**Substance name: 4-(1,1,3,3-tetramethylbutyl)phenol,
4-tert-octylphenol
EC number: 205-426-2
CAS number: 140-66-9**

MEMBER STATE COMMITTEE
SUPPORT DOCUMENT FOR IDENTIFICATION OF

4-(1,1,3,3-TETRAMETHYLBUTYL)PHENOL,
4-TERT-OCTYLPHENOL

AS A SUBSTANCE OF VERY HIGH CONCERN BECAUSE ITS ENDOCRINE DISRUPTING PROPERTIES CAUSE PROBABLE SERIOUS EFFECTS TO THE ENVIRONMENT WHICH GIVES RISE TO AN EQUIVALENT LEVEL OF CONCERN

Adopted on 9 December 2011

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Substance Name: 4-(1,1,3,3-tetramethylbutyl)phenol, 4-tert-octylphenol

EC Number: 205-426-2

CAS number: 140-66-9

- *The substance is identified as a substance of very high concern according to Article 57 (f).*

4-(1,1,3,3-tetramethylbutyl)phenol, (4-tert-octylphenol) is identified as a substance of very high concern in accordance with Article 57 (f) of Regulation (EC) 1907/2006 (REACH) because it is a substance with endocrine disrupting properties for which there is scientific evidence of probable serious effects to the environment which gives rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of REACH.

This conclusion is based on the fact that there is strong evidence from high quality studies of adverse effects in two fish species, which are estrogen mediated. Similar evidence is available for a third fish species but it is based on tests without statistics. In all other tested fish species the endpoints affected are known to be influenced by estrogen activity and, with one exception, an estrogen mediated mode of action was observed. According to the OECD draft guidance document for endocrine disruptors (OECD, 2011) 4-tert-octylphenol is an endocrine disruptor based on these results.

Based on the widely accepted IPCS definition for endocrine disruptors (WHO/IPCS, 2002) 4-tert-octylphenol is considered to be an endocrine disruptor in fish.

Based on the above conclusion, evidence that the substance is of an equivalent level of concern includes:

- Similar to certain other substances of very high concern it is difficult to quantify a safe level for 4-tert-octylphenol and therefore also the risks, using traditional risk assessment methods.
- Impairment of reproduction due to the endocrine disrupting properties of 4-tert-octylphenol may already occur after a transient short term exposure as observed in two fish species. This provides some indication that exposure in one area might influence population stability in another area (e.g. for migratory fish). With respect to 4-tert-octylphenol there is evidence of internal distribution toward embryos in viviparous fish species.
- Exposure to estrogens such as 4-tert-octylphenol may cause long lasting effects. A change in the endocrine feedback system during sensitive life stages may result in effects during the entire life. Such changes were observed after exposure to 4-tert-octylphenol with respect to development of the reproduction system and changes in sex-ratio in fish.
- The evidence presented indicates that 4-tert-octylphenol may have the potential to cause adverse effects in a range of species across different taxonomic groups.
- Exposure to 4-tert-octylphenol may result in effects that relevantly influence ecosystems with respect to the community structure and function. Comparable to other estrogens, 4-tert-octylphenol influences reproduction parameters as well as sexual development (including changes in sex-ratio) and growth and thus endpoints are affected that may impair population stability and recruitment.

In addition to the endocrine disrupting properties, the concern is increased by evidence that 4-tert-octylphenol biodegrades very slowly in the environment and tends to adsorb to sediment and soil.

Registration (s) submitted for the substance: Yes

PART I

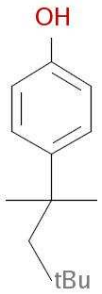
JUSTIFICATION

1 IDENTITY OF THE SUBSTANCE AND PHYSICAL AND CHEMICAL PROPERTIES

1.1 Name and other identifiers of the substance

Chemical Name: 4-(1,1,3,3-tetramethylbutyl)phenol
EC Name: 4-(1,1,3,3-tetramethylbutyl)phenol
CAS Number: 140-66-9
IUPAC Name: 4-(2,4,4-trimethylpentan-2-yl)phenol

1.2 Composition of the substance

Chemical Name:	4-(1,1,3,3-tetramethylbutyl)phenol
EC Number:	205-426-2
CAS Number:	140-66-9
CAS Name	Phenol, 4-(1,1,3,3-tetramethylbutyl)-
IUPAC Name:	4-(2,4,4-trimethylpentan-2-yl)phenol
Molecular Formula:	$C_{14}H_{22}O$
Structural Formula:	
Molecular Weight:	206.32 g/mol
Typical concentration (% w/w):	Min. > 82.7 % (w/w) [1]
Concentration range (% w/w):	

Further details on the composition of the substance are confidential and can be found in the technical dossier.

[1] Based on the minimum typical content indicated in the registration dossiers (downloaded on 21/12/2010)

1.3 Physico-chemical properties

Table 1: Summary of physico- chemical properties

REACH ref Annex, §	Property	IUCLID section	Value	Reference
VII, 7.1	Physical state at 20°C and 101.3 kPa	4.1	Solid	OECD SIDS Dossier, 1994
VII, 7.2	Melting/freezing point	4.2	79 – 82 °C	OECD SIDS Dossier [1], 1994
VII, 7.3	Boiling point	4.3	280 – 283 °C pressure not indicated	OECD SIDS Dossier [2], 1994
VII, 7.5	Vapour pressure	4.6	0.001 kPa at 20 °C	OECD SIDS Dossier [3], 1994
VII, 7.7	Water solubility	4.8	19 mg/L at 22 °C	OECD SIDS Dossier [5], 1994
VII, 7.8	Partition coefficient n-octanol/water (log value)	4.7 partition coefficient	Log POW 4.12 At 20.5°C (OECD 107, shake flask method) log P_{OW} 3.7 temperature not indicated	(Environment Agency UK, 2005) OECD SIDS Dossier [4], 1994
XI, 7.16	Dissociation constant	4.21	pKa 10.33 at 25 °C (calculated)	OECD SIDS Dossier [41], 1994
VII, 7.4	Density	4.4	950 kg/m³ temperature not indicated	OECD SIDS Dossier [22], 1994

The OECD SIDS document cited for the above mentioned data the following sources:

[1] Occupational Health Service Inc., NY/USA, Rev. 21.2.91 (CD-ROM)

[2] Sax, Dangerous properties of ind. Materials, 7th Edition, 1989

[3] ICI, Material Safety Data Sheet, January 1988

[4] McLeese D.W., Zitko V., Sergeant D.B., Burridge L., Metcalfe C.D., Lethality and Accumulation of Alkylphenols in Aquatic Fauna, Chemosphere 10 (7), 723-730, 1981

[5] Analytical Bio-Chemistry Laboratories, Inc. Method Validation and Solubility of Octylphenol in Aquatic Test Waters, unpublished test report # 31914, December 1984

[22] Sicherheitsdatenblatt HUELS AG vom 4.10.93

[41] Ahel et al.: Photochemical Degradation of Nonylphenol and Nonylphenol Polyethoxylates in Natural Waters, Chemosphere Vol. 28 No.7 pp. 1361-1368, 1994

2 CLASSIFICATION AND LABELLING

4-tert-octylphenol is listed in Regulation (EC) No 1272/2008 (1st ATP)[2] as follows:

Table 2: Classification and labelling of 4-tert-octylphenol according to part 3 of Annex VI, Table 3.1 of Regulation (EC) No 1272/2008

Index-No	International Chemical Identification	EC No	CAS-No	Classification		Labelling		Specific concentration limits, M-factors
				Hazard Class and Category Code(s)	Hazard Statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	
604-075-00-6	4-(1,1,3,3-tetramethylbutyl)phenol; 4-tert-octylphenol	205-426-2	140-66-9	Skin Irrit. 2 Eye Dam. 1 Aquatic Acute 1 Aquatic Chronic 1	H315 H318 H400 H410	GHS 05; GHS 09 Dgr	H315 H318 H410	M=10

Table 3: Classification and labelling of 4-tert-octylphenol according to part 3 of Annex VI, Table 3.2 of Regulation (EC) No 1272/2008

Index-No	International Chemical Identification	EC No	CAS-No	Classification	Labelling	Concentration limits
604-075-00-6	4-(1,1,3,3-tetramethylbutyl)phenol; 4-tert-octylphenol	205-426-2	140-66-9	Xi: R38-41 N: R50-53	Xi; N R:38-41-50/53 S:(2-)26-37/39-60-61	N; R50-53: C ≥ 2.5% N;R51-53: 0.25 % ≤ C ≤ 2.5% R52-53: 0.025% ≤ C ≤ 0.25%

[2] Regulation (EC) No 1272/2008 as amended and adapted to technical and scientific progress by Regulation (EC) No 790/2009

3 ENVIRONMENTAL FATE PROPERTIES

3.1 Degradation

3.1.1 Stability

This chapter describes abiotic degradation test results according to the Risk Evaluation Report (Environment Agency UK, 2005).

Hydrolysis

Due to the chemical structure of 4-tert-octylphenol it is expected that hydrolysis normally not will occur under environmental conditions and therefore it is supposed hydrolysis is no relevant path of abiotic degradation.

Photolysis in water

The rates of photochemical transformation of 4-tert-octylphenol in natural waters were assessed by exposing their solutions in filtered lake water to sunlight (Ahel et al., 1994). Sunlight phototransformation of 4-tert-octylphenol was performed in 50 ml quartz tubes which were suspended in a shallow flat-bottomed container filled with tap water or in a creek at a depth of 20-25 cm. The photolysis rate was 0.1 $hours^{-1}$ in the flat-bottomed container and 0.05 $hours^{-1}$ in the creek, resulting in an assumed half-life of 13.9 hours and 6.9 hours respectively (Environment Agency UK, 2005).

In the environment the exposure occurs in the whole water column. Because of the substance's behaviour it will predominantly adsorb at suspended organic matter and sediment. Photodegradation of 4-tert-octylphenol is expected to be a relevant degradation process only in very shallow clear waters and in the first few centimetres layer of the water column. Therefore aquatic photodegradation is not considered to have relevant impact on the overall persistency of 4-tert-octylphenol in the aquatic environment.

Atmospheric photodegradation

No measured data are available. 4-tert-octylphenol released to the atmosphere is likely to be rapidly degraded by reaction with hydroxyl radicals. The rate constant for this fate process has been estimated using the AOP program (v1.91, in EPISUITE, 2004) as $42 \cdot 10^{-12}$ cm^3 s^{-1} $molecules^{-1}$, assuming a hydroxyl radical concentration of 1.5·106 OH^- cm^{-3}. From this rate constant the estimated half-life for the reaction of 4-tert-octylphenol with hydroxyl radicals in the atmosphere was 0.25 days. Long-range transport would not occur because of previous degradation (Environment Agency UK, 2005).

Summary:

Even if photodegradation (DT_{50}= 0.25 days) and photolysis in water (DT_{50}= 13.9 hours) may occur in the environment, overall the abiotic degradation is a negligible removal process.

3.1.2 Biodegradation

This chapter describes biodegradation test results according to the Risk Evaluation Report and MITI-List (Environment Agency UK, 2005; MITI-List, 2002).

3.1.2.1 Biodegradation estimation

3.1.2.2 Screening tests

Table 4: Summary of Screening tests (Reliability according to (Klimisch et al., 1997))

Test	Result	Reliability	Reference
OECD 301 C	0% after 14 days	2	(MITI-List, 2002)
OECD 301 B	62.1 % after 28 days (10 day window was failed)	2	(Gledhill, 1999)
BODIS (ISO 10708)	20 % after 28 days	4[1]	(OECD SIDS, 1994)
OECD 302 C	0 % after 28 days	4[1]	(OECD SIDS, 1994)

[1] not sure about test substance identity (OECD SIDS 1994: p-octylphenol; Environment Agency UK, 2005: 4-tert-octylphenol)

In a 14 day ready biodegradability test (MITI I, OECD 301C) using 100 mg/l of the substance and 30 mg/l sludge no biodegradability was detected (MITI-List, 2002).

Gledhill et al. determined the potential of biodegradation of 4-tert-octylphenol following OECD Test Guideline 301B (Gledhill, 1999; Staples et al., 2001). Activated sludge from a WWTP (Waste Water Treatment Plant), showing a high nonylphenol ethoxylate concentration, was used for this study. With the end of the test on day 35, 69.9% $ThCO_2$ was measured. After day 28 (62.1% $ThCO_2$) the pass level for ready biodegradability (>60 % $ThCO_2$) was fulfilled, but the 10 day window was failed. This study suggests that the microorganisms may need a period of adaption (lag time ~ 6 days).

The BODIS test (ISO 10708) with non-adapted activated sludge microorganisms showed a biodegradation of 20% after 28 days (OECD SIDS, 1994). This study was carried out to GLP.

In a 28-day inherent biodegradability test (MITI II, OECD 302C), using a mixed population of non-adapted microorganisms from activated sludge and 30 mg/l octylphenol, no degradation was seen after 28 days (OECD SIDS, 1994). The reference substance (Aniline 100 mg/l) showed a degradation of 74% after 14 days and 87 % after 28 days. The result suggests that octylphenol is not inherently biodegradable. The study was not carried out to GLP.

The screening tests show contradictory results. Overall the data suggest that 4-tert-octylphenol is not readily biodegradable. The tests indicate a potential of biodegradation after a period of adaption.

3.1.2.3 Simulation tests

Table 5: Summary of Simulation tests (Reliability according to (Klimisch et al., 1997))

Environmental Compartment	Conditions	Result	Reliability	Reference
River water	aerobic	DisDT50 = 8-54 days	2	(Johnson et al., 2000)
Bed sediment	anaerobic	No elimination after 83 days		
Seawater	aerobic with bubbling	DisDT50 ~ 11 days	2	(Ying and Kookana, 2003)
	aerobic without bubbling	DisDT50 ~ 30 days		
Marine sediment	aerobic	DisDT50 > 21 days		
	anaerobic	No elimination after 70 days		

Using laboratory microcosms, Johnson et al. studied the potential for 4-tert-octylphenol to disappear in some English rivers (Johnson et al., 2000). DisDT50 – the time when 50 percent of a substance dissipates from a single media – of 8 to 54 days (zero-order reaction) were obtained for the water samples. Shorter half-lives were generally seen in more urban and industrialised rather than upland and rural areas. Although 4-tert-octylphenol could be eliminated in river samples taken from a range of urban and rural reaches, processes such as sorption to bed and suspended sediment would primarily reduce the concentration of 4-tert-octylphenol in river water. Consequently, 4-tert-octylphenol is admittedly eliminated from the water samples but removed to another environmental compartment. An experiment with bed sediments (spiked with 4-tert-octylphenol) which was incubated under anaerobic conditions showed no elimination over 83 days. This suggests that 4-tert-octylphenol would accumulate in anaerobic bed sediments.

Ying and Kookana studied the elimination of 4-tert-octylphenol in marine environment using seawater taken from a coastal area near Adelaide, Australia (Ying and Kookana, 2003). The initial concentration of 4-tert-octylphenol in water was 5 µg/L (incubation at 20 ± 3°C). The solutions were aerated by bubbling air through them. Rapid initial losses were seen in the non-sterile solutions and the sterile control, indicating that these losses resulted from abiotic processes. After this initial removal, the concentration in the non-sterile solutions continued to decrease steadily to 0.03 µg/L after 42 days ($DisDT_{50}$ ~11 days). The concentration in the sterile control remained stable with little change. Experiments which were carried out without bubbling air through the solutions, showed a slower rate of removal ($DisDT_{50}$ ~30 days). In the study a $DisDT_{50}$ of 60 days was specified, but the figure in the report clearly shows a half-life of around 30 days and complete removal by ~50 days.
Studies were also carried out on marine sediments, collected from close to the same area. 5 g of marine sediment with 5 ml of seawater were used to make slurry during all experiments. In addition to 1µg/g 4-tert-octylphenol, four other substances (including 4-*n*-nonylphenol) were added. Under aerobic conditions complete degradation of 4-tert-octylphenol was seen within 70 days. There was a period of adaption of around 3 weeks, with a concentration of 0.84 µg/g remaining after 21 days, but then a decrease to 0.09 µg/g within a further week. The $DisDT_{50}$ is >21 days. Under anaerobic conditions no degradation was occurred.

The simulation tests showed mainly dissipation and degradation only to a very limited extent. Hence, the elimination of 4-tert-octylphenol in the water samples could mainly be caused by strong adsorption to sediment, soil and sludge. In sediment no elimination was observed under anaerobic conditions.

3.1.3 Summary and discussion of persistence

Considering all available information together, the abiotic degradation of 4-tert-octylphenol is negligible.

The screening tests for ready biodegradability show contradictory results. In one test using adapted activated sludge, the pass level for ready biodegradability was fulfilled but the 10 day window was failed. (Gledhill, 1999). Further screening tests on ready and inherent biodegradability showed no biodegradation after 28 days (MITI-List, 2002). (OECD SIDS, 1994). In summary of all findings, 4-tert-octylphenol is not readily biodegradable with some indications for a certain degree of biodegradation following a period of adaption.

Only limited data are available for simulations tests. In the studies mainly dissipation and degradation only to a very limited extent was observed. 4-tert-octylphenol strongly adsorbs to soil, sludge and sediment. Thus dissipation is probably caused by adsorption and reflects a shift in media, only. As an overall picture 4-tert-octylphenol shows a little potential of biodegradation under aerobic conditions in aquatic media ($DisDT_{50}$=8-54 days), but only if an adaption of microbial populations has occurred. Under anaerobic conditions no degradation was observed in sediment ($DisDT_{50}$>83 days). Consequently, if the whole environment is considered, 4-tert-octylphenol biodegrades very slowly.

3.2 Environmental distribution

This chapter describes environmental distribution of 4-tert-octylphenol according to the Risk Evaluation Report, OECD SIDS and Simple Treat 3.0 (Environment Agency UK, 2005; OECD SIDS, 1994).

3.2.1 Adsorption/desorption

Based on a log K_{OW} of 4.12, the organic carbon–water partition coefficient (K_{OC}) for 4-tert-octylphenol is estimated as 2740 l/kg (Environment Agency UK, 2005). Based on a log Kow of 3.7 a Koc of 1376 l/kg is estimated by Episuite version 4.1 (EPISUITE, 2011). However, it is an indication for a high tendency to adsorb at organic material.

Johnson et al. studied the sorption of 4-tert-octylphenol to different river sediments using laboratory batch techniques (Johnson et al., 1998). After sufficient time or mixing, 4-tert-octylphenol will adsorb to bed sediments with K_{OC} of 3466-18500 l/kg. The sediments that adsorbed the highest quantities of 4-tert-octylphenol had higher total organic carbon levels and a greater proportion of clay and silt particles. The study predicted that suspended sediments might also play a key role in the fate of 4-tert-octylphenol in industrialised areas. In the rural areas a higher proportion of 4-tert-octylphenol might be predicted to remain free in solution.

These K_{OC} (experimental and calculated) suggest that 4-tert-octylphenol strongly adsorbs to soil, sludge and sediment.

4-tert-octylphenol is a weak acid, because of this pH might have an effect on its adsorptive behaviour. The pK_a is thought to be around 10. Hence, in the environment, the substance will be present in the un-dissociated and more hydrophobic form (Environment Agency UK, 2005).

3.2.2 Volatilisation

Henry´s Law constant (H) has been measured as 0.52 Pa·m^3/mol. An air–water partitioning coefficient ($K_{air\text{-}water}$) is calculated as $2.1 \cdot 10^{-4}$ m^3/m^3. The $K_{air\text{-}water}$ and H are low and suggest that volatilisation is unlikely to be a significant removal mechanism for 4-tert-octylphenol from water systems (Environment Agency UK, 2005).

Based on the Henry´s Law constant of 0.52 Pa m^3/mol, the volatilization half-life from a model river (1 m deep flowing at 1 m/sec with a wind velocity of 3 m/sec) can be estimated to be about 250 hours (EPISUITE, 2004).

3.2.3 Distribution modelling

The equilibrium distribution of 4-tert-octylphenol in a closed environment was calculated with Fugacity Level I (FUGMOD V1.0) (OECD SIDS, 1994). The Level I model is based on physical-chemical properties and does not include degradation processes, advective processes and intermedia transport processes.

Table 6: Distribution of 4-tert-octylphenol in the environment (Fugacity Level I)

	Distribution
Air	29.4 %
Water	12.7 %
Soil	56.6 %
Sediment	1.3 %
Suspended Soil	<0.1 %
Fish	<0.1 %

This model shows that more than half of the 4-tert-octylphenol would be found in soil and only minor amounts in sediment, suspended soil and fish.

According to Fugacity Level III calculations (FUGMOD V1.0) 4-tert-octylphenol will, after release to a specific compartment, be distributed in the environment as follows (OECD SIDS, 1994):

Table 7: Distribution of 4-tert-octylphenol in the environment (Fugacity Level III)

	release to air	**release to water**	**release to soil**
Air	26.0 %	1.2 %	<0.1 %
Water	5.1 %	77.9 %	0.3 %
Soil	67.7 %	3.1 %	99.6 %
Sediment	1.2 %	17.8 %	0.1 %

If 4-tert-octylphenol is released to air, most of the substance will partition to soil (67.7 %) followed by air (26.0 %). After release to water and soil most of the substance will stay in the respective compartment (release to water: 77.9 % in water; release to soil: 99.6 % in soil). 17.8 % of the 4-tert-octylphenol that was released to water will be found in sediment.

Both distribution models show that 4-tert-octylphenol will primarily partition to soil, when 4-tert-octylphenol is released to air or soil. When released to water the predominant amount of 4-tert-octylphenol will remain in the aquatic compartment with adsorption to organic suspended matter.

Distribution in waste water treatment plant:

The modelling of the distribution in a municipal waste water treatment plant was conducted with SimpleTreat 3.0 (debugged version, 7 Feb 1997). The conclusions on the screening tests (section 3.1.2.2) indicate that the substance is potentially biodegradable after a period of adaption. Therefore the rate constant of K=0.1/h was used for the distribution modelling.

Table 8: Distribution of 4-tert-octylphenol in the waste water treatment plant (SimpleTreat 3.0)

	Distribution
To air	2.0 %
To water	45.1 %
Via primary sludge	18.0 %
Via surplus sludge	4.3 %
Degraded	30.6 %
Total	100 %

Based on the rate constant of K=0.1/h (inherently biodegradable + period of adaption) 45.1 % of 4-tert-octylphenol will leave the waste water treatment plant with the effluent, 30.6 % will be degraded, 22.3 % adsorbed to sludge and 2.0% released to air.

3.3 Bioaccumulation

3.3.1 Aquatic bioaccumulation

This chapter describes aquatic bioaccumulation test results according to the Risk Evaluation Report, MITI-List and OECD SIDS (Environment Agency UK, 2005; MITI-List, 2002; OECD SIDS, 1994).

3.3.1.1 Bioaccumulation estimation

If a log K_{OW} of 4-tert-octylphenol of 4.12 is used a BCF of 634 is calculated (Environment Agency UK, 2005). Based on a log Kow of 3.7 the corresponding BCF is 290, calculated with Episuite version 4.1 (EPISUITE, 2011).

3.3.1.2 Measured bioaccumulation data

Table 9: Bioaccumulation factors of 4-tert-octylphenol in fish (Reliability according to (Klimisch et al., 1997))

Species	Tissue	BCF	Lipid content	Reliability	Reference
Oncorhynchus mykiss	Whole fish	471	not given	2	(Ferreira-Leach and Hill, 2001)
	Liver	1020±165			
	Fat	1190±214			
	Blood	91±12			

	Gills	187±19			
	Muscle	101±14			
	Kidney	176±57			
	Bile	68794±15432			
	Faeces	13659±4063			
Zacco platypus		129	not given	3	(Tsuda et al., 2000)
Plecoglossus altivelis		297±194	5.8-7.2 %		
Zacco temminckii		200	not given		
Micropterus salmoides		46	not given		
Oryzias latipes		261±62	2.2 %	2	(Tsuda et al., 2001)
Cyprinus carpio		12-469	not given	2	(MITI-List, 2002)

Ferreira-Leach and Hill reported about biotransformation, bioconcentration and tissue distribution of 4-tert-octylphenol in juvenile rainbow trout (*Oncorhynchus mykiss*) (Ferreira-Leach and Hill, 2001). In a flow-through system the fish was exposed with a concentration of 4 µg/l of ^{14}C 4-tert-octylphenol for 10 days. Steady state conditions in the whole fish were reached after 4 days. In certain tissues accumulation of the substance was still increasing by day 10. The BCF for the whole fish was 471. The bioconcentration was tissue dependent. The BCF in liver and fat were 1020±165 and 1190±214. In Blood (91±12), Gills (187±19), Muscle (101±14) and Kidney (176±57) a low BCF were observed, whereas the highest BCF values occurred in bile (68794±15432) and faeces (13659±4063). This study suggests that exposure to water-borne alkylphenols results in rapid conjugation and elimination of the chemical by the liver/bile route, but the high amount of the parent substance can accumulate in a variety of other fish tissues.

In another study four fish species in eight rivers were tested for 4-tert-octylphenol tissue concentrations (Tsuda et al., 2000). Water and fish samples were collected every two months for one year. In the water 4-tert-octylphenol was detected between the limit of detection (0.01 ng/ml) and 0.09 ng/ml. The whole body BCF amount to 129 for Pale chub (*Zacco platypus*), 297 for Ayu sweetfish (*Plecoglossus altivelis*), 200 for Dark chub (*Zacco temminckii*) and 46 for large-mouth bass (*Micropterus salmoides*). These field values were nearly equal to the laboratory BCF value of 261 (Killifish; *Oryzias latipes*) (Tsuda et al., 2001).

CEFAS tested the effect of 4-tert-octylphenol on the food chain of water organisms (CEFAS, 1997). A number of herring, haddock and dab, near North Sea offshore installations (alkylphenolic compounds are used as production chemicals), were analyzed for 4-tert-octylphenol. The concentrations in muscle and liver were below the limit of detection (herring muscle and dab muscle <0.004 mg/kg, haddock liver <0.04 mg/kg and haddock muscle <0.1 mg/kg).

A bioaccumulation test in carp (*Cyprinus carpio*) resulted in BCF of 12-135 (10 µg/l 4-tert-octylphenol) and 113-469 (100µg/l 4-tert-octylphenol) (MITI-List, 2002).

3.3.2 Terrestrial bioaccumulation

No data available

3.3.3 Summary and discussion of bioaccumulation

The bioaccumulation potential in aquatic organisms is low to moderate. The experimentally determined BCF ranges between 46 and 471.

3.4 Secondary poisoning

Not assessed for the identification of this substance as SVHC in accordance with Article 57(f).

4 HUMAN HEALTH HAZARD ASSESSMENT

For this chapter of the dossier studies were taken into consideration for which the substance that had been used in tests was identified as 4-tert-octylphenol (CAS No 140-66-9).

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

Oral application

Gavage

Groups of 6 male Wistar rats received 0, 50 or 200 mg/kg bw 4-tert-octylphenol in polypropylene by single gavage application (Certa et al. 1996). In animals treated with 50 mg/kg bw 4-tert-octylphenol could be detected at the earliest sampling time point (10 min after application). Blood concentration reached a maximum of 40 ng/ml within 20 min and decreased to 3-7 ng/ml after 4-6 h. An AUC of 0.086 (µg*h)/ml and a bioavailability of 2 % were calculated by the authors. In animals treated with 200 mg/kg bw individual 4-tert-octylphenol blood concentrations varied within the animals. 4-tert-octylphenol was detectable after 48 h. An AUC of 1.778 (µg*h)/ml and a bioavailability of 10 % were calculated.

Groups of 6 female DA/Han rats were treated with single doses of 50 or 200 mg/kg bw in 1,2-popanediol by gavage (Upmeier et al. 1999). Blood samples for determination of 4-tert-octylphenol concentrations were taken after 10, 30 and 60 min, 2, 4, 8 and 32 h in one subset and after 20, 45 and 90 min, 3, 6, 24 and 48 h in the second subset. Blood of control animals, which received the vehicle only, was sampled after 2 h. In animals treated with 50 mg/kg bw 4-tert-octylphenol was detected after 10 min and mean maximum blood concentrations of 181 ng/ml were reached 90 min after administration. 4-tert-octylphenol was hardly detectable after 32-48 h. In animals treated with 200 mg/kg bw the maximum blood concentration was about 419 ng/ml. After 48 h a mean blood concentration of 14 ng/ml was detected. After oral application, the time course of 4-tert-octylphenol blood levels may point to enterohepatic cycling owing to the fact that the increase and subsequent decrease after gavage application was followed by a second increase in 4-tert-octylphenol blood concentration in some but not all animals. Among the experimental animals, interindividual variations were evident. Based on the toxicokinetic parameters of the single i.v. (intravenous) and oral gavage applications, bioavailabilities of 12.3 and 8.4% for the doses of 50 and 200 mg/kg bw, respectively, were calculated.

Two male Sprague-Dawley rats received a single dose of 100 mg/kg bw 4-tert-octylphenol in propylene glycol by gavage (Hamelin et al. 2008). Blood samples were collected for up to 5 h after administration. Untreated animals served as controls. After 1h 4-tert-octylphenol blood concentration was determined to be 730 ng/ml (C_{max}), which was decreased to about 400 ng/ml after 5 h.

Groups of 5 male or female Sprague-Dawley rats (n=5, each) received single oral doses of 4-tert-octylphenol (purity 97%) of 50, 125 or 250 mg/kg bw in propylene glycol (Hamelin et al. 2009). Blood was sampled up to 24 h after administration. The maximum blood concentrations were measured after 2 h in the 50 mg/kg group and after 1h in groups receiving 125 or 250 mg/kg bw. In male and female rats C_{max} of 133, 238 or 386 ng/ml and 106, 290 or 272 ng/ml, respectively were determined. In male and female rats AUC of 1235, 2300, or 4264 and 1503, 4501, or 7838 (ng*h)/ml were determined, respectively. Bioavailability ranged from 26-38% in male animals and 46-55% in females. 4-tert-octylphenol half life ranged from 5-16.6 h in male animals and 8.3-37.9 in females. The authors concluded that there may remain uncertainties regarding the half-life determined after oral application due to relatively high 4-tert-octylphenol blood concentrations at the last sampling time point. In the second part of the study groups of male or female Sprague-Dawley rats (n=5, each) received daily doses of 25, 50 or 125 mg/kg bw/d 4-tert-octylphenol for 57 (male) or 33 (female) consecutive days. Blood samples were collected on day 1, 1 hour after administration and on the respective last day of treatment, 1 and 4 h after administration. After repeated exposure, blood 4-tert-octylphenol concentrations were higher at the end of the exposure period in both female (mean 2.26-fold, not significant compared to controls) and male (mean 3.47-fold, significant) rats. Blood 4-tert-octylphenol concentrations were higher in male than in female rats 1 h after the end of exposure on the first day of exposure (mean 1.69-fold, not significant). Tissue concentrations of 4-tert-octylphenol were determined in male and female Sprague-Dawley rats (n=5) 4 and 24 hours after administration of a single oral dose of 125 or 250 mg/kg, and after repeated doses of 25, 50 or 125 mg/kg bw/d for 60 d (males) or 35 d (females). The tissue concentrations appeared to be within a single-digit µg/g tissue range. After single oral administration the highest concentration was found in liver, followed by fat, kidneys and ovaries. Lowest concentrations were found in muscle tissue. 4-tert-octylphenol tissue concentrations appeared to be higher in female animals compared to the males. After repeated oral administration a dose-dependent increase of tissue 4-tert-octylphenol concentrations were observed. The highest concentrations were found in fat and liver. The tissue concentrations of animals treated with repeated doses of 125 mg/kg bw/d were compared to those of animals which received a single oral dose of 125 mg/kg bw. No significant differences occurred between the tissue concentrations from single and repeated treatment indicating no bioaccumulation of 4-tert-octylphenol.

Groups of 5 male Wistar rats were treated with 0, 50 or 200 mg/kg bw 4-tert-octylphenol in polypropylene by gavage once per day for 14 consecutive days (Certa et al. 1996). On the first and last day of application 4-tert-octylphenol blood concentrations were determined several times. 4-tert-octylphenol was rapidly absorbed. The mean blood concentrations in the 50 or 200 mg/kg bw group reached a maximum of 50-70 ng/ml or 80-100 ng/ml, respectively, within 2 h after application. Blood concentrations decreased within 24 h but were still detectable before the subsequent application. 24 h after the 14th administration, 4-tert-octylphenol blood concentration was 3 times higher than 24 h after single administration in the 50 mg/kg bw group but not in the 200 mg/kg bw group. After termination of the treatment 4-tert-octylphenol was determined in brain, liver, lung, kidney, testes, muscle and fat. In fat and liver of 3 animals of the 50 mg/kg group 4-tert-octylphenol concentrations of 10 and 7 ng/g tissues, respectively, were found. In the 200 mg/kg bw group 4-tert-octylphenol was detected in all analysed tissues except in testes. In fat tissue 4-tert-octylphenol levels up to a concentration of 1285 ng/g tissue were determined, lower average concentrations of 87, 71 or 47 ng/g tissue were determined in liver, kidney, and , respectively. In brain and lung 4-tert-octylphenol concentrations of 9 or 7 ng/g tissue were measured.

Table 10: Toxicokinetic parameters of 4-tert-octylphenol after oral application

Species	**Dose**	**C_{max}**	**t_{max}**	**$t_{1/2}$**	**Bioavaila-bility**	**Reference**
Wistar rat, male	50, 200 mg/kg bw Single dose gavage	40 ng/ml Not reported due to high variability	20 min	No data	2% 10%	Certa et al. 1996
Da/Han rat, female	50, 200 mg/kg bw Single dose Gavage	181 ng/ml 419 ng/ml	90 min	No data	12.3% 8.4%	Upmeier et al. 1999
Species	**Dose**	**C_{max}**	**t_{max}**	**$t_{1/2}$**	**Bioavaila-bility**	**Reference**
Sprague-Dawley rat, male	100 mg/kg bw Single dose Gavage	730 ng/ml	60 min	No data	No data	Hamelin et al. 2008
Sprague-Dawley rat, male	50 125 250 mg/kg bw Single dose gavage	133 238 386 ng/ml	2 h	5.0 h 8.5 h 16.6 h	38% 28% 26%	Hamelin et al. 2009
Sprague-Dawley rat, female	50 125 250 mg/kg bw Single dose gavage	106 290 272 ng/ml	2 h	8.3 h 10.6 h 37.9 h	46% 55% 48%	Hamelin et al. 2009
Wistar rat, male	50, 200 mg/kg bw/d 14 d, gavage	50-70 ng/ml[1] 80-100 ng/ml $C_{4\text{-tert-octylphenol, blood}}$ determined after administration on day 14	2 h	No data	Not relevant	Certa et al. 1996

Drinking water

Male Wistar rats received 4-tert-octylphenol as a saturated solution in drinking water (about 8 mg/l) for 14 (5 animals) or 28 days (10 animals) (Certa et al. 1996). Based on the water consumption a daily dose of about 800 µg/kg bw was calculated by the authors. 4-tert-octylphenol concentrations were determined in brain, liver, lung, kidney, testes, muscle, and fat. Apart from one animal 4-tert-octylphenol was not detected in tissues of either of the treatment groups. In that single animal 4-tert-octylphenol concentration were 23 ng/g in kidney and 68 ng/g in muscle. 4-tert-octylphenol was not detected in the blood over a period of 28 d.

Intravenous application

A group of 6 male Wistar rats received a single i.v. dose of 5 mg 4-tert-octylphenol (purity 98%) in polypropylene into the tail vein (Certa et al. 1996, Hüls 1996). A group of animals receiving vehicle alone served as negative control. In the 4-tert-octylphenol treated group the maximum blood concentration of 1970 ng/ml blood was reached immediately after injection, decreased within 30 min and was not detectable after 6-8 h. An AUC of 0.433 (µg*h)/ml and a half-life of about 310 min were calculated. Based on the time concentration curve after i.v. application the authors assumed that the substance is rapidly distributed into the richly perfused organs, resulting in a rapid decrease in blood levels. Distribution into fat and other

slowly perfused tissues resulted in a phase with a slower decrease of the blood concentration, which is determined by excretion from the body.

A Group of 12 female DA/Han rats was treated with a single i.v. dose of 5 mg/kg bw 4-tert-octylphenol (purity 98%) in 1,2-popanediol (Upmeier et al. 1999). Blood samples for determination of 4-tert-octylphenol concentrations were taken 1, 10 and 40 min and 2, 4, 8 and 32 h after administration in one subset and 5, 20 and 60 min and 3, 6, 24 and 48 h after administration in the second subset. Blood of control animals, which received the vehicle only, was sampled after 2 h. In animals with intravenous application blood concentrations were about 1600 ng/ml 1 or 5 min after injection (4/12 animals). Due to difficulties in finding the veins because of the black pigmentation of the test animals, the remaining animals (8/12) were treated 'paravenously' as stated by the authors. Within one hour after dosing, blood concentrations were decreased to 100 ng/ml. After 48 h 4-tert-octylphenol blood concentrations ranged from 1 to 20 ng/ml. A half-life of 36.1 h was calculated from this study.

Two male Sprague-Dawley rats received a single i.v. dose of 10 mg/kg bw 4-tert-octylphenol in alkamuls (Hamelin et al. 2008). Blood samples were collected for up to 3 h after injection. Untreated animals served as control. Intravenous treatment resulted in an 4-tert-octylphenol blood concentration of 1313 ng/ml after 15 min, which rapidly declined to about 100 ng/ml within 3 h.

Groups of male and female Sprague-Dawley rats (n=5) were treated with single i.v. injections of 2, 4, or 8 mg/kg bw 4-tert-octylphenol (purity 97%) in alcamuls (Hamelin et al. 2009). Blood was sampled up to 4 h after dosing. For determination of 4-tert-octylphenol content in tissues animals receiving the same treatment were sacrificed 1 and 3 h after 4-tert-octylphenol administration. T_{max} was achieved 10 min after injection. For male and female animals C_{max} of 160, 323 or 947 ng/ml and 119, 296 and 841 ng/ml were determined in animals treated with 2, 4 or 8 mg/kg bw, respectively. The following AUC values were obtained after i.v. application animals: 106, 262, or 662 and 100, 263, 756 (ng*h)/ml in male and female animals, respectively. The 4-tert-octylphenol half life ranged from 1.1 2.4 h. 4-tert-octylphenol in tissues was determined 1 and 3 h after a single i.v. injection of 4 or 8 µg/kg bw. 4-tert-octylphenol was found in all examined tissues (muscle, brain, liver and fat as well as in uterus and ovaries in females and testes and epididymis in male animals). The highest concentrations were found fat, ovaries and testes. No gender specific differences occurred after i.v. administration. The tissue concentrations appeared to be within a single-digit µg/g tissue range.

Table 11: Toxicokinetic parameters of 4-tert-octylphenol after i.v. injection

Species	**Dose**	**C_{max}**	**$t_{1/2}$ (h)**	**Reference**
Wistar rat, male	5 mg/kg bw	1970 ng/ml	5.2	Certa et al. 1996, Hüls 1996
DA/Han rat, female	5 mg/kg bw	1600 ng/ml	36.1	Upmeier et al. 1999
Sprague-Dawley rat, male	10 mg/kg bw	1313 ng/ml		Hamelin et al. 2008
Sprague-Dawley rat, male	2 4 8 mg/kg bw	160, 323 or 947 ng/ml	2.1 1.1 1.2	Hamelin et al. 2009
Sprague-Dawley rat, female	2 4 8 mg/kg bw	119, 296 and 841 ng/ml	2.4 1.7 1.6	Hamelin et al. 2009

Subcutaneous application

Groups of five female Crj:Donryu rats were treated with 4-tert-octylphenol in dimethylsulfoxide at doses of 6.25, 12.5, 25, 50, 100 or 200 mg/kg bw/d for 2 days or 6.25, 12.5, 25, 50, or 100 mg/kg bw/d for 14 consecutive days by subcutaneous injection (Katsuda et al. 2000). The experiment was terminated 24 h after the last s.c. injection and blood was sampled immediately after killing of the animals. Dose-dependent serum concentrations of 70-190 ng/ml or 60-190 ng/ml were detected after the 2-days or the 14-days treatment, respectively. Related to the applied dose, 4-tert-octylphenol blood levels were approximately 2-fold higher after 14 days compared to 2 days of treatment. 4-tert-octylphenol was not detectable in the serum of animals treated with 6.25 or 12.5 mg/kg bw/d for two days, or 6.25 mg/kg bw/d for 14 days.

Groups of 10 female Fisher 344 and female Crj:Donryu rats were treated with 12.5, 25, 50 or 100 mg/kg bw/d 4-tert-octylphenol in DMSO for 28 days (Yoshida et al. 2000). Control groups (n=12) were treated with DMSO alone. Animals treated with 4-tert-octylphenol showed scab and abscess formation at the sites of injection, the severity being dependent on applied doses and duration of treatment. Seven days after the first treatment half of the animals (n=5 treated, n=6 control) were sacrificed and blood samples were collected. On the day after the last treatment, all surviving animals in the treated groups were sacrificed and blood samples were collected. 4-tert-octylphenol was not detected in control animals. In 4-tert-octylphenol treated animals, mean blood concentrations ranged from approximately 20 to 160 ppb (according to the published figure) and appeared at the same level after 7 or 28 days of treatment, or when comparing Donryu and Fischer rats.

Groups of 5 male or female Sprague-Dawley rats were treated with 125 mg/kg 4-tert-octylphenol (purity 97%) in DMSO by subcutaneous injection (Hamelin et al. 2009). Blood samples were collected up to 24 h after application and analysed for unchanged 4-tert-octylphenol. 4-tert-octylphenol blood concentration reached a maximum after 6 h (females) or 8 h (males). In male and female rats C_{max} was shown to be 181.5 and 206.3 ng/ml and AUC to be 2375.1 and 3654.5 (ng*h)/ml, respectively. 4-tert-octylphenol half life was 9.8 h in male and 39.6 in female animals. A bioavailability of 29% in male and 44% in female animals was calculated for the s.c. route of administration.

