Committee for Risk Assessment

RAC

Annex 1

Background Document

to the Opinion proposing harmonised classification and labelling at Community level of **Hexabromocyclododecane (HBCDD)**

ECHA/RAC/ CLH-O-0000001050-94-03/A1

HEXABROMOCYCLODODECANE (HBCDD)
EC number: 247-148-4 and 221-695-9
CAS number: 25637-99-4 and 3194-55-6

Adopted

8 December 2010

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TABLES

PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

Substance Name: Hexabromocyclododecane

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

CAS number: 25637-99-4 and 3194-55-6

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 total 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 Regulation EC 1272/2008 criteria:

Repr. 2 - H361 (Suspected of damaging fertility or the unborn child.)

Justification is based on some evidence of adverse effect on development from experimental studies on animals, while the evidence on fertility effects is not sufficient for classification. In accordance with the guidance on the application of CLP criteria for cases in which there is some evidence for developmental effects, but fertility effects cannot be fully excluded it is proposed to communicate hazard posed by HBCDD with the hazard statement H 361 without specifying whether it is caused by fertility or developmental toxicity.

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

The evidence to demonstrate that HBCDD may cause adverse effects on or via lactation comes from animal studies. The increased pup mortality during lactation, particularly increased between postnatal day 4 (PND 4) and PND 21, in the F2 generation in a 2-generation study on rats, indicates that HBCDD may act on or *via* lactation on pup development (Ema *et al*., 2008). In order for this increased pup mortality to occur during the lactation period a rather long exposure before pregnancy is required.

The detected concentrations in human milk were in a range of 0.13 – 5.4 ng HBCDD /g of milk lipids (Polder *et al*., 2008a, Thomsen *et al.*, 2003; Fängström *et al*., 2008; Colles *et al.*, 2008; Lignell *et al*., 2003, Polder *et al*., 2008b; Kakimoto *et al.*, 2008; López *et al.*, 2004) up to 188 ng HBCDD/g of milk lipids (Eljarrat *et al* (2009). At present the potential of HBCDD to affect child development at the observed levels is unknown, however these data indicate occurrence of HBCDD in human milk.

The above data fulfil the classification criteria for the additional category for effects on or via lactation (CLP Regulation) as they provide some evidence from experimental animal studies.

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

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

The justification is based on some evidence of developmental effects in animal studies while the evidence for fertility effects is not sufficient to classify. In accordance with the hazard communication criteria within DSD system it is proposed to assign risk phrase R63 (Possible risk of harm to the unborn child).

R64 (May cause harm to breastfed babies)

The evidence to demonstrate that HBCDD may cause adverse effects on or via lactation comes from animal studies. The increased pup mortality during lactation, particularly increased between postnatal day 4 (PND 4) and PND 21, in the F2 generation in a 2-generation study on rats, indicates that HBCDD may act on or via lactation on pup development (Ema et al., 2008). In order for this increased pup mortality to occur during the lactation period a rather long exposure before pregnancy is required.

The detected concentrations in human milk were in a range of 0.13 – 5.4 ng HBCDD /g of milk lipids (Polder et al., 2008a, Thomsen et al., 2003; Fängström et al., 2008; Colles et al., 2008; Lignell et al., 2003, Polder et al., 2008b; Kakimoto et al., 2008; López et al., 2004) up to 188 ng HBCDD/g of milk lipids (Eljarrat et al (2009). At present the potential of HBCDD to affect child development

at the observed levels is unknown, however these data indicate occurrence of HBCDD in human milk.

Proposed labelling based on Regulation (EC) 1272/2008:

GHS08, Wng, H361, H362

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

R-phrases: R63 and R64
Symbol(s): Xn
S-phrases: S 36/37-53

Proposed specific concentration limits (if any): none

Proposed notes (if any): none

JUSTIFICATION

1 IDENTITY OF THE SUBSTANCE AND PHYSICAL AND CHEMICAL PROPERTIES

1.1 Name and other identifiers of the substance

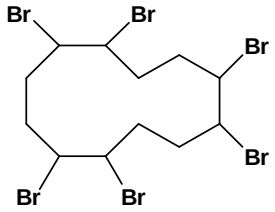
Chemical Name:	hexabromocyclododecane (HBCDD)
EC Numbers:	247-148-4; this number refers to hexabromocyclododecane (without specifying the bromine positions) and is used by industry for the commercial substance. 221-695-9[a]; this number refers to 1,2,5,6,9,10-hexabromocyclododecane and is thus the most 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.

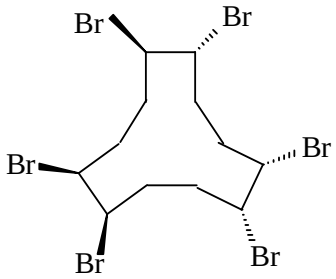
Additional information on the three main constituents of technical hexabromocyclododecane

CAS Number:

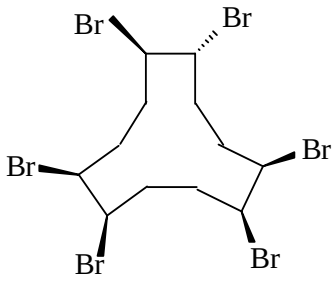
Technical HBCDD is made up of three main chiral diastereomers. Each of these have a specific CAS number, namely:

- (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]

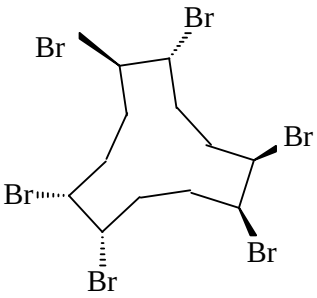
Structural Formula:



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 VI of Regulation (EC) 1272/2008.

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 2001), 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 pmol/g lipid (0.39ng/g lipid) and 0.93 pmol/g (0.60ng/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 in human serum, 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 µg/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.

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, 2001; 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. The other effects noted after long-term high exposure to HBCDD were hair loss, uncertain gait, and reduced body weight gain (EU Risk Assessment Report, 2008). In addition, in one 90 day study in rats (Chengelis, 2001), minimal to mild hepatocellular vacuolisation was observed in both sexes at all dose groups, as well as minimal to mild hepatocellular hypertrophy in females in the high dose group. It should be noted that absorption of HBCDD in the gastrointestinal tract might be incomplete and it depends upon particle size. According to EU Risk Assessment Report (2008) in the 90 days study by study of Chengelis (2001) with a mean particle size of HBCDD equal 142 μm the estimated absorption of orally administered dose was estimated to be 10-20 %, indicating that effective dose was much smaller than administered dose.

In the other 90 day study in rats (Zeller and Kirsch, 1970), hepatic lipoid phanerosis was observed in many animals. Also in a lifetime study (Kurokawa et al., 1984) in mice, liver lesions, such as hepatocytic swelling, degeneration, necrosis, vacuole formation and fatty infiltration were observed, although the dose-response relationships were not clear-cut. Although some questions regarding some of these studies remain, it cannot be stated that no clear pathological signs were observed in the liver.

The prostate weight was statistically increased in a 90-day study (Chengelis, 2001) at oral 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 oral 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.

The liver and the thyroid system are target organs, and the mechanism behind the thyroid effects are discussed below. When it comes to effects on the liver, enzyme induction clearly occurs. In addition, histological effects have been described in some studies, including hepatocellular vacuolisation, hepatocellular hypertrophy, lipoid phanerosis, hepatocytic swelling, degeneration, necrosis, and fatty infiltration.

5.6.1. Evaluation of the effects HBCDD on the thyroid system and progeny development

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 of HBCDD 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 of HBCDD is not clear, and will be discussed below basically using a structure that has been proposed by the IPCS when analyzing the mode of action for carcinogens. The discussion 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.

Evaluation of liver 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 HBCDD equal 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.

Postulated mode of action of HBCDD on thyroid function

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.

This mode of action (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 of thyroid system

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.

Effects of thyroid dysfunction on fertility and progeny development

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).

Effect on fertility. Hypothyroidism has been associated with various reproductive abnormalities in females including menstrual disorders, amenorrhea, infertility and frequent abortions (Longcope, 1991). Hypothyroidism induced by propylthiouracil and thiourea in female neonatal ICR mice resulted in a decrease in the number of primordial follicles, multilaminar follicles and Graffian follicles in ovary (Chan and Ng, 1995). In neonatal ICR male mice the drug-induced hypothyroidism was associated with a decrease in the number of seminiferous tubules with developing spermatids. The mating between hypothyroid and euthyroid males and females or females and males did not affect fertility, litter size and sex ratio of offspring (Chan and Ng, 1995).

Effect on progeny development. The 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). 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.

5.7 Mutagenicity

Not evaluated for this dossier.

5.8 Carcinogenicity

Not evaluated for this dossier.

5.9 Toxicity for reproduction

For the purpose of classification the hazard class Reproductive Toxicity is differentiated according to regulation CLP No. 1272/2008 into:

1. adverse effects
   - on sexual function and fertility, or
   - on development;
2. effects on or via lactation

5.9.1 Effects on sexual function and fertility

Effects of HBCDD was assessed based on the results of two studies (Ema et al. 2008; and van der Ven *et al.* 2009)

First study:
Two-generation reproductive study (Ema, et al. 2008) according to OECD guideline 416 and GLP.
Material:
5-week old males and females, Crl:CD(SD) rats

Method:

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.

