controls. Immunofluorescence analysis of 3β-HSD and MIS/AMH in testes • The expression of 3 β-HSD and MIS/AMH in the testes of DCHP treatment groups decreased as compared to control levels.

The authors summarize the study in the abstract as follows:

"*This study investigated the effects of di-n-hexyl phthalate (DHP) and dicyclohexyl phthalate (DCHP) on male reproductive development in utero. Pregnant rats were exposed to DHP and DCHP at doses of 0 (vehicle), 20, 100 and 500mg/kg/day, by gavage, on gestational days (GD) 6-19. A significant decrease in the anogenital distance (AGD) of male fetuses was observed at all doses of DHP and DCHP. The AGD/cube root of body weight ratio in male fetuses was also significantly reduced compared to control group. The litters with resorption, percentage of resorptions and inhibin B levels increased in treatment groups. Moreover, testosterone and MIS/AMH levels in all treatment groups decreased. Although FSH and inhibin B levels of male pups exposed to DHP and DCHP increased, FSH/inhibin B ratio decreased in treatment groups. Reduced testosterone production in response to DHP and DCHP exposure appeared to be related to changes in testosterone metabolism, as shown by decreased 3β-HSD immunoexpression. The percentages of large Leydig clusters increased after exposure to DHP and DCHP in utero. Histopathological examination of the testis on GD20 revealed changes at all doses. Relative integrated immunodensities of 3β-HSD, MIS/AMH, PCNA and AR decreased after DHP and DCHP exposures. Altered fetal Sertoli cell development and function may be caused by disrupted PMC*[12] *function revealed by reduced AR production in these cells in treatment groups.*"

[12] Peritubular myoid cells (explanation added by dossier submitter)

Annex II - Mode of Action framework[13] applied to dicyclohexyl phthalate (DCHP)

1. Problem formulation

The aim of the analysis is to establish the **Mode of Action of DCHP for endocrine mediated irreversible effects to the male reproductive system with the potential to affect male reproductive function and its relevance to Humans.**
The analysis needs to cover if the effects observed in experimental animals with DCHP are:

- **Species specific**
- **Endocrine mediated**
- **Causing irreversible damage to the male reproductive system and likelihood for adverse effects on fertility**
- **Relevant for humans**

The MoA/HRF (Mode of action / Human Relevance Framework) will be used for the Weight of Evidence analysis following consecutive steps:

1. Hypothesised mode of action statement on the basis of available information (section 2 & 3)
2. Establishment of hypothesised mode of action in experimental species (section 5a, 5b, 5c)
3. Establishment of human relevance of the established experimental species mode of action (section 5c)

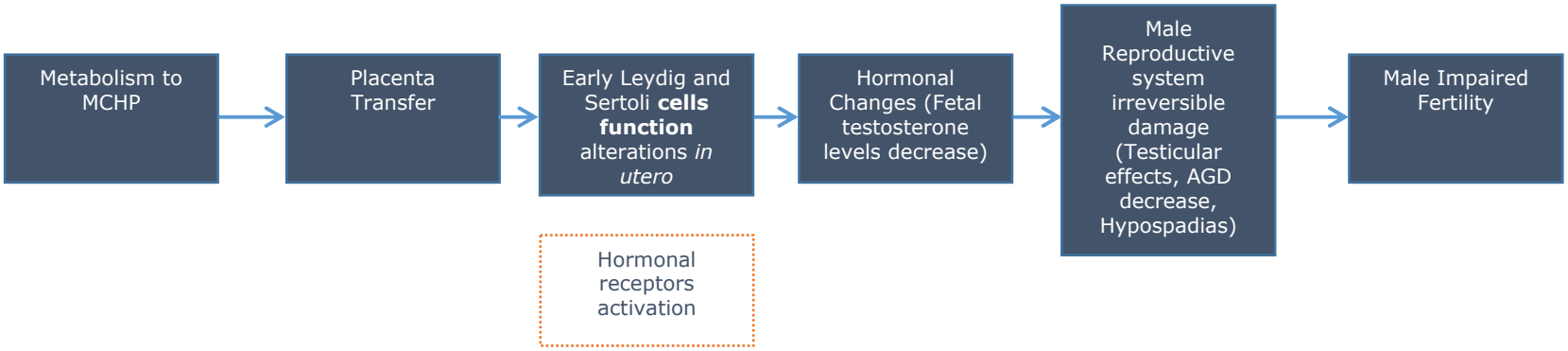
Alternative modes of action will be considered (section 6) and remaining uncertainty will be recorded (section 7).

[13] Information about the MoA framework is available at: http://echa.europa.eu/support/guidance-on-reach-and-clp-implementation/formats

2. Hypothesised Mode of action Statement

A hypothesis for the potential mode of action of DCHP in male rat reproductive system and the establishment of human relevance includes the following elements:

- DCHP once absorbed is metabolised to the mono-ester MCHP
- MCHP interferes with the Leydig cell testosterone production pathway and/or Sertoli cell function
- Decreased fetal testosterone levels cause male reproductive system effects (Leydig cells, Sertoli cells alterations, epididymis and prostate effects, decreased anogenital distance (AGD))
- The effects seen during *in utero* exposure are also present when the dosing stops (irreversible effects)
- The information available for DCHP follows the same pattern of toxicological effects observed with other phthalates that cause rat male reproduction disorders.
- Considerations of additional modes of action for the low testosterone levels after *in utero* exposure to DCHP, e.g. causality involving the hormone-receptor mediated pathways as an alternative/parallel mode of action.
- The rat male reproductive system is similar to humans. Establishment of plausibility of key events occurring in humans based on biological relevance across species.
- The following schema presents the hypothesised mode of action:



3. Summary of data for use in Mode of Action Analysis

This section summarises the main information used for the establishment of the proposed mode of action for DCHP endocrine mediated male reproductive organ effects (for more information see the following sections).

The data used for the establishment of the hypothesised mode of action of DCHP endocrine mediated reproductive organ effects and male fertility, including human relevance, are available in the Reference section (Section 9 of this Annex). It includes short description of the information, reliability, and the references.
In summary, the available experimental data cover:

- Metabolism of DCHP and species similarities
- Placenta transfer of phthalate metabolites
- Cellular functional alterations of testosterone mediated pathways following *in utero* exposure to DCHP
- Fetal testosterone decrease following *in utero* exposure to DCHP
- Male reproductive tract adverse effects following *in utero* exposure to DCHP (testicular effects, decrease in AGD, hypospadias)
- Male fertility effects (semen quality)
- Estrogen and androgen receptor assays with DCHP
- Similarity of pattern of adverse effects with similar compounds (phthalates)
- Hormonal control of male reproductive system development across species and relevance for DCHP
- Correlation of experimental data for male reproductive organ/system adverse effects with male reproductive system function

The evidence used for establishing the mode of action in experimental species and the human relevance has been collected using the information from the CLH dossier, the registration dossier, as well as a search strategy described in Annex III. For the main *in vivo* experimental studies reliability categories have been assigned as described in Annex III. For some evidence we have only used the information available in the abstract from publications (see section 9), this as well as the combination of all lines of evidence have been weighted using the Bradford Hill considerations described in section 5 of this Annex. In this way levels of confidence using criteria such as consistency, specificity and biological plausibility have been derived. The confidence levels are used as a metric of expert judgment in concluding whether the hypothesised mode of action and human relevance have been established.

4. Listing of key events identified for a specific Mode of Action

The hypothesised mode of action as presented in Section 2 above consists of six main key events. All the key events that are listed for the hypothesised mode of action need to be measurable, and are considered essential for the establishment of plausibility and human relevance.

Key Event 1	**Metabolism of DCHP to MCHP**
Key Event 2	**Placenta Transfer**
Key Event 3	**Early Leydig and Sertoli cells function alterations *in utero***
Key Event 4	**Hormonal changes (**Fetal testosterone levels decrease**)**
Key Event 5*	**Male Reproductive system irreversible damage** (Testicular effects, Prostate effects, Sperm effects, AGD decrease, Hypospadias)
Key Event 6	**Male impaired Fertility**

*For the Key event 5, a number of distinct effects described in the studies available, are grouped together under the general title "male reproductive system irreversible damage"

**The key event 6 includes effects related to semen quality and the finding of hypospadias and AGD decrease in experimental species with expected adverse effects in humans regarding infertility (as part of the testicular dysgenesis syndrome).