Table 12: Toxicokinetic parameters of 4-tert-octylphenol after s.c. injection

Species	**Dose**	**$C_{4\text{-tert-octylphenol, blood}}$**	**t_{max}**	**Bioavailability**	**Reference**
Crj:Donryu rat, female	6.25-200 mg/kg bw/d 2-14 days	60-190 ng/ml 24 h after last treatment	No data	No data	Katsuda et al. 2000
Crj:Donryu rat, female Fisher rat, female	12.5-100 mg/kg bw/d 7 or 14 d	~20-160 ng/ml	No data	No data	Yoshida et al. 2000
Sprague-Dawley rat, male	125 mg/kg bw	182 ng/ml (C_{max})	8 h	29%	Hamelin et al. 2009
Sprague-Dawley rat, female	125 mg/kg bw	206 ng/ml (C_{max})	6 h	44%	Hamelin et al. 2009

In vitro studies

Liver preparations from male Wistar rats were used to characterize the glucuronidation and sulphatation of 4-tert-octylphenol *in vitro* (Certa et al. 1996; Hüls 1996). A V_{max} and k_m of 11.24 nmol/min/mg and 8.77 µM were determined for glucuronidation, respectively. For sulphatation a V_{max} of 2.85 nmol/min/mg protein and a k_m of 11.35 µM were determined. There was no information on the isoforms of enzymes involved in either case.

The biotransformation of radiolabelled 4-tert-octylphenol (purity 99%) was investigated in isolated hepatocytes of Sprague-Dawley rats (Pedersen et al. 2000). Hepatocytes were incubated with 10, 30 or 50 µM 4-tert-octylphenol for 5, 15 or 60 min. Metabolites were detected by HPLC and gas-chromatography. Metabolites were identified as 2-hydroxy-4-(1',1',3',3'-tetramethylbutyl)phenol, 4-(1',1',3',3'-tetramethylbutyl)phenoxy-β-glucuronide, 2-methoxy-4[5]-(1',1',3',3'-tetramethylbutyl)phenoxy-β-glucuronide, 2-hydroxy-4-(1',1',3',3'-tetramethylbutyl)phenoxy-β-glucuronide and it's positional isomer 2-hydroxy-5-(1',1',3',3'-tetramethylbutyl)phenoxy-β-glucuronide, 2-methoxy-4[5]-(2'-β-glucuronoxy-1',1',3',3'-tetramethylbutyl)phenol, 4-(2'-β-glucuronoxy-1',1',3',3'-tetramethylbutyl)phenol, 2-methoxy-4-(2'-hydroxy-1',1',3',3'-tetramethylbutyl)phenoxy-β-glucuronide, 4-(2'-hydroxy-1',1',3',3'-tetramethylbutyl)phenoxy-β-glucuronide and 2-β-glucuronoxy-4-(2'-hydroxy-1',1',3',3'-tetramethylbutyl)phenol. Further β-glucuronide and sulphate conjugates were reported as minor metabolites but not specified. In this study 94% of 4-tert-octylphenol was metabolized.

In an *in vitro* study rat liver microsomes were incubated with 4-tert-octylphenol (purity 97%) at concentrations of 0.1 to 1000 µmol/l (Hanioka et al. 1999). Interference of 4-tert-octylphenol with several isoforms of CYP was determined using photometric methods or HPLC. 4-tert-octylphenol had a dose-dependent inhibitory effect on 7-ethoxycumarin-O-deethylase, testosterone 16β-hydroxylase, testosterone 2α-hydroxylase and testosterone 6β-hydroxylase with IC_{50} values of 76.2, 16.1, 19.8 and 84.3 µM, respectively, and an almost complete inhibition at 1000 µM. In 4 further tests IC_{50} values ranged between 176 and >1000 mM and no complete inhibition was achieved. A competitive inhibition was proposed for testosterone 2α-hydroxylase and testosterone 6β-hydroxylase, for the further tested enzymes a non-competitive inhibition was proposed.

Hanioka et al. (2000) investigated protein levels and enzymatic activities of cytochromes P450 in hepatic microsomes of male 4-tert-octylphenol treated Sprague-Dawley rats. Three animals per group were treated with 5, 10 or 20 mg/kg bw of 4-tert-octylphenol (purity 97%) in propylene glycol or the vehicle alone on day 1 and 3. Animals were killed on day 5 and liver microsomes were prepared. Treated animals showed a dose-dependent decrease in body weight gain. In the 20 mg/kg group the relative thymus weight was decreased as well. Activities of several CYP isoforms were determined photometrically. The activity of testosterone 2α-hydroxylase (CYP2C11) was decreased by 33, 65 or 78% in the 5, 10 or 20 mg/kg bw group, respectively, when compared to control. In the highest dose group enzyme activities of ethoxycoumarin-O-deethylase, bufuralol 1'-hydroxylase as well as testosterone 6β-hydroxylase were decreased by 32, 80 or 51%, respectively. The microsomal protein levels of CYP2C11/6 and 3A2/1 were determined by immunoblotting. CYP2C11/6 showed a decreased protein level in the 10 and 20 mg/kg bw group, the protein level of CYP3A3/1 was decreased in the 20 mg/kg bw group.

Hanioka et al. (2000b) investigated protein levels and enzymatic activities of cytochromes in liver microsomes of male 4-tert-octylphenol (purity 97%) treated Sprague Dawley rats. Three animals were treated with 16 mg/kg bw (80 µmol/kg bw) 4-tert-octylphenol in propylene glycol or the vehicle alone on day 1 and 3, and were killed on day 5. CYP was determined

spectrophotometrically and was decreased in hepatic microsomes of 4-tert-octylphenol treated animals compared to control, whereas the total protein content was unchanged. Enzymatic activities of CYP isoforms were determined photometrically. Activities of CYP dependent testosterone 2α-hydroxylase (CYP2C11) and testosterone 6β-hydroxylase (CYP3A) were decreased to 12% and 49% of the activities in controls, respectively. Activities of other CYP isoforms remained unchanged.

Kinetic parameters of 4-tert-octylphenol sulphatation were determined using purified recombinant human sulphotransferase isoforms (SULT1A1, 1A3, 1E1 and 2A1) (Nishiyama et al. 2002). Km and K_{cat} for sulphatation of 4-tert-octylphenol were 27, 7.8 or 5.1 µM and 1.9, 3.7 or 1.1 min^{-1} for SULT 1A1, 1E1 or 2A1, respectively. No conjugation of 4-tert-octylphenol by SULT 1A3 was detected. Based on the relatively high quotient of k_{cat} and k_m, 4-tert-octylphenol appears to be sulphated rather by SULT 1E1 and 2A1 than by SULT 1A1. The authors concluded that this might be due to the highly branched side chain of 4-tert-octylphenol.

Metabolism of 4-tert-octylphenol was investigated using liver perfusion in male Sprague-Dawley (SD)rats and Eisai hyperbilirubinemic rats (EHBR) with a solution containing 0.05 mM 4-tert-octylphenol (Nomura et al. 2008). Metabolites were detected using HPLC and LC/MS. In this study 4-tert-octylphenol was shown to be metabolized by hydroxylation and subsequent glucuronidation or glucuronidation alone. The metabolites were identified as hydroxyl-tert-4-tert-octylphenol glucuronide, hydroxyl-tert-4-tert-octylphenol, tert-octylcatechol-glucuronide, 4-tert-4-tert-octylphenol-glucuronide, 4-tert-octylcatechol unchanged 4-tert-4-tert-octylphenol. Glucuronides were shown to be excreted into the bile (38% of the perfused substrate) and were detected in liver tissue in SD rats. In EHBR rats only 32 % of perfused 4-tert-octylphenol were recovered almost all of which (~68 %) was found in the hepatic vein (and not in the bile or the livers).
In a second part or the study an UDP-glucuronosyltransferase assay was performed. Yeast cells expressing several isoforms of UDP-glucuronosyltransferase (UGT1A1, UGT1A6, UGT1A7, and UGT2B1) were incubated with 4-tert-octylphenol. In this test only the UGT2B1 isoform metabolized 4-tert-octylphenol with a V_{max} of 11 nmol/min/mg and K_m of 94 µM. Conversion of 4-tert-octylphenol was also measured *in vitro* in microsomes of liver, kidney, intestine and testis of rats as well as in human liver microsomes. Liver, rat and human, as well as rat intestine showed the highest conversion rate. V_{max} and k_m were determined to be 7.7, 3.8 and 2.75 nmol/min/mg or 57, 24 and 125 µM, respectively.

Degradation of 4-tert-octylphenol was determined using liver microsomes from untreated male and female Sprague-Dawley rats (Hamelin et al. 2009). Three concentrations of 4-tert-octylphenol (71, 213 and 533 ng/mg protein) were incubated together with microsomes for up to 60 min. After 15 min incubation, unchanged 4-tert-octylphenol was decreased by 98% (71 ng/mg), 91% (213 ng/mg), and 94% (533 ng/mg) in liver microsomes from male rats compared to initial concentrations, whereas in female rats, the reduction was of 50% (71 ng/mg), 26% (213 ng/mg), and 21% (533 ng/mg), respectively.

In silico studies

Hamelin et al. (2010) developed a physiologically based pharmacokinetic (PBPK) model for 4-tert-octylphenol to simulate the kinetics of 4-tert-octylphenol in male and female rats exposed to it. The compartments regarded were the liver, richly and poorly perfused tissues, fat and reproductive organs plus a subcutaneous space to reflect s.c. application. In the model it was assumed that the majority of 4-tert-octylphenol is bound to plasma proteins and the free fraction is about 0.1 %. For oral application blood concentrations of 53.3-271.2 ng/ml (males)

and 87.4- 449.7 ng/ml (females) for administration of 50-250 mg/kg bw were predicted. For a single s.c. dose of 125 mg/kg bw, 4 h after application 4-tert-octylphenol blood concentrations of 111.3 ng/ml (males) and 121.6 ng/ml (females) were predicted. Simulation of repeated exposure resulted in predicted blood concentrations of 59.8 and 150.6 ng/ml (male) and 98.2 and 248.5 ng/ml (female) (50 and 125 mg/kg bw) 4 h after the last dosing. The authors stated that the model fitted best with an assumption of a 45% bioavailability for oral and of 15 % for dermal administration. Based on blood flow and plasma:tissue partition the uptake of 4-tert-octylphenol in various tissues was described. The tissue concentrations were similar to those experimentally determined and were in the single-digit µg/g tissue range with females showing approximately 1.5-2-fold higher concentrations compared to male animals. Experimental results and model simulations varied between 20 and 40% with the model rather underestimating the actual blood and tissue concentrations in this study.

Conclusion

After oral application 4-tert-octylphenol is rapidly absorbed and quickly released into the blood. Within 10 min 4-tert-octylphenol is present in blood and C_{max} is reached between 20 min (male Wistar rats) and 2 h (Sprague Dawley rats) after administration. Doses of 50 to 250 mg/kg bw resulted in C_{max} of 40 to about 400 ng/ml blood. Repeated exposure to 4-tert-octylphenol of up to 125 mg/kg bw/d resulted in 2 to about 4-times higher blood concentrations in male and female animals than after single exposure. Indication for enterohepatic cycling was seen in some animals of one study, but not in two other studies. C_{max} varies within the several tests depending on the applied dose and the strain used. Also the bioavailability varies between the strains. In male Wistar rats and female Da/Han rats oral bioavailabilties of about 10 % were determined. In male and female Sprague Dawley rats bioavailabilities were about 35 and 55 %, respectively.

Toxicokinetic investigations with dermal application are not available. In an acute dermal toxicity study no systemic toxic effects were observed after application of 2000 mg/kg bw. Based on the water solubility, the partition coefficient of 4.12 and the molecular weight of 4-tert-octylphenol of 206 g/mol a high dermal bioavailability can be assumed. Single subcutaneous injection of 4-tert-octylphenol (125 mg/kg bw) resulted in C_{max} of 182 ng/ml in male and 209 ng/ml in female Sprague-Dawley rats at a T_{max} of6 or 8 h, respectively. Half lifes of 9.8 h and 29.6 h were calculated. Similar blood concentrations were found in female Donryu rats after 2 d s.c. injections of 4-tert-octylphenol (200 mg/kg bw). After repeated application over 14 d the blood concentrations in these animals were approximately 2-fold higher than after 2 days. Compared to oral administration the maximum concentrations are less high, and elimination is slower.

Oral doses of 50 and 200 mg/kg bw/d resulted in low 4-tert-octylphenol concentrations in lung and tissue of male Wistar rats (7-9 ng/g tissue). 4-tert-octylphenol was detected at concentrations of 43-87 ng/g tissue in muscle, kidney and liver and a concentration of 1285 ng/g in fat, but not in testes. In Sprague Dawley rats 4-tert-octylphenol could be detected in a dose-dependent manner at concentrations in the single-digit microgram range per gram tissue after administration of 25 to 125 mg/kg bw/d. Tissue concentrations were highest in liver and fat and also reaches reproductive organs such as uterus, ovaries, testes and the epididymis. After repeated doses no significant differences occurred between the tissue concentrations from single and repeated treatment indicating no bioaccumulation of 4-tert-octylphenol.

From experiments using rat liver perfusion or primary rat hepatocytes it can be concluded that 4-tert-octylphenol undergoes a rapid first pass metabolism by phase I and phase II enzymes in the liver. Detoxification pathways include hydroxylation, glucuronidation and sulphatation. Enzymes involved in phase II metabolism include rat and human UGT2B1 and human SULT

1E1 and 2A1, as shown in *in vitro* experiments. In an *in vitro* test with untreated rat liver microsomes up to 94 % of 4-tert-octylphenol was metabolized within 15 min. Further studies showed that 4-tert-octylphenol may have a direct inhibitory effect on cytochrome P450 activities, and can decrease protein levels of testosterone hydroxylating CYP activities in the liver, when rats were fed 4-tert-octylphenol. In a liver perfusion assay 38% of the applied 4-tert-octylphenol dose was directly excreted into the bile of Sprague Dawley rats as glucuronide.

In Sprague Dawley rats some gender differences were observed in terms of a higher oral bioavailability and an increased terminal half life of 4-tert-octylphenol in females compared to males after oral application. This leads to a slower degradation in vivo and is in line with an *in vitro* investigation that showed a slower degradation of 4-tert-octylphenol by liver microsomes of female SD rats compared to males.

4.2 Acute toxicity

Not assessed for the identification of this substance as SVHC in accordance with Article 57(f).

4.3 Irritation

Not assessed for the identification of this substance as SVHC in accordance with Article 57(f).

4.4 Corrosivity

Not assessed for the identification of this substance as SVHC in accordance with Article 57(f).

4.5 Sensitisation

Not assessed for the identification of this substance as SVHC in accordance with Article 57(f).

4.6 Repeated dose toxicity

4.6.1 Repeated dose toxicity: oral

The majority of studies reported in this chapter have not been performed according to standard toxicity test guidelines. For some of the non-publicly available regulatory studies that were considered in the according OECD SIDS Dossier, it was not possible to make the test reports available to the dossier submitter. Thus for these studies, the available information is directly imported from the OECD dossier.

Oral diet

3 months

In a well documented 3 month oral feeding study in BOR/WISW (SPF Cpb) rats toxicity of 4-tert-octylphenol after repeated exposure was investigated (Suberg et al. 1982). In this study doses of 30, 300, or 3000 ppm (corresponding to ≈2.3, 23, 230 mg/kg bw/d) 4-tert-octylphenol (purity 93.1 %) were fed daily to 20 male and 20 female animals/dose. No treatment related death occurred and no clinical signs were observed throughout the study. Food consumption was unaffected. Slightly increased water consumption was observed in

females receiving the highest 4-tert-octylphenol dose. The body weight gain was slightly decreased in male and female animals receiving 300 ppm and markedly decreased in animals receiving 3000 ppm 4-tert-octylphenol. Absolute organ weights were decreased in the highest dose group in male animals (thyroid, thymus, heart, lung, spleen; but not kidney, adrenals, testes or brain) and female animals (thymus, heart, lung, liver, spleen, kidney, adrenals; but not thyroid, ovaries or brain). Histopathological investigations were carried for organs of 5 male and 5 female animals each in the control and highest dose group no treatment related effects were observed.

Clinical chemistry parameters were investigated after one month and at the end of the study. Inorganic phosphate in blood plasma was significantly decreased from 300 ppm 4-tert-octylphenol upwards in female rats (1.22 vs. 1.72 mmol/l in controls) whereas in male rats phosphate was slightly increased (2.06 vs. 1.81 in control). Further effects in the highest dose group comprised increased urea and creatinine plasma concentrations in females (9.94 vs. 8.41 mmol/l in control and 58 vs. 52 µmol/l in control, respectively), increased potassium (5.1 vs. 4.3 mmol/l in control) and decreased calcium (2.67 vs. 2.83 mmol/l in control) in male animals. At 1 month, mean plasma thyroxin (T4) concentration was statistically significantly increased ($p \leq 0.01$) in the females of the 3000 ppm group (155 ± 94 vs. 67 ± 4 nmol/l in controls, respectively), however not at 3 months. Since this increase was attributed to two females and since no histopathological findings on the thyroid glands of animals from this group were observed, the finding of an elevated mean T4 plasma level was not considered to be of toxicological significance. In urine total protein was decreased after 1 and 3 month in male animals in the highest dose group. No changes were observed in enzymatic activities of alkaline phosphatase, glutamate dehydrogenase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and N-acetylglucosaminidase in blood or urine or in blood glucose, triglycerides, sodium, chloride and iron concentrations.

NOEL: 30 ppm (corresponding to 2.3 mg/kg bw/d)

LOEL: 300 ppm (corresponding to 23 mg/kg bw/d)

Oral drinking water

4 months

In a study on Fisher 344 rats effects of 4-tert-octylphenol on the reproductive system were investigated in adult (2 month old) males (Blake et al. 2004). Groups of 12 rats each received either tap water or water containing different concentrations of 4-tert-octylphenol ($1x10^{-5}$, $1x10^{-7}$, or $1x10^{-9}$ M). Water and food consumption were monitored and animals terminated after an exposure period of 4 months (110-125 days). The test was performed as two subsets. Subset 1 (n=6/group) was performed on intact males with animals terminated by decapitation. From this subset blood samples were taken for subsequent determination of LH, FSH, PRL and testosterone. Body weight and organ weights were taken from pituitary gland, left kidney, left and right testes, left epididymis, seminal vesicles, ventral prostate gland, and coagulating gland. The left testis was used for flow cytometric analyses of spermatogenic cells. Subset 2 animals (n=6/group) were castrated at the end of the 4 month exposure period and maintained on their respective drinking water regimen for an additional 3 weeks to assess effects of 4-tert-octylphenol on the castration-induced rise in gonadotropin secretion. At castration organ weights of the left and right testes and epididymides were taken and sperm numbers per gram testis and epididymal sperm count determined and epididymal sperm morphology examined. At final termination also blood samples were taken for subsequent determination of LH and FSH and organ weights of left kidney, spleen and pituitary gland recorded. Based on water consumption data the daily intake of 4-tert-octylphenol was calculated to approximately 0.020-0.035, 2.0-3, 5 and 200-350 µg/kg bw/day for the drinking water concentration of 10^{-9},

10^{-7} and 10^{-5} M. Mean water and food consumption did not differ between groups. Mean hematocrit, body and organ weight also did not differ between groups. In addition, organ to body weight ratios did not change. Similarly, mean serum LH, FSH, PRL and testosterone concentrations did not differ between groups. No effects were seen on total mixed germ cell yield, on flow cytometric distribution of spermatogenic cells or on testicular sperm concentration per g testis or per organ. Mean epididymal sperm head count per gram tissue was slightly decreased to ~700 Mio in comparison to controls (~800 Mio) at 10^{-5} M drinking water concentration. Mean percentage of sperm tail abnormalities (n=6 males/group) was slightly higher (~10-12 %) in treated groups as compared to the control group (~7 %). As expected, mean serum LH and FSH concentrations were higher in the castrated males compared to those of the intact males; however, there was no difference in mean gonadotropin concentrations between orchiectomised groups. Given that no positive control was included, no information on normal variability of epididymal sperm count and of sperm tail abnormalities from historical controls is provided and data derived from small animal numbers only, no conclusions can be drawn on the observations obtained from this study.

Oral gavage

28 days

Species/strain:	rat/Crj:CD (SD)
Sex:	6 male, 6 female/group
Route of administration:	oral (gavage)
Exposure period:	28 days
Frequency of treatment:	daily
Dose:	0, 15, 70, 300 mg/kg/day
Control group:	yes; vehicle
NOEL:	15 mg/kg/day
LOEL:	not available

Results:

Salivation was observed in the medium- and high-dose females and males after test substance administration. Body weight gain was reduced in the high-dose males. Water intake was increased in males and females of the high dose group. The test substance did not cause any consistent changes in food consumption and haematological parameters. For the high dose group, blood chemical analyses showed an increase in Na for females and males, a decrease in cholesterol and an increase in BUN[3] and triglyceride for females, and a decrease in albumin in females. A/G[4] ratios were lowered in the medium- and high- dose. Increased urine volume was evident in females and males at the high dose. Urinalysis showed decreases in specific gravity and in Na, Cl and K. Slight but statistically significant increases in kidney weights were found in the high-dose males and females and in liver weights in the high-dose females. The high-dose males and females showed greyish kidney patch as gross findings, and regeneration of renal tubules as microscopic findings. All changes attributable to the test substance disappeared or tended to recover after discontinuing the administration.

[3] Blood Urea Nitrogen

[4] A/G - Albumin/Globulin

Method:	Other: Guidelines for 28-day dose toxicity test of chemicals (Japan)
Year:	1994
GLP:	YES
Test substance:	p-tert-octylphenol, purity 98.24 %

cited from: OECD SIDS PHENOL, 4-(1,1,3,3-TETRAMETHYLBUTYL)- CAS No: 140-66-9 and

Chemical Investigation Promoting Committee, Toxicity Testing Reports of Environmental Chemicals, Vol.1, 1994;

29 days

Species/strain:	albino rat (Sprague-Dawley)
Sex:	5 male, 5 female/group
Route of Administration:	oral (gavage)
Exposure period:	29 days
Frequency of treatment:	daily
Post exp. observation period:	no
Dose:	0, 15, 150, 250 mg/kg/day
Control group:	Yes, concurrent vehicle (corn oil)
NOEL:	15 mg/kg/day
LOAEL:	150 mg/kg/day

Results:

A dose of 250 mg/kg caused the following effects:

- slightly higher food consumption in males and females
- markedly higher water consumption of male and female rats
- lower cholesterol-levels in female rats
- rel. liver and kidney weights were significantly higher in females
- minimal centrilobular hepatocyte enlargement in female rats
- interstitial inflammation in kidneys of males
- basophilic epithelium occasionally with mitotic figures in proximal convoluted tubules in male and female rats

A dose of 150 mg/kg led to the following symptoms:

- slightly higher food consumption in females
- higher water consumption of females
- lower cholesterol levels in female rats

- basophilic epithelium occasionally with mitotic figures in proximal convoluted tubules in male rats

Method: OECD 407; Year: 1994

GLP: Yes

cited from: OECD SIDS PHENOL, 4-(1,1,3,3-TETRAMETHYLBUTYL)- CAS No: 140-66-9

Reference:

Huntingdon Research Centre Ltd., Huntington, England: Twenty-Eight Day Oral Toxicity Study in the Rat; HRC Report no. SAZ 464/942419 (1994);

25 days

In a study comparing estrogenic activities of various environmental estrogens (Laws et al. 2000) intact adult female Long Evans rats (7-14/group) were treated orally (gavage) with 0, 50, 100 or 200 mg 4-tert-octylphenol/kg bw or 0.01 or 0.1mg ethinylestradiol/kg bw for a period of 25 days for cycling studies or dosed by subcutaneous injection for 25 days with 5 µg 17β-estradiol/kg bw. All animals were monitored for 3 weeks prior to treatment, and only those animals displaying consistent 4-to 5-day oestrous cycles were used in the study. Changes in vaginal epithelial cells were monitored by taking daily vaginal smears. If a female displayed an extended dioestrous smear for 7 or more days, blood samples were taken for a progesterone assay in order to further identify the underlying ovarian status (e.g., pseudopregnancy or anoestrous). 17β-estradiol and ethinylestradiol significantly reduced the number of 4- to 5-day cycles during the exposure period. Most animals in these treatment groups initially displayed an extended dioestrous vaginal smear. Many of these animals remained in dioestrous for 10-14 day, suggesting that they were pseudopregnant. Continued exposure for more than 18-20 days resulted in periods of extended oestrous in some animals. Oral exposure to 200 mg 4-tert-octylphenol/kg bw induced a similar response. The number of 4-to 5-day cycles in the animals of this treatment group was decreased to 2.2 $\pm$ 0.4 (statistically significantly reduced in comparison to the vehicle controls (4.8 $\pm$ 0.3, resp. 5.2 $\pm$ 0.2), the 50-mg treated (5.1 $\pm$ 0.3) or 100-mg treated (4.6 $\pm$ 0.5) groups). In the group exposed to 200 mg 4-tert-octylphenol/kg bw the number of days of dioestrous was statistically significantly increased.

30 days

In a study focusing on effects on testicular functions (Bian et al. 2006) groups of male Sprague Dawley rats (n=12 each) were given daily gavage administration of 0, 50, 150 or 450 mg 4-tert-octylphenol for 30 days. In the animals of the high dose group (450 mg/kg bw/d) body weight gain was suppressed (306.83 ± 19.15 g versus 325.08 ± 21.37 g in controls). Weights of testes, epididymis and prostate were statistically significantly lower ($p < 0.05$) in comparison to controls. Histopathological examinations of testes revealed alterations in rats administered 450 mg/kg bw/d with seminiferous tubules markedly reduced in size and disturbance of normal spermatogenic cell organization and total number of germ cells inside the tubules markedly reduced. Electronic micrographs of testicular cells revealed more intracellular vacuoles, lipofuscin and showed degeneration. Testicular sperm counts revealed statistically significant decreases ($p < 0.05$) of sperm head count and daily sperm production in rats treated with 450 mg/kg bw/d. Assessment of sperm motility from 7 males/group with

computer assisted Sperm analyzer revealed some minor changes for the 450 mg/kg bw/d dose group in LIN (% linearity) and VSL (straight line velocity) with other sperm motility parameters (BCF, VCL, STR, VAR[5]) unaffected. The evaluation of several testicular marker enzymes (ACP, ALP, LDH, γ-GT, G-6-PDH, SDH) revealed some minor changes ($p < 0.05$) in the testicular alkaline phosphatase activity (3.23 + 0.99 U/g versus 4.00 + 0.73 U/g in controls).

LOAEL/syst tox:	450 mg/kg bw/d (↓ body wt and repro organ wt)
NOAEL/ syst tox:	150 mg/kg bw/d
LOAEL/reproorgan tox:	450 mg/kg bw/d
NOAEL/reproorgan tox:	≥ 150 mg/kg bw/d
LOAEL/spermatotox:	450 mg/kg bw/d
LOEL/spermatotox:	≥ 150 mg/kg bw/d

1 month

In a study examining the strain-dependent differences in sensitivity of male rats towards 4-tert-octylphenol (Hossaini et al. 2003) groups of 7 week old male Wistar rats or of 7 week old male Fisher rats were allocated to the following schedule (n = 9-10): oral gavage treatment with vehicle, or with 400 mg /kg bw 4-tert-octylphenol, or s.c. injection of 40 µg/kg bw estradiol benzoate (used as positive control) every monday, wednesday and friday for 1 month. Since s.c. injections of 400 mg /kg bw 4-tert-octylphenol had induced abscesses at the injection sites of both strains, these animals were excluded from the study. Consequently, the 400 mg /kg bw 4-tert-octylphenol dose was administered by the oral route. At necropsy, terminal body weight, organ weights (liver kidneys, adrenal glands, testes, left epididymis, ventral prostate and levator ani/bulbocavernosus muscle), epididymal sperm counts (with CASA), and hormone levels (LH, FSH, testosterone, prolactin, inhibin-B) from trunk blood were determined and histopathology of the testes including occurrence of apoptosis was performed. Terminal body weights of the 4-tert-octylphenol treated males were comparable to controls in both strains, whereas body weights of the estradiol benzoate treated males were significantly reduced in both strains. In males treated with estradiol benzoate (positive control) relative organ weights of liver, kidneys and adrenals were increased. Relative organ weights of testes, left epididymis, ventral prostate and levator ani/bulbo-cavernosus muscle were significantly reduced, with the effect on testes organ weight more pronounced in the Wistar strain (reduced to 73 % of the controls) than in the Fisher strain (reduced to 31 % of the controls). In males treated with 4-tert-octylphenol relative organ weights of liver and kidneys were increased in both strains. Relative organ weight of the adrenals were statistically significantly increased and relative organ weights of seminal vesicles and the levator ani/bulbocavernosus muscle were statistically significantly reduced in the Fisher strain, whereas in the Wistar strain only the relative weight of the levator ani/bulbocavernosus muscle was decreased. No weight changes in either strain were observed for testes, epididymis or ventral prostate weight. In males of both strains treated with estradiol benzoate (positive control) almost no sperm cells were observed in the cauda epididymis, whereas no differences were observed in the number of sperm/g cauda epididymis in males treated with 4-tert-octylphenol of both strains. In males of both strains treated with estradiol benzoate (positive control) serum testosterone levels were reduced (to 0.02 and 0.004% of control

[5] beat-cross frequency, curvilinear velocity, straightness, average path velocity

levels), whereas no changes were observed for males treated with 4-tert-octylphenol. In males of both strains treated with estradiol benzoate (positive control) serum LH and FSH levels were reduced (to less than 50% of control levels), whereas no changes were observed for males treated with 4-tert-octylphenol. In males treated with estradiol benzoate (positive control) serum prolactin levels were increased (3 and 5 fold) as was observed in males treated with 4-tert-octylphenol (to 140 - 150%). In males of both strains treated with estradiol benzoate serum inhibin-B levels were reduced (to about 60 %of control levels), whereas no changes were observed for males treated with 4-tert-octylphenol. No effect on testes histopathology was observed in males treated with 4-tert-octylphenol of either strain in comparison to those of the positive control, where diameters of seminiferous tubules were decreased and spermatid retention and disorganized and atrophic seminiferous epithelium including germ cell apoptosis were observed.

LOAEL/syst tox:	400 mg/kg bw/d (increased organ weights)
NOAEL/repro organ & spermatotox:	400 mg/kg bw/d
NOAEL/endocrine (hormonal) effects:	400 mg/kg bw/d

35-41 days

In a study on Sprague Dawley rats (Sahambi et al. 2010) groups (n=7) of 35 day old females were given daily gavage administration of saline (negative control), propylene glycol (vehicle control), or 4-tert-octylphenol at dosages of 25, 50, or 125 mg/kg bw for 35-41 days (to sacrifice all at the same stage of oestrous cycle). Body weights were taken and oestrous cycle monitored. Upon sacrifice, serum was collected for determination of estradiol concentrations and organs were taken for determination of ovaries, uterus, liver and kidneys. One ovary and part of the uterus were processed for histopathological evaluations, while the rest of the uterus and parts of the liver were taken and processed for exploratory gene expression analysis. No significant differences were seen on body weights and on organ weights across groups. There were no significant differences in serum estradiol concentrations and all animals continued to cycle throughout the monitoring period. Animals of the 25-mg and 125-mg groups but not of the 50-mg group had a slight decrease in the number of cycles during the monitoring period. Ovarian histopathology did not reveal any morphological alterations or differences in numbers of ovarian follicles of any stage. Uterine histopathology and morphometry did not reveal any differences across groups. Gene expression analysis from microarray analysis and real-time PCR did not reveal any consistent changes.

60 days

Because of the discrepancies in effects, and differences in doses, route of exposure, and exposure period, a further study had been performed to determine the effects of chronic in vivo exposure to various doses of 4-tert-octylphenol on male reproductive parameters (Cyr and Gregory 2006**;** Gregory et al. 2009). Five groups of 10 male adult Sprague Dawley rats each were administered saline (control), vehicle alone (propylene glycol), 25, 50, or 125 mg 4-tert-octylphenol /kg/d dissolved in propylene glycol by gavage for 60 days, representing approximately 1.5 cycles of spermatogenesis. Animals were terminated 24 hours following the last dose, serum collected and various organs (testes, epididymis, ventral prostate, seminal vesicles, kidneys, liver) taken and weighed and submitted to histopathological evaluations, evaluation of epididymal sperm count and sperm motility, gene expression profiles. In the 4-tert-octylphenol treated rats there was a tendency toward decreased body weight, relative to controls, with a statistically significant decrease in mean body weight of 444.4 ± 11.8 g versus

535.6 ± 9.9 g in controls at the highest dose (125 mg/kg/day). There were no effects on organ weights of testes, epididymis, ventral prostate, and seminal vesicles between experimental groups. Histopathological examination of the seminiferous tubules and interstitium of the testes did not reveal any apparent morphological changes. Likewise, observations of the epididymal epithelium indicated normal morphology, and no morphological aberrations were observed in efferent ducts. Analysis of caput epididymal sperm motility did not reveal differences in any of the motility parameters between groups. Analysis of cauda epididymal sperm motility parameters revealed a 13 % lower ($p \leq 0.5$) total percent motility relative to untreated controls in the intermediate dose group (50 mg/kg/d) and correspondingly a higher percentage (28 %) of static cells ($p \leq 0.5$) relative to untreated controls (15.7 %). However, any dose-relationship was not established as such changes were not observed for the lower, respectively for the higher dose group. There were no statistically significant differences in cauda epididymal sperm count at any dose group. Gene expression profiles in the testes of vehicle and high dose treated groups (125 mg/kg/d) revealed that of the 20 245 rat genes on the arrays, 512 genes were differentially expressed between vehicle controls and the 4-tert-octylphenol treated group. Of these 512 differentially expressed genes, 16 exhibited a twofold or greater differential expression, although the effects were not statistically significantly different. Real-time PCR was performed on arbitrarily selected genes to verify changes in gene expression, however, none of the selected genes showed a statistically significant difference between the vehicle control and the treated rats.

In summary, results indicated that body weight in male adult rats was significantly decreased by 4-tert-octylphenol treatment over the 60-d exposure period with a trend towards decreasing body weight in all 4-tert-octylphenol treated groups. There were, however, no effects on the weights of the testes, epididymides, ventral prostate, and seminal vesicles or on histopathology of testes and epididymides. Tissue concentrations of 4-tert-octylphenol in the testes after the 60-d exposure period were below levels of detection in the 0-, 25-, and 50-mg/kg groups and relatively low (29.3 ± 16.1 ng/mg tissue) in the 125-mg/kg group. Tissue concentrations in the epididymides after the 60-d exposure period were below levels of detection in the 0-, 25-mg/kg groups, and 18.4 ± 5.9 ng/mg, respectively 56.3 ± 43.3 ng/mg in the 50- and 125-mg/kg groups. Analysis of caudal sperm data showed that total percent motility were statistically significantly lower at the intermediate dose (50 mg/kg/d) with a corresponding increase in percent static cells suggesting minimal if at all effects of 4-tert-octylphenol.

LOAEL/syst tox: 125 mg/kg bw/d (↓ body weight)

NOAEL/syst tox: 50 mg/kg bw/d

NOAEL/reproorgan & spermatotoxicity: > 125 mg/kg bw/d

Table 13: Compilation of studies with repeat oral administration

Species/sex (age) /n	Duration	Dosing	Results	Reference
Oral Gavage				
Sprague Dawley rat m+f n=6/group	28 days	0, 15, 70, 300 mg/kg bw/d	300 mg: ↓ bw gain (m); ↑ water intake (f+m); ↑ kidney weight (m+f) ↑ liver weight (f) NOEL: 15 mg/kg bw/d	OECD SIDS, Japanese Study

Sprague Dawley rat m+f n=5/group	29 days	0 15 150 250 mg/kg bw/d	<u>> 150 mg</u>: slightly ↑ food intake (f);↑ water intake (f); ↓ cholesterol levels (f); basophilic epithelium occasionally with mitotic figures in proximal convoluted tubules (m) <u>250 mg</u>: ↑ food and water intake (f+m), ↓ cholesterol levels (f), ↑ rel. liver and kidney weights (f), minimal centrolobular hepatocyte enlargement (f), interstitial inflammation in kidneys (m), basophilic epithelium occasionally with mitotic figures in proximal convoluted tubules (m) NOEL: 15 mg/kg bw/d LOAEL: 150 mg/kg/d	OECD SIDS, Huntingdon Study
Fisher 344 rat CrJ:Donryu rat f (11 week old)	14 days (preceding dose finding study)	100 200 mg/kg be/d	<u>up to 200 mg:</u> oestrous cyclicity and uterine weight not affected	Yoshida et al. 2000
Sprague Dawley rat m (5 week old) n=10/group	21 days (mechanistic study for spermatotoxicity	10 50 250 mg/kg bw/d	<u>up to 250 mg:</u> testes histopathology unaffected	Baek et al. 2007
Long Evans rat (adult females) n=7-14/group	25 days	50 100 200 mg/kg bw/d	<u>200 mg:</u> reduced number of 4 to 5-day cycles in cycling females with relative increase in days of dioestrous	Laws et al. 2000

Species/sex (age) /n	**Duration**	**Dosing**	**<u>Results</u>**	**Reference**
Sprague Dawley rat m (9 week old) n=12/group	30 days	0 50 150 450 mg/kg bw/d	<u>450 mg</u>: ↓ bw gain; ↓ testes, epididymis and prostate organ weight; histopathological changes in testes (with reduction in number of germ cells), ↓ testicular sperm count LOAEL/syst. tox.: 450 mg/kg/d NOAEL/syst. tox.: 150 mg/kg/d LOAEL/reproductive organ toxicity& spermatotoxicity: 450 mg/kg/d NOAEL/reproductive organ toxicity & spermatotoxicity: 150 mg/kg/d	Bian et al. 2006

Wistar rat Fisher rat m (7 week old) n=9-10/group	1 month - with three administrations per week	400 mg/kg bw/d (positive control: 40 µg estradiol benzoate/kg bw s.c.)	400 mg: ↑ relative organ wts of livers and kidneys in both strains, testis, epididymis and ventral prostate wt unaffected in both strains; ↑ relative organ weights of adrenals and ↓ relative organ weights of seminal vesicles and levator ani/bulbocavernosus in the Fisher strain; ↓ relative organ weights of levator ani/bulbo-cavernosus in the Wistar strain; caudal epididymal sperm unaffected in both strains ↑ in serum prolactin levels (to 140-150% of the controls), serum testosterone, LH or FSH levels unaffected	Hossaini et al. 2003
Sprague Dawley rat f (35 days old) n=7/group	35-41 days	0 25 50 125 mg/kg bw/d	125 mg: no effects on body and organ wts; no effects on serum estradiol and on cycling; no effects on ovarian histopathology/follicles; no effects on uterine histopathology and morphometry NOAEL/syst. tox.: > 125 mg/kg/d NOAEL/reproductive toxicity: > 125 mg/kg/d	Sahambi et al. 2010

Species/sex (age) /n	**Duration**	**Dosing**	**Results**	**Reference**
Sprague Dawley rat m n=10/group	60 days	0, 25 50 125 mg/kg bw/d	125 mg: ↓ mean body weight; no effects on reproductive organ weight and histopathology, no effects on epididymal sperm count; LOAEL/syst tox: 125 mg/kg/d NOAEL/syst tox: 50 mg/kg/d NOAEL/repro organ tox: ≥ 125 mg/kg/d	Gregory et al. 2009; Cyr and Gregory 2006
Oral diet				
Wistar rat m+f n=20/sex/group	3 months	0 30 300 3000 ppm equivalent to 2.3 23 230 mg/kg bw/d	≥ 300 ppm: ↓ weight gain 3000 ppm: ↓ hematocrit; ↑ thyroxin in females; no histopathological findings LOEL: 300 ppm NOEL: 30 ppm	Suberg et al. 1982
Oral drinking water				

Fisher 344 rat m (2 month old) n=6/group	4 months	10^{-9}M 10^{-7}M 10^{-5}M	10^{-5}M: ↓ mean epididymal sperm head count (700×10^6 versus 800×10^6) in controls	Blake et al. 2004

Subcutaneous injection

2 weeks

In a study on female Sprague Dawley rats (Blake and Ashiru 1997) effects of 4-tert-octylphenol administered to10 week old females on oestrous cyclicity were investigated. Rats showing at least two regular 4-day oestrous cycles were used. 6 females were injected s.c. corn oil (vehicle control), 6 animals with 20 mg and 21 animals with 40 mg 4-tert-octylphenol. Injections were given three times weekly for 2 weeks. 16/21 rats of the 40 mg-group entered persistent oestrous during two weeks of treatment. Five of these 16 rats continued the treatment schedule for an additional three weeks. Vaginal smears were prepared and examined daily throughout the experiment. In a further set of females the effects of 4-tert-octylphenol on ovulation during proestrus were investigated. Rats showing at least two regular 4-day oestrous cycles were used on proestrus. One group of control rats was injected i.p. with sodium pentobarbital (35 mg/kg bw) at 14:00. Ovulation was blocked in 5 of 5 rats indicating that sufficient LH was not released prior to 14:00 to induce ovulation. A total of 5 rats were injected s.c. with corn oil vehicle or with 40 mg 4-tert-octylphenol in corn oil at 13:00, 14:30, and 16:00 hr. Another 5 rats were injected s.c. with 100 mg 4-tert-octylphenol in 30 % ethanol at 13:00, 14:30, and 16:00 hr. Multiple injections of 4-tert-octylphenol were given, and 4-tert-octylphenol administered in two different vehicles in attempts to uncover any acute effect that 4-tert-octylphenol might have on ovulation. The occurrence of ovulation was determined between 08:00 and 10:00 hr the next morning. 4-tert-octylphenol treatment induced persistent oestrus within 3 days in 2 of the 6 females of the 20-mg group and in 16 of the 21 females of the 40-mg group. The remaining females had 4-or 5-day oestrous cycles during the 2-week injection period. Both, rats injected with corn oil or with 4-tert-octylphenol ovulated, and there were no differences between groups with respect to number of ova shed.