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. Haematological 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:

One F0 male at 15,000 ppm was euthanized at 13 weeks of dosing because of a moribund condition resulting from accidental injury in the home cage. . One F0 male at 15,000 ppm and one F1 male at 1500 ppm died without any apparent clinical signs of toxicity at 5 and 7 weeks of dosing, respectively. One F1 male at 1500 ppm was dead from accidental injury in the home cage.

In F0 females at 15,000 ppm, one was euthanized during the pre-mating period because of a moribund condition, and one died on day 22 of pregnancy due to dystocia.

No significant difference was seen according to Ema at al (2008) between control and HBCD-treated groups in the incidence of clinical signs of toxicity in either male or female F0 and F1 rats during the pre-mating, mating, gestation, or lactation period.

Body weights and food consumption

F0 generation.

In F0 males, the mean body weight and/or body weight gain were significantly higher than those of controls almost throughout the dosing period at 1500 ppm and in the first 5 weeks of dosing at 15,000 ppm.

In F0 females, the mean body weight gain was significantly increased on days 0–4 of lactation at 150 ppm and during weeks 0–3 of dosing at 15,000 ppm compared to controls, and the mean body weight was significantly increased on week 2 of dosing at 15,000 ppm. The body weight gain was significantly decreased on days 0–14 of pregnancy at 15,000 ppm compared to controls.

Food consumption was generally paralleled to the body weights/body weight gains during most of the study (data not provided in the Ema et al. 2008 publication).

F1 generation

Information provided in section 5.9.1 Developmental toxicity

Effects on fertility of F0 animals:

HBCD produced no significant deviations in estrous cycles, although a few control and HBCD-treated rats had extended estrus or diestrus. Number of female rats mated, successfully copulated (inseminated), pregnant and delivering live litters in parental (F_0) generation as well as fertility indexes are given in table 5.4.1

Table 5.4.1. Effects of HBCDD in a 2-generation study on fertility parameters of females in F_0 generation

	Dose (ppm)			
	0	**150**	**1500**	**15,000**
No. of mated females	24	24	24	23
No. inseminated females in 3-weeks period	24	24	22	21
No. non-inseminated females in 3-weeks period	0	0	2	2
No. females mated after 3-weeks period	0	0	2	2
No. of inseminated after 3weeks period	0	0	0	1
Total No. of inseminated females	24	24	22	22
No. of pregnant animals(delivery of fetuses or presence of resorptions in uterus	24	22	20	19
No. of females delivering litters	24	21	20	18[a]
No. of females inseminated/ mated	24/24	24/24	22/24	22/23
Copulation index (female)(%) [1]	100	100	91,7	95,7
No. of females pregnant /mated	24/24	22/24	20/24	19/23*
No. of delivering litters/ mated	24/24	21/24	20/24	18/23*

No. of females pregnant/inseminated	24/24	22/24	20/22	19/22
Fertility index (female) (%)[2]	100	91,7	90,9	86,4
No. of litters/pregnant	24/24	21/22	20/20	18/19
Gestation index (%)[3]	100	95,5	100	94,7

[a] One female died during delivery
[1]Copulation index (%)= (no. of animals inseminated/no. of animals mated) x 100
[2]Fertility index (%) = (no. of animals that were pregnant/ no. of animals inseminated) x 100
[3]Gestation index (%)=(no. of animals delivering litters/no. of animals pregnant) x 100
* Significantly different from controls, p= 0.05 or p<0.05.

According to Ema *et al.* (2008) no significant differences between HBCDD-exposed and control animals were seen in copulation index, fertility index, gestation index, pre-coital interval, number of implantations, delivery index or number of F1 pups delivered or viability of F1 pups during lactation.

However, when assessed with Fisher exact test, the proportion of pregnant females to total number of mated females equal to 19/23 (82,6%) in the 15,000ppm group was significantly lower than the corresponding proportion of females in the control group – 24/24 (100%), (p=0.05). Similarly the proportion of dams which deliver live litters to the total number of mated females equal to 18/23 (78,3%) in the 1363mg/kg/day group was significantly lower than the corresponding proportion of females in the control group – 24/24 (100%), ($p < 0.05$). However, these differences were statistical only when all mated females were considered, and not statistical reduction were noted when copulation, fertility and gestation indexes of F_0 females were compared in control and treated groups (table 5.4.1.).

Using linear-by-linear association test (SPSS ver.14.0) to assess the relationship of the proportion of pregnant females to total mated number of females in the control and experimental groups it was possible to find significant linear trend both in two sided test (p=0,032) and one-sided test (p=0,022). The calculations above were done on request of the RAC rapporteur using original data from Ema study – see table 5-4. The statistical calculation performed by the authors of dossier submitted by Sweden using the Cohran-Armitage test on the number of pregnant females 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.
Although some results suggest the effect of HBCDD on fertility of F0 females, such effect was biologically marginal, and did not resulted in significant reduction of successful copulation, fertility and gestation indexes in the treated groups. Therefore these data on fertility of F0 females do not provide sufficient evidence that HBCDD affect fertility.

Effects on fertility of F1 animals:

In F1 females, there were extended diestrus vaginal smears in a few control and HBCD treated rats, but no significant effect of HBCD was found on the incidence of females with normal estrous cycles. Number of female rats mated, successfully copulated (inseminated), pregnant and delivering live litters in first (F_1) generation as well as copulation, fertility and gestation indexes are given in table 5.4.2.

According to Ema *et al.* (2008) no significant differences between HBCDD-exposed and control animals were seen in copulation index, fertility index, gestation index, pre-coital

interval, gestation length, number of implantations, delivery index or number of F2 pups delivered or sex ratio of F2 pups.

Table 5.4.2. Effects of HBCDD in a 2-generation study on fertility parameters of females in F1 generation

	Dose (ppm)			
	0	**150**	**1500**	**15,000**
No. of mated females	24	24	24	24
No. inseminated females in 3-weeks period	24	24	24	24
No. of pregnant animals(delivery of fetuses or presence of resorptions in uterus)	23	23	21	21
No. of females delivering litters	23	23	20	21
No. of females inseminated/ mated	24/24	24/24	24/24	24/24
Copulation index (female)(%) [1]	100	100	100	100
No. of females pregnant /mated	23/24	23/24	21/24	21/24
No. of delivering litters/ mated	23/24	23/24	20/24	21/24
No. of females pregnant/inseminated	23/24	23/24	21/24	21/24
Fertility index (female) (%)[2]	95,8	95,8	87,5	87,5
No. of litters/pregnant	23/23	23/23	20/21	21/21
Gestation index (%)[3]	100	100	95,2	100

[1]Copulation index (%)= (no. of animals inseminated/no. of animals mated) x 100
[2]Fertility index (%) = (no. of animals that were pregnant/ no. of animals inseminated) x 100
[3]Gestation index (%)=(no. of animals delivering litters/no. of animals pregnant) x 100

No significant effect of HBCDD was seen on copulation and fertility indexes of male rats in F0 and F1 generation (Table 5.4.3). No significant differences in serum testosterone, estradiol, progesterone and LH levels were noted in F0 and F1 adults of both sexes between control and HBCDD-treated groups. Levels of FSH, although statistically different, in some treated groups, were not apparently dependent upon level of exposure (Ema et al. 2008).

Table 5.4.3. Effects of HBCDD in 2-generation study on fertility parameters (Males, F0 and F1 generation)

	Dose (ppm)

	0	150	1500	15,000
F_0 Generation				
No. of males in F0 generation	24	24	24	23
No. males that have inseminated a female in F0	24	24	22	21
No. of males that impregnated a female in F0	24	22	20	18
Copulation index (male)(%) [1]	100	100	91,7	91,3
Fertility index (male) (%)[2]	100	91,7	90,9	85,7
F_1 Generation				
No. of males in F1 generation	24	24	24	24
No. males who has inseminated a female in F1	24	24	24	24
No. of animals who impregnated a female in F1	23	23	21	21
Copulation index (male)(%) [1]	100	100	100	100
Fertility index (male) (%)[2]	95,8	95,8	85,7	85,7

[1]Copulation index (%)= (no. of animals who inseminated/no. of animals mated) x 100
[2]Fertility index (%) = (no. of animals that impregnated/ no. of animals that inseminated) x 100

Necropsy and histopathology (F0, F1 generation):

No compound-related gross lesions or microscopic pathological alterations were observed in reproductive organs in male and female F0 and F1 adults showing reproductive difficulties, in animal of the highest dose group and in dead animals before scheduled sacrifice. There were no compound related gross lesions or remarkable microscopic alterations in other tissues and organs, except for the thyroid, in male and female F0 and F1 adults.

Decreased size of follicles in the thyroid was found in F0 and F1 adults at 1500 ppm and higher, and in F1 females at 1500 ppm as well (Table 5-5). A significant increased incidence of rats with decreased follicle size was noted in F0 males (87%) and females (48%) and F1 males (46%) and females (54%) exposed at 15,000 ppm and in F0 males (25%), F0 females (21%) and F1 females (21%) exposed at 1500 ppm compared to controls (0%). Background incidence of decreased follicle size in the laboratory performed current study was 0%in a total of 56 males and 56 females in 6 studies (5–12/sex/study) form 1998 to 2004.