5. Bradford Hill Considerations for Weight of Evidence Analysis of available data/information for Mode of Action Analysis in experimental species

5a. Dose Response Relationships and Temporal Association

The following table and figure summarise dose response elements from the available data, where it was possible to provide association of the key events with dose response, and temporal association. As the information comes from six different studies, the potency of effects observed indicated with "+" is relative indicating the occurrence of an effect and the severity (not necessarily exactly double or three times higher than the control); the effects reported in this table represent effects that are statistically significant when compared to the control groups.

Dose response relationship*:* The key events (Key events 3-5) are observed at doses below or similar to those associated with the (adverse) effect. In some cases, as the results are from different studies, not the same parameters have been examined, and therefore information only on one or two key events may be available.

Temporal association*:* The key events are presented in hypothesised order, starting with effects occurring at fetal stage during *in utero* exposure and continuing with manifestations at prepubertal, pubertal and adult stage even without continuation of exposure to the substance after delivery.

The information from the dose response and temporal association is supportive to the fact that the key events (3-5) occur according to the hypothesised order (from earlier to latest key event) in a dose response manner and are irreversible. The information available indicate good quantitative concordance for the experimental species (rat). This information is further used in section 5c of this document.

	Key Event 3	Key Event 4	Key Event 5	Key Event 5	Key Event 5	Key Event 5	Key Event 5	Key Event 5
Study type	Gestation (treatment)	Gestation (treatment)	Gestation (treatment)	Gestation (treatment)	Prepubertal (recovery)	Pubertal (recovery)	Adult (recovery)	F1/F2 Treatment (two generation study)
Observation at	Gestation day (GD) 20	GD 18/ 20	GD 20	GD 20	Postnatal day (PND) 20	PND 32	PND 90	F1: PND 21 and ≥17 wks/F2: PND 21
Dose mg/kg bw/day	**Early Leydig and Sertoli cells function alterations in utero** [a]	**Fetal testosterone levels decrease** [b]	**Male Reproduction system irreversible damage** Testicular effects (Leydig cells, Sertoli cells, Epididymis) [c]	**Male Reproduction system irreversible damage** [d]	**Male Reproduction system irreversible damage** Testicular/Epididymis effects/Prostate [e]	**Male Reproduction system irreversible damage** Testicular/Epididymis effects/ Prostate [e]	**Male Reproduction system irreversible damage** Testicular/Epididymis effects/ Prostate [e]	**Male Reproduction system irreversible damage** Testicular effects, Sperm effects, Decrease in AGD, increase in areolae mammae [f]
0								
10								
20	+	++	++	++	++	++	++	
80								
100	++	+++	+++	++	++	++	++	+
250								
300		+++						
400								+
500	++	+++	+++	++	++	++	++	
750								
900		+++						

a) Including dose response related reduction in 3β-HSD (Leydig cell function biomarker) and MIS (Sertoli function biomarker) (Aydogan and Barlas 2015)

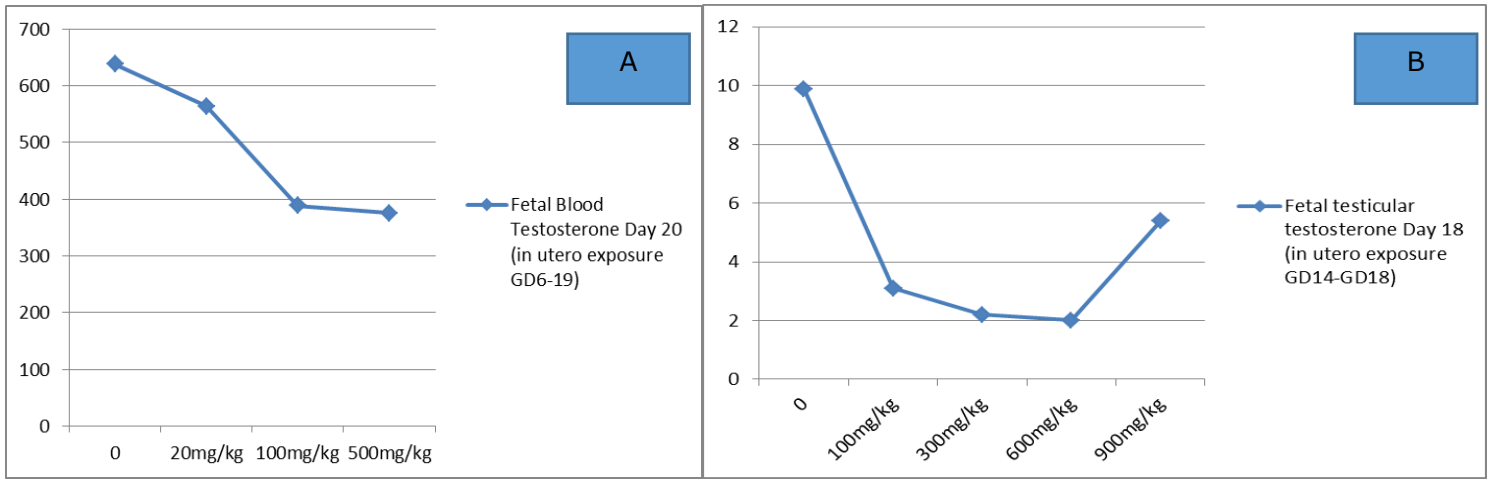
b) Aydogan and Barlas (2015) for gestation date 20 and Furr et al. (2014) for gestation date 18

c) Fetal Testicular effects: Atrophic and small seminiferous chords, decreased germ cell numbers in chords, **Sertoli cell** only chords, detached cells from chord wall, multinucleated gonocytes. **Leydig cells**: Increase of medium and large clusters with increasing doses (Aydogan and Barlas 2015).

d) Decrease in AGD (Saillenfait et al. 2009, OECD 2013), Prostate weight reduction (Yamasaki et al. 2009)

e) Aydogan and Barlas (2013): a number of testicular effects are evident in prepuberal and pubertal stages, whereas in the adult stage for some of the observations the incidencies are lower. The treatment occurred only *in utero*. Attached seminiferous tubules and effects on epididymis are however evident also in adult stage, indicating irreversible damage from the *in utero* exposure. In addition, prostate related effects are seen in prepubertal, pubertal and adult stage with atrophic tubules and prostatic intraepithelial neoplasia.
f) AGD and areola mammae effects occur at lower dose levels in F2 than in F1. (Hoshino et al. 2005, OECD 2013).

The following figure illustrates dose response results for the decrease of fetal testosterone following *in utero* exposure to DCHP. In Fig A fetal blood testosterone concentrations (pg/ml) are shown (data from Aydogan and Barlas (2015)) whereas Fig B depicts fetal testosterone amounts (ng/testis), data from Furr et al. (2014). Treatment with DCHP occurred at gestational day (GD) 6-19 and 14-18, respectively. Blood testosterone levels were measured at GD 20 (A) and fetal testicular testosterone production *ex vivo* was measured at GD 18 (B).



5b. Consistency & Specificity – Biological Plausibility

Consistency & Specificity

A number of experimental studies with DCHP indicate that the effects in male reproduction system are irreversible following *in utero* exposure. This section addresses that the effects observed for each key event separately are consistent and specific for DCHP as well as that there is causal relationship/linking of the key events with each other and with the toxicological response (key events relationships). It also addresses whether the pattern of effects across species is consistent with the hypothesised mode of action.

Key Event 1 & Key Event 2

Metabolism of DCHP to MCHP

Placenta Transfer

The data available, taking into account data and assessments from other phthalates, indicate that metabolism to the monoester is needed for effects to occur. This is supported by available evidence that metabolism to the monoester is likely to occur in humans. According to Lake et al. (1977) a study on the hydrolysis of phthalates, including that of DCHP, showed that there is species similarity in the metabolism of phthalate diesters between man, rodent, non-rodent and nonhuman primate species. In addition, the data indicate that the metabolism to the mono-ester (MCHP) would take place primarily in the intestine. The intestinal metabolism of DCHP to MCHP by both rats and human intestine is further confirmed by a study with 16hr incubation of 1% DCHP *in vitro* (IUCLID datasheet, ECB). MCHP but not cyclohexanol produced marked testicular atrophy in a rat study (Lake et al, 1982). There is evidence that phthalate monoesters can cross the human placenta and reach the human fetus (Mose et al. 2007).

