Expert Review:

Comparison of potential endocrine disrupting properties of di-isononyl phthalate (DINP), di-isodecyl phthalate (DIDP), and di-n-butyl phthalate (DNBP)

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Executive Summary

This review identifies differences between di-isononyl phthalate (DINP), di-isodecyl phthalate (DIDP), and di-n-butyl phthalate (DNBP) in reproductive effects mediated by an endocrine mechanism. "Endocrine disruptors" and "adverse effects" in the context of this review are defined as by WHO/IPCS. Adverse effects of DNBP on the endocrine system were seen both in repeated-dose toxicity and in reproductive toxicity studies. Although mechanistic data are not totally consistent, *in vivo* studies suggest that the adverse effects of DNBP on reproductive parameters are due to anti-androgenic mechanisms. Thus, DNBP is an "endocrine disruptor" in the rat. After oral exposure to DINP, adverse effects on robust endpoints indicative of reproductive toxicity or impaired fertility were not detected in repeated-dose and in reproductive toxicity studies. DIDP did not affect fertility or induce clear-cut adverse reproductive effects in rats in one- and two-generation studies. Therefore, while DNBP is a reproductive toxicant with a mode of action compatible with endocrine mediated mechanisms, both DINP and DIDP do not cause **adverse** effects on the male reproduction tract in intact organisms and thus are not "endocrine disruptors".

Using the ECHA and ECETOC Guidance on Assessment Factors, DNEL values for women of child-bearing age were calculated as 1.4 mg/kg bw/day for DINP (based on NOEL of 56 mg/kg bw/day) from recent studies, and 0.95 mg/kg bw/day for DIDP.

Contents

Definitions of Endocrine Disruptors

Chemicals with "endocrine disrupting" properties are targeted in REACH (EC 1907/2006). Identification as "endocrine disrupters" (EDs) based on the criteria in article 57(f)[*] may result in inclusion in the list of "substances of very high concern". A number of definitions have been proposed during the past two decades to establish what characterizes an endocrine disruptor (Kavlock *et al.*, 1996; Weybridge, 1996; NRDC, 1998; EDSTAC, 1998), however, at the present time there is no clear set of criteria by which to identify EDs for regulatory purposes. This review uses the widely accepted definition *"An ED is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes **adverse** effects in an **intact organism**, or its progeny,…."* (WHO/IPCS, 2002). This definition contains two key elements: **adversity** and **observations in intact organisms**. An adverse effect is a *"change in the morphology, physiology, growth, development, reproduction, or life span of an organism, … that results in an **impairment of functional capacity**,….."* (WHO/IPCS, 2004). Consequently, *"Contrasted to adverse effects, nonadverse effects can be defined as .. biological effects that do not cause biochemical, morphological, or physiological changes that affect the general well-being, growth, development or life span …."* (Lewis *et al.*, 2002). Thus, a potential for perturbation of endocrine homeostasis such as *in vitro* interactions of a chemical with hormone receptors or small and temporary changes in hormone levels not resulting in structural or functional changes are not adverse and are not indicators of toxicity. In addition, short-term *in vivo* screens for endocrine activity also only indicate a potential for a pharmacological response rather than an adverse effect (Karbe *et al.*, 2002; BfR, 2011, OECD, 2009). Therefore, endocrine perturbation is a mode of action and not a toxicological endpoint (BfR, 2011). In consequence, any evaluation of EDs needs to start with the assessment of "adverse effects potentially related to ED in intact organisms". In the absence of such effects, a chemical is "not an ED for regulatory purposes" (COT, 2010). ED-mediated toxicity can be detected in repeated-dose toxicity studies and in the assessment of reproductive and developmental toxicity; a detailed evaluation of such studies and mechanistic studies are necessary to establish a relationship between an observed adverse effect and an ED mode of action (OECD, 2002, 2010).

In the following, available toxicological and mode of action data on di-n-butyl phthalate (DNBP), di-isononyl phthalate (DINP), and di-isodecyl phthalate (DIDP) are reviewed regarding adverse effects on the endocrine system in intact animals.

Does exposure to DNBP, DINP or DIDP result in adverse effects on the endocrine system?