Table 5-5. Histopathological findings in the thyroid of F0 and F1 rats

HBCD (ppm)	**0 (control)**	**150**	**1500**	**15 000**

F0 males				
No. of males examined	24	24	24	23[a]
Decreased size of thyroid follicle[b]	0	0	6*	20**
Hypertrophy of thyroid follicular cells[b]	0	0	3	1
F0 females				
No. of females examined	24	24	24	23[a]
Decreased size of thyroid follicle[b]	0	0	5*	11**
Hypertrophy of thyroid follicular cells[b]	0	0	2	0
F1 males				
No. of males examined	24	24	22a	24
Decreased size of thyroid follicle[b]	0	0	2	11**
Hypertrophy of thyroid follicular cells[b]	0	0	0	0
F1 females				
No. of females examined	24	24	24	24
Decreased size of thyroid follicle[b]	0	1	5*	13**
Hypertrophy of thyroid follicular cells[b]	0	0	0	0

[a] The number of animals examined was 23 or 22 due to autolysis.
[b] Values are given as the number of animals that showed abnormal findings.
* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.

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 5-6).

Table 5-6. 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 4 studies with 10 animals each performed 2005-2006.

The interpretation of these findings is given in section **Effect of HBCDD on ovaries** on page 29.

There were no compound-related gross lesions and histopathological changes in male and female F1 and F2 pups and weanlings including dead pups.

Organ weights (F0)

The mean body weight of males at scheduled sacrifice was significantly heavier at 1500 ppm in males compared to controls.
The absolute and relative weights of the liver of F0 males exposed at 1500 ppm and 15 000 ppm and of the F0 males' thyroid at 15,000 ppm were significantly increased.
In F0 males, there were a significantly decreased relative weight of the seminal vesicle at 1500 ppm and 15000 ppm and decreased relative weight of the brain at 1500 ppm.

In F0 females, significant increases were found in the absolute weight of the thyroid, liver and adrenal, and relative weight of the liver at 15,000 ppm when compared with controls (Ema et al. 2008; data not shown).

Semen evaluation:

Sperm parameters were determined for all F0 and F1 male adults on the day of the scheduled sacrifice. In F1 male adults no significant changes in the sperm counts, the percentage of motile sperm and progressively motile sperm, swimming speed and pattern, and the percentage of morphologically abnormal sperm were observed between control and HBCD-treated groups (data not shown). However, F0 males significantly lower number of epididymal sperm was observed only in a 150 ppm group and in a 15,000 ppm group only higher mean amplitude of lateral head displacement (LHD) was found in comparison with controls.

Serum hormone levels:

There were significantly increased levels of TSH in F0 females, but not in males, at 150, 1500 and 15 000 ppm (~35-100%), and in F1 females, but not in males, 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 F0 and F1 adult exposed and control animals

of both sexes in serum testosterone, estradiol, progesterone and LH levels.

Hematology :

Total protein and globulin were significantly higher in F0 males at 1500 and 15,000 ppm, in F0 females at 150 and 15,000 ppm and in F1 males at 15,000 ppm than those in controls (data not shown).

In male F0 and F1 and female F1 adults, no significant difference was noted in the total white blood cells (WBC) or differential leukocyte count between control and HBCD-treated groups.

Effect of HBCDD on ovaries

The primordial follicles in ovaries are formed just after birth in rodents and, it is believed, that they are not renewable (Mc Gee & Hsueh, 2000; Skinner, 2005). However, some studies provide evidence supporting the hypothesis of postnatal primordial follicle renewal in postnatal and adult ovaries of rodents (Kerr et al. 2006). The assembly of primordial follicles from nests of primary oocytes occurs in the later stages of fetal development for a human and in the early postnatal period (2-3 PND) for the rodent. The assembly process occurs in a wave of apoptosis of some oocytes, while the surviving oocytes surrounded by squamous pregranulosa cells come into being as the primordial follicles. In rodents the process is completed within first 4 days of postnatal rat (Kezele and Skinner, 2003). Primordial follicles consist of a single oocyte surrounded by an incomplete layer of squamous (i.e. flattened) pre-granulosa cells. The assembly of primordial follicles is induced by drop of the ovarian steroid hormones (estrogen, progesterone) in blood at the end of pregnancy in the primate or after birth in rodents and it is controlled by several local and systemic factors. High levels of these hormones inhibit coordinated oocytes apoptosis (Kezele and Skinner, 2003; Skinner, 2005). Abnormalities in primordial follicle assembly and transition to primary follicles may lead to a number of pathophysiologies [e.g. premature ovarian failure (POF)] and female infertility (Kezele and Skinner, 2003; Skinner, 2005).

The repeated dose toxicity 28-day study (Zeller and Kirsch, 1969), demonstrated that HBCDD at extremely high level of oral exposure to 4700 mg/kg/day can affect number of oocytes in ovaries of adult females. The exposed females showed signs of inhibited oogenesis in most of the follicles and sparse ripening follicles in the ovaries. These effects were not observed at other high dose levels of 940 and 2400mg/kg/day in 28-days study (Zeller and Kirsch, 1969). Thus the toxic effect on oocytes in adult rat ovaries were made at the exposure level four times higher that the limit dose of 1000 mg/kg/day. These results demonstrate that HBCDD only in extremely high, over toxic doses can reduce number of primordial follicles in adult female rats, which does not warrant classification. Neonate female rats, in which the assembly of primordial follicles occurs, could be much more sensitive than adult rats to this toxic action of HBCDD, however there are no studies investigating such potential effect.

The data from Ema et al. study (2008) have shown a significant reduction of primordial follicles in ovaries of adult female of F1 generation (approximately 5 months old) exposed at 138 and 1363mg/kg/day (around 30% in each group approximately equal to a value of one standard deviation). No reduced fertility indexes were observed in these females. The reduction in number of primordial follicles was approximately the same in the 138 and 1363mg/kg/day group, suggesting apparent lack of dose response relationship. At the time of that observation these females were approximately 5 -6 months old (they were started to

be mated most probably at the age of 2,5 – 3 months, plus 3 weeks of mating, 3 weeks of gestation and 3 weeks until weaning of offspring. The examined females were adult rats having weaned one litter. At the time of examinations the ovaries of these females contained thus not only primordial follicles (in much reduced number in comparison with their original number at the age of 4 days due to age), but also primary, secondary and other developing follicles and deteriorating corpora lutea, as they have been recently pregnant. Calculation of the number of primordial follicles in such ovaries is much more difficult than in ovaries of neonatal rats containing predominantly primordial follicles. It is reflected in relatively high variation in number of primordial follicles in ovary of the individual females.

The interpretation of this reduction of number of primordial follicles in adult rats exposed both prenatally and postnatally to HBCDD for the classification of reproductive toxicity is not easy and straight forward. First, there is methodological problem in establishing a normal range of a number of primordial follicles in rodent ovary (ovaries) at this age, because such a number is highly dependent upon approach and method used to determine it (Tilly, 2003). The difference in mean number of primordial follicles in ovary of mice can differ 2-10 folds depending upon method of measurements (Tilly, 2003). In rats the reported number of primordial follicles in ovaries of young female rats vary from ca. 20 (Yucebilgin et al. 2004) till over to 2000 (Meredith and Doolin, 1997), so the difference is 100- fold. It should always be taken into account that number of primordial follicles significantly decreases with age of female (Meredith and Doolin, 1997, Shirota et al. 2003), thus number of follicles in adult female of F1 generation, examined in Ema et al. study, i.e. 21 days post partum of F2 generation, were much lower than their number after birth of these females.

In the study of Ema et al., it is noted a relatively high variability of numbers of primordial follicles in individual females in the concurrent control group as can be judged based on the proportion of one standard deviation to a mean (119.5 to 316.5). In the treated groups a value of standard deviation was close or over one third of a value of mean. The historical control values from the same laboratory and species of rats also confirm relatively high variability of this parameter with the means in 4 studies using 10 females per study (the same number as in study of Ema et al., 2008) from 189.5 to 353.4. So the numbers of follicles in two high treated groups were still within a range of historical control values, thus they occurrence by random is biologically plausible.

The study of Ema et al. did not evaluate number of other follicles such as primary and secondary follicles or corpora lutea present in ovaries of females, which gave birth 3 weeks earlier. The criteria how primordial follicles were distinguished from primary and other follicles in ovary were not provided in the study of Ema et al. ((2008), although lack of sufficient distinction between follicles could be a source of relatively high variability. It is uncertain whether number of primordial follicles alone reflects well a number of oocytes in ovaries of adult females which gave birth.

In the light of the above considerations the observed changes in the number of primordial follicles do not warrant classification of HBCDD as suspected human reproductive toxicant affecting fertility, although potential effect on fertility cannot be fully excluded.

Conclusions from Ema et al. study:

Effect of HBCDD on fertility

1. In a two-generation reproductive study the exposure to HBCDD at the highest dose level of 15 000 ppm (ca. 1363mg/kg bw/day) did not resulted in reduction of commonly used fertility indexes in both assessed F0 and F1generations , although some marginal effects (reduction by ca. 10% in comparison with controls) were seen in F0 generation in the 1363mg/kd/day group in the proportion of pregnant females to total number of mated females and the proportion of dams which deliver live litters to the total number of mated females. These effects were not however seen in females of F1 generation exposed at the same level, much longer than females of F0 generation, thus they were not treatment related. These marginal effects observed only in F0 generation do not constitute sufficient evidence that HBCDD may have inherent property to affect fertility of rats. They might be rather non-specific, secondary consequence of thyreotoxicity and hepatotoxicity of HBCDD at high dose level.

2. The mean number of primordial follicles in the ovary in F1 females was significantly reduced in the groups exposed to HBCDD at the 1500 and 15 000 ppm. However, due to a fact that these reduced mean values were well within historical control mean values of the same laboratory and animal species, lack of clear dose-response relationship, large individual variability in number of primordial follicles and methodological uncertainty in their assessment they do not warrant classification.