In utero exposure is most sensitive for the elicitation of the adverse effects in the male reproductive system (Hoshino et al. 2005, Yamasaki et al. 2009, Saillenfait et al 2009, Aydogan and Barlas 2013 and 2015, Furr et al. 2014). Lake et al. (1982) showed testicular damage in adult animals exposed to higher doses during adulthood. The Hershberger assay has been negative indicating absence of effects at the AR/5-alpha-reductase level after short term exposure of adult animals (METI 2002). In summary, a higher sensitivity for damage is present for *in utero* exposure.

Overall, key event 1 and key event 2 are considered essential for the elucidation of the subsequent key events since metabolism and placenta transfer are required to initiate the next key events.

Key Event 3 & Key Event 4

Early Leydig and Sertoli cells function alterations *in utero*

Hormonal changes (Fetal testosterone levels decrease**)**

Testosterone is synthesised within testis from Leydig cells that have a central role in endocrine control of reproduction and the paracrine control of spermatogenesis in mammalian species. The latter is enabled with the contribution of the Sertoli cells in the testis. Functional alterations in Leydig and Sertoli cells (either at cellular or biochemical level) can result in altered testosterone biosynthesis and interference with the normal development of the male reproductive system and its function. Testosterone produced in fetal rat testis by Leydig cells is first detectable at GD15 and reaches a maximum at GD 18-19.
DCHP causes a decrease in fetal testosterone with increasing doses of DCHP, decrease of 3β-HSD enzyme (responsible for testosterone production), as well as decrease in MIS (responsible for regression of Mullerian ducts) and Sertoli cells function biomarker in rats (Aydogan and Barlas 2015). Increasing doses of DCHP also caused impairment of Sertoli cells function and increase of medium and large clusters of Leydig cells in rats (Aydogan and Barlas 2015).
The effect of DCHP on testosterone levels linked to Leydig cells functionality is further supported by *in vitro* data that indicate that DCHP can interfere with testosterone biosynthesis in rats and humans via inhibition of specific enzymes (3β-hydroxysteriod dehydrogenase (3β-HSD) and 17β- hydroxysteroid dehydrogenase type 3 (17β-HSD3)) involved in the biosynthesis of androgens in testis (Yuan et al. 2012).
Foetal testosterone production is dose-dependently reduced in testis tissue examined ex vivo at GD 18 after *in utero* exposure to DCHP (0, 100, 300, 600, 900 mg/kg bw/day) GD14 to GD 18 (Furr et al. 2014). Supportive data from other phthalates (DBP) link fetal testosterone insufficiency and abnormal proliferation of Leydig cells in rats (Mylchreest et al. 2002). In addition it is proposed that in general phthalates alter gene expression for cholesterol transport and steroidogenesis in Leydig cells (Report to the US Consumer Product Safety Commission, July 2014). Furthermore exposure of rats to DEHP and DHP have also been shown to cause decrease in fetal testicular testosterone levels (Borch et al. 2006, Borch et al. 2004, Hannas et al, 2012), providing further supporting evidence of the essentiality of this key event (hormonal changes) in the mode of action analysis that is observed also with DCHP. The effect of another similar substance, DPeP (dipentyl phthalate), on CD mice regarding reduction in fetal testosterone levels (Furr et al. 2014) further supports the absence of significant species differences in relation to key event 4.
Equivocal results on blood hormonal levels (Testosterone, Inhibin B, MIS, FSH, LH) are shown in the Aydogan and Barlas (2013) publication following *in utero* exposure to DCHP, but measuring the hormones at postnatal stages when exposure has ceased. Significant changes are found but not in a dose dependent manner and varying for the different age groups (PND 20, 32 and 90), However, for Inhibin B a reduction in a dose dependent manner in all dose groups and significantly different from the control group in the 100 and 500 mg/kg bw/day adult group (PND 90) are seen. Likewise, the testosterone levels in the 2-generation study (Hoshino et al. 2005) were significantly increased in the F0 middle dose group, but not in the low and high groups and not in the F1. No firm conclusions can be

drawn when these parameters are examined after exposure ceases; as these are most likely earlier key events and their examination within the experimental design should occur at the stage of actual exposure to understand their role in the toxicological adverse pathway. Furthermore, in a study by Moody et al. (2012) using mice exposed to dibutylphthalate (0, 1, 10, 50, 100, 250 and 500 mg/kg bw/day) prepubertally (PND 4 – PND 7, 14 or 21) lowered testosterone levels were detected in the high dose group, however endocrine effects at lower dose levels were indicated by effects on AGD already at 1 mg/kg bw/day. Reversibility of testosterone levels in adult stage has been shown following *in utero* exposure to dibutyl phthalate, but with male reproductive adverse effects being irreversible in adulthood (Thompson et al. 2004, Barlow et al. 2004). The overall evidence indicates that only fetal testosterone appears to be a sensitive biomarker of the likely initiating events of antiandrogenic properties of DCHP, as changes in serum testosterone outside the critical window of susceptibility (GD 14-18) appear not detectable in a dose response manner.
Overall, there is consistency with the available evidence on the occurrence of key event 3 and 4 caused by DCHP *in utero*, as an early key event required to trigger the next key event.

Key Event 5

Male Reproductive system irreversible damage (testicular effects, prostate effects, sperm effects, AGD decrease, hypospadias)

Testosterone is essential for the male reproductive tract differentiation in mammalian species (fetal testicular development, epididymis, prostate) (Nef, 2000). Changes of the normal fetal testosterone levels (Key event 4) can result in effects like hypospadias, and decrease in AGD (androgen dependent in male rodents) (Wolf et al. 1999, Bowman et al. 2003)
Fetal Testicular effects following *in utero* exposure to DCHP (0, 20, 100, 500 mg/kg bw/day) include:

- Atrophic and small seminiferous chords, decreased germ cells in chords, Sertoli cell only chords, detached cells from chord wall, presence of multinuclear gonocytes (Aydogan and Barlas 2015).

Decrease in AGD, has been observed in male pups following *in utero* exposure to 0, 250, 500 and 750 mg/kg bw/day of DCHP in a dose dependent manner (Saillenfait et al. 2009).
A number of **testicular effects** are evident in prepubertal and pubertal stages, whereas in the adult stage for some of the observations recovery is observed. The treatment occurred only *in utero*. Attached seminiferous tubules and effects on epididymis, and increased presence of abnormal sperm are however evident also in adult stage, indicating **irreversible damage** from the *in utero* exposure (Aydogan and Barlas 2013).
The sequence of events does not seem to be reversible when dosing is stopped as indicated by the presence of male reproductive organ effects even at adult stage following only *in utero* exposure (Aydogan and Barlas 2013 and 2015)
The following observations are indicative of irreversible male reproductive systems adverse effects:

- Incidence of **testicular effects** (tubular atrophy, germinal cell debris, increase in apoptotic cells) in prepubertal and pubertal stages increase with increasing doses of *in utero* exposure to DCHP. Reversibility in adult stage.

- Incidence of **testicular effects** (attached seminiferous tubules) increase in prepubertal, pubertal and adult stage with increasing *in utero* doses.
- Incidence of **epididymis effects** (atrophic tubules) increase in prepubertal, pubertal and adult stage with increasing *in utero* doses.
- Incidence of **prostate related effects** are seen in prepubertal, pubertal and adult stage with atrophic tubules and prostatic intraepithelial neoplasia (Aydogan and Barlas, 2013).

The two-generation study showed atrophy of seminiferous tubules and atrophy of testicular changes at 400 mg/kg bw/day in F1 generation and **decrease of AGD** in F1 and F2 generation at 400 and at 100 and 400 mg/kg bw/day, respectively (Hoshino et al. 2005). The two generation study showed **reduction of spermatids in testis** at 100 and 400mg/kg bw/day in F1 generation. DCHP also affected the **sperm production and maturation** in rats in a dose-dependent manner (Aydogan and Barlas 2013).

In a supportive study, 2 cases of **hypospadias** were found in the high dose group (500 mg/kg bw) (Yamasaki et al. 2009) as well as a **decreased AGD**, further pointing to effects on the development of the male reproductive organs.