Many assessments of DNBP, DINP, and DIDP were performed (NICNAS, 2008a; NICNAS, 2008b; NICNAS, 2008c; ECB, 2003a; ECB, 2003b; ECB, 2004; EFSA, 2005a; EFSA, 2005b; EFSA, 2005c; SCCP, 2007; SCHER, 2008; CSTEE, 2001a; CSTEE, 2001b; CSTEE, 2001c; COT, 2011; ATSDR, 2001; NTP-CERHR, 2000; NTP-CERHR, 2003a; NTP-CERHR, 2003b; US CPSC, 2010a; US CPSC, 2010b; US CPSC, 2010c; US EPA, 2006). This review integrates these documents and more recent information.

Di-n-butyl phthalate (DNBP)

Adverse effects of DNBP (also commonly referred to as DBP in scientific and other literature) on the endocrine system with the testes as a target organ were seen both in repeat-dose toxicity and in reproductive toxicity studies. When rats were administered DNBP by gavage from gestational day (GD) 13.5 to 20.5 or 21.5, a dose dependent decrease in fertility of the male offspring was noted at doses > 20 mg/kg bw/day. Ninety percent of the animals at 500 mg/kg bw/day showed cryptorchidism and a significant decrease in testicular weight at GD 21.5 and in adulthood. At GD 21.5, testicular testosterone was significantly decreased at 100 or 500 mg DNBP/kg bw/day. In testes, a significant increase in occurrence of multinucleated gonocytes and a decrease in Leydig cell number at 100 mg/kg bw/day and higher, and an increase in Leydig cell size at 500 mg/kg bw/day was observed. Malformed seminiferous tubules with intratubular Leydig cells and immature Sertoli cells were statistically significant at 500 mg/kg bw/day.

A study designed to assess the effect of chronic exposure to DNBP on female fertility in rats found a significant decrease in the number of live pups delivered by treated dams which mated with treated or untreated males. Although DNBP did not affect maturation, estrous cyclicity, or percentage of females mating or pregnant, the percentage of females delivering live pups was reduced by more than 50% at 500 mg/kg bw/day and by 90% at 1000 mg/kg bw/day in the absence of maternal toxicity.

[*] „substances - such as those having endocrine disrupting properties or those having persistent, bioaccumulative and toxic properties or very persistent and very bioaccumulative properties, […] for which there is scientific evidence of probable serious effects to human health or the environment"

Consistently, the male reproductive system was the main target with a lowest reported NOAEL of 50 mg/kg bw/day and a LOAEL of 100 mg/kg bw/day for effects on male reproductive development in the F1 generation, based on decreased pup weight and male reproductive tract malformations. In females, there were no treatment-related changes or altered gross morphology.

A developmental toxicity study in the rat (Lee *et al.*, 2004) with dietary exposure to DNBP from GD 15 to PND 21 revealed reduction of testicular spermatocyte development and mammary gland changes at low incidences in both sexes at PND 21 at the lowest dose of 1.5-3.0 mg/kg bw/day, with dose-dependent increases in incidence or/and severity. While EFSA (2005a) did not derive a NOAEL, it should be noted that the effects on mammary glands in male rats observed at the lowest dose most likely reflect an androgenic activity, whereas DNBP is anti-androgenic. Moreover, the testicular effects observed at this dose were reversible and lacked a clear dose-dependence. Also, the apparent LOAEL is much lower than the NOAELs in other developmental studies with DNBP (COT, 2011). Based on a weight of evidence approach, the NOAEL of 14-29 mg/kg bw/day (NICNAS, 2008a) for significant reduction in testicular spermatocyte development, aggregations of Leydig cells and decreased epididymal duct cross section on PND 21 is better supported.

In a 2-generation rat study (continuous breeding protocol), a LOAEL of 52 mg/kg bw/day for embryotoxicity was observed based on reductions in F0 litter size and F2 pup weight. In mice, male fertility was not affected in a one-generation study while female fertility was clearly affected. The NOAEL in this study in mice was 420 mg/kg bw/day in the diet based on effects on maternal fertility and embryotoxicity.