3. In the interpretation of the above findings it has been taken into account that HBCDD at the level of 1500 and 15 000 ppm exerted general, systemic toxicity to animals as can be inferred based on:

    - Significant increases in some exposure periods in the body weight/body weight gain of F0 males exposed at 1500ppm and 15 000ppm, and significant decreases of body weight and body weight gain of F1 males during first 6 weeks of exposure to HBCDD at 15 000ppm

    - Significant decrease in body weight gain of F0 dams on day 0-14 of pregnancy at 15 000 ppm, in F1 dams exposed at the 15 000ppm it was observed a significantly lowered body weight during weeks 3 and 6-10 of dosing, during whole gestation period and days 0-14of lactation, and a reduced mean body weight gain on weeks 0-10 of dosing

    - significantly increased absolute and relative weights of the liver at 1500 ppm and higher and of the thyroid in F0 males exposed at 15,000 ppm, decrease of relative weight of the brain of F0 males at 1500 ppm, , significant increases in the absolute weight of the thyroid, liver and adrenal, and relative weight of the liver in F0 females at 15,000 ppm

    - significant increase of total protein and globulin in F0 males at 1500 and 15,000 ppm, in F0 females at 150 and 15,000 ppm and in F1 males at 15,000 ppm

    - significant increase in proportion of rats with decreased size of thyroid follicles in F0 and F1 males and females exposed at the 15000 ppm and F0 males and females and F1 females exposed to HBCDD at 1500 ppm

- significantly increased levels of TSH in F0 females, but not in males, at 150 ppm and higher (~35-100%), and in F1 females, but not in males, at 1500 ppm and higher (~80%); Serum FSH levels were significantly decreased in F0 males at 1500 ppm, and significantly increased in F0 females at 15,000 ppm.
- Significantly lower levels of T4 in F0 males and females compared to controls at 15,000 ppm.

Additional Comments:

Potential effect of particle size on the absorbed dose of HBCDD

The calculated oral 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 absorbed at the top doses are uncertain, but most probably lower than the calculated, administered doses.

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).

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

Second study of HBCDD effect on fertility (Van der Ven *et al.* 2009):

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. The animals were distributed among a larger number of dose groups than advised in the guideline with lower number of animals per group, in order to improve assessment of dose–response relationships in accordance with a benchmarking design.

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 of F1 generation, five animals/sex and dose group of F1 generation 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:

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.

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.

Conclusion:

1. HBCDD at the dose levels of 0.1 - 100mg/kg bw/day, in this benchmark study design (van der Ven *et al.*, 2009), did not induced any effects on the sexual function and fertility in parental male and female rats.

2. At the highest dos levels HBCDD induced a parental toxicity as can been concluded based on the reduced food intake by parental females during third week of gestation, reduced growth of parental males during first three weeks of exposure. These effects were initiated near the top dose, reflected in BMDLs between 88 and 95.4 mg/kg bw, at a benchmark dose response (BMR) of 10%.
3. The developmental effects or effect on lactation of HBCDD observed in this study (van der Ven *et al.* 2009) are described in section 5.9.2.

Additional comments:

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.

The developmental effects of HBCDD observed in this study (van der Ven *et al.* 2009) are described in section 5.9.2.

5.9.2 Developmental toxicity

Developmental toxicity includes, in its widest sense, any effect which interferes with normal development of the conceptus, either before or after birth, and resulting from exposure of either parent prior to conception, or exposure of the developing offspring during prenatal development, or postnatally, to the time of sexual maturation (Regulation (EC) No 1272/2008).

However, it is considered that classification under the heading of developmental toxicity is primarily intended to provide a hazard warning for pregnant women, and for men and women of reproductive capacity. Therefore, for pragmatic purposes of classification, developmental toxicity essentially means adverse effects induced during pregnancy, or as a result of parental exposure (Regulation (EC) No 1272/2008).

These effects can be manifested at any point in the life span of the organism.
The major manifestations of developmental toxicity include (1) death of the developing organism, (2) structural abnormality, (3) altered growth, and (4) functional deficiency.

5.9.2.1. Studies focused on evaluation of developmental effects manifested at the end of pregnancy

Study type:

Prenatal development toxicity study (Murai *et al*., 1985, as in EU Risk Assessment Report) 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; 0.01; 0.1 and 1% HBCDD in the diet during gestation from GD 0 till GD20.

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

visible 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 new-borns, 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 (Stump, 1999, as in EU Risk Assessment Report) 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)

Conclusions

The HBCDD did not apparently affected the prenatal development of rats as result of maternal exposure in the doses up to 1000mg/kg/day during pregnancy

5.9.2.2. Studies focused on evaluation of developmental effects manifested after birth or induced on or via lactation

Study type:

Two-generation reproductive study (Ema et al. 2008) according to OECD guideline 416 and GLP

Material:

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

Levels of exposure:

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.

Assessment of developmental landmarks

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

Results:

Effect HBCDD on viability of F1 and F2 generation

F1 generation
The viability of F1 generation during 21 day after parturition was not affected by HBCDD at any exposure level.

F2 generation
The viability of F2 generation was not affected on Day 0, but it was significantly reduced on Day 4 and 21 of lactation in the 15000 ppm (1363mg/kg bw/day) group, although it was not significantly reduced at other exposure levels – 138 and 14,3 mg HBCDD/kg bw/day. The reduction of viability was mainly due to a total loss of 8 litters by the F1 dams exposed at 15000 ppm (ca. 1365, 995 and 1724 mg HBCDD/kg bw/day during pre-mating, gestation and lactations periods, respectively). Only one dam experienced total litter loss by day 4 of lactation in the control group and one by day 2 of lactation in the 150 ppm group (19,6 mg HBCDD/ kg bw/ day). The litters in 15 000ppm group were lost on day 4, 5, 7, 9, 11, 13 and 18 of lactation.

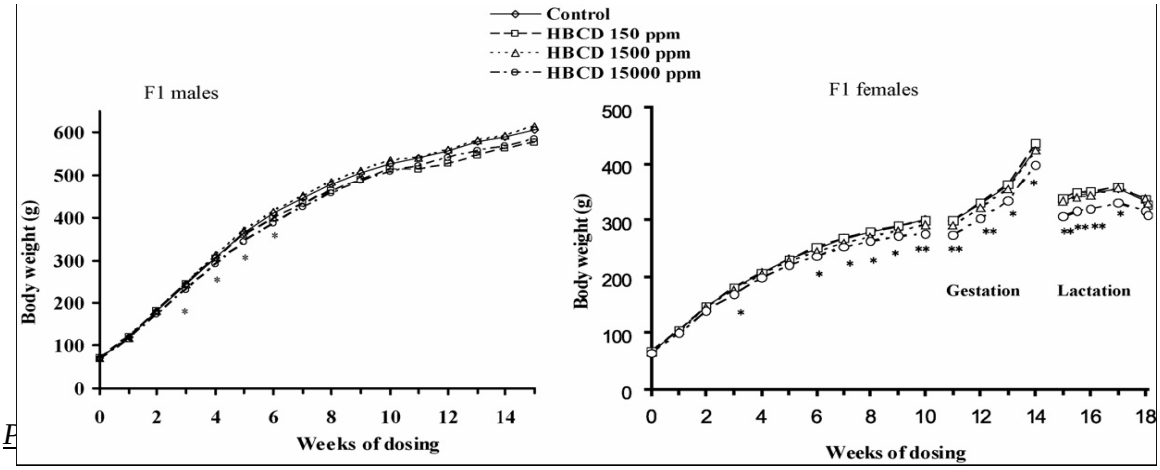
Table 5.6. Viability index (%) in F1 and F2 pups, in control and HBCDD-exposed animals

	Dose (ppm)			
	0	**150**	**1500**	**15,000**
F0 parent/F1 offspring				
Viability during lactation (in %)[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
No. of live pups in day 0	312	273	262	241
No. of live pups in day 4	296(191)	269(168)	258(157)	231(142)
No. of live pups in day 21	178	167	154	133
Viability index (%)[3]	94,9	98,5	98,5	95,6
Lactation index (%)[4]	93,2	99,4	98,1	93,7
F1 parent/F2 offspring				

Viability 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, **]
No. of live pups in day 0	301	312	256	270
No. of live pups in day 4	256(166)	283(175)	233(160)	187(136)
No. of live pups in day 21	140	157	114	74
Viability index (%)[3]	85,0	90,7	91,0	69,3*
Lactation index (%)[4]	84,3	89,7	71,3	34,6**

[1] Viability during lactation (in %) = (No. of alive 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 or p<0.05.
** Significantly different from controls, p<0.01.
[3]Viability index (%) =(total no. of pups alive on day 4/total no. of pups alive on day 0)
[4]Lactation index (%)=(total no. of pups alive on day 21/total no. of pups alive on day 4)

It should be noted that F1 dams, mothers of F2 generation, which were exposed in the diet at the 15 000 ppm demonstrated signs of maternal toxicity as can be judged based on significantly lowered mean body weight during weeks 3 and 6–10 of dosing, the whole period of gestation and days 0–14 of lactation, and a significantly reduced mean body weight gain during weeks 0–10 of dosing (Fig.1).
Figure 1. Body weights of F1 male and female rats. (*) Significantly different from the control, $P < 0.05$. (**) Significantly different from the control, $P < 0.01$ (from Ema *et al.* 2008)



F

Body weight of F1 generation

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.

The mean body weight of male weanlings at scheduled sacrifice was significantly lowered at 15,000 ppm compared to controls. 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).

Changes of F1 males weight during postnatal exposure: significant decreases compared to controls were observed in the body weight during weeks 3–6 of dosing and body weight gain during the first 6 weeks of dosing at 15,000 ppm (Fig.1).

