



European Council for
Plasticisers and Intermediates
COMMITTED TO THE SCIENCE OF SAFETY

Received CPSC

2011 APR -5 A 11: 28

March 30, 2011 Office of the Secretary
FOI

Office of the Secretary
Consumer Product Safety Commission
4330 East-West Highway
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Fao: Office of the Secretary - Consumer Product Safety Commission

Dear Sirs,

The European Council of Plasticisers and Intermediates (ECPI) is providing this information in response to the request of the Consumer Product Safety Commission (CPSC) Chronic Hazard Advisory Panel (CHAP) for comment on issues relating to the hazard, exposure, and risk posed by phthalates and phthalate substitutes from all sources of exposures, especially children's products. (1)

In July 2010, the ECPI representative, Dr. Nina Hallmark, presented a review of information on classified low molecular weight (LMW) phthalates (DBP, CAS RN 84-74-2) and non-classified high molecular weight (HMW) phthalates (DINP, CAS RN 68515-48-0 and CAS RN 28553-12-0; and DIDP, CAS RN 68515-49-1 and CAS RN 26761-40-0), in accordance with the OECD Conceptual Framework for Endocrine Data.

The full review of all relevant data conducted in accordance with the OECD Conceptual Framework for Endocrine Data has now been completed for a classified LMW phthalate (DBP) and for two non-classified HMW phthalates (DINP, DIDP) and the full report is attached to this submission. The report has been written by ECPI toxicological experts from member companies and cites all relevant published studies. Evaluating all relevant data using the five level OECD Framework leads to the clear conclusion that the two HMW phthalates, DINP and DIDP, do not produce adverse health effects mediated via an endocrine mechanism, and hence are not endocrine disrupters by WHO IPCS, Weybridge and REACH guidance definitions. The key Level 5 studies supporting this conclusion are the comprehensive 2-generation studies which have been conducted on DINP and DIDP. With respect to DBP using the five level OECD Framework for Endocrine Data leads to the conclusion that the adverse reproductive effects which lead to classification of DBP, and observed in the 1-, 2- and multi-generation studies are mediated via an endocrine mechanism, and hence DBP does meet the definition of an endocrine disrupter. The conclusions on the endocrine mediated reproductive effects for DBP are included in the EU Risk Assessment.

Following the ECPI presentation of July 26, Professor Kortenkamp raised questions on the applicability of the OECD Conceptual Framework to phthalates. Professor Kortenkamp stated that "there's no assay that would look at say reduction of fetal androgen synthesis etc., etc they are not validated at OECD level in vivo". It is correct that there is no validated test method for fetal androgen synthesis and the work

(1) Notice of Meeting of Chronic Hazard Advisory Panel on Phthalates and Phthalate Substitutes and Opportunity for Public Comment, 75 Fed. Reg. 31426 (June 3, 2010).



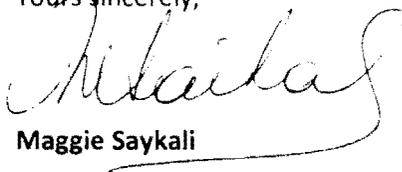
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which has been done in this area is experimental research which does require validation for regulatory purposes. In addition it is important to note there is a validated screening assay for anti-androgenic related effects, the Hershberger Assay, and this assay is specifically listed in the OECD Conceptual Framework. Furthermore, the OECD Conceptual Framework is a flexible approach which allows all relevant in vitro and in vivo work to be evaluated according to the five level approach. The experimental research which has been published relevant to rodent fetal testosterone and rodent biological markers (ano-genital distance and nipple retention) has been included in the full weight of evidence, ECPI Endocrine Data Evaluation Report. The more detailed conclusions of the report are:

DINP and DIDP do not cause the adverse health effects mediated via an endocrine mechanism of cryptorchidism, hypospadias and testicular pathology in comprehensive 2-generation rodent reproductive studies (OECD Level 5 studies). These effects are seen with DBP. With respect to in vivo screening studies (OECD Level 3 studies) DINP and DIDP do not show consistent significant adverse effects in the Hershberger Assay for anti-androgenic effects. The Hershberger Assay is a validated OECD test method for anti-androgenic related effects and is specifically included in the OECD Framework. DBP shows some evidence of effects in a Hershberger Assay, and shows clear effects on testosterone, sperm counts and causes testicular effects (OECD Level 4 studies). In non-validated research studies for anti-androgenic effects DINP has shown no, minor or inconsistent effects at high doses in rodents, and with no or limited evidence of a dose response (ano-genital distance, nipple retention). Studies on rodent fetal testosterone have reported some evidence of reduction at high doses but this change was not associated with adverse health effects. In the OECD Conceptual Framework these results in screening studies lead to the conclusion that it is appropriate to assess OECD Level 4 and OECD Level 5 studies to determine if adverse health effects are resulting from these changes. As noted above a review of OECD Level 5 studies leads to the conclusion that adverse health effects mediated via an endocrine mechanism are not observed for DINP or for DIDP.

As evidenced by this report, ECPI is committed to a robust scientific evaluation and interpretation of all relevant data, and is ready to address any further questions which the CPSC and the CHAP may have relevant to this topic.

Yours sincerely,



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Enclosed: Endocrine Data Evaluation Report

For selected high molecular weight (HMW) phthalates (DINP, DIDP) and a low molecular weight (LMW) phthalate (DBP) Using the OECD Conceptual Framework Volume I - Mammalian Data European Council for Plasticisers and Intermediates Scientific Report 110301 March 2011

March, 2011

Endocrine Data Evaluation Report

For selected high molecular weight (HMW) phthalates (DINP, DIDP) and a low molecular weight (LMW) phthalate (DBP)

Using the OECD Conceptual Framework

Volume I – Mammalian Data

DINP – CAS numbers 68515-48-0 and 28553-12-0/ EINECS numbers 271-090-9 and 249-079-5

DIDP – CAS numbers 68515-49-1 and 26761-40-0 / EINECS numbers 271-091-4 and 247-977-1

DBP – CAS number 84-74-2/EINECS number 201-557-4

**European Council for Plasticisers and Intermediates
Scientific Report 110301
March 2011**

Executive Summary

Following extensive regulatory reviews, it has been confirmed that neither DINP nor DIDP warrant classification as dangerous to human health or the environment. In particular, EU Risk Assessments have been conducted for DINP and DIDP (ECB, 2003) including the review of the available endocrine data at that time. Additional endocrine endpoint studies have been conducted since the EU Risk Assessment and this report provides an in-depth review of all endocrine data relevant to human health for selected high molecular weight (HMW) phthalates (DINP, DIDP) and a low molecular weight phthalates (DBP) using the OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals ("OECD Conceptual Framework"). This framework represents the state of the art approach for assessing endocrine data on chemical substances. This report uniquely reviews all the relevant scientific data in mammals and presents the comprehensive weight of the evidence, establishing a robust conclusion on these chemical substances in the context of the Weybridge, IPCS, and REACH Guidance definitions of endocrine disruptors and the OECD Conceptual Framework.

The three chemicals reviewed here are all high tonnage chemical substances that have been in use for a considerable time. Two (DINP, DIDP) are used principally as PVC plasticisers. DBP has some use as a PVC plasticiser, but is more commonly used as a gelling aid, as a solvent, as an antifoam agent or as a lubricant. Each of these three chemicals has a comprehensive toxicology dataset available and have all been individually evaluated by international regulatory authorities for their potential risk to human and environmental health. In Europe, hazard based classification conclusions have been reached for each of these chemicals during the regulatory risk assessment process under the EU Existing Substances Regulation (793/93 (EEC)) and the hazard classification process under the EU Dangerous Substances Directive (67/548 (EEC)), and now the EU Classification, Labelling and Packaging (CLP) Regulation (EC 1272/2008). The classification conclusions pertaining to human health, under the CLP Regulation are summarised below:

- | | | |
|-------------------------|-------------------------------|----------------------------------|
| • Di-n-butyl phthalate | CAS RN 84-74-2 | Classified: Repr.1B ¹ |
| • Di-isononyl phthalate | CAS RN 68515-48-0/ 28553-12-0 | Not classified |
| • Di-isodecyl phthalate | CAS RN 68515-49-1 | Not classified |

Unlike DBP, the adverse health effects mediated via an endocrine mechanism of cryptorchidism, hypospadias, and significant testicular pathology are not seen with either DINP or with DIDP. This conclusion is consistent with the Category 1B classification for reproductive effects of DBP, and with the statements in the EU Risk Assessments for DBP that some of these effects are mediated via an endocrine mechanism.

The comprehensive evaluation of all of the relevant mammalian endocrine data (laboratory animal data and available human epidemiology), including full references, are provided in an Appendix for each substance. Based on this information the following conclusions have been made when data are evaluated according to the OECD Conceptual Framework and using the commonly recognized definitions of an endocrine disruptor:

- There are sufficient data to conclude that DINP is not an endocrine disrupting substance for mammals
- There are sufficient data to conclude that DIDP is not an endocrine disrupting substance for mammals.

¹ DBP is also classified as Aquatic Acute 1

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1 Introduction

1.1 Background

This ECPI report provides a comprehensive review of the available mammalian endocrine data on Di-isononyl Phthalate (DINP) and Di-isodecyl Phthalate (DIDP). The large majority of the data included for DINP and DIDP have previously been submitted to regulatory authorities including for example in the REACH registration dossier and in submissions to the US Consumer Product Safety Commission. Many of the studies have been published in peer reviewed journals. There are a small number of peer reviewed research papers included in this report which have been published which were not included in prior regulatory submissions. Also, various publications are available that consider selected aspects of this issue but this report uniquely reviews all the relevant scientific data in mammals (laboratory animal data and human epidemiology studies) and weighs up this evidence to establish a robust conclusion about the endocrine disrupting potential of these non-classified high molecular weight (HMW) plasticisers. The standard against which these data are evaluated is the OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals (CF).

This paper will address the definitions of an endocrine disruptor and then consider the relevant data available for DINP and DIDP and finally conclude on their endocrine disrupting potential. For comparison, DBP is also evaluated since it is classified under the EU Classification, Labelling and Packaging (CLP) Regulation as Category 1B Reproductive agents. The three plasticisers and their classification are listed below in Table 1.

Chemical Name (abbreviation)	CAS RN	EU Classification, Labelling, Packing Regulation (CLP) classification Regulation (EC) No 1272/2008
Di-isononyl phthalate (DINP)	68515-48-0 28553-12-0	Not classified
Di-isodecyl phthalate (DIDP)	68515-49-1	Not classified
Di-n-butyl phthalate (DBP)	84-74-2	Repr. 1B Aquatic Acute 1

Table 1: Summary of compounds evaluated in this report for their endocrine disrupting potential

1.2 Not all phthalate plasticisers are the same

Alkyl ortho-phthalates ("phthalates") constitute a broad class of chemicals with a range of physical, chemical and toxicological properties. These properties are structure-dependent. This means that phthalates are not all the same; they do not all have the same uses and they are not all toxicologically equivalent.

These differences are expanded below using the three phthalate plasticisers that differ in terms of their structure and toxicological profiles.

1.2.1 Definition of Low Molecular Weight (LMW) Phthalates and High Molecular Weight (HMW) Phthalates

Phthalate esters are a diverse group of substances produced by the reaction of phthalic anhydride with aliphatic and aromatic alcohols to produce di-esters. Certain phthalate esters are

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used extensively as PVC plasticisers and also in rubber products, paints and coatings and printing inks. Since the term “phthalates” constitutes a broad class of chemicals with a wide range of physical and chemical properties, it follows that not all phthalates are toxicologically equivalent. The major commercial products used in PVC, rubber products, paints, coatings and printing inks, can be divided into two main groups – Low Molecular Weight Phthalates (LMW) and High Molecular Weight Phthalates (HMW).

1.2.2 Low Molecular Weight (LMW) Phthalates

Low molecular weight (LMW) phthalates are those with alkyl side chains of C4 – C8 total carbon number and with carbon backbones in the side-chains of C4 – C6. Members of this group include Di(2-ethylhexyl) Phthalate (DEHP, also known commonly as DOP), Di-Butyl Phthalate (DBP), Butyl Benzyl Phthalate (BBP), Di-IsoButyl Phthalate (DIBP), Di-IsoPentyl Phthalate (DIPP), Di-IsoHeptyl Phthalate (DIHP). These LMW phthalates are classified as reproductive and developmental toxins (Category 1B under the UN Globally Harmonized System and the EU Classification, Labeling and Packaging Regulation) due to significant adverse health effects observed in rodent studies.

Note: The very low molecular weight phthalates, Di-Methyl Phthalate (DMP – carbon side chains of one carbon) and Di-Ethyl Phthalate (DEP – carbon side chains of two carbons) used in cosmetics and toiletries are not classified for reproductive effects.

The specific low molecular weight phthalate which is addressed in detail in this report is:
DBP – CAS number 84-74-2/EINECS number 201-557-4

1.2.3 High Molecular Weight (HMW) Phthalates

High molecular weight (HMW) phthalates are those with carbon side chains of C9 and greater total carbon (typically to C13). The carbon backbones in the side-chains of HMW phthalates are C7 and greater. Members of this group include Di-IsoNonyl Phthalate (DINP), Di-IsoDecyl Phthalate (DIDP) and Di-(2-PropylHeptyl) Phthalate (DPHP). Based on comprehensive data and evaluations these substances are not classified as reproductive and developmental toxins as they do not produce adverse reproductive or developmental effects in laboratory animal studies.

The specific high molecular weight phthalates which are addressed in detail in this report are:
DINP – CAS numbers 68515-48-0 and 28553-12-0/EINECS numbers 271-090-9 and 249-079-5
DIDP – CAS numbers 68515-49-1 and 26761-40-0/EINECS numbers 271-091-4 and 247-977-1

In summary

	<u>Total Carbon in alkyl side chains</u>	<u>Carbon backbone in alkyl side chains</u>	<u>Classification CLP Regulation</u>
LMW Phthalates	C4 – C8	C3 – C6	Category 1B Repro
HMW Phthalates	C9 – C13	C7 – C13	Not Classified

1.2.4 General purpose and speciality plasticisers

The primary use of these substances is as plasticisers for making flexible PVC articles. Plasticisers can be divided in two application categories:

- *General purpose* = suited to a very wide range of applications and processing techniques where they bring an optimised balance between cost, versatility and performance and
- *Specialty* = impart one or more special properties; they are suited to a narrow range of applications and are in general produced in smaller quantities than general purpose plasticisers.

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DINP and DIDP are general purpose plasticisers and DBP is a specialty plasticiser which has been used in flooring. DBP use has declined significantly in use in recent years due to its classification as Category 1B Reproductive Agents.

Figure 1 shows the split between rigid and flexible PVC, the split between phthalates and other plasticisers and the split between durable goods and sensitive applications. Figure 2 shows the major uses of plasticised PVC (flexible vinyl) in Europe:

Figure 1 Global Flexible PVC market

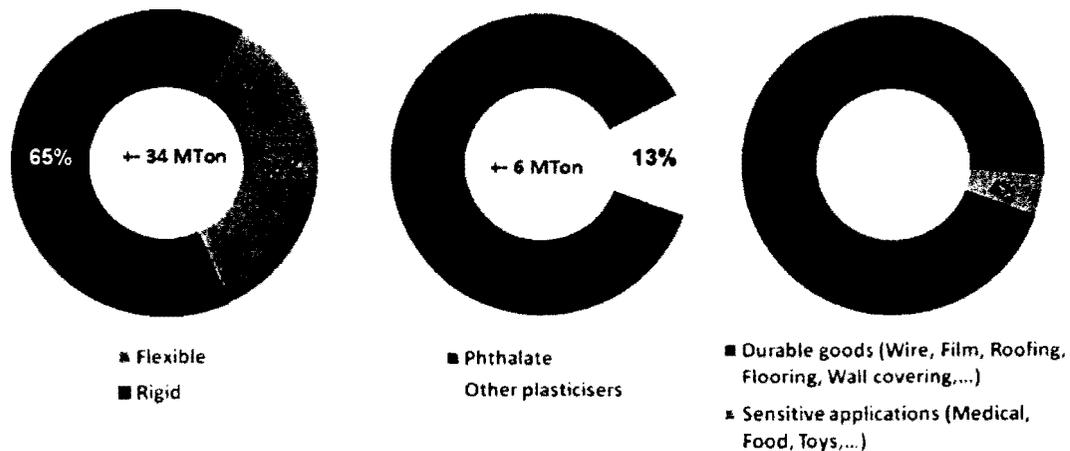
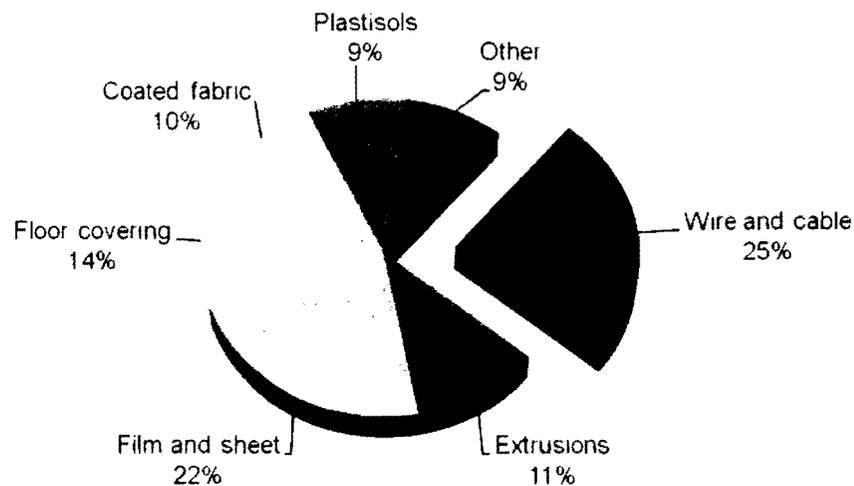


Figure 2 Major use of plasticised PVC in Europe



1.2.5 Toxicological profiles of DINP and DIDP vs. DBP

Toxicological profiles of DINP and DIDP are markedly different to those for DBP. Evaluations by regulatory agencies have shown that neither DINP nor DIDP are considered as dangerous chemicals. Therefore, neither DINP nor DIDP are classified as dangerous to human health or the environment, unlike DBP which is classified as dangerous.

A substantial amount of toxicology data exist for most phthalates. These data have shown that the different phthalates family members are toxicologically differentiated by their effects on reproductive and/or developmental parameters. Significant adverse effects are associated with phthalates with an "alcohol backbone" of between three and six carbons long.

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It is known that all the phthalates are rapidly metabolized and excreted following ingestion and absorption. None of them are acutely toxic, irritating or sensitizing. Phthalates are not mutagenic but do induce rodent specific cancers which are not relevant to humans.

A summary of the toxicological profiles of three phthalate plasticisers is outlined below, demonstrating the difference in hazard potential between these chemicals.

1.2.6 Toxicological profile for DBP

Under the new Regulation (EC) No 1272/2008 (CLP), DBP is classified as Repr. 1B, H360 (May damage the unborn child. Suspected of damaging fertility). This is consistent with the following classification of DBP under the previous legislation: Dangerous Substances Directive (67/548/EEC) = Repr. Cat. 2; R61 (May cause harm to the unborn child.) and Repr. Cat. 3; R62 (Possible risk of impaired fertility). The toxicology summary below is adapted from the European Commission Risk Assessment Report Vol. 29. Report no. EUR 19840 EN.¹

According to the EC criteria, DBP does not need to be classified on the basis of its acute toxicity, respiratory irritation or skin irritation/sensitisation. No genotoxic effects for DBP were observed in *in vivo* studies detecting chromosomal aberrations.

In reproductive and developmental toxicity studies, the key effects reported include reduced fertility in male and female laboratory rodents at 500 and 1,000 (males only) mg/kg bw. Infertility in males was related to testicular atrophy and reduced sperm production, while treated females cycled and mated successfully, but many treated females (500 mg/kg bw) aborted their litters around mid-pregnancy. In the F1 offspring (data only from F1 animals from dams treated with 0, 250 and 500 mg DBP/kg bw) urogenital malformations/ abnormalities including a low incidence of agenesis of the epididymis, hypospadias, ectopic testis, renal agenesis and uterine abnormalities (partial agenesis or lack of implants in one uterine horn) were seen. Furthermore, F1 males exhibited reduced cauda epididymal sperm numbers. The results of these and other studies indicate that DBP does not possess estrogenic activity but rather shows adverse anti-androgenic effects. No observed adverse effects levels (NOAELs) for these effects were determined in the EU Risk Assessment Report. These NOAELs have been used for risk assessment purposes for DBP.

1.2.7 Toxicological profile for DINP

Under the new Regulation (EC) No 1272/2008 (CLP), DINP is not classified as dangerous to human health or the environment. This is consistent with the non-classification of DINP under the previous regulations (Dangerous Substances Directive (67/548/EEC)). The toxicology summary below is adapted from the European Commission Risk Assessment Report Vol. 35. Report no. EUR 20784 EN.²

DINP is considered to show low acute oral, dermal and inhalation toxicity. Therefore, no classification is indicated according to the EU criteria for acute toxicity. DINP may be considered as a very slight skin and eye irritant, with effects reversible in a short time in animal studies. In humans there is no indication of an irritating, corrosion or sensitisation potential. Thus no classification is indicated according to the EU criteria for these different end points.

DINP is not mutagenic *in vitro* in bacterial mutation assays or mammalian gene mutation assay (with and without metabolic activation) and is not clastogenic in one cytogenetic assay *in vitro* on CHO cells and in one *in vivo* assay on bone marrow cell of Fisher 344 rats. These studies demonstrate

¹ ECB (2004). dibutyl phthalate (DBP). Risk Assessment. European Chemicals Bureau, Institute for Health and Consumer Protection, Joint Research Center of the European Commission.

² ECB (2003). 1,2-Benzenedicarboxylic acid, di-C8-10branched alkyl esters, C9 rich and di-isononyl phthalate (DINP). Risk Assessment. European Chemicals Bureau, Institute for Health and Consumer Protection, Joint Research Center of the European Commission.

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that DINP is not genotoxic *in vivo* or *in vitro*. Additionally, based on the overall evaluation of the available carcinogenicity data, including consideration of relevance to humans of increased incidence of liver tumours and an increase in the incidence of kidney tumours and mononuclear cell leukaemia in male rats, no classification for carcinogenicity was considered necessary as part of the EU Risk Assessment process. Of interest, IARC categorised MNCL as “an unclassified leukemia with no known human counterpart” and substances which increase MNCL frequency as “not classifiable as to carcinogenicity in humans” (IARC, 1990). Finally, pertaining to kidney tumours, the species and sex-specific alpha 2 μ globulin mechanism likely responsible for kidney tumours seen in male rats is not regarded as relevant to humans.

Unlike DBP, data show that DINP does not cause reproductive effects in rodent two generation reproductive toxicity studies. No changes in reproductive indices are observed demonstrating no adverse effects on fertility (Waterman et al, 2000). This study provides a comprehensive basis on which to evaluate reproductive and developmental effects, including anti-androgenic effects in male rat pups. As such, DINP was not classified as a reproductive toxin as part of the EU Risk Assessment. It has been proposed by Carruthers et al (2005) that there is a critical window of susceptibility for the developing male foetal reproductive system for LMW phthalates in rodent studies (gestation day 16 – 19). This critical window is fully assessed in the 2-generation reproductive studies. The 2-generation study design assesses the effects of continuous exposure in the F1 and F2 generations. In the DINP two-generation study the parameters of anogenital distance and nipple/areola retention were not specifically part of the test protocol (not included in the test guidelines in effect at the time of the study). Nevertheless, detailed clinical observations were included and if there had been evidence of changes in these parameters then this would have been detected during these observations. Anogenital distance is used as the endpoint to determine the gender of the pups; there were no clinical observations reporting that this assessment was problematic indicating that there was no biologically significant effect on AGD following DINP exposure. There are reports that DINP minimally modulated the androgenic endocrine system in developing rats (Gray et al, 2000). The usefulness of these data for hazard and risk assessment is limited as a single high dose was utilized, effects were pooled to achieve statistical significance, and the only clearly statistically significant finding is suspect based on high incidence of this finding in other control groups. The authors, too, questioned the significance of the statistical power of their analysis. These studies were considered in the recently published EU risk assessments for DINP. The EU Scientific Committee for Toxicology, Ecotoxicology and the Environment (CSTEE) evaluated the endocrine disrupting properties of DINP to be “very low”; CSTEE took into account the slight effects seen at very high dose levels. Overall, regarding fertility and development, the effects observed in the available studies do not justify classification according to the EU classification criteria. This conclusion was reached by the Technical Progress Committee for Classification and Labelling in 2000.