28 days

In a study on female Fisher 344 (F344) rats and female Crj:Donryu rats (Yoshida et al. 2000) effects of 4-tert-octylphenol were investigated using 11 week old healthy and normally cycling animals. Dose and route of administration were determined by a preliminary 14-day repeated toxicity study by gavage or s.c. treatment, in which animals of both rat strains were given 4-tert-octylphenol at 100 and 200 mg/kg bw. The gavage treatment at these doses did not affect oestrous cyclicity or uterine weights, whereas s.c. application resulted in persistent oestrus within several days after the start of administration in both strains. Thus, the subcutaneous route was selected to minimize the metabolism during first pass, because 4-tert-octylphenol is known to be metabolised via the liver, and daily doses for the 28 days study were set at 12.5, 25, 50 or 100 mg/kg bw. Twelve animals were assigned to the vehicle control (DMSO) group and 10 animals each to treatment groups. Seven days after the first treatment half of the animals (n=5) in each 4-tert-octylphenol treated group were sacrificed and examined for effects at an early stage. On the day after the last treatment, all surviving animals in the treated groups were sacrificed, and in the control group two to four animals were sacrificed at each oestrous cycle stage. Blood samples were taken for determination of serum concentrations of 17ß-estradiol (E2) and progesterone. Reproductive organs, livers, kidneys, lungs, spleen, adrenals and pituitary were taken for histopathological evaluation. The groups given 100 mg/kg bw in both strains showed significant body weight suppression throughout the treatment period. All animals of the 4-tert-octylphenol treated groups showed

scab or abscess formation at their treatment sites, the severity being dose- and duration-dependent. In the 100 mg/kg bw dose groups normal regular cyclicity was disrupted in rats of both strains within 7 days after beginning of the treatment changing to persistent oestrus. In the 50 mg/kg bw dose groups, cyclicity was normal for the first 7 days, whereas after 7 days abnormal cycling was observed more often. In the 12.5 and 25 mg/kg dose groups regular cyclicity was observed throughout the study. Relative organ weights of the spleen were dose- and duration-dependently significantly increased in all treated groups of both strains due to extramedullary haematopoiesis. Absolute organ weights of the liver and relative organ weights of the kidney were dose- and duration dependently significantly increased in F344 rats (without treatment-related microscopic changes) but not in the Donryu rats. Ovarian weights after treatment with 100 mg/kg for 28 days was significantly decreased in F344 rats (but not in Donryu rats) and histologically diagnosed as atrophic with cystic follicles and absent or a few atrophic corpora lutea in all F344 rats and one of the Donryu rats. Uterine morphology in persistent oestrous rats of both strains deviated from the normal for each oestrous stage of the cycling rats, and proliferation in the endometrium was slightly increased. In the other organs, such as lung, adrenals or pituitary, no histological differences were evident between controls and treated groups. E2 serum concentrations in treated animals with persistent oestrous or animals with abnormal cycling (of the $\geq$ 50 mg/kg bw treatment groups) showed a tendency for decline with markedly low values in the 100mg /kg groups of F344 but not Donryu rats. There were no major differences between the two rat strains in serum 4-tert-octylphenol concentrations derived from effective s.c. dosages ($\geq$ 50 mg/kg bw) indicating that certain 4-tert-octylphenol levels in serum might be necessary to appear as estrogenic action.

LOAEL/systemic toxicity:	12.5 mg/kg bw (organ wts/histopath [spleen], local effects)
LOAEL/oestrous cyclicity:	50 mg/kg bw (vaginal cytology)
NOAEL/oestrous cyclicity:	25 mg/kg bw

4 weeks

In a study on Fisher rats (Kim S.-K. et al. 2004) male animals at the age of 4 weeks (n=5/group) received s.c. injections of olive oil (vehicle control), 20, 40, or 80 mg 4-tert-octylphenol, or 0.8 µg ß-estradiol 17-valerate (EV, positive control) thrice weekly for a month and were terminated three days after the last injection and body weights and organ weights of testes, epididymis and seminal vesicles recorded and blood taken for determination of serum LH and testosterone concentrations. Length and widths of testes were measured and volumes of testes calculated. Testes were subjected to histopathological evaluations including evaluation of germ cell apoptosis and analyses of the expression of bcl-2, bcl-xl, and bax mRNA. Terminal Body weights were significantly reduced in comparison to the vehicle control in the EV treated and in all 4-tert-octylphenol treated groups. Testis weights and volumes were also were significantly reduced in comparison to the vehicle control in the EV treated and in all 4-tert-octylphenol treated groups with a dose-dependent trend. Marked decreases were also seen for epididymis and seminal vesicle weights in rats treated with EV and in all three doses of 4-tert-octylphenol. Serum testosterone concentrations were markedly reduced to about 60 % of the vehicle controls in EV and in all 4-tert-octylphenol treated groups. Serum LH concentrations were slightly reduced in the EV group, and significantly elevated in rats treated with 20 and 40 mg 4-tert-octylphenol, however, unchanged in comparison to the control in the 80-mg group. Seminiferous tubules in the EV and in all 4-tert-octylphenol treated groups were markedly reduced in size and showed disruption of normal spermatogenic cell organization. The total number of germ cells inside the tubules was dramatically decreased and there were no mature spermatozoa or late stage- developing

spermatids. An apoptotic index (percentage of seminiferous tubules containing apoptotic germ cells x number of apoptotic germ cells per tubule) was about 8 fold increased in rats treated with EV compared to controls and about 2-3 fold increased in rats treated with 4-tert-octylphenol. The expression of bcl-xl mRNA was significantly decreased in rats treated with 40 and 80 mg of 4-tert-octylphenol and EV, whereas there was no change between groups for the expression of bax mRNA.

1 or 2 months

In a study on male Fisher rats (Blake & Boockfor 1997; Boockfor & Blake, 1997) animals were injected with 0.2 ml corn oil containing 20 or 80 mg 4-tert-octylphenol for a period of 25-28 days ("1 month") or 54-61 days ("2 months"). There was a second series of identically treated rats with a 1-mo exposure period of 30-37 days and a 2-mo exposure period of 56-67 days. Groups of 6 rats were treated for each of the 1-month trials, whereas groups of 5 rats were treated for each of the 2-month trials. Injections were given trice weekly at different sites in the nape or inguinal region. It was reported that the injections of 4-tert-octylphenol caused reactions at the injection sites with temporary (several days) swelling and hardening of the area around each injection site and a mild skin necrosis that disappeared with the swelling. Rats from the first series of 1-mo and 2-mo exposures were decapitated at the end of the treatment period 1-3 days after the last injection, whereas the rats from the second series of 1-mo and 2-mo exposures were left untreated for 1-3 days, subsequently orchiectomised then, a cardiac blood sample taken 24 hours after orchidectomy and finally terminated three weeks after orchidectomy.

Effects following 4-tert-octylphenol treatment were studied on (i) body weight gain, food consumption, organ weights (spleen, kidney, pituitary gland) and hematocrit, on (ii) testes and male accessory sex organ weights (seminal vesicles, ventral prostate, coagulating glands) and histology, testicular sperm count and epididymal sperm concentration and sperm morphology, as well as on (iii) serum and anterior pituitary gland (APG) concentrations of LH, FSH, and prolactin (PRL) and serum testosterone concentrations.

In animals of the 80-mg exposure groups the final body weights were 12-18% lower ($p<0.05$) after 1-mo of exposure and 24-28% lower after 2-mo of exposure in comparison to their vehicle controls. Lower body weights were also seen in the 20-mg exposure group after 2-mo of exposure. It is reported that the rats administered the high dosage (80-mg) did no longer gain weight beyond study day 10, respectively study day 17, during the 1-mo, respectively the 2-mo exposure period. Food intake did not vary initially (days 0-3) but was about 13-22 % lower at later stages. Rats treated with 80-mg had significantly higher spleen weights in the 1- and 2-mo studies with an increase of about 65 % in the animals of the 2-mo study. The mean hematocrit (%) of rats treated with 80-mg were significantly ($p< 0.05$) lower in the 1-mo (45.3 ± 0.5 versus 48.6 ± 0.4) and in the 2-mo (4.3 ± 0.9 versus 46.7 ± 1.0) in comparison to their vehicle controls. Further, animals of the 80-mg exposure group had decreased absolute and relative reproductive organ weights and reductions in organ size that were more pronounced in the 2- mo trial. Treatment with 20-mg for 1-mo or 2-mo and treatment with 80-mg for 1-mo had no observable effects on the histology of the reproductive tract, whereas in rats administered 80-mg for 2-mo sizes, weights, and histological structures of the testes, epididymis, ventral prostate glands, seminal vesicles and coagulating glands had been markedly altered. Testicular histopathology revealed seminiferous tubules to be markedly reduced in size with irregular epithelial organisation and absence of mature spermatozoa or other steps of germ cell development. Testicular sperm count was reduced in animals of the 20-mg group after 2-mo exposure and nearly absent in the animals treated with 80-mg. Epididymal sperm count was unaffected after 1-mo and 2-mo exposure in the 20-mg group

animals, but was largely decreased in the 80-mg group animals after 1-mo exposure and almost completely eliminated when administered for 2-mo. An increase in the incidence of sperm head morphological abnormalities (pin heads, detached heads, absence of hooks) was already seen at the low dosage (20-mg) after 1-mo exposure period. APG and serum LH and FSH concentrations were decreased and APG and serum PRL concentrations were increased in animals of the 80-mg groups. Mean serum testosterone concentrations were lower in rats treated with 80-mg for 1-mo or for 2-mo. According to the authors, the pattern of effects observed in the males administered 80 mg 4-tert-octylphenol very much resembled those observed in concomitant groups of males that had been similarly treated with 8 µg Estradiol valerate.

5 weeks

In a study on Wistar rats (Herath el al. 2004) male animals at the age of 50 days (7 weeks) received daily s.c. injections of either DMSO (vehicle control) (n=8) or 3 mg/kg bw 4-tert-octylphenol (n=11). After 2 weeks of treatment blood was collected before and after LH-releasing hormone (LHRH) injection to the treated animals to investigate effects on pituitary and gonadal responses (plasma LH, testosterone and progesterone levels). After 5 weeks of treatment animals were terminated, final body weights taken and organ weights determined of testes, seminal vesicles, right epididymis and ventral prostate. Epididymal sperm count and sperm motility was measured and plasma levels of LH, progesterone, testosterone, immunoreactive inhibin (ir-inhibin) and insulin-like growth factor (IGF-I) determined. After 5 weeks of treatment, no differences were seen between 4-tert-octylphenol treated and control groups for body and organ weights, except slightly higher weight of ventral prostate ($p \leq 0.05$) in the 4-tert-octylphenol treated group (0.51 ± 0.03 g versus 0.42 ± 0.03 in controls). No differences were observed between 4-tert-octylphenol treated and control groups for terminal plasma concentrations of LH, whereas testosterone levels were reduced and progesterone and IG-F levels were elevated in 4-tert-octylphenol treated animals when compared to the controls. Epididymal sperm head counts were slightly reduced ($p \leq 0.05$) in 4-tert-octylphenol treated males (~ 160×10^6 versus ~ 190×10^6 in controls) with no effects on sperm motility parameters. Basal plasma concentrations of LH in 4-tert-octylphenol males after 2 weeks of treatment were lower in comparison to DMSO controls, and responded to LHRH stimulation with a relative increase in comparison to the control. Basal plasma testosterone concentrations after 2 weeks of treatment did not differ between groups, and was elevated after LHRH stimulation in DMSO controls but unchanged in 4-tert-octylphenol males. Basal plasma concentrations of progesterone in 4-tert-octylphenol males after 2 weeks of treatment were higher in comparison to DMSO controls, and responded to LHRH stimulation with a relative decrease in comparison to the control. Any dose-relationship of the observed effects was not established as only one dose had been tested.

Intraperitoneal injection

5 days

In a study on ICR mice (Kim S.-K. et al. 2007) effects of 4-tert-octylphenol on the expression of steroidogenic enzymes and on testosterone production were investigated on two different developmental stages. In one set juvenile 15-day-old males (n=5/group) were untreated (control), or injected intraperitoneally corn oil (vehicle control), 2 or 20 mg/kg bw 4-tert-octylphenol on five consecutive days. In the other set adult 8-week-old males (n=5/group) were injected intraperitoneally corn oil (vehicle control), 2, 20 or 200 mg/kg bw 4-tert-octylphenol, or 2 µg ß-estradiol 17-valerate (EV, positive control) on five consecutive days. Animals were terminated 2 days after the final injection and body weights and organ weights

of spleen and testes recorded and blood taken for determination of serum testosterone concentrations. Testes were subjected to histopathological evaluations including evaluation of germ cell apoptosis and analysis of testicular gene expression. Terminal body weight was significantly reduced in the juvenile animals exposed to 20 mg/kg bw 4-tert-octylphenol. Testis organ weight was significantly reduced in the juvenile animals exposed to 2 and 20 mg/kg bw 4-tert-octylphenol. Body and organ weights were unaffected in the adult animals in either treatment groups. Testes histopathology of the juvenile animals from the 20mg/kg bw dose group showed slight reduction in the lumen formation of seminiferous tubules with an increase in number of pyknotic germ cells and the overall number of germ cells inside the tubules markedly reduced. The serum testosterone concentration in the animals exposed to 20 mg/kg bw 4-tert-octylphenol during the juvenile stage was reduced to 30 % of the value observed in the control group, while in contrast to this, no significant changes were observed in the testosterone concentrations of adult mice exposed to EV or to 4-tert-octylphenol. In addition mRNAs for steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (P450scc), and 17α-hydroxylase/C_{17-20} lyase (P45017α) were down-regulated in testes of the juveniles after exposure to 4-tert-octylphenol, whereas mRNA expression of aromatase was unchanged. Animals exposed to EV or to 4-tert-octylphenol at the adult stage showed no significant changes in the gene expression of steroidogenic enzymes. P450scc was mainly detected in interstitial Leydig cells by immunohistochemical staining, and a slightly reduced expression of P450scc protein was observed in the testis of juveniles exposed to 20 mg/kg bw 4-tert-octylphenol. Histological staining for lipids with oil red O revealed a significantly lower number of positive Leydig cells in juvenile mice exposed to 20 mg/kg bw 4-tert-octylphenol with the size and number of lipid vacuoles in Leydig cells also reduced compared to those of the control testis. In summary, it was shown that juvenile exposure to 4-tert-octylphenol inhibits steroidogenesis by decreasing the expression of steroidogenic enzymes in the testis, hence resulting in decreased testosterone synthesis. Diminished lipid content in Leydig cells together with reduced transcriptional expression of the cholesterol transport gene, StAR, also support altered cholesterol metabolism and/or transport as a potential mechanism for decreased testosterone production following exposure to 4-tert-octylphenol.

Table 14: Compilation of studies with s.c. and i.p. administration

Species/sex/n	Duration	Dosing	Results	Reference
Subcutaneous injection				
Fisher 344 rat Crj:Donryu rat f (11 week old) n=12 (control) n=10/dose group	14 days (preceding dose finding study)	100, 200 mg/kg/d	<u>> 100 mg</u>: persistent oestrus within several days	Yoshida et al. 2000
Sprague Dawley rat f (10 week old) n=6 (control) n=6 (20-mg) n=21 (40-mg)	2 weeks - with three administrations per week	20, 40 mg/kg bw	<u>40 mg</u>: persistent oestrus within 2 weeks in 16/21 animals <u>20 mg</u>: persistent oestrus within 3 d in 2/6 animals; no effect on ovulation during proestrus by pre-treatment with 4-tert-	Blake and Ashiru, 1997

			octylphenol	
Fisher 344 rat Crj:Donryu rat f (11 week old) n=12 (control) n=10/dose group	28 days	12.5, 25, 50, 100 mg/kg bw/d	<u>in all 4-tert-octylphenol treated groups</u>: dose- and duration-dependent scab or abscess formation at the injection sites, spleen weight ↑ dose- and duration-dependently in all 4-tert-octylphenol treated groups due to extramedullary haematopoiesis, ↑ liver and kidney weight (F344) <u>100 mg</u>: ↓ body weight, regular cyclicity within 7 days changed to persistent oestrous, ↓ ovary weight (F344) with histopathological changes, ↓ serum estradiol (F344) <u>50 mg</u>: after a latency period of 7 days abnormal cycling observed LOAEL/syst tox: 12.5 mg/kg bw/d LOAEL/cyclicity: 50 mg/kg bw NOAEL/cyclicity: 25 mg/kg bw	Yoshida et al. 2000

Species/sex/n	**Duration**	**Dosing**	**Results**	**Reference**
Fisher rat m (4 week old) n=5/dose group	4 weeks - with three administrations per week	20, 40, 80 mg/kg bw positive control: 0.8 µg 17β-estradiol valerate (17β-EV)	in all 4-tert-octylphenol treated groups (and 17β-EV): terminal body weights ↓, epididymis and seminal vesicle weight ↓, histopathological changes in testes (with reduction in number of germ cells), serum testosterone concentrations ↓ 20, 40 mg/kg bw (and 17β-EV): LH serum concentrations ↑ LH unchanged at 80 mg/kg bw 40, 80 mg/kg bw (and 17β-EV): expression of bcl-xl mRNA ↓	Kim S.-K. et al. 2004
Fisher rat m (11 week old) n=5-6/group	25-28 days (1 month) 54-61 days (2 month) with three administrations per week	20, 80 mg/kg bw	in all 4-tert-octylphenol treated groups: local reactions with temporary swelling and hardening and mild skin necrosis 80 mg/kg bw: ↓ body weight gain (1+2 month), ↑ spleen weight (1+2 month), ↓ hematocrit (1+2 month), ↓ reproductive organ weight and size (1+2 month) with histopathological changes (2 month), testicular sperm nearly absent (2 month), epididymal sperm almost completely eliminated (2 month) serum concentrations of LH and FSH ↓ and of prolactin ↑; serum concentrations of testosterone ↓ anterior pituitary gland concentrations of LH and FSH ↓ and of prolactin ↑ 20 mg/ kg bw: ↓ body weight gain (2 month), ↓ testicular sperm count (2 month), changes in sperm morphology (1 month)	Blake and Boockfor, 1997, Boockfor and Blake, 1997

Species/sex/n	Duration	Dosing	Results	Reference
Wistar rat m (7 week old) n=8 (control, DMSO) n=11	5 weeks	single dose study 3 mg/kg bw	in comparison to DMSO controls: slightly ↑ ventral prostate weight, slightly ↓ epididymal sperm count serum LH unchanged, serum testosterone ↓, basal serum progesterone and IGF ↑ after 2 weeks on treatment: basal plasma testosterone concentrations did not differ between groups, testosterone concentrations elevated after LHRH stimulation in DMSO controls but unchanged in 4-tert-octylphenol treated males basal progesterone plasma concentrations elevated in 4-tert-octylphenol treated males after LHRH stimulation relative decrease in progesterone concentrations in 4-tert-octylphenol treated males comparison to DMSO controls	Herath et al. 2004
Intraperitoneal injection				
ICR mouse m (8 week old) n=5/group	5 days	2, 20, 200 mg/kg bw positive control: 2 µg 17β-estradiol valerate (17β-EV)	4-tert-octylphenol treated and 17β-EV: body and organ weights unaffected, no change in gene expression of steroidogenic enzymes no change in serum testosterone concentrations	Kim S.-K. et al. 2007

Mechanistic studies - spermatotoxicity/testicular toxicity

Yon et al. (2007) To investigate whether 4-tert-octylphenol affects spermatogenesis through an SGP-2 dependent mechanism effects on the expression of *Sulphated Glycoprotein-2 (SGP-2)* mRNA, a biomarker for spermatogenesis in Sertoli cells of rat testes, daily oral doses of 10, 50, and 250 mg 4-tert-octylphenol/kg bw were administered to 5 week-old male Sprague-Dawley rats (n=10/group) for three weeks. Testicular expression of SGP-2 mRNA was analyzed using reverse transcription-polymerase chain reaction. SGP-2 mRNA expression was decreased ($p<0.05$) in testes at all doses (15-40 % of the SGP-2 mRNA expression as compared to the vehicle control group).

Baek et al. (2007) To investigate whether 4-tert-octylphenol affects spermatogenesis via abnormal enhancement of phospholipid hydroxyperoxidase glutathione peroxidase (PHGPx)

expression in testes, an antioxidative selenoprotein, which interacts directly with peroxidised phospholipid, cholesterol and cholesteryl esters (and gene and enzyme activity of which are hormone-dependent), daily oral doses of 10, 50, and 250 mg 4-tert-octylphenol/kg bw were administered to 5 week-old male Sprague-Dawley rats (n=10/group) for three weeks. Histological examination of the testes did not reveal any morphological abnormalities in seminiferous tubules. PHGPx mRNA expression was slightly stimulated by about 37 % of the control level, whereas it was stimulated to about 160 % of the control level after treatment with nonylphenol and up to 250 % after treatment with DES. Since earlier studies had shown stimulation also by estradiol and tamoxifen, the authors concluded that stimulation of PHGPx expression might occur via the ER pathway.

Kim H.-H. et al. (2007) To investigate whether 4-tert-octylphenol affects Leydig cells via impairment of 3 ß-hydroxysteroid dehydrogenase/lyase (3ß-HSD), a key enzyme in steroidogenesis and molecular marker for androgen biosynthesis, daily oral doses of 10, 50, and 250 mg 4-tert-octylphenol/kg bw were administered to 5 week-old male Sprague-Dawley rats (n=10/group) for three weeks. Testicular expression of 3ß-HSD mRNA was analyzed using reverse transcription-polymerase chain reaction. 3ß-HSD mRNA was decreased ($p<0.05$) in testes at all doses (up to 27 % of the control level) indicating that 4-tert-octylphenol may influence androgen biosynthesis in rat testes through an abnormal change in 3ß-HSD mRNA expression.

Raychoudhury et al. (1999) primary rat spermatogenic and rat Sertoli cells cultivated *in vitro*, ↓ cell viability by $\geq 10^{-8}$ M 4-tert-octylphenol but not by 10^{-6} M 17ß-estradiol and 10^{-6} M dexamethasone, direct cytotoxic effect of 4-tert-octylphenol appeared to be exerted by Ca^{2+}-independent apoptosis.

Qian et al. (2006) primary rat Sertoli cells cultivated *in vitro*, ↓ cell viability and changes in gene expression (of bcl-2 and bax mRNA as well as caspase-3 protein) by 2-6 x 10^{-6} M 4-tert-octylphenol due to increased apoptosis.

Mechanistic studies - ootoxicity

Pocar et al. (2003) primary bovine oocytes cultivated *in vitro* with 1-0.0001 µg 4-tert-octylphenol/ml, ↓ in oocyte maturation, ↓ in oocyte fertilization, ↓ in oocyte ERα, but ERß and PR mRNA not affected.

4.6.2 Repeated dose toxicity: inhalation

Not assessed for the identification of this substance as SVHC in accordance with Article 57(f).

4.6.3 Repeated dose toxicity: dermal

Not assessed for the identification of this substance as SVHC in accordance with Article 57(f).

4.6.4 Other information

Not assessed for the identification of this substance as SVHC in accordance with Article 57(f).

4.6.5 Summary and discussion of repeated dose toxicity:

There are numerous studies available with repeat administration of 4-tert-octylphenol to adult rats of both sexes and with various routes (oral, subcutaneous and intraperitoneal injection) of

administration. The only route relevant with regard to human health assessment and exposure is the oral route of exposure.

During the studies with repeat oral administration for 28 to 60 days (Table 15) systemically toxic effects (e.g. reductions in body weight gain, changes in liver and kidney organ weights) were observed at daily dosages of 125 - 450 mg 4-tert-octylphenol/kg bw/day. Two studies with particular focus on the male reproductive system (Bian et al., 2006; Hossaini et al., 2003) revealed effects on male reproductive organ weights including accessory androgen-dependent organs, morphological defects in testes and reduced testicular sperm count as well as endocrine effects in terms of increased serum prolactin levels. These effects, however, were observed at the higher dosages (400 - 450 mg 4-tert-octylphenol/kg bw/day) with systemically toxic side effects. Two studies with particular focus on females (Yoshida et al., 2000; Sahambi et al., 2010) did not reveal effects up to and including daily doses of 125 - 200 mg 4-tert-octylphenol/kg bw/day on ovarian and uterine organ weight and histopathology or on oestrous cyclicity during the monitoring period or on serum estradiol concentrations, whereas a further study with focus on females (Laws et al., 2000) revealed a decrease in the number of regular cycles with an increase in the number of days of dioestrous at the highest tested dose of 200 mg 4-tert-octylphenol/kg bw/day.

Table 15: Overview on effect/ no-effect levels after oral (gavage) repeat dose administration

	systemic toxic effects		**adverse effects possibly related to endocrine MoA**		
Exposure	**Effect level (mg/kg bw/d)**	**No-effect level (mg/kg bw/d)**	**Effect level (mg/kg bw/d)**	**No-effect level (mg/kg bw/d)**	**Reference**
14 days	not reported	not reported		200, 100 (f) no effect on oestrous cyclicity or uterine weight	Yoshida et al. 2000
21 days	not reported	not reported		250 (m) no effect on testes histopathology	Baek et al. 2007
28 days	300	15		300 (f, m)	OECD SIDS, Japanese Study
29 days	150	15		150 (f, m)	OECD SIDS, Huntingdon Study
30 days	450	150	450 (m) testes, epididymis & prostate organ wt (↓) and testicular sperm count affected (↓)	150	Bian et al. 2006
1 month	400		400		Hossaini et

	(single dose study)		(m) androgen dependent organ wt (↓) and serum prolactin (↑) affected		al. 2003
25 days	not reported	not reported	200 (f) change in cycle (dioestrous) with decrease in number of regular cycles	100	Laws et al. 2000
35-41 days	not determined	125		125 (f) no effect on uterine & ovarian histopathology; serum estradiol unaffected	Sahambi et al. 2010
60 days	125	50		125 (m) no effect on histopathology or organ weights of reproductive organs, epididymal sperm count unaffected	Gregory et al. 2009

Further, a series of studies with rats is available in which the substance has been administered via routes that are not relevant for exposure of humans, such as test substance administration via subcutaneous injection Table 16). Some studies specifically indicate that this particular route of administration was used to minimize metabolism during first pass in order to maximize effects to be expected. Most studies used an application regimen of three injections/week for periods of 2, 4 or 8 weeks presumably due to clear-cut local effects and skin damage at the injection site, such as swelling, hardening, skin necrosis, scab and also abscess formation. Besides local effects induced at the injection site, signs for systemically toxic effects (e.g. reductions in body weight gain, changes in liver, kidney and spleen organ weights, extramedullary haematopoiesis) were seen after repeat s.c. administration of doses of ≤ 12.5 and ≤ 20 mg 4-tert-octylphenol/kg bw. Two studies performed with female rats (Blake and Ashiru 1997; Yoshida et al, 2000) revealed endocrine effects, such as persistent oestrous or abnormal cycling (and serum estradiol levels affected) after repeat s.c. administration of doses of ≤ 20 and ≤ 50 mg 4-tert-octylphenol/kg bw. Two studies performed with male rats (Blake and Bookfor 1997; Kim et al., 2004) revealed effects on testes, such as weight reduction and histopathological changes, and after repeat s.c. administration of doses of ≤ 20 mg 4-tert-octylphenol/kg bw. Changes in serum estradiol in females were observed after repeat s.c. administration of doses of 100 mg 4-tert-octylphenol/kg bw (e.g. at persistent oestrous), changes in serum testosterone, FSH, LH and prolactin in males were observed after

repeat s.c. administration of doses of ≤ 80 mg 4-tert-octylphenol/kg bw. It is obvious, however, that any effects on the male/female reproductive system and on plasma hormone levels were induced at 4-tert-octylphenol administrations that also displayed systemically toxic and severe local side effects. Data from a study performed with male mice indicate, that this species might be less sensitive/responsive to 4-tert-octylphenol than the rat species.

Table 16: Overview on effect/ no-effect levels after repeat dose administration with s.c. and i.p. injection

	systemic toxic effects		**adverse effects possibly related to endocrine MoA**		
Exposure	**Effect level (mg/kg bw)**	**No-effect level (mg/kg bw)**	**Effect level (mg/kg bw)**	**No-effect level (mg/kg bw)**	**Reference**
rat, subcutaneous					
2 weeks - with three administrations per week	not reported		$\underline{\geq}$ 20 (f) oestrous cyclicity affected		Blake and Ashiru, 1997
28 days	≥ 12.5		$\underline{\geq}$ 50 (f) ovary weight and histopathology, oestrous cyclicity and serum estradiol (↓) affected in F344 strain	25	Yoshida et al. 2000
1 and 2 month with three administrations per week	≥ 20		$\underline{\geq}$ 20 (m) testicular sperm count (↓) and sperm morphology affected $\underline{\geq}$ 80 (m) serum LH, and FSH (↓), prolactin (↑) and testosterone (↓)concentrations affected anterior pituitary gonadotropin and prolactin concentrations affected		Blake and Boockfor, 1997, Boockfor and Blake, 1997

	systemic toxic effects		**adverse effects possibly related to endocrine MoA**		
Exposure	**Effect level (mg/kg bw)**	**No-effect level (mg/kg bw)**	**Effect level (mg/kg bw)**	**No-effect level (mg/kg bw)**	**Reference**
4 weeks - with three administrations per week	≥ 20		≥ 20 (m) organ weight (↓) and testes histopathology affected ≥ 20 (m) serum LH (↑) and testosterone (↓) concentrations affected		Kim S.-K. et al. 2004
mouse, intraperitoneal					
5 days	≥ 200			≥ 200 (m) serum testosterone and gene expression of steroidogenic enzymes unaffected	Kim S.-K. et al. 2007

4.7 Mutagenicity

Not assessed for the identification of this substance as SVHC in accordance with Article 57(f).

4.8 Carcinogenicity

Not assessed for the identification of this substance as SVHC in accordance with Article 57(f).

4.9 Toxicity for reproduction

4.9.1 Effects on fertility

Studies with oral administration

There is one reproduction toxicity screening test available that was conducted according to OECD TG 421. The test protocol involves parental dosing from two weeks pre-mating to 4 days post natal for dams, and a total of 28 days exposure for the males. According to Annex VIII of the REACH regulation (EC No 1907/2006) this test is requested as standard information requirement at the 10 – 100 t/year tonnage level.

Type: Reproduction/developmental screening test

Species:	rat
Sex:	12 females, 12 males
Route of administration:	oral (gavage)
Exposure period:	2 week prior mating, 2 week mating, until day 4 post partum
Frequency of treatment:	daily
Duration of the test:	6 weeks
Doses:	125, 250, 500 mg/kg bw/day
Control group:	Yes
NOAEL systemic:	125 mg/kg

NOEL reproduction/offspring: 250 mg/kg

Results:

500 mg/kg produced severe toxic effects resulting in the death of 13 adult animals (9 males, 4 females) during the treatment period. Other toxicity signs were:

- post dose salivation, wet coats, matted fur, brown stained urogenital region, loose faeces, hunched posture, emaciation, lethargy and abnormal gait.

- bodyweight gain and food consumption reduction, water consumption markedly increased.

- increased number of white blood cells and platelets, increase level of plasma urea nitrogen, creatinine, bilirubin and GPT. Lower level of electrolytes and

circulating albumin.

- increased liver, kidney and adrenal weights.

- decreased weights of specific reproductive organs (testes, epididymides, ovaries and combined prostate/seminal vesicles/coagulating gland).

- minor microscopic changes in the testes and epididymides.

Effects on reproduction were:

- impaired mating performance with only 4 of the 8 paired females conceiving.

- amongst females that did conceive, mating performance was unaffected although the duration of pregnancy was longer than expected.

- libido unaffected in males, but fertility lowered.

- reduced implantation rate and increased pre and post natal mortality resulting in lower litter size.

- reduced litter weight and suggestion of impaired pup growth to day 4.

- no gross abnormalities amongst the offspring.

250 mg/kg induced reactions including:

- post dose salivation, wet coats, loose faeces.

- reduced bodyweight gain for all animals, for females bodyweight gain also affected at the end of pregnancy/early lactation.

- increased water consumption.

- no obvious haematological or biochemical changes, but increased liver and slightly increased kidney weights.

- microscopic examinations of testes and epididymides of males revealed no abnormalities.

There were no effects of treatment on mating performance or development of the litter at this dosage.

125 mg/kg induced only post dose salivation and slightly

elevated water consumption.

Method:		OECD guideline 421 (dated 12 January 1993)
GLP:	Yes	
Test substance:		4 (1, 1, 3,3-tetramethyl-butyl) phenol; purity 98.7%

cited from: OECD SIDS PHENOL, 4-(1,1,3,3-TETRAMETHYLBUTYL)- CAS No: 140-66-9

Reference: Huntington Research Centre Ltd., Huntingdon, England: Reproduction/Developmental Toxicity Screening Test; HRC Report no. SAZ 462/942750 (1995)

In a further study (Piersma et al. 1998a, b) also applying the OECD TG 421 test design 4-tert-octylphenol was tested within a series of six xenobiotic compounds displaying various endocrine properties. Compound dosage had been chosen on the basis of relative potency at the estrogen receptor level. Except for ethinylestradiol all compounds including 4-tert-octylphenol were tested at dosages much higher than any likely exposure to be expected in man. Five of the six compounds were clearly scored as reproductive toxicants, affecting one or more parameters such as fertility, luteinisation, spermatogenesis, and fetal development, however, 4-tert-octylphenol appeared lethal at the dose selected (1000 mg/kg/d). As a consequence new rats were enrolled into the study which were dosed from 12 days premating onward, at 100 mg/kg/d. Whereas the protocol recommends 10 animals/sex, in this study 4 animals (Wistar rats)/sex/group had been used, yet in addition hormone measurements (estradiol, progesterone, testosterone, LH, FSH) had been performed.

At 100 mg/kg/d, 4-tert-octylphenol affected food consumption and body weight gain of both sexes. The only reproductive effect observed was a questionable increase in corpora lutea.

Given the extreme toxicity at the 10 fold higher dose of 4-tert-octylphenol, it has been assumed, that other toxicity than endocrine-mediated reproductive effects will be more important.

In a guideline according 2-generation reproduction toxicity study (870.3800; USEPA 1996) with several enhancements (Tyl et al. 1999) Sprague Dawley rats were fed with dietary concentrations of 0, 0.2, 20, 200, and 2000 ppm 4-tert-octylphenol in their diet leading to a daily intake of 0,034-0.011, 3.3-1.05, 32.6-10.9, and 369-111 mg/kg/d depending on the age

and sex of the animals and the phase of the study (e.g., consumption was highest for weanlings and for dams during lactation). The highest test concentration (2000 ppm) was chosen to provide daily 4-tert-octylphenol intake at or in excess of a level shown to saturate liver metabolic capacity (200 mg/kg/day) and intake of young animals that would exceed this dose. This kind of test (Two-generation Reproduction Toxicity Test according to OECD TG 416) is also part of the standard information requirements according to the REACH regulation (EC No 1907/2006) for high production volume substances of > 1000 t/year (Annex X) respectively at the lower tonnage level for substances, for which adverse effects on the reproductive organ system were revealed from studies with repeat administration (Annex IX).

The test protocol involved parental dosing of the F0 generation (30 animals/sex/dose group) during the 10 weeks pre-breeding, mating and gestation period. Clinical signs for toxicity, body weights, and feed consumption were monitored according to guidelines. For the last 3 weeks of the prebreed exposure period, vaginal smears for oestrous cyclicity and normality were taken for all F0 females. The animals were mated (1:1) following the 10-week prebreed exposure with no change in mating partners. On postnatal day (pnd) 4, the size of each F1 litter was adjusted to ten pups by eliminating extra pups by random selection to yield, as nearly as possible, five males and five females per litter. On pnd 21, each litter was weaned, and at least one F1 male and one F1 female pup per litter, if possible, were randomly selected (30/sex/group) to produce the F2 generation. Following this selection, three weanlings/sex/litter, if possible, were randomly selected for necropsy. Selected animals of the F1 generation were administered 4-tert-octylphenol in the diet at their respective formulations for 10 weeks and then mated to produce the F2 generation following the same study design as described for the F0 generation. Selected weanling females of the F2 generation (30/group) were administered 4-tert-octylphenol in the diet at their respective formulations until acquisition of vaginal patency, then all were terminated. To allow for evaluation of sperm parameters, selected F2 weanling males (30/group) were maintained through acquisition of preputial separation and until 111 ± 5 days of age. Selected F1 and F2 weanling animals, all F0 and F1 parental animals, and retained F2 male offspring were subjected to a complete gross necropsy. The stage of oestrus at necropsy was determined for all F0 and F1 females. For weanling animals, the brain, spleen, thymus, ovaries (2), uterus with cervix and vagina, testes (2), epididymides (2), and seminal vesicles (2) were weighed. For parental animals (and retained F2 male offspring) the brain, liver, kidneys, adrenal glands, spleen, ovaries, uterus, testes, epididymides, seminal vesicles with coagulating glands, and the prostate and dorsal prostate were weighed. Specific attention was focused on the examination of the parental reproductive organs, including determining the weight of the prostate and dorsal prostate for all males and ovarian follicle counts for high dose and control F0 and F1 females. At the time of sacrifice of F0 and F1 parental males and retained F2 male offspring, testicular homogenization-resistant spermatid head count and calculation of daily sperm production and efficiency of daily sperm production were determined from one frozen testis/male for all males. In addition, number, motility, and morphology of sperm from one cauda epididymis were evaluated in these same animals. Histopathologic evaluation of the ovaries with oviducts (2), testis (1), vagina, epididymis (1), uterus with cervix, seminal vesicles (2), and prostate was conducted on the F0 and F1 parental animals and retained F2 male offspring from high dose and control groups.

Treatment-related systemically toxic effects were limited to consistent and persistent reductions in body weights and weight gains in both sexes in the F0, F1, and F2 generations at 2000 ppm. Feed consumption was unaffected. There were no clinical observations. Body weights during gestation were unaffected and were reduced during lactation in F0 and F1 females at 2000 ppm. At necropsy, F0 and F1 parental and F2 retained male absolute and relative organ weights were unaffected for liver, kidneys, adrenal glands, spleen, and brain.

There were no treatment- or dose related gross or microscopic findings for the examined organs, for F0 and F1 parental animals, and for F2 retained adult males.

There were no treatment-related effects in F0 or F1 females on mating, fertility, pregnancy, gestational indices, number of implants, total pups, live or dead pups per litter, percentage postimplantation loss (prenatal mortality index), or gestational length (in days). Oestrous cycle length in days and stage of oestrus at necropsy were equivalent across all groups. There were no treatment-related effects on absolute or relative reproductive organ weights or on gross or histological examinations of the reproductive organs. Paired ovarian follicle counts were similar between high dose and control F0 and F1 females. There were no effects of treatment in F0 or F1 males on mating or fertility indices. There were also no treatment-related effects in F0, F1, and retained F2 males on absolute or relative weights of the testes, epididymides, prostate, dorsal prostate, or seminal vesicles plus coagulating glands, and no effects on epididymal sperm concentration, percentage motile or progressively motile sperm, testicular homogenization-resistant spermatid head counts, daily sperm production, or efficiency of daily sperm production. Percentage abnormal sperm was also unaffected for parental F0 and F1 males and for retained F2 males. There were no treatment-related gross or microscopic findings on reproductive organs for F0, F1, or F2 adult males.

As to the offspring, pup body weights per litter were reduced at 2000 ppm for both F1 and F2 offspring during the later period of lactation (pnd 14 and 21). There were no significant changes in organ weights at weaning. The mean age of acquisition of vaginal patency in F1 females ranged from 30.5 to 31.8 days, with the mean body weight at acquisition ranging from 97.83 to 91.91 g. The mean age of acquisition of preputial separation in F1 males ranged from 43.1 to 44.7 days, with the mean body weight at acquisition ranging from 220.07 to 207.01 g. When the age at acquisition was statistically analyzed by analysis of covariance (ANCOVA), with body weight as the covariate, only the ages at acquisition of vaginal patency and preputial separation at 2000 ppm were statistically significantly delayed ($P < 0.01$) from the control group values. The same statistically significant minor delays in vaginal patency were observed at 2000 ppm in F2 females (31.3 days) versus the control value (30.6 days), and in preputial separation at 2000 ppm in F2 males (43.6 days) versus the control value (42.2 days), with no statistically significant effects on body weights at acquisition. The delays in vaginal patency in females and in preputial separation in males at 2000 ppm were relatively minor (both less than 2 days), and were considered related to the lower body weight, starting late in lactation and continuing through the post wean exposure period. The statistically significant effect on acquisition of reproductive landmarks in F1 offspring required measurement of anogenital distance in newborn (pnd 0) F2 offspring, as specified in the US EPA guidelines. Anogenital distance in males was equivalent (statistically and biologically; approximately 2 mm) across all groups, with F2 male pup body weights per litter also statistically equivalent across all groups. Anogenital distance in the newborn F2 females was significantly longer in all 4-tert-octylphenol-exposed groups, with mean values of 0.79, 0.81, 0.85, and 0.79 mm at 0.2, 20, 200, and 2000 ppm, respectively, compared to the control group mean value of 0.76 mm, with no significant differences among groups for female body weight/litter at birth.

