Proposal for Harmonised Classification and Labelling

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

Substance Name: *Hexabromocyclododecane*

EC Number: ***247-148-4 and 221-695-9***

CAS Number: ***25637-99-4 and3194-55-6***

(the first EC/CAS numbers are the ones used for HBCDD in society, although the latter ones are the most correct from a chemical point of view)

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The first EC/CAS numbers are the ones used for HBCDD in society, and the latter ones are the most correct ones from a chemical point of view.

Registration number (s): HBCDD is not yet registered

Purity: The content of the different stereoisomers of HBCDD is usually in the range of 90-100%.

Impurities: Mainly tetra- and pentabromocyclododecane

According to IUCLID the impurities in HBCDD are less than 4% w/w. The stated impurities are tetrabromocyclododecane and other brominated cyclododecanes. Technical products with a reported lower purification grade are present in the literature and have been used in some studies, e.g. Hexabromid S (92% purity). Further information on those products is not available. The occurrence of polybrominated dibenzofurans (PBDFs) and polybrominated dibenzodioxins (PBDDs), in a technical HBCDD product has been measured (mono- to octabromo congeners) (Brenner, 1993). The result, presented in Table 1-1 in the EHC document on dioxins and furans (International Programme on Chemical Safety, 1998) show low amounts of tetra- and penta-BDFs, 20 ppb and 30 ppb, respectively, and no detectable PBDDs (the detection limit was >10 ppb for both PBDFs and PBDDs). Technical HBCDD is manufactured in two forms, high-melting (HM) and low-melting (LM). The LM HBCDD consists of 70-80% γ-, 20-30% of α- and β-HBCDD. The HM HBCDD consists of 90% or more of γ-HBCDD. According to Material Safety Data Sheets from Great Lakes there are three technical products of hexabromocyclododecane (Great Lakes Chemical Corporation, 2002a-c). One product is 100% pure, another product contains an inorganic stabilizer, and a third product contains 40-60% HBCDD, water and a component A. The Dead Sea Bromine Group has two technical products, one which is 99.5% pure and another which is heat stabilized grade (Dead Sea Bromine Group, 2000, 2002). Albemarle also has two technical products, one of high-purity grade available in powder or granular form, the other one of high-purity grade is ground to a fine particle size (Albemarle Corporation, 2000a-b). All producers supply a stabilised grade of HBCDD. The nature of the particular stabiliser may vary between companies.

(EU Risk Assessment Report)

Proposed classification based on Directive 67/548/EEC criteria:

Repr Cat 3; R62 (Possible risk of impaired fertility)

> The justification is based on a significantly decreased number of primordial follicles together with a decreased fertility index (seen as a significant trend in the F0 generation) in a 2-generation study in rats.

Repr Cat 3; R63 (Possible risk of harm to the unborn child)

The justification is based on pup mortality during lactation in the F2 generation of a 2-generation study in rats, and by developmental effects in a 1-generation study in rats (impaired hearing and decreased weights of testis and prostate in male weanlings, and delayed vaginal opening in female weanlings).

R64 (May cause harm to breastfed babies)

The justification is based on animal data showing pup mortality during lactation in the F2 generation of a 2-generation study in rats and the occurrence of HBCDD in human breast milk.

Proposed classification based on GHS criteria:

Repr. 2 H361fd (Suspected of damaging fertility. Suspected of damaging the unborn child.)

Lact. Effects H362 (May cause harm to breast-fed children)

Proposed labelling:

Xn; R62; R63 R64
S 36/37/53

Proposed specific concentration limits (if any):

Proposed notes (if any): -

JUSTIFICATION

1 IDENTITY OF THE SUBSTANCE AND PHYSICAL AND CHEMICAL PROPERTIES

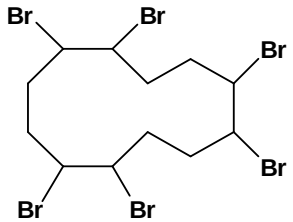
1.1 Name and other identifiers of the substance

Chemical Name:	Hexabromocyclododecane (HBCDD)
EC Numbers:	247-148-4; this number refers to hexabromocyclododecane (without specifying the bromine positions) and is used by industry for the commercial substance. 221-695-9[a]; this number refers to 1,2,5,6,9,10-hexabromocyclododecane and is thus the most correct one from a chemical point of view.
CAS Numbers:	25637-99-4; this number refers to hexabromocyclododecane (without specifying the bromine positions) and is used by industry for the commercial substance. 3194-55-6[a] ; this number refers to 1,2,5,6,9,10-hexabromocyclododecane and is thus the most correct one from a chemical point of view.
IUPAC Name:	Hexabromododecane (HBCDD), cyclododecane, hexabromo-

a: None of these CAS numbers are specific in terms of the diastereomeric composition of the substance (1,2,5,6,9,10-HBCDD; see below). The information of the stereochemistry of the α-, β- and γ-1,2,5,6,9,10-HBCDD and the concomitant CAS No can be seen below. However, as the former numbers are the numbers currently used by industry (e.g. in SDS) for technical HBCDD, the dossier needs to cover both numbers.

1.2 Composition of the substance

Chemical Name:	Hexabromocyclododecane and 1,2,5,6,9,10-hexabromocyclododecane
EC Number:	247-148-4; 221-695-9[a]
CAS Number:	25637-99-4[b] ; 3194-55-6[a]
IUPAC Name:	Hexabromocyclododecane
Molecular Formula:	$C_{12}H_{18}Br_6$
Structural Formula:	Structural formula for 1,2,5,6,9,10-HBCDD, i.e., CAS no 3194-55-6[a]



Note that CAS no 25637-99-4 is also used for this substance, although not being correct from a chemical point of view as this number is not specifying the positions of the bromine atoms. As additional information, the structures and CAS numbers for

	the diastereomers making up 1,2,5,6,9,10-HBCDD are given below, although these diastereomers always occur as mixtures in the technical product.
Molecular Weight:	641.7
Synonyms	Cyclododecane, hexabromo; HBCDD; Bromkal 73-6CD; Nikkafainon CG 1; Pyroguard F 800; Pyroguard SR 103; Pyroguard SR 103A; Pyrovatex 3887; Great Lakes CD-75P™; Great Lakes CD-75; Great Lakes CD75XF; Great Lakes CD75PC (compacted); (Dead Sea Bromine Group Ground FR 1206 I-LM; Dead Sea Bromine Group Standard FR 1206 I-LM; Dead Sea Bromine Group Compacted FR 1206 I-CM)[c]; FR-1206; HBCDD ILM; HBCDD IHM
Concentration range (% w/w):	Depending on the producer, technical grade HBCDD consists of approximately 70-95% γ-HBCDD and 3-30% of α- and β-HBCDD due to its production method (European Commission, 2007). Two additional diastereoisomers, δ-HBCDD and ε–HBCDD have been found by Heeb *et al*. (2005) in commercial HBCDD in concentration of 0.5% and 0.3%, respectively. The information on composition available in the EU RAR (European Commission, 2007), concerns a composite used for most testing purposes. The composite was prepared by mixing equal amounts of technical HBCDD obtained from the three manufacturers being on the EU market, giving a composite composition of 80-90% γ-HBCDD, 5-10% of α-HBCDD, 5-10% of β-HBCDD, and 5-10% unknowns. The composition is likely to differ between products from the different manufacturers, but also to differ between different products of a single manufacturer (e.g., HBCDD-ILM (high-melting) and HBCDD-IHM (low-melting). Thus, depending on producer, the production process, and purpose of use, the ratio between the three main stereoisomers can vary. From a strict substance ID point of view, the following substance compositions could be viewed as 4 different "substances"; >80% γ-HBCDD, 70-80% γ-HBCDD and >10% α-HBCDD, 70-80% γ-HBCDD and >10% β-HBCDD, and 70-80% γ-HBCDD, >10% α-HBCDD, >10% β-HBCDD.

a: None of these CAS numbers are specific in terms of the diastereomeric composition of the substance (1,2,5,6,9,10-HBCDD; see below). The information of the stereochemistry of the α-, β- and γ-1,2,5,6,9,10-HBCDD and the concomitant CAS No can be seen below. However, as the former numbers are the numbers currently used by industry (e.g. in SDS) for technical HBCDD, the dossier needs to cover both numbers.

b: This number refers to unspecific isomer composition.

c: Historical names of the products of ICL-IP. Current names of ICL-IP products are FR-1206, HBCDD ILM and HBCDD IHM.

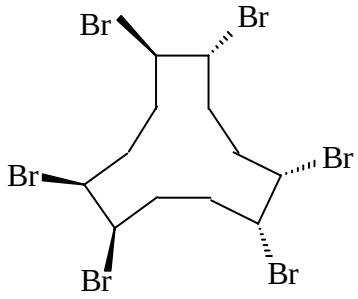
Additional information on the three main constituents of technical hexabromocyclododecane

CAS Number:

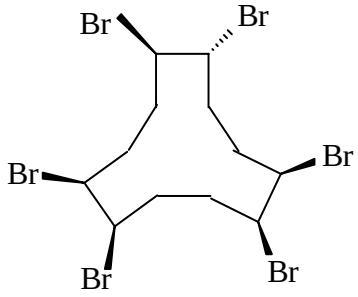
Technical HBCDD is made up of three main chiral diastereomers. Each of these have a specific CAS number, namely:

- (1R,2S,5R,6R,9R,10S)-rel-1,2,5,6,9,10-hexabromocyclododecane [beta-hexabromocyclododecane;CAS No 134237-51-7].
- (1R,2R,5S,6R,9R,10S)-rel-1,2,5,6,9,10-hexabromocyclododecane [alpha-hexabromocyclododecane;CAS No 134237-50-6]
- (1R,2R,5R,6S,9S,10R)-rel-1,2,5,6,9,10-hexabromocyclododecane [gamma-hexabromocyclododecane.;CAS No 134237-52-8]

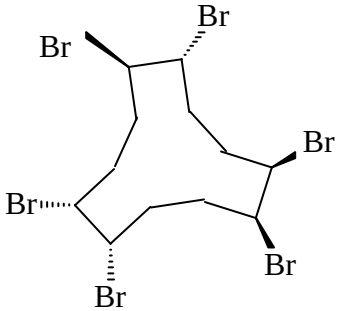
Structural Formula:



alpha-HBCDD CAS No: 134237-50-6



beta-HBCDD CAS No: 134237-51-7



gamma-HBCDD CAS No: 134237-52-8

1.3 Physico-chemical properties

Table 1-1. Summary of physico-chemical properties

REACH ref Annex, §	Property	IUCLID section	Value
VII, 7.1	Physical state at 20°C and 101.3 Kpa	3.1	White odorless solid
VII, 7.2	Melting/freezing point	3.2	Ranges from approximately: 172-184°C to 201-205°C 190°C, as an average value, is used as input data in EUSES. 179-181°C α-HBCDD 170-172°C β-HBCDD 207-209°C γ-HBCDD
VII, 7.3	Boiling point	3.3	Decomposes at >190°C
VII, 7.4	Relative density	3.4 density	2.38 g/cm^3 2.24 g/cm^3
VII, 7.5	Vapour pressure	3.6	6.3·10-5 Pa (21°C)
VII, 7.7	Water solubility	3.8	66 µg/l (sum of α-, β- and γ-HBCDD) 48.8 µg/l* α-HBCDD 14.7 µg/l* β-HBCDD 2.1 µg/l* γ-HBCDD
VII, 7.8	Partition coefficient n-octanol/water (log value)	3.7 partition coefficient	Log Kow = 5.62 (technical product) 5.07 ± 0.09 α-HBCDD 5.12 ± 0.09, β-HBCDD 5.47 ± 0.10 γ-HBCDD
VII, 7.9	Flash point	3.11	Not applicable
VII, 7.10	Flammability	3.13	Not applicable (flame retardant)
VII, 7.11	Explosive properties	3.14	Not applicable
VII, 7.13	Oxidising properties	3.15	Not applicable
	Auto flammability	3.12	Decomposes at >190°C

*Determined for the isomers present as a mixture not for the pure isomers.

(EU Risk Assessment Report)

2 MANUFACTURE AND USES

The German company BASF used HBCDD for the first time in their production of flame retarded polystyrene foams in the late 1980s. However, the substance has been on the world market since the 1960s. Hexabromocyclododecane was named Hexabromid with the CAS No 3194-55-6 when it was synthesised by BASF.

Hexabromocyclododecane is used industrially in the life cycle steps: production, formulation and industrial use with the aim to increase the flame resistance of different end-products. The end-

products are used both professionally and by consumers, have a relatively long service life and are disposed of by different means; incinerated, recycled, put on landfill or left in the environment.

It is not possible to give an exact tonnage for HBCDD since information on production and import were given by industry in ranges and for different years (see Table 2-1).

Table 2-1. EU production and import of HBCDD

Production and import country	Quantity produced (t/a)	Quantity imported (t/a)	Year
The Netherlands	500-1,000		1996
	>1000- <5000		1999-2002
	5000-7000		2002
United Kingdom + import from the USA	1000-5000	100-500	1996
	0		2003-
Import from the USA	0	1000 - 5000	1995
Germany	0	0	1997[d]

d: Second half of 1997. Information on import of HBCDD to the EU from other countries than the USA has not been reported. Import of amounts less than 1,000 t/a by one or several companies cannot be excluded.