1.2.8 Toxicological profile for DIDP

Under the new Regulation (EC) No 1272/2008 (CLP), DIDP is not classified as dangerous to human health or the environment. This is consistent with the non-classification of DIDP under the previous legislation (Dangerous Substances Directive (67/548/EEC). The toxicology summary below is adapted from the European Commission Risk Assessment Report Vol. 36. Report no.: EUR 20785 EN.¹

DIDP is considered to show low acute oral, dermal and inhalation toxicity. Therefore, no classification is indicated according to the EU criteria for acute toxicity. In humans there is no indication of an irritating, corrosion or sensitisation potential. Thus no classification is indicated according to the EU criteria for those different end points.

DIDP is not mutagenic *in vitro* in bacterial mutation assays (with and without metabolic activation) and is negative in a mouse lymphoma assay. It is not clastogenic in a mouse micronucleus assay *in vivo*. This indicates that DIDP is a non-genotoxic agent.

¹ ECB (2003). 1,2-benzenedicarboxylic acid, di-C9-11- branched alkyl esters, C10-rich and di-“isodecyl” phthalate (DIDP). Risk Assessment. European Chemicals Bureau, Institute for Health and Consumer Protection, Joint Research Center of the European Commission.

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A two year bioassay was conducted in which DIDP was administered in the diet at concentrations of 400, 2000, and 8000 ppm to F344 rats (Cho *et al.*, 2008). The average daily doses of DIDP were reported to be calculated from the body weights and feed consumption data using the concentrations of DIDP in the diet. Actual exposures for male rats were 21.9, 110.3 and 479.2 mg/kg-bw/day and for female rats 22.9, 128.2 and 619.6 mg/kg-bw/day.¹ No treatment related neoplastic lesions were observed in the internal organs, including the liver. Therefore, DIDP was considered to be not carcinogenic at doses up to 8000 ppm in rats.

Data show that DIDP does not cause reproductive effects in rodent two generation reproductive toxicity studies. There were no statistically significant differences in male mating, male fertility, female fertility, female fecundity, or female gestational indices between treated and control animals in the F1 or F2 generation (Hushka *et al.*, 2001). Mean length of gestation and mean litter size and of the treated and control groups were similar. There were no statistically significant differences in the mean sex ratio of the treated offspring compared with controls. There were no statistically significant effects on nipple retention or anogenital distance. No changes in reproductive indices were observed hence based on those assays, no adverse effects on fertility may be anticipated. These studies provide a comprehensive basis on which to evaluate reproductive and developmental effects, including anti-androgenic effects in male rat pups. No effects were seen on fertility thus no classification according to the EU is needed. DIDP did produce a small, statistically significant decrease in postnatal survival indices which was observed in the second generation of both of the two-generation studies leading to the NOAEL of 0.06% (33-76 mg/kg/d). These effects were found in association with maternal toxicity: reduced body weight, instances of increased kidney weight, and /or liver enlargement. Therefore, the effects on post-natal survival were considered as secondary rather than a direct effect of DIDP on the rat pups. Cross fostering studies demonstrated that post natal body weight effects observed were reversible when post natal exposure to DIDP via the dams stopped.

Overall, regarding fertility and development, the effects observed in the available studies do not justify classification according to the EU classification criteria. This conclusion was reached by the Technical Progress Committee for Classification and Labelling in 2000.

1.2.9 Conclusion on the comparative toxicological profiles

All three of the plasticisers listed have been extensively reviewed for their toxicological properties. DINP and DIDP are of low toxicity, and are not classified for any health or environmental hazard under EU legislation. This conclusion is supported by multiple regulatory agencies that have examined all available data and determined no further risk management measures are needed for DINP or DIDP. Together with information about the exposure of humans to these plasticisers, these data were the basis for the EU Risk Assessment conclusion that risk reduction is not required for DINP and DIDP in current applications. In contrast DBP is classified as Category 1B Reproductive Agents under the EU Classification, Labelling and Packaging Regulation based on developmental and reproductive effects in rodents. The EU Risk Assessments have also concluded that risk reduction is not required in many consumer applications for DBP, and that risk reduction is required in certain situations.

Some of the adverse developmental and reproductive effects which result in classification of DBP are likely mediated via an endocrine mechanism. In the EU Risk Assessments, ECB identifies these adverse effects mediated via an endocrine mechanism as hypospadias (malformed penis), cryptorchidism (undescended testes) and significant testes pathology. It should also be noted that DBP also causes other significant malformations in standard developmental tests in laboratory animals. Further, these adverse health effects were not observed in rodent studies with DINP and DIDP. This demonstrates a clear differentiation between the toxicological profiles of the low molecular weight phthalate, DBP and the high molecular weight phthalates, DINP and DIDP.

¹ Cho, W.-S., Han, B. S., Ahn, B., Nam, K. T., Choi, M., Oh, S. Y., Kim, S. H., Jeong, J., and Jang, D. D. (2010). Corrigendum to "Peroxisome proliferator di-isodecyl phthalate has no carcinogenic potential in Fischer 344 rats" [Toxicol. Lett. 178 (2008) 110-116]. Toxicology Letters 197, 156-156.

2 Endocrine Disruption

Endocrine disruption is a functional change that leads to adverse effects and not a hazard endpoint per se. Data are available that investigate selected aspects of endocrine disruption but this report uniquely reviews all the relevant scientific data in mammals and weighs up this evidence to establish a robust conclusion about the endocrine disrupting potential of these non-classified HMW phthalates (DINP, DIDP), and a classified LMW phthalate (DBP).

The standard against which these data are evaluated is the OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals (CF). The remainder of this chapter will explain this framework and comprehensive data for the three phthalate plasticisers is then provided in three Appendices.

2.1 Endocrine disruption – Regulatory Context

Identification of a substance as a Substance of Very High Concern (SVHC) leads to listing on the REACH Candidate List. The REACH regulation identifies CMR 1 and 2 substances (as defined by the EU Dangerous Substances Directive, 67/548/EEC), PBT, and vPvB substances, and Substances of Equivalent Concern under the Substance of Very High Concern category. Endocrine disrupting substances are identified under Substances of Equivalent Concern (Article 57(f) under the REACH regulation) which are defined as substances for which there is scientific evidence of probably serious effects to human health or the environment which give rise to an equivalent level of concern as CMR 1 and 2, PBT, vPvB substances. The precise process for evaluating and defining endocrine disruptors for REACH and other regulatory purposes is still being developed by EU Member States and the European Commission. REACH Guidance on Authorisation includes a definition of an endocrine disruptor (see below).

2.2 Definition of an endocrine disruptor

Endocrine disruption is not considered a toxicological end point per se but a functional change that leads to adverse effects. One of the earliest consensus definitions was developed during a multi-stakeholder conference in Weybridge, England during 1996. This led to further conferences and considerations within respected scientific programs such as the World Health Organization's International Program for Chemical Safety (IPCS). The OECD has based its Framework for the Testing and Assessment of Endocrine Disrupting Chemicals on the IPCS definition. Further details on this five-level framework are provided in the next section. The definitions are:

- Weybridge definition (1996): "An endocrine disruptor is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function."
- WHO IPCS definition (2002): "An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations."
- The REACH Guidance on Authorisation provides a definition of endocrine disruptor which is very similar to the above definitions:

"An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations."

These definitions consistently require that only substances that cause adverse effects in whole organisms via endocrine modes of action should be categorised as having 'endocrine disruptor properties'.

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2.3 European Community Strategy on Endocrine Disruptors

As part of the European Community Strategy on Endocrine Disruptors, DG Environment requested consultants to draw up a priority list of substances for further evaluation of their role in endocrine disruption. The full background, documentation and references for the European Community Strategy can be found at

http://ec.europa.eu/environment/endocrine/strategy/substances_en.htm.

564 different candidate substances were considered, with 146 being eventually grouped into one of three categories:

- **Category 1** - evidence of endocrine disrupting activity in at least one species using intact animals;
- **Category 2** - at least some in vitro evidence of biological activity related to endocrine disruption;
- **Category 3** - no evidence of endocrine disrupting activity or no data available.

DINP (CAS no. 28553-12-0/EINECS no. 249-079-5) was included in Category 2 (in vitro evidence) and DINP (CAS no. 68515-48-0/EINECS no. 271-090-9) in Category 3 (no evidence of endocrine disrupting activity) for potential health effects. DINP is available commercially under two CAS / EINECS numbers and the two numbers were assigned to the different categories. The basis for the listing in Category 2 is most probably related to the studies in human and yeast cells using di-esters (in vivo the diesters are metabolized to mono-esters so in vitro tests using di-esters are not valid). Repeat tests using mono-esters have not confirmed these initial results. As can be seen in the current report this type of test falls under Level 2 of the OECD Framework for Endocrine data and this is only one of five elements of the framework. Using the full weight of evidence approach and all five levels of the OECD Framework it can be clearly seen that DINP is not an endocrine disruptor. DINP has already been "further evaluated" in extensive tests (Levels 3, 4 and 5 of the OECD Framework and it is unclear at this stage what additional tests could be done to further evaluate DINP). Based on all the evidence DINP should be assigned to Category 3 (no evidence of endocrine disrupting activity).

DIDP was assigned also to Category 2 (in vitro evidence) for potential health effects. As can be seen in the current report there is no scientific basis for this and when all relevant data is evaluated using the OECD Framework the conclusion is that DIDP is not an endocrine disruptor. Extensive data exists for DIDP including OECD Level 5 studies which support this conclusion. It is therefore difficult to see what additional testing for potential health effects should be done.

DBP was included in Category 1 (evidence of endocrine disrupting activity in at least one species using intact animals) and as shown in the current report, ECB (2003) in the EU Risk Assessment concludes that DBP produces adverse health effects including endocrine disrupting effects.

In fact it is now understood that the Priority List of Substances for Further Evaluation of their role in endocrine disruption will be converted fully to a database of information to be managed by the European Union Joint Research Centre. DG Environment has confirmed that the list/database should be used as a source of information and not for regulatory action.

2.4 The United States Environmental Protection Agency (USEPA) Endocrine Disruptor Screening Program (EDSP)

The United States Environmental Protection Agency (USEPA) Endocrine Disruptor Screening Program (EDSP) is charged with using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have hormonal effects in humans. Currently, USEPA is developing requirements for the screening and testing of pesticides, commercial chemicals, and environmental contaminants for their potential to disrupt the endocrine system.

A two tier approach for identifying endocrine disruptors is proposed:

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1. Through Tier 1, EPA hopes to identify chemicals that have the potential to interact with the endocrine system.
2. Through Tier 2, EPA will determine the endocrine-related effects caused by each chemical and obtain information about effects at various doses.

The Tier 1 battery's suite of in vitro and in vivo screening assays includes the following:

In vitro:

Estrogen receptor (ER) binding – rat uterine cytosol

Estrogen receptor - (hER α) transcriptional activation - Human cell line (HeLa-9903)

Androgen receptor (AR) binding – rat prostate cytosol

Steroidogenesis – Human cell line (H295R)

Aromatase – Human recombinant microsomes

In vivo

Uterotrophic (rat)

Hershberger (rat)

Pubertal female (rat)

Pubertal male (rat)

Amphibian metamorphosis (frog)

Fish short-term reproduction

Tier 2 tests are to include: amphibian 2-generation, avian 2-generation, fish lifecycle, invertebrate lifecycle, mammalian 2-generation, and *in utero* through lactation. All of these tests except the mammalian 2-generation are still in the developmental stage.

Phthalates currently selected for Tier 1 testing include the low molecular phthalates BBP, DBP, Di-sec-octyl phthalate (DEHP) and the very low molecular weight phthalates DEP and DMP.

2.5 The OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals

The OECD conceptual framework was developed to provide a framework for the testing and assessment of potential endocrine disruptors. It is intended to apply to both new and existing substances as well as different chemical sectors such as pharmaceuticals, industrial chemicals and pesticides. The one-page Framework is accompanied by a short set of notes, both of which are replicated in Figure 3 for convenience. Further details can be found on the OECD website (<http://www.oecd.org>).

Figure 3: OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals

Note. Document prepared by the Secretariat of the Test Guidelines Programme based on the agreement reached at the 6th Meeting of the EDTA Task Force

<p>Level 1 Sorting & prioritization based upon existing information</p>	<ul style="list-style-type: none"> Physical & chemical properties, e.g., MW reactivity, volatility, biodegradability Human & environmental exposure, E.g., production, volume, release, use patterns Hazard, e.g., available toxicological data 	
<p>Level 2 <i>In vitro</i> assays providing mechanistic data</p>	<ul style="list-style-type: none"> ER, AR, TR receptor binding affinity Transcriptional activation Aromatase and steroidogenesis <i>in vitro</i> Arylhydrocarbon receptor recognition / binding QSARs 	<ul style="list-style-type: none"> High Through Put Prescreens Thyroid function Fish hepatocyte VTG assay Others (as appropriate)
<p>Level 3 <i>In vivo</i> assays providing data about single endocrine Mechanisms and effects</p>	<ul style="list-style-type: none"> Uterotrophic assay (estrogenic related) Hershberger assay (androgenic related) Non-receptor mediated hormone function Others (e.g. thyroid) 	<ul style="list-style-type: none"> Fish VTG (vitellogenin) assay (estrogenic related)
<p>Level 4 <i>In vivo</i> assays providing data about multiple endocrine Mechanisms and effects</p>	<ul style="list-style-type: none"> Enhanced OECD 407 (endpoints based on endocrine mechanisms) Male and female pubertal assays Adult intact male assay 	<ul style="list-style-type: none"> Fish gonadalhistopathology assay Frog metamorphosis assay
<p>Level 5 <i>In vivo</i> assays providing data on effects from endocrine & other mechanisms</p>	<ul style="list-style-type: none"> 1-generation assay (TG415 enhanced)¹ 2-generation assay (TG416 enhanced)¹ Reproductive screening test (TG421 enhanced)¹ Combined 28 day / reproduction screening test (TG422 enhanced)¹ <p>¹ Potential enhancements will be considered by VMG mamm.</p>	<ul style="list-style-type: none"> Partial and full life cycle assays in fish, birds, amphibia & in vertebrates (developmental and reproduction)

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Notes to the OECD Framework

Note 1: Entering at all levels and exiting at all levels is possible and depends upon the nature of existing information needs for hazard and risk assessment purposes

Note 2: In level 5, ecotoxicology should include endpoints that indicate mechanisms of adverse effects, and potential population damage

Note 3: When a multimodal model covers several of the single endpoint assays, that model would replace the use of those single endpoint assays

Note 4: The assessment of each chemical should be based on a case by case basis, taking into account all available information, bearing in mind the function of the framework levels

Note 5: The framework should not be considered as all inclusive at the present time. At levels 3, 4 and 5 it includes assays that are either available or for which validation is under way. With respect to the latter, these are provisionally included. Once developed and validated, they will be formally added to the framework.

Note 6: Level 5 should not be considered as including definitive tests only. Tests included at that level are considered to contribute to general hazard and risk assessment

The Task Force on Endocrine Disrupter Testing and Assessment (EDTA) was established by OECD in 1996. This activity was launched at the request of the Member countries and the Business and Industry Advisory Committee to the OECD (BIAC) to ensure that testing and assessment approaches for endocrine disrupters would not substantially differ among countries. This conceptual framework was initially developed to guide the Task Force's deliberations in deciding which tests were suitable for OECD test development and validation work. It was developed taking into account:

- The views of Member countries as expressed through answers to a Questionnaire and the OECD's Appraisal of Test Methods for Sex Hormone & Disrupting Chemicals (OECD Monograph No. 21).
- Proposed testing schemes such as those developed at relevant workshops notably the European Workshop on the Impact of Endocrine Disruptors on Human Health and Wildlife (the Weybridge Workshop) and the Joint SETAC & Europe/OECD/EC Expert Workshop on Endocrine Modulators and Wildlife: Assessment and Testing (the EMWAT Workshop);
- The work of national activities such as the US EPA's Endocrine Disruptor's Screening and Testing Advisory Committee (EDSTAC) and research activities in Japan;
- Industry initiatives such as being undertaken by the European Chemical Industry (CEFIC).

The initial framework has been revised by the EDTA Task Force at its meetings to reflect OECD member countries' views. The conceptual framework agreed by the EDTA6 in 2002 is not a testing scheme but rather a tool box in which the various tests that can contribute information for the detection of the hazards of endocrine disruption are placed so that a weight of evidence assessment can be made. The tool box is organised into five compartments or levels each corresponding to a different level of biological complexity (for both toxicological and ecotoxicological areas). A series of important notes are attached to the framework. Even though the conceptual framework may be full of testing tools this does not imply that they all will be needed for assessment purposes. Tools (assays and tests) will be added as they are validated in future. The conceptual framework is subject to further elaboration and discussion as the work on endocrine disruptors proceeds.

It is known that the OECD EDTA is currently developing a guidance document (GD) to support the evaluation of existing data using the Framework. This GD was not available at the time this document was prepared, and consideration will be given to updating this ECPI report if required once the GD is available.

3 Conclusions

The tables below show the key conclusions taken from the detailed appendices for each of the three substances for which endocrine data has been evaluated using the OECD Conceptual Framework. The detailed reviews supporting these conclusions can be found in Appendix A – DINP, Appendix B – DIDP, and Appendix C – DBP.

3.1 DINP (see Appendix A for detailed review)

The key conclusions from each level of the OECD Conceptual Framework for DINP are:

OECD CF Level	Conclusions DINP
1	DINP has a rich safety dataset available. These data have previously been collated and evaluated by international regulatory authorities and assessed for their potential risk to human health and the environment. The EU Risk Assessment concluded that DINP should not be classified as hazardous under EU regulations, and that risk reduction is not required for any current use. CSTEE concluded that the available data indicates that the potency of DINP for anti-androgenic effects is very low.
2	No significant responses were observed with DINP in any of the <i>in vitro</i> assays. Taken as a whole, the available data indicate that DINP does not have significant interactions with the estrogenic or androgenic receptors. It is noteworthy that <i>in vitro</i> data need to be evaluated very carefully as the tests may have involved either substances which for all practical purposes do not exist under <i>in vivo</i> conditions or may have employed non-physiological conditions.
3	Taken as a whole, these data support the conclusion that DINP does not cause adverse endocrine effects in <i>in vivo</i> screening studies. DINP shows no significant adverse effects in the Uterotrophic Assay (for oestrogenic effects), and no consistent significant adverse effects in the Hershberger Assay (for anti-androgenic effects). In non-validated research studies for anti-androgenic effects DINP showed no, minor or inconsistent effects at high doses, and with no or limited evidence of a dose response. While one animal study shows no effects on fetal testicular testosterone, one study shows variable effects with no dose response, and one study shows reduced fetal testicular testosterone at a single high dose, this was not associated with any adverse health effects in the animals. If there is an effect on testosterone this would appear to be occurring at high doses only and without adverse health effects being seen in animals. Given this potential effect it is appropriate to assess Level 4 and Level 5 studies and confirm whether or not adverse health effects are being seen.
4	Taken as a whole these data support the conclusion that DINP does not induce endocrine mediated chronic toxicity in rodents or non-human primates.
5	Based on the comprehensive 1-generation, 2-generation reproductive studies and the developmental studies it can be concluded that DINP is not an endocrine disruptor in OECD guideline <i>in vivo</i> studies. The adverse health effects mediated via an endocrine mechanism of cryptorchidism, hypospadias, and significant testicular pathology which are seen with DBP in laboratory animals are not seen with DINP.

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Overall conclusion

There are sufficient data to conclude that DINP is not an endocrine disrupting substance for mammals when evaluated according to the OECD Conceptual Framework and using the commonly recognized definitions of an endocrine disruptor. Available human and mixtures data do not lead to any change in this conclusion.

3.2 DIDP (see Appendix B for detailed review)

The key conclusions from each level of the OECD Conceptual Framework for DIDP are:

OECD CF Level	Conclusions DIDP
1	DIDP has a rich safety dataset available. These data have previously been collated and evaluated by international regulatory authorities and assessed for their potential risk to human and environmental health. These reviews have concluded that DIDP is not dangerous so should not be classified as hazardous under current EU regulations
2	No significant responses were observed with DIDP in any of the <i>in vitro</i> assays. Taken as a whole, the available data indicate that DIDP does not significant interactions with the estrogenic or androgenic receptors. It is noteworthy that <i>in vitro</i> data need to be evaluated very carefully as the tests may have involved either substances which for all practical purposes do not exist under <i>in vivo</i> conditions or may have employed non-physiological conditions.
3	These data support the conclusion that DIDP does not cause adverse endocrine effects in <i>in vivo</i> screening studies. DIDP shows no significant adverse effects in the Uterotrophic Assay (for oestrogenic effects), and no consistent significant adverse effects in the Hershberger Assay (for anti-androgenic effects).
4	Sufficient <i>in vivo</i> data exist for DIDP to demonstrate that DIDP does not induce endocrine mediated chronic toxicity to non-reproductive tissues in rodents or non-human primates.
5	Based on the comprehensive 2-generation reproductive studies and the developmental study it can be concluded that DIDP is not an endocrine disruptor as defined by the Weybridge, IPCS and REACH guidance definitions. The adverse health effects mediated via an endocrine mechanism of cryptorchidism, hypospadias, and significant testicular pathology which are seen with DBP in laboratory animals are not seen with DIDP.

Overall Conclusion

There are sufficient data to conclude that DIDP is not an endocrine disrupting substance for mammals when evaluated according to OECD Conceptual Framework and using the commonly recognized definitions of an endocrine disruptor. Available human and mixtures data do not lead to any change in this conclusion.