Changes of F1 females weight during postnatal exposure: Compared with control group, a significantly lowered mean body weight was observed during weeks 3 and 6–10 of dosing, the whole period of gestation and days 0–14 of lactation, and a significantly reduced mean body weight gain was observed during weeks 0–10 of dosing at 15,000 ppm in F1 females (Fig.1).

Food consumption was generally paralleled to the body weights/body weight gains during most of the study (data not provided in the Ema *et al.* 2008 publication).

Body weight of F2 generation

Mean body weights were significantly lowered compared to controls in male F2 pups on PNDs 7, 14 and 21 and in female F2 pups on PNDs 4, 7, 14 and 21 at 15,000 ppm.
At the time of sacrifice at weanling both males and females had significantly reduced body weight, from 82.2±17.1 g and 75.3±12, 5 g in controls to 64.7±11.2 and 57.9±11.6 g, respectively.

Organ weights (F1weanlings and adults)

F1 weanlings
In males, there were significant increases in absolute and relative weight of the liver at 1500 ppm and 15 000ppm. The absolute, but not relative, weights of the brain and kidney were significantly decreased at 15,000 ppm, but the relative weights of these organs were not reduced in comparison to controls.
In F1 male weanlings, relative testis weight was dose-dependently and significantly increased as from 150 ppm (control=565; 614/615/631 in low/mid/high dose), whereas no effects were noted on the weights of the epididymis and ventral prostate.

In F1 female weanlings significantly increased absolute and relative weights of the liver at 1500 ppm and 15000, and decreased absolute weights of the brain and kidney at 15,000 ppm were observed.

F1 adults

In F1 adult males relative weights of the brain and pituitary were significantly higher at 150 ppm compared to controls. At 15,000 ppm, absolute weight of the brain was significantly decreased, and absolute and relative weights of the thyroid and liver were significantly increased compared to control.

The F1female adults exposed to HBCDD at 15,000 ppm, had a significant decrease in the absolute weight of the brain and a significant increase in absolute and relative weights of the thyroid and liver.

No effect on testis weight was seen in adult F1 males (control 0.60; 0.61/0.58/0.59 in low/mid/high dose).

Organ weights (F2 weanlings)

The body weight of male F2 weanlings at sacrifice was significantly reduced at 15,000 ppm compared to controls.
In male weanlings, significant decrease was observed in absolute weights of the brain, kidney, spleen, adrenal, epididymis and ventral prostate and increased relative weight of the brain at 15,000 ppm. A significant decrease was observed in the relative weight of the kidney at 150 ppm, and significant increase in relative weight of the liver at 1500 and 15000ppm.

In F2 female weanlings exposed at 15,000 ppm, a significant decrease of the body weight at sacrifice was found compared to controls. The absolute and relative weights of the ovary were significantly higher at 150 ppm. At 15,000 ppm, there were significantly reduced absolute weight of the brain, thymus, kidney, spleen, adrenal and uterus and increased relative weight of the brain, liver and ovary.

Developmental landmarks and behavioral effects

There was no significant difference in the incidence of male and female F1 and F2 pups that displayed pinna unfolding, or incisor eruption between the control and HBCDD-treated groups. The incidence of male and female F1 pups showing completion of eye opening was increased compared to controls at 1500 ppm. In F2 pups, the incidence of pups showing eye opening was lowered compared to controls in males at 15,000 ppm and in females at 1500 and 15,000 ppm. The AGD were not significantly different between control and HBCDD-treated groups in male and female F1 and F2 pups (table 5-8).

All male and female F1 pups in all groups completed the surface righting reflex, negative geotaxis reflex and mid-air righting reflex. No significant changes were observed in reflex response time in F1 pups of in HBCDD-treated groups, except for faster response in the surface righting in males at 15,000 ppm. In F2 pups, a few pups failed to complete the reflex response in HBCDD-treated groups, and a significantly lower incidence of females completed mid-air righting (76.9% vs. 100% in controls) was noted at 15,000 ppm (Table 5-9)

Table 5-7. Physical development in F1 and F2 pups

HBCDD (ppm)	**0 (control)**	**150**	**1500**	**15,000**
F1 pups				
No. of litters examined	24	21	20	18
Pinna unfolding PND 3 (in %)[a,b]				
Male	86.0±26.5	92.5±16.5	93.6±15.7	81.3±27.9
Female	85.8±29.5 (23)[c]	94.7±14.7	97.3±7.5	86.4±23.8
Incisor eruption PND 11 (%)[a,b]				
Male	91.6±17.6 (23)[c]	96.4±12.0	92.1±17.0	89.7±19.9 (17)[c]
Female	94.9±11.4 (23)[c]	95.2±10.1	92.5±20.0	92.2±15.4 (17)[c]
Eye opening PND 14 (%)[a,b]				
Male	48.2±41.5 (23)[c]	56.7±37.9	77.1±36.3[*]	45.8±34.6 (17)[c]
Female	49.3±37.8 (23)[c]	66.7±41.3	82.9±33.5[**]	54.9±41.4 (17)[c]

AGD[a] on PND 4				
Male pup AGD (mm)	5.37±0.41	5.44±0.36	5.38±0.32	5.20±0.51
Female pup AGD (mm)	2.60±0.23 (23)[c]	2.67±0.16	2.62±0.18	2.57±0.23
F2 pups				
No. of litters examined	23	22	20	21
Pinna unfolding PND 3 (in%)[a,b]				
Male	79.9±36.4 (22)[c]	90.5±22.8	82.1±29.8	70.1±39.2 (20)[c]
Female	73.6±39.6	90.6±22.8	81.5±31.1	66.8±40.9
Incisor eruption PND 11 (%)[a,b]				
Male	86.4±25.3 (22)[c]	92.8±19.6	97.2±11.8 (18)[c]	86.3±27.7 (14)[c]
Female	85.7±26.9 (21)[c]	90.9±26.2	97.5±11.2	90.0±28.0 (15)[c]
Eye opening PND 14 (%)[a,b]				
Male	72.7±40.0 (22)[c]	62.5±40.6	47.2±44.8 (18)[c]	33.9±34.7 (14)[c,**]
Female	82.9±26.8 (21)[c]	72.7±37.7	53.8±40.3[*]	48.1±42.0 (13)[c,*]
AGD[a] on PND 4				
Male pup AGD *(mm)*	5.12±0.54 (22)[c]	5.12±0.41	5.04±0.42	4.84±0.39 (19)[c]
Female pup AGD (mm)	2.69±0.30 (22)[c]	2.71±0.24	2.71±0.29	2.54±0.21 (20)[c]

[a] Values are given as the mean±S.D.
[b] Incidence of animals that displayed pinna unfolding, incisor eruption or eye opening (%).
[c] Data were obtained from the numbers of litters in parentheses because females that had no male and/or female pups and/or experienced total male and/or female
pup loss during lactation were excluded.
[*] Significantly different from the control, $P < 0.05$.
[**] Significantly different from the control, $P < 0.01$.

Table 5-8. Development of reflexes in F1 and F2 pups

HBCDD (ppm)	**0 (control)**	**150**	**1500**	**15,000**
F1 pups				
No. of pups examined (male/female)	24/23	21/21	20/20	17/17
Surface righting reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/100
Surface righting reflex response time (s)[a]				
Male	2.3±1.1	2.0±0.6	1.8±0.5	1.6±0.3[**]
Female	3.1±1.8	2.4±1.5	2.9±2.6	2.6±2.6
Negative geotaxis reflex completion rate (%)				

Male/female	100/100	100/100	100/100	100/100
Negative geotaxis reflex response time (s)[a]				
Male	17.7±7.1	16.8±8.0	15.2±7.8	19.4±5.9
Female	13.9±6.2	11.5±6.2	12.7±6.3	17.0±6.9
Mid-air righting reflex completion rate (%)				
Male/female	100 (23)b/100	100/100	100/100	100/100
F2 pups				
No. of pups examined (male/female)	22/22	22/22	19/20	19/18
Surface righting reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/88.9
Surface righting reflex response time (s)[a]				
Male	2.1±1.7	2.0±1.5	2.8±2.5	2.2±2.3
Female	2.3±0.9	2.4±1.7	2.1±0.9	3.7±3.7 (16)[b]
Negative geotaxis reflex completion rate (%)				
Male/female	100/100 (21)[b]	95.5/100	100/100	13 (16)[b]/88.2 (17)[b]
Negative geotaxis reflex response time (s)[a]				
Male	17.3±8.6	14.7±6.8 (21)[b]	15.2±6.4	14.1±6.7 (13)[b]
Female	12.4±5.3 (21)[b]	12.0±5.2	16.7±6.4	14.6±6.6 (15)[b]
Mid-air righting reflex completion rate (%)				
Male/female	100/100 (21)[b]	100/100	94.4 (18)[b]/90.0	100 /76.9 (13)[b,*]

Surface righting reflex on postnatal day 5 (three trials), negative geotaxis reflex on postnatal day 8 (one trial) and mid-air righting reflex on postnatal day 18 (three trials) were examined. Completion rate (%) = (no. of animals showing all positive responses of the trials/no. of animals examined)×100.

[a] Values are given as the mean±S.D.

[b] Data were obtained from the numbers of pups in parentheses.

[*] Significantly different from the control, $P < 0.05$.

[**] Significantly different from the control, $P < 0.01$

No significant differences between control and HBCDD-treated groups were noted in the age at preputial separation in males or vaginal opening in females, or body weight at the age of preputial separation or vaginal opening.