Mitchell et al. (2012) and Heger et al. (2012) reported that DBP exposure of human fetal testes, that were xenografted into castrate male nude mice, produced no effects on testosterone production compared to rat fetal testis xenografts that were exposed to DBP as a positive control. However, this information does not contradict the hypothesised mode of action for DCHP as the xenograft studies do not take into account *in utero* exposure to phthalates throughout the male reproductive organ development (the male programming window) and therefore is not considered strong enough to disregard the proposed mode of action as relevant for humans. In addition, the study from Furr et al. (2014) further confirms that fetal testicular decrease in rats and mice is an early key event with no qualitative species differences observed, but only with some different sensitivity as a result of dose levels of dipentyl phthalate required to trigger the effect.

Overall, there is consistency at the doses at which the later key events appear in relation to earlier key events (see also section 5a). There is consistency in the effects observed (taking also into account similar chemicals e.g. other phthalates) in other species.

Key Event 6

Male Impaired Fertility

Adverse effects in relation to the development of the male reproductive system and its function are likely to have effects on fertility.
The two generation study with DCHP indicates effects in reproductive organs and function parameters but these effects were not severe enough to render effects on fertility, however, it is known that histopathological changes are a more sensitive indicator of reproductive toxicity than are reduced fertility. Decreased fertility as revealed by effect on fertility index is a rather insensitive endpoint in rats. This may be explained by the rather high sperm reserve available in rats compared to humans (OECD 2008).

Although the experimental evidence of direct impact of DCHP on male fertility is not very strong, there is strong evidence that male reproductive system irreversible effects (e.g. hypospadias, sperm quality effects, decrease in anogenital distance) observed in key event 5 are linked to fertility adverse effects in mammalian species (Asklund et al. 2010, Bracka 1989, Kurzrock and Karpman 2004, Silver 2004, Skakkebaeck et al,.2001). Overall, fetal disturbance of the developing male reproductive system can have multiple effects in mammalian species as described by Skakkebaeck et al. (2001) and summarised as the testicular dysgenesis syndrome (TDS). The disturbed fetal development resulting in alterations in Sertoli cell function and decreased Leydig cell function cause impaired germ cell differentiation and androgen insufficiency; which in turn can lead to reduced semen quality, hypospadias and testicular effects (testicular cancer and impaired testicular descent). The testicular dysgenesis syndrome summarises a number of potential adverse effects due to fetal male reproduction development disturbances but not all of them are always likely to occur concurrently (Akre and Richiardi 2009, Wohlfahrt-Veje et al. 2009).

The available experimental evidence with DCHP point in the direction of potential effects on fertility rather than testicular cancer. This is also further supported by the experimental evidence from similar chemicals (e.g. di-n-butyl phthalate), where testicular cancer in experimental species has not been reported.

The experimental evidence with DCHP on functional alterations of Leydig and Sertoli cells, the fetal testosterone decrease, the testicular histopathological changes, the decrease in AGD, hypospadias and alterations in sperm quality strongly support the hypotheses that impairment of male fertility is likely to occur.

Evidence from human epidemiological studies also show that fetal male reproductive systems development related effects correlate well with low semen quality, disturbances in testosterone levels and histopathological effects, and there are links between infertility observed in men and adverse effects on Sertoli cells and Leydig cells (Juul et al. 2014). Consistency of positive correlation of AGD with sperm count, fertility, testis size and testosterone levels has been reported between human and rats (Dean and Sharpe 2013) further supported

by findings that longer AGD in humans can be predictive of normal male reproductive performance in humans (Eisenberg et al. 2011).

Overall, the experimental evidence is consistent with the order of hypothesised key events.

There is consistency in the effects observed (taking also into account similar chemicals e.g. other phthalates) in other species.

Biological Plausibility

The hypothesised mode of action is supported by general biochemical and pharmacology knowledge on the essentiality of testosterone for the normal development of the male reproductive system in all mammalian species (e.g. rodents and humans). Disturbances in the normal levels of fetal testosterone can cause adverse effects in human male sexual development (hypospadias, decrease of AGD, lower semen quality, testicular histopathological changes) which in turn are likely to cause fertility effects (Nef 2000, Dean and Sharpe 2013, Juul et al. 2014, Kurzrock and Karpman 2004, Silver 2004).

The data available does not allow to distinguish whether the target for the metabolite of DCHP is the Leydig cells and/or the Sertoli cells. As the most relevant data are from prenatal toxicity studies, the measurement of the parameters is performed at the final stage of gestation.

It might be that following *in utero* exposure to DCHP, MCHP interferes directly with the biochemical pathway of testosterone biosynthesis in Leydig cells or the initial target is the Sertoli cells (via interference with MIS) which can affect the function of Leydig cells and production of testosterone.

The following table summarises the evidence based analysis for consistency, specificity and biological plausibility that allows establishing the mode of action in experimental species.

	Metabolism of DCHP to MCHP	**Placenta Transfer**	**Early Leydig and Sertoli cells function alterations *in utero***	**Hormonal changes (Fetal testosterone levels decrease)**	**Male Reproduction system irreversible damage Testicular effects (Leydig cells, Sertoli cells, Epididymis)**	**Male Impaired Fertility**
Consistency & Specificity	Consistent; Evidence supporting the hypothesised key event	Some evidence provides indication of placenta transfer of formed metabolite.	Consistent; Available data indicating changes at cellular level.	Consistent: Available data shows decrease of fetal testosterone, further supported from knowledge on other phthalate substances	Consistent: Histopathological evidence of testicular effects, decrease of AGD, testicular effects observed even at recovery phases, hypospadias in one supportive study	Consistent with general knowledge on impact of male reproduction abnormalities (hypospadias, sperm quality) on fertility; not supported by DCHP treated rat specific information

Biological Plausibility	Plausible	Plausible	Plausible	Plausible	Plausible	Plausible

5c. Qualitative and Quantitative Human Concordance

In this section, the established mode of action is presented in a Weight of Evidence process indicating the confidence levels, as derived by the use of the Bradford Hill considerations (see previous sections). In addition it presents the likelihood (plausibility) of human relevance in a qualitative manner and quantitative manner (for some of the key events in experimental species) (in the absence of DCHP specific human data).

	Qualitative Concordance			***Quantitative Concordance***	
Key Event (name)	***(Evidence in Experimental Species)***	***(Evidence in Humans)***	***Confidence***	***(Evidence in Experimental Species)***	***Confidence***
Metabolism of DCHP to MCHP	Evidence based	Plausible and some evidence based	High		
Placenta Transfer	Likely, sufficient evidence available	Plausible	Medium*		
Early Leydig and Sertoli cells function alterations *in utero*	Evidence based	Plausible and some evidence based	Medium **	Evidence based (see section 5a)	Medium
Hormonal changes (Fetal testosterone levels decrease)	Evidence based	Plausible	High	Evidence based (see section 5a)	High
Male Reproduction system irreversible damage Testicular effects (Leydig cells, Sertoli cells, Epididymis)	Evidence based	Plausible	High	Evidence based (see section 5a)	High
Male Impaired Fertility	Likely, not chemical specific (DCHP) based	Plausible	Medium***		

Overall, the weight of evidence is sufficient to establish the hypothesised mode of action in experimental species.

There are no fundamental qualitative differences in key events observed in experimental species and those expected in humans. Human relevance cannot be excluded due to kinetic and dynamic factors.

*Some remaining uncertainty as there is not extensive experimental evidence on quantitative aspects of placental transfer; however the presence of the treatment related adverse effects is clear. *In vitro* evidence suggestive of interference of DCHP with steroidogenic enzyme activity in testis both in rats and in humans (Yuan et al. 2012).
** Some remaining uncertainty on whether the adverse effect is due to interference with testosterone biosynthesis and/or with functional changes of Leydig or Sertoli cells. It may also be through a combination of effects on these cell types.
*** Some remaining uncertainty regarding the evidence available for impairment of fertility in experimental species and human relevance. Although there are no effects observed in fertility parameters in a two generation reproductive study the observed effects on sperm count are indicative of an adverse effect in humans (OECD 2008). Considering the reproductive capacity of rats, decreased fertility as revealed by effect on fertility index is a rather insensitive endpoint. This may be explained by the rather high sperm reserve available in rats compared to humans (OECD 2008). Difference in outcomes for the sperm parameters between the 2-generation study (Hoshino et al. 2005) and the Aydogan and Barlas studies (2013 and 2015) may depend on the different periods of exposure and use of different species of rats. It could be hypothesised that the longer exposure time in the 2-generation study caused defective germ cells to completely disappear, while the more short term exposure (GD 6-19) left damaged cells still viable to produce sperm of lower quality. The available evidence for qualitative correlation of male impaired fertility with the previous key events indicate plausibility on the basis of strong evidence (not DCHP specific) as described for Key event 6 in section 5b above.