According to industry the consumption of HBCDD in Eastern Europe, for instance in Poland, is considerable. Countries outside the EU known to produce HBCDD are the USA, Israel, and Japan. The annual consumption of HBCDD in Japan has increased from 600 tonnes in 1986 to 1600 tonnes in 1994. Data from Japan indicate that the consumption of HBCDD is about 2000 t/a. Some information on the worldwide consumption is available on the website of Bromine Science and Environmental Forum (BSEF) that indicates a worldwide consumption of 16.700 tonnes/year in 2001.

(EU Risk Assessment Report)

3 CLASSIFICATION AND LABELLING

3.1 Classification in Annex I of Directive 67/548/EEC

Classification of HBCDD with N; R50/53 was agreed at a Technical Committee for Classification & Labelling (TC C&L) meeting on 11-12 June, 2003. Classification for health effects has not yet been discussed and HBCDD is therefore not included in Annex I to Directive 67/548/EEC.

3.2 Self classification(s) –

4 ENVIRONMENTAL FATE PROPERTIES

Not evaluated for this dossier.

5 HUMAN HEALTH HAZARD ASSESSMENT

5.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

Study type: A guideline 90-day oral (gavage) study (Chengelis 2000), with analysis of HBCDD residues performed on control and top dose animals. Body fat was analysed for concentration of HBCDD as individual α-, β-, and γ-diastereoisomers.

Material: 40 Crl:CD(SD)IGS BR rats
20 animals/sex

Method: HBCDD: 0-1000 mg/kg/day for 90 days. It should be noted that the animals were dosed with a suspension of HBCDD-particles in corn oil. Because of dosing HBCDD-particles, the absorption kinetics is likely dependent on particle size and amount of particles administered, and the actual internal doses in this study are therefore uncertain.
Control: corn oil
Dosage volume: 5 ml/kg
Two animals/sex and group were euthanized on study days 2, 6, 9, 13, 20, 27, 55, 89, 104, and 118.

Results: The highest concentration of HBCDD was observed in fatty tissue on day 89. The α concentration was then 8-12 times higher than the concentration of β, and 6-8 times higher than the concentration of the γ stereoisomer, thus indicating a 100-fold higher relative bioaccumulation of the α-diastereomer than of the major γ- diastereomer.

Table 5-1. Relative bioaccumulation factors (BAF) for the three HBCDD diastereomers in an oral 90 days toxicity study (a total dose of 1000 mg 'technical' HBCDD/kg/day by gavage).

	α-HBCDD	**β-HBCDD**	**γ-HBCDD**
Composition of administered dose (%)	6.4	4.5	79.1
Administered dose (mg/kg/day)	64	45	791
Concentration in females day 89 (µg/g fat)	4340	357	544
Apparent bioaccumulation factor [e]	68	7.9	0.69
Relative BAF (γ-HBCDD set to1)	99	11	1

e: The administered dose is normally expressed as concentration in the diet (in the same unit as the concentration in the adipose tissue is expressed), and the BAF is calculated as concentration in fat divided by the concentration in the diet. Since the dose is given by gavage in the study above, only an apparent relative bioaccumulation factor can be calculated.

Conclusion: At the dose of 1000 mg/kg/day, there is a 100-fold higher relative bioaccumulation of the α-diastereomer of HBCDD in fat tissue than of the major γ- diastereomer.

(EU Risk Assessment Report)

For a lipophilic substance such as HBCDD, an important kinetic aspect is whether the substance can pass over to milk. There are no data on milk transfer of HBCDD in animals. However, human data show that HBCDD is indeed transferred to breast milk, and these human studies are presented below.

In 1986, 1993 and 2001, Norwegian breast milk samples were obtained from 10-12 primiparous mothers living in a coastal area in the North (Tromsø), in a rural inland area (Hamar), and in an industrialized area in the South of Norway (Skien/Porsgrunn). Samples collected in 1993 and 2001 in Tromsø, Hamar and Skien/Porsgrunn were pooled. From the 1986 study, only two individual samples from Tromsø were available. HBCDD was found in all samples, but at very varying levels, range 0.25-2 ng/g lipids (Thomsen *et al.*, 2003). Polder et al (2008a) have also reported on levels of HBCDD in women from Tromsø in 2000-2001. They found HBCDD in one out of ten samples, at a level of 0.13 ng/g lipid.

Colles et al (2008) reports on the concentration of HBCDD in 197 Belgian mothers' milk in 2006, but only state that the levels were "just above the detection limit". In a pooled sample made up of milk from 178 of the mothers a value of 1.5 ng/g lipid is given.Eljarrat et al (2009) analysed HBCDD in milk from A Coruna in Spain in 2006-2007. They found HBCDD in 30 out of 33 samples, at concentrations ranging between 3 and 188 ng/g lipid. The mean and median values were 47 and 27 ng/g lipid. Based on the assumptions of a lipid concentration of 3.7% in the milk, a body weight of 4.1 kg and milk consumption of 702 ml/day for 1-month-old infants, a median daily intake of 175 ng HBCDD/kg body weight/day was calculated. The intake in the most highly exposed infant would be approximately 7-fold higher (approximately 1.2 ug/kg/day based on the maximum concentration of 188 ng/g instead of the median value of 27 ng/g lipid).

A study with the objective to assess the temporal trends of polybrominated diphenyl ethers and HBCDD in mothers' milk from the Stockholm area shows an increase of HBCDD in mothers' milk over time. Milk was collected from healthy native Swedish mothers. Equal amounts of milk from individual mothers were pooled, from years 1980, 1984/85, 1988-2002, 2003 and 2004, with each pool representing milk from 116, 102, 20, 15 and 20 mothers respectively. The average age of the mothers was 27-28 years in 1980 and 1984/85, and between 29-31 years in 1988-2004. Fourteen milk samples were taken out from 1980 to 2004 for analysis. From 1980 the average concentrations of HBCDD in mothers' milk has increased from 0.13 pmol/g (0.084 ng/g) to 0.60 pmol/g (0.39 ng/g) lipid in 2004. The highest values were found in 2001 and in 2002 (0.83 and 0.93 pmol/g). During the last years the concentrations have stabilized at concentrations between 0.6 and 0.93 pmol/g lipid (Fängström *et al.*, 2008).

The National Food Administration in Sweden has analyzed HBCDD and other substances in mother's milk from Swedish women in two different studies. The first study (Aune *et al*., 2001), detected HBCDD in 12 of 33 samples. The samples were taken from primiparous mothers, aged 19-40 and living in Uppsala County, Sweden, two weeks after delivery. The limit of detection was <15 pg HBCDD/g mothers milk (fresh weight). On a lipid weight basis, the mean and max concentrations were 0.45 and 2.4 ng/g fat, respectively. In the second study (Lignell *et al*., 2003) breast milk

was sampled from 30 primiparous mothers who delivered at Uppsala University Hospital from March 2002 to February 2003. The milk was sampled during the third week after delivery (day 14-21 post partum). The limit of detection was 0.006 ng/g milk, which corresponds to 0.20-0.37 ng/g/milk fat. On a lipid basis, the mean and max concentrations were 0.42 and 1.5 ng/g fat.

Blood and mothers milk samples from Mexico and Sweden were screened for both PBDEs and HBCDD. The Mexican samples were taken from women living in an urban environment and from indigenous rural women. Blood was donated by five women from San Luis Potosi City, and milk was donated by seven women from La Huastecha Potosina, located 300 km east of San Luis Potosi City. Swedish milk (5 individuals) was bought from the mothers' milk central at a hospital pharmacy in Stockholm. The HBCDD from the blood plasma was extracted and analyzed by GC/MS. The study shows the presence of HBCDD in blood and in mothers' milk, both from Mexican and Swedish women. The mean concentration in Mexican plasma was 1.2 ng/g lipid weights (range 0.7-2.5), in Mexican milk 2.1 ng/g (range 0.8-5.4) and in Swedish milk 1.1 ng/g (range 0.3-3.2). The sample levels varied but may indicate somewhat higher levels in the indigenous Mexican women. The study shows that HBCDD will be transferred via the mothers' milk to the nursing child (López *et al.*, 2004).

Polder et al (2008b) studied the occurrence of HBCDD in breast milk from northern Russia and found HBCDD concentrations above the limit of quantification in 8/14 and 3/23 samples from Murmansk and Arkhangelsk, respectively. The overall range was 0.2-1.7 ng/g lipid.

Kakimoto et al (2008) measured the concentration of HBCDD in pooled milk of Japanese mothers between 1973 and 2006. HBCDD was below the limit of detection 1973-1983, and then increased. As from 1993, concentrations between 1.0-4.0 ng/g lipids are reported, and it is concluded by the authors that the concentrations seem to follow the Japanese consumption of HBCDD.

HBCDD and PBDE levels in serum from mothers and infants from a Dutch cohort were investigated in a study, which also had the aim to establish a clean-up method for HBCDD analysis in human serum. A total of 90 human serum samples were analyzed. Serum samples were obtained from the Dutch-Groningen-PCB-Infant-Cohort, and contained 8 samples from mothers at the 20th week of pregnancy, 70 samples from mothers at the 35th week of pregnancy and 12 cord blood samples. HBCDD was detected in almost all samples, with concentrations up to 6.9 ng/g lwt. The HBCDD concentrations were similar to serum concentrations in Mexican and Swedish women (López *et al.*, 2004). HBCDD concentrations, on a lipid weight basis, were similar in maternal and cord blood (infant level). But if the relative fat content is considered, i.e. 0.23% lipids in cord blood and 0.77% in maternal serum, the total exposure to HBCDD is lower for an infant than for the mother. The mean concentration of HBCDD in cord blood was 2.4 ng/g lwt (median 0.32 and range 0.16-4.2) and in mothers serum 1.1 ng/g (median 0.72 and range 0.16-6.9) at pregnancy week 20 and 35. Concentrations of HBCDD were within the same range as PBDE congeners in both cord and maternal serum (Weiss *et al.*, 2004).

Table 5-2. Measured levels in breast milk**.**

Country	Year	Number of samples	Concentration of HBCDD in breast milk (ng/g fat)[a]
Sweden		12	0.45-2.4
Sweden	2003	30	0.42-1.5
Norway	1993-2001	10	0.25-2
Sweden	1980-2004	14 [b]	0.084-0.93
Sweden	2004	5	0.3-3.2
Mexico	2004	7	0.8-5.4
Belgium	2006	1 pooled sample	1.5
Spain	2006	33	3-188
Russia	2000-2002	37	0.2-1.7
Japan	1993-2006	Pooled samples	1-4

a: non-detects not included

b: Equal amounts of milk from individual mothers were pooled from years 1980, 1984/85, 1988-2002, 2003 and 2004, with each pool representing milk from 116, 102, 20, 15 and 20 mothers respectively

HBCDD levels in plasma from 10 pregnant women living in Bodø, Norway and from 10 women living in Taimyr, Russia were analysed by LC-MS. The samples were collected in August-December 2002. The women's ages were 20-35 and they had all given birth to one child before. None of the locations had any known local HBCDD source. HBCDD was detected in more than half of the samples but at low concentrations, close to the limit of detection. The Norwegian samples median and range values were (pg/ml plasma): α-HBCDD 19 (<11-345), β-HBCDD 7 (5-343), γ-HBCDD 23 (7-317) and the Russian samples median and range values were: α-HBCDD 21(<11-51), β-HBCDD 8 (<5-126), γ-HBCDD 33 (13-160) (Odland *et al.*, 2005).

Blood samples were taken from 47 members of the European Parliament, representing 17 European countries, in Brussels in December 2003. The samples were weighed and dried by mixing with sodium sulphate and then extracted by Soxhlet extraction with hexane:acetone as solvent. HBCDD in blood extracts were measured by gas chromatography/mass spectrometry (GC/MS). HBCDD was detected in one individual. The concentration, 0.063 ng/g blood, was too low to allow identification of the separate HBCDD diastereomers (Brandsma *et al.*, 2004). However, Weiss *et al*. (2006), have made a stereoisomeric analysis of HBCDD in human serum using LC/MS-MS. Two serum pools were analysed, each based on serum from 25 individuals (elderly women being married to fishermen). The study shows that (-) α-HBCDD was the dominating diastereomer, with only a few percents contribution from γ-HBCDD (Weiss *et al.*, 2006).

A calculation of breast-feeding intake levels in the EU Risk Assessment Report gives the following estimates (based on 3.2 ng/g fat of HBCDD in breast milk):

- 0-3 months old: 0.015 µg/kg bw and day
- 3-12 months old: 0.0056 µg/kg bw and day

For further information on e.g. exposure estimates, see EU Risk Assessment Report.

(EU Risk Assessment Report)

As noted above, a recent Spanish breast milk study calculated much higher exposure levels than the EU Risk Assessment report, i.e., a median daily intake, for 1 month-old Spanish infants, of 0,175 µg HBCDD/kg bw and day(Eljarrat et al, 2009). The variation in concentrations were high among the Spanish mothers, and it is noted that the intake in the most highly exposed infant would be approximately 7-fold higher (approximately 1.2 ug/kg/day).