3.3 DBP (see Appendix C for detailed review)

OECD CF Level	Conclusions DBP
1	DBP has an extensive toxicology dataset available. These data have previously been collated and evaluated by international regulatory authorities and assessed for their potential risk to human and environmental health. Based on these data, classification for developmental or reproductive toxicity was concluded by the EU Risk Assessment and Regulatory Committees according to the general classification and labelling requirements for dangerous substances and preparations (Directive 67-548-EEC) (R61 Category 2 and R62 Category 3) and the classification, labelling, and packaging (CLP) regulation (EC) No 1272/2008 Annex VI (Reproductive Toxin Category 1B)
2	No significant responses were observed with DBP in any of the <i>in vitro</i> assays. Taken as a whole, the available data indicate that DBP does not have significant interactions with estrogenic or androgenic receptors. It is noteworthy that <i>in vitro</i> data need to be evaluated very carefully as the tests may have involved either substances which for all practical purposes do not exist under <i>in vivo</i> conditions or may have employed non-physiological conditions.
3	Taken as a whole, DBP does not modulate estrogenic but Lee et al (2007) conclude that DBP does modulate androgenic endocrine systems. DBP and its major metabolite MBP are devoid of estrogenic activity <i>in vitro</i> ; they show no ability to bind to rodent or human estrogen receptors or to induce estrogen receptors-mediated gene expression. <i>In vivo</i> assays demonstrated that DBP does not increase uterine wet weight and does not give rise to vaginal epithelial cell cornification.
4	Sufficient <i>in vivo</i> data exist for DBP to demonstrate that DBP does induce endocrine mediated chronic toxicity to non-reproductive tissues in rodents or non-human primates.
5	Based on a range of developmental and one and two generation studies in laboratory animals the EU Risk Assessment Report for DBP concludes that DBP causes teratogenicity and effects on fertility such that DBP is classified as a Category 2 Developmental Agent and a Category 3 Fertility Agent under the EU Dangerous Substances Directive (67/548/EC). This conclusion was confirmed by the relevant EU Technical Progress Committee at the time. The EU Risk Assessment also concluded that certain effects were indicative of an anti-androgenic mechanism of action. The adverse health effects mediated via an endocrine mechanism of cryptorchidism, hypospadias, and significant testicular pathology are seen in laboratory animals. Under the Classification, Labelling and Packaging Regulation the equivalent classification is Category 1B

Overall Conclusion

Based on a range of developmental and one and two generation studies in laboratory animals the EU Risk Assessment Report for DBP concludes that DBP causes teratogenicity and effects on fertility such that DBP is classified as a Category 2 Developmental Agent and a Category 3 Fertility Agent under the EU Dangerous Substances Directive (67/548/EC). This conclusion was confirmed by the relevant EU Technical Progress Committee at the time. The EU Risk Assessment also concluded that certain effects were indicative of an anti-androgenic mechanism of action. The adverse health effects mediated via an endocrine mechanism of

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cryptorchidism, hypospadias, and significant testicular pathology are seen in laboratory animals. Under the Classification, Labelling and Packaging Regulation the equivalent classification is Category 1B.

Appendices

The available information on the endocrine activity of the investigated phthalate plasticisers is collated below. Data per chemical is organised into tables of references, as per the levels of the OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals.

Data gathering process

The data gathering process started with a search using the on-line literature searching tool "PubMed"¹. The output of these searches is available in the Appendices. For each substance, each reference was reviewed and allocated to an appropriate level in the framework. Occasionally the information in a reference could not be included in the framework. In this case, the reference is included in the Appendix but not brought forward into the table.

Where the authors were aware of additional relevant information, these references are also included in the data summary tables e.g. EU Risk Assessment Report.

Data evaluation process

A detailed technical comment is provided for each reference for DINP and DIDP and for key references for DBP. These are followed by a concluding statement on the overall contribution of that data to the assessment of the potential endocrine activity of that chemical. The final outcome of this robust weight of evidence approach for all the chemicals evaluated is presented at the end. The outcome of this review may be any one of the following options:

- Sufficient data exist to conclude that the chemical is not an endocrine disruptor (indicate tested biological mechanism(s))
- Sufficient data exist to conclude that the chemical is an endocrine disruptor (indicate tested biological mechanism(s))
- Insufficient data exist to conclude that the chemical is or is not an endocrine disruptor

Appendix A - Evaluation of endocrine data on DINP

This Appendix will collate and review the available scientific data relating to the potential endocrine activity of DINP. The large majority of the data included for DINP have previously been submitted to regulatory authorities including for example in the REACH registration dossier and in submissions to the US Consumer Product Safety Commission. There are a small number of research papers included in this report which have been recently published and which have not been included in prior regulatory submissions. In addition to these data already known to the authors a literature search of published literature was carried out which identified 18 publications. These have been tabulated according to the appropriate framework level and a review of each reference documented.

A.1 OECD Conceptual Framework: Level 1 – DINP

The first step to evaluate the potential hazard of an existing chemical is to collate the already existing information about its chemistry, uses and any available toxicological data. These data are then sorted, prioritised and evaluated to screen whether the chemical is a high priority for further investigation.

¹ <http://www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed>

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A summary of the Level 1 endocrine data available for DINP is tabulated below. Detailed comments on these datasets are available in the pages following the table.

Level 1 Endpoints	DINP: CAS RN 68515-48-0/ 28553-12-0
physical & chemical properties, e.g., MW, reactivity, volatility, biodegradability	<ul style="list-style-type: none"> • ECB (2003). 1,2-Benzenedicarboxylic acid, di-C8-10branched alkyl esters, C9 rich and di-isononyl phthalate (DINP). Risk Assessment. European Chemicals Bureau, Institute for Health and Consumer Protection, Joint Research Center of the European Commission. http://ecb.jrc.ec.europa.eu/documents/Existing-Chemicals/RISK_ASSESSMENT/REPORT/dinpreport046.pdf
human & environmental exposure, e.g., production volume, release, use patterns	<ul style="list-style-type: none"> • ECB (2003). 1,2-Benzenedicarboxylic acid, di-C8-10branched alkyl esters, C9 rich and di-isononyl phthalate (DINP). Risk Assessment. European Chemicals Bureau, Institute for Health and Consumer Protection, Joint Research Center of the European Commission. http://ecb.jrc.ec.europa.eu/documents/Existing-Chemicals/RISK_ASSESSMENT/REPORT/dinpreport046.pdf
hazard, e.g., available toxicological data	<ul style="list-style-type: none"> • ECB (2003). 1,2-Benzenedicarboxylic acid, di-C8-10branched alkyl esters, C9 rich and di-isononyl phthalate (DINP). Risk Assessment. European Chemicals Bureau, Institute for Health and Consumer Protection, Joint Research Center of the European Commission. http://ecb.jrc.ec.europa.eu/documents/Existing-Chemicals/RISK_ASSESSMENT/REPORT/dinpreport046.pdf • CSTE (2001). Opinion on the results of the Risk Assessment of: 1,2-Benzenedicarboxylic acid, di-C8-10-branched alkyl esters, C9-rich and di-"isononyl" phthalate CAS No.: 68515-48-0 and CAS No.: 28553-12-0 EINECS No.: 271-090-9 and EINECS No.: 249-079-5 REPORT VERSION (HUMAN HEALTH EFFECTS):Final report, May 2001 • National Toxicology Program. NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Di-isononyl Phthalate (DINP). NTP CERHR MON. 2003 Mar;(2):i-III90. • Kamrin MA. Phthalate risks, phthalate regulation, and public health: a review. J Toxicol Environ Health B Crit Rev. 2009 Feb; 12(2):157-74. Review. • McKee RH, El-Hawari M, Stoltz M, Pallas F, Lington AW. Absorption, disposition and metabolism of di-isononyl phthalate (DINP) in F-344 rats. J Appl Toxicol. 2002 Sep-Oct; 22(5):293-302. • McKee RH, Butala JH, David RM, Gans G. NTP center for the evaluation of risks to human reproduction reports on phthalates: addressing the data gaps. Reprod Toxicol. 2004 Jan-Feb; 18(1):1-22. Review. • European Chemicals Agency (ECHA), July 2010. Evaluation of New Scientific Evidence Concerning the Restrictions Contained in Annex XVII to Regulation (EC) No. 1907/2006 (REACH). Review of new available information for di-"isononyl" phthalate (DINP). CAS No. 28553-12-0 AND 68515-48-0, EINECS No. 249-079-5 AND 271-090-9. Review Report. July 2010.

Table 2: OECD CF Level 1 endocrine data available for DINP

A.1.1. Detailed comments on key publications - DINP

- *European Chemicals Bureau (2003) EU Risk Assessment on DINP*: concluded that DINP does not require classification as dangerous. None of the endpoints identified for risk characterization purposes was related to endocrine effects. The conclusion for workers, consumers and the environment was Conclusion II: There is at present no need for further information and/or testing and for risk reduction measures beyond those which are being applied already.
- *Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) Opinion on the Draft EU Risk Assessment (2001)*. CSTEE in reviewing the EU risk assessment disagreed with the choice of no observed adverse effect level based on liver effects in rodents. While the ECB and Member States did not agree with CSTEE, due to this difference of opinion and uncertainties on exposure data the European Commission invoked the precautionary principle for DINP. This led to restrictions on its use in toys and childcare articles which can

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be placed in the mouth. The use of the precautionary principle was not based on endocrine related data. In the opinion CSTEE stated that the available data indicates that the potency of DINP for anti-androgenic effects is very low.

- *McKee et al (2002)*: Demonstrates that following exposure via the oral route, DINP is rapidly metabolized to the monoester which are eliminated from the body within 24 – 48 hours.
- *NTP-CERHR Monograph (2003)*: reviewed toxicology data available for DINP relating to the potential reproductive and developmental toxicity of DINP. The expert panel identified two areas of research that required further data: 1) a perinatal developmental study including examination of sexual maturation in a rodent and maybe a non-rodent species and 2) increased information on human exposure levels, through biomonitoring, especially determining DINP exposures to young children, ideally developing data to include 3-12 month old children. In conclusion, the expert panel had minimal concern for unborn children due to ambient maternal exposure to DINP and minimal concern about DINP resulting in reproductive toxicity in humans.
- *McKee et al (2004)*: Addresses data gaps identified in the NTP CERHR report which for DINP was a better understanding of the exposure of young children to DINP through the use of toys. This has been assessed by the responsible regulatory authorities e.g. the US Consumer Product Safety Commission which concluded that exposure to DINP from toys was well below effect levels in animals, and, therefore, there was no risk.
- *Kamrin et al (2009)*: Summarizes recent evaluations of the risks of various phthalates. The analysis considers biomonitoring studies and epidemiological research in addition to laboratory animal evidence. Analysis of all of the available data leads to the conclusion that the risks are low, even lower than originally thought, and that there is no convincing evidence of adverse effects on humans.
- *European Chemicals Agency (ECHA), July 2010*. ECHA concluded that new endocrine information is available and that the new information would need a more in-depth assessment to evaluate the reliability and relevance of the studies. ECHA state that all the studies evaluating the potential endocrine effects of DINP should be assessed together, including the studies in the EU Risk Assessment Report.

A.1.2. Conclusions: Level 1 - DINP

DINP has a rich safety dataset available. These data have previously been collated and evaluated by international regulatory authorities and assessed for their potential risk to human health and the environment. The EU Risk Assessment concluded that DINP should not be classified as hazardous under EU regulations, and that risk reduction is not required for any current use. CSTEE concluded that the available data indicates that the potency of DINP for anti-androgenic effects is very low.

A.2. OECD Conceptual Framework: Level 2 - DINP

The second step to evaluate the potential hazard of an existing chemical is to summarise available and suitable toxicological data generated in non-animal test systems e.g. using *in vitro* and / or *in silico* models. The purpose of this summary is to enable an expert to examine the existing *in vitro* assays to investigate detailed mechanistic data.

A summary of the Level 2 endocrine data available for DINP is tabulated below. It should be noted that it is not a requirement of the OECD Conceptual Framework that data is available for all sections within a Level. Detailed comments on these datasets are available below.

Level 2 Endpoints	DINP: CAS RN 68515-48-0/ 28553-12-0
ER, AR, TR receptor binding affinity	<ul style="list-style-type: none"> • Akahori, Y; Nakai, M; Yakabe, Y; et al. (2005) Two-step models to predict binding affinity of chemicals to the human estrogen receptor α by three-dimensional quantitative structure-activity relationships (3D-QSARs) using receptor-ligand docking simulation. SAR & QSAR in Environmental Research 16(4):323–337. • Ghisari, M; Bonefeld-Jorgensen, EC. (2009) Effects of plasticizers and their mixtures on estrogen receptor and thyroid hormone functions. Toxicol Lett 189(1):67–77. • Harris CA, Henttu P, Parker MG, Sumpter JP. The estrogenic activity of phthalate esters <i>in vitro</i>. EHP. 1997 Aug;105(8):802-11. • Mlynarcikova, A; Fickova, M; Scsukova, S. (2007) The effects of selected phenol and phthalate derivatives on steroid hormone production by cultured porcine granulosa cells. Altern Lab Anim 35(1):71–77. • Takeuchi, S; Iida, M; Kobayashi, S; et al. (2005) Differential effects of phthalate esters on transcriptional activities via human estrogen receptors α and β, and androgen receptor. Toxicology 210(2–3):223–233. • Zacharewski TR, Meek MD, Clemons JH, Wu ZF, Fielden MR, Matthews JB. Examination of the <i>in vitro</i> and <i>in vivo</i> estrogenic activities of eight commercial phthalate esters. Toxicol Sci. 1998 Dec;46(2):282-93.
High Through Put Prescreens	<ul style="list-style-type: none"> • Not required to have data in all sections of a particular level
Transcriptional activation	<ul style="list-style-type: none"> • Ghisari M, Bonefeld-Jorgensen EC. Effects of plasticizers and their mixtures on estrogen receptor and thyroid hormone functions. Toxicol Lett. 2009 Aug 25;189(1):67-77.
Thyroid function	<ul style="list-style-type: none"> • Breous, E; Wenzel, A; Loos, U. (2005) The promoter of the human sodium/iodide symporter responds to certain phthalate plasticisers. Mol Cell Endocrinol 244(1–2):75–78. • Ghisari, M; Bonefeld-Jorgensen, EC. (2009) Effects of plasticizers and their mixtures on estrogen receptor and thyroid hormone functions. Toxicol Lett 189(1):67–77. • Wenzel, A; Franz, C; Breous, E; et al. (2005) Modulation of iodide uptake by dialkyl phthalate plasticisers in FRTL- 5 rat thyroid follicular cells. Mol Cell Endocrinol 244(1–2):63–71.
Aromatase and steroidogenesis <i>in vitro</i>	<ul style="list-style-type: none"> • Not required to have data in all sections of a particular level.
Fish hepatocyte VTG assay	<ul style="list-style-type: none"> • Not relevant to this mammalian assessment
Aryl hydrocarbon receptor recognition/binding	<ul style="list-style-type: none"> • Kruger, T; Long, M; Bonefeld-Jorgensen, EC. (2008) Plastic components affect the activation of the aryl hydrocarbon and the androgen receptor. Toxicology 246(2–3):112–123.
QSARs	<ul style="list-style-type: none"> • Akahori, Y; Nakai, M; Yakabe, Y; et al. (2005) Two-step models to predict binding affinity of chemicals to the human estrogen receptor α by three-dimensional quantitative structure-activity relationships (3D-QSARs) using receptor-ligand docking simulation. SAR & QSAR in Environmental Research 16(4):323–337. • Akahori Y, Nakai M, Yamasaki K, Takatsuki M, Shimohigashi Y, Ohtaki M. Relationship between the results of <i>in vitro</i> receptor binding assay to human estrogen receptor alpha and <i>in vivo</i> uterotrophic assay: comparative study with 65 selected chemicals. Toxicol <i>In vitro</i>. 2008 Feb;22(1):225-31.
Others (as appropriate)	<ul style="list-style-type: none"> • McKee RH, El-Hawari M, Stoltz M, Pallas F, Lington AW. Absorption, disposition and metabolism of di-isononyl phthalate (DINP) in F-344 rats. J Appl Toxicol. 2002 Sep-Oct;22(5):293-302.

Table 3: OECD CF Level 2 endocrine data available for DINP

A.2.1. Detailed comments on key publications – Level 2 - DINP

- *Akahori et al (2005): Abstract only.* Models indicated that phthalates show weak if any affinity for binding to the human ER α receptor. Unclear whether DINP was one of the phthalates tested.
- *Akahori et al (2008):* Investigated the relationship between the *in vitro* ER binding and *in vivo* uterotrophic assays. The authors compared the results from these assays for 65 chemicals spanning a variety of chemicals classes. Resulting DINP binding affinity (log RBA) value of -3.49 was the lowest reported and far below the cut-off level (-2.63) that could induce estrogenic/ anti-estrogenic activities in the uterotrophic assay, indicating that DINP does not have estrogenic/ anti-estrogenic properties.
- *Breous et al (2005):* Investigated possible effects DINP on the transcriptional activity of sodium/iodide symporter (NIS) which mediates the active transport of I- in the thyroid. No effect was observed with DINP on the transcriptional activity of NIS.
- *Ghisari et al (2009):* Investigated *in vitro* the potential for thyroid hormone-like and estrogenic activities of a range of widely used plasticizers, alone or in mixtures. Thyroid hormone disrupting potential was determined by the effect on the TH-dependent rat pituitary GH3 cell proliferation (T-screen). Estrogenicity potential was assessed using MVLN cells, stably transfected with an estrogen receptor (ER) luciferase reporter vector. Results were variable with DINP being reported as causing a slight inhibition of GH3 growth and DIDP causing a small increase in GH3 proliferation at one concentration only. Neither DINP or DIDP had any effect on ER transactivation. The abstract of the paper is confusing because it states that all the tested compounds (except 2-phenylphenol) significantly affected the GH3 cell proliferation. Based on the full results presented in the paper this is not the case for DINP and DIDP as noted above. Further DINP did not induce a reportable effect in the ER assay at doses up to 5x10⁻⁵M. The chemical mixture tested in this paper did not include DINP. It should be noted that these *in vitro* experiments used the phthalate diester (DINP) whereas under *in vivo* conditions the diester is metabolized to its monoester (MINP). These *in vitro* data should be evaluated carefully as the test compounds, for all practical purposes, do not exist under *in vivo* conditions.
- *Kruger et al (2008):* Investigated the potential of a range of widely used plasticizers (alone or in mixtures) to mediate AhR and AR function using CALUX (chemically activated luciferase gene expression) bioassays. DINP had no significant effect on either assay at doses up to 10⁻⁴M. It should be noted that these *in vitro* experiments used the phthalate diester (DINP) whereas under *in vivo* conditions the diester is metabolized to its monoester (MINP). These *in vitro* data should be evaluated carefully as the test compounds, for all practical purposes, do not exist under *in vivo* conditions or the test conditions may have employed non-physiological conditions.
- *Mlynarcikova et al (2007):* Investigated the effect on progesterone and oestradiol production by primary cultures of porcine ovarian granulosa cells. Cells were incubated in the presence or absence of 3 phenols, 3 phthalates (up to 10⁻⁴M) or human recombinant Follicle Stimulating Hormone (hFSH, 1g/ml). Steroid levels were measured by radioimmunoassay (RIA). DINP did not induce any significant effect on basal or hFSH stimulated progesterone production. Further, DINP did not induce any significant effect on basal oestradiol production. A decrease in hFSH stimulated oestradiol production was reported associated with DINP at all dose levels, but the authors were not able to provide a biologically plausible explanation for this observation. It should be noted that these *in vitro* experiments used the phthalate diester (DINP) whereas under *in vivo* conditions the diester is metabolized to its monoester (MINP). These *in vitro* data should be evaluated carefully as the test compounds, for all practical purposes, do not exist under *in vivo* conditions or the test conditions may have employed non-physiological conditions.
- *Takeuchi et al (2005):* Investigated the potential receptor activities of 22 phthalates, including 3 metabolites, by highly sensitive reporter gene assays using CHOK1 cells transfected with expression vectors for human Androgen receptor (hAR), estrogen receptor alpha (hER) and estrogen receptor beta (hER) which were incubated (24h) with up to 10⁻⁵M per test compound. Separately, 10⁻¹⁰M dihydrotestosterone (DHT), 10⁻¹¹M oestradiol (E2) or 10⁻¹⁰M E2 were added, respectively, to the transfected cells to assess receptor antagonism potential of the test compounds. Results indicated that DINP did not induce an agonistic or antagonistic effect on hER, hER or hAR activity. It should be noted that these *in vitro* experiments used the phthalate diester (DINP) whereas under *in vivo* conditions the diester is metabolized to its monoester (MINP). These *in vitro* data should be evaluated

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- carefully as the test compounds, for all practical purposes, do not exist under *in vivo* conditions or the test conditions may have employed non-physiological conditions..
- *Harris et al (1997)*: A series of phthalate esters, including DINP, were screened for estrogenic activity using a recombinant yeast screen. The recombinant yeast screen, a gene for a human estrogen receptor was integrated into the main yeast genome and was expressed in a form capable of binding to estrogen response elements, controlling the expression of the reporter gene lac-Z (when receptor is activated, the lac-Z is expressed). DINP was tested at concentrations ranging from 10^{-3} M to $5 \cdot 10^{-7}$ M. DINP produced inconsistent results in the yeast screen. DINP was also tested for the ability to stimulate proliferation of human breast cancer cells (MCF-7 and ZR-75 cells). DINP produced no effects in the MCF-7 assay. In the ZR-75 cells, DINP induced proliferation to a significantly greater extent than the control, which is in contrast to the findings for this chemical using the yeast screen. It should be noted that these *in vitro* assays have investigated one mechanism of action only, the ability of phthalates to act as estrogen agonists. More importantly, it should also be noted that these were tests of phthalate diesters. Under *in vivo* conditions the diesters are metabolized to monoesters which are not estrogen receptor agonists. The *in vitro* data need to be evaluated very carefully as the tests may have involved either substances which for all practical purposes do not exist under *in vivo* conditions or may have employed non-physiological conditions.
 - *Wenzel et al (2005)*: Investigated potential of six phthalates to modulate basal iodide uptake mediated by the sodium/iodide symporter (NIS) in a rat thyroid cell line, FRTL-5. Results indicated that DINP showed no cytotoxicity at levels $<10^{-2}$ M but high concentrations (10^{-4} M) enhanced basal iodide uptake by these cells. The authors were not able to provide a biologically plausible explanation for this observation. It should be noted that these *in vitro* experiments used the phthalate diester (DINP) whereas under *in vivo* conditions the diester is metabolized to its monoester (MINP). These *in vitro* data should be evaluated carefully as the test compounds, for all practical purposes, do not exist under *in vivo* conditions or the test conditions may have employed non-physiological conditions.
 - *Zacharewski et al (1998)*: The estrogenic activities of DINP were investigated *in vitro* using estrogen receptor (ER) competitive ligand-binding and mammalian- and yeast-based gene expression assays. No significant responses were observed with DINP in any of the *in vitro* assays.

A.2.2. Conclusions: Level 2 - DINP

No significant responses were observed with DINP in any of the *in vitro* assays. Taken together and consistent with a weight of evidence approach, the available data indicate that DINP did not have significant interactions with the estrogenic or androgenic receptors. It is noteworthy that *in vitro* data need to be evaluated very carefully as the tests may have involved either substances which for all practical purposes do not exist under *in vivo* conditions or may have employed non-physiological conditions.

Evaluation of Level 2 data are indicative of possible mechanisms of action and are not sufficient to provide conclusive evidence on the potential endocrine activity of a chemical *in vivo*. As specified by the OECD Conceptual Framework further data are needed to support conclusions on endocrine activity and these are considered in the next levels of the framework.

A.3. OECD Conceptual Framework: Level 3 - DINP

The third step to evaluate the potential hazard of an existing chemical is to summarise available and suitable toxicological data generated in animal test systems that are limited in that they are only able to investigate single endpoint mechanisms and effects e.g. Hershberger assay (androgenic related) or Uterotrophic assay (estrogenic related). The purpose of this summary is to enable an expert to examine existing *in vivo* assays to investigate detailed biological activity.

A summary of the Level 3 endocrine data available for DINP is tabulated below. It should be noted that it is not a requirement of the OECD Conceptual Framework that data is available for all sections within a Level. For the purposes of this review, only available data relevant to mammalian systems has been collated and tabulated. Comments on these datasets are available below.