HBCDD did not affect the spontaneous locomotors activity of F1 males and females at the age of 4 weeks at any exposure level.

On the first day of the T-maze test, the pre-test swimming trials in the straight channel revealed that all male and female F1 rats in each group could swim satisfactorily, and no significant changes were observed in the elapsed time to traverse the straight channel. In males, there were a significantly shorter elapsed time at 1500 and 15,000 ppm and fewer number of errors at 15,000 ppm on day 3 of the T-maze. In females, there was no significant difference in the elapsed time or number of errors of the T-maze between control and HBCDD-treated groups (Ema *et al.* 2008)).

Conclusion:

The results of the two-generation study (Ema *et al.* 2008) indicate that HBCDD given orally in feed has affected postnatal development of F1 and F2 generation inducing the following alterations:

1. Increased mortality of F2 generation from day 0 till day 21 post partum in a group of mothers exposed at the dose of 1363 mg/kg bw/day, but not at the doses of 14 and 141 mg/kg bw/day.
2. Reduced body weight of F1 weanlings and adults exposed at 1363 mg/kg bw/day and F2 pups of both sexes of the 1363 mg/kg bw/day group.
3. Induced the following internal organ weight alterations:

    a. Increase in absolute and relative weight of liver of F1 male/female adults exposed at 115/138 and 1142/1363 mg/kg bw/day, as well as that of F2 male weanlings
    b. Increase in absolute and relative weight of thyroid in F1 adults of both sexes at dose levels of 1142-1363 mg/kg bw/day
    c. Increase of relative weight of testis of F1 weanlings at all exposure levels, however no effect was seen on weight of testis of F1 adults
    d. Decrease in the absolute weight of brain in F1 male/female weanlings and F1 male/female adults exposed at 1142/1363 mg/kg bw/day, as well as F2 female weanlings
    e. Decrease in absolute weight of kidneys in F1 male/female weanlings exposed at the dose levels of 1142 - 1363 mg/kg bw/day and F2 male and female weanlings

    (Note: The decreases of absolute weight of internal organs are closely related to reduce body weight of affected animals)

4. HBCDD has delayed the physical development of F2 pups as can be inferred based on reduced incidence of pups showing eye opening on PND 14 in males exposed at the highest dose and in females at the medium and highest doses, with visible dose-related trend.
5. The development of basic reflexes during rats development was also affected by the HBCDD at the highest dose level leading to:
    a. shorter time response in the surface righting reflex in F1 male pups on PDN 5 at 1142 mg/kg bw/day
    b. significantly lower incidence of females completed mid-air righting (76.9% vs. 100% in controls) at 1363 mg/kg bw/day
6. The development of the nervous system in rats could have been affected as can be judged based on the observation of a significantly shorter elapsed time on day 3 of the T-maze test in F1 males in the age of 6 weeks exposed to HBCDD at 115 and 1142 mg/kg bw/day and fewer number of errors at 1142 mg/kg bw/day.

While interpreting these data one should take into account that the majority of adverse effects on development of progeny were induced at the highest dose levels of 1363 mg/kg bw/day for F0 and F1 females or of 1008-1142 mg/kg bw/day for F0 and F1 males, which were also toxic to parental organisms. Some of these effects were observed when offspring were able to eat feed available to their mother i.e. shortly before and after weaning. Increased absolute and relative weight of offspring

liver and thyroid suggest the same toxic mechanisms of HBCDD in offspring as in parental animals.

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

Study type:
One-generation reproductive study in rats (van der Ven et al , 2009) 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, concomitant with a decreased body weight. 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. As the body weights were decreased at necropsy at the top dose, it would be interesting to also analyse the relative organ weights. The relative organ weights are, however, not given in the publication. If comparing body weights and organ weights between the control and the highest dose, one has to acknowledge that there is only 5 animals per group (because of the benchmark dose testing design) and that the comparison has little statistical value. However, except for the large decrease in the prostrate weight (-36%), it otherwise appears that organ weights and body weights are decreased to a similar magnitude (10-15%). 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.

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).

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.

Conclusion:

1. 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.
2. Delayed vaginal opening was seen in females, concomitant with a decreased body weight.
3. marked dose-dependent decreases of apolar retinoid levels in liver of F1 animals
4. 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

5. a decrease of trabecular bone mineral density in F1 females

Additional comments: 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. 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:

In addition to the main publication by Van der Ven *et al.* (2009) there is separate publication reporting on additional study conducted on animals (Lilienthal *et al.* 2009) originated from the 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:

1. 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 are not understood.
2. 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 (Saegusa *et al*. 2009) 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

Method:

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).

Main results in offspring

1. Female offspring body weight at puberty was decreased by 9% in the high dose group (day 34).
2. Relative liver weight was increased by 27-28% in both sexes in the high dose group. 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 also observed in the high dose males at week 11

3. The level of T3 in serum in male offspring was decreased (-15%) and the level of TSH increased (+30%) at PND 20 in the high dose group (females were not examined)
4. 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.
5. 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:

1. In one-generation developmental toxicity study (Saegusa *et al.* 2009) with exposure of female rats from mid-gestation through lactation to HBCDD in a diet at concentration of 100, 1000 and 10 000 ppm (stated to be equivalent to 8-21, 81-213, or 803-2231 mg/kg/day this chemical at the highest dose level induced degeneration of liver of female and male offspring, in male offspring a decrease of T3 and increase of TSH in serum and increase of relative weight of thyroid, and reduction in brain oligodendrocytes.

2. 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.

3. 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:
Neurotoxicity study (Eriksson et al., 2006, as in EU Risk Assessment Report) of HBCDD exposure during brain development. Spontaneous behaviour, learning and memory capabilities were studied.

Material: Neonatal male NMRI mice

Method:
In this study, neonatal NMRI mouse pups were given by gavage either a single dose of 0.9 mg HBCDD/kg body weight, 13.5 mg HBCDD/kg body weight, or a 20% fat emulsion vehicle on postnatal day 10 (8-10 animals/dose group). At the age of 3 months, the mice were observed regarding spontaneous behavior and concerning learning and memory capability.

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.

Some of the results have later been published more properly by Roze el at, (2009). The study was part of the prospective Groningen infant COMPARE (Comparison of Exposure-Effect Pathways to Improve the Assessment of Human Health Risks of Complex Environmental Mixtures of Organohalogens) study. It included 62 children in whose mothers the following compounds had been determined in the 35th week of pregnancy: 2,2´-bis-(4 chlorophenyl)-1,1´-dichloroethene, pentachlorophenol (PCP), polychlorinated biphenyl congener 153 (PCB-153), 4-hydroxy-2,3,3´,4´,5-pentachlorobiphenyl (4OH-CB-107), 4OH-CB-146, 4OH-CB-187, 2,2´,4,4´-tetrabromodiphenyl ether (BDE-47), BDE-99, BDE-100, BDE-153, BDE-154, and hexabromocyclododecane. The median concentration of HBCCD in blood samples taken on 35 week of pregnancy from 62 women living in the Northern provinces of the Netherlands amounted according Rose et al. (2008) to 0.8 ng/g of lipids with a range of 0.3 – 7.5 ng/g of lipids. Thyroid hormones were determined in umbilical cord blood. When the children were 5–6 years of age, they were invited for assessment of their neuropsychological functioning: motor performance (coordination, fine motor skills), cognition (intelligence, visual perception, inhibitory control, verbal memory, and attention), and behavior. The final cohort consisted of 38 boys and 24 girls. Parents gave their informed consent for themselves and their children to participate in the follow-up program before the study. The study was approved by the Medical Ethical Committee of the University Medical Center Groningen and complied with all applicable international regulations. Children were examined using standardized tests of motor skills, short form of the Wechsler Preschool and Primary Scale Intelligence test and other tests measuring cognitive outcomes and verbal memory. Children's competencies and their behavioral and emotional problems were assessed in standardized tests applied to their parents and teachers. According to authors brominated flame retardants correlated with worse fine manipulative abilities, worse attention, better coordination, better visual perception, and better behavior. The concentration of HBCDD in maternal blood was positively correlated with motor coordination ($p < 0.05$), total intelligence ($p < 0.05$) and verbal intelligence ($p < 0.01$). These findings on humans corresponds well with the results of animal study (Ema *et al.* 2008), which revealed better motor and memory performance of F1 male rats exposed to HBCDD, which had a significantly shorter elapsed time and fewer number of errors on day 3 of the T-maze (Ema *et al.* 2008).

In the Rose *et al.* study (2009) the levels of thyroid hormones from the umbilical cord blood were related to behavioral outcome in children at 5–6 years of age. Thyroid stimulating hormone (TSH) correlated with worse motor skills and worse attention. Reverse triiodothyronin (rT3) correlated with better fine manipulative abilities. Triiodothyronin (T3) correlated with better visuomotor integration and better behavior. Tyroxin (T4) correlated with better sensory integrity and less ADHD (Attention Deficit/Hyperactivity Disorder). However, no correlation was reported between HBCDD concentration in maternal blood and levels of thyroid hormones in the umbilical cord blood, although symptoms of HBCDD-induced hypothyroidism were reported in animals (Ema *et al.* 2008).
The results of Rose *et al.* study (2008), having in mind relatively small number of investigated children, low environmental exposure to many various organohalogen compounds, are not

sufficient to assess the effects of HBCDD on development of children, however they might be used as supportive evidence while drawing conclusions on animals studies. These results call for further studies on the neurodevelopment in humans exposed to environmental brominated flame retardants.