In summary, effects related to 4-tert-octylphenol administration in adult animals were limited to decreased body weights and weight gains in the high dose group. There were no treatment-related effects in reproductive measurements, reproductive organs, or extensive evaluations of sperm measurements in three generations of males. Effects in offspring occurred only at the high dose and were limited to reduced body weights and slightly delayed vaginal opening and preputial separation, considered related to reductions in body weight gain. The reduced pup body weight occurred not before the later part of the lactational period, when the pups were self-feeding and therefore directly exposed to high daily doses of 4-tert-octylphenol in the diet

by ingestion. No effects on reproductive parameters, testes weights or morphology, epididymal sperm counts, or morphology, daily sperm production, efficiency of daily sperm production, or prostate or dorsal prostate weights or histopathology were observed. Further, no estrogen-like effects on males or females and no low dose effects were evident.

NOAEL systemic toxicity/postnatal toxicity: 200 ppm (11-33 mg/kg/d)

NOAEL reproductive toxicity: 2000 ppm (111-369 mg/kg/d)

In a further study (Yoshida et al. 2001) male Crj:Donryu neonatal rats were exposed to 4-tert-octylphenol receiving a first s.c. injection of 100 mg/kg bw 24 h after birth. Pups were then treated every other day until pnd 15 (for a total of eight times: pnd 1, 3, 5, 7, 9, 11, 13, and 15). Body weights in control and 4-tert-octylphenol treated animals were measured throughout the study. All animals were checked for clinical signs, development and abnormalities of the external reproductive tract. At 13 weeks of age, seven males from both the control and the 4-tert-octylphenol treated group were selected and mated with untreated females to examine reproductive ability. After 21 days of gestation numbers of implanted live and/or dead pups and their body weights were examined. At 18 weeks of age theses males were terminated. From these animals (n=7) epididymal sperm head numbers were determined and brains prepared for morphometry of the sexually dimorphic nucleus of the preoptic area (SDN-POA). There were no differences between animals of the control and the treated group in mating success and fertility including average number of live embryos per litter.

4.9.2 Developmental toxicity

Studies with oral administration to the dams – in utero exposure

In a study (Harazono et al. 2001) aiming at the evaluation of effects on early pregnancy and maintenance of pregnancy groups of 16 sperm positive female Wistar rats each were given 4-tert-octylphenol daily by gastric intubation at a dose of 15.6, 31.3, 62.5, 125, 250 (n=6), and 500 (n=7) mg/kg bw on pregnancy days 0 (sperm plugs detected) through day 8 of pregnancy. The vehicle control group received olive oil. Maternal body weight, food consumption and clinical signs of toxicity were recorded daily. Dams were terminated on day 20 of pregnancy and the numbers of live and dead fetuses and the numbers of uterine implantation sites and resorptions were recorded. Live fetuses were sexed, weighed and inspected for external malformations and malformations within the oral cavity. In the high dose group, all seven rats died by day 6 of pregnancy and two of the six rats treated with 250 mg/kg bw died during the administration period. Clinical signs such as diarrhea and loss of fur were in animals treated with $\geq$ 62.5 mg/kg bw. The body weight gains from dose groups > 31.5 mg/kg during the treatment period and on days 0-20 were significantly lower than those of control groups. Net weight gain of the dams, however, did not differ significantly from that of the control group. The food consumption on days 0-9 and on days 0-20 of pregnancy was significantly decreased in all treated groups ($\geq$ 15.6 mg/kg bw) compared with control values. There were no significant differences in the pregnancy rate between treated animals and the control group. The numbers of corpora lutea, implantation sites and pre-implantation loss per litter in the treated animals were not significantly different from the control. A significant decrease in the numbers of live fetuses per litter was observed at 31.5 and 125 mg/kg, and a significant increase in the incidence of post-implantation loss at dosages of $\geq$ 31.3 mg/kg bw. The sex ratio of live fetuses was comparable across all groups. Body weights of male and female fetuses was comparable across groups. No significant increases in the incidences of fetuses with external malformations were observed.

LOAEL/maternal toxicity: 15.6 mg/kg bw/day (based on reduced food intake)

NOAEL/develop.toxicity: 15.6 mg/kg bw/day (based on stat. sign. increased percentage of post-implantation loss)

In a study (Veeramachaneni, 2006) aiming at the relationship of atypical germ cell development and abdominal location of testes groups of Dutch-Belted rabbit does (n=4-6 per group) were treated on alternate days between gestation days 15 and 30 with various chemicals including 4-tert-octylphenol at a dose of 150 mg/kg (orally in corn syrup). At 6 weeks of age male pups were weaned, caged individually and monitored weekly for testicular descent to a scrotal position. At 24-26 weeks of age rabbits were killed and testes, epididymides and sex accessory glands weighed and evaluated for any gross abnormalities and left and right testes processed for light and transmission electron microscopy. As a result, one of four 4-tert-octylphenol pups was found unilaterally cryptorchid. Atypical germ cell resembling gonocytes and pre-spermatogonia were found in the undescended testis but not in testes of pups surgically treated on pnd 21 to remain cryptorchid. The authors concluded from their study that abnormal development of germ cells could result from direct actions of chemicals when exposed during critical periods of gonadal differentiation but not from abdominal location of the testis per se.

In a study (Sharpe et al. 1995) aiming at the evaluation of effects on testicular size and spermatogenesis in adulthood (e.g. at 90-95 days of age) in male Wistar rats after fetal/neonatal exposure to 4-tert-octylphenol dams were exposed to several chemicals via drinking water either during lactation only, e.g. days 1-22 after birth (study 1) or 2 weeks before mating, throughout mating and gestation up to day 22 after birth with their litters culled to eight pups on the day of birth (study 2) as in study 1, or with full litter size maintained (study 3). This pre-/postnatal exposure scheme was thought to cover in particular the proliferation period of Sertoli cells (study 1, to cover the postnatal period of Sertoli cell proliferation period, study 2 and 3 to cover the whole period), which are considered to determine the ultimate testicular size in mammals. 4-tert-octylphenol was offered to the animals at drinking water concentrations of 10, 100 and 1000 µg/l, DES served as a positive (estrogenic) control at concentrations of 100 µg/l.

Prenatal exposure to 4-tert-octylphenol did not affect litter size and composition. For none of the treatments any adverse effects on the testicular morphology or on the cross-sectional area of the lumen or seminiferous epithelium at stages VII-VIII of the spermatogenesis had been observed. Testicular morphology was indistinguishable in animals from the control and treatment groups, and no obvious abnormalities in the seminiferous tubules, interstitium, or vasculature were evident. Given that litters had been either culled or fully maintained during the studies any treatment-related effects on body weight at weaning or at adulthood could not be assessed with certainty Also, there was an obvious variance in the body weight of the control groups (for weanlings as for adults) during the 3 studies, and no consistent effects (on body and relative testes weights) across the studies was revealed for DES. At drinking water concentrations of 1000 µg 4-tert-octylphenol /l relative testes weights were found to be slightly but statistically significantly reduced (5-13 %) and relative kidney weights slightly but statistically significantly increased in adult males. Further, daily sperm production (determined in some animals from study 3) was significantly reduced for about 10%. The effects observed for DES and 4-tert-octylphenol during this study, however, could not be reproduced from the performing laboratory at a different instant, when also problems with testicular weights and body weights in the control male animals were reported (Sharpe et al. 1998). Therefore, results of the study should be considered cautiously.

In a further study (Kamei et al. 2008) aiming at bone growth in vivo groups of 3 pregnant ICR mice each were exposed to 4-tert-octylphenol in their drinking water at concentrations of either 1 mg/ml (LD group) or 10 mg/ml (HD group) beginning on day 10 of pregnancy. The control group received drinking water containing 0.2% ethanol (vehicle control). Based on water intake determined on days 13 and 16 of gestation the average intake of the LD group was estimated to 0.20 mg/kg bw and of the HD group was estimated to 1.96 mg/kg bw. Dams were allowed to litter and drinking water exposure continued during lactation. After weaning, the pups were continued on the according drinking water concentration and terminated on postnatal day 30. Offspring body weights and weights of parametrial (females) and epididymal (males) adipose tissue were taken, length of femurs determined, and serum osteocalcin measured. Peripheral quantitative computed tomography (pQCT) was performed to investigate bone growth of the (right) femur, and the expression of alkaline phosphatase (ALP) was examined in periostal cells (of the left femur) by immunohistochemical staining. 4-tert-octylphenol exposure via drinking water did not affect numbers of pups/litter in the control, LD or HD dose group. There were no differences between groups in body weights, weight of adipose tissue, length of femur and osteocalcin, except for females pups of the LG group (n=12) and the HD group (n=19) with lower levels of serum osteocalcin in comparison to females of the control group (n=19) and except for females from the HD group displaying statistically significantly ($p<0.05$) slightly higher body weights (20.9 ± 0.4 g) in comparison to females from the control group (19.2 ± 0.4 g). pQCT analysis of the femoral diaphysis in females did not show differences between groups (controls: n=19; LD: n=12, HD: n=19) for cortical bone density, cortical bone area, cortical thickness and various strength indices. Compared to the controls periosteal circumference and endosteal circumference was slightly smaller for the LD group but not for the HD group. pQCT analysis of the femoral distal metaphysis in females revealed slightly higher total bone density and an increase in one of the strength indices in the HD group but no change between groups for the other parameters. No information/data were provided for pQCT analysis of femurs from males. The authors concluded from their investigation that periostal bone formation was inhibited from exposure to 4-tert-octylphenol during pre- and postnatal periods with the inhibitory effect more pronounced in females than in males.

In a study on Wistar rats (Pocock et al. 2002) effects of maternal dietary exposure to 4-tert-octylphenol were investigated on a variety of behavioural endpoints (in their offspring). The study also incorporated determinations of ultrasound vocalization. It is reported that 4-tert-octylphenol was dissolved in wax at known concentration before mixing with the rodent diet. Wax blocks that were fed to the animals consisted of 105 g paraffin wax (octylphenol carrier) to 195 g powdered rodent diet and was produced by melting wax (with a known content of 4-tert-octylphenol) and mixing with preheated powdered diet. No reliable data are provided on the quantitative intake of the test compound. In a first step, palatability was examined with increasing doses (0, 25, 75, 750, 3000, and 7500 µg 4-tert-octylphenol/g wax) in the diet for consecutive 2-day periods in three male and three female rats. Three females were maintained on the diet with 3000 µg 4-tert-octylphenol/g wax throughout pregnancy and lactation. The highest dose was discontinued after the first day because the animals would not eat it. In a second step, groups of 4-5 ovarectomised females each were given 4-tert-octylphenol in the diet (0, 25, 75, 750, and 3000 µg 4-tert-octylphenol/g wax) over 72 hours and estrogenic activity determined by cell proliferation in the uterine and vaginal luminal epithelium as measured by the number cells in mitosis in these tissues. There were no significant differences in uterine weight in the 4-tert-octylphenol-exposed animals in comparison to the controls. In comparison to the controls, slightly increased mitotic indices were seen in the uterine luminal epithelium of animals of the highest dietary concentration and in vaginal luminal epithelium

higher mitotic indices in animals exposed to 750 or 3000 µg 4-tert-octylphenol/g wax. In a third step, two groups of 11 males and females each were maintained (time period not reported) either on control diet or on a diet containing 3000 µg 4-tert-octylphenol/g wax (authors roughly estimated gross intakes of 95 - 250 mg 4-tert-octylphenol/kg bw/d) and mated. The dosage of 3000 µg 4-tert-octylphenol/g wax was chosen, because it was shown to be the highest dosage to be both estrogenic and palatable and should therefore induce the maximal effects of 4-tert-octylphenol through the physiologically relevant route of dietary exposure. After mating, the animals were maintained on the same diet throughout pregnancy and lactation. On postnatal day 21, the offspring were weaned and maintained on a normal diet and subjected to various behavioural tests for a study period of up to postnatal days 325-327. There were no differences between groups in food consumption. All mated females produced litters with numbers of litters and sex ratio similar in control and 4-tert-octylphenol-treated groups. At birth, there were no significant differences between the weights of offspring of the 4-tert-octylphenol-exposed groups compared to controls. 4-tert-octylphenol-exposed animals were significantly lighter than controls at weaning and throughout the entire observation period despite no significant differences in food consumption. Absolute organ weights of kidneys and of spleens were significantly reduced in females (at pnd 345) and in males (at pnd 265). Evaluation of prepubertal effects: There were no significant effects of 4-tert-octylphenol exposure on anogenital distance at pnd 3 in males or females or on uterus or ovary weights or the mitotic indices in these tissues in female pups at pnd 7. There were no significant effects of 4-tert-octylphenol exposure on testicular weights or the diameter of seminiferous tubules in male pups at pnd 19. The age at vaginal opening was similar in 4-tert-octylphenol-exposed females compared to controls (35 days) despite their difference in growth rate. The average age at preputial separation was the same for the 4-tert-octylphenol-exposed male pups and the control animals (43 days). Evaluation of postpubertal effects: Cycle monitoring at the age of 2-3 months showed regular cycles of 4-5 days in both 4-tert-octylphenol-exposed and control animals. By 6-7 months of age, cycles were irregular in the 4-tert-octylphenol-exposed animals with longer periods of oestrous smears and a higher proportion of cornified smears compared to the controls. Absolute ovarian weight was less in 4-tert-octylphenol-exposed females at pnd 215. Histological appearance was normal and showed antral follicles and corpora lutea. The 4-tert-octylphenol-exposed males had lighter absolute testes weights at pnd 240 with smaller diameter seminiferous tubules than controls, although relative testes weights were heavier than controls. Mating of the first generation (no details provided) did not reveal any differences between groups in number of days until the appearance of vaginal plugs, subsequent litter sizes and litter weights. Ultrasound vocalisation by both male and female 4-tert-octylphenol-exposed pups at pnd 7 was significantly reduced compared to controls. However, at pnd 12, ultrasound vocalization, both number and duration of calls was similar in the two groups. In adult animals there were no significant effects of pre-/postnatal 4-tert-octylphenol-exposure on ultrasound vocalization. It was reported that in sexual orientation tests females spent more time in front of the female teaser and a greater percentage of visiting time in front of the female teaser and that sexual behaviour testing in males indicated increased sexual arousal (mount and intromission latencies significantly shortened).

Studies with s.c. administration to the dams – in utero exposure

In a study aiming at potential effects of in utero exposure to 4-tert-octylphenol on rat prenatal testosterone surge (Haavisto et al. 2003) sperm-positive (gestation day 0.5) female Sprague-Dawley rats were injected s.c. on days 13.5, 15.5, and 17.5 of pregnancy with corn oil (vehicle), DES (0.01, 0.1, or 0.2 mg/kg bw), or 4-tert-octylphenol (0.1, 1, 10, or 100 mg/kg bw). On gestation day 19.5, when the prenatal testosterone surge should occur in the Sprague-

Dawley rats, dams were terminated and numbers of fetuses, their weights and gender recorded. Testes from a total of 73 in utero exposed fetuses were taken, part of them were ex vivo cultivated for period of 3 h. Testes and culture media were collected and stored for hormone measurements. In utero exposure to 4-tert-octylphenol had no effect on fetal body weight, whereas fetuses exposed in utero to DES (at maternal dosages of 0.1 and 0.2 mg/kg) had significantly lower body weights. DES at 0.01 mg/kg bw had no effect on fetal testicular testosterone levels, whereas 0.1 and 0.2 mg/kg bw significantly reduced fetal testicular testosterone levels from 349 ± 138 pg/testis in controls to 31 ± 55 pg/testis, respectively 12 ± 9 pg/testis. 4-tert-octylphenol at 100 mg/kg bw had a tendency to lower fetal testicular testosterone levels, but the effect was not statistically significant. When testes exposed in utero to 4-tert-octylphenol were taken into ex vivo tissue culture, testosterone secretion was similar to that of controls, whereas testes exposed to DES showed a steep depression in secreted testosterone concentrations.

In a study aiming at potential effects of prenatal exposure to 4-tert-octylphenol on the reproductive tract of male rats (Aydogan and Barlas, 2006) pregnant Wistar rats (n=4/group) were given daily s.c. injections of corn oil (vehicle control), 100-mg, or 250-mg 4-tert-octylphenol/kg bw during pregnancy (day 1-20). Maternal body weight, food consumption and clinical signs of toxicity were recorded daily. Dams were allowed to litter and offspring grown up until adulthood (2.5 months of age). At termination final body weights were taken and organ weights of testes, epididymis, caput epididymis and prostate. Besides histopathological evaluations also morphometry of seminiferous and epididymal tubules and sperm counts and morphology of epididymal sperm was performed. No data were provided on the performance of the dams or on pregnancy outcome and data from 16, 14, and 11 male pups only were finally reported. Final body weights of the male offspring from the 250-mg group were statistically significantly higher (228.9 ± 6.9 g) than those of the controls (203.7 ± 5.5 g) and the 100-mg group (204.5 ± 7.1 g). There were no changes in left and right testes weights between groups. Absolute right epididymis and prostate weights were increased in the 250-mg group; however, there were no differences between groups in relative organ weights. Organ histopathology revealed some changes in the epididymis of the 250-mg group with increased epithelial height (29.4 ± 1.9 versus 22.2 ± 0.9 µM in controls) and mononuclear cell infiltration. There were no changes between groups in organ weight of the caput epididymis and in epididymal sperm count. However, sperm morphology revealed a slight increase in percentage of abnormal sperm (of 24 versus 16 % in controls) in the offspring of the 250-mg group mostly attributable to an increase in coiled tail.

In a similar study (Götekin and Barlas, 2007, 2008) aiming at potential effects of prenatal exposure to 4-tert-octylphenol on organs of the endocrine system 4, 5, and 5 pregnant Wistar rats respectively were given daily s.c. injections of corn oil (vehicle control), 100-mg, or 250-mg 4-tert-octylphenol/kg bw during pregnancy. Maternal body weight was recorded weekly. Dams were allowed to litter and raise their offspring. After weaning male and female offspring were allowed to grow until 2.5 months of age, with food and water intake recorded daily and body weights recorded weekly. At sacrifice adrenal, hypophysis, pancreas, thyroid and parathyroid were removed and investigated morphologically and histopathologically. For the dams there were no differences between groups in food consumption and water intake. However, dams from the 250-mg group revealed a statistically significantly ($p>0.05$) lower body weight gain (19.3 % as compared to 30.8 % in controls). Litter data, however, were not provided. For the offspring there were no differences between groups in food consumption and water intake. Data from 12 female/14 male, 10 female/18 male, and 15 female/12 male pups respectively were reported for the vehicle control and the 100-mg and 250-mg treatment

groups. Only final body weights at the age of 2.5 months were provided, indicating significantly higher body weights of females (201.7 ± 10.5 g) and males (239.2 ± 9.7 g) of the 250-mg treatment group when compared to controls (156.7 ± 3.6, resp. 167.8 ± 9.9 g) and to the 100-mg group (152.2 ± 4.9 g, resp. 156.8 ± 7.4 g). There were no differences between groups in absolute and relative organ weights of adrenals, pancreas and thyroid+parathyroid, except some decrease in relative organ weights of adrenals and thyroid+parathyroid in males of the 250-mg group. Histopathological findings were reported in particular for the 250-mg group offspring for adrenals (cellular degeneration, thickening of capsula, oedema, congestion), pancreas (Langerhans cell degeneration), thyroid (damaged follicles, fatty tissue increasing, expanded follicles, mononuclear cell infiltration) and for pituitary gland (cellular degeneration, congestion). Clinical chemistry or any functional parameters were not evaluated.

In a further similar study (Barlas and Aydogan, 2009) aiming at potential effects of prenatal exposure to 4-tert-octylphenol on liver, kidney, spleen and hematologic parameters pregnant Wistar rats (n=8/per group) were given daily s.c. injections of corn oil (vehicle control), 100-mg, or 250-mg 4-tert-octylphenol/kg bw during pregnancy (P1-20). Dams were allowed to litter and offspring grown up until adulthood (2.5 months of age). At termination final body weights and organ weights of kidneys, liver and spleen were taken, and blood samples were collected from the heart. Concerning pregnancy data there were no differences between groups in length of pregnancy, initial and final dam body weights and in body weight gain during pregnancy, and in mean litter size. Data from 15 to 28 offspring females/group and from 21 to 30 offspring males/group were reported. Body weight gain of males and females during growth to adulthood did not differ between groups. Final body weight of the males of the 250-mg treatment group was significantly higher (233.5 ± 6.5 g) when compared to controls (191.9 ± 4.7 g). Among males, there were no differences between groups in relative organ weights of kidneys and livers; however, relative spleen weight was increased in the 100-mg group but not in the 250-mg group. Among females, relative organ weights of kidneys and livers were decreased ($p < 0.05$) in the 250-mg group in comparison to the controls, relative spleen weights unaffected. Haematological analysis revealed changes in offspring from the 250-mg group with increases in percentage of granulocytes in females, decreases in platelet counts in males and females, slight decreases in red blood cell counts in males and females and increases in micro-red blood cell counts in males and females of the 250 – and 100-mg group**.** Histopathological evaluations revealed changes in offspring from the 250-mg group in liver tissue (lipid deposition, degeneration of hepatic parenchyma, mononuclear infiltration), kidney tissue (oedema, tubular cell degeneration) and in spleen (degeneration of splenic tissue, increased hemosiderin deposition).

Given that dams from these three studies were repeatedly subcutaneously injected with high doses of 4-tert-octylphenol and for a long period (20 x 250 mg/kg bw) and not any information provided on local, on systemic or on pregnancy side effects, results of these studies should be considered with care.

In a study aiming at effects of prenatal exposure to 4-tert-octylphenol on oocytes in newborn females (Sonne-Hansen et al. 2003) groups of pregnant NMRI mice were treated with s.c. injection of either peanut oil (vehicle control), 100 µg DES/kg bw, 1 mg 4-tert-octylphenol/kg bw or 250 4-tert-octylphenol mg/kg bw from embryonic day (ED) 11.5 to ED 16.5 (day of plug=ED 0.5). On the day of birth gonads were taken from newborn females, and one ovary from each individual prepared for counting the numbers of prefollicular, follicular, and atretic oocytes in the ovaries used the optical fractionator stereologic method. Eight ovaries each

were examined from the DES, 4-tert-octylphenol-1mg and 4-tert-octylphenol-250mg groups, whereas 9 ovaries were examined from the control group. The general ovarian morphology did not differ between groups. All germ cells had reached the oocyte stage and were present throughout the ovaries with highest density in the outer cortex. Developing follicles were primarily seen in the medulla and the inner cortex. Neither prenatal exposure to DES nor to 4-tert-octylphenol affected the total number of oocytes or the percentage distribution of atretic, prefollicular, and follicular oocytes indicating, that fetal female germ cell proliferation and survival, or early follicle formation had not been impaired.

Studies with s.c. administration – direct pup exposure

In a study analyzing the potency of three differently endocrine-active compounds to acutely interfere with growth and steroid synthesis of infant rat testis (Mikkilä et al. 2006) randomly selected neonatal male rat (Sprague-Dawley) pups received a first s.c. injection of either 4-tert-octylphenol, DES (as a nonsteroidal estrogen) or flutamide (FLU, as a non-steroidal antiandrogen) 6 h after birth and then serial doses until pnd 4, i.e. five doses altogether. Groups of 11, 10, and 20 pups received s.c. dosages of 10, 50, and 100 mg 4-tert-octylphenol/kg , respectively; groups of 12, 18, and 12 pups received s.c. dosages of 0.1, 0.5, and 1.0 mg DES/kg, respectively; groups of 22, 25, and 30 pups received s.c. dosages of 2, 10, and 25 mg FLU/kg, respectively; a total of 30 pups received s.c. injections of the according vehicle (DMSO for 4-tert-octylphenol and DES, corn oil for FLU). Pups then grew until the day of analysis (pnd 14) with the dam to which they were allocated and measurements of circulating testosterone and gonadotropin (LH, FSH) levels, testicular testosterone and progesterone production (by testis culture ex vivo) as well as steroidogenic acute regulatory protein (StAR) and 3ß-hydroxy-steroid-dehydrogenase (3ß-HSD) type I protein expression levels were performed as well as histological analysis and measurement of the seminiferous cord diameter in testes. 4-tert-octylphenol treatment had no effects on body weight or on testis weight of the 14-day-old male rat infants. Compared to controls, the testes of 4-tert-octylphenol treated males showed no overt histological changes in Leydig cells and seminiferous cords. Plasma testosterone, LH and FSH levels were not significantly altered in 4-tert-octylphenol treated animals, whereas in contrast in the DES treated animals plasma testosterone levels were suppressed by 82-91% and an increase in LH levels was seen. In 3-h ex vivo culture, basal as well as hCG-stimulated testosterone secretion of testes from 4-tert-octylphenol treated males did not differ from that of the controls. Similarly, basal intratesticular testosterone concentrations as well as testicular testosterone concentration after hCG-stimulation did not differ from that of the controls. Basal progesterone secretion was not affected in testes from 4-tert-octylphenol treated males. However, in testes from animals that were treated with 100 mg 4-tert-octylphenol/kg the hCG-stimulated progesterone secretion was slightly elevated ($p < 0.5$). 4-tert-octylphenol treatment did not alter the pattern of StAR protein expression, whereas in DES treated groups StAR expression was suppressed by 41-44 %. No changes were found for 3ß-HSD type I protein expression.

In a study aiming at effects of exposure to estrogenic chemicals to the developing male reproductive system, in particular to effects on the excurrent ducts of the rat testis as a specific estrogen sensitive target (Fisher et al. 1999), male rat pups (Wistar) received s.c. injections of 2 mg 4-tert-octylphenol (in oil) at serial doses from pnd 2-12, i.e. 11 doses altogether. This dosing approached the maximum solubility in oil and is considered an approximate equivalent exposure to 150 mg/kg bw/d (Williams et al. 2001). DES was administered at doses of 0.1, 1, or 10 µg/kg from pnd 2 -12 every other day and ethinylestradiol was administered at a dose of 10 µg/kg from pnd 2 -12 every other day. Groups of animals were sampled on days 10, 18, 25, 35, and 75 of age and testis and epididymis taken. Testes were processes and examined for

the morphology of the efferent ducts and rete testis (rete testis distension) and for epithelial cell height in the efferent ducts by immunocytochemistry for the water channel protein aquaporin-1 (AQP-1) and subsequent image analysis. Animals treated with 4-tert-octylphenol showed minor reductions in AQP-1 immunostaining (epithelial cell height of the efferent ducts) at days 18 and/or 25. In animals that were followed through to day 35 days and/or adulthood, these changes were no longer obvious; other parameters were either unaffected or were affected marginally and transiently. Animals treated with DES showed dose-dependent changes in testis weight and in all parameters. These effects were most pronounced at days 18 and 25 and appeared to lessen with time, although some persisted into adulthood. Neonatal treatment with ethinylestradiol caused changes broadly similar to those induced by 10 µg DES.

In a study aiming at effects of exposure to estrogenic chemicals to the developing male reproductive system, in particular pubertal development of the seminiferous cords/tubules of the testis, respectively on the first wave of spermatogenesis at puberty (Atanassova et al. 2000), male rat pups (Wistar) received s.c. injections of 2 mg 4-tert-octylphenol at serial doses from pnd 2-12, i.e. 11 doses altogether. This dosing was considered an approximate equivalent exposure of 150 mg/kg bw/d (Williams et al. 2001). DES was administered at doses of 0.01, 0.1, 1, or 10 µg/kg from pnd 2 -12 every other day. Since treated animals were maintained on a standard soy-containing diet, additional groups remained untreated but were kept on a soy-free diet. Animals were evaluated on pnd 18 (and pnd 25) for plasma levels of testosterone, inhibin and FSH, seminiferous tubule lumen formation and germ cell apoptotic index, testis weight and Sertoli cell nuclear volume, and for pubertal spermatogenesis (indicated by the nuclear volume of spermatocytes per unit Sertoli cell nuclear volume). Adult animals (pnd 80-100) were evaluated for adult testis weight and morphology and for mating and fertility. DES administration caused dose-dependent retardation of pubertal spermatogenesis on pnd 18, evidenced by decreases in testis weight, lumen formation, and spermatocyte nuclear volume per unit Sertoli cell and elevation of germ cell apoptotic index. In adulthood testes weight was decreased dose dependently in rats treated with DES neonatally with DES with only the lowest dose group showing evidence for mating and fertility. Animals treated neonatally with 4-tert-octylphenol as adults had normal testis weights and exhibited reasonably normal mating and fertility. Males from the 4-tert-octylphenol group on pnd 18 in comparison to their controls, however, showed higher testis weight and slightly increased Sertoli cell nuclear volume as well as some advance in lumen formation and an increase in spermatocyte nuclear volume per unit Sertoli cell nuclear volume. The latter was also seen in the animals from the two lower DES groups (0.01 and 0.1 µg/kg) and in animals from the soy-free diet group. These effects, however, were not seen when animals were evaluated on pnd 25 and therefore considered a kind of transient stimulatory effect on these processes.

In a further study (Williams et al. 2001, 2001**a**) a systematic analysis of the impact of the estrogen/androgen environment of the neonatal rat on sex steroid receptor expression was performed with the seminal vesicles as a target tissue of the male reproductive system and with 4-tert-octylphenol included to represent a weak estrogenic challenge. Altered expression of androgen receptor (AR, reduction) and/or progesterone receptor (PR, induction) was considered defining features of estrogen induction of abnormalities of the developing male reproductive system, as well as altered expression of estrogen receptors (ER), in particular increased expression of ERα. Male rat pups (Wistar) received s.c. injections of 2 mg 4-tert-octylphenol at serial doses from pnd 2-12, i.e. 11 doses altogether. This dosing was considered an approximate equivalent exposure of 150 mg/kg bw/d (Williams et al. 2001). DES was administered at doses of 0.1, 1, or 10 µg/kg and EE was administered at a dose of 10 µg/kg from pnd 2 -12 every other day. Animals were evaluated on pnd 18 and seminal

vesicles taken for evaluation of gross morphology and processed for immunolocalisation (including semiquantitation) of stromal and epithelial estrogen ERβ, ERα, AR and PR. Treatment with 10 µg DES/kg induced loss of epithelial and stromal AR expression coincident with induction of stromal PR expression and upregulation of stromal expression of ERα. These changes were associated with gross distorsions (increase) of the normal stromal:epithelial tissue proportions in the seminal vesicles. Treatment with EE or the lower dosage of DES (1 µg/kg) induced similar but less pronounced changes. Treatment with 4-tert-octylphenol did not cause any detectable change in sex steroid receptor expression or in seminal vesicle tissue composition.

In a further study (Sharpe et al. 2003) a systematic analysis of the impact of the estrogen/androgen environment of the neonatal rat on the development of Leydig cells was performed with 4-tert-octylphenol included to represent a weak estrogenic challenge. Male rat pups (Wistar) received s.c. injections of 2 mg 4-tert-octylphenol at serial doses from pnd 2-12, i.e. 11 doses altogether. This dosing was considered an approximate equivalent exposure of 150 mg/kg bw/d (Williams et al. 2001). DES was administered at doses of 0.1, 1, or 10 µg/kg from pnd 2 -12 every other day. Groups of animals were sampled on pnd 18, 25 , 35 and as adults (pnd 75-93) with blood taken for the determination of plasma testosterone levels and with testes taken for organ weights determinations and determinations of Leydig (3β-HSD immunostaining positive) cell volume and number per testis. Treatment with DES caused largely dose-dependent suppression o testis growth, Leydig cell (nuclear) volume per testis and testosterone levels up to pnd 35. By adulthood, Leydig cell volume/number per testis was comparable with controls in the DES treated rats, although testosterone levels remained subnormal. Treatment with 4-tert-octylphenol had no effect on testis growth or on testosterone levels (except significantly elevated testosterone levels on pnd 18) and no effects on Leydig cell development or on final volume per testis.

In a further study (Yoshida et al. 2001) the time–course of possible alterations of the male reproductive system as well as pituitary and gonadal hormones were investigated in male Crj:Donryu rats neonatally exposed to 4-tert-octylphenol. Newborn pups (number of pups not provided) received a first s.c. injection of 100 mg/kg bw 24 h after birth. Pups were then treated every other day until pnd 15 (for a total of eight times: pnd 1, 3, 5, 7, 9, 11, 13, and 15). Body weights in control and 4-tert-octylphenol treated animals were measured throughout the study. All animals were checked for clinical signs, development and abnormalities of the external reproductive tract. At pnd 6, 10, 14, 21, and 28, and at 5, 7, 8, and 18 weeks of age four to eight animals per group were randomly selected from different litters and terminated, blood samples taken, organ weights determined from testes, ventral prostate, epididymides, liver, kidney, and thymus and organs processed for histopathological examination. Cross sections of testes were evaluated for spermatology. At 13 weeks of age, seven males from both the control and the 4-tert-octylphenol treated group were selected and mated with untreated females to examine reproductive ability. After 21 days of gestation numbers of implanted live and/or dead pups and their body weights were examined. At 18 weeks of age theses males were terminated. From these animals (n=7) epididymal sperm head numbers were determined and brains prepared for morphometry of the sexually dimorphic nucleus of the preoptic area (SDN-POA). All blood samples were examined for serum FSH, LH, inhibin, E2 and testosterone. It is reported that s.c. injections of 4-tert-octylphenol caused inflammatory changes of skin or subcutis of the pups resulting in scab formation. Body weight development was comparable between the controls and the treated groups. Slight but statistically significantly lower relative organ weights were observed for testes at the age of 10 days until 5 weeks, for epididymis at 4 weeks of age, and for prostate at 7 weeks of age. No such differences between groups were observed at the later age (8 and 18 weeks). No differences between groups were detected for the organ weights of pituitary, adrenals, liver,

kidney and spleen at any age. No treatment-related changes were observed in any reproductive organs including those with depressed weights during histopathological examination. Apparently normal fetal Leydig cells were found in both control and treated rat testes at pnd 6 and 10. Developing and mature Leydig cells were visible in the interstitium of both control and treated groups at 21 days and thereafter. Data from morphometrical stage analysis of testicular spermatogenesis did not reveal any significant differences in the number of germ cells, Sertoli cells or Leydig cells between the control and the treated groups. There were no differences between animals of the control and the treated group in mating success and fertility including average number of live embryos per litter. Epididymal sperm head numbers at 18 weeks, however, were slightly reduced ($p<0.05$) in treated animals (145 ± 22 Mio/epididymis) in comparison to the controls (190 ± 17 Mio/epididymis). No differences between groups were found in the area of the SDN-POA or in its length or width. In the 4-tert-octylphenol treated group prepubertal FSH levels were lower than in the controls until pnd 14 and markedly increased thereafter with values being significantly elevated from 5 to 8 weeks. LH levels did not reveal any consistent differences between groups. The testosterone levels in the 4-tert-octylphenol treated animals up to 7 weeks of age were significantly lower than in the controls.

In a study on Wistar rats (Bicknell et al. 1995) the ability of 4-tert-octylphenol to induce estrogen-dependent sex differences in the sexually dimorphic nucleus of the preoptic area (SDN-POA) of the rat was investigated and compared with DES. Pregnant rats on the 4 last days of gestation were injected s.c. with either ethyl oleate (vehicle control), 20 µg DES or 40 mg 4-tert-octylphenol. Following delivery of litters, all pups received a daily s.c. injection on postnatal days 1-4 of either the vehicle, 1 µg DES or 2 mg 4-tert-octylphenol. Litters were weaned at 21 days and sexes caged separately from 24 days of age. At 60 days of age, a maximum of 6 male and female rats per treatment group were sacrificed, perfused for whole body fixation and their brains removed for morphometry of the SDN-POA. SDN-POA area was calculated using computer-based image analysis. Sex-dependent differences in the size of the SDN-POA were confirmed in vehicle controls with males displaying a 115 % greater area than females. 4-tert-octylphenol treatment was found to have no effect on SDN-POA morphology, whereas DES significantly increased the SDN-POA area in females by 46 %.

In a study on Crj:Donryu rats (Katsuda et al. 2000a) the time–course of possible alterations of the female reproductive system as well as pituitary and gonadal hormones were investigated in female offspring neonatally exposed to 4-tert-octylphenol. A preliminary *pilot study* was included and performed beforehand to identify the most effective dose and dosing schedule for inducing persistent oestrus. For this purpose, female pups (number of pups not provided) were given s.c. injections of 12.5, 25, 50, and 100 mg/kg bw on pnd 1, 3, 5, 7, 9, 13, and 15. Oestrous cyclicity was checked by vaginal smears until rats were 10 weeks of age. The incidences of persistent oestrous that occurred in rats given s.c injections of 100 mg/kg bw once (on pnd 1), three times (pnd 1, 3, and 5), and eight times (pnd 1, 3, 5, 7, 9, 11, 13, and 15) were also determined. Dosing of 12.5-50 mg/kg bw on every other day until pnd 15 did not cause persistent oestrus. Dosing with 100 mg/kg bw on pnd 1,3, and 5 or every other day until pnd 15 resulted in persistent oestrus in 14 %, respectively 100 % of the animals.

For the *main study*, newborn female pups selected from different litters received a first s.c. injection of 100 mg/kg bw 24 h after birth. Pups were then treated every other day until pnd 15 (for a total of eight times: pnd 1, 3, 5, 7, 9, 11, 13, and 15). After weaning, animals were checked daily for vaginal opening. Oestrous cyclicity was monitored by examination of vaginal smears, and in animals of 10 weeks of age ovulation was confirmed by counting ova in the oviducts from some 4-tert-octylphenol treated persistent oestrus and control rats in oestrus. At pnd 6, 10, 14, 21, 28, 56, and 77 groups of animals were terminated at the oestrous stage, blood samples taken (and pooled for groups before weaning) for determination of FSH,

LH, E2 and progesterone determinations, organ weights determined from ovaries, uterus, vagina, liver, kidneys, spleen, adrenals, heart and thymus and organs processed for histopathological examination. Treatment with 100 mg/kg bw at every other day until pnd 15 significantly accelerated vaginal opening (30.6 + 0.4 days in control animals versus 26.0 + 0.4 days in treated animals; $p< 0.01$). None of the treated animals showed a regular oestrous cycle throughout the study, and persistent oestrus was ultimately observed. At 10 weeks of age, in comparison to the controls no ova were found in the oviducts of treated animals. Body weights of the treated rats were statistically significantly lower compared to controls at pnd 6 and 14 and thereafter remained at slightly lower levels without statistical significance. Relative weights of the uterus (0.45 + 0.06 g) of the treated groups at pnd 10 were statistically significantly increased ($p< 0.01$) compared to controls (0.20 + 0.03 g). Relative weights of the ovaries of the treated groups were significantly lower than those in controls, in particular at pnd 56 and 77. Other organ weights were not different from control values. Macroscopically, animals from the treated groups displayed enlarged cleft and a hypertrophic mucosal fold of the clitoris at vaginal opening. Histologically, treatment with 4-tert-octylphenol was associated with a reduced number of uterine glands during the immature period before weaning and significantly reduced uterine gland ontogeny thereafter. In treated animals terminated at pnd 56 and 77 changes were observed in both uterine lining epithelium and glands (tall columnar cells, intraepithelial small gland formation) diagnosed as endometrial hyperplasia. Cell proliferation assays revealed increased activities in the glands of the lining epithelium, and ERα mRNA (evaluated by in situ hybridization) was strongly expressed in particular in the lining epithelium. In the ovaries of animals from treated groups from pnd 56 and 77, atrophic changes characterized by follicular cysts and a complete absence of corpora lutea were found. In the 4-tert-octylphenol treated groups prepubertal FSH and LH levels were consistently lower than in the controls. In particular, serum FSH levels of treated animals remained low, whereas in the controls FSH levels increased significantly between pnd 14-28 and thereafter decreased again. Prepubertal LH levels were also consistently lower than the controls, however, compared to the levels of controls increased after weaning and remained high until termination. Serum E2 levels of the treated animals demonstrated essentially the same pattern and levels as in the controls. Serum progesterone pattern and levels were comparable in treated animals and in controls until weaning, thereafter levels in treated animals were about half those of the control values.

In a study on Wistar rats (Herath et al. 2001) effects of administration of 4-tert-octylphenol to neonatal females on estrogen-induced daily surges of pituitary gonadotropins were investigated. Fourteen to 20 pups per group were s.c. injected with either 100 mg 4-tert-octylphenol/kg bw, 500 µg 17β-estradiol or with DMSO (vehicle control) from pnd 1-15 every other day (total of eight injections). Dose selection was based on the study of Katsuda et al. (2000a) using a dose high enough to induce persistent vaginal oestrous. Pups were weaned at pnd 26 and checked daily for vaginal opening and thereon vaginal smears were taken daily to check cyclicity until day 110. In a subset on pnd 78, spontaneous preovulatory LH surge was investigated in plasma of DMSO treated rats in the 4-tert-octylphenol and estradiol treated rats with persistent oestrous (n=4/group) on four consecutive days. In a further subset on pnd 107, DMSO, 4-tert-octylphenol as well as estradiol treated animals underwent bilateral ovarectomy (ovx) with implantation of silastic tubules containing 17β-estradiol (1mg/ml) eight days later (n=5-7). Subsequently blood sampling was performed in the morning and in the afternoon on three consecutive days. In a further subset on pnd 186, DMSO and 4-tert-octylphenol treated animals (n=4-5/group) underwent bilateral ovarectomy (ovx) and six days later received s.c. injections of estradiol benzoate and 24 h later s.c. injections of progesterone – a treatment regime shown previously to induce receptive behaviour in ovx females in response to stimulus males – and behavioural testing was performed 4 h after progesterone treatment. Sexual receptivity was quantified by lordosis quotient and lordosis rating.