Level 3	DINP: CAS RN 68515-48-0/ 28553-12-0
Uterotrophic assay (estrogenic related)	<ul style="list-style-type: none"> • Akahori Y, Nakai M, Yamasaki K, Takatsuki M, Shimohigashi Y, Ohtaki M. Relationship between the results of <i>in vitro</i> receptor binding assay to human estrogen receptor alpha and <i>in vivo</i> uterotrophic assay: comparative study with 65 selected chemicals. <i>Toxicol In vitro</i> . 2008 Feb;22(1):225-31. • Zacharewski TR, Meek MD, Clemons JH, Wu ZF, Fielden MR, Matthews JB. Examination of the <i>in vitro</i> and <i>in vivo</i> estrogenic activities of eight commercial phthalate esters. <i>Toxicol Sci</i>. 1998 Dec;46(2):282-93.
Hershberger assay (androgenic related)	<ul style="list-style-type: none"> • Lee BM, Koo HJ. Hershberger assay for antiandrogenic effects of phthalates. <i>J Toxicol Environ Health A</i>. 2007 Aug; 70(15-16):1365-70. 2009 Jul;28(1):66-74.
Non-receptor mediated hormone function	<ul style="list-style-type: none"> • Adamsson A, Salonen V, Paranko J, Toppari J. Effects of maternal exposure to diisononylphthalate (DINP) and 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,p'-DDE) on steroidogenesis in the fetal rat testis and adrenal gland. <i>Reprod Toxicol</i>. • Boberg J, Christiansena, S Axelstada M, Seidler Kledala T, Vinggaarda AM, Daigaarda M, Nellemanna C and Hass U (2011). Reproductive and behavioral effects of diisononyl phthalate (DINP) in perinatally exposed rats. <i>Reproductive Toxicology</i> (Article in Press). • Borch J, Ladefoged O, Hass U, Vinggaard AM. Steroidogenesis in fetal male rats is reduced by DEHP and DINP, but endocrine effects of DEHP are not modulated by DEHA in fetal, prepubertal and adult male rats. <i>Reprod Toxicol</i>. 2004 Jan-Feb;18(1):53-61. • Gray LE Jr, Ostby J, Furr J, Price M, Veeramachaneni DN, Parks L. Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. <i>Toxicol Sci</i>. 2000 Dec; 58(2):350-65. • Hass U, Filibska M, and Kledal TSA. (2003). Antiandrogenic effects of diisononyl phthalate in rats. <i>Reprod Toxicol</i>. 17, 475. • Masutomi N, Shibutani M, Takagi H, Uneyama C, Takahashi N, Hirose M. Impact of dietary exposure to methoxychlor, genistein, or diisononyl phthalate during the perinatal period on the development of the rat endocrine/reproductive systems in later life. <i>Toxicology</i>. 2003 Nov 5;192(2-3):149-70. • Masutomi et al (2004) Alteration of pituitary hormone immunoreactive cell populations in rat offspring after maternal dietary exposure to endocrine-active chemicals, <i>Arch Toxicol</i>; 2004; 78: 232 - 240 • Ostby JS, Hotchkiss AK, Furr R, and Gray LE Jr. (2001) Investigation of the ability of diisononyl phthalate (DINP) to alter androgen-dependent tissue development in Sprague-Dawley rats. <i>The Toxicologist</i> 60, 225.
Others (e.g. thyroid)	<ul style="list-style-type: none"> • Lee HC, Yamanouchi K and Nishihara M (2006) Effects of perinatal exposure to phthalate/adipate esters on hypothalamic gene expression and sexual behaviour in rats. <i>J. Reprod. Dev</i>. 52: 343-352.

Table 4: OECD CF Level 3 endocrine data available for DINP

A.3.1. Detailed comments on key publications – Level 3 - DINP

- *Adamsson et al (2009)*: Maternal exposure to DINP at 250 or 750 mg/kg on embryonic days (EDs) 13.5–17.5 did not down-regulate the activity of steroidogenesis in ED 19.5 male rat fetus. Protein expression levels of testicular and adrenal StAR, P450scc, 3β-HSD and androgen receptor (AR) did not show any changes. Further no morphological change in the testis was noted. Therefore, no effect on testosterone synthesis, or expression of the genes and proteins associated with testosterone synthesis were observed in this study.

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- *Akahori et al (2008)*: investigates the relationship between the *in vitro* ER binding and *in vivo* uterotrophic assays by determining the meaningful binding potency from the ER binding assay by comparing the results from these assays for 65 chemicals spanning a variety of chemicals classes. Resulting DINP binding affinity (log RBA) value of -3.49 was the lowest reported and far below the cut-off level (-2.63) that could induce estrogenic/ anti-estrogenic activities in the uterotrophic assay, indicating that DINP does not have estrogenic/ anti-estrogenic properties.
- *Boberg et al (2011)*: Maternal rat exposure to DINP at 0, 300, 750 and 900 mg/kg/day from gestation day 7 to post-natal day 17, via oral gavage in corn oil. Authors reported effects on testes histopathology, testosterone levels, nipple retention, anogenital distance, sperm motility and sperm count. However, data presented do not show any consistent dose response with statistical significance. Increases in testosterone and sperm counts were also reported. The data presented do not therefore support the authors' conclusions that DINP causes anti-androgenic effects on reproductive development in rats. There were no effects on the weights of androgen sensitive tissues such as testes, prostate, epididymis, seminal vesicle, LABC, and bulbourethral gland. In addition behavioural tests were run using a Morris Water Maze Test. The data showed improved swimming times for high dose females on memory day 1 compared to female controls, but this was not confirmed on memory day 2 or when the platform was moved. In view of these inconsistent findings, the lack of any dose response and the lack of effects on male rats, the authors' conclusions that these results show masculinisation of females are not supported by the data presented. In addition the authors' hypothesis that a substance is causing both anti-androgenic and androgenic effects is not scientifically plausible.
- *Borch et al (2004)*: Maternal exposure to DINP at 750 mg/kg on gestation days (EDs) 7-21 induced reduced ex vivo testicular testosterone production and *in vivo* testosterone levels in testes and plasma of male fetuses at ED 21. However, the utility of this study for hazard identification and risk assessment is limited by several factors. First, the study utilized only one very high dose of DINP. Second, there were no adverse phenotypic effects reported in the study, therefore it is unclear if the observed decrease in testosterone content is in-fact a toxicologically significant response. Finally, the authors measured the testosterone levels on gestation day 21, a time point after the developmental surge of testosterone that occurs during gestation day 16-18 in the rat. After gestation day 18, plasma testosterone levels are naturally declining in the fetal rat, thus, conclusions regarding reductions in testosterone synthesis are unreliable when assayed at this timepoint.
- *Gray et al (2000)*: Conducted a study during the late gestational period: time-mated rats were gavaged daily with DINP at single dose of 750 mg/kg/d in corn oil as vehicle from gestational day 14 through postnatal day 3. DINP was reported as producing slight equivocal changes in phenotypic expression of anti-androgenic effects. No effect was observed on anogenital distance or testis weight following DINP treatment. The authors reported a small statistically significant increase in malformations of the genital tract in male rat exposed in utero to DINP (7.7%). This statistical result is questionable because statistical significance was achieved only by pooling several different effects and treating them as a single effect. Further, the statistical unit was the pup as opposed to the litter, the commonly accepted statistical unit for developmental toxicity studies. An increase in percentage of males with retained areolas was observed in the DINP dose group at day 13 of age (22% vs. 0% in controls). However, subsequent publications from this lab indicated that retained areolas in control animals ranged as high as 14% (Ostby et al., 2001). The usefulness of these data for hazard and risk assessment is limited as a single high dose was utilized, effects were pooled to achieve statistical significance, and the only clearly statistically significant finding is suspect based on high incidence of this finding in other control groups. The authors, too, questioned the significance of the statistical power of their analysis.
- *Hass et al (2003 Abstract only)*: Anti-androgenic parameters were also evaluated in this study, reported in 2003 as an abstract. Groups of 12 mated female Wistar rats were gavaged from gestation day 7 to PND 17 with 0, 300, 600, 750, or 900 mg/kg/day DINP. The study has now apparently been published again as an abstract in 2010 (Boberg et al) and as a full paper (Boberg et al, 2011 – see discussion above under Boberg et al).
- *Ostby et al (2001)*: Conducted a study during the late gestational period: time-mated rats were gavaged daily with DINP at single dose of 1000 or 1500 mg/kg/d in corn oil as vehicle

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from gestational day 14 through postnatal day 3. DINP produced slight equivocal changes in phenotypic expression of anti-androgenic effects.

- *Lee et al (2006)*: Investigated perinatal phthalate exposure effects on hypothalamic gene (granulin, grn and p130) expression in rats. Pregnant rats were fed diet containing 0, 40, 400, 4000 or 20000 ppm DINP from gestational day 15 daily until weaning (post natal day 21). Post natal examinations including ano-genital parameter assessments were inconclusive, as males and females exhibited opposite biological effects. DINP did not induce a consistent dose-response effect on grn or p130 mRNA expression. These results indicate that perinatal exposure to high doses of DINP does not overtly affect hypothalamic gene expression.
- *Lee and Koo (2007)*: Reported a study designed similar to the Hershberger bioassay screen to test the anti-androgenic properties of DINP. DINP did not induce consistent changes in the androgen sensitive tissues therefore these data indicate that DINP does not meet the OECD criteria for androgen antagonists as the weights of the sex accessory tissues from the administered groups showed no consistent statistically significant differences from the testosterone-only animals.
- *Masutomi et al., (2003)*: DINP was administered to Sprague-Dawley rats at concentrations of 400, 4000, and 20,000 ppm from gestational day 15 to post natal day 10. Maternal intake as estimated for both the gestational and lactational phases, up to 2656.7 mg/kg/day lactation, 20000 ppm. DINP, at 20,000 ppm (~1165 - 2657 mg/kg/day) did not cause any developmental alterations, despite maternal toxicity. DINP did not alter any parameters in the females except for slight ovarian changes in the adult stage (i.e. marginal decrease in the number of corpora lutea). In males, slight degeneration of Sertoli cells and meiotic spermatocytes was noted at 11 weeks of age. In summary, no anti-androgenic effects were observed on the developing male reproductive tract in this study.
- *Masutomi et al (2004)*: In another study with a similar exposure, no effects of DINP on luteinising hormone (LH), follicle stimulating hormone (FSH) or prolactin levels were found.
- *Zacharewski et al (1998)*: The estrogenic activities of DINP were investigated In an *in vivo* study, 20, 200, 2,000 mg/kg/d of DINP was administered by oral gavage once daily for a period of 4 days to ovariectomised rats. DINP did not produce any reproducible, dose-dependant effect on uterine wet weight relative to vehicle control at any of the dose tested. DINP did not induce a vaginal cornification response at any of the doses tested. Accordingly, it can be concluded that DINP is not estrogenic under *in vivo* conditions.

A.3.2. Conclusions: Level 3 - DINP

Taken as a whole, these data support the conclusion that DINP does not cause adverse endocrine effects in *in vivo* screening studies. DINP shows no significant adverse effects in the Uterotrophic Assay (for oestrogenic effects), and no consistent significant adverse effects in the Hershberger Assay (for anti-androgenic effects). In non-validated research studies for anti-androgenic effects, DINP showed no, minor or inconsistent effects at high doses and with no or limited evidence of a dose response. While one animal study shows no effects on fetal testicular testosterone, one study shows variable effects with no dose response, and one study shows reduced fetal testicular testosterone at a single high dose, this was not associated with any adverse health effects in the animals. If there is an effect on testosterone this would appear to be occurring at high doses only and without adverse health effects being observed. Given this potential effect, it is appropriate to assess Level 4 and Level 5 studies and confirm whether or not adverse health effects are being induced.

A.4. OECD Conceptual Framework: Level 4 - DINP

The forth step to evaluate the potential hazard of an existing chemical is to summarise available and suitable toxicological data generated in animal test systems that can inform the reviewer on multiple endpoint mechanisms and effects e.g. Male and female pubertal assays. The purpose of this summary is to enable an expert to examine existing *in vivo* assays to investigate multiple endocrine mechanisms and effects.

A summary of the Level 4 endocrine data available for DINP is tabulated below. It should be noted that it is not a requirement of the OECD Conceptual Framework that data is available for all sections within a Level. For the purposes of this review, only available data relevant to

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mammalian systems has been collated and tabulated. Comments on these datasets are available below.

Level 4	DINP CAS RN 68515-48-0/ 28553-12-0
enhanced 407 (endpoints based on endocrine mechanisms)	<ul style="list-style-type: none"> Kwack SJ, Kim KB, Kim HS, Lee BM. (2009) Comparative Toxicological Evaluation of Phthalate Diesters and Metabolites in Sprague-Dawley Male Rats for Risk Assessment Toxicity of Phthalate Diesters and Metabolites. <i>Journal of Toxicology and Environmental Health, Part A</i>, 72: 1446–1454.
Male and female pubertal assays	<ul style="list-style-type: none"> Not required to have data in all sections of a particular level. Endpoints addressed by 2-generation studies – see Level 5
Adult intact assay	<ul style="list-style-type: none"> Not required to have data in all sections of a particular level. Endpoints addressed by 2-generation studies – see Level 5
Other	<ul style="list-style-type: none"> Moore, M (1998a). Oncogenicity Study in Rats with Di(isononyl) phthalate including ancillary hepatocellular proliferation and biochemical analyses. Testing laboratory: Covance Laboratories Incorporated, Vienna, Virginia. Report no.: 2598-104. Owner company: Aristech Chemical Corporation, study now owned by BASF Corp. Report date: 1998-05-12. Moore, M (1998b). Oncogenicity Study in Mice with Di(isononyl) phthalate including ancillary hepatocellular proliferation and biochemical analyses. Testing laboratory: Covance Laboratories Incorporated, Vienna, Virginia. Report no.: 2598-105. Owner company: Aristech Chemical Corporation, study now owned by BASF Corp. Report date: 1998-01-28. Lington AW, Bird MG, Plutnick RT, Stubblefield WA, Scala RA. Chronic toxicity and carcinogenic evaluation of diisononyl phthalate in rats. <i>Fundam Appl Toxicol</i>. 1997 Mar;36(1):79-89. Pugh, G, Jr; Isenberg, JS; Kamendulis, LM; et al. (2000) Effects of di-isononyl phthalate, di-2-ethylhexyl phthalate, and clofibrate in cynomolgus monkeys. <i>Toxicol Sci</i> 56(1):181–188.

Table 5: OECD CF Level 4 endocrine data available for DINP

A.4.1. Detailed comments on key publications - DINP

- Kwack et al (2009)*: Comparative assessment of the systemic toxicity and sperm parameters for DINP - administered orally to Sprague Dawley male rats at 500 mg/kg body weight (bw)/d for 4 wk by oral gavage. Liver weights were significantly increased compared to the control. Testes weights were not significantly reduced compared to the control but Kwack et al did report that DINP lowered sperm counts by approximately 25% and lowered sperm motility of epididymal sperm, detected by a change in the sperm motion parameters. Given the observed liver toxicity, body weight loss, and lack of a dose response (only a single high dose used) it is unclear if these reported effects are the result of direct effects of DINP. In addition no effects on sperm counts were observed for DIDP. For LMW phthalates (DEHP, DBP) effects were reported on testes weight and on sperm counts and sperm motility (without any dose response since only a single high dose was used).
- Lington et al (1997)*: DINP was administered to Fischer 344 rats (110/sex) at dietary concentrations of 0, 0.03, 0.3, and 0.6% (w/w) for 2 years. The mean daily intakes over the 2 years were 15, 152, and 307 mg/kg/day for male rats and 18, 184 and 375 mg/kg/day for female rats, corresponding to the 0.03, 0.3 and 0.6% dose levels, respectively. High dose males exhibited a statistically significant, dose-related decrease in body weight beginning at 12 months of treatment and persisting until termination. This was not noted for the females. Males and females from the mid and high-dose groups exhibited a statistically

significant, dose related increase in relative kidney and liver weights throughout most of the treatment period; the absolute liver and kidney weights demonstrated a similar trend. Statistically significant changes in organ weights consisted of dose-related increased absolute and relative spleen weights of the high-dose males, increased relative spleen weights of the high-dose females and a relative increased adrenal weight in both sexes as well as relative increased testes weights in high-dose males. At 18 and 24 months, non-neoplastic lesions were observed in the liver and kidney of high-dose rats. Ultrastructural examination of liver specimens from representative rats of each sex from the four groups did not reveal any treatment-related peroxisome proliferation. An increased incidence of spongiosis hepatis, a degenerative change, was noted in males receiving 0.3 and 0.6% DINP in the diet, and of hepatocellular enlargement in both sexes at the high dose. Focal necrosis was increased in both sexes from 0.3%, but was only significant in males of the high-dose group. Hepatic pathology was significantly increased only in males from 0.3% and at 0.6% in females. Spongiosis hepatis is a spontaneous lesion in the livers of ageing rats, appearing most often in the second year of life, with a strong predilection for male animals. The incidence of spongiosis hepatic can reach 34% in male Fischer rats as a spontaneous lesion and it can be increased and the age of onset reduced by exposure to a diverse number of chemicals. Some of these chemicals are genotoxic, but also a number of non-genotoxic agents as well, including di(2-ethylhexyl) phthalate (DEHP), a known peroxisome proliferator, have been shown to produce spongiosis hepatis (Karbe and Kerlin, 2002; David et al., 2000; Moore et al., 1998; Lington et al., 1998).

- *Moore, 1998(a)*: In this study, DINP was administered daily to rats in the diet for at least 104 weeks at dietary concentrations of 0, 500, 1500, 6000, and 12000 ppm. Rats in the recovery group were administered DINP at a dietary concentration of 12000 ppm for 78 weeks, followed by a 26-week recovery period during which they were administered the basal diet alone. Administration of DINP for at least 104 weeks at levels of 6000 and 12000 ppm resulted in compound-related histomorphologic alterations in the liver and kidneys. Liver changes consisting of increased cytoplasmic eosinophilia and hepatocellular enlargement were observed only in the animals of the 12000 ppm group. An increased incidence of hepatocellular neoplasia was observed in rats of both sexes of the 12000 ppm group, but was not present in the high-dose recovery group. Kidney changes at 104 weeks consisted of mineralization of the renal papilla and increased pigment in tubule cells at 6000 and 12000 ppm. Increased mineralization was noted in the renal papilla of the males of the 6000, 12000 ppm and recovery groups but was not present in the females. Mononuclear cell leukemia occurred with increased frequency in rats of the 6000, 12000 ppm and recovery groups, and renal tubule cell carcinomas were noted in two and four males of the 12000 ppm and recovery groups. No evidence for sustained cell proliferation associated with the peroxisome proliferation induced by DINP was observed. Based on the test results, the NOAEL for systemic toxicity was found to be 1500 ppm (88.3 and 108.6 mg/kg bw/d for males and females, respectively) based on liver and kidney changes.
- *Moore, 1998(b)* In mice, a NOAEL of 1,500 ppm (276 mg/kg/d) can be derived from a 104-week study based on testicular weight decrease observed from 4,000 ppm (742 mg/kg/d). It is likely that the effect on testicular weight is a secondary consequence associated with reduced body weight and is not the result of any underlying toxicological process.
- *Pugh et al(2000)*: Investigated the effect on non-human primate liver of high doses of either DINP (500mg/kg/day), DEHP(500mg/kg/day), or clofibrate (250mg/kg/day). No significant effects were seen after 14d exposure. No treatment related effects were observed; suggesting that rodent livers are not good at predicting phthalate induced hepatic effects in primates.

A.4.2. Conclusions: Level 4 - DINP

The No Observed Adverse Effects Levels for the above referenced chronic toxicology studies in rodents are based on liver and kidney changes and these effects are not related to an endocrine mode of action. In the chronic mouse study effects on testicular weight are reported but this is likely to be secondary consequence of reduced body weight. One study using a single high dose of DINP (Kwack et al, 2009) does report a reduction of sperm counts and effects on sperm motility. Given the lack of a dose response, and other effects on bodyweights and liver weights, and the results of the chronic toxicology studies it cannot be concluded that these are direct effects of DINP. Taken as a whole these data support the conclusion that DINP does not induce endocrine mediated chronic toxicity in rodents or non-human primates.

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The Level 5 reproductive studies will determine if there are any effects on reproductive performance.

A.5. OECD Conceptual Framework: Level 5 - DINP

The fifth and final step to evaluate the potential hazard of an existing chemical is to summarise available and suitable toxicological data generated in all whole animal test systems that can together inform the reviewer on all endpoint mechanisms and effects e.g. Multi-generation assay. The purpose of this summary is to enable an expert to examine these robust *in vivo* assays to investigate all endocrine mechanisms and other mechanisms.

A summary of the Level 5 endocrine data available for DINP is tabulated below. It should be noted that it is not a requirement of the OECD Conceptual Framework that data is available for all sections within a Level. For the purposes of this review, only available data relevant to mammalian systems has been collated and tabulated. Comments on these datasets are available below.

Level 5 Endpoints	DINP: CAS RN 68515-48-0/ 28553-12-0
2-generation assay (TG416 enhanced)	<ul style="list-style-type: none"> Waterman, SJ; Keller, LH; Trimmer, GW; et al. (2000) Two-generation reproduction study in rats given di-isononyl phthalate in the diet. <i>Reprod Toxicol</i> 14(1):21–36.
reproductive screening test (TG421 enhanced)	<ul style="list-style-type: none"> Not required to have data in all sections of a particular level. Endpoints addressed by more comprehensive, higher level study – see 2-generation study.
combined 28 day/ reproduction screening Test (TG 422 enhanced)	<ul style="list-style-type: none"> Not required to have data in all sections of a particular level. Endpoints addressed by more comprehensive, higher level study – see 2-generation study.
Other	<ul style="list-style-type: none"> Waterman, SJ; Ambroso, JL; Keller, LH; et al. (1999) Developmental toxicity of di-isodecyl and di-isononyl phthalates in rats. <i>Reprod Toxicol</i> 13(2):131-136. 47 Hellwig J., et al. Differential prenatal toxicity of branched phthalate esters in rats. <i>Food and Chemical Toxicology</i> vol 35 (1997)

Table 6: OECD CF Level 5 endocrine data available for DINP

A.5.1. Detailed comments on key publications - DINP

- Waterman et al., (1999)*: Administered DINP by gavage to rats at doses of 0, 40, 200, 500 or 1000 mg/kg/day on gestation day (GD) 6 through day 15. On GD 21, dams were terminated and uteri removed and examined. All live fetuses were weighed, sexed, and examined externally for morphologic abnormalities. Overt signs of maternal toxicity were not apparent at any dose level. Transient signs of maternal toxicity at 1000 mg/kg/d, as indicated by slight reductions in body weight gain and food consumption were observed; however, normal weight and food consumption patterns were observed during the late gestation period, after exposure ceased, possibly indicating a recovery effect. A significant increase in fetuses with skeletal lumbar rudimentary ribs and with visceral (dilated renal pelvis) variations at 1,000 mg/kg/d on a per litter basis was observed. The maternal and fetal NOAELs were determined to be 500 mg/kg/day. There were no changes observed in fetal morphology or maternal response indicative of endocrine mediated toxicity.
- Hellwig et al., (1997)*: Administered three types of DINP compounds by gavage at 0, 40, 200, and 1000 mg/kg/day to 8-10 sperm-positive Wistar females/group on gestation day 6 through day 15. The results below apply to the DINP 1 (CAS number 68515-48-0, EC number 271-090-9) and DINP 2 (CAS number 28553-12-0, EC number 249-079-5) which are still produced in the EU and which are REACH registered. DINP 3 (highly branched) was decommercialized in 1995. On GD 20, dams were terminated and uteri removed and examined. All live fetuses were weighed, sexed, and examined externally for morphologic abnormalities. Maternal toxicity at the high dose consisted of reduced food consumption and increased relative liver and kidney weights. There were no treatment-related effects on the number of live fetuses/dam or fetal weight. The only foetal effects were evident at the

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highest dose by a statistically significant increase in percent fetuses per litter with variations. These variations consisted of rudimentary cervical and/or accessory 14th ribs. A modest increase in dilated renal pelvis in the high-dose group was also noted. There were no maternal or foetal effects at 40 or 200 mg/kg/day. A maternal and fetal NOAEL of 200 mg/kg/day was determined. There were no changes observed in fetal morphology or maternal response indicative of endocrine mediated toxicity.