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 by Mariussen & Fonnum, 2002, as presented in EU Risk Assessment Report

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 two generation study of Ema et al. (2008) HBCDD did not significantly reduced such reproduction parameters as copulation, fertility and gestation indexes, neither in F_0 nor in F_1 generation. HBCDD at any of applied dose levels (14-14,3; 141-138 and 1363 mg/kg/day) did not affect also litter size and number of implantation sites.

In the group of F_0 females exposed to HBCDD in diet at dose level of 1363 mg/kg/day the proportion of pregnant females to total number of mated females was significantly lower (82,6%) than the corresponding proportion of females in the control group (100%), (p=0.05). Similarly the proportion of F_0 dams which deliver live litters to the total number of mated females equal to 18/23 (78,3%) in the 1363 mg/kg/day group was significantly lower than the corresponding proportion of females in the control group – 24/24 (100%). However both these reductions were marginal and could not be treatment-related. It is not probable that they are evidence of inherent property of HBCDD since in the F_1 females exposed at the same dose level, but much longer, through various phases of their development and life (in utero, during lactation and since weaning to maturation) these effects were not observed. The difference is thus more probably due to biological variability in proportion of pregnant females to total number of mated (cohabitated with male) females, which in the Sprague Dawley strain used in this study vary from 72% to 100%, with mean value of 85% (Parker, 2006).

Although linear trend to reduce proportion of pregnant females to total mated number of females in the control and experimental groups were observed, but the slope of this trend was very low and not proportional to the slope of dose levels increases. With a dose increase 10-fold and 100-fold the observed fertility was respectively reduced from 91, 7% at the lowest dose level to 90,9% and 86,4% in two higher dose levels, respectively.

The mean number of primordial follicles in the ovary in F1 females was significantly reduced in the groups exposed to HBCDD at the 1500 and 15 000 ppm. However, due to a fact that these reduced mean values were well within historical control mean values of the same laboratory and animal species, lack of clear dose-response relationship, large individual variability in number of primordial follicles and methodological uncertainty in their assessment they do not warrant classification. However, the potential effect of HBCDD on fertility cannot be fully excluded.

While the data collected in the study of Ema et al. (2008) do not provide sufficient evidence that HCBDD affects fertility of rats, but they provide the evidence of toxic effects of HBCDD in liver and thyroid at two higher dose levels of 101 -115 and 1008-1142 mg/kg/day in male rats and 141-138 and 1363 mg/kg/day in female rats. Those levels are too high to consider classification of HBCDD for repeated specific organ toxicity. However, these effects demonstrate that at two higher dose levels HBCDD was toxic to rats.

In a one-generation study, using the benchmark study design, at the dose levels of 0.1 - 100 mg/kg bw/day (van der Ven *et al.*, 2009) HBCDD did not induce effects on fertility of parental male and female rats. In this study HBCDD did not affect the blood levels of thyroid hormones. There were also no histopatological changes in the thyroid gland.
The analyzed data from one-generation and two generation studies do not provide sufficient evidence which fulfils the criteria of classification of HBCDD into category of substances suspected of damaging fertility) (Regulation (EC) 1272/2008 (CLP) or posing a possible risk of impaired fertility (Directive 67/548/EEC (DSD).

Development:

In a study Murai *et al.* 1985 (as presented in as in EU Risk Assessment Report) no prenatal or postnatal developmental toxicity was observed in offspring of female rats given 0; 0.001; 0.1 and 1% HBCDD in the diet during from Day 0 till Day 20 gestation. The doses were approximately equivalent to 0; 7.5; 75 and 750 mg/kg/day, respectively. Lack of effects could be due to low number of litters observed during lactation (6 per group), relatively short exposure time lasting only during gestation and low dose level of HBCDD.

In a study of Stump, 1999 (as presented in EU Risk Assessment Report effects) HBCDD administered once daily from gestation day 6 till gestation day 19 at doses of 0, 500, or 1000 mg HBCDD per kg and day orally in corn oil (suspension with a mean particle size of 142 µM) did not induced signs of prenatal toxicity in rats.
Two studies of prenatal toxicity of HBCDD (Murai et al., 1985; Stump et al.. 1999) performed according to OECD 414 guidelines, although with some deviations, which do not decreased reliability of these studies, did not provide evidence of alteration of prenatal development of rats, however taking into account that exposure time to reach steady-state is in rats in order of months prenatal toxicity in animals exposed long before gestation and during gestation cannot be excluded.

The other studies did provide sufficient evidence of alterations in postnatal developmental effects.

The results of the two-generation study (Ema *et al.* 2008) indicate that HBCDD given orally in feed has affected postnatal development of F1 and F2 generation inducing the following alterations:

1. Increased mortality of F2 generation from day 0 till day 21 post partum in a group of mothers exposed at the dose of 1363 mg/kg bw/day, but not at the doses of 14 and 141 mg/kg bw/day.
2. Reduced body weight of F1 weanlings and adults exposed at 1363 mg/kg bw/day and F2 pups of both sexes of the 1363 mg/kg bw/day group.
3. Induced the following internal organ weight alterations:
    a. Increase in absolute and relative weight of liver of F1 male/female adults exposed at 115/138 and 1142/1363 mg/kg bw/day, as well as that of F2 male weanlings
    b. Increase in absolute and relative weight of thyroid in F1 adults of both sexes at dose levels of 1142-1363 mg/kg bw/day
    c. Increase of relative weight of testis of F1 weanlings at all exposure levels, however no effect was seen on weight of testis of F1 adults
    d. Decrease in the absolute weight of brain in F1 male/female weanlings and F1 male/female adults exposed at 1142/1363 mg/kg bw/day, as well as F2 female weanlings
    e. Decrease in absolute weight of kidneys in F1 male/female weanlings exposed at the dose levels of 1142 - 1363 mg/kg bw/day and F2 male and female weanlings

    (Note: The decreases of absolute weights of internal organs are closely related to reduced body weights of affected animals)

4. HBCDD has delayed the physical development of F2 pups as can be inferred based on reduced incidence of pups showing eye opening on PND 14 in males exposed at the highest dose and in females at the medium and highest doses, with visible dose-related trend.
5. The development of basic reflexes during rats development was also affected by the HBCDD at the highest dose level leading to:
    a. shorter time response in the surface righting reflex in F1 male pups on PDN 5 at 1142 mg/kg bw/day
    b. significantly lower incidence of females completed mid-air righting (76.9% vs. 100% in controls) at 1363 mg/kg bw/day
6. The development of the nervous system in rats could have been affected as can be judged based on the observation of a significantly shorter elapsed time on day 3 of the T-maze test in F1 males in the age of 6 weeks exposed to HBCDD at 115 and 1142 mg/kg bw/day and fewer number of errors at 1142 mg/kg bw/day.

While interpreting these data one should take into account that the majority of adverse effects on development of progeny were induced at the highest dose levels of 1363 mg/kg bw/day for F0 and F1 females or of 1008-1142 mg/kg bw/day for F0 and F1 males, which were also moderately toxic to parental organisms. Some of these effects were observed when offspring were able to eat feed

available to their mother i.e. shortly before and after weaning. Increased absolute and relative weight of offspring liver and thyroid suggest the same toxic mechanisms of HBCDD in offspring as in parental animals.

In the one-generation study by van der Ven *et al.* (2009) using the lower dose levels of HBCDD ranging from 0,1 to 100mg/kg bw/day and few animals per group the following alterations of the postnatal development were observed:

1. 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.

2. Delayed vaginal opening was seen in females, concomitant with a decreased body weight.

3. Marked dose-dependent decreases of apolar retinoid levels in liver of F1 animals

4. Decrease of trabecular bone mineral density in F1 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.

In one-generation developmental study (Saegusa *et al.* 2009) the following alterations of the postnatal development of offspring were observed:

1. Exposure of female rats from mid-gestation through lactation to HBCDD in a diet at concentration of 100, 1000 and 10, 000 ppm (stated to be equivalent to 8-21, 81-213, or 803-2231 mg/kg/day this chemical at the highest dose level induced degeneration of liver of female and male offspring, in male offspring a decrease of T3 and increase of TSH in serum and increase of relative weight of thyroid, and reduction in brain oligodendrocytes.

2. 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.

3. 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).

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

In the study by Lilienthal *et al.* (2009), effects of HBCDD on hearing function and dopamine-dependent behaviour were observed. In the 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 3-month old mice, which have received by gavage a single dose of HBCDD on postnatal day 10.

In several other studies no effects of HBCDD on development of animals were observed (Murai *et al.* 1985, Stump, 1999; as in EU Risk Assessment Report

Parental toxicity

In the study of Ema *et al.* (2008) HBCDD, at the dose level affecting fertility of female rats and postnatal development of offspring and lower, exerted general systemic effects most probably resulting from alterations of liver and thyroid functions. Besides the temporary decreases of body weight gain in F0 female generation and in F1 female generation (mothers of affected F2 generations) exposed at 15 000 ppm (ca. 1363 mg/kg bw/day) there was significant increase of absolute and relative weights of the liver at dose levels of 1500 ppm and higher and of the thyroid in F0 males exposed at 15,000 ppm, significant increases in the absolute weight of the thyroid, liver and adrenal, and relative weight of the liver in F0 females at 15,000 ppm. The histopathology revealed significant increase in proportion of rats with decreased size of thyroid follicles in F0 and F1 males and females exposed at the 15000 ppm and F0 males and females and F1 females exposed to HBCDD at 1500 ppm. The evidence of altered hormonal function of thyroid of parental animals was provided by finding of significantly increased levels of TSH in F0 females, but not in males, at 150 ppm and higher (~35-100%), and in F1 females, but not in males, at 1500 ppm and higher (~80%); Serum FSH levels were significantly decreased in F0 males at 1500 ppm, and significantly increased in F0 females at 15,000 ppm. Significantly lower levels of T4 in F0 males and females compared to controls at 15,000 ppm. It is noteworthy that serum levels of testosterone, estradiol, progesterone and LH levels were not altered in F0 and F1 adults, which is consistent with lack of changes in sexual cycle of exposed animals. A significant increase of total protein and globulin in F0 males at 1500 and 15,000 ppm, in F0 females at 150 and 15,000 ppm and in F1 males at 15,000 ppm

In the study of van der Ven *et al.* (2009), using the lower dose levels of HBCDD ranging from 0,1 to 100mg/kg bw/day and few animals per group, alterations of liver or thyroid weight or functions in parental organism were not detected, a well as no reduced fertility of parental animals or increase mortality of offspring during lactation were noted. However, developmental toxicity was identified as reduced weight of testes and prostate and delayed vaginal opening not seen in the study of Ema *et al.* 2008.