Proceptive behaviour was recorded by noting ear-wiggling and hopping. Animals from this latter subset also served for tracing of the SDN-POA areas in the brain (pnd 200) in two brain sections/animal. In the 4-tert-octylphenol and 17β-estradiol treated animals vaginal opening was significantly advanced (pnd 17.1 and 17.0) in comparison to the DMSO controls (pnd 32.9) with 17β-estradiol treated animals entering persistent oestrous at about pnd 38 and 4-tert-octylphenol treated animals at about pnd 68 (preceded with irregular cyclicity). Spontaneous LH surge in the intact animals was seen in the DMSO controls in the afternoon of day 3 of the 4-day monitoring period, but failed in animals pretreated with 4-tert-octylphenol or with 17β-estradiol. 17β-estradiol-induced surges in plasma LH, FSH and PRL levels in ovx animals revealed significant increases in plasma LH, FSH and PRL levels in the afternoon of day 2 and day 3 of the 3-day monitoring period, but failed in animals pretreated with 4-tert-octylphenol or with 17β-estradiol. Animals of the 4-tert-octylphenol treated group (n=5) in comparison to animals of the DMSO control (n=4) had a lower lordosis quotient and rating, and the relative area of the SDN-POA was reported to be larger than in the DMSO group. These observations, however, are based on the investigation of very few animals only.

In a further study (Myllymäki et al. 2005b) effects of prepubertal s.c. injections of 4-tert-octylphenol on plasma FSH, LH, estradiol and progesterone in Sprague Dawley female rat pups was investigated at the age of their prepubertal peak values (14-day-old). Groups of female pups (animal numbers not reported) received s.c. dosages of 10, 50, and 100 mg 4-tert-octylphenol/kg on days 6, 8, 10, and 12 of postnatal life. Body weights and survival of animals were recorded. One set of animals was terminated on postnatal day 14 and blood samples taken for plasma hormone analysis. A second set of animals was grown until postnatal day 35 and checked daily for the occurrence of vaginal opening. Ovaries were taken for (i) follicle culture (mainly primary and secondary follicles and measurement of basal secretion of estradiol, testosterone and progesterone, and of hFSH-stimulated production of cAMP production) and (ii) Western Blot analysis of ovarian StAR and FSH receptor (FSHr) protein levels in pooled ovaries. It is reported that treatment with 4-tert-octylphenol had no effects on pup survival and on body weight. The age at vaginal opening was determined in 4, 2, and 5 animals of the control, 50-mg and 100-mg dosage group, indicating that postnatal s.c. treatment with 100 mg 4-tert-octylphenol significantly advanced vaginal opening (29.4 ± 1.3 days versus 34.5 ± 0.3 days in the control group). On pnd 14, in the group exposed to 100 mg 4-tert-octylphenol/kg the plasma levels of FSH were significantly reduced (19.9 ± 3.1 ng/ml versus 41.4 ± 3.3 ng/ml in the control group) and progesterone levels were significantly increased (1.51 ± 0.2 ng/ml versus 1.03 ± 0.1 ng/ml in the control group), whereas plasma levels of LH and estradiol remained unaffected. On pnd 35 plasma FSH, LH, progesterone and estradiol levels did not differ between groups. On pnd 14, in the group exposed to 100 mg 4-tert-octylphenol/kg a decrease ($p \leq 0.001$) in ovarian StAR protein expression was observed. No effects were observed on the expression of full-length, FSHr or two splicing variants of the receptor. On pnd 35 no effects were observed on both ovarian StAR protein and FSHr protein expression.

In a study aiming at the development of reproductive function after neonatal exposure to 4-tert-octylphenol (Willoughby et al. 2005) groups of (6-8) newborn female pups (Sprague Dawley rat) received s.c. injections of 5 or 50 mg/kg bw on days 1-10 after birth. Vehicle controls received corn oil and positive controls received 0.5 mg DES/kg bw. There is no information upon the assignment of the newborn pups to the various treatment groups, however, it is reported that litter sizes were kept at eight with male pups present until weaning at 25 d. Body weights were taken weekly and day of vaginal opening recorded. Some of the pubescent animals were terminated and their ovarian and uterine weights were recorded. Their

ovaries were examined visually for the presence of corpora lutea for determination of ovulation. Pubertal LH release in terms of plasma LH levels were determined before and after onset of puberty (generally the day before vaginal opening) and in females that had been implanted sc on day 27 after birth with an empty or estradiol-filled silastic capsule. Vaginal smears of rats were performed between 40 and 60 days of age in order to determine effects on the oestrous cycle. Between 60 and 65 d of age, animals were terminated and ovaries collected for investigation of presence and numbers of corpora lutea, preantral, antral and atretic follicles in order to assess reproductive function. No significant differences were seen for body growth curves among the treatment groups. The age at vaginal opening was significantly shorter in rats treated with DES and with 50 mg 4-tert-octylphenol/kg bw. At time of puberty, most of the animals that had been treated with DES or with 50 mg 4-tert-octylphenol/kg bw did not show corpora lutea. Like wise, DES- and high-dose 4-tert-octylphenol-treated animals showed lower ovarian organ weights than the controls and higher uterine weights than the controls. Animals of the control group revealed a prepubertal LH surge, whereas animals treated with 50 mg 4-tert-octylphenol/kg bw did not exhibit a preovulatory LH surge, they rather had consistently higher basal serum LH levels than the controls. Treatment with an estradiol capsule increased afternoon release of LH in plasma of control rats, whereas control rats treated with an empty capsule did not have increased LH release. High-dose 4-tert-octylphenol-treated animals treated with an estradiol capsule failed to increase LH levels. After vaginal opening, all DES (5/5) and all 50 mg 4-tert- 4-tert-octylphenol-treated animals (14/14) exhibited persistent oestrous, whereas all control animals had regular 4-5-d cycles. Histopathology of the ovaries revealed in the DES- and 4-tert-octylphenol-treated animals significantly reduced numbers of corpora lutea, and significantly higher numbers of preantral and atretic follicles**.**

In a study on Sprague Dawley rats (Blake and Ashiru, 1997) effects of 4-tert-octylphenol (4-tert-octylphenol) administered to female neonates on vaginal opening and oestrous cyclicity were investigated. Newborn pups derived from 12 litters were mixed together on the day after birth and randomly distributed to form 12 new groups of pups. Three “new litters” each were assigned to four treatment groups, and pups at the age of one day were injected s.c. with either corn oil (vehicle control), or a single dose of 1.0 mg 4-tert-octylphenol (according to about 170 mg/kg bw assuming a pup body weight of about 6 g [Katsuda et al. 2000]), 1.7 mg methoxychlor (considered as weak estrogenic), or 1.0 mg 2,4,5-trichlorphenol (TXP) (considered as not estrogenic). The study does not report on any further evaluations of the animals, except that after weaning, the females were checked daily for vaginal opening. At 40 days of age, 9-11 females were selected at random from each of the three “new” litters to be kept for monitoring of vaginal oestrous cyclicity starting at 3 months after birth. At that time vaginal smears were prepared and examined daily for three weeks. In a total of 13 females that were raised from the 4-tert-octylphenol treated group and examined for day of vaginal opening no differences were observed with regard to vaginal patiency in comparison to the vehicle control. Eleven of these 13 females had been monitored for oestrous cyclicity and revealed 9/11 females in persistent oestrous as exhibited by the presence of epithelial and/or cornified cells daily during the 3-week examination period.

Studies with intraperitoneal injection – direct pup exposure

In a study on ICR mice (Kim S.-K. et al. 2007) effects of 4-tert-octylphenol on the expression of steroidogenic enzymes and on testosterone production were investigated on two different developmental stages. In one set juvenile 15-day-old males (n=5/group) were untreated (control), or injected intraperitoneally corn oil (vehicle control), 2 or 20 mg/kg bw 4-tert-

octylphenol on five consecutive days. In the other set adult 8-week-old males (n=5/group) were injected intraperitoneally corn oil (vehicle control), 2, 20 or 200 mg/kg bw 4-tert-octylphenol, or 2 µg ß-estradiol 17-valerate (EV, positive control) on five consecutive days. Animals were terminated 2 days after the final injection and body weights and organ weights of spleen and testes recorded and blood taken for determination of serum testosterone concentrations. Testes were subjected to histopathological evaluations including evaluation of germ cell apoptosis and analysis of testicular gene expression. Terminal body weight was significantly reduced in the juvenile animals exposed to 20 mg/kg bw 4-tert-octylphenol. Testis organ weight was significantly reduced in the juvenile animals exposed to 2 and 20 mg/kg bw 4-tert-octylphenol. Body and organ weights were unaffected in the adult animals in either treatment groups. Testes histopathology of the juvenile animals from the 20mg/kg bw dose group showed slight reduction in the lumen formation of seminiferous tubules with an increase in number of pyknotic germ cells and the overall number of germ cells inside the tubules markedly reduced. The serum testosterone concentration in the animals exposed to 20 mg/kg bw 4-tert-octylphenol during the juvenile stage was reduced to 30 % of the value observed in the control group, while in contrast to this, no significant changes were observed in the testosterone concentrations of adult mice exposed to EV or to 4-tert-octylphenol. In addition mRNAs for steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (P450scc), and 17α-hydroxylase/C_{17-20} lyase (P45017α) were down-regulated in testes of the juveniles after exposure to 4-tert-octylphenol, whereas mRNA expression of aromatase was unchanged. Animals exposed to EV or to 4-tert-octylphenol at the adult stage showed no significant changes in the gene expression of steroidogenic enzymes. P450scc was mainly detected in interstitial Leydig cells by immunohistochemical staining, and a slightly reduced expression of P450scc protein was observed in the testis of juveniles exposed to 20 mg/kg bw 4-tert-octylphenol. Histological staining for lipids with oil red O revealed a significantly lower number of positive Leydig cells in juvenile mice exposed to 20 mg/kg bw 4-tert-octylphenol with the size and number of lipid vacuoles in Leydig cells also reduced compared to those of the control testis. In summary, it was shown that juvenile exposure to 4-tert-octylphenol inhibits steroidogenesis by decreasing the expression of steroidogenic enzymes in the testis, hence resulting in decreased testosterone synthesis. Diminished lipid content in Leydig cells together with reduced transcriptional expression of the cholesterol transport gene, StAR, also support altered cholesterol metabolism and/or transport as a potential mechanism for decreased testosterone production following exposure to 4-tert-octylphenol.

Table 17: l.Compilation of available studies with pre-/peri-/postnatal exposures

Species	Duration	Dosing	Results	Reference
Oral administration – in utero exposure				
Wistar rat	Dosing of dams: gd 0-8 evaluation at gd 20	Oral gavage 15.6 31.3 62.5 125 250 500 mg/kg bw/d	500 mg: maternal death (6/6) 250 mg: maternal death (2/6) ≥ 62 mg: clinical signs, loss of fur, diarrhea ≥ 31.3 mg. body weight gain ↓ ≥ 15.6 mg: food intake ↓ 125/31.3 mg:	Harazono et al. 2001

			no of live fetuses/litter ↓ ≥ 31.3 mg: ↑ post-implantation loss	
Dutch-belted rabbit	Dosing of dams: g.d. 15-30 every other day	Oral gavage 150 mg/kg bw	1/4 pups unilaterally cryptorchid with atypical germ cells in undescended testis exhibiting morphological hallmarks of CIS	Veeramachaneni 2006
Wistar rat	Dosing of dams: pnd 1-22 (study 1) 2 weeks premating, during mating, gestation and until pnd 22 with /without culling (studies 2/3)	Oral drinking water, 4-tert-octylphenol concentrations of 10 µg/l 100 µg/l 1000 µg/l DES concentrations of 100 µg/l	no effects on testes morphology including seminiferous tubule epithelium in any of the treatment groups at 1000 µg/l 4-tert-octylphenol: rel. testes wt slightly decreased (5-15%), rel. kidney wt slightly increased, daily sperm production decreased for 10 % in study 3	Sharpe et al. 1995
ICR mouse	Dosing of dams: gd 0 until end of weaning with pup dosing continued until pnd 30	Oral drinking water 1 mg/ml (≈ 0.20 mg/kg bw/d) 10 mg/ml (≈ 1.96 mg/kg bw/d)	Inhibition of periostal bone formation (females > males)	Kamei et al. 2008
Wistar rat	Dosing of dams: during mating gestation and lactation with pup dosing continued until pnd 325-327	Oral diet containing wax with 3000 µg 4-tert-octylphenol/ g wax	body wt reduction in progeny throughout entire study period and final kidney and spleen organ wts ↓ no estrogenic effects in prepubertal males and females postpubertal females: irregular cycles and smears by the age of 6-7 months and ovarian organ weight ↓ with normal histopathology postpubertal males: absolute testes weights & seminiferous tubule diameter ↓ mating of F1 offspring unaffected ultrasound vocalisation	Pocock et al. 2002

			↓ on pnd 7, yet unaffected at pnd 12 and in adult offspring sex specific behaviour reported to be affected	
subcutaneous injection – in utero exposure				
Sprague Dawley rat	Dosing of dams: injected at g. d. 13.5, 15.5, 17.5 (in total 3x) evaluation at g.d. 19.5	4-tert-octylphenol: 0.1 1.0 10 100 mg/kg bw DES: 0.01 0.1 0.2 mg/kg bw	Fetal body weight unaffected 4-tert-octylphenol in contrast to DES did not affect fetal testicular testosterone concentrations or fetal testicular testosterone production in culture after explantation	Haavisto et al. 2003
Wistar rat	Dosing of dams: daily injected at g.d. 1-20 with offspring (m) grown up until 2.5 months of age	4-tert-octylphenol: 100, 250 mg/kg bw	<u>250 mg</u>: male progeny body wt ↑, histopathological changes in epididymis, slight ↑ in abnormal sperm no effects on testes, epididymis , prostate organ wts or on epididymal sperm count	Aydogan and Barlas 2006
Wistar rat	Dosing of dams: daily injected at g.d. 1-20 with offspring (m+f) grown up until 2.5 months of age	4-tert-octylphenol: 100, 250 mg/kg bw	<u>250 mg</u>: maternal body wt gain ↓, progeny body wt (m+f) ↑, relative organ wts of adrenals and thyroid+parathyroid ↓ in male progeny, histopathological changes in adrenal, pancreas and pituitary gland	Götekin and Barlas 2007, 2008
Wistar rat	Dosing of dams: daily injected at g.d. 1-20 with offspring grown up until 2.5 months of age	4-tert-octylphenol: 100, 250 mg/kg bw	length of pregnancy, dam body wt, litter size unaffected <u>250 mg</u>: histopathological changes in liver, kidney, spleen and changes in hematologic parameters m: body wt ↑ f: relative liver and kidney wts ↓ <u>100 mg:</u> (m) relative spleen wt ↑	Barlas and Aydogan 2009
NMRI mouse	Dosing of dams: daily injected	4-tert-octylphenol: 1	Fetal gonads: general ovarian morphology did not differ between	Sonne-Hansen et al. 2003

	at embryonic days 11.5-16.5 evaluation at birth	250 mg/kg bw DES: 0.1 mg/kg bw	groups, all germ cells had reached the oocyte stage, total number of oocytes and percentage distribution of follicular stages unaffected	
subcutaneous injection – direct pup exposure				
Sprague Dawley	male pups (10-30 per group) injected at age of 6h, and pnd 1-4 (in total 5 x) evaluations: pnd 14	4-tert-octylphenol: 10 50 100 mg/kg bw DES: 0.1 0.5 1.0 mg/kg bw flutamide: 2 5 25 mg/kg bw	4-tert-octylphenol treated pups: body and testis weight unaffected, Leydig cells and seminiferous cords unaffected 4-tert-octylphenol in contrast to DES had no effect on plasma testosterone, LH and FSH no effect on testicular basal and hCG stimulated testosterone concentration and secretion in culture after explantation no effect on testicular basal and hCG stimulated progesterone secretion no effect on the pattern of StAR and 3β-HSD type I protein expression	Mikkilä et al. 2006
Wistar rat	male pups injected pnd 2-12 (in total 11x) evaluations pnd 10 pnd 18 pnd 25 pnd 35 pnd 75	4-tert-octylphenol: 2 mg/pup DES: 0.1 1.0 10 mg/kg bw ethinylestradiol: 10 µg/kg bw	4-tert-octylphenol in contrast to DES and EE did not affect testis weight or the other endpoints except minor transient (day 18 and 25) changes in epithelial cell height of efferent ducts	Fisher et al. 1999
Wistar rat	male pups injected pnd 2-12 (in total 11x) evaluations pnd 18 pnd 25 pnd 80-100	4-tert-octylphenol: 2 mg/pup DES: 0.01 0.1 1.0 10 mg/kg bw	4-tert-octylphenol in contrast to DES did not affect testis weight and pubertal spermatogenesis or mating and fertility	Atanassova et al. 2000
Wistar rat	male pups	4-tert-	4-tert-octylphenol in	Williams et al.

	injected pnd 2-12 (in total 11x) evaluations pnd 18	octylphenol: 2 mg/pup DES: 0.1 1.0 10 mg/kg bw ethinylestradiol: 10 µg/kg bw	contrast to DES and EE had no effect on seminal vesicles morphology, AR and PR expression and on differential steroid receptor expression in stroma and epithelium	2001, 2001a
Wistar rat	male pups injected pnd 2-12 (in total 11x) evaluations pnd 18 pnd 25 pnd 35 pnd 90	4-tert-octylphenol: 2 mg/pup	4-tert-octylphenol in contrast to DES had no effect on testes growth or on plasma testosterone levels octylphenol in contrast to DES and GnRHa had no effect on Leydig cell development or on final Leydig cell volume per testis	Sharpe et al. 2003
Crj:Donryu rat	male pups injected pnd 1-15 every other day (in total 8x) evaluations pnd 6, 10, 14, 21, 28 and pnweek 5, 7, 8, 18	4-tert-octylphenol: 100 mg/kg bw	local effects at the skin (inflammation, scab formation) no effects on body wt, slight ↓ in testis wt from pnd 10 until age of 5 weeks (reversible) without histopathological changes, no effects on fetal, developing and mature Leydig cells, no effects on stage analysis of spermatogenesis (number of germ cells, Sertoli or Leydig cells), no effects on other organ wts nd histopathology, FSH levels ↓ than controls until pnd 14 and ↑ than controls from 5 to 8 weeks, LH levels unaffected, testosterone levels up to 7 weeks ↓ than in controls	Yoshida et al. 2001
Wistar rat	male and female pups injected pnd 1-4 (their dams had been treated for the last 4 days of gestation)	4-tert-octylphenol: 2 mg to pups; 40 mg to dams DES: 1 µg to pups; 20 µg to dams	brain morphometry on pnd 60 confirmed sex-dependent differences in size of SDN-POA area, 4-tert-octylphenol exposure in contrast to DES did not have any effect on morphology of the SDN-POA area	Bicknell et al. 1995

Crj:Donryu rat	female pups injected pnd 1-15 every other day (in total 8x)	4-tert-octylphenol: pilot study 12.5, 25, 50, 100 mg/kg bw main study 100 mg/kg bw	oestrus cyclicity checked until 10 weeks of age 12.5-50-mg: no persistent oestrous 100-mg: 100 % persistent oestrous lower body weight, vaginal opening accelerated, persistent oestrous, empty oviducts, uterine wt ↑ with endometrial hyperplasia, ovary wt ↓ with atrophic changes, other organ wts unaffected, FSH and LH levels lower than in controls with different pattern after weaning, progesterone levels ↓ after weaning	Katsuda et al. 2000a
Wistar rat	female pups injected pnd 1-15 every other day (in total 8x)	4-tert-octylphenol: 100 mg/kg bw 17ß-estradiol: 500 µg/kg bw	vaginal opening accelerated and persistent oestrous in 4-tert-octylphenol treated and in 17ß-estradiol treated animals, in contrast to controls, spontaneous LH surge in intact femals was seen in controls but failed in 4-tert-octylphenol treated and in 17ß-estradiol treated animals, 17ß-estradiol-induced surges in ovx females were seen in controls but failed in animals pretreated with 4-tert-octylphenol or with 17ß-estradiol, 4-tert-octylphenol treated animals displayed lower lordosis quotient and rating and relatively larger SDN-POA area when compared to controls	Herath et al. 2001
Sprague Dawley rat	female pups injected pnd 6, 8, 10, 12 (in total 4x) evaluations pnd 14 pnd 35	4-tert-octylphenol: 10 50 100 mg/kg bw	survival and body weight unaffected, at 100-mg vaginal opening advanced, 100-mg at pnd 14: plasma FSH ↓ and plasma progesterone ↑, plasma estradiol and	Myllymäki et al. 2005b

			plasma LH unaffected, StAR protein expression ↓ 100 mg at pnd 35: plasma hormone levels and Star expression pattern unaffected	
Sprague Dawley	female pups injected pnd 1-10 (in total 10x)	4-tert-octylphenol: 5 or 50 mg/kg bw DES: 0.5 mg/kg bw	no differences between groups in growth, vaginal opening advanced in DES and in 50-mg 4-tert-octylphenol treated animals with no signs of ovulation, uterine wt ↑ and ovary wt ↓ (with histopathological changes) in DES and in 50-mg 4-tert-octylphenol treated animals, failure of preovulatory LH surge in DES and in 50-mg 4-tert-octylphenol treated animals with higher basal LH levels than controls, estradiol capsule implant increased LH release in controls but failed with 50-mg 4-tert-octylphenol treated animals, persistent ostrous after vaginal opening in all DES and all 50-mg 4-tert-octylphenol treated animals	Willoughby et al. 2005
Sprague Dawley rat	female pups injected pnd 1	4-tert-octylphenol: 1 mg/kg bw methoxychlor: 1.7 mg/kg bw 2,4,5-trichlorphenol: 1 mg/kg bw	no differences for age at vaginal opening between controls and 4-tert-octylphenol treated animals, after vaginal opening 11/13 4-tert-octylphenol treated animals revealed persistent oestrous	Blake & Ashiru 1997
Intraperitoneal injection – direct pup exposure				
ICR mice	male pups injected on pnd 15 (juvenile) or at 8 weeks of age (adult)	juvenile: 4-tert-octylphenol: 2 or 20 mg/kg bw adult: 4-tert-octylphenol: 2, 20, 200 mg/kg	juvenile treatment: body wt ↓ at 20-mg 4-tert-octylphenol testis wt ↓ at 2- and at 20-mg 4-tert-octylphenol (with histopathological changes) serum testosterone level	S.-K. Kim et al. 2007

		bw 17ß-estradiol valerate: 2 µg/kg bw	↓ to 30 % of controls mRNA in testes of StAR, P450scc and P45017α ↓ lipid content of Leydig cells ↓ adult treatment: gene expression of steroidogenic enzymes unaffected	

4.9.3 Human data

No data.

4.9.4 Other relevant information

No data.

4.9.5 Summary and discussion of reproductive toxicity

4-tert-octylphenol was proposed to be classified regarding toxicity for reproduction and to be labelled with R 62-63, however, it was decided *not* to classify the subststance (ECBI/60/05 Rev. 3).

Effects on fertility resulting from oral treatment with 4-tert-octylphenol were investigated in studies which were in accordance with OECD TG 421 and 416. In one reproduction toxicity screening study evidence of fertility impairment in terms of reduced mating performance, reduced implantation rate and litter size as well as reduced weights of reproductive organs and minor microscopic changes in the testes and epididymis were observed. However these effects were only seen in the dosing group where also severe systemic toxicity including death (75 % for males and 33 % for females) occurred. Lower doses resulted also in systemic but not fertility effects. A second reproduction toxicity screening test resulted in an equivocal increase in corpora lutea at a dose that also affected food consumption and body weight gain. In a two generation study rats, no effects on any parameters of mating, fertility, pregnancy or parturition in the F0 or F1 generation were observed. Treatment related effects were observed at the highest treatment dose. This dose caused systemic toxicity which resulted in reductions of body weights and body weight gains in the F0, F1 and F2 generation. Pup body weights per litter were reduced in both F1 and F2 offspring during the later period of lactation when the pups were self-feeding and exposed to high doses of 4-tert-octylphenol. Minor delays in vaginal patency in females and preputial separation in males were observed, effects which can be contributed to the lower pup body weight. No effects on reproductive parameters, testes weights or morphology, epididymal sperm counts, or morphology, daily sperm production, efficiency of daily sperm production, or prostate or dorsal prostate weights or histopathology were observed. Furthermore, no estrogen-like effects on males or females and no low dose effects were evident. These findings are supported by the results from repeated dose studies where effects on reproductive organs were observed at the dose which also caused systemic toxicity. Overall, the effects observed in fertility studies are considered to represent systemic toxic effects or be related to general systemic toxicity and instead of being due to substance related target specific effects.

No guideline compliant studies on developmental toxicity are available.

In the available studies developmental effects of 4-tert-octylphenol were investigated resulting either from intrauterine exposure (oral administration/subcutaneous injection) or from exposure of newborns (subcutaneous injection) during the postnatal period.

4-tert-octylphenol was investigated for developmental effects in offspring in several studies using oral application to pregnant dams in various species. In rats, intrauterine exposure to 4-tert-octylphenol caused a significant decrease in the numbers of live fetuses and a significant increase in the incidence of post-implantation loss (NOAEL 15.6 mg/kg bw). But these effects occurred at doses that also caused maternal toxicity, including death, reduced body weight gain and reduced food consumption (LOAEL 15.2 mg/kg bw). Slightly reduced relative testes weights and increased relative kidney weights, as well as a 10 % decrease in daily sperm production were observed in a further study in rat adult progeny after exposure of pregnant dams to the highest tested dose of 1000 µg/l in drinking water. These results could not be reproduced in a second study, which was preformed in the same laboratory. In a further rat study with dietary exposure of pregnant dams no effects were observed in male and female offspring at the prepubertal stage. Postpubertal effects comprised irregularity of cycles with longer periods of oestrous smears and of a higher proportion of cornified smears. Mating of these animals was normal and litter sizes and weights of their offspring were not affected. None of these effects were observed in the guideline compliant two generation study with oral (dietary) administration.

Several studies investigating developmental effects of 4-tert-octylphenol used subcutaneous injection to pregnant dams with the rationale of inducing or maximising effects that with oral administration to pregnant dams could not be achieved and/or investigated. In comparison to DES, which clearly affected postnatal testosterone surge in male rat offspring, no effects were observed on postnatal testicular testosterone levels or on testicular ex vivo *in vitro* testosterone secretion after s.c. administration of 4-tert-octylphenol to rats. In a series of further studies, male and female rat offspring final body weights at 2.5 months of age were increased after intrauterine exposure to the highest applied s.c. injection (of 250 mg 4-tert-octylphenol/kg bw/d) together with findings of some histopathological changes in epididymis, liver, kidney, spleen, adrenals, thyroid, some hematological parameters and in sperm morphology. However, the results from these studies are not supported from the findings of a guideline compliant two-generation study or from the findings in rat offspring that had been directly exposed (s.c. injected) neonatally. Further, in a mouse study, s.c. administration of 4-tert-octylphenol to pregnant dams did not reveal any effects on oocytes and general ovarian morphology in newborn female offspring.

A further set of studies aimed at *directly* exposing newborns for the investigation of possible developmental effects. In these studies newborn pups were treated during the postnatal period with multiple s.c. injections using various dosages of 4-tert-octylphenol.

In comparison to DES applied subcutaneously, which clearly affected testis development and steroid synthesis, seminal vesicle tissue (including sex steroid receptor expression), as well as pubertal spermatogenesis in male pups, no effects were observed in testis morphology including Leydig cell development, plasma testosterone or gonadotropin levels, testicular testosterone and progesterone production, seminal vesicle morphology and sex steroid receptor expression at subcutaneously applied 4-tert-octylphenol dosages calculated to equal to approximately 150 mg/kg bw. A study on the *time-course* of possible alterations of 4-tert-octylphenol to the male reproductive system including various other organs as well as

pituitary and gonadal hormones did not reveal effects at subcutaneously applied 4-tert-octylphenol doses of 8 x 100 mg/kg bw, except slightly reduced epididymal sperm head numbers at 18 weeks of age. Also, the FSH levels at the prepubertal stage were lower than in controls and at the postpubertal stage were higher than in controls. Further, testosterone levels in the treated pups were found to be lower up to 7 weeks of age than in controls. Mating and fertility of these treated animals was normal and did not show differences in comparison to controls in number of live embryos/litter. Further, in comparison to subcutaneously applied DES, which induced changes in the sexually dimorphic nucleus of the preoptic area (SDN-POA) in the brains of females, no such changes were detected in the same study after s.c. application of 4-tert-octylphenol, whereas an other study reported the relative area of SDN-POA to be larger than in concurrent controls.

In female newborns repeat subcutaneous administration did not affect sexual maturation (time of vaginal opening) at doses of (4 x) 50 mg 4-tert-octylphenol/kg, whereas administration of (4 x) 100 or (10 x) 50 mg 4-tert-octylphenol/kg significantly accelerated time of vaginal opening.

Repeat s.c. administration of dosages of up to (8 x) 50 mg 4-tert-octylphenol/kg did not induce oestrous cycle irregularities in one study, whereas repeat administration of (10 x) 50 mg 4-tert-octylphenol/kg in another study induced persistent oestrous in all females. The repeat subcutaneous application of high dosages of 4-tert-octylphenol (8 x 100, respectively 10 x 500 mg/kg bw) was shown to be sufficient to completely and irreversibly disrupt oestrous cyclicity and induce persistent oestrous at a ratio of 100 %.

Repeat s.c. administration of dosages of (4 x) 50 mg 4-tert-octylphenol/kg did not interfere with pre- or postpubertal serum levels of pituitary or gonadal (E2) hormones, whereas administration of (4 x) 100 mg 4-tert-octylphenol/kg revealed changes in prepubertal FSH (decreased) and progesterone (increased) levels, changes no longer seen after puberty.

Further studies with repeat s.c. application of cycle disrupting high dosages of 4-tert-octylphenol (10 x 50 mg/kg or 8 x 100 mg/kg bw) to newborns were used to investigate additional effects in females already in persistent oestrous and revealed failure to ovulate, atrophy of ovaries, changes in uterus morphology including uterus gland development, as well as permanent changes in serum gonadotropin levels and failure of spontaneous and estradiol-induced preovulatory LH-surge. In Wistar rats, the effect pattern of such high 4-tert-octylphenol dosages (800 mg/kg bw for the period of pnd 1-15) was similar to that observed after multiple s.c. injections of (8 x) 0.5 mg 17β-estradiol/kg bw and in Sprague Dawley rats (500 mg/kg bw for the period of pnd 1-10) similar to that observed after multiple s.c. injections of (10 x) 0.5 mg DES/kg bw.

In summary, the data base regarding developmental toxicity of 4-tert-octylphenol is very heterogenious. No guideline compliant studies are available. The designs of the published studies were often aimed to investigate particular effects or mechanisms and in most studies subcutaneous injection was the preferred route of application in order to maximise effects and to circumvent systemic toxic effects, which may prevail when 4-tert-octylphenol is administered orally. As a result, the outcomes of these studies are heterogenious and often contradictory. Overall, it can be concluded that, if at all, 4-tert-octylphenol has some inherent potential of being toxic for reproduction, probably in relation to female sexual maturation and female fertility. It needs to be emphasised that such indication is derived exclusively from studies using routes of administration that are not relevant for humans, such as subcutaneous injection and administration of unrealistic high doses. The evidence from the available information, however, does not suggest that 4-tert-octylphenol after oral administration causes severe adverse effects.

4.10 Other effects

4.10.1 Estrogenic activity of 4-tert-octylphenol - *in vitro* and *in vivo* screening assays

Results of *in vitro* and *in vivo* screening of estrogenic activity of 4-tert-octylphenol concerning *environmental* species and hazard assessment are reported in chapter 5.1.2.

Table 18: *In vitro* Proliferation assays

Cell type	NOEC/NOEL	LOEC/LOEL or EC50	Relative potency	Remarks	Reference
MCF-7 cell proliferation	10^{-8} M	10^{-7} M	0.001	PC: 17β-estradiol	White et al. 1994
MCF-7 cell proliferation	10^{-8} M	10^{-7} M	$< 10^{-5}$	PC: 17β-estradiol	Dodge et al. 1996
MCF-7 cell proliferation	10^{-7} M	10^{-6} M	no data	screening of several chemicals	Desaulniers et al. 1998
MCF-7 cell proliferation			no data	2-fold higher cell count (maximum mean proliferation) than control at 10^{-7} M	Jones et al. 1998
MCF-7 cell proliferation		10^{-8} M		induction of apotosis	Diel et al. 2002
MCF-7 cell proliferation	10^{-8} M	10^{-7} M		cytotoxic at 10^{-5} M	Kwack et al. 2002
MCF-7 cell proliferation		EC50=10^{-7} M	0.0001		Rajapakse et al. 2004
MCF-7 cell proliferation		EC50=$5x10^{-6}$ M	0.00012		Olsen et al. 2005
MCF-7 cell proliferation	10^{-9} M	10^{-8} M	0.0003	PK 17β-estradiol	Isidori et al. 2010
MCF-7 cells		EC=$2x10^{-7}$ M	$6x10^{-6}$		Sahambi et al. 2010

Table 19: Receptor binding assays

Cell system/ receptor type	NOEC/NOEL	LOEC/LOEL or EC50	Relative potency	Remarks	Reference
RUC[6] of ovarectomised rats		IC50=2.1 µM (ER)	0.0007		Laws et al. 2000

6 Rat uterine cytosol

Cell system/ receptor type	NOEC/NOEL	LOEC/LOEL or EC50	Relative potency	Remarks	Reference
cytosol of RUCA-1 cells			0.001		Strunck et al. 2000
MUC[7]		EC50=0.16 µM	0.001		Yoon et al. 2000
RUC		IC50=12 µM	0.0004		Laws et al 2006
Yeast/ER		IC50=1.72 µM	0.004		Sheeler et al. 2000
MCF-7 cell/hER		IC50=3,8x10^{-5} M	0.0064		Olsen et al. 2005
bacterial expressed GST-ERα fusioned proteins		IC50=2.4 µM (human ERα) IC50=1.6 mM (mouse ERα)	0.012 0.17		Matthews et al. 2000
bacterial expressed ERα/β proteins			0.0001 (ERα) 0.0025 (ERβ)		Routledge et al. 2000
bacterial expressed ERα/β proteins		IC50=2.08 µM (ERα) IC50=3.52 µM (ERβ)	0.003 0.001		Sahambi et al. 2010
HEK293/ERα HEK293/ERβ			0.0002 0.0007		Kuiper et al. 1998
HELN[8] cells		IC50=0.04 µM	0.07		Paris et al. 2002
MCF-7 cells competitive binding	10^{-8} M	10^{-7} M	0.0001		Kwack et al. 2002

Table 20: Reporter gene assays

Cell type	NOEC/NOEL	LOEC/LOEL or EC50	Relative potency	Remarks	Reference
MCF-7 and CEF cells		10^{-7} M			White et al. 1994
MCF-7 cells		1.15 x 10^{-7}M (EC50)	8.4 x 10^{-5}	GFP induction	Kuruto-Niwa et al. 2005
MCF-7 cells/ ER-α	10^{-8} M		No data		Charles et al. 2007

[7] Mouse uterine cytosol

[8] HeLa transfected with ERα or β

Cell type	NOEC/NOEL	LOEC/LOEL or EC50	Relative potency	Remarks	Reference
MCF-7 cells	10^{-9} M	10^{-8} M		luciferase cytotoxicity at $3x10^{-5}$ M	Ghisari et al. 2009
MCF-7		1.25×10^{-5} M	no data	luciferase	Wu and Safe 2007
MCF-7 and MDA-MB-231 cells		2.5×10^{-5} M (wt-ERα; ERαΔZF1 or ΔZF2; ERα 1-553; ERα 1-537)	no data	transfected with wt or mutant ERα; single 4-tert-octylphenol concentration; mutant ERs are slightly less capable of transactivation	Wu and Safe 2007 Wu et al. 2008
MCF-7 derived MVLN cells		10^{-8} M/ $1.9x10^{-5}$ M	1.4×10^{-6}	luciferase	Ghisari et al. 2009
primary pituitary cells /ER	10^{-7} M	10^{-6} M		Prolactin gene expression	Abraham, 1997
HEK293/ERα and ERβ			0.7 (ERα) 0.5 (ERβ)	luciferase induction with 1 µM 4-tert-octylphenol compared to 1µM E2 (E2 luciferase activity suspected to be in the saturated range at that concentration)	Kuiper et al. 1998
HELN[8] cells		10^{-7} M		HeLa/ERα or β transfected cells luciferase LOEC lowest tested dose	Paris et al. 2002
HeLa		1.2×10^{-7} M (REC10)	0.001		Yamasaki et al. 2002
COS-1 cells	10^{-8} M	10^{-7} M	0.001		Yamakoshi et al. 2000
HepG2	1 µM ERα-AF1 10 µM wtERα and ERα-AF2	10 µM ERα-AF1		transfected with wt and mutant ERα	Yoon et al. 2000
U2 cells	1 µM ERα-AF2 10 µM wtERα and ERαAF1	10 µM ERα-AF2		transfected with wt and mutant ERα	Yoon et al. 2000

Cell type	NOEC/NOEL	LOEC/LOEL or EC50	Relative potency	Remarks	Reference
HepG2	1 µM ERα-AF1 10 µM wtERα and ERα-AF2	10 µM ERα-AF1	0.008	transfected with wt and mutant ERα	Yoon et al. 2000
U2 cells	1 µM ERα-AF2 10 µM wtERα and ERαAF1	10 µM ERα-AF2	0.008	transfected with wt and mutant ERα	Yoon et al. 2000
HepG2; U2 cells; MDA-MB-231 cells		10 µM (wt-ERα, ERα-AF2 and –AF2)	0.0025	transfected with wt and mutant ERα	Yoon et al. 2001
Yeast/hER			0.001	β-Gal	Routledge and Sumpter 1997
Yeast/ER			0.001	β-Gal no effect with AR	Moffat et al. 2001
Yeast RMY326/hERα			0.00016	β-Gal	Isidori et al. 2006
Yeast/ERα		$2x10^{-7}$ M (EC20)		β-Gal	Li et al. 2010
Yeast/ERα		$2x10^{-7}$ M (REC10)	0.0015	β-Gal	Nishihara et al. 2000
Yeast		10^{-5} M	0.0002	β-Gal	Rehmann et al. 1999
Yeast		EC50=$4.3x10^{-7}$ M	0.0003	β-Gal	Sheeler et al. 2000
Yeast		EC=0.7 µM	0.0002	enhanced estrogen response element dependent transcriptional activatio	Sheeler et al. 2000

Table 21: *In vivo* screening assays

Test system/ model	NOEC/NOEL	LOEC/LOEL	Relative potency*	Remarks	Reference
Uterotrophic assay Rat, ovex	50 mg/kg bw	200 mg/kg bw	~0.0005 compared to ethinylestradiol	oral 3 d treatment	Diel et al. 2000
Uterotrophic assay Rat, ovex Rat, immature	50 mg/kg bw	100 mg/kg bw	0.00005 compared to E2	oral 3 d treatment in prepubertal rats and ovx adult rats	Laws et al. 2000

Test system/ model	**NOEC/NOEL**	**LOEC/LOEL**	**Relative potency***	**Remarks**	**Reference**
Uterotrophic assay Mouse, immature		56 mg/kg/d		oral gavage potency not determined, no data on positive control	Charles et al. 2007
Uterotrophic assay Rat, immature	250mg/kg bw			oral 3 d treatment PC: ethynylestradiol	Sahambi et al. 2010
Uterotrophic assay Rat, immature		10 mg	~0.0005 compared to DES	s.c., only one dose tested	Bicknell, 1995
Uterotrophic assay Rat, ovex	25 mg/kg bw	50 mg/kg bw	< 0.00005 compared to E2	2 d of s.c. treatment	Katsuda et al. 2000
Uterotrophic assay Rat, ovex	12.5 mg/kg bw	25 mg/kg bw	~0.00005 compared to E2	14 d of s.c. treatment	Katsuda et al. 2000
Uterotrophic assay Rat,ovex Rat, immature	50 mg/kg bw	100 mg/kg bw	0.00005 compared to E2	s.c. 3 d treatment	Laws et al. 2000
Uterotrophic assay Rat, ovex	10 mg/kg bw	50 mg/kg bw		s.c. application 3 d of treatment PC: E2	Kwack et al. 2002
Uterotrophic assay Rat, immature	20 mg/kg bw	200 mg/kg bw		s.c. application 3 d of treatment, 2 and 20 µg/kg/d ethynyl estradiol caused increased uterine weight.	Yamasaki et al. 2002
Uterotrophic assay	1 mg/kg bw	10 mg/kg bw	~0.01 compared to E2	unknown route of exposure	Dodge, 1996
Uterine vascular permeability			ca. 0.00002 compared to E2	single dose Swiss albino mice n=6 10^{-6}, 10^{-5}, 10^{-4} mol (206µg-20.6 mg) increased uterine vascular permeability	Milligan et al. 1998

Test system/ model	**NOEC/NOEL**	**LOEC/LOEL**	**Relative potency***	**Remarks**	**Reference**
CaBP-9k mRNA	50 mg/kg	200 mg/kg ↑	$< 5 \times 10^{-6}$ compared to E2	female Sprague-Dawley rat n=6/group 10, 50, 200, 400 mg/kg bw/d s.c. 3 d treatment sampling 24 h after last treat-ment PC: 1 µg/kg E2	Kwack et al. 2002
CaBP-9k mRNA	maternal uterus: 400 mg/kg fetal uterus: 200 mg/kg extraembryonic membrane: 200 mg/kg	maternal uterus: 600 mg/kg ↑ fetal uterus: 400 mg/kg ↑ extraembryonic membrane: 400 mg/kg ↓	ca. 0.0001 compared to E2 ca. 0.001 compared to DES	female Sprague Dawley rats n=5/group 200, 400 or 600 mg/kg bw/d s.c. treatment on GD 17, 18, 19 sampling on GD 20 no significant effect in placenta positive controls did not cause consistent effects the relative potencies were estimated based on the applied doses resulting in a similar effect no information on systemic toxicity	Hong et al. 2003
CaBP-9k protein expression				positive controls did not cause consistent effects according to the authors it is unclear whether the differences in CaBP-k9 protein levels are substance related or due to chance	

Test system/ model	**NOEC/NOEL**	**LOEC/LOEL**	**Relative potency***	**Remarks**	**Reference**
CaBP-9k mRNA	maternal uterus: 400 mg/kg pup uterus: 400 mg/kg	maternal uterus: 600 mg/kg ↑ pup uterus: 600 mg/kg ↑		female Sprague Dawley rats n=5/group 200, 400 or 600 mg/kg bw/d s.c. treatment on GD 17, 18, 19 sampling on d 5 after parturition substance ID not unequivocal, however the author published on 4-tert-octylphenol before. no information on systemic toxicity/clinical signs	Hong et al. 2004a
CaBP-9k protein expression	maternal uterus: 400 mg/kg	maternal uterus: 600 mg/kg ↑		DES: increase in protein expression in uteri of pups, no effect of 4-tert-octylphenol	
ERα mRNA Epression	maternal uterus: 200 mg/kg pup uterus: 400 mg/kg	maternal uterus: 400 mg/kg ↓ pup uterus: 600 mg/kg ↓		E2 but not DES caused a decrease ERα mRNA	

Test system/ model	NOEC/NOEL	LOEC/LOEL	Relative potency*	Remarks	Reference
CaBP-9k mRNA and protein expression		100 mg/kg: ↑ CaBP-9k protein in uterus 250 mg/kg: ↑ CaBP-9k mRNA in uterus 500 mg/kg: inhibition of 4-tert-octylphenol induced CaBP-9k mRNA and protein expression under concurrent treatment with RU486 or ICI		immature female Crj:CD-1 mice n=5/group3 d, 100, 250 and 500 mg/kg bw/d sampling 24 h after last treatment or 3, 6, 12, 24, 48 or 72 h after last treatment ↑ CaBP-9k mRNA in uterus, max. after 6-24 h, decreasing after 48 h when treated with 500 mg/kg bw/d alone	Jung et al. 2005
Delayed implanting rat model	300 mg/kg	400 mg/kg			Cummings et al. 2000
Growth hormone and prolactin mRNA and protein expression in pituitary gland	100 mg/kg	600 mg/kg ↑ GH and PRL expression (mRNA and protein)		immature female Sprague-Dawley rats ER dependency shown by co-treatment with ER inhibitor ICI	Dang et al. 2009

* the relative potencies were estimated based on doses resulting in a similar effect size.