It is not always clear in the study reports when the milk has been sampled after delivery, but the samples are usually taken a few weeks after delivery. For instance, in the study by Eljarrat et al (2009) the samples were taken 40 days after delivery. HBCDD accumulates in fat and the fat concentration in breast milk is much higher just after delivery than later on during the lactation period. The concentration of HBCDD is therefore likely to be highest just after birth and then decrease with time, both as a consequence of the decreasing fat concentration and of decreasing depots of HBCDD in the mothers. The measured concentrations of HBCDD in milk may therefore underestimate the early lactation exposure and overestimate the late lactation exposure.

Comments
In many studies, including the above one, the animals were dosed with a suspension of HBCDD-particles in corn oil. Because of dosing HBCDD-particles, the absorption kinetics is likely dependent on particle size and amount of particles administered, the actual doses received are more uncertain the higher the dose is. There is also one study were dosing was done by mixing HBCDD particles into an appropriate amount of powdered basal diet for each dietary concentration (Ema et al 2008), and also in this case the actual exposure will depend on the particle size of the HBCDD used, and the actual doses received are uncertain. Especially at the top dose, the internal dose is likely much lower than the nominal dose.

The only available studies, were properly dissolved HBCDD has been administered to the animals, are the studies by van der Ven at al (2006, 2008), with some data from these studies also presented by Germer et al (2006), Canton et al (2008), and Lillienthal et al (2009).

5.2 Acute toxicity

Not evaluated for this dossier.

5.3 Irritation

Not evaluated for this dossier.

5.4 Corrosivity

Not evaluated for this dossier.

5.5 Sensitisation

Not evaluated for this dossier.

5.6 Repeated dose toxicity (this information is only given as supporting information)

No repeated dose studies with inhalation or dermal exposure as route of administration are available. Five repeated dose studies with oral administration of HBCDD in rats have been conducted; three 28-day studies (Chengelis 1997; van der Ven *et al.*, 2006; and Zeller & Kirsch, 1969) and two 90-day studies (Chengelis, 2000; and Zeller & Kirsch, 1970). (For further details, see 4.1.2.6 Repeated dose toxicity in the EU Risk Assessment Report.)

In the first four studies, the rats were dosed with HBCDD particles in suspension, and doses ≥100 mg/kg/day resulted in a dose-dependent (but reversible) increase in liver weight not accompanied by any clear pathological signs. In addition, the prostate weight was statistically increased in a 90-day study (Chengelis, 2001) at exposure up to 1000 mg/kg/day, and in a 28-day study (Zeller and Kirsch, 1969) females showed signs of inhibited oogenesis in most of the follicles and sparse ripening follicles in the ovaries at exposure to 4700 mg/kg/day. However, the effects in the latter study were only seen at a very high dose and the study design did not comply with today's standards. All four studies showed effects on the thyroid hormone system. A two-generation reproductive toxicity (Ema *et al*., 2008) study has also shown the liver and thyroid system to be target organs. Also in this study HBCDD-particles were administered to the rats, although this time mixed into ground food. Because of dosing HBCDD-particles, with the absorption kinetics likely being dependent on particle size and amount of particles administered, the actual doses received at the top doses are uncertain (EU Risk Assessment Report).

Evaluation of the effects on the thyroid system

Effects on the thyroid hormone system and the mode of action analysis are also of relevance for the evaluation of the reproductive toxicity (chapter 5.9). Thus, thyroid hormone (TH) is essential for normal brain development and the maternal thyroid function during early pregnancy plays a fundamental role in foetal brain development as synthesis of thyroid hormone does not begin until the 20th week of gestation in humans. A lack of thyroid hormone during early development results in multiple morphological and functional alterations in the developing brain in both humans (Lazarus 2005) and rats (Wijk et al 2008) and the effects of this have been shown i several studies. In a rat study by van Wijk *et al*. (2008) neuromotor competence, locomotor activity and cognitive function were monitored in the offspring until postnatal day 71. They found that early neuromotor competence (assessed in the grip test and balance beam test) was impaired by both chronic and

perinatal hypothyroidism. Also, testing of locomotor activity in the open field test showed hyperactivity in chronic hypothyroid animals. In the Morris water maze test, chronic hypothyroidism affected spatial memory in a negative manner. In contrast, perinatal hypothyroidism was found to impair spatial memory in female rats only. In general, the effects of chronic hypothyroidism on development were more pronounced than the effects of perinatal hypothyroidism, suggesting the early effects of hypothyroidism on functional alterations of the developing brain to be partly reversible and to depend on developmental timing of the deficiency.

Evaluation of enzyme induction as a potential Mode of Action It has been suggested that the liver weight increase is caused by hepatic enzyme induction, as indicated by histopathology (proliferation of SER; (Chengelis, 2001) and induced hepatic enzyme activities/mRNA/protein (van der Ven *et al.*, 2006); (Germer *et al.*, 2006). However, there is no consistent difference in sensitivity towards hepatic enzyme induction between males and females. It is noteworthy that in spite of similar enzyme induction in females and males, the concentration of HBCDD was higher in females than in males, indicating little relationship between enzyme induction and accumulation of HBCDD in the animals. Enzyme induction is clearly occurring, and is likely the most important reason for the liver weight increase, but it cannot be ruled out that other mechanisms also are involved in causing the thyroid effects.

The existence of other mechanisms is very likely in view of the study by Canton et al (2008), who studied the hepatic gene expression profile in rats from the 28 days study by van der Ven et al (2006). Canton et al (2008) studied the gene expression at the doses of 30 and 100 mg/kg/day, and found at the low dose 148 and 999 affected genes ($\geq$1.5-fold up-or down regulation) in males and females, respectively. At 100 mg/kg/day, the numbers were 422 and 2297, respectively, with 2185 genes only affected in females. The affected genes were broadly grouped into genes regulating the following pathways; cholesterol biosynthesis, estrogens metabolism, glutathione metabolism/conjugation, PPAR regulation of lipid metabolism, and triacylglycerol metabolism.

With regard to effects on the thyroid system, the studies have shown either no effects, effects only in females, or effects in both sexes. However, in the early studies, the thyroid system was not studied that thoroughly. Chengelis (2001) and van der Ven *et al.* (2006) showed effects on the thyroid weight (increases) only in females, whereas Ema et al (2008) found weight increases in both sexes and van der Ven (2009) no effects in any sex. In contrast, Chengelis (2001) indicated decreased serum T4 and increased serum TSH in both sexes, Ema et al (2008) decreased serum T4 in both sexes but increased serum TSH only in females, van der Ven *et al.* (2006) only observed effects in females, and van der Ven et al (2009) no effects in either sex.

The mechanism for the thyroid effects is not clear, and will be discussed below basically using a structure that has been proposed by the IPCS when analysing the mode of action for carcinogens. The discussion below will solely be based on the data presented by (van der Ven *et al.*, 2006), as only this study has been designed to allow this analysis.

Postulated mode of action

Hepatic enzyme induction (T4 conjugation) leads to increased excretion of T4, compensatory activation of the pituitary, increased serum TSH concentration and activation of the thyroid. Depending on the magnitude of the T4-decrease, the feed-back system may manage to produce sufficient amounts of T3, or if the reduction is severe, lead to a condition of hypothyroidism.

Key events

This MoA is based on the assumption that hepatic enzymes involved in the metabolism of T4/T3 are induced by HBCDD. There should be a reasonable relationship between induction of hepatic enzymes and thyroid effects in both sexes, acknowledging that females are somewhat more sensitive to thyroid effects than males. The van der Ven study(van der Ven *et al.*, 2006) has indeed identified induction of T4-UGT transferase by HBCDD, and the induction occurred in both sexes. However, the gene expression study (Canton et al 2008) on animals from the van der Ven study (2006) shows that genes involved in phase I and II metabolism were up-regulated predominantly in males.

Dose-response

The hypothesis postulates that the first effect (occurring at the lowest exposure level) is enzyme induction, followed by activation of the pituitary (resulting in TSH synthesis), activation of the thyroid (hyperactive cells/weight increase), and if the thyroid is incapable of producing sufficient amounts of T4/T3, effects in other tissues/systems regulated by T4/T3.

In females, the BMD-L for induction of T4-UGT is 4.1 mg/kg/day (and LBD/CYP3A4 is induced as from 10 mg/kg/day), the BMD-L for pituitary weight 29.9 mg/kg/day, the BMD-L for thyroid weight 1.6 mg/kg/day (this BMDL seems too conservative when looking at all data, but the weight increase seems undisputable at 30 mg/kg/day), and there are histological signs of thyroid hyperactivity at 25, 47, and 177 mg/kg/day for nuclear size, cell height, and vacuolization, respectively. Considering the variation in all data, this sequence of events could support the theory with regard to females.

In males, enzyme induction occurs (the BMD-L for T4-UGT transferase was calculated to 0.1 mg/kg/day but is very uncertain because of high variation in the data; PROD/CYP2B is induced as from 10 mg/kg/day), there are no effects of T4 or the pituitary, but there are histological signs of thyroid hyperactivity at 39, 90, and 199 mg/kg/day for nuclear size, vacuolization, and follicle size, respectively. There are no effects on the thyroid weight.

Strength, Consistency, and Specificity of association of response with key events

There is always some variation in biological data, making firm conclusions difficult. However, the key event (enzyme induction) seems to occur at low exposure in both males and females although the gene profile of phase I and II pathways was more affected in males. In females, the chain of events could support the theory, although the data are not clear-cut. The effect on the pituitary would be expected to appear before effects in the thyroid are evident, but it is acknowledged that if only some pituitary cells are affected, the weight of the whole pituitary will be a rather insensitive parameter. Still, the occurrence of only few and mild effects in males are unexpected, even when considering that the male thyroid system is known to be less affected than the female by chemicals.

Other modes of action

Considering that *in vitro* and *in vivo* data indicate an interaction of HBCDD with many different hormone systems, it is possible that HBCDD also could affect the thyroid system via other mechanisms than hepatic enzyme induction.

In vivo, genes for fatty acid metabolism and cholesterol biosynthesis are decreased in females, which could be related to hypothyroidism, and estrogens metabolism is increased in both sexes (Canton et al, 2008).

In cell cultures, HBCDD was found to exert antagonistic effects at the progesterone receptor, androgen receptor, and estrogen receptor (IC_{50} 1-5 µM for γ-HBCDD). A low binding of α-HBCDD to the thyroxin-binding transport protein TTR was also indicated *in vitro* (IC_{50} 12 µM). α-HBCDD was a T3-agonist (21 % of maximal T3-effect at 1 µM α-HBCDD) and enhanced T3-dependent effects both in rat cells and *ex vivo* in tadpole tail tips (Hamers *et al.* 2006, Yamada-Okabe *et al.* 2005, Schriks 2006a). These *in vitro* effects could theoretically explain the effects seen *in vivo* on the thyroid hormone homeostatis.For instance, the binding of HBCDD (or metabolites of HBCDD) to TTR could displace T4 from TTR, making T4 more susceptible to metabolism and excretion. Binding of chemicals to TTR is usually increased by hydroxy-groups (the endogenous ligand thyroxin is hydroxylated), and one may speculate that hydroxy-HBCDD could have a higher binding affinity to TTR than HBCDD itself. OH-HBCDD is formed from HBCDD by microsomes in vitro (Zegers *et al.*, 2004), but it is not known if there is any sex-dependent difference in formation of OH-HBCDD.

A T3-potentiating effect could possibly occur simultaneously as the concentration of T4 is decreased, masking some of the hypothyroidogenic symptoms.

Assessment of postulated MoA

The postulated mode of action is a plausible mechanism, but the lack of effects (other than some effects on thyroid histology) in males in spite of enzyme induction in males is surprising, even though females are known to be more sensitive than males. The big difference between males and females in sensitivity towards effects on the thyroid system could possibly be explained if HBCDD has other effects that contribute to the thyroid effects in females or antagonize the effects in males.

In conclusion, HBCDD affects the thyroid hormone system in experimental animals, most likely through several mechanisms of action. Relevance to human beings can therefore not be dismissed.

5.7 Mutagenicity

Not evaluated for this dossier.

5.8 Carcinogenicity

Not evaluated for this dossier.

5.9 Toxicity for reproduction

5.9.1 Effects on fertility

Also see 5.6. Repeated dose toxicity.

Study type: Two-generation reproductive study according to OECD guideline 416 and GLP.

Material: 5-week old males and females, Crl:CD(SD) rats

Method: For further details, the reader is referred to the scientific publication by Ema *et al.* (2008).

The test substance was a composite of HBCDD commercial products from three leading producers and the preparation was a mixture of three enantiomers, HBCDD-α, -β and –γ, and their respective proportions in the used batch were 8.5, 7.9 and 83.7%. The test substance was 99.7% pure.

Animals: One hundred and ninety-two Crl:CD(SD) rats were randomly assigned in four groups, 24/sex/group, as F0 animals. Animals were housed individually, except during acclimatization, mating and nursing periods.