6. Other potential Modes of Action

The following modes of action have been considered as part of the weight of evidence analysis within the context of establishing specificity and biological plausibility of the observed adverse effects. It is concluded that the following modes of action are not likely to occur based on the available data and the existing knowledge on these pathways.

- *Receptor Mediated Pathways*

Endocrine mediated effects involved in the male reproductive system development could include receptor mediated pathways. However in the case of DCHP, the available *in vivo* data (Hersberger (METI 2002) and Uterotrophic assays (Yamasaki et al. 2002; METI 2002)) indicate that this mode of action is not plausible in the absence of any significant (anti)estrogen or (anti)androgen effects. The Hershberger assay is a screening assay intended to identify substances having agonistic or antagonistic effects on the AR receptor and/or inhibit the enzyme 5-alpha-reductase. DCHP shows *in vitro* estrogenic, antiestrogenic and antiandrogenic effects through interaction with human ERα, ERβ and AR in a transcriptional reporter assay (Takeuchi et al. 2005). However, since negative results from one Uterotrophic assay (Yamasaki et al. 2002) and one Hershberger assay (METI 2002) have been reported, effects mediated directly through receptor binding are less likely to be the main source of the adverse effects observed.

- *Cholesterol Biosynthesis/Availability Pathway*

Competition of cholesterol related pathways during fetal development is more likely to occur at a stage relevant only to the normal development of the fetal male reproductive system. Cholesterol is essential during pregnancy for the development of the fetus as it is the precursor of steroidogenesis. However since no effects are observed in females in the 2-generation study (Hoshino et al. 2005), it is less likely that DCHP interferes either with the availability/transfer of cholesterol via the placenta, or with the first biochemical reactions of conversion to pregnenolone to a greater extent. However this would not exclude potential interference with cholesterol/testosterone biochemical pathway triggered through interference with fetal male specific receptors or pathways; for the latter there is no conclusive evidence for DCHP to support the elaboration of key events that would occur earlier to the functional changes at cellular level or testosterone biosynthesis (key events 3 and 4 of the hypothesised mode of action). Cholesterol lowering drugs have been shown to lower fetal testosterone levels in rat with additive effect when co administered with dipentyl phthalate (Beverly et al. 2014) as well as linking with reproductive tract development alteration in rats (Beverly et al. 2015).

- *Pituitary / Hormonal regulation*

We have not found any information indicating that DCHP affects pathways that include pituitary regulation.

- *Genotoxicity Mediated Pathways*

Consideration of genotoxicity on testicular cells of male rats following *in utero* exposure to DCHP has also been investigated (Ahbab et al. 2013). However it is unlikely that a genotoxic mode of action for DCHP is involved as the observations on DNA damage are observed at the same time points as the ones involved in the testosterone biosynthesis mode of action proposed. It is likely that the genotoxicity is secondary to the cellular effects observed. There are no other data from similar substances to support genotoxic potential as an alternative mode of action. In addition, the overall data set including the two generation reproductive toxicity assay do not reveal any results that could be associated with an irreversible genotoxic mode of action.

7. Uncertainties/Inconsistencies and Identification of Data Gaps

There are no major data gaps identified in establishing the mode of action. The data and the analysis indicate that DCHP *in utero* exposure interferes with steroidogenesis of the male reproductive system development, and an endocrine mediated mode of action is responsible for the adverse effects observed that are of irreversible nature. The mode of action is based on evidence in experimental species and is found plausible with high confidence for humans.
Remaining uncertainty regarding the exact temporal relation of the cellular key events involved could be resolved with further research *in vitro* to identify if MCHP competes with the regular biochemical pathway of testosterone biosynthesis (cholesterol male mediated pathways) or the effects are due to functional changes of Sertoli cells that in turn affect the testosterone production from Leydig cells or other modes of action.

8. Conclusions in relation to problem formulation

The hypothesised mode of action of DCHP for endocrine mediated irreversible effects to the male reproductive system with the potential to affect male reproductive function and its relevance to humans has been established with medium/high confidence. The overall weight of evidence analysis shows that the male reproductive effects observed following *in utero* exposure to DCHP are mediated via an endocrine (antiandrogenic) mode of action that involves irreversible effects induced by alterations in steroidogenesis in fetal life. This is supported by the available experimental evidence, the biological plausibility for human relevance and the absence of inconsistent evidence.

9. References

Dicyclohexyl phthalate (DCHP) and its monoester				
Species	**Route/Dose**	**Incidence**	**Comments**	**References / Reliability**
Rat, Baboon, Ferret, human	Intestinal preparations (*in vitro*)	Species similarity in the metabolism of phthalate diesters between man, rodent, non-rodent and nonhuman primate species. In addition, the data indicates that the metabolism to the monoester (MCHP) would take place primarily in the intestine.	Abstract only available	Lake BG, Phillips JC, Linnell JC, Gangolli SD. *The in vitro hydrolysis of some phthalate diesters by hepatic and intestinal preparations from various species.* Toxicology and Applied Pharmacology, 1977, 39(2): 239-48 **Used in a WoE approach as supporting study**
Rat, Human	Intestinal preparations	The intestinal metabolism of DCHP to MCHP by both rats and human intestine is further confirmed by a study with 16 hr incubation of 1% DCHP *in vitro.*	Only summary of finding available	IUCLID Datasheet, ECB (as available under ESIS/JRC, currently available within ECHA). **Used in a WoE approach as supporting study**
Rat	Oral	MCHP, but not cyclohexanol, produced marked testicular atrophy in a rat study.	Abstract only available	Lake B.G., Foster J.R., Collins M.A., Stubberfield C.R., Gangoli S.D., Srivastava S.P. *Studies on the effects of orally administered dicyclohexyl phthalate in the rat.* Acta Pharmacol Toxicol (Copenh), 1982, 51 (3):217-26 **Used in a WoE approach as supporting study**
Human	*In vitro* system	There is evidence that phthalate monoesters (MMP, MEP, MBP, MEHP) can cross the placenta and reach the fetus.	Abstract only available	Mose T., Mortensen G.K., Hedegaard M., Knudsen L.E. *Phthalate monoesters in perfusate from a dual placenta perfusion system, the placenta tissue and umbilical cord blood*. Reproductive toxicology, 2007, 23(1): 83-91 **Used in a WoE approach as supporting study**
Rat	Oral	Effects on male reproductive system after *in utero* exposure to DCHP, at prepubertal, pubertal and adult stage. Association of hormonal changes with irreversible organ adverse effects.	Article published	Aydogan A. M. & Barlas N. *Developmental effects of prenatal di-n-hexyl phthalate and dicyclohexyl phthalate exposure on reproductive tract of male rats: Postnatal outcomes.* Food and Chemical Toxicology, 2013, 51:123-136 **Reliability R1 (se also Annex III)**
Rat	Oral	Prenatal male reproductive developmental effects and endocrine association.	Article published	Aydogan A. M. & Barlas N. *Influence of in utero di-n-hexyl phthalate and dicyclohexyl phthalate on fetal testicular development in rats.* Toxicology Letters, 2015, 233: 125-137 **Reliability R1 (se also Annex III)**
Rat	Oral	Foetal testosterone reduction in GD14 to GD18 following *in utero* exposure to DCHP (0, 100, 300, 600, 900 mg/kg bw/day) with increasing doses.	Article published	Furr R.J., Lambright S. C., Wilson S. V., Foster M. P. & Gray E. L. *A Short-term in vivo screen using fetal testosterone production, a key event in the phthalate adverse outcome pathway, to predict disruption of sexual differentiation.* Toxicological Sciences 2014,