Di-isononyl phthalate (DINP)

Effects on testes weight seen in repeat-dose toxicity studies with DINP in mice or rats were minor and inconsistent. In most studies, histopathological changes in the testes were not observed. DINP had no effect on male mating, female fecundity, and gestational index or length of gestation, with a NOAEL for fertility of 622 mg/kg bw/day. In a two-generation study, DINP produced no changes in live birth index, sex ratio or offspring survival during lactation in the F1 or F2 generations (Waterman *et al.*, 2000). A dose-related decrease in offspring body weight during the postnatal period for F1 high dose males was only seen on PND 0, in males and females of the mid and high-dose levels on PND 7 and 14, and at all doses at PND 21. However, weights remained within historical control range. The LOAEL for developmental effects was estimated to be 159 mg/kg bw/day based on reduced pup weights in F1 and F2 pups during lactation. DINP exposure during early gestation induced skeletal and visceral variations, rudimentary lumbar ribs, supernumerary cervical ribs as well as dilated renal pelves. The NOAEL in a prenatal developmental toxicity study in rats was reported by the study authors as 500 mg/kg bw/day (Waterman *et al.*, 1999) and confirmed in the EU RAR for DINP.

When administered by gavage between GD 14 and PND 3 at a single dose level of 750 mg/kg bw/day (Gray Jr *et al.*, 2000), exposure to DINP was associated with retention of nipples in males and an increase in malformations of the male reproductive tract (from 0/80 animals in controls to 4/52). The dose applied resulted in maternal toxicity as evidenced by a significant decrease in maternal body weight gain.

Administration of a single dose level of 750 mg DINP/kg bw/day by gavage to rats during GD 7-21 resulted in a significant reduction in testicular testosterone content and production at GD 21 in male fetuses (Borch *et al.*, 2004). The authors stated that this dose was not expected to cause maternal toxicity, but parental toxicity seen in other studies with this dose level questions the relevance of the findings (Gray Jr *et al.* 2000; Waterman *et al.*, 2000).

Adamsson *et al.* (2009) investigated the effects of oral gavage of daily doses of 250 or 750 mg/kg bw/day of DINP to rats between GD 13.5 and 17.5 on testicular and adrenal steroidogenesis. Adverse effects on fertility, pup body weight, or testicular testosterone were not seen.

When dosed by gavage from GD 7 to PND 17, pregnant rats tolerated doses of 300, 600, 750, or 900 mg DINP/kg bw/day without overt maternal toxicity (Boberg *et al.*, 2011). Fertility and birth weights were unaffected; however, effects were seen on male AGD (reduced at highest dose), nipple retention in males (increase at 750 mg/kg bw/day and higher), body weight of male pups at PND 13 (reduced at highest dose), and testes histology (at 600 mg/kg bw/day and higher). Effects disappeared at PND 90. Testicular changes consisted of multinucleated germ cells (MNG), an increased number of gonocytes with a central location in seminiferous cords, and significantly increased cord diameters. Testes weights were unaffected when analysed at PND 90. Sperm analysis showed a significant decrease in sperm motility at the 600 and 900 mg/kg doses. Although the authors speculated that DINP does not affect testicular sperm production, but may rather affect epididymal function and sperm maturation, no changes in epididymal histopathology was observed. A NOAEL of 300 mg/kg bw/day for reproductive toxicity was determined.

Two recent studies provide a detailed assessment of the potential of DINP to induce male reproductive toxicity in the rat.

In the first study (Clewell, 2011a), male offspring from pregnant rats administered 0, 50, 250, or 750 mg/kg bw/day DINP via gavage from GD 12 to GD 19. Three robust markers of male reproductive tract development were examined in the male pups from each dose group and concurrent controls: AGD, testosterone concentrations in the fetal testes, and histopathology of the fetal testes. AGD (absolute and body weight adjusted) was not altered by DINP treatment at doses up to 750 mg/kg bw/day. Testosterone concentrations in the testes of male fetuses from the 250 and 750 mg/kg bw/day dose groups were significantly reduced at 2 hr post-dosing. At 24 hrs post-dosing, testosterone levels were not statistically different from controls, indicating that the hormone levels had recovered. Histopathological examination did not reveal effects on the average seminiferous tubule diameter. An increase in the number of multi-nucleated germ cells (MNG) was noted in the testes of rats from the 250 and 750 mg/kg bw/day dose groups. Overall, a clear no observed effect level (NOEL) of 50 mg/kg bw/day was identified for the effects on testosterone and MNG. Analysis of the changes in testosterone concentrations gave a benchmark dose of 120 mg DINP /kg bw/day regarding changes in fetal testosterone (Hamner, 2012) An increased incidence of large Leydig cell aggregates was observed in the highest dose group. Thus, a NOEL of 250 mg/kg bw/day was identified for Leydig cell aggregate formation.