Table 22: Mechanistic studies in vivo

Test system	Doses	Results	Reference
female Sprague Dawley rats n=3 treatment on GD 17, 18, 19 sampling on lactation day 5	600 mg/kg bw s.c.	cDNA microarray in neonate and maternal uteri compared to DES (500 µg/kg) increase in ER-responsive (e.g. c-fos, complement component 3, Calbindin 3) and other 'randomly selected' genes cDNA	Dang et al. 2007a
male and female Fischer 344 rat (pups) n=8-10/group treatment on d 1-5 after birth	0, 100 and 500 µg/pup/d s.c.	↑ ERα-expression in medial basal hypothalamus in f and in anterior pituitary in m ↑ ERβ expression in anterior pituitary in m	Khurana et al. 2000

blood samples on d 15, 20, 25 and 30 tissue samples on d 30			
BALB7c mice n=6/group	2 or 5 mg/kg bw	↑ in IL-4 production in antigen primed T cells in a dose-dependent manner IL-4 production in T cells via stimulation of the calcineurin/NF-AT signalling pathway through an ER-independent pathway	Lee at al. 2004

4.10.2 Endocrine activity of 4-tert-octylphenol other than estrogenic

Table 23: *In vitro* assays involving AR or PR binding/activation and TH dependent cell proliferation

Cell system/ receptor type	NOEC/NOEL	LOEC/LOEL or EC50	Relative potency	Remarks	Reference
RUC[9] of ovarectomised rats Receptor binding		IC50=11.4 µM	0.001	progesterone receptor	Laws et al. 2000
PALM[10] cells Receptor binding		IC50=3µM		androgen receptor	Paris et al. 2002
YEAST/AR YEAST/PR Receptor binding		IC50=1.6 µM (AR) IC50=1.2 µM (PR)			Li et al. 2010
transient transfected CHO cells (AR-CALUX)		10^{-5} M/1.2 x 10^{-6} M		Competitive response (compound in the presence of R1881)	Krüger et al. 2008
MCF-7 mRNA expression				no effect AR or PR mRNA expression	Diehl et al. 2002
Yeast/hPR-PRE	500 nm	1µM		inhibition of progesterone transactivation activity	Tran et al. 1996
rat pituitary GH3 cells		10-6 M		compared to T3 treatment cytotoxicity at 3x10-5 M	Ghisari et al. 2005

[9] Rat uterine cytosol

[10] Cell line expressing human androgen receptor

Cell system/ receptor type	NOEC/NOEL	LOEC/LOEL or EC50	Relative potency	Remarks	Reference
rat pituitary GH3 cells		10-8 M	0.036	compared to T3 treatment cytotoxicity at 3x10-5 M	Ghisari et al. 2009

Table 24: *In vivo* assays involving PR

Test system	Doses	Results	Reference
female ovarectomised Wistar rats n=6/group	10 mg/kg bw s.c.	↑ progesterone receptor mRNA in frontal cortex	Funabashi et al. 2004

4-tert-octylphenol was screened for androgenic/antiandrogenic activity in the Hershberger bioassay in rats (Yamasaki et al. 2003). Seven-week old castrated male Brl Han: WIST Jcl (GALAS) rats were orally administered via stomach tube for 10 consecutive days 4-tert-octylphenol doses of 50, 200 or 600 mg/kg bw. Decreased spontaneous locomotion was seen in rats given 200 and 600 mg/kg bw, and all rats given 600 mg/kg bw died during the administration period. Significant decrease in body weight was observed in rats given 200 mg/kg bw. No androgen agonistic effects were seen at doses of 50 or 200 mg/kg bw.

The impact of 4-tert-octylphenol on testosterone biosynthesis/testicular steroidogenic competence was investigated *in vitro* in neonatal Leydig cells derived from 6-7 days old neonatal rats (Sprague Dawley) as donors of testicular cells (Murono et al. 1999). Following exposure of cultured cells for 24 h to 4-tert-octylphenol concentrations of 0, 1, 10, 100, 500, and 2000 nM 4-tert-octylphenol together with 10 mIU/ml human chorionic gonadotropin (hCG), the lower concentrations (1 and 10 nM) consistently enhanced testosterone levels (approximately 10 to 70 % above control), whereas higher concentrations (100 to 2000 nM) progressively decreased testosterone from peak levels to approximately 40 to 80 % below control at the highest concentration. Increasing concentrations of 17ß-estradiol (1 to 1000 nM) were without effect on testosterone biosynthesis under the same conditions. The biphasic pattern of testosterone biosynthesis elicited by increasing 4-tert-octylphenol concentrations was unaffected by concomitant treatment with a pure estrogen antagonist (treatment with either 10 or 100 nM ICI 182,780). Therefore, the actions of 4-tert-octylphenol on testosterone biosynthesis by cultured neonatal Leydig cells do not appear to be mediated through classic estrogen receptor α or β pathway. Although the increase in testosterone levels after exposure to lower 4-tert-octylphenol concentrations and to 0.1 and 1.0 mM 8-Br-cAMP was attenuated, suggesting that lower 4-tert-octylphenol concentrations may alter cellular cAMP levels, because hCG-stimulated cAMP levels were unaffected by any of the 4-tert-octylphenol concentrations evaluated, it appears that its main site(s) of action occurs after the generation of cAMP. In addition, because pre-treatment of cells with increasing 4-tert-octylphenol concentrations and hCG had no effect on the conversion of steroid precursors (22(R)-hydroxycholesterol, pregnenolone, progesterone, or androstenedione) to testosterone, it seems that the main actions of 4-tert-octylphenol under the present conditions occur before the mitochondrial cholesterol side-chain cleavage step. Furthermore, because concomitant treatment of cells with various antioxidants (a-tocopherol, butylated hydroxyanisole, or ascorbic acid) did not alter the biphasic pattern of testosterone response to increasing concentrations of 4-tert-octylphenol and hCG, it seems that 4-tert-octylphenol is not acting as an anti- or pro-oxidant in producing these effects.

The impact of 4-tert-octylphenol on testosterone biosynthesis/testicular steroidogenic competence was investigated *in vitro* in precursor and immature Leydig cells derived from 23 days old prepubertal rats (Sprague Dawley) as donors (Murono et al. 2000). Exposure to increasing 4-tert-octylphenol concentrations (1 to 2000 nM) progressively decreased hCG-stimulated testosterone formation in both precursor and immature Leydig cells at higher 4-tert-octylphenol concentrations (100 or 500 to 2000 nM). Testosterone levels were reduced approximately 30 to 70% below control at the highest concentration in both cell types. Similar reductions in testosterone associated with 4-tert-octylphenol exposure were observed in cells stimulated with 1 mM 8-Br-cAMP, suggesting that the main actions of 4-tert-octylphenol occur after the generation of cAMP. Increasing concentrations of 17ß-estradiol (1 to 1000 nM) had no effect on hCG-stimulated testosterone formation in both precursor and immature Leydig cells and the inclusion of 100 nM ICI 182,780, a pure estrogen antagonist, in precursor and immature Leydig cells exposed to 4-tert-octylphenol and hCG, did not alter the inhibition by higher 4-tert-octylphenol concentrations of testosterone formation in both cell types. These results suggest that 4-tert-octylphenol is a hormonally active agent, but that some of its actions are distinct from those of 17ß-estradiol and are not mediated through the estrogen receptor α or β pathway. To further localize the potential site(s) of action of 4-tert-octylphenol, cultured precursor and immature Leydig cells were exposed to increasing concentrations of 4-tert-octylphenol and hCG for 24 h. Next, fresh media containing 1 mM 22(R) hydroxycholesterol, 1 mM pregnenolone, 1 mM progesterone, or 1 mM androstenedione was added, and the conversion of each substrate to testosterone was determined after incubation for 4 h. The conversion of androstenedione to testosterone was unaffected by exposure to 4-tert-octylphenol, suggesting that the 17ß-hydroxysteroid dehydrogenase step is not inhibited. However, the conversion of 22(R)-hydroxycholesterol, pregnenolone and progesterone all were inhibited by prior exposure to 4-tert-octylphenol and hCG. This finding suggests that the 17a-hydroxylase/c17–20-lyase step, which converts progesterone to androstenedione, is inhibited by 4-tert-octylphenol, and that the cholesterol side-chain cleavage and 3b-hydroxysteroid dehydrogenase-isomerase steps, which convert cholesterol to pregnenolone and pregnenolone to progesterone, respectively, are other potential sites of 4-tert-octylphenol action. Because concomitant exposure to the antioxidants a-tocopherol or ascorbate did not alter the inhibition of testosterone formation by higher 4-tert-octylphenol concentrations, it does not appear that 4-tert-octylphenol is acting as a pseudosubstrate for the generation of free radicals, which can damage P450 enzymes.

The impact of 4-tert-octylphenol on testosterone biosynthesis/testicular steroidogenic competence was investigated *in vitro* in adult Leydig cells derived from rats of 55-65 days of age (Sprague Dawley) as donors (Murono et al. 2001). Exposure to increasing concentrations of 4-tert-octylphenol (1 to 2000 nM), 17ß-estradiol (1 to 1000 nM), endosulfan (1 to 1000 nM) or BPA (1 to 1000 nM), alone or with 10 mIU/mL hCG for 4 or 24 h, did not lower ambient testosterone levels, although cells exposed to higher 4-tert-octylphenol concentrations + hCG for 24 h often had modest declines in testosterone (10 to 20%). Of interest, exposure to the highest concentration 4-tert-octylphenol (2000 nM) alone for 4 or 24 h increased testosterone levels (2-fold in 4-h exposed cells). Whether prior exposure to 4-tert-octylphenol + hCG for 24 h affects the subsequent conversion of steroid substrates to testosterone over 4 h was evaluated. Progressive declines in 1 mM 22(R) hydroxycholesterol, 1 mM pregnenolone, or 1 mM progesterone conversion to testosterone was observed beginning at 100 to 500 nM 4-tert-octylphenol exposure (maximal declines of 40 to 12 % of controls were observed); however, the conversion of 1 mM androstenedione to testosterone was not affected by 4-tert-octylphenol. These results suggested that 24-h exposure to 4-tert-octylphenol + hCG has no effect on 17ß-hydroxysteroid dehydrogenase, which converts androstenedione to testosterone, but that it inhibits the 17αhydroxylase/C17–20 lyase step, which converts progesterone to androstenedione. In addition, potentially, 4-tert-octylphenol

could inhibit cholesterol side/chain cleavage activity, which converts cholesterol to pregnenolone, and/or 3ß-hydroxysteroid dehydrogenase, which converts pregnenolone to progesterone. Of interest, exposure to increasing concentrations of 17ß-estradiol (1 to 1000 nM), endosulfan (1 to 1000 nM), or BPA (1 to 1000 nM) 1 hCG for 24 h had no effect on subsequent conversion of 22(R)hydroxycholesterol to testosterone. Furthermore, the inhibiting effects of 4-tert-octylphenol + hCG exposure on subsequent conversion of progesterone to testosterone was unaffected by concomitant exposure to the pure estrogen antagonist, ICI 182,780, or the antioxidants, ascorbate or dimethyl sulfoxide, suggesting that the actions of 4-tert-octylphenol are not mediated through binding to estrogen receptor α or β or by free radical induced damage to steroidogenic enzymes, respectively. These results demonstrate that direct exposure of adult Leydig cells to 4-tert-octylphenol may have subtle effects on their ability to produce testosterone, which may not be detected by measuring ambient androgen levels. In addition, the effects of 4-tert-octylphenol on Leydig cell testosterone formation appear to be different from those of the native estrogen, 17ß-estradiol, and from other reported weak xenoestrogens such as endosulfan and BPA.

Mechanisms for the increased testosterone levels in cultured rat adult Leydig cells was further investigated (Murono et al. 2002). The increase in testosterone was both dose and time sensitive, and this response was observed in medium lacking both calcium and magnesium and containing a membrane-permeable calcium chelator, suggesting that the increase in testosterone was not mediated by an increase in the permeability of extracellular calcium into cells or the redistribution/release of calcium from intracellular stores, respectively. Cellular cAMP levels also were unaffected by 4-tert-octylphenol alone in cultured Leydig cells. Furthermore, initial exposure to 2000nM 4-tert-octylphenol alone for 4 h did not alter the subsequent conversion of endogenous cholesterol or exogenously added 22 (R)hydroxycholesterol to testosterone, suggesting that the increase in testosterone was not due to the enhanced availability of endogenous cholesterol or an increase in cholesterol side-chain cleavage activity, respectively. The increase in testosterone also was observed in the presence of the pure estrogen antagonist, ICI 182,780, or a 5 α-reductase inhibitor, suggesting that this effect of 4-tert-octylphenol was not mediated through the estrogen receptor α or ß pathway or by inhibition of Leydig cell testosterone metabolism, respectively. In addition, exposure of cells to comparable concentrations of two different detergents, Triton X-100 or sodium cholate, did not increase testosterone levels, suggesting that this effect of 4-tert-octylphenol was not due to its potential detergent qualities. Although these studies did not identify specific mechanism(s) that increase constitutive testosterone levels by 4-tert-octylphenol, they identify specific pathways that appear not to be involved. The physiological relevance of this observation is not known; nevertheless, they illustrate potential diverse actions of 4-tert-octylphenol in modulating the level of androgen secreted by Leydig cells, and they emphasize that some actions of 4-tert-octylphenol do not appear to be mediated through the estrogen receptor α or ß pathway.

Haavisto et al. (2003): *ex vivo – in vitro* tissue culture of intact fetal testes (ED 19.5). Basal testosterone, progesterone, cAMP production and hCG-induced testosterone levels were determined during and after a 3-h culture period. The addition of DES and of 4-tert-octylphenol to the culture medium resulted in different performance on the *in vitro* testicular steroid hormone production. DES (100 mg/l) did not alter testosterone production but caused a two-fold increase in progesterone, whereas 4-tert-octylphenol (10, 100, 500 mg/l) significantly increased testosterone and progesterone levels. The mechanism through which 4-tert-octylphenol stimulates *in vitro* testicular steroid hormone production is unknown and would need further investigation.

Nikula et al. (1999): Test system: mLTC-1 cells – cell line of mouse Leydig tumour cells. The effects of 4-tert-octylphenol on steroidogenesis in Leydig cells by measuring the LH receptor-

mediated cAMP and progesterone (P) production in cultured mouse Leydig tumour cells (mLTC-1 cells) was investigated. After preincubation for 48 h in the presence of 4-tert-octylphenol in micromolar concentration, the hCG stimulated cAMP and progesterone (P) formation in these cells was inhibited. 4-tert-octylphenol could neither inhibit cAMP nor P formation stimulated by forskolin (Fk) or cholera toxin (CT) nor steroidogenesis stimulated by 8-BrcAMP. The preincubation of mLTC-1 cells with estradiol or diethylstilbestrol (DES) at the concentration of 1028 mol/liter had no inhibitory effect on cAMP formation stimulated by hCG or Fk, however, P production was inhibited. Similarly, both estrogens inhibited P production stimulated by 8-Br-cAMP. 4-tert-octylphenol had no effect on 125I-hCG binding to Leydig cell LH-receptors. From these results it was concluded that the chemical appears to inhibit cAMP formation and steroidogenesis in mLTC-1 Leydig tumour cells by preventing the coupling between LH receptor and the adenylate cyclase. Since estradiol did not inhibit hCG-stimulated cAMP production, it was concluded that the effects of 4-tert-octylphenols may not be estrogen related.

Nakajin et al. (2001): H295R cells – cell line derived from human adrenocortical cells – model of human steroidogenic cells. 4-tert-octylphenol (4-*t*-octylphenol) produced significant decreases in the dibutyryl cAMP-stimulated cortisol secretion by 34 % at 50 µM. Reductions in cortisol secretion were dose-dependent. To elucidate the inhibitory effects 4-tert-octylphenol on cortisol secretion from H295R cells, the effects on various steroidogenic enzymes, such as C20,22-lyase (CYP11A), 3ß-hydroxysteroid dehydrogenase type II (3ß-HSDII), 17α-hyroxylase/C17,20-lyase (CYP17), 21-hydroxylase (CYP21B) and 11ß-hydroxylase (CYP11B1), were investigated. 4-tert-octylphenol strongly inhibited CYP11A activity at 12.5 and 25 µM, and inhibited CYP17 and CYP21B at 25 µM. No effects were seen on 3ß-HSDII activity.

Akgul et al. (2008): *ex vivo – in vitro* culture of rat ovarian cells and effects on progesterone production. In contrast to 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane [HPTE], a metabolite of methoxychlor, concentrations of up to 1000 nM 4-tert-octylphenol did not have any effect on ovarian progesterone production.

Myllymäki et al. (2005a): *ex vivo – in vitro* culture of rat ovarian follicles from 14 day old rats. Concentration-dependent *in vitro* inhibition of estradiol and testosterone secretion by 4-tert-octylphenol (10^{-8}, 10^{-7}, 10^{-6} M), no effect on aromatase activity, decrease in forskolin-induced cAMP levels. DES (10^{-8}, 10^{-7}, 10^{-6} M) caused strongest decline of estradiol and testosterone secretion with no effect on either cAMP production or aromatase activity.

Myllymäki et al. (2005b): *ex vivo – in vitro* culture of rat ovarian follicles from 14 day old rats after treatment with 4-tert-octylphenol on pnds 6, 8, 10, and 12. Determination of basal and hFSH-induced secretion of estradiol, testosterone and progesterone and determination of effects of 4-tert-octylphenol on follicle growth and survival, and on follicular steroid (estradiol, progesterone, testosterone) and cAMP production. Compared to follicles from untreated rats the *ex vivo – in vitro* hormone and cAMP production was decreased in follicles from all 4-tert-octylphenol dosed animals. The authors resumed, that the actual mechanisms of 4-tert-octylphenol-induced *in vitro*-results remains to be resolved.

4.10.3 Mechanistic studies other than endocrine mechanisms

Table 25: mechanistic studies *in vitro*

Cell type/test system	Doses	Results	Reference
SerW3 cell line (sertoli cell derived)	ca. 0,1-30 µM for 24h	↓ protein levels of occludin, N-cadherin, Cx43	Fiorini et al. 2004

Cell type/test system	**Doses**	**Results**	**Reference**
human liver microsomes	0.5-50 µM	IC50=8.9 µM for inhibition of 11β-hydroxysteroid dehydrogenase type 2 (enzyme activity: 0.76 pmol/min/mg)	Ohshima et al. 2005
TE671 cell line (human medullablastoma derived)	0.005-0.5 µM for 24 h	↓ mRNA of sulfite oxidase (SUOX) and phosphoadenosine phosphosulfate synthetase 1 (PAPSS1)	Harris et al. 2007
Human liver cytosol		inhibitory effect on sulfatation of human SULT 2A1 K_i=2.8 µM	Harris et al. 2007
Primary naïve thymocytes from MHC double knockout mice	up to ca.10 µM	minor effect on T cell differentiation in this system	Iwata et al. 2004
human embryonic stem cell lines	12.5-200µM for 24-48 h	50µM: induction of apoptosis (TUNEL), ↑ protein level of Fas/FasL 100µM: cytotoxicity (MTT) ↓ protein level of markers for pluripotent status after differentiating hES to neural progenitor cells: apoptosis at 12.5 µM	Kim et al. 2006
MCF-7 cells	25 µM	4fold increase in luciferase activity as indicator of activation of MAPK, PI3K, PKA/PCK pathways compared to control	Li et al. 2006
CaCo-2 cells	44 µg	permeability of 4-tert-octylphenol through Ca-Co-2 monolayer and cycling via P glycoprotein	Yoshikawa et al. 2002
RUCA-1 cells	10 nM – 1 µM	doubled induction of complement C3 mRNA and protein expression at 100 nm and 1µM	Strunck et al. 2000
Ishikawa cells (human endometrial adenocarcinoma cell line)	100 pM-10µM	alkaline phosphatase activity NOEC 1 µM LOEC 10 µM	Wober et al. 2003
primary murine and rat splenocytes cultivated in vitro	10-4 - 10-20M (rat) 10-4 - 10-16M (mouse)	↓ cell viability by 4-tert-octylphenol and 4-tert-octylphenol-ETOs, 4-tert-octylphenol less toxic than 4-tert-octylphenol-ETOs, toxic effects not shared by 17ß-estradiol, direct cytotoxic effect of 4-tert-octylphenol appeared to be exerted by Ca2+-dependent apoptosis	Nair-Menon et al. 1996

Cell type/test system	Doses	Results	Reference
primary murine splenocytes cultivated *in vitro*	5 x 10^{-5} or 5 x 10^{-7}M	incubation of splenocytes with 17ß-estradiol prior to incubation with 4-tert-octylphenol can prevent 4-tert-octylphenol-induced ↓ of cell viability but not dexamethasone-induced ↓ of cell viability incubation of splenocytes with testosterone prior to incubation with 4-tert-octylphenol cannot prevent 4-tert-octylphenol-induced ↓ of cell viability	Blake et al. (1997)
primary murine splenocytes cultivated *in vitro*	10^{-5} - 10^{-9}M	incubation of splenocytes with tamoxifen prior to incubation with 4-tert-octylphenol can prevent 4-tert-octylphenol-induced ↓ of cell viability	Nair-Menon et al. (1999)
3T3-L1 cells (cell line of mouse fibroblasts) model for cell proliferation assays model for adipocyte formation of fully differentiated 3T3-L1 cells	45 µM	treatment with 4-tert-octylphenol revealed to be less potent than nonylphenol in stimulating cell proliferation treatment with 4-tert-octylphenol failed to accelerate terminal adipocyte formation of fully differentiated cells	Masuno et al. (2003, 2005)
C3H10T1/2 cells (cell line established from mouse embryonic tissue) model for the investigation of osteoblast differentiation	15 µg/mL	treatment with 4-tert-octylphenol revealed inhibition of osteoblast differentiation, causing a lineage shift towards adipocytes with expression of peroxisome proliferator-activated receptor r (PPARr) found to be higher in than in control cultures	Miyawaki et al. (2008)

5 ENVIRONMENTAL HAZARD ASSESSMENT

5.1 Aquatic compartment (including sediment)

5.1.1 Acute toxicity test results

This chapter provide a short summary of acute toxicity test results in order to be able to compare between acute and chronic effects for 4-tert-octylphenol.

Several acute data for fish, invertebrates and algae are available, though some of them are use with care studies. For fish acute toxicity test results are in the range of 170 – 720 µg/L, for invertebrates between 13 and 620 µg/L and for algae within the range of 300 – 1900 µg/L (lowest 72h EC_{10} = 300 µg/L nominal for *Scenedesmus subspicatus* (IUCLID, 2000). All results are included in the IUCLID-file.

5.1.2 Toxicity test results concerning endocrine disruption

In this chapter information about the endocrine mode of action of 4-tert-octylphenol and subsequent adverse effects is summarized. As effects are described in order to assess the endocrine disrupting properties of 4-tert-octylphenol, all types of information is summarized.

Conclusion on whether or not 4-tert-octylphenol is a substance of very high concern according to these data is provided in chapter 6.2.1.

5.1.2.1 General approach

No criteria are available yet on how to assess whether or not a substance has endocrine disrupting properties and/or is actually an endocrine disruptor. However, a widely accepted definition of an endocrine disruptor by the IPCS is available:

"An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations" (WHO/IPCS, 2002).

As it is assumed in this report, that a substance should fulfil at least this definition in order to be of equivalent concern (see chapter 6), information available is assessed based on the following questions:

- Does the substance influence the endocrine system?
- Are adverse effects observed likely to be a consequence of this alteration?

Whether or not information available indicates that 4-tert-octylphenol is an endocrine disruptor of equivalent concern is discussed in chapter 6.

As 4-tert-octylphenol is described in the literature as showing estrogen agonist activity (estrogen receptor activation and other estrogen like activity), information has been analysed with a focus on this mode of action. However, other modes of action have been analysed too, if information was available.

Information is summarized by organism groups. In addition in *vitro-tests* are summarized as supporting information.

5.1.2.2 *In vitro* data

In vitro results may provide information about a specific mechanism of action, in this case estrogen receptor binding. They may also provide information about the potency of this mechanism but do not consider whether or not effects may occur in intact organisms and do not provide information on the potency *in vivo* as this is influenced by pharmaco-kinetic processes such as uptake, distribution, metabolisation and excretion.

Vitellogenin induction as an indicator of an estrogen mechanism of action was examined in hepatocytes from 6 fish species. In addition, receptor binding was examined in three fish species and one amphibian species.

Table 26 summarizes the main *in vitro* systems providing mechanistic information on 4-tert-octylphenol.

Table 26: Summary of *in vitro* test results for 4-tert-octylphenol using cells from aquatic organism VTG = vitellogenin; E2 = 17ß-estradiol; EE2 = Ethinylestradiol

Vitellogenin gene expression						
Species	**Reference**	**Cell type**	**Test condition**	**Endocrine mediated measurement parameters**	**Potency (relative to 17ß-estradiol=1)** (ED [E2] / ED [OP])	**Comment**
Ameriurus nebulosus, brown bullhead catfish	(Toomey et al., 1999)	Hepato-cytes	10 - 25 - 50 - 100 µM, (2060 - 5650 - 10300 - 20600 µg/L)	- 10µM (2060µg/L) moderate induction of VTG (appr. 14ng/mL) after 24h exposure; - 50µM (10300µg/L) max. VTG (appr. 70ng/mL) after 24h exposure; - 100µM (20600µg/L) cell death (early stages of apoptosis) after 3h. Complete Inhibition by tamoxifen (1µM) at 4-tert-octylphenol concentration of 10 and 25µM; and nearly completely at 50µM 4-tert-octylphenol	No data	
Ictalurus punctatus, Channel catfish	(Monteverdi and Giulio, 1999)	Hepato-cytes	10nM – 1µM – 10µM (2.1 – 206 – 2060µg/L)	- 10nM (2.06µg/L) 4-tert-octylphenol induced VTG synthesis of 65ng/mL, - 1µM (206µg/L) induced 100ng/mL VTG, - 10µM (2060µg/L) induced 303ng/mL VTG, (duration 4-6 days, not specified) At 4-tert-octylphenol concentration of 10nM and inclusion of tamoxifen (1µM) VTG synthesis was completely inhibited. At 4-tert-octylphenol concentration of 1µM and tamoxifen (1µM) VTG synthesis was only 22 ng/mL.	No data	10 pM E2 induced consistent quantities of VTG

Species	**Reference**	**Cell type**	**Test condition**	**Endocrine mediated measurement parameters**	**Potency (relative to 17ß-estradiol=1)** (ED [E2] / ED [OP])	**Comment**
Cyprinus carpio	(Segner et al., 2003)	Hepato-cytes	Not given	- EC50: 38.2µM (7869 µg/L) after 3 days exposure	0.0031	ED50 (E2): 90nM (24.5µg/L)
Oncorhynchus mykiss	(Jobling and Sumpter, 1993)	Hepato-cytes	0.1 – to 100µM (20.6 – 20600µg/L)	- ED50: 2.11µM (434µg/L) duration not specified (2 or 4 days), - 10µM (2060µg/L): significant increase (90-fold, relative to controls) in VTG after 96 h exposure - 100µM (20600µg/L): cytotox. effect after 48h exposure,	0.000037 calculated from regression lines, 0.0011 calculated from ED values	Pos. Control: 17β-estradiol. ED50 (E2): 1,81nM (0.492µg/L)
Oncorhynchus mykiss	(Navas and Segner, 2000)	Hepato-cytes	10^{-3} to 10 µM (0.21-2060 µg/L)	- 1µM (206µg/L) significant increase (relative to controls) in VTG after 72h exposure; - At 10µM (2060 µg/L) 4-tert-octyphenol became cytotoxic.	No data	
Oncorhynchus mykiss	(Olsen et al., 2005)	Hepato-cytes	Not given	- EC50: 3.1µM (640µg/L) after 4 days exposure	0.000032	EC50 (E2): 0.1nM
Oncorhynchus mykiss	(Segner et al., 2003)	Hepato-cytes	Not given	- EC50: 41.4µM (8528µg/L) after 3 days exposure	0.00083	ED50 (E2): 26nM (7.08µg/L)
Oncorhynchus mykiss	(White et al., 1994)	Hepato-cytes	10^{-7} to 10^{-5} M (20.6 to 2060 µg/L)	- 0.1µM (20.6 µg/L): sign. increase in VTG secretion relative to controls	No data	Pos. Control: 17β-estradiol
Salmo salar	(Tollefsen et al., 2003)	Hepato-cytes	1nM to 1µM (0.206–2.06–20.6-206 µg/L)	- EC50: 0.29µM (59.7µg/L) - Acute toxicity of 4-tert-octylphenol concentrations exceeding 10µM (2060µg/L). Inhibition of VTG production by the anti-estrogen ZM 189.154 in a dose-dependent manner.	0.000091	EC50 (E2): 26pM

Species	**Reference**	**Cell type**	**Test condition**	**Endocrine mediated measurement parameters**	**Potency (relative to 17ß-estradiol=1)** (ED [E2] / ED [OP])	**Comment**
Zoarces viviparous, eelpout	(Andreassen and Korsgaard, 2000)	Cytoso-lic fraction of hepatic extracts	Injection volume: 2 mL/kg fish; 10 mg/kg 4-tert-octylphenol per injection. E2: (injection volume: 2 mL/kg fish; 0.2 mg/kg 17ß-estradiol).	Significantly elevated levels in VTG after 48h. After 336 h exposure VTG in the range mg/ml. 0.02-fold VTG level in comparison to E2 after 48 and 336 h	No data	Fish were injected intraperitoneally (i.p.) with peanut oil (control) or 4-tert-octylphenol dissolved in peanut oil in comparison with E2.
Reporter gene assays						
Chicken	(White et al., 1994)	CEFs	10^{-7} to 10^{-5} M (20.6 to 2060 µg/L)	- At 10-7 M (20.6 µg/L) 4-tert-octylphenol stimulated transcription of reporter gene (appr. 2.5-fold).	No data	cells were cotransfected with the mouse estrogen receptor
Receptor binding experiments						
Cyprinus carpio	(Segner et al., 2003)	Liver cytosol	10^{-9} to 10^{-3}M (0.206 to 206000 µg/L)	- EC50: 32 µM (6602 µg/L), (concentration where 50% of specific binding is displaced, determined from competitive displacement experiment)	0.0013	EC50 (E2): 32nM (8,72 µg/L)
Oncorhynchus mykiss	(Olsen et al., 2005)	Hepato-cytes		- IC50: 8.4 x 10-4 M (173000 µg/L) binding affinity	0.000076	IC50 (E2): 6.6 x 10^{-9} M (1.798µg/L)
Oncorhynchus mykiss	(White et al., 1994)	Hepato-cytes	10^{-10} to 10^{-3} M (0.0206 to 206000µg/L)	Kd of 1.1 x 10^{-5} M 3H-Labelled 17β-estradiol were displaced from receptor by 4-tert-octylphenol	No data	Kd-value: dissociation constant

Species	**Reference**	**Cell type**	**Test condition**	**Endocrine mediated measurement parameters**	**Potency (relative to 17ß-estradiol=1)** (ED [E2] / ED [OP])	**Comment**
Xenopus laevis	(Lutz and Kloas, 1999)	Liver cytosol	10^{-9} to 10^{-3} M (0.206 to 206000µg/L)	- Significant increase in competitive displacement of [3H]estradiol binding at 10µM (2060 µg/L), - IC50: 78320 nM (16134 µg/L); - At 10-3M (206000 µg/L) 4-tert-octyphenol was able to compete completely with [3H]E2 binding to estrogen receptors.	0,00054	IC50 (E2): 42nM Identity of test substance unclear (4-octylphenol)
Zoarces viviparus	(Andreassen and Korsgaard, 2000)	Cytoso-lic fraction of hepatic extracts from male fish	Injection volume: 2 mL/kg fish; 10 mg/kg 4-tert-octylphenol per injection. E2: (injection volume: 2 mL/kg fish; 0.2 mg/kg 17ß-estradiol).	Kd increased from 0.64 to 1.48 nM; in comparison E2 treated fish: Kd 2.62 nM after 48 h exposure. Affinity for the estrogen binding site was sign. reduced by 4-tert-octylphenol treatment at 48 h. 4-tert-octylphenol upregulated (2.5-fold) the abundance of estrogen binding sites at 48 h.	No data	Fish were injected intraperitoneally (i.p.) with peanut oil (control) or 4-tert-octylphenoldissolved in peanut oil in comparison with E2. Kd-value: dissociation constant
Zoarces viviparus	(Andreassen and Korsgaard, 2000)	Cytoso-lic fraction of hepatic extracts from female fish	10^{-10} to 10^{-4} M (0.0206 µg/L to 20600 µg/L)	- IC 50: 5900 nM (1215µg/L) IC50 is the concentration of competitor inhibiting specific binding of [3H]estradiol by 50%.	0.0011 (relative binding affinity of 4-tert-octylphenol to estrogen binding sites in comparison to estradiol, IC50 (E2)/ IC50 (OP))	IC50 (estradiol): 6.2nM

To summarize the *in vitro* estrogenic data, a competitive binding of 4-tert-octylphenol was observed in all tests. Similar to human cells effects were inhibited by tamoxifen, if examined. 4-tert-octylphenol induces *in vitro* VTG production in fish hepatocytes and binds competitively to estrogen receptors (hER and rtER). Effects started at concentrations between 2 and 8528 µg/L. 4-tert-octylphenol concentrations required to initialize this effect are 3 to 6 orders of magnitude higher than those of 17ß-estradiol, which activates estrogen receptors at very low concentrations (starting at 2.7 ng/L).

The observed effects *in vitro* support an estrogen agonist mode of action.

5.1.2.3 Fishes

With regard to the question whether or not a substance is an actual endocrine disruptor in fish a draft OECD guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption is available (OECD, 2011). Although it focuses on validated OECD test guidelines, some general information on how to assess endocrine disrupting properties can be extracted. In addition several test guidelines are available focusing on the assessment of substances with possible endocrine mode of action (OECD test guideline 229 for the fish short term reproduction assay (OECD, 2009a); OECD test guideline 230, 21d short term screening assay (OECD, 2009b) and OECD 234, fish sexual development test (OECD, 2011)) Information provided in this document is supplemented by information from other guidance documents (e.g. OECD guidance document on the diagnosis of endocrine related histopathology in fish gonads (OECD, 2010)) and information from literature (e.g. (IPCS, 2002; Kendall et al., 1998; Knacker et al., 2010; OECD, 2004)) In general two different types of effects are considered and analysed separately:

- Indicators of an endocrine mode of action and
- Effects on apical endpoints that are considered to provide evidence that a substance results in adverse effects owing to its endocrine mode of action.

Indicators of endocrine mode of action:

Indicators of an endocrine mode of action may be provided by biomarkers that are known to indicate a specific mode of action as well as by histological changes that are likely to be a direct response to an estrogen mode of action.

One of the most common biomarkers indicating an estrogen endocrine mode of action is vitellogenin (VTG). Vitellogenin is naturally produced by female fishes as a precursor of yolk proteins that are incorporated in eggs (IPCS, 2002). Induction of vitellogenin in female and (more pronounced) in male fishes is a known indicator of an estrogen agonist mode of action (IPCS, 2002; Kendall et al., 1998; Knacker et al., 2010; OECD, 2004, OECD, 2009b, OECD 2011).

With respect to histological changes according to the OECD test guideline 229 for the fish short term reproduction assay (OECD, 2009) and the guidance document on the diagnosis of endocrine related histopathology in fish gonads (OECD, 2010), the following endpoints are diagnostic for endocrine activity:

- Male: increased proportion of spermatogonia (early sperm cells), presence of testis-ova, increased testicular degeneration, interstitial (Leiydig) cell hyperplasia/hypertrophy
- Female: increased oocyte atresia, perifollicular cell hyperplasia/hypertrophy, decreased yolk formation (aromatase inhibition), changes in gonadal staging.

Other effects such as decreased proportion of spermatogonia, altered proportions of spermatozoa (mature sperm cells) and gonadal staging in males are of secondary diagnostic interest as they may also be influenced by other modes of action.

Changes in the gonadosomatic index (GSI) may provide additional information about the gonad maturation and spawning readiness (OECD, 2004). It describes changes in the relation of gonad to whole body mass and thus may be an indicator of the reproductive effort of organisms (Helfman et al., 1997). Although GSI might be influenced by other modes of action too, reduction of GSI in male fishes is regarded as a sensitive parameter in reproductive studies with estrogenic substances (OECD, 2004). However, care must be taken as the GSI is highly dependent on the individual fish (frequent spawners) or seasonal gonadal stage (seasonal breeders) [11].

In addition, the following apical endpoints are considered to be indicators of an estrogen agonist mode of action according to the draft OECD guidance document (OECD, 2011):

- Depression of male secondary sex characteristics in fathead minnow or medaka
- Female biased phenotypic sex-ratio during sexual development

Decrease in *secondary sex characteristics* in males may indicate an estrogenic mode of action but should be interpreted with caution and based on weight of evidence according to (OECD, 2009). Induction of female secondary sex characteristics in males such as uro-genital papillae in male zebrafish was shown to be significant after exposure to estrogenic substances (Kendall et al., 1998; OECD, 2004).

Change of sex-ratio towards females is a known result of estrogen exposure during sexual development (Kendall et al., 1998; IPCS, 2002; OECD, 2004, OECD 2011). In aquaculture this phenomenon is frequently used to generate all female or partial female populations by exposing fishes to exogenous estrogen active substances (Baroiller et al., 1999; Piferrer, 2001)

Whether or not endocrine mediated effects are observable highly depends on the life stage tested. For example testis-ova might be induced in adult males as at least in some species gonads remain bipotent, but sensitivity is usually highest during sexual development (e.g. (Nakamura et al., 1998)). Differences in development of fish species must be considered. *O.latipes* for example is a differentiated gonochorist that naturally develops either male or female gonads and sex is naturally not changed after gonadal development. Hormonal influence (especially of female hormones) in this species starts very early during pre-hatch development (OECD, 2004) and thus life stages under exposure need to be considered carefully while analysing test results. Especially if effects on gonadal staging are analysed the reproductive cycle of a species should be considered. Especially for total spawners having only one breeding season such as *O.mykiss* effects may be observed only during the process of maturing prior to spawning and may be missed at other times of the year.

[11] The size of the sexual gonades (testis and ovaries) increases when gonads mature prior to spawning. Depending on the spawning strategy of fish species (total spawners, spawning only once in a breeding season or lifetime versus repeated, batch or serial spawners) the gonadal size and thus the GSI may substantially increase during a spawning season, reaching maxima just before spawning (Helfman et al., 1997). In repeated spawners, this process recurs and, as their spawning is usually not synchronized, individual gonadal growth differs in time.

Indicators that adverse effects are endocrine mediated

Alteration of the endocrine system may cause adverse effects that are endocrine specific but may also influence endpoints that are not endocrine specific (Kendall et al., 1998; OECD, 2004; Knacker et al., 2010).

Secondary sex characteristics and sex-ratio, are apical endpoints that are considered to be estrogen specific.