Dosing: Rats were given dietary HBCDD at a concentration of 0, 150, 1500 or 15000 ppm, which numerically has been translated to 10-14, 101-141, or 1008-1363 mg/kg/day in low, mid, and high dose animals with the low end of the ranges representing males and the high females. The daily intake of food increased during lactation, leading to calculated daily intake of 20-23, 179-240, and 1724-2200 mg/kg/day in dams of the low, mid, and high dose groups, respectively. Diet preparations were formulated by mixing HBCDD particles into an appropriate amount of a powdered diet for each dietary concentration. Administration was continued for 10 weeks prior to the mating period, throughout the mating, gestation and lactation periods. Twenty-four male and 24 female weanlings in each group were selected as F1 parents on PNDs 21-25. The day on which F1 parental animals were selected was designated as 0 week of dosing for the F1 generation. F1 selected rats were administered HBCDD as described for F0 rats.

The estimated mean daily intake in mg/kg bw/day of HBCDD in the different dose groups are shown in Table 5-3.

Table 5-3. Mean daily intake (mg/kg bw/day) of HBCDD in F0 and F1 males/females in the 150, 1500 and 15,000 ppm groups, respectively.

	Dose (mg/kg bw/day)			
Dose (ppm)	**F0 males**	**F0 females**	**F1 males**	**F1 females**
150	10.2	14.0	11.4	14.3
1500	101	141	115	138
15,000	1008	1363	1142	1363

Mating: Each female was mated with a single male of the same dosage group until copulation occurred or the mating period (three weeks) had elapsed. During the mating period, vaginal smears were examined for the presence of sperm, which was considered as evidence of successful mating. Successful mating was designated as day 0 of pregnancy. For F1 matings, cohabitation of siblings was avoided.

Clinical observation: All adult rats were observed twice a day for clinical signs of toxicity and bodyweights and food consumption were recorded weekly. For females exhibiting evidence of successful mating, body weight and food consumption were recorded on days 0, 7, 14, and 20 of pregnancy and days 0, 4, 7, 14, and 21 of lactation. Daily vaginal lavage samples of each F0 and F1 female were evaluated for oestrus cyclicity.

Once insemination was confirmed, female rats were checked at least three times daily on days 21-25 of pregnancy to determine the time of delivery. The females were allowed to deliver spontaneously and nurse their pups until PND 21. The day on which parturition was completed was designated as PND 0. Total litter size and the numbers of live and dead pups were recorded, and live pups were counted, sexed, examined grossly, and individually weighed on PNDs 0, 4, 7, 14, and 21. On PND 4, litters were randomly adjusted to 8 pups comprising of 4 males and 4 females. No adjustment was made for litters with fewer than 8 pups. After weaning their pups, parental female rats were necropsied at the prooestrus stage of the oestrus cycle. For each female, the number of uterine implantation sites was recorded.

Developmental landmarks and behavioural effects: All F1 and F2 pups were observed for pinna unfolding on PND 3, incisor eruption on PND 11, and eye opening on PND 14. One male and one female F1 and F2 pup selected from each dam were evaluated for the surface righting reflex on PND 5, negative geotaxis reflex on PND 8, and mid-air righting reflex on PND 18. All F1 offspring selected as F1 parents were observed daily for male preputial separation beginning on PND 35 or female vaginal opening beginning on PND 25. Body weight of the respective F1 rats was recorded on the day of preputial separation or vaginal opening. The anogenital distance (AGD) was measured using callipers on PND 4 in all F1 and F2 pups, and the normalized value of AGD to body weight, AGD per cube root of body weight ratio, was calculated. Spontaneous locomotor activity was measured with a multi-channel activity monitoring system in 10 male and female F1 rats selected from each group at 4 weeks of age. Rats were placed individually in transparent polycarbonate cages, which were placed under an infrared sensor that detects thermal radiation from animals. Spontaneous motor activity was determined for 10 min intervals and for a total of 60 min. A test in a water-filled multiple T-maze was conducted in 10 male and 10 female F1 rats selected from each group at 6 weeks of age. The water temperature of the maze was kept 21-22°C. The elapsed time between entry into the water at the starting point and touching the goal ramp and number of errors were recorded.

Necropsy and histopathology: *Parental animals* were necropsied - males after the parturition of paired females and females after weaning of their pups. A complete necropsy was performed on all rats found dead and those killed at the scheduled sacrifice. Weights of the brain, pituitary, thyroid, thymus, liver, kidney, spleen, adrenal, testis, epididymis, seminal vesicle, ventral prostate, uterus, and ovary were recorded. Weights of the thyroid and seminal vesicle were measured after fixation.

Histopathological evaluation of F0 and F1 adults was performed on the tissue specified below after fixation, paraffin embedding, and sectioning and staining with hematoxylin and eosin: the pituitary, liver, thymus, kidney, spleen, adrenal, bone marrow, mesenteric lymph node, Peyer´s patches, testis, epididymis, seminal vesicle, coagulating gland,

ventral prostate, ovary, uterus, vagina and mammary gland of all male and females in the control and highest dose (15,000 ppm) groups. The same evaluation was done on females with abnormal oestrus cycles, males and females without evidence of copulation or insemination, and females with abnormal delivery or litters with only dead pups, in all dose groups. Any organs or tissues of F0 and F1 adults showing gross alterations were evaluated histopathologically. The thyroid in all rats in all groups was examined histopathologically. In ten F1 females of each group, the number of primordial follicles was counted. The right ovary was fixed in 10% neutral buffered formalin and dehydrated and embedded in paraffin in a longitudal orientation by routine procedures. Sections were cut serially at five µM and every 20th section was serially mounted on a slide and stained with hematoxylin and eosin. About 40 sections per ovary were used to determine the primordial follicles.

Following the adjustment of litter size on PND 4, culled pups were euthanized. No tissues from these pups were collected.

On PND 26, unselected F1 weanlings and all F2 weanlings were euthanized and necropsied as described for the adults. Organ weights of one male and one female F1 and F2 weanling selected from each dam were measured as described for adults. The weights of the pituitary, thyroid and seminal vesicle were not determined. All pups found dead before weaning were also necropsied.

In all male and female F1 and F2 weanlings whose organs were collected, histopathological evaluations of the liver, in the control and 15000 ppm groups, and thyroid, in all groups, were performed.

Haematology/serum hormone levels:

On the day of the scheduled sacrifice, blood samples were collected. Hematological examinations and blood chemical evaluations were performed for 10 males and 10 females of F0 and F1 rats randomly selected from each group. Eight males and eight prooestrus females of F0 and F1 generations from each group were selected randomly for blood collection. Serum levels of several hormones were measured with a radioimmunoassay kit.

Semen evaluation: Sperm parameters were determined for all F0 and F1 male adults on the day of the scheduled sacrifice. The right testes were used to count testicular homogenization-resistant spermatid heads. The right cauda epididymis was weighed and used for sperm analysis. Sperm motility, percentage of motile sperm, percentage progressively motile sperm and the swimming speed and pattern were determined. After recording sperm motion, the cauda epididymal fluid was diluted and the sperm were enumerated. Sperm count per gram of epididymal tissue was obtained, and the percentage of morphologically abnormal sperm was calculated

Results: General observations:

F0/F1 adults: Unscheduled deaths and euthanasia due to moribund condition were noted in a few animals, but these events were inconsistent across doses, sex and generations, and not thought to be attributed to the administration of HBCDD. Mean body weights and body weight gains were significantly affected during different periods in F0 and F1 adults, but since the data are inconsistent this is not thought to be

attributed to HBCDD exposure.

F1 pups: Pup body weight was significantly decreased compared to controls at 15,000 ppm in F1 male weanlings on PND 21, and significantly increased at 1500 ppm in F1 female weanlings on PND 0.

F2 pups: Mean body weights were significantly decreased at 15,000 ppm in F2 male and female weanlings on PND 4 (only females), 7, 14 and 21.

Reproductive effects:

F0 animals: Copulation was not observed in two males and two females at 1500 ppm, and in two males and one female at 15,000 ppm. Two of the mated females at 150 ppm and 1500 and three of the mated females at 15,000 ppm did not become pregnant (see Table 5-4.). Using the Cohran-Armitage test on the number of pregnant females (both mated and non-mated females included) vs. the total number of females for the F0 parent generation results in an exact one-sided P-value of 0.02, indicating a significant trend (the statistical calculation was performed by the authors of this dossier). It should be noted that fertility index is affected by both copulation ability and impregnation ability. Total litter loss was observed in one F0 dam at 15,000 ppm by day 5 of lactation. In the sperm analyses, F0 males at 15,000 ppm had higher mean amplitude of lateral head displacement (LHD) compared to controls.

F1 animals: All animals in this generation copulated, but one mated female each at 0 and 150 ppm, and three each at 1500 and 15,000 ppm, were not impregnated (see Table 5-4.). At 1500 ppm, one female did not deliver pups. Total litter loss was seen in one dam in the control group (by day 4 of lactation), and in one dam in the 150 ppm group (by day 2 of lactation). Eight F1 dams in the 15,000 ppm group experienced total litter loss at different days of lactation (up until day 18) without clear signs of toxicity. At 15,000 ppm, a significantly decreased viability index was seen in F2 pups on PND 4 and 21 (68 % vs. 87 %) (see Table 5-4.). The viability index was also decreased in 1500 ppm animals at 21 days (71% vs 85% in controls). This decrease is not statistically significant, but is supported by the 50% decrease at the highest dose, and is therefore considered plausible and of toxicological relevance.

No significant differences between HBCDD-exposed and control animals were seen in copulation index, gestation index, pre-coital interval, number of implantations, delivery index or number of pups delivered in either F0 or F1 animals.

Table 5-4. Fertility index (%), no. of pregnant females, no. of litters in the F0 and F1 parental generation, and viability index (%) in F1 and F2 pups, in control and HBCDD-exposed animals.

	Dose (ppm)			
	0	**150**	**1500**	**15,000**
F0 parent/F1 offspring				
Fertility index in male/female (%)	100/100	91.7/91.7	90.9/90.9	85.7/86.4
No. of pregnant females/total no. females	24/24	22/24	20/24	19/23
No. of litters/total no. females	24/24	21/24	20/24	18/23
Viability index during lactation (%)[1]				
Day 0	99.6	97.5	98.8	99.2
Day 4	95.6	98.7	98.7	95.8
Day 21	93.2	99.4	98.1	93.8
F1 parent/F2 offspring				
Fertility index (male/female) (%)	95.8/95.8	95.8/95.8	87.0/87.5	87.5/87.5
No. of pregnant females/total no. females	23/24	23/24	21/24	21/24
No. of litters/total no. females	23/24	23/24	20/24	21/24
Viability index during lactation[1]				
Day 0	98.6	97.7	96.0	97.8
Day 4	86.9	87.3	92.1	68.4*
Day 21	85.0 (22)[2]	89.6 (22)[2]	71.3	49.7 (20)[2, **]

[1] Viability index (%) = (No. of pups on PND 0, 4 & 21 resp./no. of pups delivered) x 100
[2] Data were obtained from no. of litters in parentheses because females that had only female or male pups, or that lost all female or male pups, during lactation were excluded.
* Significantly different from controls, $p<0.05$.
** Significantly different from controls, $p<0.01$.

Necropsy and histopathology:

F0, F1 and F2 animals:

In F1 females at 1500 and 15,000 ppm, the number of primordial follicles in the ovary was significantly decreased compared to controls (see Table 5-5).

Table 5-5. Number of primordial follicles in the ovary[1] in F1 females of the control and HBCDD-treated groups, respectively.

Dose (ppm)	No of follicles
0	316.3 ± 119.5
150	294.2 ± 66.3
1500	197.9 ± 76.9*
15,000	203.4 ± 79.5*
Historical controls[2]	295.6 (189.5-353.4)

* = significantly different compared to controls ($p<0.05$)

[1] The right ovary was fixed in 10% neutral buffered formalin and then dehydrated and embedded in paraffin in a longitudinal orientation by routine procedures. Sections were cut serially at five µM, every 20th section was serially mounted on a slide and stained with hematoxylin and eosin, and the number of primordial follicles was counted. About 40 sections per ovary were used to determine the primordial follicles. The value given in Table 6 is the total number of primordial follicles counted on about 40 sections.

[2] The historical control data is taken from studies performed in the same laboratory that performed the current study and is based on data from a total of 100 female rats (10 studies with 10 animals each performed 2005-2006).

In F0 males at 1500 and 15,000 ppm, the relative weight of the seminal vesicle was decreased (magnitude not given), and in F0 females at 15,000 ppm significant increases were found in the absolute weight of the adrenal (magnitude not given). In F2 male weanlings, significantly decreased absolute weights of the adrenal, epididymis and ventral prostate (16-27%) was seen at 15,000 ppm, but there were no effects when expressed relative the body weight. In F2 female weanlings, there were significantly reduced absolute weights of the adrenal and uterus (20-22%) at 15,000 ppm, but there were no effects when expressed relative the body weight.

Absolute and/or relative liver and thyroid weights were increased in all generations in both sexes. There were statistically significant increases of liver and thyroid weights by 20-30% at the top dose, and statistically significant increases in liver weight were sometimes also observed at 1500 ppm (7-10% in F0 and F1&F2 weanlings).