In the second study (Clewell, 2011b), male offspring of rats administered 0, 760, 3 800, or 11 400 ppm DINP, or 7 600 ppm DNBP in the diet from GD 12 to PND 14 were examined on PND 2, 14 and 49 for a number of effects including reduced AGD, nipple retention, testes testosterone inhibition, hypospadias, preputial separation, morphology and tissue weights. Daily target doses were 0, 50, 250, and 750 mg/kg bw/day DINP and 500 mg/kg bw/day DNBP. Estimated effective doses were 56, 288, or 720 mg/kg bw/day DINP or 642 mg/kg bw/day DNBP during gestation, and 109, 555, or 1513 mg/kg bw/day DINP or 1138 mg/kg bw/day DNBP during lactation.

With DINP, pup body weights were reduced in the 750 mg/kg bw/day dose group at PND 2, and in the 250 and 750 mg/kg bw/day dose groups at PND 14. No effects on body weight were seen at PND 49. At PND 2, no change in relative testes or epididymis weights or statistically significant changes in testicular testosterone content were seen in pups. While no change in absolute or scaled AGD or seminiferous tubule diameter was seen at PND 2, a reduced AGD was observed at the highest dose at PND 14. This effect had disappeared by PND 49. No increase in nipple retention and preputial separation was noted. The average preputial separation score indicative of delayed puberty was reduced in animals treated with DNBP, but not with DINP. The increase in animals with multinucleated gonocytes seen at PND 2 with target doses > 250 mg/kg bw/day had disappeared at PND 49, as had the increase in animals with large Leydig cell aggregates observed at the highest dose. Histopathology revealed no significant permanent alterations in epididymal development. While DNBP treatment resulted in malformations of the phallus, testis or epididymis, DINP did not cause malformations in male reproductive organs. In summary, these data indicate a clear NOEL for effects on the developing male reproductive tract in rats of 56-109 mg/kg bw/day, and a lowest observed effect level of 288-555 mg/kg bw/day DINP based on a significant increase in MNG on PND 2 and a decrease in pup body weight on PND 14. The maternal NOEL was determined to be 288-555 mg/kg bw/day based on a decrease in food consumption and body weight observed at the highest dose level.

Di-isodecyl phthalate (DIDP)

There is no indication of effects on reproductive organs from histological observation in repeated dose toxicity studies in rats and in dogs (Cho *et al.*, 2008, NTP-CERHR, 2003b). In one- and two-generation studies, DIDP did not affect fertility in rats. Decreases in postnatal survival indices were observed in two dietary two-generation studies with rats at doses of 0.2% DIDP in feed and higher (Hushka *et al.*, 2001). Increased liver and kidney weights indicative of parental toxicity were found at those dose levels, although no dose-dependent clinical signs of toxicity in either the P_1 males or females were seen. Additionally, cross-fostering satellite groups suggested a contribution of lactational exposure and/or reduced milk quality or quantity to the decreased pup survival. There were no differences in AGD or nipple retention. Skeletal variations (including rudimentary lumbar ribs and supernumerary cervical ribs) were observed in developmental studies by gavage in rats at 1,000 mg/kg bw/day with slight maternal toxicity (Waterman *et al.*, 1999).

Are the observed adverse effects plausibly related to a MoA of endocrine disruption?

Di-n-butyl phthalate (DNBP)

DNBP caused adverse effects on reproductive organs in animals on androgen-mediated endpoints in multiple studies with increased incidences of undescended testes, hypospadias, reproductive organ malformations, and nipple retention in male rats in the absence of maternal toxicity. These are clear adverse effects with NOAELs between 20 to 50 mg/kg bw/day. The pattern of effects seen suggest antiandrogenic activity. *In vitro*, MNBP induced detachment of germ cells from a Sertoli cell monolayer. Although interaction with PPARγ may conceivably play a role in the reproductive toxicity of

DNBP, the guinea pig, a non-responding species to the peroxisomal-proliferating effects of DNBP, is also susceptible to the testicular effects of DNBP.