Other endpoints such as growth, sexual maturity, reproduction and behavior are known to be sensitive to estrogens (IPCS, 2002; OECD, 2004; OECD, 2011). Fertility rate, growth, time to first spawn sex-ratio shift toward females (medaka and fathead minnow) and delay of male sexual development (zebrafish) evolved to be the most sensitive endpoints for estrogen agonists in fish full life cycle tests (Knacker et al., 2010).

Thus, in combination with indicators of endocrine activity they provide evidence of estrogen mediated effects but alone they are not diagnostic for this mode of action as they might also be influenced by other modes of action

Table 27 summarizes endpoints that are considered indicators of estrogen activity and may be affected as a result of this activity in vivo.

Table 27: Summary of endpoints that are considered during analysis of fish data

Endpoints indicating an estrogen agonist mode of action	Endpoint considered to be sensitive to an estrogen mode of action *in vivo*
• Vitellogenin induction in males • increased proportion of spermatogonia (early sperm cells), presence of testis-ova, increased testicular degeneration, interstitial (Leiydig) cell hyperplasia/hypertrophy in males • increased oocyte atresia, perifollicular cell hyperplasia/hypertrophy, decreased yolk formation (aromatase inhibition), changes in gonadal staging in females • Depression of male secondary sex characteristics in fathead minnow or medaka and induction of female secondary sex characteristics such as uro-genital papillae in zebrafish • Female biased phenotypic sex-ratio during sexual development.	• Female biased phenotypic sex-ratio during sexual development especially in medaka • Reproduction (fecundity, fertility, number of males or females with reproductive success) • Spawning behaviour • Growth of offspring

Analysis of available data for fish species:

Available data have been analyzed by summarizing information on indicators of estrogen activity and indicators of estrogen mediated adverse effects. In order to do so, exposure regime and life stages tested were considered.

Overall for 6 fish species *in vivo* data at different levels (biomarker, histology and apical endpoints) are available. In the following they are discussed species by species.

Oryzias latipes (medaka):

Overall, three tests assessing sexual development, two screening tests assessing influence of 4-tert-octylphenol on reproduction, one screening test assessing the influence of 4-tert-

octylphenol on secondary sex-characteristics and gonads after exposure of adult males and two fish full life cycle tests are available for *O.latipes*. An overview of results derived from these tests is provided in Table 28. They are discussed in the following sections by comparing results from tests with similar test design followed by an overall conclusion.

Table 28: Summary of effects of 4-tert-octylphenol in *O.latipes.* If not indicated otherwise LOEC values are reported (* study not summarized in (Environment Agency UK, 2005). VTG = vitellogenin. m = measured values. n= nominal values.. Reliability was assessed according to (Klimisch et al., 1997)

Life stage/ duration	Test condition/ conc	Vitello genin	Histology	Fertility/ Fecundity	Sex-ratio	Sec. sex charac-teristics	other	Positive control	Reference	reliability
Fish sexual developmenttest										
Eggs/ 60 d	Flow-through, 6.94; 11.4; 23.7;48.1; 94 µg/L (m)	11.4 (dose-dependency)	23.7 testis-ova (in 2 of 10 gonades) Reduced no. of spermatocytes and spermatides and increased no of early oocytes at 94 µg/L		48.1 phenotypgonads (partially reversable after 60d postexposure)		> 94 hatchability, time to hatch, growth, mortality		(Seki et al., 2003)	1
1 – 35 d posthatch / 100 d	Semi-static/ 100 µg/L (n)		indication of testis ova at 100 µg/L (6 % - 30 %, not statistically significant)		> 100 (gonadal histology)		≤ 100 µg/L (growth offspring)	E2: No testis ova at 100 µg/L	(Gray et al., 1999a)	2 – single exposure level, nom. conc.
1 d posthatch / 6 month, females	Semi-static/ 10; 25; 50µg/L (n)		indication of testis-ova at 50 µg/L (1 of 13 males) , not statistically		> 50 µg/L		,		(Gray et al., 1999b)	2 – nom. conc.

			significant							
Fish screening assays										
1 d posthatch / 6 month, exposed males mated with unexposed females	Semi-static/ 10; 25; 50 µg/L (n)		indication of testis-ova at 50 µg/L (1 of 13 males, no indication in controls) , not statistically significant)	≤ 10 fertility 25 reproductive success, courtshop behavior	> 50		≤ 10 development offspring, 25 µg/L courtship behavior, number of males with reproductive success.		(Gray et al., 1999b)	2 – nom. conc. Should not be used as key-study
Adult males / 21 d, mated with unexposed females	Flow-through 20, 41, 74; 230 µg/L (m)	≤ 20 (dose dependency)	Indication of testis-ova at 74 and 230 µg/L (one of 10 males, no indication in controls), no statistics	≤ 20 fecundity			≤ 20 embryo abnormalities		(Gronen et al., 1999)	1
Adulte male / 36 d	200 and 300 µg/L (n)		200 (testicular fibrosis) at 300 µg/L 50% of tissues affected			200 swollen urogenital papillae (4 males)		E2: swollen orogenital papillae (5 of 7) at 100µg/L , no testis-ova	(Gray et al., 1999a)	2 – single exposure level, nom. Conc.
Fish full life cycle tests										

Fertilized eggs /full life cycle	Flow-through / 0; 2; 20; 50 µg/L (m)		Indication of testis-ova in all test concentrations (2-4 %, , no testis-ova in controls), no statistic and no , no dose dependency	Indication of dose dependent reduced fertility (75% of controls in the lowest test concentration), statistically not significant.	Indication of increased number of females in all test concentrations (36% higher than in controls), no statistic, no dose dependency		≤ 2 mortality offspring	E2: At 0.1 µg/L: 4 % testis ova, Embryo toxicity, sex-ratio change, reduction of fertilisation	(Knörr and Braunbeck, 2002)	2- 4-octylphenol, some documentation missing
Fertilized eggs/ Full life cycle		9.9 (F0 and F1)	30.4 testis ova F1	82.3 fecundity,fertility			> 82.3 hatchability, time to hatch, mortality, growth, F0 and F1		(Ministry of the Environment of Japan, 2002)	4 – study report not available yet

Information from fish sexual development tests

A fully reliable fish sexual development test has been reported by (Seki et al., 2003). Although the test design does not match the draft OECD guideline for the fish sexual development test in all aspects (only 60 embryos instead of 160 embryos were used in 4 replicates and test chambers were significant smaller (1.8 L instead of 7 L), the number of replicates (4), test concentrations (at least 3) and test duration matched the OECD guideline. Validation criteria of the draft guideline were fulfilled (survival fertilized eggs in control 95%, hatchability 98%, weight and length at the end of the test 26 mm and 188 mg respectively and sex-ratio in controls 45 % males).

Vitellogenin concentration in male fish (measured as hepatic vitellogenin concentration with a medaka VTG ELISA assay kit in 9-10 fish per treatment) increased in a dose-dependent manner, being significant at 11 µg/L. At 94 µg/l vitellogenin level was nearly as high as in females. Dose-dependent increase of testis-ova was observed starting from 11 µg/L (1 of 9 male gonads) being statistically significant[12] at 23 µg/L (2 of 10 gonades). Analysis of secondary sex characteristics (shape of anal fin) revealed that sex-ratio was skewed toward females in a dose-dependent manner starting from 23 µg/L, being statistically significant at and above 48 µg/L with no males at all at 94 µg/L. In that concentration 25% of the phenotypic females still had normal male gonads while 25% had testis ova and the number of spermatocytes was reduced in nearly half of them (2 of 5). In addition, at this concentration many previtellogenic oocytes (early, premature oocytes, indicating regressed condition of the ovaries) were observed in 6 of 10 specimens of ovaries. No effects on other apical endpoints such as hatchability, time to hatch, growth, or mortality was observed.

Two other sexual development studies (Gray et al., 1999a and Gray et al., 1999b) with lower reliability (missing analytic), seem to provide contradictory data at first .Testis-ova were observed at much higher concentrations only (not even significant) and no effects on sex-ratio were observed. However, exposure started at a later stage of development than in the assay by (Seki et al., 2003) (at 1- 35d posthatch instead of directly after fertilization). For *O.latipes* it is known that female gonadal development and thus involvement of estrogens starts during the embryo stages (OECD, 2004). Thus it must be considered, that both studies by Gray et al. (Gray et al., 1999a and1999b) missed most sensitive life stages for xenobiotic estrogen agonist influence on sexual development in *O.latipes*. This conclusion is supported by results in Gray et al. (1999a) where also no testis-ova were observed at 100 µg/L 17ß-estradiol, a potent estrogen agonist.

In summary results by Seki et al. (2003) indicate that 4-tert-octylphenol influences the endocrine system in *O.latipes* during sexual development resulting in adverse effects on the sex-ratio while the two studies by Gray et al. do not allow for such an assessment because exposure did not include sensitive life stages.

Information from screening assays

A fully reliable 21d fish reproduction screening assays was reported by (Gronen et al., 1999). The test differs from OECD 229 with respect to the number of animals tested (17 males per vessel instead of 5), size of aquaria (47 fish in 20L instead of 10 fish in 2L) and age of males (6 month instead of 16 weeks) but provides sufficient treatments and replicates. It differs with regard to exposure regime (only males were exposed and mated after exposure of 21d with unexposed females) and endpoints observed, as fertility and development of offspring was included.

[12] Significance was calculated by Ministry of the Environment of Japan (2002)

Vitellogenin level in males (measured as blood VTG-concentration by Western blotting, compared to sexually mature females) increased in a dose-dependent manner with a visible vitellogenin level already at the lowest test concentration (20 µg/L). Although females were not exposed, the number of eggs laid decreased from 1108 eggs in 9 days in controls to nearly half of it in all treatment groups (no dose-dependency). In addition, the percentage of fertilized eggs was reduced significantly in all treatment groups in a dose-dependent manner from 96 % (controls) to 83 % (highest test concentration). Embryo survival decreased from 90 % in controls to 62 % in the highest test concentration in a dose-dependent manner being already significant at the lowest test concentration (survival 73.5 % at 20 µg/L). The number of embryos with abnormalities increased with increasing test concentration but was still low (max 8 of 200 embryos). Incidence of testis-ova even after exposure of adults was observed in the two highest test concentrations (in 1 of 10 gonads).

Similar effects were observed in the reproduction trial of the study by (Gray et al., 1999b). Fish were exposed 1 d posthatch for 6 month (test design described above) and after exposure was terminated, mature previously exposed males were mated with unexposed females. The number of fertilized eggs was counted (no measurement of number of eggs laid) and courtship behaviour was taped and analysed blind. The number of fertilized eggs was significantly reduced already at the lowest test concentration (10µg/L). In addition, courtship behaviour with regard to number of circles (LOEC 25µg/L), number of approaches and number of copulations (LOEC 50 µg/L) was changed and the percentage of males that fertilised eggs was reduced in a dose-dependent manner (starting at the lowest concentration, being significant at 25 µg/L with less than 30% of males with reproductive success at 50 µg/L.

In the second part of the study by Gray et al. (1999a) adult males were exposed to 4-tert-octylphenol for 36 d (4 replicates with 3 males each) and secondary sex characteristics were examined and compared with results for 100 µg/L 17ß-estradiol. Results show that 4-tert-octylphenol induces swollen urogenital papillae (in 4 males) to a similar degree as 100 µg/L E2 (5 of 7 males). Swollen urogenital papillae are female secondary sex characteristic.

In summary information from (Gronen et al., 1999) and (Gray et al., 1999b) show, that 4-tert-octylphenol influences reproduction even if males are exposed only. Changes in number of eggs laid observed by Gronen et al. (1999) as well as changes in male courtship behaviour and reproductive success observed by Gray et al. (1999b) indicate that this might be due to a change in successful copulation. But effects may also result from reduced sperm quality as observed by (Seki et al., 2003) and (Gronen et al., 1999). According to the fully valid study effects start at ≤ 20 µg/L but results by Gray et al. (1999b) indicate that even at concentrations below 10 µg/L effects may occur if males are exposed for a longer time period. Effects observed by Gronen et al. (1999) and Gray et al. (1999a) indicate, that even in adults, gonads may be affected by 4-tert-octylpenol (testis ova and inhibition of spermatogenesis) and that 4-tert-octylphenol may influence phenotypic appearance of adult medaka (changes in secondary sex characteristics) although at high concentrations.

Information from fish full life cycle tests:

Two fish full life cycle tests are available for *O.latipes*, on of which is considered as not fully reliable due to constraints in documentation and the other is scored as not assignable due to missing information. Nevertheless, effects observed in both tests fit to those observed in tests described above but differ significantly in effect concentrations.

In a study summarized by the Ministry of the Environment of Japan (2002) (Klimisch code 4), fertilized eggs were exposed until 60d posthatch of F1 generation. Changes in indicators of estrogen agonist activity were observed at similar concentrations compared to the fish sexual development test by Seki et al. (2003). Vitellogenin level in F0 generation increased in a dose-dependent manner being significant at 9.9 µg/L and above (11.9 µg/L observed by (Seki

et al., 2003)) and a dose-dependent increase of testis ova was observed being significant at 30.4 µg/L and above (5 of 7 male gonads with testis-ova while Seki et al observed testis-ova in 3 of 10 male gonads at 48 µg/L). Unfortunately sex-ratio was not reported.

In the second study provided by Knörr and Braunbeck (2002) vitellogenin was not examined, some testis-ova and some influence on sex-ratio were observed starting at 2 µg/L but both effects were not significant up to the highest concentration tested, no dose- dependency was observed and percentage of testis-ova and change in sex-ratio were in the range of historical controls (3% testis-ova, max 55 % percent of females). Dose-dependent effects were observed with regard to fertilization rate starting already at the lowest test concentration (reduced to < 75 % at 2 µg/L). Development and mortality of offspring was affected in a dose-dependent manner being significant at 20 µg/L and above if females were exposed only.

If effect concentrations are compared to those of other studies available, it becomes obvious, that the study by the Ministry of the Environment of Japan (2002) shows similar sensitivity compared to the sexual development tested by Seki et al. (2003) with regard to vitellogenin level and testis-ova. With regard to fertility it was less sensitive than the two reproduction assays available. Results by Knörr and Braunbeck (2002) with regard to testis ova and sex-ratio might provide some indication that effects may occur even at lower concentrations. They should be read with care as effects were not significant. The assay by Knörr and Braunbeck (2002) is the only test where concentrations below10 µg/L were tested. No indication of an unrealistic high sensitivity is available. Effects observed at concentrations similar to those tested in the two reproduction screening assays by Gronen et al. (1999) and Gray et al. (1999b) show a similar or even lower sensitivity (30% reduction of fertility at 20 µg/L compared to 40% at 10 µg/L observed by Gray et al. (1999b) and 50% reduction at 20 µg/L (Gronen et al., 1999).

Overall summary:

In summary at least one fully reliable sexual development tests (Seki et al., 2003) and results from two fish full life cycle tests show, that 4-tert-octylphenol influences the hormonal system of medaka with endpoints affected that are considered by the OECD draft guidance document to be estrogen agonist mediated. Apical endpoints affected in these tests fit to this mode of action. The mode of action is substantiated by *in vitro* tests and all other test results support the hypothesis that 4-tert-octylphenol influences the hormonal system of medaka by an estrogen agonist mode of action that consequently causes effects on reproduction. If compared to the criteria described in the OECD guidance document information from both type of tests (sexual development test and fish full life cycle tests) strongly indicate that 4-tert-octylphenol is an endocrine disruptor for medaka (see comparison against OECD criteria in Table 29 below).

Table 29: Summary of evidence of endocrine disrupting effects of 4-tert-octylphenol in *O.latipes*

Test sytem	Number of tests available	Indication of hormonal activity?	Apical endpoints positive?	Indication that apical endpoints fit to mode of action	OECD conclusion
Sexual development test	3	Yes, if sensitive life stages are considered. LOEC = 11 µg/L (VTG) LOEC = 23 µg/L	Yes, if sensitive life stages are considered LOEC sex-ratio 48.1 µg/L	Yes, testis ova as a first result of feminization observed at concentrations, below apical effect	Substance almost certain an actual endocrine diruptor

		(testis-ova)		concentrations, changes in sex-ratio known effect of estrogens	
Reproduction screen	2	Yes, LOEC ≤ 20 µg/L (VTG)	Yes, LOEC fertility ≤20 µg/L with some indication that it might start at 10 µg/L	Yes, reduction of fertility a result of exposure to males only, changes in courtship behavior observed	Strong evidence that the substance is an actual endocrine disruptor
Fish full life cycle	2	Yes, LOEC = 9.9 µg/L (VTG) LOEC = 30 µg/L (testis-ova)	Yes, LOEC fertility 82.3 µg/L might	Yes, reduction of fertility is a known response to estrogens, effects observed in all other tests fit to this conclusion (testis-ova, changes in sperm quality)	Substance is an actual endocrine disruptor
Overall conclusion		Yes, VTG and testis –ova if sensitive endpoints are considered	Yes, effects on sex-ratio, fertility, courtship behaviour	Yes	Substance is an actual endocrine disruptor

***Cyprinodon variegates* (sheephead minnow)**

For *C.variegates* only one, however fully reliable, fish reproduction screening assay by Karels et al. (2003) is available (see Table 31). The test design differs from OECD 212 as male fish only were exposed in two replicates for 24 d and the reproduction trial started after exposure was terminated. More replicates than required by OECD were used for the reproduction trail (10 replicates with one previously exposed male and one unexposed female each) and reproduction was assessed for 10 days. A total of 25 eggs per breading group were incubated until 3 d posthatch.

Vitellogenin level (measured as blood VTG concentration using dot/slot via immunofiltration) increased in a dose-dependent manner being significant already at the lowest test concentration (11.5 µg/L). Late sperm stages (spermatocytes) were reduced and interstitial tissue proliferation increased in a dose-dependent manner starting at 11.5 µg/L and being significant at 33.6 µg/L and above. At the same concentration (33.6 µg/L) a significant reduction of viable eggs was observed after exposure of males only (< 30% viable eggs compared to > 80 % in controls).

In summary the study shows, that 4-tert-octylphenol influences indicators of hormonal activity that are known to be estrogen agonist sensitive (VTG level in male, reduced number of late sperm stages, interstitial degradation). It influences an apical endpoint (reproduction, measured as viable eggs after exposure of males only) which is not diagnostic for estrogen activity but seems likely to be a results of the hormonal influence observed. (Summary see Table 30 below).

Table 30: Summary of evidence for endocrine disrupting effects of 4-tert-octylphenol in *C.variegatus*

Test sytem	Number of test systems	Indication of hormonal activity?	Apical endpoints positive?	Indication that apical endpoints fit to mode of action	OECD conclusion
Reproduction test	1	Yes,. LOEC = 11.5 µg/L (VTG) LOEC = 33.6 µg/L (sperm-stages, tissue changes)	Yes, LOEC viable eggs 33.6 µg/L	Yes, changes in sperm stages indicate reduced reproductive capacity of males and this fits to reduced number of viable eggs after exposure of males only.	Strong evidence that the substance is an actual endocrine disruptor

Table 31: Summary of effects of 4-tert-octylphenol in *C.variegatus.* If not indicated otherwise LOEC values are reported (* study not summarized in (Environment Agency UK, 2005). VTG = vitellogenin. m = measured values. n= nominal values. Reliability was assessed according to (Klimisch et al., 1997)

Life stage/ duration	Test condition/ conc.	Vitellogenin	Histology	Fertility/Fecundity	Sex-ratio	Sec. sex charac-teristics	other	Positive control	Reference	reliability
Adult males / 24 d	Intermittent flow-through/ 11.5; 33.6; 68.1 µg/L (m)	≤ 11.5	33.6 missing late sperm stages, testicular fibrosis	33.6 % viable eggs After mating with unexposed females					(Karels et al., 2003)	1

***Oncorhynchus mykiss* (rainbow trout)**

With respect to *O. mykiss* only two screening tests assessing endocrine activity are available which do not address apical endpoints. On the other hand, two early life stage tests address apical endpoints (growth and mortality) but do not consider endpoints assessing endocrine activity (see summary in Table 33).

A 60d post-hatch early life stage toxicity study has been carried out to an American Society for Testing and Materials (ASTM) protocol according to GLP (IUCLID, 2000). No endpoints indicating endocrine activity were analyzed. Endpoints observed were mortality, hatchability of eggs, growth of fry with the most sensitive endpoint being growth of fry (LOEC = 11 µg/L).

In a second use with care early life stage test females from an all-female population were exposed starting from post-hatch for 22 (first experiment) and 35 d in two groups of 200 individuals. Growth was monitored 86 d after exposure was terminated (experiment 1) and on day 24 of exposure and further dates until 431 days after exposure (experiment 2). In the first experiment, 86 days after the end of exposure weight of fish was significantly reduced in all treatment groups (LOEC $\leq$ 1 µg/L) but not in a dose-dependent manner. In the second experiment results are more difficult to interpret as no effects were observed until day 84 on which weight in the lowest test concentration was higher, compared to controls but during further development it changed to be significant lower compared to controls (day 300 and 466). Again effects were not dose-dependent. No changes in the gonado-somatic index were observed. However, this index should be used with care as it is highly dependent on the reproduction status of fishes.

In a fully valid screening assay by Jobling et al. (1996), adult males (seven groups with 12- 15 individuals) were exposed for 21 d and vitellogenin level and histological changes were examined but no apical endpoints. In an initial experiment vitellogenin level as well as gonadosomatic index (GSI) and sperm stages were examined while in the actual dose-response test vitellogenin level and GSI were measured only. According to the dose response experiment the LOEC for vitellogenin induction was 4.8 µg/L and no changes in GSI was observed. However, results for GSI should be considered with care as experiments were carried out in August and November and thus during a time period without reproductive acitivty of *O.mykiss*. In the initial experiment, vitellogenin induction at the only concentration tested (30 µg/L) was comparable to the induction at similar concentrations in the dose-response trial (vitellogenin concentration of 10^7 ng/ml compared to a similar concentration at 14.6 µg/L and a little bit above at 43.9 µg/L). In addition, a reduction in growth of tests compared to control was observed. This trial was started during the reproductive phase of the rainbow trout. As expected, GSI of control fish increased during the experiment while this growth was inhibited by 50% by 30 µg/L 4-tert-octylphenol. This influence on testis growth was accompanied by an increased percentage of early sperm cells (spermatocyts A) and a reduced level of later sperm stages compared to the control.

In a second fully reliable screening assay by Routledge et al. (1998), vitellogenin induction was measured in adult males after exposure for 21 d. The LOEC for vitellogenin induction was 10 µg/L.

Influence on vitellogenin level was supported by two other studies which are not further described as they used only single exposure level at relatively high test concentration (Pedersen et al., 1999; Van den Belt et al., 2003).

In summary results from single tests alone do not allow for conclusion on the endocrine activity of 4-tert-octylphenol towards rainbow trout. According to the OECD draft guideline (OECD, 2011) a positive result with regard to indicators of endocrine activity, such as

vitellogenin level, in fish screening assays indicate, that a substance is a possible endocrine disruptor, especially if positive *in vitro* data and other *in vivo* data are available. No tests are available that clearly show, that 4-tert-octylphenol does result in adverse effects in *O.mykiss* due to its estrogen activity. However, weighing the evidence it seems, that adverse effects observed in the two early life stage tests are endocrine mediated:

- Vitellogenin was induced at similar concentrations as in other fish species indicating that 4-tert-octylphenol influences the hormonal system in rainbow trout at similar concentrations as in other fish species.
- Effects observed by Jobling et al. (1996) on sperm development are comparable to effects observed in other fish species with regard to the observed effect type and the effect concentrations. For *O.latipes* and *C.variegatus* the results show, that this impact on sperm development might result in decreased reproduction. It seems likely that this also holds true for *O.mykiss.*

Data show that 4-tert-octylphenol influences the endocrine system of *O.mykiss* but life stages that proofed to be most sensitive in other fish species (reproduction) were not tested. Thus, potentially more sensitive life stages of *O. mykiss* might be affected at concentrations below those reported in Table 33.

In summary no clear conclusion can be drawn as to whether 4-tert-octylphenol is an actual endocrine disruptor in rainbow trout. However, it cannot be excluded that the effects observed are endocrine mediated and it seems likely that, similar as in other fish species, 4-tert-octylphenol influences reproduction at even lower concentrations than observed in the described tests (summary see Table 32 below).

Table 32: Summary of evidence for endocrine disrupting effects of 4-tert-octylphenol in *O.mykiss*

Test system	Number of test systems	Indication of hormonal activity?	Apical endpoints positive?	Indication that apical endpoints fit to mode of action	OECD conclusion
Short term screening test / ELS	2 / 1	Yes,. LOEC 4.8 µg/L (VTG, adult males) (LOEC ≤ 39 µg/L (increased percentage of early sperm stages (spermatogonia))	Yes, LOEC = 11 µg/L (reduced growth ELS) Some indication that effects might start at ≤ 1 µ/L (no dose-response)	No apical endpoints diagnostic for endocrine activity assessed. No conclusion with respect to growth possible	Stronge evidence for *in vivo* endocrine activity, no conclusion with respect to actual ED possible

Table 33: Summary of effects of 4-tert-octylphenol in *O.mykiss.* If not indicated otherwise LOEC values are reported (* study not summarized in (Environment Agency UK, 2005). VTG = vitellogenin. m = measured values. n= nominal values. Reliability was assessed according to (Klimisch et al., 1997)

Life stage/ duration	Test condition/ conc.	**Vitello-genin**	**Histology**	**Fertility/ Fecundity**	**Sex-ratio**	**Sec. sex charac-teristics**	**others**	**Positive control**	**Reference**	**reliability**
ELS /60 d	Flow-through/ 6.1; 11; 22; 51; 91 µg/L (measured)						11 (growth)		(IUCLID, 2000)	4 -secondary source)
Posthatched females / 22 and 35 d)	Flow-through/ 1.0; 10; 30; 50 µg/L (nominal)		> 50 (GSI)				≤ 1 (body weight) No dose-response		(Ashfield et al., 1998)	2- nom. Conc.
Adult males / 21 d	Flow-through/ 0.3; 0.6; 1.6; 4.8; 14.6; 43.9 µg/L (measured) and 39 µg/L (m) initial experiment	4.8 (dose-response experiment), ≤ 39 µg/l (initial experiment)	≤ 39 µg/L increased percentage of early sperm stages (spermatogonia), reduced GSI in initial experiment					EE2: 0.0002: VTG, increased early sperm stages	(Jobling et al., 1996)	1
Adult males / 21 d	Flow-through/ 1; 10; 100 µg/L (nominal, but verified by analytic)	10							(Routledge, 1998)	1

Zoarces viviparous (eel pout)

For *Z. viviparous* one short term screening assay and a modified early life stage test is available. Both tests include endpoints that are indicators of estrogen activity but the only apical endpoint measured was growth of offspring in the modified early life stage test. Results are summarized in Table 35.

In a study by Rasmussen et al. (Rasmussen et al., 2002) pregnant females were taken from wild and exposed during the late yolk-sack phase of embryos. *Z. viviparous* is a viviparous species which carries its progeny for about five month. Sexual differentiation takes places during yolk sack phase of the pregnancy. After exposure vitellogenin induction in females and its embryos was analyzed on the transcriptional levelas well as gonadal development of embryos and uptake of 4-tert-octylphenol. Results show that 4-tert-octylphenol bioaccumulated in the plasma of the adult females (BCF up to 550) and resulted in a transfer of 4-tert-octylphenol to the ovarian fluid. Vitellogenin induction in females, being significant already at the lowest test concentration (14 µg/L) showed that 4-tert-octylphenol acts via an estrogen mode of action in this fish species and induction in embryos show that they were exposed via the maternal ovarien fluid. While embryos from control females showed about 50 % normal female gonads and 50% presumably male gonads, in the highest test concentration only 22 % of embryos had normal presumptive male gonads and 32 % of embryos showed abnormal gonads that resembled testis-ova. Weight and length of offspring was significantly reduced at 14µg/L and above.

In another study by Rasmussen et al. (Rasmussen et al., 2005) adult males taken from wild (two replicates with 6- 7 fish) were exposed for 3 weeks and analyzed for histological changes. The experiment was performed in spring during active spermatogenesis. Vitellogenin induction was significant at 35 µg/L and above with an increase in induction similar to 0.5 µg/L 17ß-estradiol. Disruption of lobular arrangement started at 9 µg/L and resulted in severe effects in all testes comparable to effects after exposure to 0.5 µg/L 17ß-estradiol. While spermatogenesis was almost completed in control groups, spermatogenesis was impaired starting at 35 µg/L. Effects included testicular degeneration and interstitial (Leiydig) cell fibrosis increases which are known to be estrogen mediated. In addition, gonadal growth as observed in controls due to maturing of sperms was inhibited starting from 9 µg/l but being significant at 35 µg/L and above.

In summary, both tests show, that 4-tert-octylphenol alters the endocrine function both in adult as well as in embryo eel pout if the embryo is exposed via the mother. The only apical endpoint assessed does not allow for a conclusion as to whether this results in adverse effects, but it seems likely that it is a direct or indirect result of the estrogen activity observed. According to the OECD guideline, an indication of estrogen activity without knowledge of the influence on clearly estrogen mediated endpoints would result in the conclusion that the substance is a possible endocrine disruptor with strong evidence for *in vivo* endocrine activity in fish (summary see Table 34 below).

Table 34: Summary of evidence for endocrine disrupting effects of 4-tert-octylphenol in *Z.viviparous*

Test sytem	Number of test systems	Indication of hormonal activity?	Apical endpoints positive?	Indication that apical endpoints fit to mode of action	OECD conclusion
Short term screening test /	2	Yes, LOEC $\leq$ 14 µg/L	Yes, LOEC = 14 µg/L	No apical endpoints	Strong evidence for

modified ELS		(VTG, adult females) LOEC = 35 µg/L (sperm-stages, adult males). Some indication that testis might be affected at lower concentrations (≤9 µg/L, no statistics)	(reduced weight and length offspring)	diagnostic for endocrine activity assessed. No conclusion with respect to growth possible	*in vivo* endocrine activity, no conclusion with respect to actual ED possible

Table 35: Summary of effects of 4-tert-octylphenol in *Z.viviparous.* If not indicated otherwise LOEC values are reported (* study not summarized in (Environment Agency UK, 2005). VTG = vitellogenin. m = measured values. n= nominal values.. Reliability was assessed according to (Klimisch et al., 1997)

Life stage/ duration	Test condition/ conc.	**Vitello-genin**	**Histology**	**Fertility/ Fecundity**	**Sex-ratio**	**Sec. sex charac-teristics**	**others**	**Positive control**	**Reference**	**reliability**
Pregnant female and their embryos / 35 d	Flow-through / 14; 65 µg/L (measured)	≤ 14 µg/L in adult females (dose dependency)	Indication of testis-ova at the highest test concentration (32 % gonads observed were abnormal gonads, no abnormal gonads in controls), no statistic				14 µg/L (reduced weight and length offspring)	E2: no testis-ova at 0.5 µg/L, reduced weight and length offspring (not significant)	(Rasmussen et al., 2002)	2- test organisms taken from wild*
Adulte male / 3 weeks	Flow-through / 9; 35; 63 µg/L (measured)	35 µg/L (dose-dependent)	Indication of severly affected abnormal testis in all test concentrations (> 50% at the lowest concentration 100% in all other concentrations), no statistics. GSI.: LOEC= 35 µg/L					E2: similar effects on vitellogenin, abnormal testis, gondal staging and GSI at 0.5 µg/L	(Rasmussen et al., 2005)	2- test organisms taken from wild*

Danio rerio (zebra fish)

With respect to the zebra fish, one modified reproduction screening assay as well as a full life cycle study are available (see summary in Table 37). Both tests are considered as fully reliable according to Klimisch et al. (1997). Effects on indicators of estrogen activity were measured and with the endpoints fertility and fecundity, apical endpoints that might be estrogen mediated are recorded.

In the reproduction screening assay by Van den Belt et al. (2001) adult fish were exposed to 4-tert-octylphenol for 21 days. After this exposure period exposure was terminated and fish were held in breeding pairs for 5 days. No vitellogenin induction was observed up to the highest test concentration (100 µg/L). However, it should be kept in mind, that vitellogenin level was measured 5 days after the end of exposure and thus vitellogenin level might have decreased during this time period. Some effects on the gonadal size was observable in females exposed to 25 µg/L and above if only not yet spawned females (females that are just about to spawn and should have well developed gonads) are considered. No effects on fertility or fecundity was observable in all test concentrations while ethinyl-estradiol resulted in a reduced number of females starting from 0.005 µg/L.

In the fish full life cycle test conducted by Wenzel et al. (2001) and published by Segner et al. (2003) fertilized eggs were exposed and the influence on apical endpoints such as fertilization, time to first spawn, growth of offspring were examined but no potential indicators of estrogen activity. Most sensitive endpoints were fertilization rate, time to first spawn and body length of offspring with a LOEC of 35 µg/L. Effects observed were comparable to effects observed for 0.001 µg/L ethinyl estradiol.

In summary no clear conclusion about possible endocrine mediated effects can be drawn. Due to the test design (vitellogenin measurement at day 5 after the end of exposure) they do not allow to conclude whether or not endpoints indicating estrogen activity were affected or not. However, fertilization a sensitive endpoint for exposure to 4-tert-octylphenol in other fish species with clear influence on the endocrine system was affected in this species too. In addition time to first spawn, known to be a sensitive endpoint for estrogen active substances (Knacker et al., 2010) was affected (summary see Table 36 below). Thus, in a weight of evidence approach considering information for other fish species, it seems likely that effects observed are endocrine mediated.

Table 36: Summary of evidence for endocrine disrupting effects of 4-tert-octylphenol in *D.rerio*

Test sytem	Number of test systems	Indication of hormonal activity?	Apical endpoints positive?	Indication that apical endpoints fit to mode of action	OECD conclusion
FLC /modified reproduction screening	2	Not available. VTG measurement not appropriate, no other diagnostic endpoints measured (LOEC = 25 µg/L GSI adult females)	Yes, LOEC = 35 µg/L (fertility, time to first spawn, body length offspring but no effects on sex-ratio)	Yes, Effects on fertility in FLC fit to reduced GSI in screening assay. Effects observed are known to be sensitive to estrogens	No conclusion possible

Table 37: Summary of effects of 4-tert-octylphenol in *D.rerio.* If not indicated otherwise LOEC values are reported (* study not summarized in (Environment Agency UK, 2005). VTG = vitellogenin. m = measured values. n= nominal valuesReliability was assessed according to (Klimisch et al., 1997)

Life stage/ duration	Test condition/ conc.	**Vitello-genin**	**Histology**	**Fertility/ Fecundity**	**Sex-ratio**	**Sec. sex charac-teristics**	**others**	**Positive control**	**Reference**	**reliability**
Adult fish / 21 d	Semi-static/ 12.5; 25; 50; 100 µg/L (measured)	≥ 100[1]	25 µg/L (reduced GSI in females) with regard to not yet spawned females (no effects in individuals after spawing)	> 100 µg/L				EE2: number of females spawning starting at 0.005 µg/L	(Van den Belt et al., 2001)	1
Fertilized eggs / full life cycle	Flow-through/ 1.2; 3.2; 12; 35 µg/L (measured)			35 µg/L (fertilization)	> 35 µg/L		35 µg/L (time to first spawn, body length offspring)	EE2: 0.001 µg/L (body length offspring, time to first spawn, fertilization capacity)	(Wenzel et al., 2001)	1

[1] Vitellogenin was measured 5 days after the end of exposure

***Poecilia reticulata* (guppy)**

For *P.reticulata* one partial life cycle test, one screening study which included exposure of embryos during pregnancy and three additional prolonged short term endocrine screening assays are available. Results are summarized in Table 39.

In the three endocrine screening assays adult males were exposed for 60d ((Toft and Baatrup, 2001; Kinnberg and Toft, 2003), both fully reliable) or 28 d (Kinnberg et al., 2003) and analyzed for changes in spermatogenesis and secondary sex characteristics. All tests revealed effects on spermatogenesis however they were evaluated differently and started at different concentrations. Both tests analyzing histological changes in spermatogenesis found an increased number of late sperm cells and a reduced number of early stages indicating a reduced sperm mitosis however at different test concentrations. While effect started at 900 µg/L in the experiment by Kinnberg and Toft (2003), effects were already observable at 26 µg/L in the experiment by Kinnberg et al. (2003). However results should be used with care as no statistics was provide. Effects on the number of mature sperm cells (fish were stripped and sperms ejaculated) was significant at all test concentrations (LOEC ≤ 100 µg/L) observed by Toft and Baatrup (2001).

Effects on secondary sex characteristics after exposure of adults was analyzed by two of these studies (Kinnberg et al., 2003 and Toft and Baatrup, 2001). No effect on the gonopodium length was observed at 26 µg/L (Kinnberg et al., 2003) after exposure of 28 days. But males showed a reduced body coloration being significant at 300 µg/L and above after exposure of 60d (Toft and Baatrup, 2001).

In an additional screening study adult males (1 replicate, 20 individuals) were exposed for 28d to 150 µg/L 4-tert-octylphenol and changes in sexual display was examined 10 days after the exposure had stopped (Bayley et al., 1999). Sexual display of males was video taped in two aquaria with one male and one female guppy for 10 min each. Exposure to 4-tert-octylphenol reduced sexual display to half of the number of cycles and half of the duration observed in controls. Similar but even more pronounced effects were observed after exposure to 20 µg/L 17ß-estradiol.

Effects of 4-tert-octylphenol exposure on developing guppy were analyzed in two studies. Embryos in pregnant females were exposed via the mother in one study (Kinnberg et al., 2003) and sex-ratio as well as growth of offspring were analyzed. In the second study (Toft and Baatrup, 2003) newly born larvae (max 5 days after birth) were exposed for 90d and growth, sex-ratio, number of sperms and sexual behavior were analyzed. If exposed via the mother, embryos did not show significant effects with regard to development, growth and sex-ratio (Kinnberg et al., 2003). However, an increased number of early sperm stages in offspring, and an increased number of late oocyte stages were observed at the only test concentration (26 µg/L, no statistics). If exposed after birth for 90 days (Toft and Baatrup, 2003) an increased number of sperms produced by male *P.reticulata* was observed even at the lowest test concentration (LOEC ≤ 1.7 µg/L, no dose-response curve). Body coloration was reduced and gonopodium length increased in males (LOEC 200 µg/L and 149 µg/L respectively). A change in sexual behavior was observable but not statistically relevant starting at the lowest test concentration (nearly double number of cycles compared to controls). No effect on sex-ratio was observed but body length was significantly increased at 200 µg/L. A reduced number of mature oocytes or embryos was observed and corresponded to a reduced ovarian weight for females (no statistics). As *P.reticulata* is a viviparous species and males and females were exposed together a reduced number of mature oocytes and embryos in pregnant females indicate a reduced reproduction capacity of females.

In summary indication of effects on female oocyte staging in *P. reticulata* as observed by Kinnberg et al. (2003) and Toft and Baatrup (2003) provide some evidence of endocrine activity in females after exposure to 4-tert-octylphenol (starting at 26 µg/L, no statistics). Results observed for males do not allow for a definitive decision as to whether the effects observed are endocrine mediated. However, some results do indicate that 4-tert-octylphenol elicits endocrine activity in *P.reticulata*:

- Effects observed by Kinnberg and Toft (2003) and Kinnberg et al. (2003) on sperm development (reduced proportion of early stage spermatogonia and increased proportion of late sperm stages (spermatocytes) are (secondary) indicators for endocrine activity in zebra fish, medaka and fathead minnow according to (OECD, 2010). Although not definitively diagnostic for an endocrine activity these effects provide some evidence of such an effect**.**

- Changes in courtship behavior were observed in two studies after exposure of adults (Bayley et al., 1999) and new born larvae (Toft and Baatrup, 2003). Although not unambiguous as type of effects differed between these two studies, this also provides some evidence of endocrine activity.

- Similar changes in body coloration in two studies (Toft and Baatrup, (2001); Toft and Baatrup, (2003)) – although starting at rather high concentrations – might be influenced by other modes of action too but may also provide some evidence of endocrine activity. Increased gonopodium length at similar test concentrations as observed by Toft and Baatrup (2003) provides some evidence of an androgenic mode of action but is at least partially induced by estrogens too (Toft and Baatrup, 2003).

In addition,indication of effects on reproduction were observed after exposure of adults (Toft and Baatrup, 2001) and new born larvae until sexual maturity (Toft and Baatrup, 2003), starting at 900 µg/L (Number offspring/female) and 1.7 µg/L (number of mature oocytes and embryos) (no statistics).

In summary evidence of estrogen activity is available and apical endpoints affected fit to these the estrogen activity. Thus, although none of the key studies is fully reliable and statistical analysis is missing, it seems to be possible to conclude in a weight of evidence approach that 4-tert-octylphenol is likely to result in adverse effects due to an estrogen agonist mode of action (summary seeTable 38 below).