Haematology/serum hormone levels:

There were significantly increased levels of TSH in F0 females at 150 ppm and higher (~35-100%), and in F1 females at 1500 ppm and higher (~80%). In F0 males and females, lower levels of T4 compared to controls were seen at 15,000 ppm. There were no effects on T3 in any groups. Serum FSH levels were significantly decreased in F0 males at 1500 ppm, and significantly increased in F0 females at 15,000 ppm. Levels of dihydrotestosterone (DHT) were significantly higher in F1 males at 1500 ppm. There were no significant differences between exposed and control animals in serum testosterone, estradiol, progesterone and LH levels. The effect on TSH levels is consistent through dose groups and generations, and is considered an effect of HBCDD-exposure, while no clear pattern could be seen for other changes in hormone levels.

Conclusion: The main effects seen were a significantly reduced number of primordial follicles in the mid and high dose groups (~30%; only measured in F1 females), and a dose-related decrease in fertility index in both generations, although only being significant in the F0 generation (both mated and non-mated females included in the calculations; the statistical evaluation was performed by the authors of this dossier). In the F0 generation, some animals did not copulate and this influenced the decrease in fertility index, while in the F1 generation all females were mated. A high and dose-dependent pup mortality during lactation was also observed in the F2 generation (increased by ~35% in the high dose group and ~15% in the mid dose group), and this was statistically significant in the high dose group.

Comments: A low number of follicles and ripening follicles in the ovaries were reported in a 28-day-study with HBCDD (Zeller and Kirch 1969), and although the doses in that study were high, the finding could support the effects on primordial follicles seen in this study. A decrease in the number of primordial follicles neonatally can be seen as a decrease in the number of follicles later in life. As far as we know, the effects of HBCDD on the primordial follicles have not been examined in any other studies.

The calculated exposure doses in mg/kg bw/day are high in the 15,000 ppm groups, but the actual exposure depends on the particle size of the HBCDD used. In this study, dosing was done by mixing HBCDD particles into an appropriate amount of powdered basal diet for each dietary concentration. Because of dosing HBCDD-particles, with the absorption kinetics likely being dependent on particle size and amount of particles administered, the actual doses received at the top doses are uncertain, and hence the effects seen in the highest dose groups can not be discarded.

There were no signs of toxicity in adults, and the effects can therefore not be related to parental toxicity. Thus, the weight gain was either slightly increased (F0) or decreased (F1) in males. No effects were noted in F0 females, whereas the weight gain was slightly decreased in young F1 females, leading to an approximately 10% lower body weight in the high dose females at the time of copulation (approximately 280 g vs 300 g in controls). However, weight gain was not affected by HBCDD during gestation and lactation, and there is, thus, no maternal toxicity even at the top dose that could influence the reproductive success (ECBI/30/04).

(Ema *et al.*, 2008; EU Risk Assessment Report)

Study type: One-generation reproductive study according to OECD 415 with some modifications, i.e. a larger number of dose groups, fewer animals per group, and enhancements for endocrine and immunological end-points.

Material: Wistar rats (RIVMpb:WU) distributed in unisex groups of 10 animals/group.

HBCDD (technical mixture containing traces of tetra- and pentabromocyclododecane) was contained as a composite mix. The preparation was a mixture of three diastereoisomers (α-, β- and γ-HBCDD) and their respective proportion in the batch was 10.3; 8.7 and 81%. The test substance was completely dissolved in corn oil, and mixed in the feed. The mixture of HBCDD in oil and the feed was then pelleted (for further details, see van der Ven *et al.*, 2009). An additional control groups was included to monitor the potential effects of corn oil in feed and received food with the standard

lipid concentration. Target dosing was 0; 0.1; 0.3; 1; 3; 10; 30 and 100 mg/kg bw/day.

Method: Parental (P) animals: Exposure started 70 (males) and 14 days (females) before mating, and was continued throughout mating (males), pregnancy and weaning (females), where after the animals were euthanized. In females, number of uterus implantation sites was recorded.

F1 animals: All F1 animals were maintained and litter size was not standardized. At the day of birth, number of living pups and their weights, sex and anogenital distance (AGD) were recorded, and a macroscopic evaluation was done.

During the lactation period, early mortality and the time to vaginal opening or balano-preputial separation were monitored, and pup weights and AGD were recorded at PND 4, 7, 14 (only weights), and 21. During lactation, pups were exposed via the milk, and also had access to the feed of the dam. At weaning (PND21), two male and two female F1 animals from each litter were euthanized for inspection and weighing of reproductive organs. The remaining F1 animals were identified with microchip transponder implants and maintained under the same dosing regime as their parents. Near the end of the study, animals were assigned to groups for either neurobehavioral tests (Lilienthal *et al*., 2006; see below), for an immunization assay, or for necropsy.

Necropsy: In addition to the necropsies at weaning, five animals/sex and dose group were assigned to necropsies at 11 weeks of age. The necropsy protocol included, in summary, sperm sampling from the cauda epididymis for direct analysis, sampling of whole blood, bone marrow, and a standardized part of the spleen for fresh analysis of (immune) cell subpopulations and/or natural killer (NK) cell activity (only males), and sampling of a complete set of organs for gross inspection and weighing. Defined parts of the liver, intestines, brain, one of each pair of adrenals, testes and ovaries, and samples of muscle and fat were snap frozen in liquid nitrogen and stored at −80 °C. Plasma aliquots were stored at −20 °C for analysis of thyroid hormones and further clinical chemistry. Samples of liver were similarly stored for analysis of HBCDD. A distal body preparation including one intact hind limb was frozen at −20 °C for analysis of bone parameters. All remaining dissected organs/tissues were fixed in standard formalin for further histological processing and histopathological assessment.

Compound analysis: Internal dosing was verified by analysis of HBCDD diastereoisomers in liver samples of the five animals/sex/dose group used for final pathological analysis, but was not analyzed in animals used for functional assays in immunology and neurobehaviour. Only 30 female and 24 male samples of the total of 40 samples for each sex in the experimental groups were available for measurement of liver lipid.

Immunology/haematology: Haematological analysis of blood and of femoral bone marrow preparations and analysis of spleen subpopulations and of splenocyte natural killer activity was done. Immunotoxicological effects of HBCDD were further assayed by testing the immunization efficacy of sheep red blood cells (SRBC) in a separate cohort of F1 animals, starting at the age of 8 weeks. Four male rats per dose group were injected intraperitoneally with 2×10^9 SRBC on day 0 and day 15 (booster). Blood was drawn on days 0 (control), 7 (for IgM analysis) and 21 (for IgG analysis) by orbital punction or terminal bleeding, and antibody titers in the serum were determined.

Clinical chemistry: Albumin, alkaline phosphatase, alanine aminotransferase, total cholesterol, creatinine, glucose, total protein and urea were measured. Haemolytic, icteric and lipemic indices were simultaneously measured to exclude interferences with the targeted analyses, and these indices were within acceptable limits in all cases.

Thyroid hormone analysis: Total concentrations of circulating thyroid hormones thyroxin (TT4) and triidothyronin (TT3) were determined in plasma.

Steroid hormone synthesis: Activity of 17-hydroxylase/17,20-desmolase (CYP17) in rat adrenals and of aromatase (CYP19) in ovaries was measured in microsomes isolated from these organs. These enzymes play an important role in the formation of androgens and estrogens, respectively.
Apolar retinoid analyses: Apolar retinoids were extracted from liver homogenates (20%, w/v in water) using diisopropyl ether. All analyses were carried out in duplicate, and the summarized levels of apolar retinoids were computed as sum of retinol and retinylesters.

Bone analyses: Bone analyses were done to determine cortical bone parameters.

Statistical analysis: The dose–response data were analyzed using the BMD (Bench Mark Dose) approach, which enables an integrated evaluation of a complete data set by fitting a dose–response model over the entire dose range. After fitting the optimal dose–response model, a BMD (or critical effect dose, CED) was calculated at a benchmark response (BMR, or critical effect size, CES) of 5–20% (see Table 1 in van der Ven *et al*., 2009). CES is the size of the effect that is considered to be close to the border between adverse and non-adverse, which was defined for each individual parameter on the basis of its known physiological homeostatic variation and of its pathophysiology, including irreversibility or adverse follow-up effects. Most effects were assessed at a CES of 10%, while parameters considered affected during development were assessed at CES of 5%, and immune and liver parameters at CES of 20%. A calculation of a 90% confidence interval (two-sided) was done, enabling the calculation of a 95% (one-sided) lower confidence bound of the BMD estimate. This value may be considered as a BMDL (BenchMark Dose Lower confidence bound) for continuous data. BMDL values expressed as external doses were converted to internal doses (i.e. liver concentrations). For more precision of this value for critical effects, i.e. effects at low doses, BMDLs as internal dose were recalculated directly based on internal doses. A conventional Student's t-test was used to test for possible differences between the high oil vehicle and standard diet control groups. However, the statistical power of the experimental setup (only 5 replicates per dose group) to detect differences between groups is limited.

For further details, see van der Ven *et al*. (2009).

Results: HBCDD analysis: Analysis of HBCDD-α and -γ concentrations in the liver of F1 animals at termination of the experiments showed a dose-dependent increase. The HBCDD concentrations were higher in the liver of females compared to males over the entire dose range (4–6x for the HBCDD-α and -γ, respectively, in the highest doses). This was also seen in the 28-day study by van der Ven *et* al. (2006), indicating a sex difference in kinetics.

Carrier (vehicle) oil effect: There was no difference in liver lipid concentrations between carrier control diet and standard low fat diet controls (5.4±1.7% and 5.1±1.3% in females, and 5.1±0.9% and 5.5±2.6% in males, respectively). However, there were some differences between these groups, e.g. increased mortality of F1 pups during lactation, decreased weight of liver (males) and decreased weight of the adrenals (females) in the carrier oil group. Interaction of the carrier oil in the feed with exposure to HBCDD was suggested e.g. for the parameters food intake and body weight in the dams during gestation, body weights of F1 animals during lactation (except male pups on PND7), decreased weight of adrenals (males), and increased weight of the thymus (males). An increased pup mortality related to a high-fat diet has been seen in other studies as well, while most of the other effects have not.

Food intake & body weight: Food intake was reduced in parental females during the third week of gestation. Reduced growth was observed in parental males during the first 3 weeks of exposure, i.e. weeks 9 - 7 before mating. These effects were initiated near the top dose, reflected in BMDLs between 88 and 95.4 mg/kg bw, at a BMR of 10%. The higher food consumption during lactation of dams compared to previous stages in the experiment should partly be contributed to food intake by the pups.

Reproductive endpoints: There were no significant dose-response effects of HBCDD treatment on endpoints of reproduction, i.e. mating success, time to gestation, gestation duration, number of implantation sites and litter size, nor was pup mortality during lactation affected. Mortality was higher in male pups (in all groups) and the overall mortality rates in male and female pups were 19.3% and 14.9%, respectively.

Developmental end-points: *F1 weanlings:* There was no change in sex ratios in F1 litters. HBCDD exposed male pups had an increased AGD on PND4, but not on PND7 and 21. The increased AGD in males on PND 4 is not thought to be of toxicological relevance since the effect disappeared with age. Time to vaginal opening in females was delayed by 12 % at the highest dose. There was no effect on preputial separation in male F1 pups, on AGD of female F1 pups, or on reproductive organ weights at the time of weaning. F1 body weights were measured on PND 4 and then every week from PND7 until necropsy, and a dose-dependent decrease (~ 7-36% in males and ~10-20% in females; average over the entire observation period ~49 mg/kg bw) was seen. These decreases were first detected at PND4 and persisted until the final recording at week 11 of age. After weaning, the decreased body weights may be related to lower food intake, which was statistically significant at some stage. Food intake varied between life stages, and the average intake decreased from 125 g/kg bw in females and 123 g/kg bw in males in the first week after weaning down to 67 g/kg bw in F1 females and 80 g/kg bw in F1 males at the end of the exposure period.

Sperm parameters & organ weights: No effects were seen on cauda epididymis sperm counts or on morphology, except for a reduction in the ratio of separate sperm heads. The weight of the testes was decreased (≤13.1 %) at a low BMDL. Effects with higher BMDLs (>30 mg/kg/day) were decreased kidney and thymus weights both in males and females, and decreased weight of the adrenals, prostate, heart and brain in F1 males. Further analysis of testis weight, which was the most sensitive effect in the set of affected organ weights, against individual body burden of HBCDD (i.e. concentrations in the liver) also showed a significant dose-response according to the authors (data not shown). No remarkable histopathological changes in either of these organs were seen.

Clinical chemistry: A decrease in the concentration of plasma alkaline phosphatase was seen in females. When re-analyzing this effect against individual body burden of HBCDD, no significant dose-response was seen. There were no effects in any of the other tested plasma parameters in females, nor were there any effects in males.

Endocrinology: No effects were seen on thyroid hormones, total T4 and total T3, in either males or females, nor were there any histopathological changes in the thyroid gland. Total T4 was also analyzed in control and top dose P animals after mating (males) or after lactation (females). The recorded values were suggestive of a reduction in the top dose group, although not statistically significant. There was no significant dose–response in CYP17 activity in the adrenals and CYP19/aromatase activity in the ovary. However, based on group averages, the authors claim that there was a strong correlation between CYP19 and internal concentration of γ-HBCDD (with a linear correlation coefficient of 0.90)(data not shown).