Several studies have documented that the underlying key event for the toxicological effects of DNBP in the male fetus is a sustained decrease in fetal testicular testosterone in Leydig cells. Additional studies have established a decrease in cholesterol metabolism and in cholesterol transport genes and down-regulation of most of the genes in the testosterone biosynthesis in the fetus. Gubernacular malformations and the resulting cryptorchidism appear to be due to a separate mode of action involving reduced insulin-like growth factor 3 (Insl3) and reduced testosterone. However, this mode of action in the rat appears to require higher doses of DNBP. Although the relevance of findings in rats with DNBP for human risk assessment seems to be questionable (Mitchell *et al.*, 2012), DNBP can be considered to be an "endocrine disruptor" in the rat according to the WHO/IPCS (2002) definition, since adverse effect are induced by an endocrine mode-of-action in intact animals.

Di-isononyl phthalate (DINP)

The results of the toxicity studies show, when applying a weight of evidence approach, that DINP does not impair male fertility, onset of puberty and male mating behavior, and does not induce cryporchisdism, hypospadias, or general reproductive tract malformations (CSTEE, 2001b; ECB, 2003a; EFSA, 2005b; NICNAS, 2008b; NTP-CERHR, 2003a, Clewell, 2011b)

When observed, changes indicative of potential anti-androgenic activity (occurrence of female-like areolas/nipple retention in male pups, reduced AGD) were slight, questionable, occurred only at high doses without a clear dose-response, and did not result in permanent changes considered to be adverse. In one study, nipple retention was observed at doses of 750 mg/kg bw/day. Numbers of nipples per male were between 0.11 (versus 0 in controls, Gray Jr *et al.*, 2000) and 3.17 (versus 1.98 in controls, Boberg *et al.*, 2011). Although permanent nipples in males constitute a permanent structural change, the development of fur in the animals results in difficulties to see the areolas, and only the presence or absence should be registered in the males instead of the numbers per male. Usually, the frequency in control male rats is low (e.g., below 5%); but control values up to 30% have been reported (OECD, 2008). As no historical laboratory ranges for nipple retention were given, the importance of the findings from this study is difficult to assess. For low incidence data, the range of historical values is important to differentiate a genuine effect from a spurious difference (Lewis *et al.*, 2002).

Reduced sperm motility at high DINP doses was also observed in one study (Boberg *et al.*, 2011). However, only 1-3 animals per litter were examined, and no dose-response was established. According to OECD (2008), there is no generally accepted standard for the extent of a change in motility to be considered adverse, especially without dose-response and changes in testes histology. Furthermore, sperm motility of the control group in this study was at the upper range of the historical values (Jarfelt *et al.*, 2005; Taxvig *et al.*, 2007).

AGD was reduced slightly but significantly on PND 1 at 900 mg/kg bw/day (Boberg *et al.*, 2011) and on PND 14 at 750 mg/kg bw/day (Clewell, 2011). While Boberg *et al.* (2011) do not give information on AGD in adulthood, Clewell (2011 b) did not observe changes in AGD on PND 49. Only a permanent change in AGD (i.e., at birth and adulthood) is considered adverse (OECD, 2008). The biological significance of MNG (Boberg *et al.*, 2011; Clewell, 2011) is unclear. Reduced intratesticular testosterone levels could be involved in the etiology of MNG formation in rats, but this is not supported by parallel findings from the AR knockout mice and testicular feminized mice because neither of these end points were affected in these animals (Scott *et al.*, 2007). In the same vein, a study performed in mice by Gaido *et al.* (2007) indicates that altered seminiferous cord formation and gonocyte multinucleation may not be mechanistically linked to lowered testosterone, thus suggesting that MNG formation is not androgen dependent. Additionally, no sustained reduction in testicular testosterone was seen in several studies (Adamsson *et al.*, 2009; Boberg *et al.*, 2011; Clewell, 2011b). In conclusion, due to the lack of adverse effects with biological relevance in intact animals and the absence of alterations in fertility or reproductive performance in a comprehensive 2-generation study in rats, DINP is not an "endocrine disruptor" according to the WHO/IPCS (2002) definition.