- *Waterman et al (2000)*: Describes both a one generation and a two generation reproductive toxicity study. In the one generation study, groups of 30 male or female Crl:CDBR, VAF Plus rats were administered DINP in the feed at doses of 0, 0.5, 1.0, or 1.5% w/w for 10 weeks prior to mating. The females were exposed throughout mating, gestation, and lactation until post natal day (PND) 21. The males were killed immediately after the mating period. Parental effects included a statistically significant lower mean body weight, as well as suppression in body weight gain, primarily observed in the mid and high-dose groups. The greatest decrease from controls was observed during the postpartum period. Similarly statistically significant lower mean food consumption was observed primarily in the mid and high-dose groups. Statistically significant increases in the mean and absolute and/or mean relative liver and kidney weights of both male and female animals at all dose levels tested were observed. Males in the high dose group exhibited a statistically significant increase in the mean absolute and relative right testis weight, left testis and right epididymis weights and the mean relative left epididymis and seminal vesicle weights. High dose females showed a significant decrease in the mean absolute and relative right ovarian and mean absolute left ovarian weights. No significant differences in male mating, male fertility, female fertility, female fecundity, or female gestational indices were noted. Mean days of gestation were unaffected by treatment as well as the mean sex ratio of the treated offspring when compared with controls. Offspring effects were noted for a number of parameters. The mean live birth index, day 4 survival index, day 14 survival index and lactation index of the high-dose offspring were statistically significantly decreased. Dose related decreases in mean offspring body weight were observed during the postnatal period (PND 0-21). There were statistically significant lower mean body weights in the high-dose males and females, mid dose females at all weighing intervals and in mean offspring body weight of the mid dose males on PND 0, 1, 7, 14 and 21. Statistically significant lower mean body weights in the low-dose males on PND 0, 1, 14, and 21 and low-dose females at all weighing intervals was also observed. Based on increases in liver and kidney weights from 0.5%, no NOAEL could be determined for parental systemic toxicity. No effect was observed on fertility parameters indicating a reproductive NOAEL of 1000 mg/kg/day; however, a decrease of live birth and survival indices occurred at 1.5% which led to a NOAEL of 1% (622 mg/kg/day for parental males during pre-mating). A two generation study was designed based on the results of the one generation range finding study. Crl:CDBR VAF Plus rats (30/group) were fed DINP in the diet at 0.2, 0.4, or 0.8% (w/w) for 10 weeks prior to mating, and through gestation and lactation. There were no treatment-related deaths and no clinical signs which were judged to be directly related to treatment with DINP in P1 and P2 animals. During gestation, significantly lower mean food consumption in the P2 high-dose females compared with controls was noted without an associated decrease of the body weight change during gestation days 0-21. During the postpartum period, parental toxicity was limited to a lower mean body weight in the high dose P1 females on post partum days 14 and 21 which corresponded to significant body weight gain suppression during the overall postpartum interval and was associated with decreased mean food consumption. Lower mean body weights were observed in the P2 high-dose females with an associated decrease of mean food consumption but without an associated decrease of the body weight gain. Statistically significant increases in the mean absolute and mean relative liver weights in P1 and P2 in both sexes at 0.4% and 0.8% were observed. Microscopic hepatic changes were noted from 0.2% in P1 and P2 animals. High-dose males exhibited a statistically significant increase of relative right and left epididymal weights in P2 animals with a concurrent increase (not statistically significant) of absolute epididymis weight. There were no statistically significant differences in male mating, male fertility, female fertility, female fecundity or female gestational indices in P1 generation. A slight decrease, not statistically significant, of male mating, male fertility, female fertility, and female fecundity indices was observed in P2 generation. Mean days of gestation of the P1/P2 treated and control animals

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were equivalent. No treatment-related clinical findings and no biologically significant differences in the F1 or F2 offspring survival indices were observed between the treated and control offspring or gross post-mortem findings. There were statistically significant, dose-related, lower mean offspring bodyweights in all treatment groups compared with controls during the F1 or F2 generations. However, when the litter size was taken into account (Waterman et al., 2000), effects were only significant in high-dose males on PND 0, in males and females of the mid and high-dose levels on PND 7 and 14 and in all treated animals on PND 21. In addition, the weights of all F1 and F2 treated offspring were within the historical control range of the laboratory with the exception of the F2 high-dose males and females on PND 0 and the F2 high-dose males on PND 1 (considering litter size). These findings were considered by the laboratory to be a result of maternal stress and/or direct effects of DINP via exposure through lactation. Studies with other phthalates concluded that these decreases were apparently due to decreased food consumption by the dams and changes in the quality or quantity of milk (Dostal et al., 1987). Thus the laboratory concluded that the lower body weights in the pups might have resulted from decreased milk consumption. No statistically significant differences were observed in reproduction indices indicating a reproductive NOAEL of 0.8% (1000 mg/kg/day). Based on the microscopic liver changes observed from 0.2%, the NOAEL for parental systemic toxicity is considered to be lower than 0.2% (114 to 395 mg/kg bw/day seeing that received doses are widely dependent on the period considered). No NOAEL can be derived from this study, but a LOAEL for offspring might be considered as 0.2%, emphasizing a trend observed similarly in males and females, based on the dose dependent reduced mean body weights of the treated offspring. The LOAEL remained approximate since pups switched diet from milk to solid food between PND 14 and 21 but may be estimated to be 159 mg/kg/d, the lowest dose of the maternal estimated range (159 - 395 mg/kg/d) during post-partum. Together, these robust study data indicate that DINP does not affect male reproductive development or fertility at doses up to approximately 1000 mg/kg/day (the highest dose tested). It has been proposed by Carruthers et al (2005) that there is a critical window of susceptibility for the developing male foetal reproductive system for LMW phthalates in rodent studies (gestation day 16 – 19). This critical window is fully assessed in the 2-generation reproductive studies. The 2-generation study design assesses the effects of continuous exposure in the F1 and F2 generations. In the DINP 2-generation study the parameters of anogenital distance and nipple/areola retention were not specifically part of the test protocol (not included in the test guidelines in effect at the time of the study). Nevertheless, detailed clinical observations were included and if there had been evidence of changes in these parameters then this would have been detected during these observations. Anogenital distance is used as the endpoint to determine the gender of the pups; there were no observations reported indicating that this assessment was problematic indicating that there was no biologically significant effect on AGD following DINP exposure.

A.5.2. Conclusions: Level 5 - DINP

Based on the comprehensive 1-generation, 2-generation reproductive studies and the developmental studies it can be concluded that DINP is not an endocrine disruptor in OECD guideline in vivo studies. The adverse health effects mediated via an endocrine mechanism of cryptorchidism, hypospadias, and significant testicular pathology which are seen with DEHP and DBP in laboratory animals, are not seen with DINP.

A.6. Human data - DINP

Human data are not included in the OECD CF as the OECD does not include human tests among its Guidelines for the testing of chemicals. However, if relevant data are available, it is appropriate to include them in the overall assessment of the toxicity of the chemical of interest. Some data are available on the exposure of humans to DINP; however none of these data indicate any endocrine mediated effects on DINP on human health.

An outline of key human data available for DINP is provided below.

Several studies in humans in which development of the male reproductive system has been evaluated with respect to phthalate exposure during pregnancy or early childhood have been

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published. Of those studies, only one, Main *et al.*, 2006, has evaluated exposure to DINP in an attempt to associate phthalate monoester levels with reproductive hormone levels and cryptorchidism in male infants. Pooled milk samples were obtained from each of 130 women when their children were 1-3 months old. Milk was analysed using HPLC-MS for the monoesters of di-(2-ethylhexyl) phthalate, di-methyl phthalate, di-n-butyl phthalate, butylbenzyl phthalate and DINP. There were no significant differences in milk phthalate concentrations between the 62 mothers of sons with cryptorchidism and the 68 controls. The children had venous blood sampled at 3 months of age for determination of sex hormone-binding globulin, total and free testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH), and inhibin B. Individual hormone levels were used to calculate LH/testosterone, LH/free testosterone, and FSH/inhibin B ratios. MINP was found in all milk samples. Of the parameters tested, MINP was significantly associated with increased serum LH levels. The authors implied that testosterone levels were likely decreased relieving the negative feedback to the pituitary and thereby increasing LH levels. However, no alteration in free or total testosterone was observed (in fact an increase in free testosterone was observed). Further, this association could simply have been a statistical consequence of multiple comparisons to a common control. Overall, the authors concluded that there were "subtle, but significant, dose-dependent associations between neonatal exposure to phthalate monoesters in breast milk and levels of reproductive hormones in boys at three months of age." In 2006, the NTP-CERHR evaluated this study and indicated a number of weaknesses including confounding and possible contamination of breast milk samples. According to Calafat *et al.*, 2004, a special treatment of the milk is required upon sample collection to denature milk enzymes and avoid overestimating the concentrations of phthalate metabolites in milk caused by contamination from the phthalate contaminants that may have been incorporated in the milk during the collection, storage, and measurement process. These considerations limited the usefulness and there is no basis for concluding that DINP or MINP is associated with endocrine effects in humans.

The report of Swan *et al.*, 2005 investigated the link between metabolites of certain phthalates (DMP, DEP, DBP, BBP, DiBP, DnOP, DEHP) in the urine of pregnant women and changes in the reproductive systems of the male infants they later gave birth to. DINP was not assessed in this study. The author was careful to state that no adverse health effect was detected just changes in the infants' ano-genital distance (AGD) index, a correction of ano-genital distance (AGD) of age and size of the infant at time of measurement. Contrary to media reports, she reported no correlation between changes in penis size and maternal phthalate exposure, but only to the changes in the AGD measure.

A.7. Mixtures Studies - DINP

Borch *et al.* (2004) examined hormonal effects in male rat fetuses exposed to DEHP, DINP, or a combination of the two. Thirty-two dams were dosed with either 300 mg DEHP/kg bodyweight per day, 750 mg DINP/kg bodyweight per day, or a combination of these doses. Male fetuses were examined on gestation day 21, and blood and testes were collected for hormone analysis. Reduction in *in vitro* testosterone synthesis was observed following DINP treatment, but in the absence of observation of adverse phenotypic outcomes. The authors report that a factorial statistical analysis revealed no statistically significant interaction between the effects of DEHP and DINP.

Ghisari and Bonefeld-Jorgensen (2009) reported a series of *in vitro* experiments examining the potential of BBP, DBP, DIDP, DINP, DEHP, tOP, CMP, 2,4-DCP, 2-PP resorcinol and DEHA to affect the thyroid hormone (TH) system and estrogen receptor (ER) function, alone and in combination. DINP and DIDP, which were not included in the mixtures experiment, did not have any effect in the ER transactivation assay and only slight effect in the TH assay which occurred at the maximal dose tested. However, the utility of this information is questionable since phthalate diesters are rapidly metabolized to monoesters in humans (Silva *et al.*, 2006a; Koch *et al.*, 2007; Silva *et al.*, 2007). Conclusions drawn from diesters *in vitro* have no basis for extrapolation to *in vivo* systems.

A.8. Conclusions - DINP

The key conclusions from each level of the OECD Conceptual Framework for DINP are:

OECD CF Level	Conclusions DINP
1	DINP has a rich safety dataset available. These data have previously been collated and evaluated by international regulatory authorities and assessed for their potential risk to human health and the environment. The EU Risk Assessment concluded that DINP should not be classified as hazardous under EU regulations, and that risk reduction is not required for any current use. CSTEER concluded that the available data indicates that the potency of DINP for anti-androgenic effects is very low.
2	No significant responses were observed with DINP in any of the <i>in vitro</i> assays. Taken as a whole, the available data indicates that DINP does not have significant interactions with the estrogenic or androgenic receptors. It is noteworthy that <i>in vitro</i> data need to be evaluated very carefully as the tests may have involved either substances which for all practical purposes do not exist under <i>in vivo</i> conditions or may have employed non-physiological conditions.
3	Taken as a whole, these data support the conclusion that DINP does not cause adverse endocrine effects in <i>in vivo</i> screening studies. DINP shows no significant adverse effects in the Uterotrophic Assay (for oestrogenic effects), and no consistent significant adverse effects in the Hershberger Assay (for anti-androgenic effects). In non-validated research studies for anti-androgenic effects DINP showed no, minor or inconsistent effects at high doses, and with no or limited evidence of a dose response. While one animal study shows no effects on fetal testicular testosterone, one study shows variable effects with no dose response, and one study shows reduced fetal testicular testosterone at a single high dose, this was not associated with any adverse health effects in the animals. If there is an effect on testosterone this would appear to be occurring at high doses only and without adverse health effects being seen in animals. Given this potential effect it is appropriate to assess Level 4 and Level 5 studies and confirm whether or not adverse health effects are being seen.
4	Taken as a whole these data support the conclusion that DINP does not induce endocrine mediated chronic toxicity in rodents or non-human primates.
5	Based on the comprehensive 1-generation, 2-generation reproductive studies and the developmental studies it can be concluded that DINP is <u>not</u> an endocrine disruptor in OECD guideline <i>in vivo</i> studies. The adverse health effects mediated via an endocrine mechanism of cryptorchidism, hypospadias, and significant testicular pathology which are seen with DBP in laboratory animals, are not seen with DINP.

Overall conclusion

There are sufficient data to conclude that DINP is not an endocrine disrupting substance for mammals when evaluated according to the OECD Conceptual Framework and using the commonly recognized definitions of an endocrine disruptor. Available human and mixtures data do not lead to any change in this conclusion.

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A.9. Source of references and output from PubMed searches

In addition to already identified references from regulatory submissions including the REACH registration dossier an on-line literature search for published studies was carried out with a search date of 07 October 2010 using the on-line tool "PubMed"¹.

A.9.1. "DINP and endocrine"

The PubMed search resulted in 18 hits using the search terms "DINP and endocrine". All 18 references are listed below, in order of publication, most recent first.

All 18 were reviewed. Four included information that was either not relevant to Framework Levels 2-5 e.g. human biomonitoring data or no novel data relevant for this chemical e.g. mention of DINP in the introduction/ conclusion but no experimental data. Non-mammalian data were excluded as the focus of this paper is a review of relevant mammalian data. The excluded references are indicated with *italics text*. The references from the PubMed search in bold below are included in the relevant Appendices tables for the appropriate level of the OECD Conceptual Framework tables, together with other already identified references.

1. *Correlations between phthalate metabolites in urine, serum, and seminal plasma from young Danish men determined by isotope dilution liquid chromatography tandem mass spectrometry. Frederiksen H, Jørgensen N, Andersson AM. J Anal Toxicol. 2010;34(7):400-10.*
2. **Effects of maternal exposure to di-isononylphthalate (DINP) and 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,p'-DDE) on steroidogenesis in the fetal rat testis and adrenal gland. Adamsson A, Salonen V, Paranko J, Toppari J. Reprod Toxicol. 2009 Jul;28(1):66-74. Epub 2009 Mar 20.**
3. **Effects of plasticizers and their mixtures on estrogen receptor and thyroid hormone functions. Ghisari M, Bonefeld-Jørgensen EC. Toxicol Lett. 2009 Aug 25;189(1):67-77. Epub 2009 May 20.**
4. *A critical evaluation of the environmental risk assessment for plasticizers in the freshwater environment in Europe, with special emphasis on bisphenol A and endocrine disruption. Oehlmann J, Oetken M, Schulte-Oehlmann U. Environ Res. 2008 Oct;108(2):140-9. Review.*
5. *Phthalates: metabolism and exposure. Wittassek M, Angerer J. Int J Androl. 2008 Apr;31(2):131-8. Epub 2007 Dec 7. Review.*
6. **Hershberger assay for antiandrogenic effects of phthalates. Lee BM, Koo HJ. J Toxicol Environ Health A. 2007 Aug;70(15-16):1365-70.**
7. *Metabolism of phthalates in humans. Frederiksen H, Skakkebaek NE, Andersson AM. Mol Nutr Food Res. 2007 Jul;51(7):899-911. Review.*
8. **The effects of selected phenol and phthalate derivatives on steroid hormone production by cultured porcine granulosa cells. Mlynarciková A, Ficková M, Scsuková S. Altern Lab Anim. 2007 Mar;35(1):71-7.**
9. *Internal phthalate exposure over the last two decades—a retrospective human biomonitoring study. Wittassek M, Wiesmüller GA, Koch HM, Eckard R, Dobler L, Müller J, Angerer J, Schlüter C. Int J Hyg Environ Health. 2007 May;210(3-4):319-33. Epub 2007 Mar 30.*
10. **Effects of perinatal exposure to phthalate/adipate esters on hypothalamic gene expression and sexual behavior in rats. Lee HC, Yamanouchi K, Nishihara M. J Reprod Dev. 2006 Jun;52(3):343-52. Epub 2006 Feb 22.**
11. **Modulation of iodide uptake by dialkyl phthalate plasticisers in FRTL-5 rat thyroid follicular cells. Wenzel A, Franz C, Breous E, Loos U. Mol Cell Endocrinol. 2005 Dec 1;244(1-2):63-71. Epub 2005 Nov 10.**
12. *Impact of maternal dietary exposure to endocrine-acting chemicals on progesterone receptor expression in microdissected hypothalamic medial preoptic areas of rat offspring. Takagi H, Shibutani M, Lee KY, Masutomi N, Fujita H, Inoue K, Mitsumori K, Hirose M. Toxicol Appl Pharmacol. 2005 Oct 15;208(2):127-36.*
13. **Hazard evaluation of diisononyl phthalate and diisodecyl phthalate in a Japanese medaka multigenerational assay. Patyna PJ, Brown RP, Davi RA, Letinski DJ, Thomas PE, Cooper KR, Parkerton TF. Ecotoxicol Environ Saf. 2006 Sep;65(1):36-47.**
14. **Steroidogenesis in fetal male rats is reduced by DEHP and DINP, but endocrine effects of DEHP are not modulated by DEHA in fetal, prepubertal and adult male rats. Borch J, Ladefoged O, Hass U, Vinggaard AM. Reprod Toxicol. 2004 Jan-Feb;18(1):53-61.**
15. **Impact of dietary exposure to methoxychlor, genistein, or diisononyl phthalate during the perinatal period on the development of the rat endocrine/reproductive systems in later life.**

¹ <http://www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed>

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- Masutomi N, Shibutani M, Takagi H, Uneyama C, Takahashi N, Hirose M. Toxicology. 2003 Nov 5;192(2-3):149-70.**
16. *The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat.* Parks LG, Ostby JS, Lambright CR, Abbott BD, Klinefelter GR, Barlow NJ, Gray LE Jr. *Toxicol Sci.* 2000 Dec;58(2):339-49.
 17. **Two-generation reproduction study in rats given di-isononyl phthalate in the diet.** Waterman SJ, Keller LH, Trimmer GW, Freeman JJ, Nikiforov AI, Harris SB, Nicolich MJ, McKee RH. *Reprod Toxicol.* 2000 Jan-Feb;14(1):21-36.
 18. **Chronic toxicity and carcinogenic evaluation of diisononyl phthalate in rats.** Lington AW, Bird MG, Plutnick RT, Stubblefield WA, Scala RA. *Fundam Appl Toxicol.* 1997 Mar;36(1):79-89.

A.9.2. Additional references

- CSTE (1998) EU Scientific Committee on Toxicity, Ecotoxicity and the Environment Phthalate migration from soft PVC toys and child-care articles – Data made available since the 16th of June 1998, opinion expressed at the CSTE 6th plenary meeting, Brussels, 26/27 November 1998.
- CSTE (2001) Position paper on Margins of Safety (MOS) in human health risk assessment, expressed at the 22nd CSTE plenary meeting, Brussels, 06/07 March 2001.

Appendix B - Evaluation of endocrine data on DIDP

This Appendix will collate and review the available scientific data relating to the potential endocrine activity of DIDP. The large majority of the data included for DIDP have previously been submitted to regulatory authorities including for example in the REACH registration dossier and in submissions to the US Consumer Product Safety Commission. There are a small number of research papers included in this report which have been recently published and were not included in prior regulatory submissions. In addition to these data already known to the authors, a search of published literature was carried out which identified 6 additional publications. These have been tabulated according to the appropriate framework level and a review of each reference documented.

B.1 OECD Conceptual Framework: Level 1 - DIDP

The first step to evaluate the potential hazard of an existing chemical is to collate the already existing information about its chemistry, uses and any available toxicological data. These data are then sorted, prioritised and evaluated to screen whether the chemical is a high priority for further investigation.

A summary of the Level 1 endocrine data available for DIDP is tabulated below. Detailed comments on these datasets are available in the pages following the table.

Level 1 Endpoints	DIDP: CAS RN 68515-49-1/ 26761-40-0
physical & chemical properties, e.g., MW, reactivity, volatility, biodegradability	<ul style="list-style-type: none"> • ECB (2003). 1,2-benzenedicarboxylic acid, di-C9-11- branched alkyl esters, C10-rich and di-"isodecyl" phthalate (DIDP). Risk Assessment. European Chemicals Bureau, Institute for Health and Consumer Protection, Joint Research Center of the European Commission. http://ecb.jrc.ec.europa.eu/DOCUMENTS/Existing-Chemicals/RISK_ASSESSMENT/REPORT/didpreport041.pdf
human & environmental exposure, e.g., production volume, release, use patterns	<ul style="list-style-type: none"> • ECB (2003). 1,2-benzenedicarboxylic acid, di-C9-11- branched alkyl esters, C10-rich and di-"isodecyl" phthalate (DIDP). Risk Assessment. European Chemicals Bureau, Institute for Health and Consumer Protection, Joint Research Center of the European Commission. http://ecb.jrc.ec.europa.eu/DOCUMENTS/Existing-Chemicals/RISK_ASSESSMENT/REPORT/didpreport041.pdf
hazard, e.g., available toxicological data	<ul style="list-style-type: none"> • ECB (2003). 1,2-benzenedicarboxylic acid, di-C9-11- branched alkyl esters, C10-rich and di-"isodecyl" phthalate (DIDP). Risk Assessment. European Chemicals Bureau, Institute for Health and Consumer Protection, Joint Research Center of the European Commission. http://ecb.jrc.ec.europa.eu/DOCUMENTS/Existing-Chemicals/RISK_ASSESSMENT/REPORT/didpreport041.pdf • NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Di-Isodecyl Phthalate (DIDP). National Toxicology Program. NTP CERHR MON. 2003 Apr;(3):i-III90. • Kamrin MA. Phthalate risks, phthalate regulation, and public health: a review. J Toxicol Environ Health B Crit Rev. 2009 Feb; 12(2):157-74. Review. • McKee RH, Butala JH, David RM, Gans G. NTP center for the evaluation of risks to human reproduction reports on phthalates: addressing the data gaps. Reprod Toxicol. 2004 Jan-Feb; 18(1):1-22. Review. • European Chemicals Agency (ECHA) (July 2010). Evaluation of new scientific evidence concerning the restrictions contained in Annex XVII to Regulation (EC) No. 1907/2006 (REACH). Review of new available information for di-isodecyl phthalate (DIDP). Review Report.