Apolar retinoids: There were marked dose-dependent decreases in liver apolar retinoid levels. The maximum decreases as compared to background levels were similar between females (32.6% and 28.5% for apolar retinoid concentrations and total amount, respectively) and males (20.6% and 24.6% for concentrations and total amount, respectively). Because these parameters had low BMDLs in females as analyzed based on HBCDD dosing, they were further analyzed based on the individual body burden of HBCDD. This analysis also resulted in low BMDLs (data not shown).

Bones: A decrease of trabecular bone mineral density in F1 females (maximal decrease 22.6%) was seen when performing a pQCT analysis of bones.

Immunotoxicology & hematology: The immunization assay against SRBC in juvenile F1 animals revealed no change in the initial immunization response as read from specific IgM after 7 days. However, there was an increased specific IgG response after 21 days. The NK activity test showed no effect, but spleen cell analysis suggested an increase of the NK cell fraction. In the peripheral blood of male littermates of the animals used for immunization, there was an increased fraction of neutrophilic granulocytes. This increase was also observed when analyzed against internal liver concentrations of HBCDD, although the high variation in the data set did not provide a valid BMDL. According to the authors, there were also significant dose-responses for decreased lymphocyte fraction, a decreased whole white blood cell count in the blood and an increased white blood cell count in the bone marrow; BMDLs for these three parameters were however not relevant for various statistical reasons. The same is true for decreased thymus weight in both sexes, and for increased weight of popliteal lymph nodes in males. There were no discernible exposure-related histopathologic changes in the thymus and popliteal lymph nodes. In the spleen, on the other hand, the marginal zone showed enlargement with a significantly higher frequency in top dose animals compared with control animals (7/10 vs. 1/8).

Conclusion: The decreased weight of the testis and prostate in males is thought to be treatment-related and not related to the decreased body weight since the effects were larger than the observed body weight decrease and occurred with lower BMDL than the BMDL for the body weight decrease. Delayed vaginal opening was seen in females. The male reproductive organ weights as well as time to vaginal opening are sex hormone dependent, and may indicate that HBCDD have endocrine disrupting effects. Also the bone effects observed may be related to disturbances in the sex hormone system. Interactions with e.g. androgen and estrogen receptors have been seen in *in vitro*

studies with HBCDD, but the mechanism seems to be complex.

As mentioned above, effects on several parameters are obvious at the top dose(s) (30-100 mg/kg/day), e.g., on testis and prostrate weight as well as on female bone mineral density, suggesting developmental toxicity in this study in the absence of any maternal toxicity. The effect on prostrate weight is corroborated by the 90 days study by Chengelis (2001), but a decreased weight of the testis has not been seen in other studies, including the 2-generation study. Bone mineral density is not normally studied, and whereas a previous 28 days study in adult rats suggested an increased density, this one-generation study shows a decreased bone density in the offspring. Potential effects of HBCDD on bone density are, thus, indicated, but needs verification in further studies.

The data are also suggestive of effects on the immune system, seen both as effects on non-functional parameters and as a stimulatory effect in functional assays, but the data are difficult to evaluate.

The liver and the thyroid have been target organs for HBCDD in all previous studies, but no effects on weights or histology of these organs were observed in the present one-generation study. Reasons for these discrepancies could be related to that the animals were dosed via the diet, or that the high dosing of the oil-vehicle is a confounding factor.

As to the effect levels, the setting of conventional LOEALs/NOAELs are hampered by the chosen benchmark study design. It is also noted that there is still limited regulatory experience in assessing and handling benchmark studies. The benchmark dose modeling in this study follows standard procedures, with a default critical effect size of 10 %. The calculated BMDLs are very much dependent on the size of the chosen critical effect size, and it is noted that also individual control animals usually fall below/over the chosen critical effect sizes. For some effects, the normal variation is so high (e.g., mean±S.D. = 0.18±0.12 for IgG response after immunization with sheep red blood cells) that the chosen 20 % critical effect size becomes meaningless. We are therefore of the opinion that the BMDLs calculated in this study should be viewed with caution.

(van der Ven *et al*., 2009)

5.9.2 Developmental toxicity

Study type: Two-generation reproductive study according to OECD guideline 416 and GLP

Material: 5-week old males and females, Crl:CD(SD) rats

Method: Rats were given dietary HBCDD at a concentration of 0, 150, 1500 or 15000 ppm, which numerically has been translated to 10-14, 101-141, or 1008-1363 mg/kg/day in low, mid, and high dose animals with the low end of the ranges representing males and the high females. The daily intake of food increased during lactation, leading to calculated daily intake of 20-23, 179-240, and 1724-2200 mg/kg/day in dams of the low, mid, and high dose groups, respectively.

For further details, see section 5.9.1and Ema *et al*. (2008).

All F1 and F2 pups were observed for pinna unfolding (PND 3), incisor eruption (PND 11) and eye opening (PND 14). One male and one female F1 and F2 pup from each dam was selected for examination of surface righting reflex (PND 5), negative geotaxis reflex (PND 8) and mid-air righting reflex (PND 18). F1 weanlings selected as F1 parents were examined daily for male preputial separation (beginning PND 35) and female vaginal opening (beginning PND 25). Anogenital distance was measured on PND 4 in all F1 and F2 pups.

Behavioural tests were performed in selected F1 animals. Spontaneous locomotor activity was measured in 10 male and 10 female F1 rats from each group (at 4 weeks of age), and a water-filled multiple T-maze test were conducted in the same number of animals (at 6 weeks of age).

Necropsy and histopathological examination of F0 and F1 parental animals and F1 and F2 weanlings were performed. Organ weights of one male and one female F1 and F2 weanling from each dam, were measured.

Results: *General observations*

See section 5.9.1for a detailed description of the general observations.

The weight gain was either slightly increased (F0) or decreased (F1) in males. No effects were noted in F0 females, whereas the weight gain was slightly decreased in young F1 females, leading to an approximately 10% lower body weight in the high dose females at the time of copulation (approximately 280 g vs 300 g in controls). However, weight gain was not affected by HBCDD during gestation and lactation, and there is, thus, no maternal toxicity even at the top dose that could influence the reproductive success.

Reproductive effects

Total litter loss was observed in one F0 dam at 15,000 ppm by day 5 of lactation, and in one F1 dam in the control group (by day 4 of lactation) and one in the 150 ppm group (by day 2 of lactation). Eight F1 dams in the 15,000 ppm group experienced total litter loss at different days of lactation (up until day 18) without clear signs of toxicity. At 15,000 ppm, a significantly decreased viability index was seen in F2 pups on PND 4 and 21. The viability index was also decreased in 1500 ppm animals at 21 days (71% vs 85% in controls). This decrease is not statistically significant, but is supported by the 49.7% decrease at the highest dose, and is therefore considered plausible and of toxicological relevance.

Organ weights

In F2 male weanlings, significantly decreased absolute weights of the adrenal, epididymis and ventral prostate was seen at 15,000 ppm, but there were no effects on relative weights. In F2 female weanlings, there were significantly reduced absolute weights of the adrenal and uterus at 15,000 ppm, but no effects on relative weights. However, the relative ovary weight was significantly increased at 150 and 15,000 ppm in F2 weanlings, and non-significant tendencies of increased relative ovary weight were observed in F1 weanlings and adults.

Developmental landmarks and behavioral effects

A significantly lower completion rate of mid-air righting reflex was seen in F2 female pups at 15,000 ppm (76.9% vs. 100% in controls). No effects were seen in the behavioural tests.

Conclusion: A high and dose-dependent pup mortality during lactation was also observed in the F2 generation (increased by ~35% in the high dose group and ~15% in the mid dose group), and this was statistically significant in the high dose group. There were no signs of toxicity in adults, and the effects can therefore not be related to maternal toxicity.

(Ema et al., 2008; EU Risk Assessment Report)

Study type: One-generation reproductive study in rats according to OECD 415 with some modifications, i.e. a larger number of dose groups, fewer animals per group, and enhancements for endocrine and immunological end-points.

Results: For further details on the conduct of this study (van der Ven et al , 2009) and the general results, the reader is referred to the description of the study above in section 5.9.1. Only effects related to development is described below.

Developmental end-points: *F1 weanlings:* There was no change in sex ratios in F1 litters. HBCDD exposed male pups had an increased AGD on PND4, but not on PND7 and 21. The increased AGD in males on PND 4 is not thought to be of toxicological relevance since the effect disappeared with age. Time to vaginal opening in females was delayed by 12% at the highest dose. There was no effect on preputial separation in male F1 pups, on AGD of female F1 pups, or on reproductive organ weights at the time of weaning. F1 body weights were measured on PND 4 and then every week from PND7 until necropsy, and a dose-dependent decrease (~ 7-36% in males and ~10-20% in females; average over the entire observation period ~49 mg/kg bw) was seen. These decreases were first detected at PND4 and persisted until the final recording at week 11 of age. After weaning, the decreased body weights may be related to lower food intake, which was statistically significant at some stage. Food intake varied between life stages, and the average intake decreased from 125 g/kg bw in females and 123 g/kg bw in males in the first week after weaning down to 67 g/kg bw in F1 females and 80 g/kg bw in F1 males at the end of the exposure period.

Sperm parameters & organ weights: No effects were seen on cauda epididymis sperm counts or on morphology, except for a reduction in the ratio of separate sperm heads. The absolute weight of the testes was decreased (≤13.1 %) at a low BMDL. Effects with higher BMDLs (>30 mg/kg/day) were decreased absolute kidney and thymus weights both in males and females, and decreased absolute weight of the adrenals, prostate, heart and brain in F1 males. Further analysis of testis weight, which was the most sensitive effect in the set of affected organ weights, against individual body burden of HBCDD (i.e. concentrations in the liver) also showed a significant dose-response according to the authors (data not shown). No remarkable histopathological changes in either of these organs were seen.

Endocrinology: No effects were seen on thyroid hormones, total T4 and total T3, in

either males or females, nor were there any histopathological changes in the thyroid gland. Total T4 was also analyzed in control and top dose P animals after mating (males) or after lactation (females). The recorded values were suggestive of a reduction in the top dose group, although not statistically significant. There was no significant dose–response in CYP17 activity in the adrenals and CYP19/aromatase activity in the ovary. However, based on group averages, the authors claim that there was a strong correlation between CYP19 and internal concentration of γ-HBCDD (with a linear correlation coefficient of 0.90)(data not shown).

Apolar retinoids: There were marked dose-dependent decreases in liver apolar retinoid levels. The maximum decreases as compared to background levels were similar between females (32.6% and 28.5% for apolar retinoid concentrations and total amount, respectively) and males (20.6% and 24.6% for concentrations and total amount, respectively). Because these parameters had low BMDLs in females as analyzed based on HBCDD dosing, they were further analyzed based on the individual body burden of HBCDD. This analysis also resulted in low BMDLs (data not shown).

Bones: A decrease of trabecular bone mineral density in F1 females (maximal decrease 22.6%) was seen when performing a pQCT analysis of bones.

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Conclusion: The decreased weight of the testis and prostate in males is thought to be treatment-related and not related to the decreased body weight since the effects were larger than the observed body weight decrease and occurred with lower BMDL than the BMDL for the body weight decrease. Delayed vaginal opening (12%) was seen in females at the top dose. The male reproductive organ weights as well as time to vaginal opening are sex hormone dependent, and may indicate that HBCDD have endocrine disrupting effects. Also the bone effects observed may be related to disturbances in the sex hormone system. Interactions with e.g. androgen and estrogen receptors have been seen in *in vitro* studies with HBCDD, but the mechanism seems to be complex.

As mentioned above, effects on several parameters are obvious at the top dose(s) (30-100 mg/kg/day), e.g., on testis and prostrate weight as well as on female bone mineral density, suggesting developmental toxicity in this study in the absence of any maternal toxicity. The effect on prostrate weight is corroborated by the 90 days study by

Chengelis (2001), but a decreased weight of the testis has not been seen in other studies, including the 2-generation study.

(Van der Ven *et al*. 2009)

Study type: Separate reporting of additional studies conducted within the above reported one-generation study by van der Ven et al (2009). The study was, thus, a one-generation reproductive study according to OECD 415 with some modifications, i.e. a larger number of dose groups, fewer animals per group, and enhancements for studies on behaviour and hearing function.

Material: Wistar rats (see further information above in relation to the van der Ven et al study (2009)).

Method: A benchmark design was used and the study was conducted to investigate the neurotoxicity of HBCDD in rats. Wistar rats received HBCDD in the diet, and the exposure started before conception and was continued throughout mating, gestation, lactation, and after weaning of the offspring. There were eight dose groups; control (vehicle), 0.1; 0.3; 1; 3; 10; 30 or 100 mg/kg bw. At the age of 90 days, 6 males and 6 females from each group were transferred from the Netherlands to another research facility in Germany for additional studies on hearing and behaviour. Four to six animals per sex per group were used for the studies.