Di-isodecyl phthalate (DIDP)

Although the results of a Hershberger assay in castrated prepubertal rats suggest that acute exposure to DIDP may cause antiandrogenic activity in rats (Lee and Koo, 2007), no nipple retention or effects on AGD were seen in male offspring of rats exposed to DIDP during gestation, suggesting lack of antiandrogenic activity in the intact animal.

The lowest NOAEL from the 2-generation reproductive toxicity study of DIDP in rats is 38 mg/kg bw/day and is considered conservative due to small effects on pup survival in the presence of maternal toxicity. The reduced pup survival was suggested to be due to decreased milk consumption by the laboratory performing the studies. The decreases in survival

indices mainly in F 2 (day 1 and day 4) in the two-generation study as well as skeletal variations in developmental studies are not sufficiently severe to justify classification (ECB, 2003b).

There is no indication of effects on reproductive organs from histological observation in repeated dose toxicity studies in rats and in dogs and in 2-generation studies in rats. Thus, DIDP cannot be considered to be an "endocrine disruptor" according to the WHO/IPCS (2002) definition.

Determination of Derived No Effect Levels

Derived no effect levels (DNEL) for the reviewed endpoints are proposed. According to the ECHA Guidance (ECHA, 2010), DNEL values for effects on fertility ($DNEL_{fertility}$) and developmental toxicity ($DNEL_{development}$) should be derived from the data on reproductive toxicity. Based on the statistical reasons discussed in ECETOC (2010), no additional assessment factor of 2.5 for 'remaining differences' will be applied to allometric scaling (as suggested by ECHA, 2010).

Di-isononyl phthalate (DINP)

A NOAEL for fertility of 622 mg/kg bw/day bw/day has been established in rats. The lowest reported developmental NOEL (from Clewell, 2011b) was 56 mg/kg bw/day.

Applying asssessment factors according to ECHA guidance for:

- extrapolation from rats to humans: 4 (allometric scaling)
- intraspecies differences: 10. As no adverse effects on organ systems and functions that are especially vulnerable under development and maturation in early life were seen, no additional intraspecies assessment factor has to be applied. Additionally, it has been shown that from age 6 to 12 months, kinetics of xenobiotics in infants are equivalent to those in adults (Renwick *et al.*, 2000; Ginsberg et al., 2002).
- differences in duration of exposure: 1
- issues related to dose-response: 1
- Due to comprehensive database, no additional factor is needed: 1

results in the following DNEL-values:

$DNEL_{fertility}$ = 15.55 mg/kg bw/day bw/day $DNEL_{development}$ = 1.4 mg/kg bw/day bw/day

The thus derived $DNEL_{development}$ is in good agreement with a DNEL-value of 1.3 mg/kg bw/day bw/day when derived from the LOAEL in the rat 2-generation study identified in the EU-risk assessment (before results from Clewell, 2011 a, b were available). This value is obtained by using the LOAEL from the two-generation study in rats using the same assessment factor as above (40) and an additional factor of three to account for the use of a LOAEL.

Di-isodecyl phthalate (DIDP)

A NOAEL for fertility of 542 mg/kg bw/day bw/day has been established in a one-generation reproduction study in rats. A developmental NOAEL of 38 mg/kg bw/day bw/day was identified for offspring survival which may be secondary to parental effects.

- extrapolation from rats to humans: 4 (allometric scaling)
- intraspecies differences: 10. As for DINP, adverse effects on organ systems and functions vulnerable in development and maturation were not observed and an additional factor to cover altered toxicokinetics in children is not necessary (Renwick *et al.*, 2000).
- differences in duration of exposure: 1
- issues related to dose-response: 1
- Due to comprehensive database, no additional factor is needed: 1
- $DNEL_{fertility}$ = 13.5 mg/kg bw/day $DNEL_{development}$ = 1 mg/kg bw/day

As outlined, the DNELs for both DINP and DIDP are based on highly conservative points of departure. DNEL values are orders of magnitude higher than for those derived for DNBP[†]. In addition, recent studies question the relevance of findings regarding male reproductive toxicity of certain phthalates in rats for human risk assessment (Mitchell *et al.*, 2012).

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[†] Using the lowest reported developmental NOAEL of 14-29 mg/kg/day and an additional intraspecies assessment factor of 10 for children younger than 6 months because of "endocrine disruptive" mode of action, the $DNEL_{development}$ for DNBP is calculated as 0.035 mg/kg/day.

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