Table 7: OECD CF Level 1 endocrine data available for DIDP

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B.1.1. Detailed comments on key publications - DIDP

- *European Chemicals Bureau (2003): EU Risk Assessment on DIDP:* concluded that DIDP did not deserve to be classified as dangerous. None of the endpoints identified for risk characterization purposes was related to endocrine effects. The outcome for workers and the environment was Conclusion (ii): There is at present no need for further information and/or testing and for risk reduction measures beyond those which are being applied already. The outcome for consumers was Conclusion (iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account. This conclusion applies in case DIDP should be used as a substitute for other phthalates in toys because of concerns for hepatic toxicity as a consequence of repeated exposure of infants and newborn babies arising mainly by the oral route from mouthing and sucking toys and baby equipment. Pertaining to reduced offspring survival, due to the uncertainty related to the relevance of this end point for newborns and infants and to the lack of experience in this particular field of transgenerational effect, no formal conclusion could be drawn. Conclusion (ii) There is at present no need for further information and/or testing and for risk reduction measures beyond those which are being applied already. This conclusion applies for all other scenarios.
- *European Chemicals Agency (2010).* ECHA conclude that some new endocrine information is available and that the new information would need a more in-depth assessment to evaluate the reliability and relevance of the studies. ECHA state that all the studies evaluating the potential endocrine effects of DIDP should be assessed together, including the studies in the EU Risk Assessment Report.
- *NTP-CERHR Monograph (2003):* reviewed toxicology data available for DIDP relating to the potential reproductive and developmental toxicity of DIDP. The expert panel identified two areas of research that required further data: 1) a perinatal developmental study including examination of sexual maturation in a rodent and maybe a non-rodent species and 2) increased information on human exposure levels, through biomonitoring, especially determining DINP exposures to young children, ideally developing data to include 3-12 month old children. In conclusion, the expert panel had minimal concern for unborn children due to ambient maternal exposure to DIDP and minimal concern about DIDP resulting in reproductive toxicity in humans.
- *McKee et al (2004):* Addresses the few data gaps identified in the NTP CERHR report which for DIDP identified exposure characterization as a critical need. New data on child behaviour are reviewed and indicate that exposures to DINP from toys are much lower than previously anticipated; therefore as DIDP is apparently not used in toys, children's exposures as a consequence of this use are unlikely to be problematic. The need for a perinatal developmental study in a non-rodent species was also raised by the Expert Panel but the authors concluded that as DEHP did not affect male reproductive parameters in primates, further studies of DIDP in primates seem unwarranted.
- *Kamrin et al (2009):* Summarizes recent evaluations of the risks of various phthalates. The analysis considers biomonitoring studies and epidemiological research in addition to laboratory animal evidence. Analysis of all of the available data leads to the conclusion that the risks are low, even lower than originally thought, and that there is no convincing evidence of adverse effects on humans. The data reviewed extend and confirm the consensus of the NTP-CERHR panel and the ECB expert group that there is minimal concern about possible adverse effects of DIDP on humans
- *ECHA (2010): European Chemicals Agency (ECHA), July 2010.* ECHA concluded that new endocrine information is available and that the new information would need a more in-depth assessment to evaluate the reliability and relevance of the studies. ECHA state that all the studies evaluating the potential endocrine effects of DINP should be assessed together, including the studies in the EU Risk Assessment Report.

B.1.2. Conclusions: Level 1 - DIDP

DIDP has a rich safety dataset available. These data have previously been collated and evaluated by international regulatory authorities and assessed for their potential risk to human and environmental health. These reviews have concluded that DIDP is not dangerous, should

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not be classified as hazardous under EU regulations, and that risk reduction is not required for any use.

B.2. OECD Conceptual Framework: Level 2 - DIDP

The second step to evaluate the potential hazard of an existing chemical is to summarise available and suitable toxicological data generated in non-animal test systems e.g. using *in vitro* and / or *in silico* models. The purpose of this summary is to enable an expert to examine the existing *in vitro* assays to investigate detailed mechanistic data.

A summary of the Level 2 endocrine data available for DIDP is tabulated below. Detailed comments on these datasets are available below.

Table 8: OECD CF Level 2 endocrine data available for DIDP

Level 2 Endpoints	DIDP: CAS RN 68515-49-1/ 26761-40-0
ER, AR, TR receptor binding affinity	<ul style="list-style-type: none"> • Akahori, Y; Nakai, M; Yakabe, Y; et al. (2005) Two-step models to predict binding affinity of chemicals to the human estrogen receptor α by three-dimensional quantitative structure-activity relationships (3D-QSARs) using receptor-ligand docking simulation. SAR & QSAR in Environmental Research 16(4):323–337. • Ghisari, M; Bonefeld-Jorgensen, EC. (2009) Effects of plasticizers and their mixtures on estrogen receptor and thyroid hormone functions. Toxicol Lett 189(1):67–77. • Harris CA, Henttu P, Parker MG, Sumpter JP. The estrogenic activity of phthalate esters <i>in vitro</i>. EHP. 1997 Aug;105(8):802-11. • Mlynarcikova, A; Fickova, M; Scsukova, S. (2007) The effects of selected phenol and phthalate derivatives on steroid hormone production by cultured porcine granulosa cells. Altern Lab Anim 35(1):71–77. • Takeuchi, S; Iida, M; Kobayashi, S; et al. (2005) Differential effects of phthalate esters on transcriptional activities via human estrogen receptors α and β, and androgen receptor. Toxicology 210(2–3):223–233. • Zacharewski TR, Meek MD, Clemons JH, Wu ZF, Fielden MR, Matthews JB. Examination of the <i>in vitro</i> and <i>in vivo</i> estrogenic activities of eight commercial phthalate esters. Toxicol Sci. 1998 Dec;46(2):282-93.
High Through Put Prescreens	<ul style="list-style-type: none"> • Not required to have data in all sections of a particular level.
Transcriptional activation	<ul style="list-style-type: none"> • Ghisari M, Bonefeld-Jorgensen EC. Effects of plasticizers and their mixtures on estrogen receptor and thyroid hormone functions. Toxicol Lett. 2009 Aug 25;189(1):67-77.
Thyroid function	<ul style="list-style-type: none"> • Breous, E; Wenzel, A; Loos, U. (2005) The promoter of the human sodium/iodide symporter responds to certain phthalate plasticisers. Mol Cell Endocrinol 244(1–2):75–78. • Ghisari, M; Bonefeld-Jorgensen, EC. (2009) Effects of plasticizers and their mixtures on estrogen receptor and thyroid hormone functions. Toxicol Lett 189(1):67–77. • Wenzel, A; Franz, C; Breous, E; et al. (2005) Modulation of iodide uptake by dialkyl phthalate plasticisers in FRTL-5 rat thyroid follicular cells. Mol Cell Endocrinol 244(1–2):63–71.
Aromatase and steroidogenesis <i>in vitro</i>	<ul style="list-style-type: none"> • Not required to have data in all sections of a particular level.
Fish hepatocyte VTG assay	<ul style="list-style-type: none"> • Not relevant to this assessment of mammalian data
Aryl hydrocarbon receptor recognition/binding	<ul style="list-style-type: none"> • Kruger, T; Long, M; Bonefeld-Jorgensen, EC. (2008) Plastic components affect the activation of the aryl hydrocarbon and the androgen receptor. Toxicology 246(2–3):112–123.
QSARs	<ul style="list-style-type: none"> • Akahori, Y; Nakai, M; Yakabe, Y; et al. (2005) Two-step models to predict binding affinity of chemicals to the human estrogen receptor α by three-dimensional quantitative structure-activity relationships (3D-QSARs) using receptor-ligand docking simulation. SAR & QSAR in Environmental Research 16(4):323–337.

	<ul style="list-style-type: none"> • Akahori Y, Nakai M, Yamasaki K, Takatsuki M, Shimohigashi Y, Ohtaki M. Relationship between the results of <i>in vitro</i> receptor binding assay to human estrogen receptor alpha and <i>in vivo</i> uterotrophic assay: comparative study with 65 selected chemicals. <i>Toxicol In vitro</i>. 2008 Feb;22(1):225-31.
Others (as appropriate)	<ul style="list-style-type: none"> • Turan N, Waring RH, Ramsden DB. The effect of plasticisers on "sulphate supply" enzymes. <i>Mol Cell Endocrinol</i>. 2005 Dec 1;244(1-2):15-9 • Harris R, Turan N, Kirk C, Ramsden D, Waring R. Effects of endocrine disruptors on dehydroepiandrosterone sulfotransferase and enzymes involved in PAPS synthesis: genomic and nongenomic pathways. <i>Environ Health Perspect</i>. 2007 Dec; 115 Suppl 1:51-4.

B.2.1. Detailed comments on key publications – Level 2 - DIDP

- *Akahori et al (2005): abstract only*. Models indicated that phthalates show weak if any affinity for binding to the human ER α receptor. Unclear whether DIDP was one of the phthalates tested.
- *Akahori et al (2008)*: Investigated the relationship between the *in vitro* ER binding and *in vivo* uterotrophic assays. The authors compared the results from these assays for 65 chemicals spanning a variety of chemical classes. Resulting DIDP binding affinity (log RBA) value of -3.46 was one of the lowest reported and far below the cut-off level (-2.63) that could induce estrogenic/ anti-estrogenic activities in the uterotrophic assay, indicating that DIDP does not have estrogenic/ anti-estrogenic properties.
- *Breous et al (2005)*: Investigated possible effects DIDP on the transcriptional activity of sodium/iodide symporter (NIS) which mediates the active transport of I⁻ in the thyroid. A slight effect was observed with DIDP on the transcriptional activity of NIS but the biological relevance of this weak effect is not clear.
- *Ghisari et al (2009)*: Investigated *in vitro* the potential for thyroid hormone-like and estrogenic activities of a range of widely used plasticizers. The TH disrupting potential was determined by the effect on the TH-dependent rat pituitary GH3 cell proliferation (T-screen). The estrogenic activities of the compounds were assessed in MVLN cells, stably transfected with an estrogen receptor (ER) luciferase reporter vector. Results were variable with DIDP being reported as causing a small increase in GH3 proliferation at one concentration only, and DINP actually causing a slight inhibition of GH3 growth. Neither DIDP or DINP had any effect on ER transactivation. The abstract of the paper is confusing because it states that all the tested compounds (except 2-phenylphenol) significantly affected the GH3 cell proliferation. Based on the full results presented in the paper this is not the case for DINP and DIDP as noted above. It should also be noted that these were tests of phthalate diesters whereas under *in vivo* conditions the diesters are metabolized to monoesters. Therefore, *in vitro* data need to be evaluated very carefully as the tests involved substances which for all practical purposes do not exist under *in vivo* conditions.
- *Harris et al (1997)*: A series of phthalate esters, including DIDP, were screened for estrogenic activity using a recombinant yeast screen. The recombinant yeast screen, a gene for a human estrogen receptor was integrated into the main yeast genome and was expressed in a form capable of binding to estrogen response elements, controlling the expression of the reporter gene lac-Z (when receptor is activated, lac-Z is expressed). DIDP was tested at concentrations ranging from 10⁻³ M to 5x10⁻⁷ M. DIDP no effects in any of these screens performed. It should be noted that these *in vitro* assays have investigated one mechanism of action only, the ability of phthalates to act as estrogen agonists. More importantly, it should also be noted that these were tests of phthalate diesters. Under *in vivo* conditions the diesters are metabolized to monoesters which are not estrogen receptor agonists. The *in vitro* data need to be evaluated very carefully as the tests may have involved either substances which for all practical purposes do not exist under *in vivo* conditions or may have employed non-physiological conditions.
- *Kruger et al (2008)*: The effects of DIDP on the aryl hydrocarbon receptor (AhR) and the androgen receptor (AR) were assessed using luciferase reporter gene. DIDP had a slight effect at the highest dose in the AhR assay but no significant effect on either assay.

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- *Mlynarcikova et al (2007)*: DIDP exposed porcine ovarian granulosa cells may exhibit slightly amplified FSH stimulated progesterone release but the biological relevance of this weak effect is not clear.
- *Takeuchi et al (2005)*: compared the ER and AR binding potential of 22 different phthalates. DIDP did not show significant binding for either receptor.
- *Wenzel et al (2005)*: Investigated possible effects of DIDP on the uptake of iodide in a thyroid cell line. Results indicated that high concentrations (10⁻⁴M) of some phthalates enhanced iodide uptake but the biological relevance of this weak effect is not clear.
- *Zacharewski et al (1998)*: The estrogenic activities of DIDP were investigated *in vitro* using estrogen receptor (ER) competitive ligand-binding and mammalian- and yeast-based gene expression assays. No significant responses were observed with DIDP in any of the *in vitro* assays.

B.2.2. Conclusions: Level 2 - DIDP

No significant responses were observed with DIDP in any of the *in vitro* assays. Taken as a whole, the available data indicate that DIDP does not have significant interactions with the estrogenic or androgenic receptors. It is noteworthy that *in vitro* data need to be evaluated very carefully as the tests may have involved either substances which for all practical purposes do not exist under *in vivo* conditions or may have employed non-physiological conditions.

Evaluation of Level 2 data are indicative of possible mechanisms of action and are not sufficient to provide conclusive evidence on the potential endocrine activity of a chemical *in vivo*. As specified by the OECD Conceptual Framework further data are needed to support conclusions on endocrine activity and these are considered in the next levels of the framework.

B.3. OECD Conceptual Framework: Level 3 - DIDP

The third step to evaluate the potential hazard of an existing chemical is to summarise available and suitable toxicological data generated in animal test systems that are limited in that they are only able to investigate single endpoint mechanisms and effects e.g. Hershberger assay (androgenic related) or Uterotrophic assay (estrogenic related). The purpose of this summary is to enable an expert to examine existing *in vivo* assays to investigate detailed biological activity.

A summary of the Level 3 endocrine data available for DIDP is tabulated below. For the purposes of this review, only available data relevant to mammalian systems has been collated and tabulated. Comments on these datasets are available below.

Level 3	DIDP: CAS RN 68515-49-1/ 26761-40-0
Uterotrophic assay (estrogenic related)	<ul style="list-style-type: none"> • Akahori Y, Nakai M, Yamasaki K, Takatsuki M, Shimohigashi Y, Ohtaki M. Relationship between the results of <i>in vitro</i> receptor binding assay to human estrogen receptor alpha and <i>in vivo</i> uterotrophic assay: comparative study with 65 selected chemicals. <i>Toxicol In vitro</i>. 2008 Feb;22(1):225-31. • Zacharewski TR, Meek MD, Clemons JH, Wu ZF, Fielden MR, Matthews JB. Examination of the <i>in vitro</i> and <i>in vivo</i> estrogenic activities of eight commercial phthalate esters. <i>Toxicol Sci</i>. 1998 Dec;46(2):282-93.
Hershberger assay (androgenic related)	<ul style="list-style-type: none"> • Lee BM, Koo HJ. Hershberger assay for antiandrogenic effects of phthalates. <i>J Toxicol Environ Health A</i>. 2007 Aug; 70(15-16):1365-70. 2009 Jul;28(1):66-74.
Non-receptor mediated hormone function	<ul style="list-style-type: none"> • Not required to have data in all sections of a particular level.
Others (e.g. thyroid)	<ul style="list-style-type: none"> • Barber ED, Cifone M, Rundell J, Przygoda R, Astill BD, Moran E, Mulholland A, Robinson E, Schneider B. <i>J Appl Toxicol</i>. 2000. Results of the L5178Y mouse lymphoma assay and the Balb/3t3 cell <i>in vitro</i> transformation assay for eight phthalate esters. <i>Jan-Feb</i>; 20(1):69-80.

Table 3: OECD CF Level 3 endocrine data available for DIDP

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B.3.1. Detailed comments on key publications - DIDP

- *Akahori et al (2008)*: investigates the relationship between the *in vitro* ER binding and *in vivo* uterotrophic assays by determining the meaningful binding potency from the ER binding assay by comparing the results from these assays for 65 chemicals spanning a variety of chemicals classes. Resulting DIDP binding affinity (log RBA) value of -3.49 was the lowest reported and far below the cut-off level (-2.63) that could induce estrogenic/ anti-estrogenic activities in the uterotrophic assay, indicating that DIDP does not have estrogenic/ anti-estrogenic properties.
- *Lee et al (2009)*: Reported a study designed similar to the Hershberger bioassay screen to test the antiandrogenic properties of DIDP. DIDP did not induce consistent changes in the androgen sensitive tissues therefore these data indicate that DIDP does not meet the OECD criteria for androgen antagonists as the weights of the sex accessory tissues from the administered groups showed no consistent statistically significant differences from the testosterone-only animals.
- *Zacharewski et al (1998)*: The estrogenic activities of DIDP were investigated In an *in vivo* study, 20, 200, 2,000 mg/kg/d of DIDP was administered by oral gavage once daily for a period of 4 days to ovariectomised rats. DIDP did not produce any reproducible, dose-dependant effect on uterine wet weight relative to vehicle control at any of the dose tested. DIDP did not induce a vaginal cornification response at any of the doses tested. Accordingly, it can be concluded that DIDP is not estrogenic under *in vivo* conditions.
- Barber (2000): Eight phthalate esters, with alcohol chain lengths of 1-11 carbon atoms and with various degrees of branching, were tested *in vitro* in the L5178Y mouse lymphoma mammalian cell mutation assay and in the Balb/3T3 cell transformation assay. DIDP did not have any indication of mutagenic potential in the mouse lymphoma assay and did not increase transformation frequency in the Balb/3T3 cell transformation assay.

B.3.2. Conclusions: Level 3 - DIDP

These data support the conclusion that DIDP does not cause adverse endocrine effects in *in vivo* screening studies. DIDP shows no significant adverse effects in the Uterotrophic Assay (for oestrogenic effects), and no consistent significant adverse effects in the Hershberger Assay (for anti-androgenic effects).

B.4. OECD Conceptual Framework: Level 4 - DIDP

The forth step to evaluate the potential hazard of an existing chemical is to summarise available and suitable toxicological data generated in animal test systems that can inform the reviewer on multiple endpoint mechanisms and effects e.g. Male and female pubertal assays. The purpose of this summary is to enable an expert to examine existing *in vivo* assays to investigate multiple endocrine mechanisms and effects.

A summary of the Level 4 endocrine data available for DIDP is tabulated below. For the purposes of this review, only available data relevant to mammalian systems has been collated and tabulated. Comments on these datasets are available below.

Level 4	DIDP CAS RN 68515-49-1/ 26761-40-0
Enhanced 407 (endpoints based on endocrine mechanisms)	<ul style="list-style-type: none"> Kwack SJ, Kim KB, Kim HS, Lee BM. (2009) Comparative Toxicological Evaluation of Phthalate Diesters and Metabolites in Sprague-Dawley Male Rats for Risk Assessment Toxicity of Phthalate Diesters and Metabolites. <i>Journal of Toxicology and Environmental Health, Part A</i>, 72: 1446–1454.
Male and female pubertal assays	<ul style="list-style-type: none"> Not required to have data in all sections of a particular level. Endpoints addressed by 2-generation studies – see Level 5
Adult intact assay	<ul style="list-style-type: none"> Not required to have data in all sections of a particular level. Endpoints addressed by 2-generation studies – see Level 5
Other	<ul style="list-style-type: none"> Cho WS, Han BS, Ahn B, Nam KT, Choi M, Oh SY, Kim SH, Jeong J, Jang DD. Peroxisome proliferator di-isodecyl phthalate has no carcinogenic potential in Fischer 344 rats. <i>Toxicol Lett.</i> 2008 May 5;178(2):110-6 Cho WS, Han BS, Ahn B, Nam KT, Choi M, Oh SY, Kim SH, Jeong J, Jang DD,* , Corrigendum to "Peroxisome proliferator di-isodecyl phthalate has no carcinogenic potential in Fischer 344 rats" [<i>Toxicol. Lett.</i> 178 (2008) 110–116]. <i>Toxicology Letters</i> 197 (2010a) 156 Cho WS, Jeong J, Choi M, Park SN, Han BS, Son WC. 26-Week carcinogenicity study of di-isodecyl phthalate by dietary administration to CB6F1-rasH2 transgenic mice. <i>Arch Toxicol.</i> 2010b Apr 1. McKee RH, Przygoda RT, Chirdon MA, Engelhardt G, Stanley M. J Di(isononyl) phthalate (DINP) and di(isodecyl) phthalate (DIDP) are not mutagenic. <i>Appl Toxicol.</i> 2000 Nov-Dec; 20(6):491-7.

Table 9: OECD CF Level 4 endocrine data available for DIDP

B.4.1. Detailed comments on key publications – Level 4 - DIDP

- Kwack et al (2009): Comparative assessment of the systemic toxicity and sperm parameters for DIDP - administered orally to Sprague- Dawley male rats at 500 mg/kg body weight (bw)/d for 4 wk by oral gavage. Liver weights were significantly increased compared to the control. Testes weights were not significantly reduced compared to the controls and Kwack et al did report that DIDP had no effect on sperm counts but did lower sperm motility of epididymal sperm, detected by a change in the sperm motion parameters. In contrast to DINP, DBP and BBP no effects on bodyweight were observed with DIDP. Increased platelets were observed with DIDP and random effects on blood parameters were observed with other phthalates (DMP, DBP). Given the inconsistent findings on bodyweight and effects on blood parameters, and the lack of a dose response (only a single high dose used) it is cannot be concluded that DIDP is having direct effects on sperm motility.
- Cho (2008): A two year bioassay was conducted in which DIDP was administered in the diet at concentrations of 400, 2000, and 8000 ppm to F344 rats (Cho et al., 2008). The average daily doses of DIDP were reported to be calculated from the body weights and feed consumption data using the concentrations of DIDP in the diet. For doses of 400, 2000, and 8000 ppm, the calculated average daily doses of DIDP over 2 years for male rats reported in the paper are incorrect. Actual exposures for male rats were 21.9, 110.3 and 479.2 mg/kg-bw/day and for female rats 22.9, 128.2 and 619.6 mg/kg-bw/day (Cho et al 2010: Corrigendum to "Peroxisome proliferator di-isodecyl phthalate has no carcinogenic potential in Fischer 344 rats" [*Toxicol. Lett.* 178 (2008) 110–116]). Rats of both sexes exhibited significant decreases in overall survival and body weights, and increases in the relative weights of kidneys and liver with 8000 ppm DIDP. No treatment related neoplastic lesions were observed in the internal organs, including the liver. In addition, measurement of catalase enzyme activity, a marker for cell peroxisome proliferating activity, suggests that DIDP can induce peroxisome proliferation at an early stage (12 weeks of treatment) but fails

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to maintain the catalase-inducing potential by 32 weeks of treatment. An increased incidence of mononuclear cell leukemia (MNCL) was observed in this study, but MNCL is a common neoplasm in F344 rats, and the observed increased incidence is likely to be a species-specific effect with little or no relevance to humans. Therefore, DIDP was considered to be non-carcinogenic at doses up to 8000 ppm in rats.

- *Cho et al., (2010a)*: is a Corrigendum to the 2008 paper above, correcting the dose levels previously published. Actual exposures for male rats were 21.9, 110.3 and 479.2 mg/kg-bw/day and for female rats 22.9, 128.2 and 619.6 mg/kg-bw/day
- *Cho et al., (2010b)*: This study examined the carcinogenic potential of di-isodecyl phthalate (DIDP) in rasH2 mice dietary levels of 0, 0.1, 0.33, or 1% and 15 wild-type mice/gender/group at dietary levels of 0 and 1% for 26 weeks. This study adds a set of results for an additional test chemical for the performance of the rasH2 short-term transgenic model to the existing database of 3 compounds (WY-14643, DEHP, and clofibrate) tested in the ILSI/HESI ACT project. Non-neoplastic changes were observed in the liver (parenchymal inflammation, fatty changes, diffuse hepatocyte hypertrophy with eosinophilic granules and focal necrosis) and kidneys (tubular basophilia and tubular hyperplasia) after administration of DIDP in the rasH2 and wild-type mice. The ILSI/HESI project papers and subsequent publications indicated the rasH2 model is an acceptable alternative to a 2-year study, even though it did not respond to most classes of non-genotoxic rodent carcinogens. There is substantial evidence that PPAR α -mediated liver tumorigenesis is not relevant to humans, and therefore the authors concluded that the rasH2 mouse liver response to this class is considered by many a "false positive" for human risk.
- *McKee (2000)*: reviewed the available literature DEHP and DINP induced liver tumours in animal models. Liver effects of DIDP were not the focus of this particular review, however the conclusions are considered pertinent given the similarity in liver effects between DINP and DIDP. The author concluded that peroxisome proliferation may be necessary but not sufficient to induce rodent liver tumours but may also require the inhibition of GJIC. These processes appear to have a key role in rodents, however strong *in vitro* and *in vivo* data indicate that this is a mechanism that is not relevant to non-human primates or humans.