Because in vitro data have indicated that HBCDD may affect dopamine uptake in cells (Mariussen & Fonnum, 2002), haloperidol-induced catalepsy was studied to test if HBCDD affects dopamine-dependent behaviour in vivo. Haloperidol is a substance that blocks a variety of receptors in the brain, particularly dopamine receptors. Therefore, haloperidol-induced catalepsy was selected for investigating if HBCDD affects the dopamine uptake in the nervous system. At the age of 110 days, catalepsy was induced in the rats by i.p. injection of haloperidol. Thirty and sixty minutes later the rats were placed in three unusual body postures, and the time until movement was measured.

Because of previously reported effects of HBCDD on the thyroid hormone system and the importance of thyroid hormones in the development of the auditory system (e.g., the cochlea), the effect of HBCDD on the auditory function was studied. A test called brainstem auditory evoked potentials (BAEPs) was used to study the hearing function. At the age of 140 days, rats were sedated with ketamine/xylazine and exposed to tone stimuli in one ear (broadband click and frequency-specific tone stimuli), with electrodes simultaneously measuring the potentials (BAEPs). Auditory thresholds as well as latencies between stimuli and potentials were recorded.

Results: In the catalepsy test, exposure to the two top doses of HBCDD resulted in decreased

latencies to movement onset in female rats in all three body postures used to measure the cataleptic behaviour. The effects were much less pronounced in males. The BMD-L values calculated by the authors are in the range of 0.6-4.4 mg/kg/day, but these values should be viewed with caution as explained above in relation to the van der Ven study (van der Ven et al, 2008). The outcome may, according to the author, be due to HBCDD-related hepatic enzyme induction, resulting in enhanced metabolism of haloperidol, to disturbances of the thyroid/steroid systems, or/and to lower dopaminergic activity in the brain. A relation to hepatic enzyme induction seems plausible, although other mechanisms are possible.

In contrast, only males were affected by effects on the auditory system, as measured by the BAEP test. Effects of HBCDD were observed as elevated thresholds in the lower frequency range and after click stimulation. The threshold was elevated by 5-9 dB at the top dose (100 mg/kg/day). There were also corresponding prolongations of latencies between stimuli and potential in the lower frequency range. Calculated BMD-L values were much lower for effects on thresholds (1-6 mg/kg/day) than for effects on latency. The authors interpret the effects as indicating a cochlear origin of the hearing impairment.

Conclusion: The study indicates that HBCDD exposure during pregnancy and lactation may cause effects that persist and can be detected in adult animals. The effects are most obvious at the top dose, but the calculated BMDLs are low (1-6 mg/kg/day). The effects on the catalepsy test could be related to hepatic enzyme induction, whereas the reasons for the effects on hearing not are understood. The parameters investigated are not part of any test guidelines, and it is therefore difficult to assess the robustness of these assays and the degree of adversity of the effects. However, the main author has been consulted, and provided further interpretation of the data. Thus, the increased hearing threshold by 5-9 dB can be translated into requiring a (4-8)-fold increase in sound intensity to pass the hearing threshold in the lower frequency range, or into requiring a (1.5-3)-fold increase in loudness to pass the threshold. Considering the importance of hearing, the effects observed at the top dose in this study on hearing have to be viewed as adverse effects.

(Lilienthal *et al*. 2009)

Study type: 1-generation developmental toxicity study in rat offspring after maternal exposure from mid-gestation through lactation. The study did not follow any internationally accepted test guidelines.

Material: Pregnant Sprague-Dawley rats

Metod: Pregnant Sprague-Dawley rats were exposed via the diet to HBCDD from gestation day 10 until weaning of the offspring on day 20. HBCDD was obtained from a Japanese company, and had a purity >95%. The test substance was mixed into the diet at concentrations of 0, 100, 1000 or 10,000 ppm, stated to be equivalent to 8-21, 81-213, or 803-2231 mg/kg/day, and given to groups of 10 dams per concentration. The

report does not give information whether HBCDD was dissolved prior to mixing in the diet or if HBCDD particles (of unknown size) were mixed into the food. The study also included 4 other groups of rats similarly exposed to tetrabromobisphenol A (TBBPA). Dams were sacrificed by exsanguination at postnatal day (PND) 20 and growth and thyroid endpoints were measured in the dams. Offspring were sacrificed and autopsied at weaning (PND 20) or at the age of 11 weeks. Offspring autopsy included measurements of body weight, organ weights, histopathology, developmental landmarks, and thyroid hormones. In addition, brain development was studied using immunohistochemistry and morphometry. Statistically significant effects ($p<0.05$) are reported below.

Results:

Effects in dams

The only observed effects on the dams, at autopsy, were a 30% increased weight of the thyroid gland and an increased incidence and severity of diffuse thyroid follicular cell hypertrophy in the high dose animals (10,000 ppm).

Effects in offspring

In pups, there were no effects on any parameters at PND 1, such as number of live offspring or body weights. At day 20, the body weight was decreased by 9% in high dose prepubertal females, and the relative liver weight was increased by 27-28% in both sexes in the high dose group. The onset of puberty was not affected in either sex, but the female body weight at puberty was decreased by 9% in the high dose group (day 34).

Serum levels of thyroid-related hormones were examined only in males, both at PND 20 and at week 11. The level of T3 was decreased (-15%) and the level of TSH increased (+30%) at PND 20 in the high dose group. At 11 weeks, T3 was decreased in the mid and high dose groups (7-8%), but there were no effects on TSH. The relative thyroid weight was dose-dependently increased in males (17, 19, 28%), with the increases being statistically significant in the mid and high dose groups. There were no effects on the weight of the female thyroid or on thyroid histology in either sex.

Histopathology revealed increased incidences of diffuse vacuolar degeneration of liver cells of both sexes in the high dose groups on PND20 but not at week 11. An increased incidence of adrenocortical vacuolar degeneration was observed in the high dose males at week 11.

The brain morphometry was only conducted on males. The parameters chosen were thought as markers for hypothyroidism since substances causing hypothyroidism (propylthiouracil and methimazole) have affected these parameters in a previous study by the same authors. The morphometry showed no effects on the distribution of hippocampal CA1 neurons, whereas effects on the oligodendroglial development was indicated by a reduction in the number of CNPase(2',3'-cyclic nucleotide 3'-phosphodiesterase)-positive oligodendrocytes in the cingulate deep cortex. The reduction in brain oligodendrocytes was statistically significant at the high dose and

was supported by a dose-dependent trend (-8, -12, -24% in the low, mid, and high dose groups, respectively).

In the parallel study on TBBPA, only negligible effects were observed, supporting that the findings in the HBCDD study were substance-related and not chance findings.

Conclusion: In summary, the study shows the thyroid hormone system and the liver being target organs for HBCDD also in offspring of rats dosed during the latter half of pregnancy and the lactation period. Thyroid effects were observed both in dams (thyroid weight increase and follicular cell hypertrophy at 10,000 ppm) and offspring (thyroid weight increase, decreased serum T3 and increased serum TSH at 1,000 and 10,000 ppm). Thus, rather persistent thyroid effects were noted in the offspring (decreased T3 and increased thyroid weight), which together with the impaired oligodendroglial development in the brain cortex and the decreased female body weight could indicate developmental hypothyroidism. The LOAEL of this study is 1,000 ppm (stated to be equivalent to 81-213 mg/kg/day), and the NOAEL 100 ppm (8-21 mg/kg/day).

(Saegusa *et al*. 2009)

Study type: Prenatal development toxicity study similar to OECD 414, but with some deviations (see comments below)

Material: 80 non-pregnant female Wistar rats, divided into four groups of 20 animals each. In the main developmental study the 20 rats of each dose group were given 0; 0.001; 0.1 and 1% HBCDD in the diet during GD 0-20.

Method: This study also covered potential effects during the preimplantation stage. The doses are approximately equivalent to 0; 7.5; 75 and 750 mg/kg/day, respectively. These calculations are based on assumptions that the animals mean weight is 200g and their food consumption is 15 g/day. On day 20 of gestation 14 rats per group were killed by cervical dislocation, and served for abdominal surgery to visual observation of major organs. Organs were weighed. Numbers of corpora lutea, implants, resorptions, and live foetuses were measured. The incidence of abnormalities on external examination was examined, sex was identified and both body weight and placental weight were measured for each live foetus. 1/3 of the foetuses were examined for visceral anomalies and 2/3 of the foetuses were examined for skeletal abnormalities. The remaining 6 dams per group were delivered naturally, and the pups monitored through weaning. Number and body weight of delivered foetuses, number of live foetuses, and abnormalities resulting from external examination was recorded. From the third week, males and females were separated, and the new-borns growth and survival was observed until the 7th week.

Results: In the highest dose group the maternal food intake was slightly suppressed, and both the absolute and relative maternal liver weights were significantly increased by 13%. The mothers showed no signs of toxicity, nor were body-weight gain affected. No significant changes in number of implants, number of resorbed, dead or live foetuses was reported, or external, visceral or skeletal anomalies of foetuses that could be

attributed to exposure to HBCDD. No difference was seen in the number of live newborns, or in the number of dead newborns, and no abnormalities were observed based on external examination. No abnormality of newborns was observed during parturition, during the weaning period, and after the weaning period. Normal body weight changes of both male and females of each administration group were observed. No significant difference in either the weaning index or the survival index, obtained when the experiment was over, was observed between administration groups and the control group.

Conclusion: No HBCDD related developmental toxicity was seen at any of the dose levels.

(Murai *et al*., 1985, as in EU Risk Assessment Report)

Study type: Prenatal developmental toxicity study according to OECD Guideline 414

Material: The test material used consisted of a composite of the commercial products from three different producers. As determined by NMR, the material consisted of 90% HBCDD diastereomers (about 6.4% α-, 4.5% β-, and 79% γ-diastereomer, plus 0.5-0.9% tetrabromocyclododecane and about 9% unknown constituents) that were found to be stable during the time of the study.

Three groups of 25 female Charles River CD rats (Crl:CD(SD)IGS BR) were administered 0, 500, or 1000 mg HBCDD per kg and day orally in corn oil (suspension with a mean particle size of 142 µM) once daily from gestation days 6 through 19.

Method: Clinical observations, body weights and food consumption were recorded during the exposure period. All animals survived until sacrifice on gestation day 20. Body weight gain and food consumption were not adversely affected at any dose level, and no significant clinical signs were observed. However, histopathological analyses of selected organs of the dams were not performed. On day 20 all maternal animals were subjected to laparohysterectomy, and uteri and ovaries were examined as well as the number of foetuses, early and late resorptions, total implantations and number of corpora lutea. Mean gravid uterine weights and net body weight changes were assessed. The foetuses were weighed and examined for external soft tissue and skeletal malformations as well as variations.

Results: At necropsy, no treatment-related clinical signs were observed at any dose level. Intrauterine growth and survival were unaffected, and no treatment-related foetal malformations or developmental variations were observed in any of the treated groups. In the 500 mg/kg/day group, one foetus had a facial cleft as well as exencephaly and another foetus exencephaly. There were no soft tissue malformations in any of the examined foetuses, but soft developmental variations were observed in one foetus in each of the 500 and 1000 mg/kg/day groups. However, these single occurrences cannot be considered to have any significance. Skeletal variations occurred in all dose groups as well as in controls and consisted primarily of unossified sternebrae (2 foetuses), ossified cervical centrum (1) as well as rudimentary ribs (2).

Conclusion: No HBCDD related developmental effects were seen at any of the dose levels.

(Stump, 1999, as in EU Risk Assessment Report)

Study type: Neurotoxicity study of HBCDD exposure during brain development. Spontaneous behaviour, learning and memory capabilities were studied.

Material: Neonatal male NMRI mice

Method: HBCDD [0.9 or 13.5 mg/kg body weight] as a single oral dose by gavage (8-10 animals/dose group)
The animals were placed in cages and locomotion (horizontal movement), rearing (vertical movement) and total activity (all types of vibrations within the test cage) were monitored for 3×20 minutes.
Learning and memory capability was measured with the Morris water maze method.

Results: *The high-dose group (13.5 mg/kg):*
All three parameters (locomotion, rearing and total activity) were significantly affected compared to controls during the first and the last 20-minute-period, where hypo- and hyperactivity, respectively were demonstrated. The locomotion and total activity during the first 20-minute-period were estimated to 50% of control values, and rearing was even more affected with approximately 6 times less activity compared to controls. The total activity during the last 20-minute-period was twice as high in the high-dose animals compared to the control mice and differences were even greater for locomotion and rearing.

The low-dose group (0.9 mg/kg):
Locomotion and rearing were significantly decreased by a factor of approximately 1.3, during the first 20-minute-period, but there were no effects in the later measurements or in total activity.

Morris water maze (Learning and memory capability)
The latency periods were estimated to be 20-30, 15 and 10 seconds, respectively, in the high-dose, low-dose and control groups. Moreover, the re-learning ability on day five was impaired in high-dose animals with significantly longer latency periods (20-30 s) compared to controls (10-20 s). Mice exposed to 0.9 mg HBCDD/kg did not differ from controls in the water maze experiment.

Comments: The study does not follow the current guidelines. The mice were exposed during the peak period of rapid brain growth, known as the “brain growth spurt” (BGS). During the BGS, the brain undergoes several fundamental phases, such as dendritic and axonal outgrowth and establishment of neural connections. Whereas this period is neonatal in rats, spanning the first 3-4 weeks of life, this period begins during the third trimester of pregnancy in humans and continues throughout the first two years of life.