In the study of Saegusa *et al.* (2009) using higher dose levels of 100, 1000 or 10,000 ppm (stated to be equivalent to 8-21, 81-213, or 803-2231 mg/kg/day) and applying exposure from 10 day of gestation till weaning of offspring there was a 30% increased weight of the thyroid gland and an increased incidence and severity of diffuse thyroid follicular cell hypertrophy in dams exposed at the high dose level (10,000 ppm). Similar changes were seen in offspring, even at lower exposure levels: (thyroid weight increase decreased serum T3 and increased serum TSH at 1,000 and 10,000 ppm). In addition there was an increase in relative weight of liver of offspring from 10 000 ppm group in both sexes.

Results of the above studies suggest the developmental toxicity of HBCDD at high doses seen during postnatal development is associated with altered function of liver and thyroid of maternal animals, although offspring seem to be more sensitive to this action of chemical than paternal animals. The potential mechanism for these effects has been described in section 5.6 under subheading *Effects of thyroid dysfunction on fertility and progeny development* (page 19).

Thus, at least to a certain extent, the developmental toxicity of HBCDD might be a secondary effect of maternal toxicity, however, this effect is mediated through induction of liver enzymes and dysfunction hormonal system (reduced level of thyroid hormones), therefore it should be considered

as specific secondary effect and taken into account in classification. 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 human relevance of effects on the thyroid hormone system is well known (Mead MN, 2004), and further discussed in section 5.6.

5.9.5.1. Comparison with classification criteria for reproductive toxicity

As described in the background document, there were observations of increased postnatal mortality, delayed physical development, and alterations in the weight of internal organs in offspring in one- and two-generation studies (Ema et al. 2008; van der Ven et al. 2009, Saegusa et al. 2009) at dose levels inducing mild maternal toxicity. The contribution of prenatal developmental alterations to these postnatally manifested effects cannot be excluded for the available data.

The observations from the study of Ema et al. (2008) suggesting potential effects of HBCDD on fertility such as reduction of primordial follicles in ovaries of F1 generation females in the medium and high dose exposure levels was not alone sufficient as a base for classification for adverse effects on fertility. This is because of the unknown significance of this observation (due to lack of clarity of the methodological procedure) and the fact that the reduced number of follicles were within a range of values observed in animals of historical control groups evaluated in the same laboratory. However, the fertility effects cannot be fully excluded in the overall assessment of potential reproductive hazard.

CLP Regulation:
HBCDD should be classified into category Repr. 2, because there is some evidence of an adverse effect on development from experimental studies on animals. Although the evidence for an adverse effect on fertility is not sufficient for classification, the possibility of this hazard cannot be excluded entirely. In accordance with the available guidance, this hazard profile merits labelling with the hazard statement: **H361 without specifying fertility or developmental toxicity.**

Dangerous Substances Directive:
HBCDD should be classified in category 3 for reproductive toxicity given the evidence in animal studies for a possible effect on development (Repr Cat 3; R63 - Possible risk of harm to the unborn child). Regarding fertility, the available evidence for this effect alone is not sufficient for any further classification and labelling.

Summing up the above considerations the following classifications is proposed:
CLP Regulation: Repr. 2 - H361 (Suspected of damaging fertility or the unborn child.)
DSD; Repr. Cat 3; Xn, R63 (Possible risk of harm to the unborn child).

Effect on or through lactation:

Adverse effects on or via lactation are included under reproductive toxicity, but for classification purposes such effects are treated separately. This is because it is desirable to be able to classify substances specifically for an adverse effect on lactation so that a specific hazard warning about this effect can be provided for lactating mothers.

Considering classification for hazard category for lactation effects it should be noted that CLP regulation [table 3.7.1(b)] require that substances which are absorbed by women and have been

shown to 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, shall be classified and labelled to indicate this property hazardous to breastfed babies. This classification can be assigned on the:

(a) human evidence indicating a hazard to babies during the lactation period; and/or

(b) results of one or two generation studies in animals which provide clear evidence of adverse effect in the offspring due to transfer in the milk or adverse effect on the quality of the milk; and/or

(c) absorption, metabolism, distribution and excretion studies that indicate the likelihood that the substance is present in potentially toxic levels in breast milk.

Thus, the one or two generation studies are recognized sources of data to be used while considering the classification for lactation effects.

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;

1. 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.
2. toxicokinetic studies that would indicate the likelihood that the substance would be present in potentially toxic levels in breast milk.
3. and/or on the basis of evidence in humans indicating a risk to babies during the lactation period.

The first criterion is at least partially fulfilled based on increasing pup mortality during lactation period in the Ema study (2008), although cross-fostering studies were not done. In F2 pups, the mortality was increased and there were decreased body weights already at day 4 but not on day 0 PND indicating that lactation exposure had affected the pups body weight gain.

The fact that mortality which occurred already at day 4 and it was markedly increased before weaning (Ema *et al*., 2008) indicates that exposure via lactation was responsible for this increased mortality. There is evidence from human studies that HBCDD is excreted with milk. Furthermore, similarly lipophilic substances (e.g., PCB and DEHP) are efficiently excreted via milk.

The exposure through milk during lactation period most probably contributed also to demonstrated alterations in postnatal development observed in one and two generations studies of Ema *et al*., 2008; Saegusa *et al.* 2009 and van der Ven *et al*. 2009.

The human monitoring data reviewed in section 5.1. 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.

Adverse effects on or via lactation - comparison with criteria

There is evidence from human studies that HBCDD is excreted with milk. It is also known that similarly lipophilic substances (e.g., PCB and phthalates) are efficiently excreted *via* milk (Patandin *et al*., 1999; Dostal *et al.,* 1987, Högberg *et al.* 2008). The detected concentrations of HBCDD in human milk were in a range of 0.8 – 188 ng/g of lipids, however, its potential to affect child development at the observed levels is unknown. HBCDD has a high capacity to bioaccumulate and it has been recognized in the EU as a PBT-substance (ECHA, 2008). Due to its bioaccumulation properties it may cumulate in mammary glands from which it may be transferred to milk. It is known that a lengthy exposure of animals (months) is required to reach steady-state.

The detected concentrations in human milk were in a range of 0.13 – 5.4 ng HBCDD /g of milk lipids (Polder *et al*., 2008a, Thomsen *et al.*, 2003; Fängström *et al.*, 2008; Colles *et al.*, 2008; Lignell *et al*., 2003, Polder *et al.*, 2008b; Kakimoto *et al.,* 2008; López *et al.*, 2004) up to 188 ng HBCDD/g of milk lipids (Eljarrat *et al* (2009). A calculation in the EU Risk Assessment Report (May 2008) of HBCDD intake by breast-feed babies gives the following estimates (based on 3.2 ng of HBCDD/g of fat in breast milk): 0.015 µg/kg bw/day for 0-3 months old and 0.0056 µg/kg bw/day for 3-12 months old. Using a recent Spanish breast milk study (Eljarrat *et al,* 2009) a calculated median daily intake for 1 month-old Spanish infants amounted to 0,175 µg HBCDD/kg bw/day, much higher than in a previous estimation. However, at present a potential of HBCDD to affect child development at the observed levels is unknown.

The evidence to demonstrate that HBCDD may cause adverse effects on or via lactation comes from animal studies. The increased pup mortality during lactation, particularly increased between postnatal day 4 (PND 4) and PND 21, in the F2 generation in a 2-generation study on rats, indicates that HBCDD may act on or *via* lactation on pup development (Ema *et al*., 2008). In order for this increased mortality of pups to occur during the lactation period a rather long exposure before pregnancy is required. This may have been the reason that the effect on or via lactation was not observed in F1 generation in Ema *et al*. study (2008) following shorter exposure before pregnancy of F0 dams or in the offspring of the one generation studies (Saegusa *et a*l. 2009 and van der Ven *et al*. 2009).

However, since cross-fostering was not done in any of these studies, it is not possible to distinguish between developmental effects induced *in utero* and those induced during lactation. In addition, those effects were observed in offspring of mothers showing mild signs of maternal toxicity (hypothyroidism).

The above data fulfil the classification criteria for the additional category for effects on or via lactation (CLP Regulation) as they provide some evidence of the adverse effect on or via lactation from experimental animal studies.

Taking into account the above considerations RAC is of the opinion that the following classification applies to HBCDD:
CLP Regulation: **Lact. - H362** (CLP Regulation) (May cause harm to breast-fed children)

DSD - **R64** (May cause harm to breastfed babies)

5.9. 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.

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