B.4.2. Conclusions: Level 4 - DIDP

The effects identified in the chronic studies are high dose effects on liver and kidney and these are not produced via an endocrine mode of action. One study (Kwack et al, 2009) using a single high dose reports that DIDP has no effects on testes weight or on sperm counts. An effect on sperm motility was reported but given the lack of any dose response and effects on bodyweight and blood parameters, it cannot be concluded that DIDP is having a direct effect on sperm motility. Taken as a whole these data support the conclusion that DIDP does not induce endocrine mediated chronic toxicity to non-reproductive tissues in rodents or non-human primates. The Level 5 reproductive studies will determine if there are any effects on reproductive performance.

B.5. OECD Conceptual Framework: Level 5 - DIDP

The fifth and final step to evaluate the potential hazard of an existing chemical is to summarise available and suitable toxicological data generated in all whole animal test systems that can together inform the reviewer on all endpoint mechanisms and effects e.g. Multi-generation assay. The purpose of this summary is to enable an expert to examine these robust *in vivo* assays to investigate all endocrine mechanisms and other mechanisms.

A summary of the Level 5 endocrine data available for DIDP is tabulated below. It should be noted that it is not a requirement of the OECD Conceptual Framework that data is available for all sections within a Level. For the purposes of this review, only available data relevant to mammalian systems has been collated and tabulated. Comments on these datasets are available below.

Level 5 Endpoints	DIDP: CAS RN 68515-49-1/ 26761-40-0
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2-generation assay (TG416 enhanced)	<ul style="list-style-type: none"> Hushka LJ, Waterman SJ, Keller LH, Trimmer GW, Freeman JJ, Ambroso JL, Nicolich M, McKee RH. Two-generation reproduction studies in Rats fed diisodecyl phthalate. <i>Reprod Toxicol.</i> 2001 Mar-Apr; 15(2):153-69.
reproductive screening test (TG421 enhanced)	<ul style="list-style-type: none"> Not required to have data in all sections of a particular level. Endpoints addressed by more comprehensive, higher level study – see 2-generation study.
combined 28 day/ reproduction screening Test (TG 422 enhanced)	<ul style="list-style-type: none"> Not required to have data in all sections of a particular level. Endpoints addressed by more comprehensive, higher level study – see 2-generation study.
Other	<ul style="list-style-type: none"> Waterman, SJ; Ambroso, JL; Keller, LH; et al. (1999) Developmental toxicity of diisodecyl and di-isononyl phthalates in rats. <i>Reprod Toxicol</i> 13(2):131-136. 47 Hellwig J, Freudenberg H, Jäckh R. Differential prenatal toxicity of branched phthalate esters in rats. <i>Food Chem Toxicol.</i> 1997 May; 35(5):501-12. NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Di-Isodecyl Phthalate (DIDP). National Toxicology Program. NTP CERHR MON. 2003 Apr;(3):i-III90.

Table 10: OECD CF Level 5 endocrine data available for DIDP

B.5.1. Detailed comments on key publications – Level 5 - DIDP

- Hushka (2001): : In two 2-generation reproductive toxicity studies, there were no changes in reproductive indices and no effects on fertility. Additionally, there were no effects on reproductive organs in the repeated dose study. Accordingly, the overall conclusion from these studies was that DIDP has no effect on fertility. DIDP did produce a small, statistically significant decrease in postnatal survival indices which was observed in the second generation of both of the two-generation studies leading to the NOAEL of 0.06% (33-76 mg/kg/d). These effects were found in association with maternal toxicity: reduced body weight, instances of increased kidney weight, and /or liver enlargement. Therefore, the effects on post-natal survival could be secondary rather than a direct effect of DIDP on the rat pups. Cross fostering studies demonstrated that post natal body weight effects observed were reversible when post natal exposure to DIDP via the dams stopped.
- Waterman et al., (1999)*: Developmental toxicity studies of DIDP conducted at doses of 100, 500, and 1000 mg/kg provided evidence of slight and transient signs of maternal toxicity at 1,000 mg/kg/d (significant reversible decrease of body weight gain and food consumption) suggesting a conservative NOAEL of 500 mg/kg/d for maternal toxicity. The only statistically significant changes were skeletal variations (supernumerary cervical and rudimentary lumbar ribs) on a per litter basis at the high dose. Rudimentary ribs are a common finding in rat fetuses and should not be regarded as associated with malformations, but may only be related to transient maternal stress. It should be noted that supernumerary ribs were located in the cervical region which is less common (*Waterman et al., 1999*), but the biological significance of cervical supernumerary ribs remains uncertain. A NOAEL of 500 mg/kg/d may be assumed for skeletal variations. There were no changes observed in fetal morphology or maternal response indicative of endocrine mediated toxicity.
- Hellwig et al., (1997)*: Administered three types of DIDP compounds by gavage at 0, 40, 200, and 1000 mg/kg/day to 8-10 sperm-positive Wistar females/group on gestation day 6 through day 15. On GD 20, dams were terminated and uteri removed and examined. All live fetuses were weighed, sexed, and examined externally for morphologic abnormalities. Maternal toxicity at the high dose consisted of reduced food consumption and increased relative liver and kidney weights. There were no treatment-related effects on the number of live fetuses/dam or fetal weight. The only foetal effects were evident at the highest dose by a statistically significant increase in percent fetuses per litter with variations. These variations consisted of rudimentary cervical and/or accessory 14th ribs. A modest increase in dilated renal pelvis in the high-dose group was also noted. There were no maternal or foetal effects at 40 or 200 mg/kg/day. A maternal and fetal NOAEL of 200 mg/kg/day was determined. There were no changes observed in fetal morphology or maternal response indicative of endocrine mediated toxicity.

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B.5.2. Conclusions: Level 5 - DIDP

Based on the comprehensive 2-generation reproductive study and the developmental study it can be concluded that DIDP is not an endocrine disruptor in comprehensive OECD guideline *in vivo* studies. The adverse health effects mediated via an endocrine mechanism of cryptorchidism, hypospadias, and significant testicular pathology which are seen with DEHP and DBP in laboratory animals, are not seen with DIDP.

B.6. Human data - DIDP

Human data are not included in the OECD CF as the OECD does not include human tests among its Guidelines for the testing of chemicals. However, if relevant data are available, it is appropriate to include them in the overall assessment of the toxicity of the chemical of interest. Some data are available on the exposure of humans to DIDP; however none of these data indicate any endocrine mediated effects of DIDP on human health.

The report of Swan *et al.*, 2005 investigated the link between metabolites of certain phthalates (DMP, DEP, DBP, BBP, DiBP, DnOP, DEHP) in the urine of pregnant women and changes in the reproductive systems of the male infants they later gave birth to. DIDP was not assessed in this study.

B.7. Mixtures Studies with DIDP

There are no known published *in vivo* studies that investigate mixtures including DIDP. Ghisari and Bonfeld-Jorgensen (2009) reported a series of *in vitro* experiments examining the potential of BBP, DBP, DIDP, DiDP, DEHP, tOP, CMP, 2,4-DCP, 2-PP resorcinol and DEHA to affect the thyroid hormone (TH) system and estrogen receptor (ER) function, alone and in combination. DIDP and DiDP, which were not included in the mixtures experiment, did not have any effect in the ER transactivation assay and only slight effect in the TH assay which occurred at the maximal dose tested. However, the utility of this information is questionable since phthalate diesters are rapidly metabolized to monoesters in humans (Silva *et al.*, 2006a; Koch *et al.*, 2007; Silva *et al.*, 2007). Conclusions drawn from diesters *in vitro* have no basis for extrapolation to *in vivo* systems.

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B.8. Conclusions - DIDP

The key conclusions from each level of the OECD Conceptual Framework for DIDP are:

OECD CF Level	Conclusions DIDP
1	DIDP has a rich safety dataset available. These data have previously been collated and evaluated by international regulatory authorities and assessed for their potential risk to human and environmental health. These reviews have concluded that DIDP is not dangerous so should not be classified as hazardous under current EU regulations
2	No significant responses were observed with DIDP in any of the <i>in vitro</i> assays. Taken as a whole, the available data indicate that DIDP does not have significant interactions with the estrogenic or androgenic receptors. It is noteworthy that <i>in vitro</i> data need to be evaluated very carefully as the tests may have involved either substances which for all practical purposes do not exist under <i>in vivo</i> conditions or may have employed non-physiological conditions.
3	These data support the conclusion that DIDP does not cause adverse endocrine effects in <i>in vivo</i> screening studies. DIDP shows no significant adverse effects in the Uterotrophic Assay (for oestrogenic effects), and no consistent significant adverse effects in the Hershberger Assay (for anti-androgenic effects).
4	Sufficient <i>in vivo</i> data exist for DIDP to demonstrate that DIDP does not induce endocrine mediated chronic toxicity to non-reproductive tissues in rodents or non-human primates.
5	Based on the comprehensive 2-generation reproductive studies and the developmental study it can be concluded that DIDP is not an endocrine disruptor as defined by the Weybridge, IPCS and REACH guidance definitions. The adverse health effects mediated via an endocrine mechanism of cryptorchidism, hypospadias, and significant testicular pathology which are seen with DBP in laboratory animals, are not seen with DIDP.

Overall Conclusion - DIDP

There are sufficient data to conclude that DIDP is not an endocrine disrupting substance for mammals when evaluated according to OECD Conceptual Framework and using the commonly recognized definitions of an endocrine disruptor. Available human and mixtures data do not lead to any change in this conclusion.

B.9. Source of references and output from PubMed searches

In addition to already identified references from regulatory submissions including the REACH registration dossier an on-line literature search for published studies was carried out with a search date of 07 October 2010 using the on-line tool "PubMed"¹.

¹ <http://www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed>

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B.9.1. "DIDP and endocrine"

The PubMed search resulted in 6 hits using the search terms "DIDP and endocrine". All 6 references are listed below, in order of publication, most recent first.

All 6 were reviewed. Two included information that was either not relevant to Framework levels 2-5 e.g. non-mammalian data. These were excluded as the focus of this paper is a review of relevant mammalian data. The excluded references are indicated with *italics text*. The references from the PubMed search in bold below are included in the relevant Appendices tables for the appropriate level of the OECD Conceptual Framework, together with other already identified references.

1. **Effects of plasticizers and their mixtures on estrogen receptor and thyroid hormone functions.** Ghisari M, Bonefeld-Jorgensen EC. *Toxicol Lett.* 2009 Aug 25;189(1):67-77
2. *A critical evaluation of the environmental risk assessment for plasticizers in the freshwater environment in Europe, with special emphasis on bisphenol A and endocrine disruption.* Oehlmann J, Oetken M, Schulte-Oehlmann U. *Environ Res.* 2008 Oct;108(2):140-9. Review.
3. **Hershberger assay for antiandrogenic effects of phthalates.** Lee BM, Koo HJ. *J Toxicol Environ Health A.* 2007 Aug;70(15-16):1365-70.
4. **The effects of selected phenol and phthalate derivatives on steroid hormone production by cultured porcine granulosa cells.** Mlynarciková A, Ficková M, Scsuková S. *Altern Lab Anim.* 2007 Mar;35(1):71-7.
5. **Modulation of iodide uptake by dialkyl phthalate plasticisers in FRTL-5 rat thyroid follicular cells.** Wenzel A, Franz C, Breous E, Loos U. *Mol Cell Endocrinol.* 2005 Dec 1;244(1-2):63-71.
6. *Hazard evaluation of diisononyl phthalate and diisodecyl phthalate in a Japanese medaka multigenerational assay.* Patyna PJ, Brown RP, Davi RA, Letinski DJ, Thomas PE, Cooper KR, Parkerton TF. *Ecotoxicol Environ Saf.* 2006 Sep;65(1):36-47. Epub 2005 Jul 18.

B.9.2. Further references

- CSTEE (1998) EU Scientific Committee on Toxicity, Ecotoxicity and the Environment Phthalate migration from soft PVC toys and child-care articles – Data made available since the 16th of June 1998, opinion expressed at the CSTEE 6th plenary meeting, Brussels, 26/27 November 1998.
- CSTEE (2001) Position paper on Margins of Safety (MOS) in human health risk assessment, expressed at the 22nd CSTEE plenary meeting, Brussels, 06/07 March 2001.

Detailed references are provided at each framework level for the specific substance. Useful websites relevant endocrine disruptor national and regional programmes.

- EU/DG Environment website on endocrine disruptors:
http://europa.eu.int/comm/environment/endocrine/index_en.htm
- EU/DG Research website on endocrine disruptor research:
http://europa.eu.int/comm/research/endocrine/activities_dg_en.html
- Japan (Ministry of Environment) Strategic programme on environmental endocrine disruptors:
<http://www.env.go.jp/en/topic/edcs.html>
- US EPA Programme on endocrine disruptors:
<http://www.epa.gov/scipoly/ospendo/index.htm>

Appendix C - Evaluation of endocrine data on DBP

This appendix will collate and review the key scientific data relating to the potential endocrine activity of DBP. In addition to these data already known to the authors a literature search of published literature for the terms "DBP and endocrine" was carried out. Key studies selected from an extensive literature database are tabulated according to the appropriate framework level and a review of these and other relevant references is documented below.

C.1 OECD Conceptual Framework: Level 1 - DBP

The first step to evaluate the potential hazard of an existing chemical is to collate the already existing information about its chemistry, uses and any available toxicological data. These data are then sorted, prioritised and evaluated to screen whether the chemical is a high priority for further investigation.

A summary of the Framework Level 1 endocrine data available for DBP is tabulated below. Detailed comments on these datasets are available in the pages following the table.

Level 1 Endpoints	DBP: CAS RN 84-74-2
1. physical & chemical properties, e.g., MW, reactivity, volatility, biodegradability	<ul style="list-style-type: none"> • ECB (2004). dibutyl phthalate (DBP). Risk Assessment. European Chemicals Bureau, Institute for Health and Consumer Protection, Joint Research Center of the European Commission.
2. human & environmental exposure, e.g., production volume, release, use patterns	<ul style="list-style-type: none"> • ECB (2004). dibutyl phthalate (DBP). Risk Assessment. European Chemicals Bureau, Institute for Health and Consumer Protection, Joint Research Center of the European Commission. • European Chemicals Agency (ECHA), July 2010. Evaluation of New Scientific Evidence Concerning the Restrictions Contained in Annex XVII to Regulation (EC) No. 1907/2006 (REACH). Review of new available information for dibutyl phthalate (DBP). CAS No. 84-74-2, EINECS No. 201-557-4 Review Report. July 2010.
3. hazard, e.g., available toxicological data	<ul style="list-style-type: none"> • ECB (2004). dibutyl phthalate (DBP). Risk Assessment. European Chemicals Bureau, Institute for Health and Consumer Protection, Joint Research Center of the European Commission. • European Chemicals Agency (ECHA) (July 2010). Evaluation of new scientific evidence concerning the restrictions contained in Annex XVII to Regulation (EC) No. 1907/2006 (REACH). Review of new available information for dibutyl phthalate (DBP). • NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Dibutyl phthalate (DBP). National Toxicology Program. NTP CERHR MON. 2000

Table 11: OECD CF Level 1 endocrine data available for DBP

C.1.1. Detailed comments on key publications - DBP

- *European Chemicals Bureau (2004): Workers:* There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account. This conclusion is reached because of: concerns for general systemic toxicity as a consequence of repeated dermal exposure arising from aerosol forming activities, concerns for adverse local effects in the respiratory tract as a consequence of repeated inhalation exposure in all occupational exposure scenarios. It is possible that in some industrial premises adequate worker protection measures are already being applied. Consumers: There is at present no need for further information or testing or risk reduction measures beyond those which are being applied already. As part of the EU Risk Assessment process classification was reviewed by the EU regulatory committees with the conclusion that classification as a Category 2 Reproductive agent is appropriate (Dangerous Substances Directive, 67/548/EEC). This was based on a developmental study in the rat showing malformations (Category 2 Developmental) and a 2-generation study in rats showing male reproductive

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effects (Category 3 Fertility). Under the Classification, Labelling and Packaging Regulation the equivalent classification is Category 1B Reproductive Agent

- *NTP-CERHR Monograph (2006)*: Based on the report developed by the expert panel, NTP concluded: there is some concern for adverse developmental effects at high exposures and negligible concern of adverse effects on reproduction in adult.
- *ECHA (2010): European Chemicals Agency (ECHA), July 2010*. ECHA focuses on new exposure information in this report and highlights the LOAEL for embryotoxicity from the EU Risk Assessment Report of 52 mg/kg/day. Reference is also made to a Danish EPA (2009) report which quotes a LOAEL of 2 mg/kg/day for effects on "gametes and mammary tissue". However no original reference is provided for this suggested LOAEL.

C.1.2. Conclusions: Level 1 - DBP

DBP has an extensive toxicological dataset available. These data have previously been collated and evaluated by international regulatory authorities and assessed for their potential risk to human and environmental health. These reviews have concluded that classification for developmental or reproductive toxicity is indicated according to the general classification and labelling requirements for dangerous substances and preparations (Directive 67-548-EEC) (R61 Category 2 Developmental Effects and R62 Category 3 Fertility Effects) and the classification, labelling, and packaging (CLP) regulation (EC) No 1272/2008 Annex VI (Reproductive Toxin Category 1B).

C.2. OECD Conceptual Framework: Level 2 - DBP

The second step to evaluate the potential hazard of an existing chemical is to summarise available and suitable toxicological data generated in non-animal test systems e.g. using *in vitro* and / or *in silico* models. The purpose of this summary is to enable an expert to examine the existing *in vitro* assays to investigate detailed mechanistic data.

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A summary of the Level 2 endocrine data available for DBP is tabulated below. Detailed comments on these datasets are available below.

Level 2 Endpoints	DBP: CAS RN 84-74-2
4. ER, AR, TR receptor binding affinity	<ul style="list-style-type: none"> • Akahori, Y; Nakai, M; Yakabe, Y; et al. (2005) Two-step models to predict binding affinity of chemicals to the human estrogen receptor α by three-dimensional quantitative structure-activity relationships (3D-QSARs) using receptor-ligand docking simulation. SAR & QSAR in Environmental Research 16(4):323–337. • Ghisari, M; Bonefeld-Jorgensen, EC. (2009) Effects of plasticizers and their mixtures on estrogen receptor and thyroid hormone functions. Toxicol Lett 189(1):67–77. • Harris CA, Henttu P, Parker MG, Sumpter JP. The estrogenic activity of phthalate esters in vitro. EHP. 1997 Aug;105(8):802-11. • Takeuchi, S; Iida, M; Kobayashi, S; et al. (2005) Differential effects of phthalate esters on transcriptional activities via human estrogen receptors α and β, and androgen receptor. Toxicology 210(2–3):223–233. • Zacharewski TR, Meek MD, Clemons JH, Wu ZF, Fielden MR, Matthews JB. Examination of the in vitro and in vivo estrogenic activities of eight commercial phthalate esters. Toxicol Sci. 1998 Dec;46(2):282-93.
5. High Through Put Prescreens	This framework does not require relevant data for all sections of a particular level to draw a valid conclusion, especially when Level 5 data are available.
6. Transcriptional activation	<ul style="list-style-type: none"> • Ghisari M, Bonefeld-Jorgensen EC. Effects of plasticizers and their mixtures on estrogen receptor and thyroid hormone functions. Toxicol Lett. 2009 Aug 25;189(1):67-77.
7. Thyroid function	<ul style="list-style-type: none"> • Breous, E; Wenzel, A; Loos, U. (2005) The promoter of the human sodium/iodide symporter responds to certain phthalate plasticisers. Mol Cell Endocrinol 244(1–2):75–78. • Ghisari, M; Bonefeld-Jorgensen, EC. (2009) Effects of plasticizers and their mixtures on estrogen receptor and thyroid hormone functions. Toxicol Lett 189(1):67–77. • Wenzel, A; Franz, C; Breous, E; et al. (2005) Modulation of iodide uptake by dialkyl phthalate plasticisers in FRTL-5 rat thyroid follicular cells. Mol Cell Endocrinol 244(1–2):63–71.
8. Aromatase and steroidogenesis in vitro	This framework does not require relevant data for all sections of a particular level to draw a valid conclusion, especially when Level 5 data are available.
9. Fish hepatocyte VTG assay	Not relevant to this assessment of mammalian data
10. Aryl hydrocarbon receptor recognition/binding	<ul style="list-style-type: none"> • Kruger, T; Long, M; Bonefeld-Jorgensen, EC. (2008) Plastic components affect the activation of the aryl hydrocarbon and the androgen receptor. Toxicology 246(2–3):112–123.
11. QSARs	<ul style="list-style-type: none"> • Akahori, Y; Nakai, M; Yakabe, Y; et al. (2005) Two-step models to predict binding affinity of chemicals to the human estrogen receptor α by three-dimensional quantitative structure-activity relationships (3D-QSARs) using receptor-ligand docking simulation. SAR & QSAR in Environmental Research 16(4):323–337. • Akahori Y, Nakai M, Yamasaki K, Takatsuki M, Shimohigashi Y, Ohtaki M. Relationship between the results of in vitro receptor binding assay to human estrogen receptor alpha and in vivo uterotrophic assay: comparative study with 65 selected chemicals. Toxicol In vitro. 2008 Feb;22(1):225-31.
12. Others (as appropriate)	<ul style="list-style-type: none"> • Turan N, Waring RH, Ramsden DB. The effect of plasticisers on "sulphate supply" enzymes. Mol Cell Endocrinol. 2005 Dec 1;244(1-2):15-9 • Harris R, Turan N, Kirk C, Ramsden D, Waring R. Effects of endocrine disruptors on dehydroepiandrosterone sulfotransferase and enzymes involved in PAPS synthesis: genomic and nongenomic pathways. Environ Health Perspect. 2007 Dec; 115 Suppl 1:51-4.

Table 12: OECD CF Level 2 endocrine data available for DBP

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C.2.1. Detailed comments on key publications - DBP

- *Akahori et al., (2005): abstract only.* Models indicated that phthalates show weak if any affinity for binding to the human ER α receptor. Unclear whether DBP was one of the phthalates tested.
- *Akahori et al., (2008):* Investigated the relationship between the *in vitro* ER binding and *in vivo* uterotrophic assays. The authors compared the results from these assays for 65 chemicals spanning a variety of chemicals classes. Results indicate that DBP does not have estrogenic/ anti-estrogenic properties.
- *Breous et al., (2005):* Investigated possible effects DBP on the transcriptional activity of sodium/iodide symporter (NIS) which mediates the active transport of I⁻ in the thyroid. DBP appeared to down-regulate hNIS.
- *Ghisari et al., (2009):* Investigated *in vitro* the thyroid hormone-like and estrogenic activities of a range of widely used plasticizers. The TH disrupting potential was determined by the effect on the TH-dependent rat pituitary GH3 cell proliferation (T-screen). The estrogenic activities of the compounds were assessed in MVLN cells, stably transfected with an estrogen receptor (ER) luciferase reporter vector. It should also be noted that these were tests of phthalate diesters whereas under *in vivo* conditions the diesters are metabolized to monoesters which are not hormone receptor agonists. Therefore, *in vitro* data need to be evaluated very carefully as the tests may have involved either substances which for all practical purposes do not exist under *in vivo* conditions or may have employed non-physiological conditions.
- *Harris et al., (1997):* A series of phthalate esters, including DBP, were screened for estrogenic activity using a recombinant yeast screen. The recombinant yeast screen, a gene for a human estrogen receptor was integrated into the main yeast genome and was expressed in a form capable of binding to estrogen response elements, controlling the expression of the reporter gene lac-Z (when receptor is activated, lac-Z is expressed). DBP showed no effects in any of the screens performed. It should also be noted that these were tests of phthalate diesters. Under *in vivo* conditions the diesters are metabolized to monoesters which are not estrogen receptor agonists. The *in vitro* data need to be evaluated very carefully as the tests may have involved either substances which for all practical purposes do not exist under *in vivo* conditions or may have employed non-physiological conditions.
- *Kruger et al., (2008):* The effects of DBP on the aryl hydrocarbon receptor (AhR) and the androgen receptor (AR) were assessed using luciferase reporter gene. DBP had no significant effect on either assay.
- *Takeuchi et al., (2005):* compared the ER and AR binding potential of 22 different phthalates. DBP did not show significant binding for either receptor.
- *Wenzel et al., (2005):* Investigated possible effects of DBP on the uptake of iodide in a thyroid cell line. Results indicated that high concentrations (10⁻⁴M) of some phthalates enhanced iodide uptake but the biological relevance of this weak effect is not clear. .
- *Zacharewski et al., (1998):* The estrogenic activities of DBP were investigated *in vitro* using estrogen receptor (ER) competitive ligand-binding and mammalian- and yeast-based gene expression assays. DBP exhibited weak ER-mediated activity.