Conclusion: The study design was not considered to be robust by the ESR TC NES expert group and no conclusion on developmental neurotoxicity can be drawn from this study alone.

However, the results may indicate that HBCDD can cause developmental neurotoxic effects at low exposure levels, and the results could potentially be correlated to the effects on thyroid hormones seen in other studies.

(Eriksson et al., 2006, as in EU Risk Assessment Report)

5.9.3 Human data

Meijer et al (2008) have studied the influence of prenatal exposure to selected environmental contaminants, including HBCDD, on infant sexual and neurological development. The Dutch cohort included 90 mothers and 90 children (56 boys and 34 girls). HBCDD and other organohalogens were analysed in 69 maternal serum samples. In the children, the following parameters were studied; sex hormones in 3 months infants, testes volume and penile length at 3 and 18 months of age, neurological development at 10 days as well as at 3 and 18 months of age.

The results are reported in an extended abstract, and the results for HBCDD are only reported in tables and not specifically discussed in the report. The tables indicate a correlation between HBCDD concentrations in the mothers and 7 different parameters; the concentration of luteinising hormone in boys (↑), free and total testosterone in girls (the direction of effect is not indicated), testes volume and penile length at 18 months of age (↑), mental classification (↓), and the motor quality score (↑).

The significance of these finding are at present not clear.

Many studies show that HBCDD is present in human breast milk. The data are presented in section 5.1 on toxicokinetics.

5.9.4 Other relevant information

Study type: In vitro study

Material: Rat brains from male Wistar rats

Method: The plasma membrane uptake of the neurotransmitters dopamine, glutamate and γ-amino-n-butyric acid (GABA) in rat brains was studied. Male Wistar rats (150-200g) were killed by decapitation and the brains were quickly removed and kept on ice. The brains were homogenised in sucrose, centrifuged, and the supernatant was mixed with sucrose and centrifuged a second time to get a crude synaptosomal pellet without myelin. The pellet was pre-incubated at 25 ºC for 15 min in absence or presence of HBCDD in Tris-Krebs buffer. HBCDD was tested in four different concentrations (2-20 μM). The reaction was started by adding substrate containing either 3H-glutamate, 3H-GABA or 3H-dopamine, and terminated by a bovine serum dilution, and rapid filtration into a glass-fibre filter mat. The filters were dissolved and counted for retained radioactivity in a liquid scintillation spectrophotometer. Blanks were treated similarly. HBCDD inhibited neurotransmitter uptake into synaptosomes and dopamine uptake into synaptic vesicles.

Results: The dopamine uptake was inhibited at low concentrations with an IC50 value of 4 ± 1μM. HBCDD also inhibited glutamate uptake at low concentrations with a 26 ± 9% inhibition at 1 μM, but it never achieved more than a 40 ± 6 and 50 ± 4% inhibition at 20 and 50 μM, respectively. The study does not report any effects concerning GABA inhibition. Glutamate uptake was inhibited to an equal extent in synaptosome fractions from cerebellum and forebrain.

Conclusion: The present study indicates that HBCDD might have a neurotoxicological potential, but it is very difficult to evaluate the in vivo relevance of this kind of in vitro studies.

(Mariussen & Fonnum, 2002, as in EU Risk Assessment Report)

5.9.5 Summary and discussion of reproductive toxicity

Fertility:

In the two-generation reproductive study (Ema *et al*., 2008) performed in rats, there was evidence of effects on fertility parameters. The main findings were a trend for a decrease in fertility index in both F0 and F1 (although only being significant in the F0 generation), and a significantly reduced number of primordial follicles in the mid and high dose groups (~30%; only measured in F1) This study indicates that when the pre-dosing period is 10 weeks, as in this study, it is possible to detect effects on fertility even in the prenatal group (F0).The primordial follicle pool is formed just after birth in rodents (Mc Gee & Hsueh, 2000; Skinner, 2005) and is, according to current knowledge, not renewable. Hence a decrease in the number of primordial follicles can lead to a non-reversible decrease in fertility. Although the number of primordial follicles in the mid- and high-dose group is within the limits of historical control data, the number of historical control animals is not considered high enough to draw conclusions from, since the number of animals in the present study examined for this end-point is equally high, and the historical control data are taken from studies with a smaller number of animals per study than in the present study.

In the high dose group, the exposure is 1008-1363 mg/kg/day. However, actual internal exposure depends on the particle size of the HBCDD used. In this study, dosing was done by mixing HBCDD particles into a powdered basal diet for each dietary concentration. Because of dosing HBCDD-particles, with the absorption kinetics likely being dependent on particle size and amount of particles administered, the actual internal doses received at the top doses are uncertain (i.e. the internal dose is likely to be lower than expected), and hence the effects seen in the highest dose groups can not be discarded. Furthermore, effects were also observed in the mid dose where the dose was 100-140 mg/kg/day, and there were no signs at any dose of any maternal toxicity that needs to be considered in relation to classification and labeling.

The EU criteria specifically mention effects on oogenesis as reason for classification (“Effects on male or female fertility, includes adverse effects on….. any aspect of spermatogenesis or oogenesis, or on hormonal activity or physiological response which would interfere with the capacity to fertilise, fertilisation itself or the development of the fertilised ovum up to and including implantation.”). Therefore, the significant decrease in number of primordial follicles together with the decrease in fertility index (seen as a significant trend in the F0 generation) indicates that HBCDD

affects fertility in rats and a classification as **Repr. Cat. 3; R62 (Possible risk of impaired fertility)** is therefore proposed.

Development:

A high and dose-dependent pup mortality during lactation was observed in the F2 generation (increased by ~35% in the high dose group and ~15% in the mid dose group; statistically significant in the high dose group) without signs of toxicity in the dams. This dose-dependent increase in pup mortality is therefore considered plausible and to be of toxicological relevance. There were no HBCDD related effects on mortality or body weights in the dams (Ema *et al.*, 2008).

In the one-generation study by van der Ven *et al.* (2009), a decrease in testis and prostrate weight in male weanlings, as well as delayed vaginal openings in females, were seen. These effects are considered treatment related and indicate that HBCDD may have endocrine disrupting properties, leading to effects on sex hormone dependent organs. HBCDD may also seem to affect bone density and the immune system, seen both as effects on non-functional parameters and as an immunostimulatory effect in functional assays.

There are also indications of developmental neurotoxic effects in two different studies.

In the study by Lilienthal *et al*. (2009), effects on hearing function and dopamine-dependent behaviour were observed.

In the other study, by Eriksson *et al.* (2006) effects on spontaneous behaviour, manifested as reduced habituation with initial hypoactivity followed by hyperactivity in a novel environment was observed.

In several repeated dose toxicity studies, as well as in the study by Ema *et al.* (2008), HBCDD-related effects were seen on the thyroid system. Thyroid hormone is essential for normal brain development and it has been shown that disturbances in the thyroid system in dams can affect the neurological development in pups, leading to effects on e.g. locomotor activity, neuromotor competence, hearing, and cognitive function after birth.

The EU criteria specifically mention many of the observed effects (noted in italics in the following citation "Developmental toxicity, is taken in its widest sense to include any effect interfering with normal development, both before and *after birth*. It includes effects induced or manifested prenatally as well as those manifested *postnatally*. This includes embrytoxic/fetotoxic effects such as reduced body weight, growth and developmental retardation, organ toxicity, *death*, abortion, structural defects (teratogenic effects), *functional defects*, peri-postnatal defects, and impaired postnatal mental or physical development up to and including normal *pubertal development*."). With regard to category 3, the criteria say; "In general, classification in category 3 …. would be assigned on an ad hoc basis where the only effects recorded are …….small differences in postnatal developmental assessments."

The observed pup mortility during lactation in the F2 generation of a 2-generation study in rats, and the other developmental effects; decreased weights of testis and prostate in male weanlings, delayed vaginal opening in female weanlings of a one-generation study and the observed developmental neurotoxicity effects indicates that HBCDD affects developmental parameters and a classification as **Repr. Cat 3; R63 (Possible risk of harm to the unborn child)** is proposed.

Table 5-6. Summary of reproductive effects

+ = observed effect; 0 = no observed effect; - = not investigated

	2-gen[1] (OECD Guideline 416)			**Extended 1-gen**[2] (OECD Guideline 415, extended with endocrine endpoints)		**Modified 1-gen**[3] (not Guideline study)		**Develop. Neurotox**[4] (not Guideline study)
Specie:	***-Crl:CD(SD) rats***			***-Wistar rats (RIVMpb:WU)***		***-Sprague-Dawley rats***		***-NMRI mice (neonatal)***
Dosegroups (mg/kg):	***-0, 10-14, 101-141, 1008-1363***			***-0, 0.1, 0.3, 1, 3, 10, 30, 100***		***-8-21, 81-213, or 803-2231***		***-0, 0.9, 13.5***
Vehicel:	***- no vehicle***			***-Corn oil (completely dissolved)***		***-?(not reported)***		***-egg lecithin and peanut oil***
Administration:	***-Mixed in the diet (particles of mean size 0.1mm)***			***-Solution mixed in the diet***		***-mixed in the diet***		***-emulsion via gavage***
Effects	***F0***	***F1***	***F2***	***F0***	***F1***	***F0***	***F1***	
Affected thyroid hormone system	+	+	-	-	+	-	+	-
Pup mortality	-	0	+	-	0	-	-	-
Decreased fertility index	+	+		-	0	-	-	-
Reduced number of primordial follicles	-	+	-	-	-	-	-	-
Decreased testis weight	0	0	0	-	+	-	-	-
Decreased prostate weight	0	0	0	-	+	-	-	-
Delayed vaginal opening	-	-	-	-	+	-	-	-
Impaired hearing	-	-	-	-	+	-	-	-
Effects on behaviour	-	0	-	-	-	-	-	+
Impaired brain development	-	-	-	-	-	-	+	-

[1]: Ema, M.,*et al*. (2008).
[2]: Van der Ven, L.T.M.,*et al*. (2009)
[2]:Lilienthal H, *et al*. (2009)
[3]: Saegusa Y., et al. (2009)
[4]: Eriksson P, (2006)

Lactation:

The pup mortality observed in the F2 generation of a 2-generation study in rats is occurring during the lactation phase. As no cross-fostering studies are performed, it is not known to what extent prenatal and/or lactational exposure contributes to the mortality. However, the fact that mortality increases during lactation indicates that exposure via lactation is important and it is highly likely that rat pups are exposed via milk as HBCDD is found in human breast milk. Furthermore, similarly lipophilic substances (e.g., PCB and DEHP) are efficiently excreted via milk. The risk phrase R64 should be used “for substances and preparations which are absorbed by women and may interfere with lactation or which may be present (including metabolites) in breast milk in amounts sufficient to cause concern for the health of a breastfed child”.

In the comments regarding the categorisation of substances toxic to reproduction in the EU criteria (Directive 67/548/EEC), it is said that classifying with R64 can be based on either of the three following reasons;

- results of one or two generation studies in animals which indicate the presence of adverse effects on the offspring due to transfer in the milk.

We believe this criterion is fulfilled based on increasing pup mortality during lactation in the Ema study (2008).

- toxicokinetic studies that would indicate the likelihood that the substance would be present in potentially toxic levels in breast milk.

- and/or on the basis of evidence in humans indicating a risk to babies during the lactation period.

We believe that human monitoring data from many countries over the world proves that HBCDD is present in human breast milk, and the time trend data indicate that the concentration in breast milk may correspond to the used volumes of HBCDD in the society. HBCDD is a very lipophilic compound which is persistent and tends to accumulate in the fat in many species including man. The milk samples have generally been collected some weeks after the baby's birth and the levels could have been much higher in the premier milk, the colostrum. The metabolic capacity of a newborn are limited and the bioaccumulation in the babies can be expected to be higher than in adults. This indicates that the substance may be present at ***potentially*** toxic levels in breast milk. In general, newborns are more vulnerable towards toxic effects. All together, these findings are of concern for the health of breastfed children and the GHS criteria for classification are met.

It is not proven that the pup mortality is caused by the HBCDD exposure via lactation, but it is likely that lactation is involved. Based on the animal data showing pup mortality during lactation in the F2 generation of a 2-generation study in rats, and the occurrence of HBCDD in human breast milk, a classification with **R64 (May cause harm to breastfed babies)** is proposed.

5.10 Derivation of DNEL(s) or other quantitative or qualitative measure for dose response

Not relevant for this dossier.

6 HUMAN HEALTH HAZARD ASSESSMENT OF PHYSICO-CHEMICAL PROPERTIES

See 5.1 and 5.9 for information on bioaccumulation potential of HBCDD.

7 ENVIRONMENTAL HAZARD ASSESSMENT

See 3.1. for current classification.

JUSTIFICATION THAT ACTION IS REQUIRED ON A COMMUNITY-WIDE BASIS

As the proposal concerns reproductive toxicity, no justification is needed. The classification with R64 is an integral part of the reproductive toxicity, and should therefore be harmonised as part of the classification for Reproductive toxicity

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