				140(2):403-24 doi:10.1093/toxsci/kfu081 **Reliability R1 (se also Annex III)**
Rat	Oral	Decrease in AGD observed in male pups following *in utero* exposure to DCHP in dose response manner.	Article published	Saillenfait, A. M., Gallissot F. & Sabata J. P. *Differential developmental toxicities of di-nhexyl phthalate and dicyclohexyl phthalate administered orally to rats,* Journal of Applied Toxicology, 2009, 29: 510-521. **Reliability R1 (se also Annex III)**
Rat	Oral	Two generation reproductive toxicity study indicating decrease AGD in F1 and F2.	Article published	Hoshino N., Iwai M. & Okazaki Y. *A Two-Generation Reproductive Study of Dicyclohexyl Phthalate in Rats*. The Journal of Toxicological Sciences, 2005, Volume 30, Special Issue, 79-96. **Reliability R1 (se also Annex III)**
Rat	Oral	Effects on DNA at prepubertal, pubertal and adult rat testis at the same concentrations as those at which testicular histopathological effects and testosterone levels changes are observed.	Article published	Ahbab M.A., Undeger U., Barlas N. & Basaran N. *In utero exposure to dicyclohexyl and di-n-hexyl phthalate possess genotoxic effects on testicular cells of male rats after birth in the Comet and TUNEL assays.* Human and Experimental Toxicology, 2014, 33(3): 230-239. **Used in a WoE approach as supporting study**
Rat	Oral	Prolonged preputial separation, reduced AGD, increased areolas/nipple retention, hypospadias, decreased ventral prostate and levator ani/bulbocavernous muscle weight and decreased testicular germ cells were observed in male offspring in the 500 mg/kg bw group.	Article published	Yamasaki K., Okuda H., Takeuchi T. & Minobe Y. *Effects of in utero through lactational exposure to dicyclohexyl phthalate p,p'-DDE in Sprague-Dawley rats*. Toxicolcogy Letters, 2009, 189, 14-20. **Reliability R2 (se also Annex III)**
Rat (castrated)	Oral (forced)	0, 10, 100, 1,000 mg/kg/day Without (androgenic) and with (antiandrogenic) testosterone propionate 0.4 mg/kg/day (s.c.). Negative outcome – neither androgenic nor antiandrogenic activity detected	Results summarised in a report	Hershberger assay – as presented in Table 3-5 page 40 in METI (2002): Current Status of Testing Methods Development for Endocrine Disrupters, Minsitry of Economy, Trade and Industry, Japan. Available at: http://www.meti.go.jp/english/report/data/gEndoctexte.pdf **Used in a WoE approach as supporting study**
In vitro – rat and human testis microsomes	Inhibitory potencies on rat and human 3β-hydroxysteriod dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase	Effect on synthesis of androgens *in vitro* at µM concentrations. 3β-HSD (IC_{50s})human = 25.5 µM 3β-HSD (IC_{50s})rat = 24.7 µM 17β-HSD3 IC_{50s} human = 8.2 µM 17β-HSD3 IC_{50s} rat= 9.1 µM The mode of action of DCHP on 3β-HSD activity was competitive with the substrate pregnelonone but non-competitive with the	Article published	Yuan K., Zhao B., Li, X.-W., Hu G.-X.,Su Y., Chu Y., Akingbemi B.T., Lian Q.-Q. & Ge R.-S. *Effects of phthalates on 3β-hydroxysteriod dehydrogenase and 17β- hydroxysteroid dehydrogenase 3 activities in human and rat testes.* Chemico-Biological Interactions, 2012, 195, 180-188. **Used in a WoE approach as supporting study**

	type 3 (17β-HSD3) activities.	cofactor NAD+. The mode of action of DCHP on 17β- HSD3 was competitive with the substrate androstenedione but non-competitive with the cofactor NADPH.		
In vitro – receptor binding to human ERα, ERβ, and AR.	Chinese Hamster ovary cell line (CHO-K1) transfected with expression vectors for human ERα, ERβ, and AR.	Estrogenic Relative effective conc. showing 20% of the agonistic activity of 10^{-9} M 17ß-estradiol via ERα was $2.8x10^{-6}$ M for DCHP. Antiestrogenic Relative inhibitory conc. showing 20% of the antagonistic activity of 10^{-10} M 17ß-estradiol (RIC20) via ERß was 2.5x10-6 M for DCHP. RIC20 by 10^{-11}M 17ß-estradiol via ERα was $2.8x10^{-6}$ M for DCHP. No androgenic activity. Antiandrogenic RIC20 of 10^{-10} M 5α-dihydrotestosterone via AR was $3.8x10^{-6}$ M for DCHP.	Article published	Takeuchi S., Iida M, Kobayashi S., Jin K., MatsudaT. & Kojima H. *Differential effects of phthalate esters on transcriptional activities via human estrogen receptors α and β, and androgen receptor*. Toxicology, 2005, 210, 223–233 **Used in a WoE approach as supporting study**
Rat	Subcutaneous injection – uterotrophic assay in immature females	2, 20 and 200 mg/kg/bw and day PND 20-22 – no effects on uterine weight. (No information why higher doses were not tested)	Article published	Yamasaki K., Takeyoshi M., Yakabe Y., Sawaki M., Imatanaka N. & Takatsuki M. *Comparison of reporter gene assay and immature rat uterotrophic assay of twenty-three chemicals*. Toxicology, 2002, 170, 21-30. **Used in a WoE approach as supporting study**
Rat (ovariectomized)	Oral (forced)	10, 100, 1,000 mg/kg/day without (estrogenic) and with (antiestrogenic) ethinyl estradiol, 30 µg/kg/day.	Results summarised in a report	Uterotrophic assay – as presented in Table 3-4 page 36 in METI (2002): Current Status of Testing Methods Development for Endocrine Disrupters, Minsitry of Economy, Trade and Industry, Japan. Available at: http://www.meti.go.jp/english/report/data/gEndoctexte.pdf **Used in a WoE approach as supporting study**

Dibutyl phthalate and other phthalates				
Rat	Oral	Supportive data from other phthalates (DBP) link fetal testosterone insufficiency and abnormal proliferation of Leydig cells in rats.		Mylchreest E., Madhabananda S., Wallace D. G. & Foster P. *Fetal testosterone insufficiency and abnormal proliferation of Leydig cells and gonocytes in rats exposed to di(n-butyl)phthalate.* Reprod Toxicol. 2002 Jan-Feb;16(1):19-28. **Used in a WoE approach as supporting study**
Rat *in vivo* and *in vitro*		Leydig and Sertoli cells as a target for phthalates	Abstract only available	Jones H.B., Garside D.A., Liu R. & Roberts J.C. *The influence of phthalate esters on Leydig cell structure and function in vitro and in vivo.* Experimental Molecular Pathology, 1993, 58(3):179-93 **Used in a WoE approach as supporting study**
Human and rat fetal testes, that were xenografted into castrate male nude mice	Oral	Dibutylphthalate produced no effects in testosterone production in human fetal testes compared to rat fetal testis xenografts.	Article publisched	Mitchell R.T., Childs A.J., Anderson R.A., van den Driesche S., Saunders P.T., McKinnell C., Wallace W.H., Kelnar C.J. & Sharpe R.M. *Do phthalates affect steroidogenesis by the human fetal testis? Exposure of human fetal testis xenografts to di-n-butyl phthalate*. Journal of Clinical Endocrinology and Metabolism , 2012, 97(3):E341-8. Available at: http://press.endocrine.org/doi/full/10.1210/jc.2011-2411 **Used in a WoE approach as supporting study**
Human, rat and mice fetal testes was xenografted into immunodeficient rodent hosts	Oral (gavage 100, 250 or 500 mg/kg bw for 1, 2 or 3 conscutive days)	Exposure to dibutylphthalate Only the rat xenograft expressed suppressed steroidogenesis, but all xenograft species exhibited multi nucleated germ cell formations. Human fetal testes xenograft did not express steroidogenic gene expression.	Article published	Heger N.E., Hall S.J., Sandrof M.A., McDonell E.V., Hensley J.B., McDowell E.N., Martin K.A., Gaido K.W., Johnson K.J. & Boekelheide K. *Human Fetal Testis Xenografts Are Resistant to Phthalate-Induced Endocrine Disruption*. Environmental Health Perspectives, 2012, 120, 1137-1143. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3440087/pdf/ehp.1104711.pdf **Used in a WoE approach as supporting study**
Mouse	Oral via pipette	Postnatal-prepubertal exposure of male mice pups. Di-n-butyl phthalate (DBP) oral doses in corn oil: 0, 1, 10, 50, 100, 250 and 500 mg/kg bw/day from PND 4 – PND7, 14 or 21. Adult mice (exposed PND4-21) were examined for remaining effects at 8wks of age. Study reports inter alia delayed spermatogenesis and impaired Sertoli cell maturation, lower serum and testis testosterone levels, and also reduced AGD.	Article published	Moody S., Goh H., Bielanowicz A., Rippon P., Loveland K.L., & Itman C. *Prepubertal mouse testis growth and maturation and androgen production are acutely sensitive to di-n-butyl phthalate.* Endocrinology. 2013 Sep;154(9):3460-75. doi: 10.1210/en.2012-2227. Epub 2013 Jun 13. http://press.endocrine.org/doi/10.1210/en.2012-2227?url_ver=Z39.88-2003&rfr_id=ori:rid:crossref.org&rfr_dat=cr_pub%3dpubmed **Used in a WoE approach as supporting study**
Rat	Oral gavage	High-dose DBP exposure leads to rapid and reversible diminution of the expression of several proteins required for cholesterol transport and	Article published	Thompson C.J., Ross S.M. & Gaido K.W., *Di(n-butyl) phthalate impairs cholesterol transport and steroidogenesis in the fetal rat testis through a rapid and reversible mechanism.,* Endocrinology, 2004,145(3):1227-37