Table 38: Summary of evidence for endocrine disrupting effects of 4-tert-octylphenol in *P.reticulata*

Test sytem	Number of test systems	Indication of hormonal activity?	Apical endpoints positive?	Indication that apical endpoints fit to mode of action	OECD conclusion
Short term screening test / partial life cycle	4/1	Yes, No VTG measurement, some indication of reduced number of oocytes (change in female gonadal staging) at 1.7 µg/L and above	Yes, some indication of reduced number of mature oocytes and embryos at 1.7 µg/L and above (no statistics)	Yes, changes in female gonadal staging fit the reduced number of mature oocytes and embryos	Evidence for *in vivo* endocrine activity with potential adverse effects (but based on tests without statistics)

		(no statistics)			

Table 39: Summary of effects of 4-tert-octylphenol in *P. reticulata.* If not indicated otherwise LOEC values are reported (* study not summarized in (Environment Agency UK, 2005). VTG = vitellogenin. m = measured values. n= nominal values. Reliability was assessed according to (Klimisch et al., 1997)

Life stage/ duration	Test condition/ conc.	**Vitello-genin**	**Histology**	**Fertility/ Fecundity**	**Sex-ratio**	**Sec. sex charac-teristics**	**others**	**Positive control**	**Reference**	**reliability**
Adult males and pregnant females (exposed directly after last birth) / 28 days and until birth (26 -36 d) offspring cultured in clear water (70 d)	Flow-through/ 26 +/- 8 µg/L (measured)		indication ofreduced early sperm stages in offspring, increases number late oocyte stages, no statistics		> 26 µg/L	> 26 µg/L (gonopodium length)		E2: 0.85 µg/L (reduced early sperm stages in offspring, increases number late oocyte stages) > 0.85 µg/L(gonopodium lemthg, sex-ratio)	(Kinnberg et al., 2003)	2- single exposure level, test substance octylphenol
Adult males / 60 d	Flow-through/ 100; 300; 900 µg/L (measured)		Indication of increased number of late sperm stages compared to controls, at the highest test concentration, no statistics				60% mortality	E2: no changes in gonadal staging at 0.03 and 0.1 µg/L E2	(Kinnberg and Toft, 2003)	1
Adult males /60 d Paired with unexposed females after	Flow-through/ 100; 300; 900 µg/L (measured)		≤ 100 µg/L (Increased number of sperms)	Number offspring/female: decrease towards 79% at 900 µg/L compared to		300 µg/L (reduced body coloration		E2: 0.03 µg/L (body coloration) 0.1 µg/L (number offspring)1	(Toft and Baatrup, 2001)	1

end of exposure				controls,no statistics				µg/L (number of sperms)		
newly born larvae (max 6 d)/ 90 d	Flow-through/ 1.7; 11.7; 149; 200 µg/L (measured)		Indication of reduced number of mature oocytes and embryo at all test concentrations (dose-dependent, 33% reduction at the lowest concentration,) , no statistics)	Indication of reduced number of mature oocytes and embryo at all test concentrations (dose-dependent, 33% reduction at the lowest concentration,)	> 200 µg/L	149 µg/L (gonopodium length) 200 µg/L (reduced body coloration) Indication of increased sexual display in all test concentrations, not significant.	Increased growth of males at 149 µg/L	E2: 0,01 µg/L (reducednumber mature oocytes, embryos, gonopodium length); 0.1 µg/L (sperm cells), 0.5 µg/L (coloration, sex-ratio)	(Toft and Baatrup, 2003)	2 – wide separation of levels
Adult males/ 28 d	Flow-through/ 150 µg/L (nominal)						≤ 150 µg/L (Rate and intensity of sexual display)	E2: Similar, but even more pronounced effects at 20 µg/L	(Bayley et al., 1999)	2 single exposure level, nominal concentration

Other fish species

In addition to the fish species described above, some further test results are available for other fish species. For four species *in vitro* test results show that 4-tert-octylphenol activates the estrogen receptor and results in vitellogenin induction (see chapter 5.1.2.2). Additionally, for two species vitellogenin induction *in vivo*, indicating estrogen activity, was observed but no other endpoints were analyzed (Table 40). Vitellogenin induction started to occur at slightly higher test concentrations than for some of the species described above.

Table 40: Summary of available tests for in vivo vitellogenin induction induced by 4-tert-octylphenol in fish species not described above. Reliability was assessed according to (Klimisch et al., 1997)

Species	Test condition/ test concentration	Life stage tested / test duration	Effect concentration [µg/L]	Reference	Reliability
Pomatoschistus Minutes (Sand goby)	Flow-through/ 3; 20; 31; 101 µg/L (measured)	Immature males / 28 d	LOEC = 31 (dose dependency)	(Robinson et al., 2004)	2 - test organisms taken from wild
Rutilus rutilis (Common roach)	Flow-through / 1; 10;100 µg/L (measured)	Adulte males / 21 d	LOEC = 100 Positive control: similar induction at 0.1 µg/l E2	(Routledge et al., 1998)	1

Overall summary

Overall, indication of estrogen mediated effects was observed in all fish species tested with the only exemption being *D.rerio.* Estrogen mediated effects started to occur at concentrations between 4.8 (*O.mykiss*) and 19.9 µg/L (*O.latipes*) with respect to increased vitellogenin level in males and between 23 µg/L (*O.latipes*, testis ova) and ≤ 39µg/L (*O.mykiss*, sperm stages) with respect to histological changes.

In three of the five species (*O.latipes, C.variegatus, P.reticulata*) the observed effects on apical endpoints are very likely to be estrogen mediated. In two other fish species (*O.mykiss* and *Z.viviparous)* no apical endpoints being diagnostic for endocrine mediated effects have been examined. The most sensitive effect observed was growth of offspring. This endpoint is often regarded as an endpoint indicating systemic toxicity rather than endocrine mediated toxicity. However, it should be kept in mind, that growth is known to be mediated by estrogen activity (OECD, 2004, IPCS, 2002) and that it was among the most sensitive endpoints for estrogen agonists in fish full life cycle tests. Thus, although for these two species no clear indicator of estrogen mediated adverse effects is available, it seems very likely that the effects observed are endocrine mediated. With respect to *D.rerio* no conclusion can be drawn as the only indicator for estrogen activity examined (sex-ratio) was negative. However, growth (the most sensitive endpoints observed) is known to be sensitive to estrogens.

In summary, the results suggest that 4-tert-octylphenol acts as an endocrine disruptor in a variety of fish species (strong evidence in five of six fish species). Clearly endocrine mediated effects start to occur between 1.7 µg/L (*P.reticulata)* and 33.6 µg/L *(C.variegatus)* (see summary Table 41 below).

Table 41: Summary of available tests for *in vivo* vitellogenin induction induced by 4-tert-octylphenol in fish species not described above. Reliability was assessed according to (Klimisch et al., 1997).

Test sytem	Indication of hormonal activity?	Apical endpoints positive?	Indication that apical endpoints fit to mode of action	OECD conclusion
O.latipes	Yes, LOEC = 9.9 µg/L (VTG) LOEC = 23 µg/L testis –ova)	Yes, LOEC ≤ 20 µgL (fertility with some indication that effects might start at ≤10µg/L)	Yes, reduction of fertility is a known response to estrogens, effects observed in all other tests fit to this conclusion (testis-ova, changes in sperm quality)	Substance is an actual endocrine disruptor
C.variegatus	Yes,. LOEC = 11.5 µg/L (VTG) LOEC = 33.6 µg/L (sperm-stages, tissue changes)	Yes, LOEC viable eggs 33.6 µg/L	Yes, changes in sperm stages indicate reduced reproductive capacity of males and this fits to reduced number of viable eggs after exposure of males only.	Strong evidence that the substance is an actual endocrine disruptor
O.mykiss	Yes,. LOEC 4.8 µg/L (VTG, adult males) (LOEC ≤ 39 µg/L (increased percentage	Yes, LOEC = 11 µg/L (reduced growth ELS) Some indication that effects might start at ≤	Yes, no apical endpoints diagnostic for endocrine activity assessed but growth is known to be influenced by estrogen	Strong evidence for *in vivo* endocrine activity, no conclusion with respect to actual ED possible

	of early sperm stages (spermatogonia))	1 µ/L (no dose-dependency)	activity.	
Z.variegatus	Yes,. LOEC ≤ 14 µg/L (VTG, adult females) LOEC = 35 µg/L (sperm-stages, adult males). Some indication that testis might be affected at lower concentrations ≤9 µg/L, no statistics	Yes, LOEC = 14 µg/L (reduced weight and length offspring)	Yes, no apical endpoints diagnostic for endocrine activity assessed but growth is known to be influenced by estrogen activity.	Strong evidence for *in vivo* endocrine activity, no conclusion with respect to actual ED possible
D. rerio	Not available. VTG measurement not appropriate, no other diagnostic endpoints measured (LOEC = 25 µg/L GSI adult females)	Yes, LOEC = 35 µg/L (fertility, time to first spawn, body length offspring but no effects on sex-ratio)	Yes, Effects on fertility in FLC fit to reduced GSI in screening assay. Effects observed are known to be sensitive to estrogens	No conclusion possible
P.reticulata	Yes, No VTG measurement, some indication of reduced number of oocytes (change in female gonadal staging) at 1.7 µg/L and above (no statistics)	Yes, Some indication of reduced number of mature oocytes and embryos at 1.7 µg/L and above (no statistics)	Yes, changes in female gonadal staging fit the reduced number of mature oocytes and embryos	Evidence for *in vivo* endocrine activity with potential adverse effects (but based on tests without statistics)

5.1.2.4 Vertebrates – Amphibians

In this chapter information about the potential endocrine mode of action of 4-tert-octylphenol in amphibians is summarized, as far as available.

While in fishes estrogen and androgen effects are the most commonly assessed modes of action, in amphibians impact on the thyroid hormone level is a known potent endocrine mode of action and information about estrogen or androgen like effects is rare.

According to the OECD guideline for the amphibian metamorphosis assay (OECD, 2009b), the following effects indicate a thyroid mode of action:

- Advanced development (according to development stages or hind limb length)
- Asynchronous development
- Remarkable histological effects

Delay in development may be induced by a thyroid antagonistic mode of action, but could also be influenced by systemic toxicity. Thus, this parameter should be regarded as indicative for an endocrine mode of action only, if no systemic toxicity (reduced growth, mortality) is observable. Similar, increased body weight is often observed for substances negatively affecting normal development but should not be used alone.

No specific guidance is available on how to identify estrogen mediated effects and knowledge of

vertebrate steroid hormones and their role in normal development and reproduction in non-mammalians is scarce (U.S.EPA, 2005; OECD, 2008). In order to identify whether or not 4-tert-octylphenol induces estrogen-like effects in amphibians, the effects observed are compared to effects observed after exposure to 17ß-estradiol.

Overall, 8 studies with 4 frog species and one salamander species are available assessing possible endocrine modulated effects on development and metamorphosis. Results are summarized in Table 42. As age and developmental stages differed among studies and were examined according to different criteria (by (Nieuwkoop and Faber, 1994) and (Gosner, 1960)) information about duration, development stage and criteria used for determination are included.

Table 42: Summary of effects on amphibians after exposure to 4-tert-octylphenol. (* study not summarized in (Environment Agency UK, 2005)). E2 = 17ß-estradiol; EE2 = Ethinylestradiol. If the test substance is not exactly known, this is indicated in the last column

Species	Life stage tested/ test duration	test conditions/ test concentration	examined parameter(s)	Effect concentration(s)	Reference	Reliability (Klimisch)
Xenopus laevis	2-3 days post-hatch larvae/ 12 weeks (up to developmental stage 38/40 - (Nieuwkoop and Faber, 1994))	2.1 and 21.0 µg/L (nominal)	sex ratio , development, growth, mortality	LOEC sex ratio = 2.1 µg/L; No significant effects on development, growth and mortality E2: LOEC sex-ratio = 0.1 µM, LOEC Vitellogenin induction = 1nM, no significant effect on development, growth and mortality,	(Kloas et al., 1999)	3 - nominal conc., reproducibility unclear, no clear identity of test substance (4-octylphenol)
Xenopus laevis	Embryos (Gosner stage 10.5 up to stage 37)/ 2 days	2 – 20 – 100 – 200 – 1000 - 2000 µg/L (nominal)	body length, abnormalities (e.g. malformed cement glands)	LOEC decreased body length = 100 µg/L LOEC abnormalities = 1000 µg/L Mortality starting at 200 µg/L (no statistics)	(Bevan et al., 2003)	2 - nominal conc.
Xenopus laevis	Males/ 28 days	single dose of 100 µg/g/week; injected intraperitoneally (IP) with the specific treatment chemical on days 1, 7, and 14 of the experiment	mean epithelium heights of nuptial, plasma VTG, body condition changes in GSIndex or HSIndex	Significant lower nuptial gland epithelium at 100 µg/L No significant changes in VTG levels and GSI and HSI. E2: lower nuptial gland epithelium and VTG induction at 10 µg/L E2. No changes in GSI and HSI	(van Wyk et al., 2003)	2 - exposure route, single dose

Species	**Life stage tested/ test duration**	**test conditions/ test concentration**	**examined parameter(s)**	**Effect concentration(s)**	**Reference**	**Reliability (Klimisch)**
Rana pipiens	Newly hatched tadpoles (Gosner stage 21)/ 10 days	0.2 and 200 µg/L (nominal) + UV-B ratiation	premetamorphic development, body weight, day at hind-limb emergence (HLE)	No effects if exposed to 4-tert-octylphenol alone LC_{50} 7 d = 577 µg/L LOEC = 200 µg/L if combined with UV B radiation (disruption of normal premetamorphic development, increased body weight and accelerated day at HLE).	(Crump et al., 2002)	2 - nominal conc. widely separated, identity of test substance, no clear identity of test substance (octylphenol)
Rana pipiens	Tadpoles (Gosner stage 25)/ 8 months (+3 months in clean water without exposure)	0.02 and 2 µg/L (nominal)	development; body weight, effect on thyroid hormone receptor (TR) alpha levels, TR beta mRNA levels	LOEC delayed development and higher body weight ≤ 0.02 µg/L (stage 29 only) no overall effect on thyroid hormone receptor (TR)α but minor but significant ~ 11% increase in TR ß mRNA levels	(Croteau et al., 2009)	2– nominal conc.
Rana pipiens	Tadpoles (Gosner stages 26 and 36)/ 2 weeks	50 - 100 - 150 - 200 - 500 - 1000 - 1500 - 2000 µg/L (nominal)	mortality, body weight	LC_{50} (stage 26) = 293 µg/L; LC_{50} (stage36) = 577.9 µg/L; LOEC increased body weight at stage 36 ≤ 50 µg/L, decrease body weight at higher concentrations. no effects on body weight after exposure of stage 26 up to 200 µg/L E2 and EE2: decreased body weight after exposure of stage 36 (LOEC 5 µM)	(Hogan et al., 2006)	2– nominal conc., because of higher densitiy in stage 36 use body weight results with care *

Species	**Life stage tested/ test duration**	**test conditions/ test concentration**	**examined parameter(s)**	**Effect concentration(s)**	**Reference**	**Reliability (Klimisch)**
Rana sylvatica	Tadpoles (Gosner stages 26)/ 2 weeks	50 - 100 - 150 - 200 - 500 - 1000 - 1500 - 2000 µg/L (nominal)	mortality, body weight	LC_{50} = 153 µg/L; no significant effect on body weight up to 200 µg/L E2 and EE2:, LOEC increased body weight 0.5 and 0.75 µM respectively	(Hogan et al., 2006)	2– nominal conc. *
Rana catesbeiana	Tadpoles (Gosner stages 32 - 36)/ 24 h	0.2, 2 and 20 µg/L (nominal)	sexual differentiation; sex ratios	LOEC earlier completion of sexual differentiation ≤ 0.2 µg/L (males 3 stages earlier, females 1 stage); no changes in sex ratios	(Mayer et al., 2003)	2– nominal conc.
Ambystoma barbouri	Eggs/ 37 days	5, 50 and 500 µg/L (measured)	time to hatch, larval survival and snout-vent length	500µg/L: significant effects on time to hatch, larval survival and snout-vent length	(Rohr et al., 2003)	2 – test substance identity not clear, wide separation of levels, no clear identity of test substance (octylphenol)

Only few studies providing information about potential endocrine modes of action in amphibians are available. None of them was performed according to the OECD Guideline for the amphibian metamorphosis assay (assay (OECD, 2009b)) or is fully reliable according to (Klimisch et al., 1997). Data provide some hints only.

For *Xenopus laevis* three studies examining different development stages and endpoints are available. None of the studies provides evidence about a thyroidal mode of action, in fact no changes in development were observed by Kloas et al. (1999) while this endpoint was not examined in the two other studies. Results by Kloas et al. (1999) indicate that 4-tert-octylphenol might influence sexual development of amphibians by a similar mode of action compared to 17ß-estradiol. A shift of sex-ratio towards females, as observed by Kloas et al. (1999) fits to effects observed in fish species after exposure to estrogens. The effect is consistent with effects observed for 17ß-estradiol in this study and is also consistent with effects observed for 17ß-estradiol in a guideline conform study performed as part of the validation of the OECD amphibian metamorphosis assay (OECD, 2006). Indication of an estrogen like mode of action is supported by *in vitro* results showing reporter gene binding in *X. laevis* cells (see chapter 5.1.2.2). However, results provided by the study by Kloas et al. (1999) should be used with care as it was a not reliable method development study. Results are not in line with results by van Wyk et al. (2003) who found no vitellogenin induction in male *X. Laevis* after intraperitoneal injection in high dosasge.

Changes in the mean height of nuptial epithelium as observed by van Wyk et al. (2003) might be an indicator for an anti-androgen mode of action according to the author. However, information about this endpoint is rare and it is difficult to assess its relevance.

For *Rana pipens* some results provide evidence that 4-tert-octylphenol might influence the thyroid axis but results are not consistent. While an increase development observed by Crump et al. (2002) indicates a thyroid agonistic mode of action at high test concentrations, development was reduced in the study by Croteau et al. (2009) at lower concentrations and no activation of the thyroid receptor was observed. Changes in body weight as observed by Crump et al. (2002) and Hogan et al. (2006) do not provide evidence about the mode of action as results are contradictory. Increase of body weight, as found by Crump et al. (2002) and Hogan et al. (2006), is commonly observed with compounds that negatively affect normal development. However, increased development rather than delay was observed by Crump et al. (2002) and studies using 17ß-estradiol showed contradictory results with respect to body weight too.

Results for *Rana catesbeiana* support the hypothesis of a thryroid agonistic mode of action in amphibians. Although no effects with regard to development stages were observed, sexual differentiation was accelerated after exposure to 4-tert-octylphenol. As no changes in sex-ratio were observed, no estrogen like mode of action is expected for this species.

No information about potential modes of action is available for the only salamander species (*Ambystoma barbouri*).

In summary, although all studies should be used with care, the overall weight of evidence suggests that organism groups other than fish may be adversely affected by exposure to 4-tert-octylphenol at low concentrations (low µg/L range and below). Comparison with effects observed for 17ß-estradiol is suggestive of being estrogene like with respect to *Xenopus laevis* while hints for an influence on the thyroid axis for two Rana species are not consistent. Thus in summary some information suggests that 4-tert-octylphenol might have endocrine disrupting properties for additional taxonomic groups other than fishes but further verification would be needed.

5.1.2.5 Invertebrates

There is only limited information available about endocrine disrupting effects of 4-tert-octylphenol on (aquatic) invertebrates. Even though this phylum is very large and diverse the knowledge on how exogenoussubstances influence invertebrate endocrine systems is up till now scare (U.S.EPA, 2005). OECD development of test methods for the detection of adverse effects on development and reproduction for several groups of invertebrates is still underway .Owing to our lack of knowledge on hormonal systems of most invertebrates, no biochemical endpoints are available. Therefore no specific mode of action can be ascertained and no conclusion can be made on the endocrine disrupting properties of chemicals on species tested.

Table 43 summarizes adverse effects on development and reproduction in invertebrates. Where possible, the observed effects are assessed in relation to knowledge on endocrine effects and effects observed for natural and synthetic estrogens.

Table 43: Summary of adverse effects on aquatic invertebrates after exposure to 4-tert-octylphenol. (* study not summarized in (Environment Agency UK, 2005)). E2 = 17ß-estradiol; EE2 = Ethinylestradiol, If the test substance is not exactly known, this is indicated in the last column

Species	Life stage tested/ test duration	Test conditions/ test concentration	Examined parameters	Effect concentrations [µg/L)	Reference	Reliablity
Crustacea						
Daphnia magna	Juveniles / 21 d	37-62-120-230-510 µg/L (measured)	Survival adults, adult length, reproduction	LOEC reproduction and body length = 120 µg/L	(IUCLID, 2000)	4 – secondary source
Daphnia magna	12-h-old neonates/ 7 days	10 – 20 – 40 µg/L (nominal)	Moulting frequence and morphology of adults	no significant change relative to the controls	(Zou and Fingerman, 1997)	2 - Use with care nominal conc. Test substance 4-octylphenol
Acartia tonsa	Eggs/ 5 days	Semistatic, saltwater / (measured)	naupliar development	EC_{10} (inhibition of naupliar development) = 5.2µg/L	(Andersen et al., 2001)	2 – use with care, concentration series not given
Tigriopus japonicas	<24 h old nauplii/ 21 days parent and F1	Semistatic, saltwater / 0.01-0.1 1 10 µg/L	Development as number of days to reach first copepodid stage in parents and F1 generation sexual maturity in P and F1-Generation, fecundity, sex ratio, survival	LOEC ≤ 0.01µg/L (F1 generation), no clear dose-response curve in parents LOEC = 1 µg/L (sexual maturity in F1 generation) no effects on survival up to 10 µg/L	(Marcial et al., 2003)	2 – nominal conc.; substance identity not clear
Echinodermata						

Species	**Life stage tested/ test duration**	**Test conditions/ test concentration**	**Examined parameters**	**Effect concentrations [µg/L)**	**Reference**	**Reliablity**
Arbacia lixula (sea urchin)	Sperm and eggs/ 3 days	5 - 10 - 20 - 40 - 80 - 160 µg/L (nominal)	developmental anomalies [normal plutei (N), retarded) plutei, pathologic malformed plutei (P1), pathologic embryos (P2) unable to differentiate up to the pluteus larval stages and dead (D) embryos/larvae]	LOEC (larval malformations) = 20 µg/L, start skeletal malformations at 5µg/L, clear dose-response relationship	*(Arslan and Parlak, 2007)	2 - - nominal conc.
Paracentrotus lividus (sea urchin)	Sperm and eggs/ 3 days	5 - 10 - 20 - 40 - 80 - 160 µg/L (nominal)	sperm fertilization success, quantitative and morphologic changes in mitotic activity, larval malformations, developmental arrest, embryonic/larval mortality	LOEC (larval malformations) = 5 µg/L Embryo: at 10 µg/L: blocked gastrula or blastula	*(Arslan et al., 2007)	2 – nominal conc.
Strongylocentrotus purpuratuns (sea urchin)	Embryos/ 4 days	0.001 - 0.001 - 0.01 - 1 - 2.5 - 5 µg/L (nominal)	normal development (5 categories: normal, delayed, abnormal, elongated, hatched)	EC_{50} (delayed development) = 0.174 µg/L	*(Roepke et al., 2005) (Roepke et al., 2005)	2 – nominal conc., test substance identity not clear , test substance 4-octylphenol
Molluscs						
Marisa cornuarietis	Adults or egg masses/ 5 months or 12 months	1 – (5 - 25 –) 100 µg/L (nominal)	mortality; production of spawning masses with number of eggs per aquaria; appearance, extension of all sex organs, imposex intensity	LOEC ≤ 1µg/L:(increase in mortalities of animals; increased numbers of eggs produced and size of spawning masses	(Oehlmann et al., 2000)	3 – nominal conc., test substance identity not clear; methodical defects, test substance octylphenol

Species	**Life stage tested/ test duration**	**Test conditions/ test concentration**	**Examined parameters**	**Effect concentrations [µg/L)**	**Reference**	**Reliablity**
Nucella lapillus	Adults/ 2-3 months	1 – 25 – 100 µg/L (nominal)	mortality; production of spawning masses with number of eggs per aquaria; appearance, extension of all sex organs, imposex intensity	LOEC ≤ 1 µg/L (significant increase in relative numbers of oocytes and in length of capsule gland and weight of female pallial glands, sign increase in length of penis and the prostate gland)	(Oehlmann et al., 2000)	3 – nominal conc., test substance identity not clear; methodical defects, test substance octylphenol
Potamopyrgus antipodarum	Adults/ 9 weeks	Semi-static; 1 – 5 – 25 – 100 µg/L (nominal)	Growth, mortality, embryo production	LOEC = 5 µg/L: (significantly more embryos than in the controls; 100 µg/L: inhibition of embro production; inverted U-shaped dose-response curve)	(Jobling et al., 2003)	3 – nominal conc., wide separation of conc., missing details conditions
Potamopyrgus antipodarum	Adults/ 8 weeks	Static 1 – 10 – 30 – 100 – 300 µg/kg dw (spiked sediment) (nominal)	Embryo production, unshelled embryos	LOEC (stimulated embryo production) = 1 µg/kg; inverted U-shaped dose-response curve	(Duft et al., 2003)	3 – nominal conc.; some results extrapolated well below lowest conc.; some methodical defects

In summary, effects on three different phyla (crustaceans, echinodermata and mollusks) were examined.

Within the group of crustaceans three species were tested (*D.magna*, and the two copepods *A. tonsa* and *T. japonicas)*. No developmental effects (molting) were observed in *D.magna* up to 40 µg/L and effects on reproduction started to occur at high test concentrations (LOEC = 120 µg/L). Effects were observed in the two copepods including delay in development of in naupilar stages (*A.tonsa* and *T.japonica*) and delay in sexual maturity (*T. japonica*) starting in the low µg/L range. As the parameters assessed are not specific, no evidence for endocrine mediated effects is available. However, for *A. tonsa*, the results obtained for 17ß-estradiol indicate that the effects are not estrogen mediated as *A.tonsa* was less sensitive towards 17ß-estradiol (EC_{10} = 370 µg/L) than to 4-tert-octylphenol.

Effects on echinodermata were assessed with three sea urchin species. In two species (*P.lividus* and *A. lixula*) larvae malformation after exposure of sperms and eggs were observed, starting to occure at concentrations of respectively 5 and 20 µg/L). In the third species (*S. purpuratuns*) delayed embryo development after exposure to 4-octylphenol (exact substance identity not specified) started at much lower concentrations (EC50 = 0.174 µg/L). Again, no information about the mode of action eliciting these effects is available. However, data for *S. purpuratuns* indicate that the observed effects of 4-tert-octylphenol are not estrogen mediated as the EC50 for 17ß-estradiol was much higher (EC50 = 14.2 µg/L).

For molluscs four tests with three species are available, all of which were scored not valid. Although they may not be used alone, due to the low reliability, they seem to provide some indication of possible effects if used in a weight of evidence approach. For two species (*M. cornuarietis, P. antipodarum*) an increased number of offspring (eggs or embryos) was observed (LOEC ≤ 1 and 5 µg/L, respectively) while for *N. lapillus* only histological effects were observed indicating changes in both female and male gonads (LOEC ≤ 1 µg/L). Again, the effects observed do not provide information about a specific mode of action. However, increase of egg numbers is a feminization effect that may be consistent with an estrogen or estrogen-like mode of action (U.S.EPA, 2005; OECD, 2010b).

In summary, the results show that 4-tert-octylphenol can affect various phyla of invertebrates like crustaceans, echinodermata and mollusks. There is some evidence that exposure of early life stages to low µg/L concentrations results in developmental delays and changes in reproductive outcome. Since for most invertebrates no biomarkers are available that could indicate a specific mode of action, no definitive link between a potential endocrine mode of action and the observed apical effects is possible. Based on low quality studies some indication exist that with respect to mollusks 4-tert-octylphenol might influence endpoints consistent withthose observed for other synthetic endocrine active substances (see e.g. (OECD, 2010b)).

5.1.2.6 Sediment organisms

No data available.

5.1.2.7 Other aquatic organisms

No data available.

5.2 Terrestrial compartment

No information available.

5.3 Atmospheric compartment

Not assessed for the identification of this substance as SVHC in accordance with Article 57(f).

6 PBT, VPVB AND EQUIVALENT LEVEL OF CONCERN ASSESSMENT

6.1 Comparison with criteria from annex XIII

Not assessed for the identification of this substance as SVHC in accordance with Article 57(f).

6.2 Assessment of substances of an equivalent level of concern

6.2.1 Environment

According to Art 57 (f) REACH, substances having endocrine disrupting properties, for which there is scientific evidence of probable serious effects to the environment which give rise to an equivalent concern to those of PBT/vPvB and/or CMR substances might be substances of very high concern, identified on a case by case basis.

Although Art 57 (f) provides no clear criteria for “equivalent concern”, starting from the legal text two questions seem to be relevant:

a) Is 4-tert-octylphenol a substance having endocrine disrupting properties?

b) Is there scientific evidence of probable serious effects to the environment which give rise to an equivalent concern compared to CMR and/or PBT substances?

Information available for 4-tert-octylphenol is structured along these two questions in order to facilitate a conclusion.

6.2.1.1 Endocrine disrupting properties

Endocrine disrupting properties are one example of inherent properties that might, if scientific evidence of probable serious effects is available, give rise to an equivalent level of concern as exerted by CMR and/or PBT/vPvB substances. Although the term “endocrine disrupting properties” is not equivalent to the term “endocrine disruptor” it is assumed in this dossier, that a substance should fulfil at least the definition of an endocrine disruptor provided by WHO/IPCS (WHO/IPCS, 2002) in order to be considered as of equivalent concern based on the endocrine disrupting properties.

“An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations” (WHO/IPCS, 2002)”.

In chapter 5.1.2.3 to 5.1.2.5 available data for fish, invertebrates and amphibians are examined against this definition. This examination is based on the criteria set out in the draft OECD guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption (OECD, 2011).

As described in chapter 5.1.2.3 the analysis of data available for fishes reveals that

- Adverse effects observed in 2 fish species (*O.latipes, C. variegates*) are clearly estrogen mediated and thus there is strong evidence from high quality data that 4-tert-octylphenol actually acts as endocrine disruptor in these species. Similar evidence is available for *P. reticulata* but based on tests without statistics.

- In all other tested fish species endpoints are affected which are known to be influenced by estrogen activity. With only one exemption, in all these species an estrogen mode of action was observed.

Thus, the available data indicate that 4-tert-octylphenol is an endocrine disruptor in fish.

For amphibians study results as well as knowledge about estrogen mediated effects in these animals do not allow for a definitive conclusion as to whether the adverse effects observed are endocrine mediated. Effects observed in one species *(X.laevis*) in a low quality study and in an *in-vitro* study are comparable to effects observed for 17ß-estradiol providing some indication that effects may be caused by an estrogen-like mode of action. Effects observed in two other species (*R. catesbeiana* and *R.pipens*) are not consistent with regard to a thyroid agonist mode of action (see chapter 5.1.2.4).

With regard to invertebrates, again no clear conclusion can be drawn. Effects observed for mollusks in low quality studies are comparable to effects observed for natural and synthetic estrogens and provide some indication that effects might be caused by an estrogen-like mode of action. In crustacean and echinodermata a delayed development in naupilar stages, sexual maturation and embryos was observed. Comparison with effects elicited by 17ß-estradiol indicated that these delays are not the result of an estrogen-like mode of action of 4-tert-octylphenol in these organisms.

In summary, available information show, that 4-tert-octylphenol acts as an endocrine disruptor in fish. Tests with other species provide some indication that 4-tert-octylphenol might also act as an endocrine disruptor in other taxonomic groups, but no clear conclusion can be drawn.

This conclusion is summarized in Table 44.

Table 44: Endocrine disrupting effects of 4-tert-octylphenol in different taxonomic groups. Indication that apical endpoints fit to the mode of action is based on studies with 4-tert-octylphenol and reference estrogens such as 17ß-estradiol and ethinylestradiol. In addition observed 4-tert-octylphenol effects were compared to known effects of xenoestrogens as summarized in a recent report by the EU Commission (Evans et al., 2011).

Taxonomic group	Number of species	Indication of hormonal activity?	Apical adverse effects observed?	Indication that apical endpoints fit to mode of action
Fishes	6	Yes, in 5 of 6 species (increased vitellogenin level in males and females, changes in female gonadal staging, changes in sperm stages in males)	Yes, in all species (fertility, viable eggs, larvae growth) Most sensitive fully reliable LOEC ≤ 20 µg/L (fecundity in *O.latipes*) with some indication that effects might start at ≤ 1.7 µg/L (fertility, *P.reticulata*)	Yes, based on studies with 4-tert-octylphenol clear link for three fishes, Effects observed in all species are known to be estrogen sensitive

Amphibians	5	Yes, in vitro receptor binding for one species, Some hints that effects might be endocrine mediated in an other species but not conclusive.	Yes, in all species (change in sex – ratio, changes in development) Most sensitive LOEC≤ 0.2 µg/L (accelerated sexual differentiation in *R. catesbeiana*, Klimisch 2)	Effects observed on sex-ratio in X.laevis in low quality studyand accelerated sexual differentiation in *R. Catesbeiana* could point to an estrogen mediated mode of action
Invertebrates	3 crustaceans	Not available	Yes (reproduction, naupliar development, sexual maturity) Most sensitive fully reliable EC_{10} = 5.2 µg/L (naupliar development *in A. tonsa*) with some indication that effects may start at 0.1 µg/L (delayed development in *T. japonicas*)	No conclusion possible due to lack of knowledge
	3 echinoderms	Not available	Yes (larval malformations, delayed development) Most sensitive LOEC = 5 µg/L (larval malformation in *P.lividus*, Klimisch 2)	No conclusion possible due to lack of knowledge
	3 molluscs	Similarity with effects observed for 17ß-estradiol for 2 mollusk species	Yes (increase in number of oocytes and embryos) Most sensitive LOEC ≤ 1 µg/L (increase of oocyte production in *N. lapillus*, Klimisch 3)	Some indication as effects observed for 4-tert-ocytlphenol are similar to those observed for 17ß-estradiol. However low quality of the underlying studies prevents clear conclusions.

6.2.1.2 Equivalence of concern based on probable serious effects in the environment

As described in the legal text, an endocrine disruptor should be regarded as of very high concern if its probable serious effects to the environment are of equal concern compared to CMR and/or PBT/vPvB substances (REACH, Art. 57 f).

How to define “probable serious effects to the environment” and how to conclude whether or not they are of equivalent concern is still subject of debate. While there is some argumentation that all substances identified as endocrine disruptors should be regarded as of equivalent of concern, some further requirements are suggested by other parties. This dossier does not suggest any criteria needed to be fulfilled in order to agree on the equivalent level of concern of a substance. However,

for the sake of transparency, the data obtained for 4-tert-octylphenol are examined against a set of criteria that has been proposed by different parties.

The so far suggested criteria address and take into account the potency of the substance and the relevance of the observed effects. In addition the level of concern compared to PBT/vPvB and/or CMR substances is examined.

4-tert-octylphenol must be considered as being of equivalent concern according to all criteria suggested so far with regard to its potency as well as with regard to the relevance of effects exerted by it (see below).

Potency

Criteria suggested so far with regard to the potency of a substance are effect concentration, comparison with non-endocrine mediated endpoints and exposure duration needed to cause effects. Analysis of effects for 4-tert-octylphenol show that 4-tert-octylphenol must be considered as a potent endocrine disruptor with respect to all these criteria suggested:

Effect concentration: As described in chapter 5.2 4-tert-octylphenol results in endocrine mediated adverse effects on apical endpoints in fish at very low test concentrations (low µg/L range). It also causes adverse effects at even lower concentrations in invertebrates and amphibians and some evidence indicates that they might be endocrine mediated.

Comparision with binding capacity and effect concentrations observed for 17ßestradiol provide some information about the relative potentcy of 4-tert-octylphenol compared to this natural highly potent estrogen. The in vitro binding and VTG induction capacity of 4-tert-octylphenol compared to 17ß-estradiol is low to moderate (> 0.001) for two fish species (*C.carpio* and *Z.viviparous*) and much lower for two additional species (< 0.00009 for *S.salar* and *O.mykiss*). In vivo data provide some evidence that at least for some species relative potency might be higher (factor 0.5 – 0.05 for *O.latipes* (Gray et al., 1999a, Knörr and Braunbeck, 2002) and 0.01 - ≥0.007 for *Z.viviparous* (Rasmussen et al., 2002 and 2005)). However, data should be used with care as studies are not fully reliable and no statistics is provided. A similar releative potency was observed in a fully reliable study for *P. Reticulata* with respect to the endpoint changes in sperm stages (Factor > 0.01) while relative potency was lower for other endpoints (Toft and Baatrup, 2001). A much lower potency compared to the synthetic ethinylestradiol (EE2) was observed for *D.rerio* and *O.mykiss* (factor 0.00003 and ≥ 0.000005) in fully reliable studies ((Van den Belt et al., 2001, Wenzel et al., 2001, Jobling et al., 1996.

Thus, 4-tert-octylphenol is a potent endocrine disruptor with respect to the effect concentration causing endocrine mediated effects, while its relative potency compared to 17ß-estradiol and ethinylestradiol seem to differ between species.

Comparison with non-endocrine mediated endpoints: This criterion focuses on the question whether or not endocrine mediated effects are the “lead” effects (the most sensitive endpoints) compared to systemic toxicity. For 4-tert-octylphenol the most sensitive endpoints are fecundity in fish, sexual differentiation in amphibians and increased fertility in mollusks (see Table 44). Algae are considerably less sensitive (EC_{10} = 300 µg/L for *S.subspicatus*, see chapter 5.1.1). As described in chapter 5.1.2.3 and summarized in chapter 6.2.1.1, the most sensitive endpoints in all fish species tested are either clearly endocrine mediated or are endpoints considered to be sensitive to endocrine mediation. With regard to amphibians and invertebrates no clear conclusion is possible due to insufficient knowledge about the endocrine system of these taxa and low data quality. But some evidence exists, that the effects seen in these taxa on the most sensitive endpoints might be elicited by an endocrine mode of action. In conclusion endocrine mediated effects are the “lead” effects i.e.

effects on the most sensitive endpoints are either clearly endocrine mediated or there is at least evidence that these effects might be endocrine mediated.

Exposure duration needed to cause effects: Most sensitive effects observed in *O. latipes* and *C. variegatus* (impaired reproduction) occurred after exposure of adult males for 21d. Results obtained for *O. latipes* in three sexual development tests indicate that exposure during a very short window (prehatch) influences sensitivity with respect to the endpoints on sexual development and sex-ratio. Thus, 4-tert-octylphenol is a potent endocrine disruptor with respect to duration of exposure needed to cause effects (even short term exposure of adults of one sex is sufficient to cause effects on reproduction).

Relevance of effects

Criteria suggested so far with regard to the relevance of effects are the type of effects observed and the number of species potentially affected. As described below, effects observed for 4-tert-octylphenol are relevant effects compared to these criteria.

Type of effect: 4-tert-octylphenol acts as an estrogen receptor agonist in fish with some indication (based on low quality data) that this might also hold true for invertebrates and amphibians.(see chapter 5.1.2.3 and 5.1.2.4).

Estrogen agonists are known to interfere with reproduction parameters as well as sexual development (including changes in sex-ratio) and growth. Specific life stages and endpoints such as sexual development and sexual maturation are especially sensitive to the influence of estrogen agonists (Kendall et al., 1998; IPCS, 2002). Effects are considered relevant as they impair population stability or recruitment.

With respect to 4-tert-octylphenol effects described above were observed in the low µg/L range for nearly all species tested (see table 45). Thus, 4-tert-octylphenol must be considered to cause relevant effects with respect to population stability and recruitment.

Number of species affected: Steroids are known to play an important role e.g. in vertebrates other than fishes (Baroiller et al., 1999) and in invertebrates (Kendall et al., 1998). In fish and mammals as well as in mollusks, arthropods, amphibians, alligators, turtles and birds estrogens, such as 17ß-etradiol and ethinylestradial, influence the endocrine system by causing changes in development, reproduction and behaviour (see summary in EU Report, (Evans et al., 2011)).

With respect to 4-tert-octylphenol effects known to be estrogen mediated were observed in all fish species tested and some indication exist for mollusk species based on low quality data. For amphibians some indication is available that the underlying mode of action might be estrogenic (based on a low quality study) while no information about the underlying mode of action is available for echinoderms and crustaceans. Due to the estrogen mode of action observed in several species it seems likely that 4-tert-octylphenol will also influence the endocrine system of other species.

In summary, it can be concluded that 4-tert-octylphenol has the potential to cause adverse effects in a range of species across different taxonomic groups, which consequently could lead to relevant impacts on community structure and function of the affected ecosystems.

Level of concern as compared to PBT/vPvB and CMR substances

Equivalence of concern compared to other substances of very high concern is one of the requirements in order to conclude whether or not a substance having endocrine disrupting properties should be regarded as substance of very high concern.

As described above, some criteria to assess the level of concern are already suggested. In addition, some aspects that were considered to be relevant for PBT/vPvB and CMR substances might be relevant with regard to endocrine disruptors too. Aspects are irreversibility, persistence of potential impact on the environment and distribution of effects among species and regional scales a well as difficulties to quantify risk using traditional risk assessment methods. For the sake of transparency and again without preempting any discussion, endocrine disruptors and 4-tert-octylphenol in particular are in the following assessed with regard to these aspects.

Irreversibility and Persistence: Substances acting as (potent) estrogen agonist are considered to cause long lasting effects with regard to impairment of development and reproduction on the individual level as well as with regard to its effects on a population level. Effects persist even after exposure has ceased:

- Endocrine modulation is a very complex feedback process that is set up during critical life stages. As summarized in (IPCS, 2002) disturbance of this set up may result in effects during the entire life.
- Effects may result in a substantial failure of recruitment and almost disappearance of population even after cessation of exposure, as observed in the wild for ethinylestradiol in fathead minow (Palace et al., 2009).
- Even transient exposure during sensitive life stages may result in severe effects on populations. Changes in male reproduction capacity might influence genetic variability of populations in the long-term, as only a part of the males may be capable to reproduce (Sumpter and Johnson, 2008).

Distribution of effects:

Distribution of effects is considered with respect to distribution among species as well as geographical distribution.

As described above, substances influencing the estrogen system might elicite adverse effects in very different taxonomic groups. Due to the conservatism of estrogen receptors it is very likely that a wide range of species with different function in ecosystems could be affected although effects may start to occur at different concentrations. Thus the impact of 4-tert-octylphenol must be regarded as potentially widespread.

As described above, exposure during a sensitive life stage might result in effects during lifetime, e.g. on reproduction. