CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

International Chemical Identification:

Diisooctyl phthalate

EC Number: 248-523-5

CAS Number: 27554-26-3

Index Number: -

Contact details for dossier submitter:

ANSES (on behalf of the French MSCA)

14 rue Pierre Marie Curie

F-94701 Maisons-Alfort Cedex

reach@anses.fr

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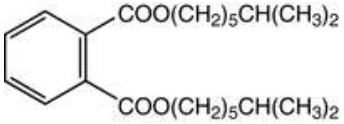
CONTENTS

1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 1: Substance identity and information related to molecular and structural formula of the substance

Name(s) in the IUPAC nomenclature or other international chemical name(s)	Diisooctyl phthalate
Other names (usual name, trade name, abbreviation)	DIOP bis(6-methylheptyl) phthalate Diisooctyl phthalate 1,2-Benzenedicarboxylic acid, 1,2-diisooctyl ester
ISO common name	-
EC number	248-523-5
CAS number	27554-26-3
Molecular formula	$C_{24}H_{38}O_4$
Structural formula *	
SMILES notation (if available) *	CC(C)CCCCCOC(=O)C1=CC=CC=C1C(=O)OCCCCCC(C)C
Molecular weight or molecular weight range	390.56
Information on optical activity and typical ratio of (stereo) isomers (if applicable and appropriate)	Not relevant
Description of the manufacturing process and identity of the source (for UVCB substances only)	Not available
Degree of purity (%) (if relevant for the entry in Annex VI)	100% (DIOP is an UVCB)

* DIOP is an UVCB substance, structural formula and SMILES refer to one of the possible isomers present in the composition of the substance.

1.2 Composition of the substance

Diisooctyl phthalate is a UVCB substance including a number of constituents having alkyl chains showing different type of branching with overall carbon number corresponding to eight. The branching type determines the length of the main alkyl chain backbone, *i.e.* the number of carbons in the backbone.

This classification dossier was not backed up by any REACH, PPP or biocide dossier, therefore composition detailled below is taken from litterature. According to information found in literature (Saillenfait, 2013), the composition of commercially available diisooctyl phthalate (DIOP) commonly includes 70-75% of isomers with C4-C6 ester backbone and less than 25% of isomers with C7 backbone or more. Examples of isomers that may be present in the substance composition are di-2-ethylhexyl phthalate (DEHP, CAS RN 117-81-7), which has a backbone of six carbons and di-n-octyl phthalate (DnOP, CAS RM 117-84-0, backbone of 8 carbons), which has linear ester chains.

No further information is available on the composition of the substance.

As the substance is an UVCB, its purity is 100%.

Table 2: Constituents (non-confidential information)

Constituent (Name and numerical identifier)	**Concentration range (% w/w minimum and maximum in multi-constituent substances)**	**Current CLH in Annex VI Table 3.1 (CLP)**	**Current self-classification and labelling (CLP)**
Diisooctyl phthalate	100%	None	See table below*

*As notified in ECHA website on February 2017

Classification		**Number of notifiers**
Hazard class and category code	**Hazard statement code**	
Repr. 1 B	H360	66
Aquatic Chronic 4	H413	81
Not classified	H335	1

Table 3: Impurities (non-confidential information) if relevant for the classification of the substance

Impurity (Name and numerical identifier)	**Concentration range (% w/w minimum and maximum)**	**Current CLH in Annex VI Table 3.1 (CLP)**	**Current self-classification and labelling (CLP)**	**The impurity contributes to the classification and labelling**
Not relevant				

Table 4: Additives (non-confidential information) if relevant for the classification of the substance

Additive (Name and numerical identifier)	**Function**	**Concentration range (% w/w minimum and maximum)**	**Current CLH in Annex VI Table 3.1 (CLP)**	**Current self-classification and labelling (CLP)**	**The additive contributes to the classification and labelling**
/					

2 PROPOSED HARMONISED CLASSIFICATION AND LABELLING

2.1 Proposed harmonised classification and labelling according to the CLP criteria

Table 5:

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific 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)		
Current Annex VI entry	No existing Annex VI entry										
Dossier submitters proposal	To be determined	Diisooctyl phthalate	248-523-5	27554-26-3	Repr. 1B	H360DF	Danger GHS 08	H360DF	-	-	-
Resulting Annex VI entry if agreed by RAC and COM	To be determined	Diisooctyl phthalate	248-523-5	27554-26-3	Repr. 1B	H360DF	Danger GHS 08	H360DF	-	-	-

Table 6: Reason for not proposing harmonised classification and status under public consultation

Hazard class	Reason for no classification	Within the scope of public consultation
Explosives	Hazard class not assessed in this dossier	No
Flammable gases (including chemically unstable gases)	Hazard class not assessed in this dossier	No
Oxidising gases	Hazard class not assessed in this dossier	No
Gases under pressure	Hazard class not assessed in this dossier	No
Flammable liquids	Hazard class not assessed in this dossier	No
Flammable solids	Hazard class not assessed in this dossier	No
Self-reactive substances	Hazard class not assessed in this dossier	No
Pyrophoric liquids	Hazard class not assessed in this dossier	No
Pyrophoric solids	Hazard class not assessed in this dossier	No
Self-heating substances	Hazard class not assessed in this dossier	No
Substances which in contact with water emit flammable gases	Hazard class not assessed in this dossier	No
Oxidising liquids	Hazard class not assessed in this dossier	No
Oxidising solids	Hazard class not assessed in this dossier	No
Organic peroxides	Hazard class not assessed in this dossier	No
Corrosive to metals	Hazard class not assessed in this dossier	No
Acute toxicity via oral route	Hazard class not assessed in this dossier	No
Acute toxicity via dermal route	Hazard class not assessed in this dossier	No
Acute toxicity via inhalation route	Hazard class not assessed in this dossier	No
Skin corrosion/irritation	Hazard class not assessed in this dossier	No
Serious eye damage/eye irritation	Hazard class not assessed in this dossier	No
Respiratory sensitisation	Hazard class not assessed in this dossier	No
Skin sensitisation	Hazard class not assessed in this dossier	No
Germ cell mutagenicity	Hazard class not assessed in this dossier	No
Carcinogenicity	Hazard class not assessed in this dossier	No
Reproductive toxicity	Harmonised classification proposed	Yes
Specific target organ toxicity-single exposure	Hazard class not assessed in this dossier	No
Specific target organ toxicity-repeated exposure	Hazard class not assessed in this dossier	No
Aspiration hazard	Hazard class not assessed in this dossier	No
Hazardous to the aquatic environment	Hazard class not assessed in this dossier	No
Hazardous to the ozone layer	Hazard class not assessed in this dossier	No

3 HISTORY OF THE PREVIOUS CLASSIFICATION AND LABELLING

There is no current harmonized classification for DIOP.

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

Available data show that DIOP has CMR property, i.e. reproductive toxicity that is not currently harmonised and justify a harmonised classification and labelling according to article 36 of CLP.

C&L inventory reported that 66/82 notifiers classify DIOP as Repr. 1B – H360. The diverging classification notifications for CMR properties renders harmonised classification necessary.

5 IDENTIFIED USES

DIOP is a phthalic acid diester which is primarily used as a plasticizer for synthetic rubber and vinyl, cellulosic and acrylate resins in a variety of consumer products. There are known uses of DIOP in the manufacture of jackets for building wire, and automotive hoses and parts. It was also identified in some children toys and in commercial milk products. The production of DIOP was estimated at about 10 000 metric tons in United States and its consumption at 15 000 metric tons in Western Europe in 2008. DIOP contributed to 1.7% of the total phthalate market (Saillenfait, 2013).

DIOP may potentially be used as an alternative of DEHP. In this context, despite a current limited use of DIOP in Europe, a classification process for this substance is needed in order to avoid possible substitution of phthalates for which harmonized classification as Repr. 1B exists, by DIOP which has no agreed classification.

6 DATA SOURCES

DIOP is only under pre-registration process in REACH regulation. Information provided in this CLH report is only issued from literature search.

Data in physico-chemical properties are issued from references found in *"handbook of physico-chemical properties and envirnemental fate for organic chemicals" second edition 2005, volume III* and, for missing data, from MSDS from an industry available on line. These data have not been assessed and should be considered as indicative only.

7 PHYSICOCHEMICAL PROPERTIES

Table 7: Summary of physicochemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
Physical state at 20°C and 101,3 kPa	liquid	MSDS Alfa Aesar	
Melting/freezing point	-46°C	Staples and al. 1997.	
Boiling point	270°C	Lide 2003	
Relative density	0.983 g/cm^3	MSDS Alfa Aesar	
Vapour pressure	7.4.10^{-4} Pa at 25°C	Howard and al. 1985	Gas saturation method
Surface tension	No data		
Water solubility	0.09 mg/L	Howard and al. 1985	Shake flask -GC
Partition coefficient n-octanol/water	8.0 ; 8.39	Staples and al. 1997	Calculated QSAR
	7.73	Cousins and Mackay 2000	Calculated QSPR
Flash point	390°C	MSDS Alfa Aesar	
Flammability	No data		
Explosive properties	Not expected to be	eCA assessment	

Property	Value	Reference	Comment (e.g. measured or estimated)
	explosive according to structure of compound		
Self-ignition temperature	> 390°C	eCA assessment	Based on flash point data
Oxidising properties	Not expected to be oxidising according to structure of compound	eCA assessment	
Granulometry	Not relevant		
Stability in organic solvents and identity of relevant degradation products	No data		
Dissociation constant	Not expected in the range 0-14 according to structure of compound		
Viscosity	No data		

8 EVALUATION OF PHYSICAL HAZARDS

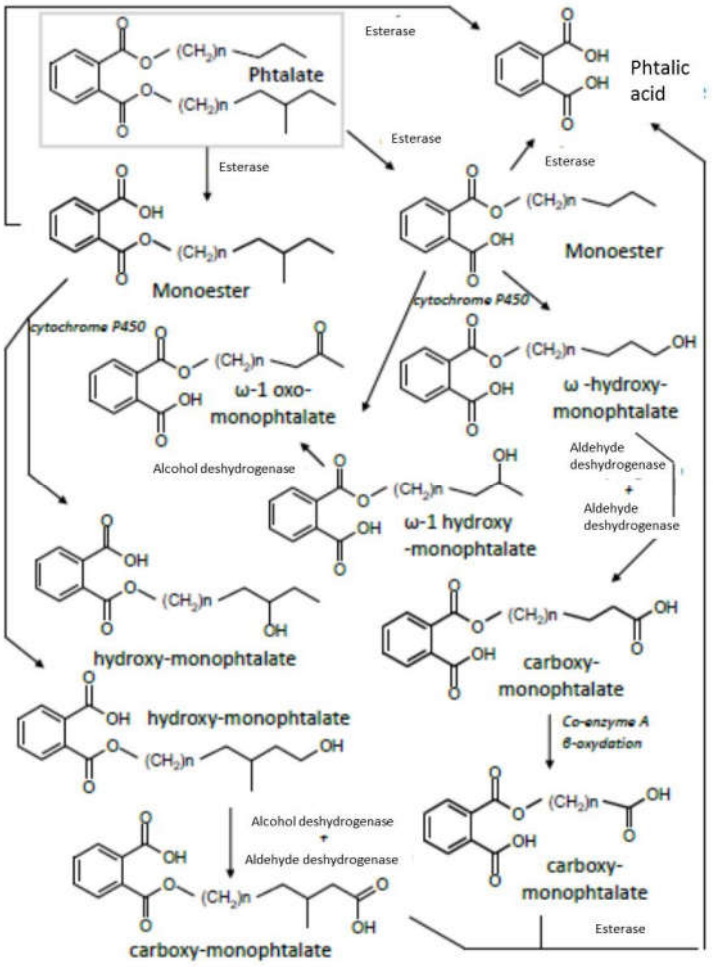
Physical hazards are not assessed in this dossier.

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Phthalates are converted to monoesters and alcohols and rapidly excreted. It is expected that DIOP would behave in the same way (ANSES, 2015).

In addition, the following toxicokinetics data on DIOP are detailed in the CPSC toxicity review for DIOP (2011). A study on Human volunteers showed that monoesters of DIOP are excreted in the urine within 24 hours (Anderson *et al*., 2001 as cited in CPSC (2011) report). After gavage administration in rats, dogs and miniature pigs, DIOP persisted in gastrointestinal tract for several days. DIOP was mainly excreted in the urine of pigs and in the faeces of dogs. In rats, the excretion was more rapid than in dogs and pigs and was equally distributed between the urine and the faeces. DIOP was highly metabolized in particular in rats in which virtually all the radioactivity was in the form of metabolites. Apart from a small amount distributed to fat within this timeframe, no significant tissue accumulation of DIOP was found in experimental animals (Ikeda *et al*., 1978 as cited in CPSC (2011) report). In another metabolism study, mono-(3-carboxypropyl) phthalate (MCPP), mono-*n*- octyl phthalate (MnOP) and mono-(3-methyl-5- dimethylhexyl) phthalate (MiNP) were found in the urine of rats orally exposed to DIOP by gavage (Calafat *et al*., 2006 as cited in CPSC (2011) report).

Figure 1: Main Phase I metabolic pathways for phthalates in rodents and humans (from INSERM Expertise "reproduction and environnement, 2011)



Details are available in Annex I to the CLH report.

10 EVALUATION OF HEALTH HAZARDS

10.1 Acute toxicity - oral route

Oral LD_{50} = 2769 mg/kg in one mouse study and > 22,000 mg/kg in two orther studies (rat and mouse) (US CPSC, 2011). Data not assessed in this CLH report.

10.2 Acute toxicity - dermal route

Dermal toxicity with LD_{50} > 3160 mg/kg were reported in two rabbits studies (US CPSC, 2011). Data not assessed in this CLH report.

10.3 Acute toxicity - inhalation route

Not assessed.

10.4 Skin corrosion/irritation

DIOP was a severe dermal irritant at high doses in one well conducted rabbit study, but only a minimal to mild irritant in two other studies (rabbit and rat) (US CPSC, 2011). Data not assessed in this CLH report.

10.5 Serious eye damage/eye irritation

Not assessed.

10.6 Respiratory sensitisation

Not assessed.

10.7 Skin sensitisation

Not assessed.

10.8 Germ cell mutagenicity

Not assessed.

10.9 Carcinogenicity

Not assessed.

10.10 Reproductive toxicity

10.10.1 Adverse effects on sexual function and fertility

Table 8: Summary table of animal studies on adverse effects on sexual function and fertility

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Experiment 1: Prenatal toxicity test, comparable to OECD guideline 414 SD female rats 10-12 time-mated females (8-12 pregnant) Klimish 2	DIOP: 0, 100, 500, 1000 mg/kg bw/day GD6-20 by oral gavage	Maternal effects: Decreased body weight and body weight gain in late gestation at 1000 mg/kg bw/day related to decrease uterine content since there was no effect in net body weights. NOAEL maternal = 1000 mg/kg bw/day Developmental effects: From 500 mg/kg bw/day: - Decreased foetal body weight but not significant when litter size used as covariable - Skeletal variations (14th supernumerary lumbar ribs, retarded ossification) At 1000 mg/kg bw/day: - Increased post-implantation loss and resorption, decreased foetal body weight - Skeletal variations (14th supernumerary lumbar ribs,	Saillenfait, 2013

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
		retarded ossification) - **Testis malpositioned** NOAEL development = 100 mg/kg bw/day	
Experiment 2 Ex-vivo testosterone production by foetal testis No guideline followed SD female rats Klimish 2	DIOP: 0, 10, 100, 500, 1000 mg/kg bw/day GD12-19 by oral gavage	**Dose-dependent decrease in testicular testosterone production from 100 emg/kg bw/day** NOAEL = 10 mg/kg bw/day	Saillenfait, 2013
Experiment 3 Peri-postnatal toxicity study No guideline followed 10-12 pregnant SD female rats Klimish 2	DIOP: 0, 100, 500, 1000 mg/kg bw/day GD12-21 by oral gavage	Maternal effects: - Decreased body weight at GD21 at 1000 mg/kg bw/day NOAEL maternal = 500 mg/kg bw/day Developmental effects: At 500 mg/kg bw/day - **Gross morphological alterations of external and internal genitalia in 3 males in 3 litters** - **Increased absolute and relative testis weight** and decreased relative right kidney weight - **Histopathological lesions in the testis (in particular hypospermatogenesis in 2 males of 2 litters)** At 1000 mg/kg bw/day: - Decreased viability (PND1-21) - **Permanent areolas and/or nipple buds at adult necropsy** - **Marked malformations of the male reproductive tract** - **Decreased relative and absolute weights of kidneys, testis and epididymis** - **Histopathological lesions in the testis (in particular hypospermatogenesis in 15 males in 8 litters)** NOAEL development = 100 mg/kg bw/day	Saillenfait, 2013

In bold effects that can be involved in further fertility impairment.

GD: Gestational Day
PND: Post-Natal Day

Detailed studies are available in Annex I to the CLH report.

10.10.2 Short summary and overall relevance of the provided information on adverse effects on sexual function and fertility

Human data

No human data is available to assess fertility and sexual function with DIOP. However, there are numerous epidemiological studies with other phthalates.

Contradictory results were found regarding an association between phthalates concentration and alteration of sperm in adult life. In particular, large internationally coordinated studies on semen quality of men from the general population found large differences between countries; especially in Denmark a large proportion of the men had semen quality in the sub-fertle range while Finnish men seem to have significantly better semen quality. However, latest studies in Finland seem to indicate that the semen quality of young Finnish men has continued to decrease and now approach the levels seen in the Dannish male population (Jørgensen *et al.,* 2011 cited in ECHA, 2016). Congenital malformation of the male genitalia and delayed sexual maturation can be involved in further fertility impairment. However, no direct link between these effects and pre and peri-natal exposure to phthalates has been proven in human even if there are some circumstantial findings indicating that phthalates could play a role. Overall, epidemiological studies are generally associated with considerable uncertainties due to exposures to many susbtances, limited study populations, uncertainties in back-calculation of urine concentrations to estimated daily exposures, behaviour and societal background, genotype, smoker/non smoker, diet, weight etc. Therefore, a clear conlusion on fertility from human data is difficult to reach (ECHA, 2016).

Non-human data

No experimental fertility study performed in one or more generations according to OECD guidelines is available with DIOP. Instead, effects on sexual function can be anticipated from three experiments where pregnant rats were exposed to DIOP during different periods of gestation to detect effect on prenatal, peri-natal and post-natal development. Male reproductive tract as a target of DIOP was evidenced in these studies.

In the first experiment, pregnant rats were exposed to DIOP from GD6-20 by oral gavage with assessment of prenatal development on GD21. In dams, only some reductions of body weight was observed at the highest dose of 1000 mg/kg bw/day. This was not considered as a maternal toxicity since net body weight was not affected. Abnormal position of the testes (i.e. abdominal or supra-inguinal) was detected in 1 male foetus at 500 mg/kg bw/day and in 10 male foetuses in 5 litters at 1000 mg/kg bw/day (statistically significant at this dose). This result is consistent with the high incidence of undescended testis found in adult male rats after *in utero* exposure reported in the third experiment. It is thus demonstrated that prenatal transabdominal migration of the testis, that is mediated by the production of Insl3 (insulin-like 3) protein by foetal Leydig cells, is affected by *in utero* exposure to DIOP. Reduction of Insl3 gene expression and/or alteration of transabdominal migration of the testis is consistently reported with various C3-C7 phthalates, showing a similar mode of action of these substances (Anses, 2015; Saillenfait *et al*., 2013).

In the second experiment, pregnant rats were exposed to DIOP by oral gavage during the critical period of male sexual differentiation (i.e. GD12-19). Testis were collected on GD19 and analysed for testosterone production. A dose-dependent decreased in *ex-vivo* testosterone production by the foetal testis at GD19 was observed from 100 mg/kg bw/day (Saillenfait *et al*., 2013). Decreased testosterone production is consistently reported with various C3-C7 phthalates, showing a similar mode of action of these substances.

In the third experiment, pregnant rats were exposed to DIOP from GD12-21 by oral gavage with assessment of peri and post-natal development before and after weaning (last necropsy on PND 82-84). Little maternal toxicity was reported with a significant decrease of body weight on GD21 (- 9%) at the highest dose of 1000 mg/kg bw/day. *In utero* exposure of DIOP induced permanent postnatal alterations in androgen-dependent structures of male offspring. They mainly consisted on retained nipples (69% of males), hypospadias (36% of males), undescended testis (74% of males), markedly underdeveloped seminal vesicles (38%) and hypospermatogenesis (88% of males) reported at 1000 mg/kg bw/day. In addition, alterations in epididymis

(thin body or absent), vasa deferentia (absent, thin or crossed) and prostate (underdeveloped) were found in some animals (between 10 and 23% of males) at this dose. Some effects on male reproductive tract were also apparent at 500 mg/kg bw/day but only in few animals: one animal had an unilaterally enlarged testis, one had an abnormal epididymis, one displayed markedly underdeveloped seminal vesicles and prostate and two presented hypospermatogenesis (Saillenfait *et al*., 2013). These effects are characteristics of a decrease of androgens, that is consistent with the result of the second experiment. Recent publications have questioned the relevance of anti-androgenic effects induced by phthalates in rats to humans. Indeed, some experimental studies using *in vitro* or xenograft models did not show any decrease of testosterone by different phthalates (such as DBP, MEHP and MBP) in human foetal testis although this effect was clearly observed in rat foetal testis (Anses, 2015). However, in the current state of knowledge, these data are not sufficiently robust to conclude that the effects in testis observed in rats will not be also found in humans.

Additional data of lower quality were found in the literature:

The following summary relative to reproductive toxicity was found on Pubchem website (July 2016) (source HSDB). In a two generation study, male/female Swiss CD-1 mice (20 animals/sex) were exposed daily to 0.0, 0.01,0.10, or 0.3% (approximately 0, 14, 140, or 420 mg/kg) of diisooctyl phthalate in their diet beginning 7 days pre-mating and throughout a cohabitation period for approximately 14 weeks. There were 40 (animals/sex) in the untreated control group. Reproductive function was assessed during this cohabitation period for number of litters per pair, number of live pups, sex, live births, and pup weight. Following the 14-week cohabitation, the pairs were separated during which any final litters were delivered and kept for assessment of the next generation fertility (F1). Due to an observed effect on fertility, a crossover mating study was performed to determine the affected sex. These mice were evaluated for body weight, organ weights, and sperm indices. When the F1 litters were sexually mature, they were mated with animals from different litters within the same group. The F2 litters were examined for litter size, survival, sex and pup weight. The F1 animals were then necropsied. A significant decrease in the number of litters/pair, live pups/litter, mean live pup weight and proportion of live pups was observed at 140 mg/kg/day. Exposure to 420 mg/kg/day resulted in significant infertility during the continuous breeding phase of the study which was seen in both sexes as identified via the crossover mating study. Exposure to the high dose in the crossover study also resulted in male specific effects including reduced testis, epididymis, prostate weights, percentages of motile sperm and abnormal sperm, and sperm concentration in the males. In females effects included reduced combined weight of ovaries, oviducts and uterus. Both sexes exhibited increased liver weights. The majority of high-dose male mice evidenced some degree of bilateral atrophy of the seminiferous tubules, however; no exposure related histopathology was observed in the females. The reference for this summary is EPA/Office of Pollution Prevention and Toxics; High Production Volume (HPV) Challenge Program's Robust Summaries and Test Plans (2007). A website link is provided but is not valid. No further details on this study can be found in the literature. Therefore, the relevance of these findings cannot be checked. In this context, this study cannot be adequately assessed for classification purpose.

In the CPSC (2011) report, reference to a study performed by Lefaux (1972) cited by NICNAS (2008) is made. According to the summary available in the CPSC (2011) report, no effect on growth was reported in this study performed in rats administered DIOP via the oral route at 0, 1000 mg/kg/day (five generations for 21 months), 300 mg/kg/day (3 generations for 21 months) or 500 mg/kg/day (3 generations for 15 months)(Lefaux, 1972 as cited in NICNAS, 2008 and ECB, 2000). However, it is also stated that although this study included multigeneration exposure, it is unclear if reproductive toxicity endpoints were evaluated (no data were provided). Due to the low level of details provided, the relevance of these findings cannot be checked.

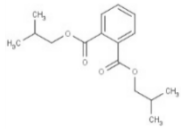
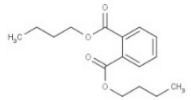
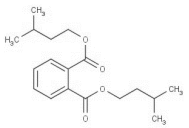
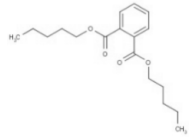
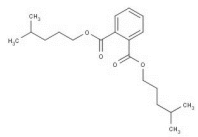
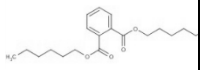
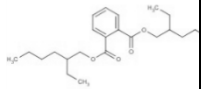
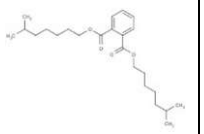
In summary, even if fertility was not appropriately assessed for DIOP, effects on male reproductive tract were reported after *in utero* administration in rats suggesting that DIOP is likely to impact sexual function and/or fertility. The effects found in the Saillenfait *et al*. (2013) study with DIOP are consistent with those observed with other phthalates of medium chain.

Category approach

The proposed category approach includes *ortho*-phthalates with alkyl side-chain length between C3 and C7 which already have a harmonized classification. Phthalates with less than 3C and with more than 8C are considered not reprotoxic (Health Canada, 2015).

The weight of evidence is based on structure, physicochemical and toxicological properties related to reproduction.

Table 8.1: Comparison of structure

	Diisobutyl phthalate (DIBP)	Dibutylphthalate (DBP)	Diisopentylphthalate (DIPP)	Pentylphthalate (DnPP)	1,2-benzenedicarboxylic acid, dihexyl ester, branched and linear (DHP)	di-n-hexyl phthalate (DnHP)	Di-(2-ethylhexyl) phthalate (DEHP)	Diisooctyl phthalate (DIOP)
CAS number	84-69-5	84-74-2	605-50-5	131-18-0	68515-50-4	84-75-3	117-81-7	27554-26-3
Chemical formula	$C_{16}H_{22}O_4$	$C_{16}H_{22}O_4$	$C_{18}H_{26}O_4$	$C_{18}H_{26}O_4$	C20H30O4	C20H30O4	C24H38O4	$C_{24}H_{38}O_4$
Side chain length *In parentheses, the total number of carbon atoms in the side chain*	3C (4C)	4C	4C (5C)	5C	5C (6C)	6C	6C (8C)	7C (8C)
Structure								
Current harmonized classification	Repr. 1B - H361Df	Repr. 1B - H361Df	Repr. 1B - H361DF	Repr. 1B - H361DF	Repr. 1B - H361DF	Repr. 1B - H361DF	Repr. 1B - H361DF	None
ATP	ATP09	CLP00	CLP00	CLP00	ATP07	ATP05	CLP00	None

All these phthalates have a common ortho phthalic acid group esterified to alkyl chain between C3 and C7. The alkyl chain is either linear or contains methyl or ethyl branching.

As given above, technical Diisooctyl phthalate is a UVCB substance that commonly includes 70-75% of isomers with C4-C6 ester backbone and less than 25% of isomers with C7 backbone. Examples of isomers that may be present in the substance composition are di-2-ethylhexyl phthalate (DEHP, CAS RN 117-81-7), which has a backbone of six carbons and di-n-octyl phthalate (DnOP, CAS RM 117-84-0, backbone of 8 carbons), which has linear ester chains. Therefore, It should be taken into account that technical DIOP contains a proportion of DEHP and probably a portion of DHP.

Table 8.2: Comparison of physicochemical properties

	DIBP	**DBP**	**DIPP**	**DnPP**	**DHP**	**DnHP**	**DEHP**	**DIOP**
Physical state	liquid	liquid	liquid	liquid	liquid	liquid	liquid	liquid
Molecular weight	278.35	278.35	306.41	306.41	334.46	334.46	390.57	390.56
Melting/freezing point	-37°C	-69°C	<-25°C	<-55°C	-27.4°C	-27.4°C	-55 or -50	-46°C
Boiling point	320°C	340°C	339°C	342°C	373°C	350°C	385°C	270°C
Density	1.038 g/cm^3	1.045 g/cm^3	1.020 g/cm^3	Not available	1.010 g/cm^3	1.011 g/cm^3	0.984 g/cm^3	0.983 g/cm^3
Vapour pressure	0.01 Pa at 20°C	0.0097 Pa at 25°C	0.025 Pa at 25°C	0.026 Pa at 25°C	3.44.10^{-4} Pa at 25°C	6.667.10^{-4} Pa at 25°C	0.34.10^{-4} Pa at 20°C	7.4.10^{-4} Pa at 25°C
Water solubility	20 mg/L at 20°C	10 mg/L at 20°C	1.1 mg/L at 20°C	0.8 mg/L at 20°C	0.159 mg/L at 25°C	0.05 mg/L at 25°C	0.003 mg/L	0.09 mg/L
Partition coefficient n-octanol/water	4.11	4.57	5.45	5.62	6	6.30	7.5	8.0 ; 8.39 7.73

Results for DBP, DEHP, DnPP, DIPP, DnHP, DHP and DIBP were extracted from the CLH report on Diisohexyl phthalate (DIHP) (05/07/2016). See CLH report on Diisohexyl phthalate (DIHP) (05/07/2016) for more details.

All products above are consider to have low volatility. Increasing side chain length shows a clear trend for water solubility (decreased with increased alkyl chain, from moderate water solubility for 4C to very low solubity for 8C), for log Pow and molecular weight (increased with increased alkyl chain) suggesting that C3 phthalates would be more absorbed than C7 phthalates.

In particular, physicochemical properties of DIOP are very close to those of DEHP.

DIHP was not included in the proposed category approach since at the time of writing the CLH report on DIOP, classification of DIHP was not discussed at RAC level. Furthermore, no data are available on fertility and developmental effects for this substance and the proposal was based only based on read-across.

Toxicity data related to reproductive function

Since there is no data available for Diisopentylphthalate (DIPP) and 1,2-benzenedicarboxylic acid, dihexyl ester, branched and linear (DHP), these substances were not included in the tables below. However, it should be noted that even if there is a data gap for fertility and developmental endpoints for these phthalates, they have an harmonized classification as Repr. 1B - H361DF. Indeed, according to RAC opinion on 7 June 2013, a classification as Repr. 1B - H361DF is required for "1,2-benzenedicarboxylic acid, dihexyl ester, branched and linear" based on a chemical category for transitional phthalates. For DIPP, the reasoning behind the harmonized classification is not publicly available in the ECHA website since the classification is old (CLP 00).

Comparison of fertility toxicity for phthalate category

The main reproductive effects were summarized in the table below, with the lowest NOAEL/LOAEL found for each specific endpoint and each phthalate.

Table 8.3: Comparison of fertility toxicity studies including effects and respective NOAEL/LOAELs

Endpoint	DIBP	DBP	DnPP	DnHP	DEHP	DIOP
Decreased fertility	Not assessed - no specific fertility studies	- / 52-80 (↓ number of live born pups per litter) 256-385 / 509-794 (↓ fertility, mating and reproductive performance in F1) Rat, oral (diet), continuous breeding protocol (RACB) (NTP, 1995) - / 250 (↓ fecundity in F1) Rat, oral (gavage), multigeneration study (Gray, 1999)	- / 760 (↓ fertility, number of litters and number of pups per litter) Mice, oral (diet), continuous breeding (RACB) (NTP, 1985)	- / 380-430 (↓ number of litters/pair, live pups/litter and proportion of pups born alive) Mice, oral (diet), continuous breeding protocol (RACB) (Lamb, 1987)	14 / 140 (↓ fertility and reproductive performance) Mice, oral (diet), continuous breeding protocol (RACB) (Lamb, 1987)	Not assessed - no specific fertility studies
Effects on testes of parental generation	No specific fertility studies but indication from developmental toxicity studies (see below)	52-80 / 256-385 (testicular atrophy and seminiferous tubule degeneration) Rat, oral (diet), continuous breeding protocol (RACB) (NTP, 1995)	250 / 1000 (histopathological lesions in testicular tissue) Rat, oral (gavage, single dose), up to 10 weeks followed by mating (Lindstrom, 1988)	na / 1670-1870 (↓ sperm motility and concentration, atrophy of seminiferous tubules) Mice, oral (diet), continuous breeding protocol (RACB) (Lamb, 1987)	7.9 / 23 (testis seminiferous tubular atrophy) Rat, oral (diet), 3-generation (Wolfe, 2003)	No specific fertility studies but indication from developmental toxicity study (see below)

Results for DBP, DEHP, DnPP, DIPP, DnHP, DHP and DIBP were extracted from the CLH report on Diisohexyl phthalate (05/07/2016) and/or from restriction dossier for 4 phthalates (ECHA, 2016).
Results expressed as "NOAEL/LOAEL" in mg/kg bw/day. "-" indicates that no NOAEL is available because effects were observed at all tested doses
RACB: Reproductive Assessment by Continuous Breeding
na: non applicable since only the high tested dose was subjected to necropsy

Effects on fertility were reported with all considered phthalates including decreased fertility when adequate data are available and effects on male reproductive system. From this table, it can be suggested that DEHP is the most potent. In the absence of adequate data with DIOP and since DIOP and DEHP are structurally similar and have physicochemical properties very close, it can be expected that DIOP would have similar effects as DEHP on fertility. However,

ranking the toxicity is difficult due to the various protocols used and the intervals between tested doses. In particular it can be noted that in some cases, no NOAEL can be set due to effects reported at all doses (DBP, DnPP and DnHP).

Comparison of developmental toxicity for phthalate category

The main developmental effects were summarized in the table below, with the lowest NOAEL/LOAEL found for each specific endpoint and each phthalate. Other developmental effects are reported in the literature (such as impairment of mammary gland development, delayed preputial separation, cryptorchidism, effect on sperm production, skeletal and visceral malformations etc) but are not described in the table below because they were not assessed for all phthalates considered.

Table 8.4: Comparison of developmental toxicity studies including effects and respective NOAEL/LOAELs

Endpoint	DIBP	DBP	DnPP	DnHP	DEHP	DIOP
Effects on testes of offspring	- / 125 (testicular damage, degeneration of seminiferous tubules) Rat, oral (gavage) GD12-21 (Saillenfait, 2008)	- / 1.5-3.0 (↓ number of spermatocytes in prepubertal male offspring) Rat, oral (diet) GD15-PND21 (Lee, 2004)	Not assessed in the developmental toxicity studies considered.	50 / 125 (degeneration of seminiferous tubules) Rat, oral (gavage), GD12-20 (Saillenfait, 2009)	4.8 / 14 (small or aplastic tested and epididymis, seminiferous tubule atrophy) Rat, oral (diet), 3-generation (Wolfe, 2003)	100 / 500 (histopathological lesions in testicular tissue) Rat, oral (gavage) GD12-21 (Saillenfait, 2013)
Lethality	500 / 750 (resorptions, ↓ number of live fetuses) Rat, oral (gavage), GD6-20 (Saillenfait, 2006)	- / 52-80 (↓ number of live born pups) Rat, oral (diet), continuous breeding protocol (RABC) (NTP, 1995)	33/100 (↓ pup viability) Rat, oral (gavage), GD8-18 (Hannas, 2011)	0 / 500 (↓ live pups) Rat, oral (gavage), GD6-20 (Saillenfait, 2009)	40 / 200 (resorption, post-implantation loss) Mice, oral (gavage), GD6-15 (Huntington, 1997)	500 / 1000 (resorption, post-implantation loss) Rat, oral (gavage), GD6-20 (Saillenfait, 2013)
Decreased AGD in males	125 / 250 Rat, oral (gavage), GD12-21 (Saillenfait, 2008)	100 / 250 Rat, oral (gavage), GD12-21 (Mylchreest, 1999)	33 / 100 Rat, oral (gavage), GD8-18 (Hannas, 2011)	125 / 250 Rat, oral (gavage), GD12-20 (Saillenfait, 2009)	3 / 10 Rat, oral (gavage), GD7-PND16 (Christiansen, 2010)	Not assessed in the developmental toxicity studies considered.
Nipple retention	125 / 250 Rat, oral (gavage), GD12-21 (Saillenfait, 2008)	50 / 100 Rat, oral (gavage), GD12-21 (Mylchreest, 2000)	100 / 300 Rat, oral (gavage), GD8-18 (Hannas, 2011)	125 / 250 Rat, oral (gavage), GD12-20 (Saillenfait, 2009)	3 / 10 Rat, oral (gavage), GD7-PND16 (Christiansen, 2010)	500 / 1000 Rat, oral (gavage), GD6-20 (Saillenfait, 2013)