		steroidogenesis in the fetal testis, resulting in decreased testosterone synthesis and consequent male reproductive maldevelopment.		**Used in a WoE approach as supporting study**
Rat	Oral	Morphology and incidence of DBP-induced testicular developmental lesion.	Article published	Barlow N.J., McIntyre B.S. & Foster P.M., *Male reproductive tract lesions at 6, 12, and 18 months of age following in utero exposure to di(n-butyl) phthalate*. Toxicol Pathol, 2004, 32(1):79-90 **Used in a WoE approach as supporting study**
Rat	Oral	Decrease in fetal testosterone levels following exposure to DEHP and DINP.	Article published	Borch, J., Ladefoged O., Hass U. & Vinggaard A. *Steroidogenesis in fetal male rats is reduced by DEHP and DINP, but endocrine effects of DEHP are not modulated by DEHA in fetal prepubertal and adult male rats*. Reproductive toxicology, 2004, 18 (1): 53-61. **Used in a WoE approach as supporting study**
Rat	Oral	Decrease in fetal testosterone levels following exposure to DEHP and DINP.	Article published	Borch J., Metzdorff S., Vinggaard A., Brokken L. & Dalgaard M. *Mechanisms underlying the anti-androgenic effects of diethylhexyl phthalate in fetal rat testis*. Toxicology, 2006, 223: 144-55. **Used in a WoE approach as supporting study**
Rat	Oral	Dose-response studies with diisobutyl (DIBP), dipentyl (DPeP), dihexyl (DHP), diheptyl (DHeP), diisononyl (DINP), or diisodecyl phthalate (DIDP) indicating that all phthalates, with the exception of DIDP, reduced fetal testicular T production.	Article published	Hannas B., Lambright C., Furr J., Evans N., Foster P., Gray E. & Wilson V. *Genomic biomarkers of phthalate induced male reproductive developmental toxicity: a targeted RT-PCR array approach for defining relative potency*. Toxicological Sciences, 2012, 125 (2): 544-57. **Used in a WoE approach as supporting study**
Rat	Oral	Effects of dipentyl phthalate on testicular testosterone production.	Abstract published	Beverly B., Lambright C., Furr J., Sampson H., Wilson V., McIntyre B., Foster P., Travlos G. & Gray L. *Simvastatin and dipentyl phthalate lower ex vivo testicular testosterone production and exhibit additive effects on testicular testosterone and gene experession via distinct mechanistic pathways in the fetal rat*. Toxicological Sciences,2014, doi: 10.1093/toxsci/kfu149. **Used in a WoE approach as supporting study**

Human relevance				
N/A	N/A	"*a statistically significant change in sperm count in a rodent study is considered to be indicative of a potentiual effect on fertility in humans*" (OECD 2008 due to a greater sperm reserve in rats than in humans.	Guidance document published	OECD. *Guidance document on mammalian reproductive toxicity testing and assessment*. OECD Environment, Health and Safety Publications. Series on Testing and Assessment. No. 43, 2008 **Used in a WoE approach as supporting information**

N/A	N/A	*"Decreased AGD in male rats is a hallmark of antiandrogenic substances (Noriega et al 2009; Christiansen et al 2010). A statistically significant change in AGD that cannot be explained by the size of the animal indicates an adverse effect of exposure and should be considered in setting the NOAEL (OECD 2008)"*	Guidance document published	OECD. *Guidance document supporting OECD test guideline 443 on the Extended One-Generation Reproductive Toxicity Test*. OECD Environment, Health and Safety Publications. Series on Testing and Assessment. No. 151, 2013 **Used in a WoE approach as supporting information**
Human	N/A	Interlinkage of endocrine mediated pathways with hypospadias	Article published	Silver R.I. *Endocrine abnormalities in boys with hypospadias*. Adv Exp Med Biol. 2004;545:45-72 **Used in a WoE approach as supporting information**
Human	N/A	Interlinkage of hypospadias with fertility	Article published	Kurzrock E.A. & Karpman E. *Hypospadias: pathophysiology and etiologic theories*. Pedriatr Endocrinol Rev. 2004 Mar;1(3):288-95 **Used in a WoE approach as supporting information**
N/A	N/A	Review of developmental toxicity of phthalates	Review	Chronic Hazard Advisory Panel on Phthalates and Phthalate Alternatives Final Report , Appendix A, Report to the US Consumer Product Safety Commission, July 2014 Appendix A Developmental toxicity available at: http://www.cpsc.gov/en/Regulations-Laws--Standards/Statutes/The-Consumer-Product-Safety-Improvement-Act/Phthalates/Chronic-Hazard-Advisory-Panel-CHAP-on-Phthalates/ **Used in a WoE approach as supporting information**
Mammals	N/A	Review of male reproductive development and endocrine pathways	Article published	Nef, S. *Hormones in Male Sexual development*. Genes and Development, 2000, 14 (24), 3075-3086. **Used in a WoE approach as supporting information**
Rat	Oral	Testosterone dependency for male reproductive system development	Article published	Bowman C., Barlow N., Turner K., Wallace D. & Foster P. *Effects of in utero exposure to finasteride on androgen dependent reproductive development in the male rat*, Toxicological Sciences, 2003, 74 (2): 393-406. **Used in a WoE approach as supporting information**
Human	N/A	Interlinkage of hypospadias with fertility	Article published	Asklund C., Jensen T., Main K., Sobotka T., Skakkebaek N., Jorgensen N., *Semen quality, reproductive hormones and fertility of men operated for hypospadias*. International Journal of Andrology, 2010, 33: 80-7. **Used in a WoE approach as supporting information**
Human	N/A	Interlinkage of hypospadias with fertility	Article published	Bracka A. *A long term view of hypospadias*. British Journal of Plastic Surgery, 1989, 42(3): 251-5. **Used in a WoE approach as supporting information**
Rat	Oral	Testosterone dependency for male	Abstract	Beverly B., Furr J., Lambright C., McIntyre B., Foster P., Travlos G., Wilson

		reproductive system development	published	V. & Gray L., *Simvastatin reduces fetal testosterone production and permanently alters reproductive tract development in the male Crl: CD (SD) Rat*, available at: http://usgov.info/2015/04/16/simvastatin-reduces-fetal-testosterone-production-and-permanently-alters-reproductive-tract-development-in-the-male-rat/ **Used in a WoE approach as supporting information**
Rat	Oral	Antiandrogenic mode of action and male reproductive adverse effects	Abstract published	Wolf C., Lambright P., Mann M., Price M., Cooper R., Ostby J. & Gray L., *Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, P,p-DDE and ketoconazole) and toxic substance (dibutyl- and diethylexyl phthalate, PCB 169 and ethan dimethane suphonate) during sexual differentiation*. Toxicology and Industrial Health, 1999, 15: 94-118. **Used in a WoE approach as supporting study**
Human	N/A	Male reproductive disorders of antenatal origin	Article published	Skakkebaek N.E., Rajpert-De Meyts E. & Main K.M. *Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects*. Human Reproduction, 2001, 16(5):972-978. Available at: http://humrep.oxfordjournals.org/content/16/5/972. **Used in a WoE approach as supporting study**
Human	N/A	Anogenital distance related effects and fertility	Article published	Eisennberg L.M., Hsieh H.M., Walters C.R., Krasnow R., Lipschultz I. L. *The relationship between Anogenital distance, fatherhood and fertility in adult men*. PLoS ONE 6(5): e18973. Doi:10.1371/journal.pone.0018973 **Used in a WoE approach as supporting study**
Human	N/A	Linking of infertility with fetal reproductive systems impairment	Article published	Juul A., Almstrup K., Andersson A., Jensen T., Jorgensen N., Main K., Raipert-De Meyts E., Toppari J. & Skakkebaek N. *Possible fetal determinants of male infertility*. Nature Reviews Endocrinology, 2014, 10: 553-562. **Used in a WoE approach as supporting study**
Human	N/A	Anogenital distance related effects and fertility	Article published	Dean A., Sharpe M.R., *Anogenital distance or digit length ration as measures of fetal androgen exposure: relationship to male reproductive development and its disorders*. J Clin Endocrinol Metabl, 2013, 98(6):2230-2238. **Used in a WoE approach as supporting study**
Human	N/A	Relevance of testicular dysgenesis syndrome for fertility effects	Article published	Wohlfahrt-Veje C., Main M.K. & Skakkebaek E.N. *Testicular dysgenesis syndrome: foetal origin of adult reproductive problems*. Clinical Endocrinology, 2009, 71:459-465. **Used in a WoE approach as supporting study**
Human	N/A	Relevance of testicular dysgenesis syndrome for fertility effects	Article published	Akre O. & Richiardi L., *Does a testicular dysgenesis syndrome exists?* Human Reproduction, 2009, 24(9):2053-2060. **Used in a WoE approach as supporting study**

Annex III – Method for collection and assessment of the data used for preparing SVHC dossier for dicyclohexyl phthalate (DCHP)

The information used in the CLH-report and the RAC-documentation for the classification of DCHP was used as the starting material. An additional search was done in PubMed July 2014, complemented in June 2015, using the search term: dicyclohexyl phthalate. Additional information on similar substances (other phthalates) as available in registration dossiers, in previous RAC opinions as well as existing regulatory reviews of these substances were considered as part of the strategy of collecting the available information.

In this Annex, the reliability of the *in vivo* studies considered for the preparation of the SVHC dossier is further elaborated. The reliability of other types of information (*in vitro* studies, bridging information from other phthalates) is addressed with confidence levels using the Weight of Evidence approach within the Mode of Action/ Human Relevance Framework on the basis of the Bradford Hill considerations, for more information see Annex II.

The studies were all considered relevant as already used by RAC and/or fulfilling the relevance criteria of the Science in Risk Assessment and Policy (Scirap) tool. The used *in vivo* studies were evaluated using Scirap tool available at www.scirap.org. The tier I and II tool was used to evaluate the reliability of the studies.

During the preparation of the dossier contact was made with authors for complementary information if the papers contained crucial information with some flaws in the reporting with regards to the study design. This made it possible to better evaluate the reliability of the study results.

The reliability categories used are adapted from the Klimisch score by Moermond et al. (submitted manuscript) and defined as follows:

R1 Reliable without restrictions: All critical reliability criteria for this study are fulfilled. The study is well designed and performed, and it does not contain flaws that affect the reliability of the study.

R2 Reliable with restrictions: The study is generally well designed and performed, but some minor flaws in the documentation or setup may be present.

R3 Not reliable: Not all critical reliability criteria for this study are fulfilled. The study has clear flaws in study design and/or how it was performed.

R4 Not assignable: Information needed to make an assessment of the study is missing. This concerns studies which do not give sufficient experimental details and which are only listed in abstracts or secondary literature (books, reviews, etc.), or studies of which the documentation is not sufficient for assessment of reliability for one or more vital parameters.

Table. Summary of the evaluations on *in vivo* DCHP publications

Reference	Study design	Scirap summary (tier II)	Comments	Final category
Hoshino N., Iwai M., Okazaki Y. *A Two-Generation Reproductive Study of Dicyclohexyl Phthalate in Rats*. The Journal of Toxicological Sciences,	OECD TG 416 (1983) + additional ED endpoints	Of the 29 applicable parts of the evaluation, 62% fulfilled, 14 % partially and 24% not fulfilled – overall good reporting, the not fulfilled includes analysis of feed and water for	Information about study design was also available in the introductory paper of the special issue: Yamasaki K., Takahashi M, Yasuda M. 2005. Two-Generation Reproductive Toxicity Studies	Over-all R1

Volume 30, Special Issue, 79-96, 2005		contaminants.	in rats with extra parameters for detecting endocrine disrupting activity: Introductory overview of results from nine chemicals. The Journal of Toxicological Sciences, Volume 30, Special Issue, 1-4, 2005	
Yamasaki K., Okuda H., Takeuchi T., Minobe Y. ***Effects of in utero through lactational exposure to dicyclohexyl phthalate and p,p'-DDE in Sprague-Dawley rats*****. Toxicolcogy Letters 189,2009,14-20.**	Mated female rats (F0) (~12 weeks old) subdivided into 4 equally sized groups • Culling at PND 4, to litter size of 8 aiming for 4 pups/sex when possible. •At weaning pups (F1) randomly subdivided into sub groups A and B A. Sacrificed at 10 weeks of age. B. 2 females and 2 males/dam mated at 12 weeks to assess reproductive performance and possible effects on early embryonic development (caesarean sections performed on gestation day 13).	Of the 28 applicable parts of the evaluation, 43% fulfilled, 18 % partially and 39% not fulfilled – the not fulfilled includes lack of detailed reporting regarding animals, housing, feed, and choice of administration route.	Important study results – no contact made with author as other studies with similar design show similar results. Results used as supportive.	R2

Saillenfait, A. M., Gallissot F., Sabata J. P., *Differential developmental toxicities of di-n-hexyl phthalate and dicyclohexyl phthalate administered orally to rats*, Journal of Applied Toxicology, 2009, 29: 510-521.	Study protocol resemble Prenatal developmental toxicity study (OECD TG 414). 20-25 pregnant dams/group. In addition, anogenital distance was measured on GD 21. Satellite study 6-9 animals/dose level, dosing interval as main study, for examination of liver effects (histopathology, enzyme activity and liver weights) on GD 21. Non-GLP study.	Of the 30 applicable parts of the evaluation, 74% fulfilled, 13 % partially and 13% not fulfilled – overall good reporting, the not fulfilled includes analysis of feed and water for contaminants.		R1
Aydogan A. M., Barlas N. *Developmental effects of prenatal di-n-hexyl phthalate and dicyclohexyl phthalate exposure on reproductive tract of male rats: Postnatal outcomes*, Food and Chemical Toxicology, 2013, 51:123-136	Pregnant rats After delivery all pups were allowed to grow with their dam for 1 month and then male pups were separated and housed 4/cage until they were killed on: - PND 20 (pre-pubertal), - PND 32 (pubertal) - PND 90 (adult).	Initial evaluation - Of the 29 applicable parts of the evaluation, 57% fulfilled, 7 % partially and 34.5% not fulfilled – reporting poor for animals, housing and feed.	Further info provided from author – renewed evaluation: Of the 28 applicable parts of the evaluation, 82% fulfilled, and 18% not fulfilled.	Over-all R1
Aydogan A. M., Barlas N. *Influence of in utero di-n-hexyl phthalate and dicyclohexyl phthalate on fetal testicular development in rats*. Toxicology Letters, 2015, 233: 125-137	Wistar rats, exposed GD 6-19 with DCHP 0, 20, 100 and 500 mg/kg bw/day. Male foetuses examined on GD 20 for testosterone, FSH, inhibin B, MIS, testis histopathology (including immunohistochemical staining	Of the 29 applicable parts of the evaluation, 69% fulfilled, and 31% not fulfilled - reporting poor for animals, housing and feed.	Further info provided from author – renewed evaluation: Of the 28 applicable parts of the evaluation, 82% fulfilled, and 18% not fulfilled.	Over-all R1

	for 3β-HSD. MIS/AMH. AR and PCNA and determination of Leydig cell numbers and clusters.			
Furr R.J., Lambright S. C., Wilson S. V., Foster M. P., Gray E. L. A *Short-term in vivo screen using fetal testosterone production, a key event in the phthalate adverse outcome pathway, to predict disruption of sexual differentiation*. Toxicological Sciences 2014, 140(2):403-24 doi:10.1093/toxsci/kfu081	*In vivo* screen to detect disruption of fetal testosterone synthesis	Of the 29 applicable parts of the evaluation, 83% fulfilled, 6.9 % partially and 10% not fulfilled		R1