C.2.2. Conclusions: Level 2 - DBP

No significant responses were observed with DBP in any of the *in vitro* assays. Taken as a whole, the available data indicate that DBP does not have significant interactions with estrogenic and androgenic receptors. It is noteworthy that *in vitro* data need to be evaluated very carefully as the tests may have involved either substances which for all practical purposes do not exist under *in vivo* conditions or may have employed non-physiological conditions. Evaluation of Level 2 data are indicative of possible mechanisms of action and are not sufficient to provide conclusive evidence on the potential endocrine activity of a chemical *in vivo*. As specified by the OECD Conceptual Framework further data are needed to support conclusions on endocrine activity and these are considered in the next levels of the framework.

C.3. OECD Conceptual Framework: Level 3 - DBP

The third step to evaluate the potential hazard of an existing chemical is to summarise available and suitable toxicological data generated in animal test systems that are limited in that they are

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only able to investigate single endpoint mechanisms and effects e.g. Hershberger assay (androgenic related) or Uterotrophic assay (estrogenic related). The purpose of this summary is to enable an expert to examine existing *in vivo* assays to investigate detailed biological activity.

A summary of the Level 3 endocrine data available for DBP is tabulated below. For the purposes of this review, only key data relevant to mammalian systems has been collated and tabulated. Comments on these datasets are available below.

Level 3	DBP: CAS RN 84-74-2
1. Uterotrophic assay (estrogenic related)	<ul style="list-style-type: none"> • Akahori Y, Nakai M, Yamasaki K, Takatsuki M, Shimohigashi Y, Ohtaki M. Relationship between the results of <i>in vitro</i> receptor binding assay to human estrogen receptor alpha and <i>in vivo</i> uterotrophic assay: comparative study with 65 selected chemicals. <i>Toxicol In vitro</i>. 2008 Feb;22(1):225-31. • Zacharewski TR, Meek MD, Clemons JH, Wu ZF, Fielden MR, Matthews JB. Examination of the <i>in vitro</i> and <i>in vivo</i> estrogenic activities of eight commercial phthalate esters. <i>Toxicol Sci</i>. 1998 Dec;46(2):282-93.
2. Hershberger assay (androgenic related)	<ul style="list-style-type: none"> • Lee BM, Koo HJ. Hershberger assay for antiandrogenic effects of phthalates. <i>J Toxicol Environ Health A</i>. 2007 Aug; 70(15-16):1365-70. 2009 Jul;28(1):66-74.
3. Non-receptor mediated hormone function	<ul style="list-style-type: none"> • This framework does not require relevant data for all sections of a particular level to draw a valid conclusion, especially when Level 5 data are available.
4. Others (e.g. thyroid)	<ul style="list-style-type: none"> • This framework does not require relevant data for all sections of a particular level to draw a valid conclusion, especially when Level 5 data are available

Table 13: OECD CF Level 3 endocrine data available for DBP

C.3.1. Detailed comments on key publications - DBP

- *Akahori et al., (2008)*: investigates the relationship between the *in vitro* ER binding and *in vivo* uterotrophic assays by determining the meaningful binding potency from the ER binding assay by comparing the results from these assays for 65 chemicals spanning a variety of chemicals classes. Results indicate that DBP does not have estrogenic/ anti-estrogenic properties.
- *Lee et al., (2007)*: Reported a study designed similar to the Hershberger bioassay screen to test the antiandrogenic properties of DBP. DBP significantly decreased ventral prostate weight.
- *Zacharewski et al., (1998)*: The estrogenic activities of DBP were investigated. In an *in vivo* study, 20, 200, 2,000 mg/kg/d of DBP was administered by oral gavage once daily for a period of 4 days to ovariectomised rats. DBP did not produce any reproducible, dose-dependant effect on uterine wet weight relative to vehicle control at any of the dose tested. DBP did not induce a vaginal cornification response at any of the doses tested. Accordingly, it can be concluded that DBP is not estrogenic under *in vivo* conditions.

C.3.2. Conclusions: Level 3 - DBP

Taken as a whole, these data support the conclusion that DBP does not modulate estrogenic endocrine systems, but Lee et al (2007) conclude that DBP does modulate the androgenic endocrine system. DBP and its major metabolite MBP are devoid of estrogenic activity *in vitro*; they show no ability to bind to rodent or human estrogen receptors or to induce estrogen receptors-mediated gene expression. *In vivo* assays demonstrated that DBP does not increase uterine wet weight or does not give rise to vaginal epithelial cell cornification.

C.4. OECD Conceptual Framework: Level 4 - DBP

The fourth step to evaluate the potential hazard of an existing chemical is to summarise available and suitable toxicological data generated in animal test systems that can inform the reviewer on multiple endpoint mechanisms and effects e.g. Male and female pubertal assays. The purpose

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of this summary is to enable an expert to examine existing *in vivo* assays to investigate multiple endocrine mechanisms and effects.

A summary of the Level 4 endocrine data available for DBP is tabulated below. For the purposes of this review, only available data relevant to mammalian systems has been collated and tabulated. Comments on these datasets are available below.

Level 4	DBP: CAS RN 84-74-2
1. enhanced 407 (endpoints based on endocrine mechanisms)	<ul style="list-style-type: none"> Kwack SJ, Kim KB, Kim HS, Lee BM. (2009) Comparative Toxicological Evaluation of Phthalate Diesters and Metabolites in Sprague-Dawley Male Rats for Risk Assessment Toxicity of Phthalate Diesters and Metabolites. <i>Journal of Toxicology and Environmental Health, Part A</i>, 72: 1446–1454.
2. Male and female pubertal assays	<ul style="list-style-type: none"> This framework does not require relevant data for all sections of a particular level to draw a valid conclusion, especially when Level 5 data are available
3. Adult intact assay	<ul style="list-style-type: none"> O'Connor, J. C., Frame, S. R., and Ladics, G. S. (2002). Evaluation of a 15-Day Screening Assay Using Intact Male Rats for Identifying Antiandrogens. <i>Toxicological Sciences</i> 69, 92-108.
4. Other	<ul style="list-style-type: none"> ECB (2008) EU Risk Assessment on DBP addresses several studies relating to the testicular toxicity of DBP:

Table 14: OECD CF Level 4 endocrine data available for DBP

C.4.1. Detailed comments on key publications - DBP

- Kwack et al., (2009)*: Comparative assessment of the systemic toxicity and sperm parameters for DBP having administered orally to Sprague- Dawley male rats at 500 mg/kg body weight (bw)/d for 4 weeks by oral gavage. Liver weights were significantly increased in groups treated with DBP, BBP, DIDP, DINP, MEHP, and MBuP compared to the control. Testes weights were significantly reduced only in DBP, BBP, and MEHP-treated groups compared to the control. Significant decreases in red blood cell (RBC) and hematocrit (Ht) levels were observed in DBP-treated rats, whereas significant increases in mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet (PLT) levels were found in the DBP-treated group. Hemoglobin (Hb) level was reduced only in the DMP group. Similar to effects on testis and epididymal weights, DBP and MEHP significantly reduced sperm numbers and motility.
- O'Conner et al (2002)*: An *in vivo* screening assay using intact adult male rats has been evaluated for its ability to detect six antiandrogenic compounds via oral administration. The test compounds included cyproterone acetate (CPA), flutamide (FLUT), p,p'-DDE (DDE), di-n-butyl phthalate (DBP), linuron (LIN), and vinclozolin (VCZ). Male rats were dosed for 15 days via oral gavage and euthanized on the morning of test day 15. DBP, a compound with antiandrogen-like activity via a nonreceptor mediated mechanism, caused hormonal alterations (decreased T, DHT, and E2; increased LH, FSH, and PRL) and induced general testicular degeneration.
- ECB (2008) EU Risk Assessment on DBP*. Rats showed characteristic testicular changes after repeated oral exposure to DBP. In special studies examining these testicular effects in rats the lowest tested dose-level of 250 mg/kg bw induced already changes in testicular enzymes associated with degeneration of spermatogenic cells and histopathology showed testicular degeneration in 5% of tubules at this dose-level (Srivastava et al., 1990). At doses of 500 mg/kg bw and higher decreases in weight of testes (atrophy) and accessory sex glands, decreased numbers of spermatocytes, degeneration of the seminiferous tubules of the testes, a reduction in testicular zinc levels and serum testosterone levels, increases in testosterone levels in the testes and an increase in urinary zinc excretion were observed (Cater et al., 1977; Gray et al., 1982, 1983; Oishi and Hiraga, 1980b; Srivastava et al.1990).

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C.4.2. Conclusions: Level 4 - DBP

ECB (2008) concludes that DBP causes effects on the testes of laboratory animals in repeat dose studies.

C.5. OECD Conceptual Framework: Level 5 - DBP

The fifth and final step to evaluate the potential hazard of an existing chemical is to summarise available and suitable toxicological data generated in all whole animal test systems that can together inform the reviewer on all endpoint mechanisms and effects e.g. Multi-generation assay. The purpose of this summary is to enable an expert to examine these robust *in vivo* assays to investigate all endocrine mechanisms and other mechanisms.

A summary of the Level 5 endocrine data available for DBP is tabulated below. It should be noted that it is not a requirement of the OECD Conceptual Framework that data is available for all sections within a Level. For the purposes of this review, only available data relevant to mammalian systems has been collated and tabulated. Comments on these datasets are available below.

Level 5 Endpoints	DBP: CAS RN 84-74-2
1. 2-generation assay (TG416 enhanced)	<ul style="list-style-type: none"> • Mylchreest E et al. (1998). Male reproductive tract malformations in rats following gestational and lactational exposure to di(n-butyl) phthalate: an antiandrogenic mechanism? <i>Toxicol. Sci.</i> 43, 47-60. • NTP (1995). National Toxicology Program. Toxicity Report Series Number 30. by DS Marsman. NTP Technical Report on toxicity studies of dibutyl phthalate (CAS No. 84-74-2). Administered in feed to F344/N rats and B6C3F1 mice. NIH Publication 95-3353. US Department of Health and Human Services. Public Health Service. National Institutes of Health. Dated April 1995. • ECB (2004). dibutyl phthalate (DBP). Risk Assessment. European Chemicals Bureau, Institute for Health and Consumer Protection, Joint Research Center of the European Commission.
2. reproductive screening test (TG421 enhanced)	<ul style="list-style-type: none"> • This framework does not require relevant data for all sections of a particular level to draw a valid conclusion, especially when Level 5 data are available – see 2-generation study above.
3. combined 28 day/ reproduction screening Test (TG 422 enhanced)	<ul style="list-style-type: none"> • This framework does not require relevant data for all sections of a particular level to draw a valid conclusion, especially when Level 5 data are available.– see 2-generation study above.
4. Other	<ul style="list-style-type: none"> • ECB (2004). dibutyl phthalate (DBP). Risk Assessment. European Chemicals Bureau, Institute for Health and Consumer Protection, Joint Research Center of the European Commission. • NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Dibutyl phthalate (DBP). National Toxicology Program. NTP CERHR MON. 2000

Table 15: OECD CF Level 5 endocrine data available for DBP

C.5.1. Detailed comments on key publications - DBP

- *Mylchreest et al (1998)*: Groups of 10 pregnant CD rats (Sprague-Dawley) received by gavage 0, 250, 500 or 750 mg DBP (purity 99.8%)/kg bw in corn oil from gestation day 3 throughout pregnancy and lactation until the offspring were at postnatal day 20 with a 2-day interruption at parturition and on the following day (postnatal day 1-2). Dams were killed at weaning (postnatal day 21). Pups were killed at sexual maturity (postnatal day 100-105). In male offspring at birth anogenital distance was decreased at 500 and 750 mg/kg bw and at sexual maturity a dose-dependent increase in the incidence of malformations of internal and external genitalia was observed at all dose-levels. Hypospadias were observed in 3, 21 and 43% of males at 250, 500 and 750 mg/kg bw, respectively. Underdeveloped or absent

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epididymis, frequently bilaterally, was observed in 9, 50 and 70% of the males at 250, 500 and 750 mg/kg bw, respectively, and was associated with atrophy of seminiferous tubules (50-100% of tubules affected in all treated groups) and abnormal or reduced spermatogenesis. At 500 and 750 mg/kg bw seminal vesicles were not developed or their weight was decreased by 16 and 32%, respectively. Mean weight of the prostate gland was decreased by 27% at 750 mg/kg bw. One animal from each of 500 and 750 mg/kg group had no prostate at postmortem examination. An increased incidence of dilated renal pelvis was observed in male offspring at all dose-levels. Mean kidney weight was significantly decreased at 750 mg/kg bw. In female offspring DBP treatment had little effect on development of the reproductive system. A NOAEL cannot be established in this study. The results of this study suggested that DBP does not possess estrogenic activity but rather shows antiandrogenic activity at these dose-levels.

- *NTP, (1995)*: In an oral reproduction study in Sprague-Dawley rats according to the continuous breeding protocol and including the production of two generations, doses of 0, 0.1, 0.5 and 1.0% in diet (0, 52, 256 and 509 mg/kg bw for males and 0, 80, 385 and 794 mg/kg bw for females) were administered to groups of 20 m and 20 f animals. In male F1 parents at 1.0% body wt. and rel. wts of all reproductive organs were lower while rel. liver and kidney wts were statistically significantly increased. Epididymal sperm count and testicular spermatid head count were statistically significantly decreased at 1.0%. Epididymides were absent or poorly developed in 12/20 F1 males at 1.0% and in 1/20 F1 males at both lower dosage levels. In 4/20 males at 1.0% and 1/20 at 0.5% in diet testicular atrophy was seen. Testes of 3/20 males at 1.0% were not descended into the scrotal sacs; 4/20 males at this dose-level had poorly developed seminal vesicles and 4/20 had an underdeveloped prepuce or penis. Histopathology showed degeneration of seminiferous tubules in 8/10 F1 males at 1.0% and in 3/10 at 0.5% DBP in the diet. 7/10 F1 males at 1.0% revealed testicular interstitial cell hyperplasia. Histopathology of seminal vesicles revealed in 1/10 F1 males at 1.0% vesiculitis with inspissated secretion. There was no indication of an effect on estrous cyclicity or duration of the estrous cycles in F1 females at all dose-levels. In this study DBP appeared to be a reproductive toxicant in rats exposed both as adults and during development. The effects on the 2nd generation were greater than on the first generation. The lowest dose-level in this study, 0.1% in the diet (52 mg/kg bw for males; 80 mg/kg bw for females) is a LOAEL for embryotoxicity. The NOAEL for maternal toxicity is 0.5% in the diet (385 mg/kg bw).

C.5.2. Conclusions: Level 5 - DBP

Based on a range of developmental and one and two generation studies in laboratory animals the EU Risk Assessment Report for DBP concludes that DBP causes teratogenicity and effects on fertility such that DBP is classified as a Category 2 Developmental Agent and a Category 3 Fertility Agent under the EU Dangerous Substances Directive (67/548/EC). This conclusion was confirmed by the relevant EU Technical Progress Committee at the time. The EU Risk Assessment also concluded that certain effects were indicative of an anti-androgenic mechanism of action. The adverse health effects mediated via an endocrine mechanism of cryptorchidism, hypospadias, and significant testicular pathology are seen in laboratory animals. Under the Classification, Labelling and Packaging Regulation the equivalent classification is Category 1B.

C.6. Human data - DBP

Human data are not included in the OECD CF as the OECD does not include human tests among its Guidelines for the testing of chemicals. However, if relevant data are available, it is appropriate to include them in the overall assessment of the toxicity of the chemical of interest. The report of Swan *et al.*, 2005 investigated the link between metabolites of certain phthalates (DMP, DEP, DBP, BBP, DiBP, DnOP, DEHP) in the urine of pregnant women and changes in the reproductive systems of the male infants they later gave birth to. The author was careful to state that no adverse health effect was detected just changes in the infants' ano-genital distance (AGD) index, a correction of ano-genital distance (AGD) of age and size of the infant at time of measurement. Contrary to media reports, she reported no correlation between changes in penis size and maternal phthalate exposure, but only to the changes in the AGD measure. In reviewing this data the EU Scientific Committee for Emerging and Newly Identified Health Risks (SCENIHR) concluded that the data was insufficient as solid evidence for an effect. In 2008

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Swan presented a revised mathematical analysis for measuring changes in AGD and applied this new methodology to a further population of infant boys. The new dataset resulted in contradictory results to some of the previous findings.

C.7. Mixtures Studies with DBP

- Ghisari, M., and Bonefeld-Jorgensen, E. C. (2009). Effects of plasticizers and their mixtures on estrogen receptor and thyroid hormone functions. *Toxicol Lett* 189, 67-77.
- Howdeshell, K. L., Furr, J., Lambright, C. R., Rider, C. V., Wilson, V. S., and Gray, L. E., Jr. (2007). Cumulative effects of dibutyl phthalate and diethylhexyl phthalate on male rat reproductive tract development: altered fetal steroid hormones and genes. *Toxicol Sci* 99, 190-202.
- Howdeshell, K. L., Wilson, V. S., Furr, J., Lambright, C. R., Rider, C. V., Blystone, C. R., Hotchkiss, A. K., and Gray, L. E., Jr. (2008). A mixture of five phthalate esters inhibits fetal testicular testosterone production in the sprague-dawley rat in a cumulative, dose-additive manner. *Toxicol Sci* 105, 153-165.
- Martino-Andrade, A. J., Morais, R. N., Botelho, G. G., Muller, G., Grande, S. W., Carpentieri, G. B., Leao, G. M., and Dalsenter, P. R. (2009). Coadministration of active phthalates results in disruption of foetal testicular function in rats. *Int J Androl* 32, 704-712.
- Rider, C. V., Furr, J., Wilson, V. S., and Gray, L. E., Jr. (2008). A mixture of seven antiandrogens induces reproductive malformations in rats. *Int J Androl* 31, 249-262.
- Rider, C. V., Furr, J. R., Wilson, V. S., and Gray, L. E., Jr. (2010). Cumulative effects of in utero administration of mixtures of reproductive toxicants that disrupt common target tissues via diverse mechanisms of toxicity. *Int J Androl* 33, 443-462.
- Rider, C. V., Wilson, V. S., Howdeshell, K. L., Hotchkiss, A. K., Furr, J. R., Lambright, C. R., and Gray, L. E., Jr. (2009). Cumulative effects of in utero administration of mixtures of "antiandrogens" on male rat reproductive development. *Toxicol Pathol* 37, 100-113.

Some of these studies are suggestive of a possible additive effect of LMW phthalates.

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C.8. Conclusions - DBP

The key conclusions from each level of the OECD Conceptual Framework for DBP are:

OECD CF Level	Conclusions DBP
1	DBP has an extensive toxicology dataset available. These data have previously been collated and evaluated by international regulatory authorities and assessed for their potential risk to human and environmental health. Based on these data, classification for developmental or reproductive toxicity was concluded by the EU Risk Assessment and Regulatory Committees according to the general classification and labelling requirements for dangerous substances and preparations (Directive 67-548-EEC) (R61 Category 2 and R62 Category 3) and the classification, labelling, and packaging (CLP) regulation (EC) No 1272/2008 Annex VI (Reproductive Toxin Category 1B)
2	No significant responses were observed with DBP or MBP in any of the <i>in vitro</i> assays. Taken as a whole, the available data indicate that neither DBP nor MBP have significant interactions with estrogenic or androgenic receptors. It is noteworthy that <i>in vitro</i> data need to be evaluated very carefully as the tests may have involved either substances which for all practical purposes do not exist under <i>in vivo</i> conditions or may have employed non-physiological conditions.
3	Taken as a whole, DBP does not modulate estrogenic receptors but Lee et al (2007) conclude that DBP does modulate the androgenic endocrine system. DBP and its major metabolite MBP are devoid of estrogenic activity <i>in vitro</i> ; they show no ability to bind to rodent or human estrogen receptors or to induce estrogen receptors-mediated gene expression. <i>In vivo</i> assays demonstrated that DBP does not increase uterine wet weight and does not give rise to vaginal epithelial cell cornification.
4	ECB (2003) concludes that DBP causes effects on the testes of laboratory animals in repeat dose studies.
5	Based on a range of developmental and one and two generation studies in laboratory animals the EU Risk Assessment Report for DBP concludes that DBP causes teratogenicity and effects on fertility such that DBP is classified as a Category 2 Developmental Agent and a Category 3 Fertility Agent under the EU Dangerous Substances Directive (67/548/EC). This conclusion was confirmed by the relevant EU Technical Progress Committee at the time. The EU Risk Assessment also concluded that certain effects were indicative of an anti-androgenic mechanism of action. The adverse health effects mediated via an endocrine mechanism of cryptorchidism, hypospadias, and significant testicular pathology are seen in laboratory animals. Under the Classification, Labelling and Packaging Regulation the equivalent classification is Category 1B

Overall Conclusion

Based on a range of developmental and one and two generation studies in laboratory animals the EU Risk Assessment Report for DBP concludes that DBP causes teratogenicity and effects on fertility such that DBP is classified as a Category 2 Developmental Agent and a Category 3 Fertility Agent under the EU Dangerous Substances Directive (67/548/EC). This conclusion was confirmed by the relevant EU Technical Progress Committee at the time. The EU Risk Assessment also concluded that certain effects were indicative of an anti-androgenic mechanism of action. The adverse health effects mediated via an endocrine mechanism of cryptorchidism, hypospadias, and significant testicular pathology are seen in laboratory animals. Under the Classification, Labelling and Packaging Regulation the equivalent classification is Category 1B

C.9. "DBP and endocrine"

Source of references and output from PubMed searches

In addition to already identified references from regulatory submissions including the REACH registration dossier an on-line literature search for published studies was carried out with a search date of 16 December 2010 using the on-line tool "PubMed"¹. Only the key references have been included in the tables in the Appendix.

The PubMed search using the search terms "DBP and endocrine".

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C.9.1. Detailed references are provided at each framework level for the specific substance. Useful websites relevant to endocrine disruptor national and regional programmes.

- EU/DG Environment website on endocrine disruptors:
http://europa.eu.int/comm/environment/endocrine/index_en.htm
- EU/DG Research website on endocrine disruptor research:
http://europa.eu.int/comm/research/endocrine/activities_dg_en.html
- Japan (Ministry of Environment) Strategic programme on environmental endocrine disruptors:
<http://www.env.go.jp/en/topic/edcs.html>

US EPA Programme on endocrine disruptors:
<http://www.epa.gov/scipoly/oscpendo/index.htm>