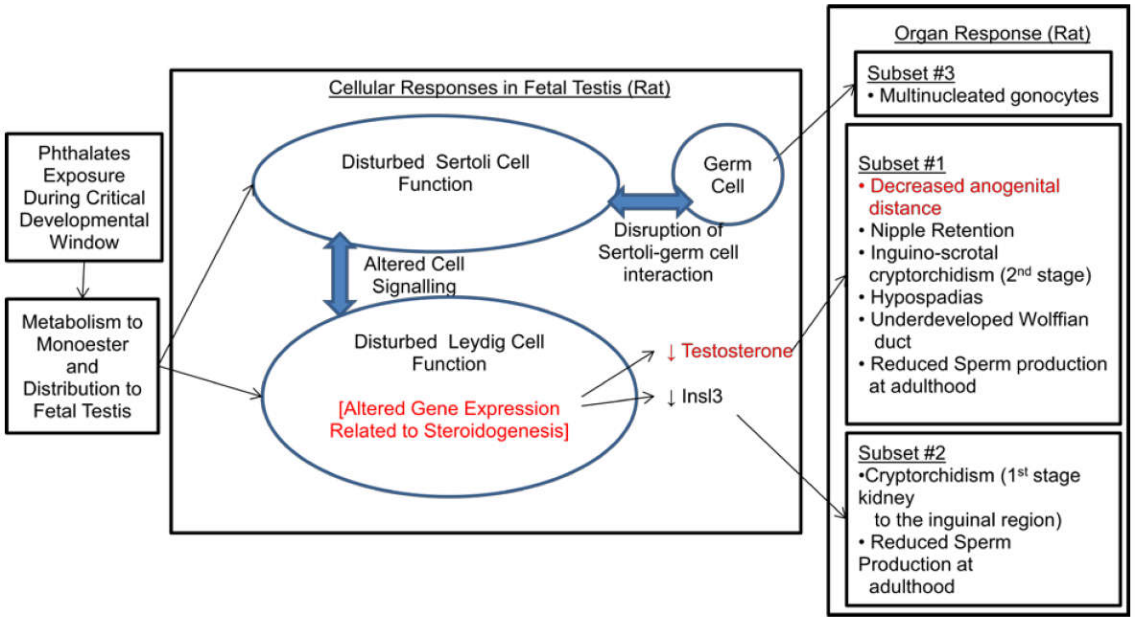
Results for DBP, DEHP, DnPP, DIPP, DnHP, DHP and DIBP were extracted from the CLH report on Diisohexyl phthalate (05/07/2016) and/or from restriction dossier for 4 phthalates (ECHA, 2016).
Results expressed as "NOAEL/LOAEL" in mg/kg bw/day. "-" indicates that no NOAEL is available since effects were observed at all tested doses
AGD: AnoGenital Distance

Clear developmental effects were reported with all considered phthalates, including fetal lethality and developmental effects on the male reproductive system mainly related to an androgen insufficiency. DBP seems to be the most potent phthalate regarding toxicity on testes based on a decreased number of spermatocytes. This effect can be due to a direct effect rather than linked to an impairment of testosterone production. Nevertheless, although the decreased number of spermatocytes seems to be the most sensitive parameter for C3-C7 phthalate reprotoxicity, it was not assessed for other phthalates. In contrast, regarding decreased AGD and nipple retention, the comparison of data suggests that DEHP would be the most potent. In the absence of adequate data with DIOP and since DIOP and DEHP are structurally similar and have physicochemical properties very close, it can be expected that DIOP would have similar effects as DEHP on development. However, for a same type of effects, it seems that DIOP is less potent. This impression can come from the use of various protocols with different treatment duration, species and doses. In addition, the number of parameters evaluated with DIOP are rather limited in comparison to those assessed with other phthalates. Therefore, ranking the toxicity of DIOP among other C3-C7 phthalates is rather difficult and depends on the effect considered. In conclusion, since data are lacking with DIOP for some effects such as decreased AGD and effects on spermatocytes, it cannot be excluded the presence of a more sensitive effect at lower doses.

Mode of action for reproductive toxicity of phthalates

Several studies have been performed to understand the mode of action of reprotoxic effects of phthalates. The proposed mode of action described as "phthalate syndrome" is linked to disturbance of Sertoli cell function and Leydig cell function.

Figure 2: Representation of the cellular targets for the "phthalate syndrome" with associated changes in gene expression and subsequent hormonal and organ responses (issued from Health Canada, 2015).



According to the figure 2, phthalates induce developmental effects by two pathways: the first consisting on an impairment of Sertoli cell function with a direct effect on germ cell, the second consisting on an alteration of Leydig cell function with a decrease of testosterone and insulin-like 3 protein (Inl3).

In their report, Health Canada (2015) evaluated the toxicity of various phthalates to male reproductive system related to the anti-androgenic mode of action. Effects of 28 phthalates on gene expression related to the steroid biosynthesis pathway in the fetal rat testis and on testicular testosterone production in the fetal rat were summarized. Only *ortho*-phthalates with harmonized classification and having a linear or branched alkyl chain between C3 to C7 are described thereafter.

Table 8.5: Selected studies for phthalate SAR analysis for gene expression in the fetal rat testes (issued from Health Canada, 2015)

	DIBP	**DBP**	**DIPP**	**DnPP**	**1,2-benzenedicarboxylic acid, dihexyl ester, branched and linear**	**DnHP**	**DEHP**	**DIOP**
SR-B1	300 / 302	1 / -				100 / 86	NM	
StAR	300 / 295	50 / -				100 / 54	500 / 443	
Cyp11a	300 / 339	50 / -				300 / 267	500 / 574	
3bHSD	300 / 538	0.1 / -				100 / 185	NM	
Cyp17a1	300 / 325	500 / -				100 / 119	NM	

Results expressed as LOEL (lowest Observed Effect Level; min $p < 0.05$)/ED_{50} (dose resulting in 50% effect) for decreased expression (mg/kg/day)
Grey column = no data on these substances reported in Health Canada report (2015)
NM = not measured according to Health Canada report (2015)

The studies for the SAR analysis were selected using the following criteria: 1) *in utero* rat studies that have at least three biological replicates (dams) per dose; 2) maternal exposure occurring during the critical development window (GD 15-17 at minimum) and; 3) where the authors analyzed the appropriate tissue at the appropriate time (fetal rat testes on GD18/19)) and expression for biologically relevant genes related to the mechanism for decreased androgen synthesis.

No data is available with DIOP. However, when data are available, all the tested phthalates reduced steroid biosynthesis gene expression, with DBP being the most potent and DEHP the less potent.

Table 8.6: Selected studies for phthalate SAR analysis for *ex vivo* testicular testosterone production in the fetal testes following gestational exposure (issued from Health Canada, 2015)

	DIBP	DBP	DIPP	DnPP	DHP	DnHP	DEHP	DIOP
Decreased testicular testosterone production (ex vivo)	300 / 305	300 / 399				100 / 75 20 / 67*	300 / 383 50 / NM* (effect observed at both tested dose)	100 / 145*

Results expressed as LOEL (lowest Observed Effect Level; min $p < 0.05$)/ED_{50} (dose resulting in 50% effect) for decreased expression (mg/kg/day)
Grey column = no data on these substances reported in Health Canada report (2015)
NM = not measured according to Health Canada report (2015)

The studies for the SAR analysis met the following criteria: (1) in utero rat studies that have at least three biological replicates (dams) per dose; (2) maternal exposure occurring during the critical development window (GD 15-17 at minimum) and testes isolated between GD18 and 19; (3) testes from male offspring incubated between 2-3 hours and incubation media tested for testosterone.

* Results from Saillenfait (2013). In separate experiments, DIOP, DnHP or DHEP were administered to pregnant SD rats during GD12-19. All these substances significantly reduced fetal testicular testosterone production compared to controls starting at 100, 20 and 50 mg/kg bw/day respectively (which was the lowest dose tested for DEHP); with magnitude of change increasing with dose for all three phthalates. The ED_{50} values were only calculated for DIOP and DnHP which were 145 and 67 mg/kg/day, respectively. From these results, DEHP and DnHP appear more potent than DIOP. As an ED_{50} value was not derived for DEHP by the study authors, the relative potency of DEHP to DnHP from these studies could not be determined.

All tested phthalates induce a decrease of testicular testosterone production with DIOP being the less potent for reducing production of testicular testosterone compared to DnHP or DEHP in a similar protocol carried out by Saillenfait *et al.* (2013 a, 2013b) as cited in Health Canada (2015). In contrast, DIOP seems to be more potent than DIBP and DBP based on ED_{50} listed in the table above.

Overall conclusion on category weight of evidence

Alteration of male reproductive system with same hypothesized mechanisms of action is reported for the C3-C7 phthalates considered. These coherent data confirm the relevance of the proposed category for C3-C7 phthalates. This category was also proposed by Health Canada (2015) which concluded that "medium chain phthalate esters (longest carbon backbone length 3 to 7)" (including DIOP) showed activity in assays for important events in the mode of action for androgen-dependent effects on the developing male reproductive system. In contrast, they concluded that phthalates with 1 to 2 carbon backbones and phthalates where the number of carbons in the longest alkyl chain is greater than or equal to 8 are not active in studies related to important mechanistic events for phthalates-induced androgen insufficiency during male reproductive development in rat. These results show that reprotoxic effects of phthalate esters appear to be structure-dependent and highly related to the length and nature of their alkyl chain.

No fertility study is available with DIOP but developmental experiments showed that it already have an effect on testes. In this context, a read-across from C3-C7 phthalates data is judged appropriate to conclude that DIOP is a toxic to fertility. In conclusion, on the basis of the experimental results with DIOP supported by a category approach with C3-C7 phthalates, a classification for fertility is judged appropriate. Ranking potency among C3-C7 phthalates is challenged by the various protocols used. Moreover, different conclusions can be made depending on the parameter considered.

10.10.3 Comparison with the CLP criteria

Classification for adverse effects on sexual function and fertility

Reproductive toxicity category 1 in the CLP Regulation is dedicated to "substances which are known or presumed human reproductive toxicant". Substances are classified in category 1 for reproductive toxicity when they are known to have produced an adverse effect on sexual function and fertility or when there is evidence from animal studies possibly supplemented with other information, to provide a strong presumption that the substance has the capacity to interfere with reproduction with humans. The classification of a substance is further distinguished on the basis of whether the evidence for classification is primarily from human data (category 1A) or from animal data (category 1B).

Reproductive toxicity category 2 in the CLP Regulation is dedicated to substances which are "suspected human reproductive toxicants". "Substances are classified in category 2 for reproductive toxicity when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function or fertility, and where the evidence is not sufficiently convincing to place the substance in category 1".

For DIOP, a classification Repr. 1A is not justified based on the lack of adequate human data with DIOP.

Based on lesions of male reproductive tract (in particular hypospadias, undescended testis and hypospermatogenesis) reported in the absence of significant maternal toxicity after *in utero* exposure to DIOP and supported by a category approach with C3-C7 phthalates showing similar toxicity on fertility, a classification Repr. 1B – H360 is required for DIOP. This proposal is consistent with the fact that DIOP alters male reproductive system with same hypothesized mechanisms of action as the C3-C7 phthalates. This approach is also judged consistent with conclusion made by the RAC on other phthalates such as "1,2-benzenedicarboxylic acid, dihexyl ester, branched and linear" based on a similar chemical category in the absence of specific adequate data.

Classification Repr. 2 is not appropriate since all data on C3-C7 phthalates confirm a similar reprotoxic mode of action based on several studies. In addition, even if there is no adequate fertility study in one or more generation with DIOP, testicular toxicity reported in the developmental toxicity experiments suggest that this substance would also impact fertility. Therefore, the category approach is sufficiently robust to conclude on a clear evidence of toxicity on fertility for DIOP.

10.10.4 Adverse effects on development

Table 9: Summary table of animal studies on adverse effects on development

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Experiment 1: Prenatal toxicity test, comparable to OECD guideline 414 SD female rats 10-12 time-mated females (8-12 pregnant)	DIOP: 0, 100, 500, 1000 mg/kg bw/day GD6-20 by oral gavage	Maternal effects: Decreased body weight and body weight gain in late gestation at 1000 mg/kg bw/day related to decrease uterine content since there was no effect in net body weights. NOAEL maternal = 1000 mg/kg bw/day Developmental effects: From 500 g/kg bw/day: - Decreased foetal body weight but not significant when litter size used as covariable - Skeletal variations (14th supernumerary lumbar ribs, retarded ossification) At 1000 mg/kg bw/day: - Increased post-implantation loss and resorption, decreased foetal body weight - Skeletal variations (14th supernumerary lumbar ribs, retarded ossification) - Testis malpositioned NOAEL development = 100 g/kg bw/day	Saillenfait, 2013
Experiment 2 Ex-vivo testosterone production by foetal testis No guideline followed	DIOP: 0, 10, 100, 500, 1000 mg/kg bw/day GD12-19 by oral gavage	Decrease in testicular testosterone production from 100 mg/kg bw/day NOAEL = 10 mg/kg bw/day	Saillenfait, 2013

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
SD female rats			
Experiment 3 Peri-postnatal toxicity study No guideline followed 10-12 pregnant SD female rats	DIOP: 0, 100, 500, 1000 mg/kg bw/day GD12-21 by oral gavage	Maternal effects: - Decreased body weight at GD21 at 1000 mg/kg bw/day NOAEL maternal = 500 mg/kg bw/day Developmental effects: At 500 mg/kg bw/day - Gross morphological alterations of external and internal genitalia in 3 males - Increased absolute and absolute testis weight and decreased relative right kidney weight - Histopathological lesions in the testis (in particular hypospermatogenesis in 2 males of 2 litters) At 1000 mg/kg bw/day: - Decreased viability (PND1-21) - Permanent areolas and/or nipple buds at adult necropsy - Marked malformations of the male reproductive tract - Decreased relative and absolute weights of kidneys, testis and epididymis - Histopathological lesions in the testis (in particular hypospermatogenesis in 15 males in 8 litters) NOAEL development = 100 mg/kg bw/day	Saillenfait, 2013

Detailed studies are available in Annex I to the CLH report.

10.10.5 Short summary and overall relevance of the provided information on adverse effects on development

Human data

No human data is available for DIOP but epidemiological data are available with other phthalates.

However, the overall evidence of human data is considered limited to conclude on the relationship between phthalates exposure and congenital malformations of the male genitalia (cryptorchidism, hypospadias) or delayed sexual maturation (Anses, 2015; RAC, 2012, RAC, 2006).

Non-human data

In the first experiment, pregnant rats were exposed to DIOP from GD6-20 by oral gavage with assessment of prenatal development on GD21 (Saillenfait, 2013). In dams, only some reductions of body weight was observed at the highest dose of 1000 mg/kg bw/day. Since net body weight was not affected, the decrease of body weight in late gestation appeared to be primarily related to a significant decrease in uterine contents.

Embryolethality was evidenced by a statistically significant increase of post-implantation loss (17.8% versus 4.7% in the control group) and resorptions per litter (16.4% versus 4% in the control group) at the highest dose of 1000 mg/kg bw/day. Retardation of foetal growth was found from 500 mg/kg bw/day and was characterized by a reduced fetal body weight and/or ossification delay.

There was no increase in the incidence of external, visceral and skeletal malformations. Short supernumerary lumbar rib was the only fetal skeletal variant to be significantly increased at 500 and 1000 mg/kg bw/day. Abnormal position of the testes was detected in 1 male foetus at 500 mg/kg bw/day and in 10 male foetuses in 5 litters at 1000 mg/kg bw/day (statistically significant at this dose). This result is consistent with the high incidence of undescended testis found in adult male rats after *in utero* exposure reported in the third experiment. It is thus demonstrated that prenatal transabdominal migration of the testis, that is mediated by the production of Insl3 (insulin-like 3) protein by foetal Leydig cells, was affected by *in utero* exposure to DIOP. Reduction of Insl3 gene expression and/or alteration of transabdominal migration of the testis is consistently reported with various C3-C7 phthalates, showing a similar mode of action of these substances (Anses 2015).

In the second experiment, pregnant rats were exposed to DIOP by oral gavage during the critical period of male sexual differentiation (i.e. GD12-19). Testis were collected on GD19 and analysed for testosterone production. A dose-dependent decreased in ex-vivo testosterone production by the foetal testis at GD19 was observed from 100 mg/kg bw/day (Saillenfait, 2013). Decreased testosterone production is consistently reported with various C3-C7 phthalates, showing a similar mode of action of these substances.

In the third experiment (Saillenfait, 2013), pregnant rats were exposed to DIOP from GD12-21 by oral gavage with assessment of peri and post-natal development before and after weaning (last necropsy on PND 82-84). Little maternal toxicity was reported with a significant decrease of body weight on GD21 (-9%) at the highest dose of 1000 mg/kg bw/day. *In utero* exposure of DIOP induced permanent postnatal alterations in androgen-dependent structures of male offspring. They mainly consisted on retained nipples (69% of males), hypospadias (36% of males), undescended testis (74% of males), markedly underdeveloped seminal vesicles (38%) and hypospermatogenesis (88% of males) reported at 1000 mg/kg bw/day. In addition, alterations in epididymis (thin body or absent), vasa deferentia (absent, thin or crossed) and prostate (underdeveloped) were found in some animals (between 10 and 23% of males) at this dose. Some effects on male reproductive tract were also apparent at 500 mg/kg bw/day but only in a few animals: one animal had an unilaterally enlarged testis, one had an abnormal epididymis, one displayed markedly underdeveloped seminal vesicles and prostate and two presented hypospermatogenesis. These effects are characteristics of a decrease of androgens, that is consistent with the result of the second experiment. Recent publications have questioned the relevance of anti-androgenic effects induced by phthalates in rats to humans. Indeed, some experimental studies using *in vitro* or xenograft models did not show any decrease of testosterone by different phthalates (such as DBP, MEHP and MBP) in human foetal testis although this effect was clearly observed in rat foetal testis (Anses, 2015). However, in the current state of knowledge, these data are not sufficiently robust to conclude that the effects in testis observed in rats will not be also found in humans.

Additional data of lower quality were found in the literature:

The following information relative to developmental toxicity was found on Pubchem website (July 2016). Male/female CD-1 mice were exposed to 0.0, 0.025, 0.05, 0.1 0, or 0.15% (0, 44, 91, 190.6, or 292.5 mg/kg bw) diisooctyl phthalate in their diet during gestation days 0-17. After a 7 day quarantine period, breeding pairs were cohabited overnight. Gestation day 0 was determined the morning a vaginal copulation plug was found. Dams were observed daily for signs of clinical toxicity and weights were taken on gestation day 0, 4, 8, 12, 16, and 17. Parameters evaluated following termination included body weight, liver weight, gravid uterine weight, number of ovarian corpora lutea of pregnancy, and status of uterine implantation sites.

Fetuses were weighed, examined for external abnormalities and received a visceral examination. Actual doses received: 0, 44, 91, 190.6, or 292.5 mg/kg based on body weights and food consumption. No dams died during gestation. Reduced maternal body weight gain was observed in the 0.10 and 0.15% treatment groups. No effects on the number of corpora lutea, implantation sites per dam, the percent pre-implantation loss, and sex ratio of live pups were observed. The number and percent of resorptions, late fetal deaths, and dead and malformed fetuses were all increased in response to 0.1 and 0.15% treatments. Female fetal weight and the number of live fetuses per litter for both sexes were significantly reduced at 0.10 and 0.15% doses. A significant increase in both the percentage of fetuses with malformations and the percentage of malformed fetuses per litter were observed with dosing as low as 0.05%. External malformations included unilateral and bilateral open eyes, exophthalmia, exencephaly, and short, constricted, or no tail. Visceral malformations were identified in the major arteries. Noted skeletal defects included fused and branched ribs and misalignment and fused thoracic vertebral centra. The reference for this summary is EPA/Office of Pollution Prevention and Toxics; High Production Volume (HPV) Challenge Program's Robust Summaries and Test Plans (2007). A website link is provided but is not valid. No further details on this study can be found in the literature. Therefore, the relevance of these findings cannot be checked. In this context, this study cannot be adequately assessed for classification purpose.

After exposure to female rats to 0, 5, or 10 mL/kg DIOP (0, 4930 or 9860 mg/kg, using the density of 986 kg/m^3 [NICNAS, 2008]) on days 5, 10 and 15 of gestation by intraperitoneal injection (Grasso, 1981, as cited in ECB, 2000), a high incidence of soft tissue abnormalities is reported in both treated groups. However quantitative data were not provided in the available summary. No increase in fetal mortality or skeletal abnormalities was observed (CPSC (2011) report). Due to the low level of details provided, the relevance of these findings cannot be checked.

In conclusion, after *in utero* administration, DIOP induced embryotoxicity (decreased pup weight and skeletal variations) from 500 mg/kg bw/day. At the higher dose of 1000 mg/kg bw/day, it induced embryolethality (post-implantation losses and resorptions) and malformations of the male reproductive tract. These effects are reported in the absence or with minimal maternal toxicity. The effects found with DIOP are consistent with those observed after exposure to other C3-C7 phthalates (see reasoning of category approach in section 10.10.2). Clear developmental effects were reported with all considered C3-C7 phthalates, including fetal lethality and developmental effects on male reproductive tract. From table 8.4, DIOP seems to be less potent than other C3-C7 phthalates. In this context, the relevance of setting specific concentration limits (SCL) is raised if DIOP can be considered as reproductive toxicant of low potency. ED_{10} were calculated for effects relevant for classification found in Saillenfait *et al.* (2013) study. According to CLP guidance, the ED_{10} value is the lowest dose which induces reproductive toxic effects fulfilling the criteria for classification for reproductive toxicity with an incidence or magnitude of 10% after correction for the spontaneous incidence.

Table 9.1: Summary of calculated ED_{10} from Saillenfait (2013) experiments

Dose (mg/kg bw/day)	**0**	**100**	**500**	**1000**	**ED_{10}**
Experiment 1					
% post-implantation loss per litter	4.70	5.00	8.00	17.8	842
% resorption per litter	4.00	5.00	8.00	16.4	858
% foetuses with testis malpositioned	0	0	1.25	17.2	774
% litter with testis malpositioned	0	0	8.33	50.0	520
Experiment 3					
Pup survival to weaning PND21 (%)	89.2	91.2	85.6	68.4	686
Permanent areolas and/or nipple buds (%)	0	0	0	69.0	572
Small penis (% foetus)	0	0	0	20.5	744

Cleft prepuce (% fœtus)	0	0	0	25.6	695
Hypospadia (% fœtus)	0	0	0	35.9	639
Cleft phallus with exposed os penis (% fœtus)	0	0	0	28.2	677
Testis undescended (uni or bilateral) (% fœtus)	0	0	0	74.4	567
Testis. undescended (bilateral) (% fœtus)	0	0	0	46.2	608
Testis absent or markedly underdeveloped (unilateral) (% foetus)	0	0	0	10.3	1000
Epididymis absent (unilateral) or markedly underdeveloped (unilateral) % fœtus)	0	0	0	10.3	1000
Epididymis thin body (unilateral) (% fœtus)	0	0	1.50	12.8	876
Vasa deferentia. crossed (% fœtus)	0	0	0	10.3	1000
Vasa deferens absent (uni or bilaterally) (% fœtus)	0	0	0	12.8	890
Seminal vesicles absent (% fœtus)	0	0	0	5.10	1480
Seminal vesicles markedly underdeveloped (% fœtus)	0	0	1.50	38.5	615
Seminal vesicles malformed (% fœtus)	0	0	0	2.60	2423
Prostate markedly underdeveloped (% fœtus)	0	0	1.50	23.1	697
Hypospermatogenesis (%foetuses)					
Grade 1	0	0	0	8.00	
Grade 2	0	0	0	8.00	
Grade 3	0	0	2.78	8.00	
Grade 4	0	0	0.00	60.0	583
Grade 5	0	0	2.78	4.00	
Hypospermatogenesis (%litter)					
Grade 1	0	0	0	22.2	
Grade 2	0	0	0.00	22.2	
Grade 3	0	0	8.33	22.2	560
Grade 4	0	0	0	88.9	
Grade 5	0	0	8.33	11.1	

All sensitive endpoints considered for potency evaluation are in the boundaries of the low potency group (≥ 400 mg/kg bw/day). Indeed, all ED_{10} are above 500 mg/kg bw/day, the lowest being 520 mg/kg bw/day for the percentage of litter with testis malpositioned reported in experiment 1. Nevertheless, the low number of litters in the study (8-12) may underestimate the ED_{10} values.

In addition, according to CLP guidance, modifying factors needs to be taken into account such as type of effect/severity, data availability, dose-response relationship, mode or mechanism of action, toxicokinetics and bioaccumulation of substances in order to confirm or not the potency group.

a) Type of effect/severity

Fetal lethality, malformations of male reproductive tract and nipple retention can be judged as severe effects.

b) Data availability

Only one reliable publication is available to assess developmental toxicity of DIOP (Saillenfait, 2013). Data from phthalates category suggest that parameters assessed with DIOP would be not the most sensitive endpoints. This is reflected by results from a study performed in rats exposed to DBP during GD15-PND21 (Lee, 2004). In this study, a LOAEL between 1.5-3.0 mg/kg bw/day was set based on a decreased number of spermatocytes in males adult offspring and by effects on mammary glands in female adult offspring. The occurrence of similar effects cannot be checked with DIOP since experiments performed by Saillenfait (2013) were limited to treatment during pregnancy and specific assessment on spermatocytes and mammary glands in adulthood was not performed. Therefore, it is unknown if DIOP would induce similar effects as DBP in a comparable protocol. For other developmental endpoints available with both substances such as lethality and nipple retention, DIOP seems to be less potent than DBP (table 8.4). However, the design of the studies are not comparable. In contrast, when comparing ED_{50} for decreased testosterone production, DIOP seems to be more potent than DBP (table 8.6).

c) Dose-response relationship

Dose-response relationship was observed for most of endpoints identified for potency evaluation, the only exception was pup survival to weaning.

d) Mode of action

Most of developmental effects reported with DIOP are characteristics of a decrease of androgens. Recent publications have questioned the relevance of anti-androgenic effects induced by phthalates in rats to humans. Indeed, some experimental studies using *in vitro* or xenograft models did not show any decrease of testosterone by different phthalates (such as DBP, MEHP and MBP) in human foetal testis although this effect was clearly observed in rat foetal testis (Anses, 2015). However, in the current state of knowledge, these data are not sufficiently robust to conclude that the effects in testis observed in rats will not be also found in humans.

Other developmental effects reported with phthalates can be considered independent of testosterone production. For example, delayed of prenatal transabdominal migration of the testis is at least partially related to an impairment of Insl3. In addition, germ cell effect can be due to a direct effect on Sertoli cells (see figure 2).

e) Toxicokinetics

No difference in toxicokinetics between tested animal and humans has been identified.

f) Bio-accumulation

There is no evidence for bioaccumulation of DIOP.

Conclusion on SCL setting:

Based on calculated ED_{10}, DIOP falls into the low potency group. However, the low number of litters in the study (8-12) may underestimate the ED_{10} values. In addition, only limited dataset is available with DIOP regarding all possible developmental effects that can be induced by C3-C7 phthalates. In particular, category assessment show that effects on germ cells and mammary gland development in adult offspring could be very sensitive parameters for phthalate toxicity. However, assessment of these parameters is not reported in Saillenfait *et al*. (2013). In this context, it cannot cannot be excluded the presence of reproductive effects at lower dose levels. In conclusion, there is too much uncertainties to set SCL above GCL (general concentration limit) even if all ED_{10} are above the threshold of 400 mg/kg bw/day. This is consistent with CLP guidance page 406 section 3.7.2.5.2.2 on data availability.

10.10.6 Comparison with the CLP criteria

Classification for adverse effects on development

Reproductive toxicity category 1 in the CLP Regulation is dedicated to "substances which are known or presumed human reproductive toxicant". Substances are classified in category 1 for reproductive toxicity when they are known to have produced an adverse effect on development in humans or when there is evidence from animal studies possibly supplemented with other information, to provide as strong presumption that the substance has the capacity to interfere with reproduction with humans. The classification of a substance is further distinguished on the basis of whether the evidence for classification is primarily from human data (category 1A) or from animal data (category 1B).

Reproductive toxicity category 2 in the CLP Regulation is dedicated to substances which are "suspected human reproductive toxicants". "Substances are classified in category 2 for reproductive toxicity when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on development, and where the evidence is not sufficiently convincing to place the substance in category 1".

A classification Repr. 1A is not justified based on the lack of adequate human data with DIOP.

A classification Repr. 1B is justified based on animal data with DIOP:

- Embryolethality (post-implantation losses and resorptions),
- Embryotoxicity (decreased foetal body weight and skeletal variations),
- Permanent post-natal alteration of male reproductive system (mainly retained nipples, hypospadias, markedly underdeveloped seminal vesicles, undescended testis and hypospermatogenesis).

Since these effects occurred in the absence or with minimal maternal toxicity, they cannot be considered as a secondary non-specific consequence of other toxic effect. Therefore, the data provide clear evidence of an adverse effect on development. The proposal is also supported by the category approach for C3-C7 phthalates showing similar effects and modes of action. In this context, the criteria for classification Repr. 1B are fulfilled.

A category 2 is not appropriate considering the severity of effects observed with DIOP and since all data on C3-C7 phthalates confirm similar reprotoxic modes of action based on several studies. Furthermore, there is no available data to support that the observed effects and proposed mechanisms are not relevant to humans. Therefore, the evidence is sufficiently robust to conclude on the developmental toxicity for DIOP.

10.10.7 Adverse effects on or via lactation

10.10.8 Short summary and overall relevance of the provided information on effects on or via lactation

There is no adequate study to assess effect of DIOP on or via lactation.

10.10.9 Comparison with the CLP criteria

No classification is proposed for effects on or via lactation in the absence of adequate study.

10.10.10 Conclusion on classification and labelling for reproductive toxicity

Based on animal studies carried out with DIOP in association with supportive data on C3-C7 phthalates showing effects on male reproductive tract and embryolethality in the absence of significant maternal toxicity, a classification **Reproductive toxicity category 1B , H360DF "May damage fertility or the unborn child**" is warranted.

10.11 Specific target organ toxicity-single exposure

Not assessed.

10.12 Specific target organ toxicity-repeated exposure

Only very limited data is available with DIOP. The following information is summarized in the CPSC (2011) report.

"The repeated-dose toxicity of DIOP was evaluated in several poorly reported animal studies. No effects were observed in rats dosed orally with 1,000 mg/kg-day DIOP for 8 days, as assessed by blood, post-mortem, and histological examinations (ICI Chemicals & Polymer, 1958, as cited in NICNAS, 2008). No effect on growth was reported in rats administered DIOP via the oral route at 0, 100 mg/kg-day (five generations for 21 months), 300 mg/kg-day (three generations for 21 months), or 500 mg/kg-day (three generations for 15 months) (Lefaux, 1972, as cited in NICNAS, 2008 and ECB, 2000). In studies conducted by the U.S. Food and Drug Administration (FDA), no effects were reported in rats or dogs dosed orally with 100 mg/kg-day DIOP for 4 or 14 weeks, respectively (Shibko and Blumenthal, 1973). No further details were provided. […] Although the study by Lefaux (1972, as cited in NICNAS, 2008) described above included multigenerational exposure, it is unclear if reproductive toxicity endpoints were evaluated in this study (no data were provided)."

Data not assessed in this CLH report.

10.13 Aspiration hazard

Not assessed.

11 EVALUATION OF ENVIRONMENTAL HAZARDS

Not assessed.

12 EVALUATION OF ADDITIONAL HAZARDS

Not assessed.

13 ADDITIONAL LABELLING

Not assessed.

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15 ANNEXE 1

ANNEX I CONTENTS

1 PHYSICAL HAZARDS

Not assessed.

2 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

2.1.1 US CPCS (2011)

Study reference: United States Consumer Product Safety Commission (CPSC). May 2, 2011. Final Toxicity Review for Diisooctyl phthalate (DIOP).

The following toxicokinetics data on phthalates including DIOP are summarized in the CPSC toxicity review for DIOP (2011). Twenty-four human volunteers were administered either a single control, low, or high dose of combined phthalate diesters (containing dibutyl phthalate [DBP], diethylhexyl phthalate [DEHP], butyl benzyl phthalate [BBP], and DIOP) labeled with isotope, spiked in margarine, and spread on toast (Anderson et al., 2001). The low dose included 168 μg of [13C]-DIOP and 190–255 μg of each of the other phthalates, while the high dose included 336 μg of [13C]-DIOP and 380–510 μg of each of the other phthalates. [13C]-DIOP was 60% pure, with isooctylalcohol (used to synthesize the labeled compound) being the major impurity. The levels of excreted monoesters in the urine were measured by liquid chromatography-mass spectrometry (LC-MS) from samples collected 1 day prior to dosing and 1, 2, and 6 days following dosing. The study design was approved by an unspecified ethics panel. Monoesters for DEHP and DIOP co-eluted when analyzed by the LC protocol and were reported as the mean for the two octyl metabolites. The mono esters had excretion yields of 14% and 12% for the low and high doses, respectively, in the 24 hour urine collection (Anderson et al., 2001). No labeled monoesters were detected in 2- and 6 day urine collections. Interestingly, background levels of the monoesters were detected in the urine of all volunteers at all sample points.

Sprague-Dawley rats, beagle dogs, and miniature pigs were administered DIOP in the diet at 50 mg/kg-day for 21–28 days prior to being administered a single radioactively [14C]- labeled dose of DIOP in corn oil via gavage (Ikeda et al., 1978). Animals were sacrificed; tissues (liver, lung, kidney, gastrointestinal tract, brain, muscle, and fat), urine, and feces were analyzed for [14C] content at 4, 8, 24, and 96 hours (all species) and 21 days (dogs and pigs) after dosing with [14C]-labeled DIOP. Radioactivity persisted in the gastrointestinal tract in all species for several days. In rats, approximately 50% of [14C] activity was excreted in urine and the remaining 50% was excreted in the feces; nearly 85% of the dose was excreted within 24 hours and 100% within 4 days. In contrast, DIOP was primarily excreted in the feces in dogs (69– 80%) and in the urine of pigs (65 86%), and excretion was slower in these species than in rats (complete excretion slightly >4 days in dogs and nearly 21 days in pigs). In each species, [14C]- DIOP was distributed to body fat; however, distribution to lipid-rich tissues such as the brain and the lung was minimal. Additional data indicate that virtually all of the [14C] in rat tissue and excreta 4 days after dosing was in the form of metabolites (metabolized DIOP as measured by the percentage of water-soluble radioactivity); in contrast, only 63 and 71% of [14C] in dogs and pigs, respectively, had been metabolized in 4 days.

In another metabolism study (Calafat et al., 2006), four female Sprague Dawley rats (75 day old; 250 g) were administered 300 mg DIOP/kg via gavage. Urine was collected from each rat for analysis 24 hours before, just before, and 24 hours after DIOP administration. Collected urine was stored at -40C until analysis for mono-(3-carboxypropyl) phthalate (MCPP), mono-nbutyl phthalate (MBP), mono-(2-ethylhexyl) phthalate (MEHP), mono-(3-methyl-5- dimethylhexyl) phthalate (MiNP), mono-(3-methyl-7-methyloctyl) phthalate (MiDP), and monon- octyl phthalate (MnOP) by the U.S. Centers for Disease Control and Prevention (CDC). Three metabolites (± standard deviatons) were detected in 24 hour urine samples, MCPP (1.9 ± 0.5 μg/mg creatinine), MnOP (1.9 ± 0.8 μg/mg creatinine), and MiNP (0.005 ± 0.004 μg/mg creatinine). For

comparison, in the same experiment, DnOP administration (300 mg/kg gavage) resulted in the production of MCPP (225 ± 1.2 µg/mg creatinine) and MnOP (0.4 ± 0.2 µg/mg creatinine) metabolites. The author has suggested that detection of MCPP (and MnOP) following DIOP administration may be from contamination of the isomeric mix with DnOP or another linear chain phthalate.

3 HEALTH HAZARDS

3.1 Acute toxicity - oral route

Not assessed.

3.2 Acute toxicity - dermal route

Not assessed.

3.3 Acute toxicity - inhalation route

Not assessed.

3.4 Skin corrosion/irritation

Not assessed.

3.5 Respiratory sensitisation

Not assessed.

3.6 Skin sensitisation

Not assessed.

3.7 Germ cell mutagenicity

Not assessed.

3.8 Carcinogenicity

Not assessed.

3.9 Reproductive toxicity

3.9.1 Animal data

3.9.1.1 AM. Saillenfait (2013)

Study reference:

AM. Saillenfait, JP. Sabaté, A. Robert, B. Cossec, AC. Roudot, F. Denis, M. Burgart. Adverse effects of diidooctyl phthalate on the male reproductive development following prenatal exposure. Reproductive Toxicology 42 (2013) 192-202

Detailed study summary and results:

Test type

Three separate studies were conducted:

- Study 1: prenatal toxicity study (comparable to guideline)

- Study 2: ex vivo testosterone production by fetal testis
- Study 3: peri-postnatal toxicity study

GLP not stated.

Test substance

- DIOP (≥ 99% pure; lot 04817PE)
- Analysis by GC/MS indicated that linear di-n-butyl phthalate, di-n-pentyl phthalate, di-n-hexyl phthalate (DnHP), di-n-heptyl phthalate, di-n-octyl phthalate, di-n-nonyl phthalate and di-n-decyl phthalate were absent from the test substance. Additional GC/MS analyses of the alkyl moieties were performed after hydrolysis of the phthalate diester by a simple reaction with a commercially available Grignard reagent. Linear or branched butanol, pentanol, hexanol, heptanol, nonanol and decanol were not identified in the crude residue of hydrolysis, nor 2-ethyl-1-hexanol (alkyl moiety of DEHP). Six primary alcohols with a C8 chain were isolated, which did not correspond to 1-octanol or 2-octanol (1-methyl-1-heptanol). Due to the lack of corresponding analytical standards, their exact structure could not be identified.

Test animals

- Sprague-Dawley female rats
- Primiparous; 180-200g; supplied by Charles River Laboratories
- Study 1: 10-12 time-mated females (8-12 pregnant)
- Study 2: not clear in the publication
- Study 3: 10 or 12 pregnant females

Administration/exposure

- Oral gavage (5mL/kg), once daily in the morning. Initial doses were based on maternal weight on the first day of treatment and adjusted on the most recently recorded body weight of the individual animal (weighted on GD 0, 6, 9, 12, 15, 18, and 21).
- Vehicle: olive oil (formulation prepared weekly and stored in a dark place at room temperature); stability of the formulation was established for up to 2 weeks by gas chromatography-mass selective detector analysis.
- Study 1: 0, 100, 500, 1000 mg/kg bw/day on gestation days (GD) 6-20
- Study 2: 0, 10, 100, 500, 1000 mg/kg bw/day on GD 12-19
- Study 3: 0, 100, 500, 1000 mg/kg bw/day on GD 12-21
- In each studies, a concurrent control group received the vehicle under the same conditions as the treated groups.

Description of test design:

After 1-2 weeks of acclimatization, females were housed overnight with adult males from the same strain and supplier. The day sperm was detected in the vaginal smear was considered to be GD0. Mated-females were housed individually.

Study 1:

Pregnant females were administrated DIOP on GD 6-20 at 0, 100, 500, 1000 mg/kg bw/day. Cage side observations were conducted at least once daily. Food consumption was measured at three-day intervals tarting on GD6. Maternal body weights were recorded on GD 6, 9, 12, 15, 18 and 21. On GD21, females were killed. Uterine contents were examined to determine the number of implantation sites, resorptions and dead and live foetuses. All live foetuses were individually examined externally, weighted and euthanized. Half of the live foetuses from each litter was examined for internal soft tissue changes. The other half was examined for skeletal changes. The sex of all foetuses was determined by internal examination of the gonads.

Study 2:

Pregnant females were administrated DIOP on GD 12-19 (critical window for masculinization in the rat) at 0, 10, 100, 500, 1000 mg/kg bw/day. Maternal weight was monitored every three days through the dosing period and on GD 19. The dams were euthanized after the last treatment on the afternoon of GD19. The foetuses were quicky removed from the uterus and killed. The right and left testes and epididymides were collected from the three first males identified in a litter (6-8 litters per group). Each testis was placed in one well of a 24-well plate containing 500 µL of Ham F12/Dubelcco modified Eagle medium in each well and incubated at 37°C for 3 hours, under gentle rocking platform. Following incubation, the media was collected, quicky frozen and stored at – 20°C until testosterone measurement by turbulent flow liquid chromatography coupled with tandem mass spectrometry (TFC-MS/MS).

Study 3:

Pregnant females were administrated DIOP on GD 12-21 at 0, 100, 500, 1000 mg/kg bw/day. Maternal body weights were monitored during gestation and lactation. Day of delivery was recorded (post-natal day PND 0). Pups were counted and weighted on PND 1, 7, 14 and 21. At weaning on PND 21-22, all males from all litters were retained for further assessment. Nursing dams were euthanized on the day of weaning and the implantation sites were counted. Male offspring were weighted weekly until euthanization. Young adult males were necropsied on PND 68-71 (postnatal week 10; all litters, three males in each litter whenever possible) and on PND 82-84 (postnatal week 12; all litters, remining males). They were examined for the presence of areolas and/or nipples on the ventral surface of the thorax and the abdomen, for gross abnormalities of external and internal genitalia and position of the testes. On PND 68-71, liver, kidneys, testes and epididymides of each male from the control and DIOP groups were weighted and histopathology was conducted on testes.

Results and discussion

Study 1: prenatal toxicity study

No clinical signs related to DIOP treatment were observed. At 1000 mg/kg bw/day, maternal weight gain was lower than control during the first three days of treatment (GD 6-9) but with no statistical significance. There was also a significant decrease in maternal weight on GD 18 and GD 21 and in weight gain over GD 15-18, GD 18-21 and GD 6-21 at the high dose. This difference in late gestation appeared to be primarily related to the significant decrease in uterine contents, since the net body weight (weight on GD 21 minus uterine weight) and net weight gain (weight gain on GD 21 corrected from uterine weights) was not significantly affected by treatment. No significant changes in maternal food intake was observed.

Table 1: Study 1: Maternal findings

	DIOP (mg/kg bw/day)			
	0	**100**	**500**	**1000**
No (%) pregnant	10 (90.9)	8 (80.0)	12 (100.0)	10 (90.9)
Body weight (g)				
GD 0	224±9[a]	223±12	223±8	222±7
GD 6	258±8	259±12	257±12	254±9
GD 9	270±10	275±13	269±12	262±9
GD 12	292±10	295±12	292±19	279±11
GD 15	315±13	319±15	313±22	299±10
GD 18	358±14	364±21	357±26	335±15*
GD 21	415±23	422±29	410±39	378±18*
Gravid uterine weight (g)	106±12	107±15	400±22	83±21*
Net body weight (g)	310±16	315±20	311±25	296±14
Body weight change (g)				
GD 0-6	34±4	36±3	34±9	33±6
GD 6-9	13±4	15±2	12±5	8±7
GD 9-12	22±6	20±5	22±8	17±6
GD 12-15	22±5	24±7	22±6	20±4
GD 15-18	44±5	45±7	43±7	36±8*
GD 18-21	57±10	58±10	54±15	44±7*
GD 6-21	158±18	163±22	153±34	124±19*
Net weight gain (g)	52±12	56±11	54±16	41±15
Food consumption				

(g/day)				
GD 0-6	23±1	24±3	23±2	22±1
GD 6-9	22±2	24±3	22±2	21±3
GD 9-12	23±3	24±2	23±3	21±3
GD 12-15	24±2	25±2	23±2	22±2
GD 15-18	26±2	28±2	26±3	24±3
GD 18-21	27±2	28±3	27±3	25±2
GD 6-21	24±2	26±2	24±2	23±2

[a]: values are expressed as mean ± SD

*: significant difference from the vehicle control, $p < 0.05$ (Dunnett's test)

The number of implantation sites was comparable across groups. The incidence of post-implantation loss and resorptions per litter was significantly increased in the high dose group. The number of live foetuses per litter was slightly, although not significantly lower than control at 1000 mg/kg bw/day. There was a dose-related reduction of foetal body weight, which was significantly different from control at 500 mg/kg bw/day (sexes combined and males). It was only significantly lower at 1000 mg/kg bw/day, when litter size was used as covariable. The decreases amounted to 3-4% and 13-15% compared to control at 500 and 1000 mg/kg bw/day. The mean percentage of male foetuses per litter was significantly increased at 1000 mg/kg bw/day. This finding was attributed to the exceptionally low incidence of males in the concurrent control group, and was not interpreted as an effect of treatment.

Table 2: Study 1: Gestational parameters following administration of DIOP on GD 6-20

	Dose (mg/kg bw/day)			
	0	**100**	**500**	**1000**
All litters[a]	10	8	12	10
No. implantation sites per litter	14.4±2.0[b]	14.5±2.1	14.4±2.7	13.9±1.7
% post-implantation loss per litter[c]	4.7±4.4	5.0±4.5	8.0±12.2	17.8±16.3**
No. litters with dead foetus	1	0	0	1
% dead foetuses per litter	0.7±2.3	0.0±0.0	0.0±0.0	1.3±2.8
No. litters with resorptions	5	5	6	9
% resorptions per litter	4.0±4.6	5.0±4.5	8.0±12.2	16.4±16.5*
Live litters[d]	10	8	12	10
No. live foetuses per litter	13.7±1.8	13.8±1.9	13.3±3.3	11.5±2.8
% male foetuses per litter	40.6±12.1	54.2±11.3	48.9±7.9	56.5±14.8*
Foetal body weight (g)				
All foetuses	5.72±0.22	5.76±0.21	5.52±0.23*	4.96±0.50**

Male foetuses	5.89±0.22	5.89±0.21	5.63±0.28*	5.12±0.49**
Female foetuses	5.60±0.22	5.61±0.20	5.42±0.22	4.74±0.56**

[a]: Includes all pregnant females at euthanization
[b]: values are expressed as litter means ± SD
[c]: Resorptions plus dead foetuses
[d]: Includes all animals with live foetuses at euthanization
*: Significant difference from the vehicle control, $p < 0.05$ (Mann-Whitney test)
**: Significant difference from the vehicle control, $p < 0.01$ (Mann-Whitney test)

External or visceral fetal malformations were not found in any dose group. Two different skeletal malformations were seen in single foetus from two different litters at the high dose. Treatment-related effects on the occurrence of several visceral and skeletal variations were observed. Malpositioned testes (i.e. abdominal or supra-inguinal) were seen in 1/39 male foetuses at 500 mg/kg bw/day, ant in 10/32 male foetuses at 1000 mg/kg bw/day. At this developmental stage, all males of the GD 21 control and 100 mg/kg bw/day foetuses were located at the bottom of the abdominal cavity near the bladder neck. The inguino-scrotal descent normally occurs postnatally. The incidence of foetuses showing 14th supernumerary lumbar ribs (mostly short) was significantly increased at 500 and 1000 mg/kg bw/day. Slight incidences in cervical ribs were also noted at the mid and high doses, but they did not reach statistical significance. Retarded ossification was evidenced by the elevated incidence of incompletely ossified sternebrae at 1000 mg/kg bw/day and the significant decreases in the number of ossified phalanges in the fore and hindlimbs at 500 and 1000 mg/kg bw/day. A few other external, visceral and skeletal common variations were found at low incidences, and were evenly distributed over the groups.

Table 3: Study 1: Fetal malformations and variations in GD 21 rat foetuses

	Dose (mg/kg bw/day)			
	0	**100**	**500**	**1000**
Total no. foetuses (litters) examined[a]				
External	137 (10)	110 (8)	160 (12)	115 (10)
Visceral	69 (10)	55 (8)	80 (12)	58 (10)
Skeletal	68 (10)	55 (8)	80 (12)	57 (10)
Malformations				
Cervical arches, fused	0	0	0	1 (1)[b]
Thoracic vertebral centra, unilateral ossifications and fused	0	0	0	1 (1)
External variations				
Club foot (unilateral)	0	0	2 (2)	0
Visceral variations				
Umbilical artery, left	1 (1)	0	1 (1)	3 (2)
Dilated renal pelvis	0	0	0	1 (1)
Distended ureter	0	0	0	2 (2)

Testis, malpositioned (uni and/or bilateral)	0	0	1 (1)	10# (5)*
Severe	0	0	0	3 (3)
Moderate	0	0	1 (1)	7 (4)
Skeletal variations				
Cervical arches, rudimentary and/or discontinuous	0	0	0	1 (1)[b]
Sternebra ossification				
Bipartite, incomplete or absent	1 (1)	0	0	12# (6)
Misshapen (two)	0	0	0	1 (1)
First and second, fused	0	0	0	2 (2)
Cervical rib(s), supernumerary (short)	1 (1)	2 (1)	6 (4)	7 (3)
14th rib(s), supernumerary (any)	5 (4)	9 (4)	3## (10)	42## (10)*
Long	0	0	1 (1)	2 (2)
Thoracic vertebral centra, ossification				
Bipartite, dumbbell, and/or incomplete (one or two)	2 (2)	2 (2)	4 (4)	2 (2)
Incomplete (four)	0	0	0	1 (1)
No. of ossification centers				
Metacarpals	4.0±0.0[d]	4.0±0.0	4.0±0.0	4.0±0.0
Forelimb proximal phalanges	3.9±0.1	3.9±0.2	3.5±0.2##	2.6±0.9##
Metatarsals	5.0±0.0	5.0±0.0	5.0±0.0	5.0±0.0
Hindlimb proximal phalanges	3.0±1.1	3.0±0.9	0.9±0.9##	0.6±1.2##
Caudal vertebral centra	6.4±0.5	6.2±0.6	6.5±0.6	5.9±0.7

[a]: Incidence of individual defect is presented as number of foetuses (number of litters)
[b]: Alterations with the same letter subscript are from the same foetus
[c]: More than one third of the length of the preceeding rib
[d]: Mean ±SD
*: Significant different from the vehicle control, $p < 0.05$ (Chi-2 test)
#: Significant different from the vehicle control, $p < 0.05$ (Mann-Whitney test)
##: Significant different from the vehicle control, $p < 0.01$ (Mann-Whitney test)

Study 2: ex-vivo testosterone production by fetal testis

DIOP induced significant decreases in testicular testosterone production at 100 mg/kg bw/day (- 34%), 500 mg/kg bw/day (- 72%) and 1000 mg/kg bw/day (- 84%).

Table 4: Study 2: Testicular production of GD 19 male rat foetuses following in utero exposure of DIOP on GD12-19.

Dose (mg/kg bw/day)	**n litters**	**ng/testis/3h**
0	8	6.46 ± 1.12[a]
10	7	6.60 ± 1.44
100	8	4.26 ± 0.41**
500	6	1.80 ± 0.60**
1000	7	1.03 ± 0.22**

a: Values are expressed as litter means ± SD
**: Significant difference from the vehicle control, $p < 0.01$ (Mann-Whitney test)

Study 3: peri-postnatal toxicity study

One dam from the 100 mg/kg bw/day dose group was found dead in the morning of GD22. The cause of death was not apparent and there was no direct evidence that this isolated death was treatment-related. All other dams survived to the end of the study and no clinical signs were noticed at any dose. No statistically significant differences in maternal body weight were observed between control and treated groups throughout the gestation and lactation periods, except for the high dose group on GD21. DIOP had no effect on gestation length or parturition, and all dams delivered live litters on GD21 or 22. The incidence of pre/perinatal loss per litter was increased at the high dose, but the effect was not statistically significant. The viability of the offspring (i.e. mean percent of pups surviving) through the lactation period (PND 1 -21) was statistically decreased at 1000 mg/kg bw/day. Congruently, the mean number of live pups per litter was reduced on PND21, although not significant. There was no statistically significant difference in animal weight at birth (PND1) and during lactation. Male survival was comparable in controls and treated groups after weaning.

Table 5: Study 3: Reproductive parameters in rats administered DIOP on GD 2-21

	DIOP (mg/kg bw/day)			
	0	**100**	**500**	**1000**
No. dams pregnant	12	12	12	11
Maternal body weight (g)				
GD12	305±14[a]	304±10	300±17	301±13
GD15	329±15	323±13	323±17	318±16
GD18	373±21	367±21	372±20	357±22
GD21	431±31	417±33	426±26	392±25**
PND1	318±19	312±13	311±21	296±21
PND21	313±16	315±22	322±20	324±18
No. dams littering	12	12	12	10
Gestation length (days)	21.2±0.4	21.5±0.5	21.1±0.3	21.5±0.5

No. implantations sites per litter	15.7±1.9	13.2±4.7	15.1±1.2	14.9±2.1
Pre- and perinatal loss per litter (%)[b]	7.5±5.5	4.4±4.3	8.5±10.4	15.4±13.8
No. live pups per litter PND1	14.5±2.0	12.5±4.4	13.8±1.5	12.8±3.4
No. live pups per litter PND21	13.0±3.1	11.6±4.5	11.7±1.5	8.9±3.6[c]
Pup survival to weaning PND21 (%)	89.2±17.1	91.2±13.2	85.6±12.8	68.4±22.9#
Pup weight during lactation (g)				
PND1	6.8±0.6	7.4±1.0	6.5±0.7	6.4±0.7
PND7	14.4±1.7	15.7±3.2	13.7±1.5	13.4±2.1
PND14	29.0±4.4	31.0±5.9	28.8±2.4	28.9±3.9
PND21	45.2±7.9	49.8±11.1	46.9±4.5	47.0±5.8

a: Values are expressed as litter means ± SD

b: (No. implantations minus No. live pups PND1)/No. implantations

c: The overall 1-way ANOVA was statistically significant at the $p < 0.051$ level and the No. live pups at $p < 0.05$ with Dunnett's post-test (*)

**: Significant difference from the vehicle control, $p < 0.01$ (Dunnett's test)

#: Significant difference from the vehicle control, $p < 0.05$ (Mann-Whitney test)

At adult necropsy, 69% of males prenatally exposed to 1000 mg/kg bw/day displayed permanent areolas and/or nipple buds. The litter mean of thoracic and abdominal areolas/nipples per affected rat was 5.6±3.2. Retained areolas/nipples were not observed in the control and other treated groups at lower doses.

No gross morphological alterations of the external and internal genitalia were observed in any male offspring from the control or at 100 mg/kg bw/day. At 500 mg/kg bw/day, different abnormalities occurred in three males from three different litter: one animal had an unilaterally enlarged testis, one had an abnormal epididymis and one displayed markedly underdeveloped seminal vesicles and prostate. The testes from all 500 mg/kg bw/day rats were descended into the scrotum. A high incidence of marked malformations of the male reproductive tract was observed at 1000 mg/kg bw/day, with 36 and 74% of the males displaying hypospadias and undescended testes, respectively. In the most severe cases (64%), hypospadias were accompanied by exposed os penis and cleft prepuce. In several instances, the penis was also reduced in size. Except for one animal which had an intra-abdominal testis, all undescended testes were located in the inguinal or supra-inguinal region. A few animals exhibited grossly abnormal epididymis (i.e. thin body), or absent testis and/or epididymis. One third of the high dose animals displayed alteration of the vasa deferentia (e.g. absent, thin, or crossed). Markedly underdeveloped seminal vesicles were present in 39% of the males and markedly underdeveloped prostate was observed in 23% of the males. In addition, two rats from two different litters had no seminal vesicles and one rat displayed unilaterally small seminal vesicle.

Table 6: Study 3. Reproductive tract abnormalities in adult male rats (PNW10 and 12) following in utero exposure to DIOP on GD12-21

	DIOP (mg/kg bw/day)			
	0	**100**	**500**	**1000**
No. males evaluated/litter (%)	84/12	74/12	67/12	39/9[a]
Small penis	0	0	0	8/4 (20.5)[b]
Cleft prepuce	0	0	0	10/5 (25.6)
Hypospadia	0	0	0	14/6 (35.9)
Cleft phallus with exposed os penis	0	0	0	11/4 (28.2)
Testis, undescended (uni or bilateral)	0	0	0	29/9 (74.4)
Testis, undescended (bilateral)	0	0	0	18/6 (46.2)
Testis, enlarged (unilateral)[c]	0	0	1/1 (1.5)	0
Testis, absent or markedly underdeveloped (unilateral)[d]	0	0	0	4/3 (10.3)
Epididymis, absent (unilateral) or markedly underdeveloped (unilateral)[d]	0	0	0	4/4 (10.3)
Epididymis, thin body (unilateral)	0	0	1/1 (1.5)[e]	5/4 (12.8)
Vasa deferentia, crossed	0	0	0	4/3 (10.3)
Vasa deferens, absent (uni or bilaterally)	0	0	0	5/4 (12.8)
Seminal vesicles, absent	0	0	0	2/2 (5.1)
Seminal vesicles, markedly underdeveloped[f]	0	0	1/1 (1.5)	15/6 (38.5)
Seminal vesicles, malformed[g]	0	0	0	1/1 (2.6)
Prostate, markedly underdeveloped[f]	0	0	1/1(1.5)	9/4 (23.1)

a: One litter had no surviving male at euthanization

b: (No. males affected/total males evaluated) x 100

c: Approximately 150% of the control weight. Scrotal. Associated with hypospermatogenesis at histological examination

d: Less than 10% of the control weight. Undescended testes are not included

e: Associated with sperm granuloma at histological examination. Marked hypospermatogenesis was observed in the ipsilateral testis

f: Approximately half of controls or less

g: Unilaterally small seminal vesicle

After weaning, the body weights of male offspring from the 1000 mg/kg bw/day group was slightly, although not significantly lower than control (4-7%) from postnatal week 6 (PNW) onward.

Table 7: Study 3. Body weight of male offspring following in utero exposure to DIOP on GD 12-21

	DIOP (mg/kg bw/day)			
	0	**100**	**500**	**1000**
No. litters	12	12	12	9

Body weight (g)				
PND 28	77±16[a]	85±20	77±13	76±6
PND 35	129±18	143±26	131±14	127±10
PND 42	189±20	203±29	190±14	181±11
PND 49	245±21	262±33	248±15	230±15
PND 56	303±26	318±35	306±16	284±21
PND 63	347±31	361±39	352±16	328±25

a: Values are expressed as litter means ± SD

On PNW10, the liver weights were similar across groups. There was a dose-related decreased in kidney weights, which were about 3 and 16% less than control at 500 and 1000 mg/kg bw/day, respectively. The weights of testes and epididymides were severly reduced at 1000 mg/kg bw/day (with and without adjustement of body weight). They were approximately 38-52% lower than control weight. All undescended testes were hypoplastic and highly contributed to these decreases. Thus, the absolute weights of non-scrotal testes were only 16-43% of the controls, and ranged from 0.28 to 0.75g. Most (84%) of the epididymides of undescended testes weighted less than half of controls. At 500 mg/kg bw/day, there was an increase in the absolute and relative weights of the testes compared to control (6-10%). No significant changes were observed in organ weight at 100 mg/kg bw/day.

Table 8: Study 3: Absolute and relative organ weights of PNW 10 male rats following in utero exposure to DIOP on GD12-21

	DIOP (mg/kg bw/day)			
	0	**100**	**500**	**1000**
No. males/litter	36/12	34/12	36/12	27/9
Body weight (g)	385±33[a]	390±43	388±21	357±29
Absolute organ weight (g)				
Liver	18.37±1.94	18.47±2.17	18.83±1.41	17.40±1.68
Right kidney	1.57±0.12	1.53±0.10	1.51±0.09	1.32±0.12##
Left kidney	1.52±0.09	1.49±0.13	1.47±0.11	1.29±0.11##
Right testis[b]	1.75±0.10	1.80±0.15	1.86±0.11#	1.02±0.36##
Right epididymis[c]	0.41±0.03	0.42±0.04	0.40±0.03	0.25±0.06##
Left testis[b]	1.74±0.10	1.82±0.17	1.91±0.16##	0.84±0.37##
Left epididymis[c]	0.40±0.03	0.42±0.04	0.40±0.03	0.24±0.07##
Relative organ weight (g)				

Liver	4.76±0.24	4.74±0.26	4.85±0.19	4.87±0.24
Right kidney	0.41±0.02	0.40±0.03	0.39±0.01#	0.37±0.02##
Left kidney	0.40±0.02	0.38±0.02	0.38±0.02	0.36±0.02##
Right testis	0.46±0.03	0.47±0.03	0.48±0.03#	0.28±0.10##
Right epididymis	0.11±0.01	0.11±0.01	0.10±0.01	0.07±0.01##
Left testis	0.45±0.03	0.47±0.03	0.49±0.04#	0.24±0.10##
Left epididymis	0.11±0.01	0.11±0.01	0.10±0.01	0.07±0.02##

a: Values are expressed as litter means ± SD

b: When only descended testes are included, the means are at 1 g/kg bw/day: 1.73±0.16 (right, n = 11) and 1.37±0.43* (left, n = 10); no non-scrotal testis in any other groups

c: When only descended testes are included, the means are at 1 g/kg bw/day: 0.36±0.05# (right, n = 11) and 0.31±0.06## (left, n = 10); no non-scrotal testis in any other groups

#: Significant differences from the vehicle control, $p < 0.05$ (Mann-Whitney test)

##: Significant differences from the vehicle control, $p < 0.01$ (Mann-Whitney test)

*: Significant differences from the vehicle control, $p < 0.07$ (Mann-Whitney test)

Histopathologically, the most frequent finding was hypospermatogenesis, which occurred in two males from two different litters at 500 mg/kg bw/day, and in 22 males from the 9 litters at 1000 mg/kg bw/day (6 and 88% of the evaluated males, respectively). Bilateral hypospermatogenesis was seen in approximately half of the most severely affected animals at the high dose level (8/15). This lesion was not observed in control or 100 mg/kg bw/day animals. It was characterized by a reduced number of spermatogenic epithelial cells or layers, and degenerate epithelial cells. Undescended testes were generally severely affected, but hypospermatogenesis could also be present in scrotal testes.

Table 9: Study 3: Histopathological lesions in the testis of PNW 10 male rats following in utero exposure to DIOP on GD12-21[a]

	DIOP (mg/kg bw/day)			
	0	**100**	**500**	**1000**
Number of males/litters examined	36/12	34/12	36/12	25/9
Hypospermatogenesis[b]				
Grade 1	0	0	0	2/2
Grade 2	0	0	0	2/2
Grade 3	0	0	1/1	2/2
Grade 4	0	0	0	15/8
Grade 5	0	0	1/1	1/1
Intratubular cell debris (uni or bilateral)	2/1	0	1/1	2/2
Tubular dilatation, minimal	0	0	1/1	0

(bilateral)				
Mineralization of tubules, marked (unilateral)	0	0	0	1/1
Lymphoid cell infiltration, slight (unilateral)	0	0	1/1	0
Leydig cell hyperplasia (minimal) and vacuolation of spermatogenic epithelium (unilateral)	0	0	0	1/1

a: Results are expressed as the number of males/litters affected

b: The lesions appeared unilaterally or bilaterally. Only the highest severity was mentioned when the lesion was bilateral.

3.10 Specific target organ toxicity – single exposure

Not assessed.

3.11 Aspiration hazard

Not assessed.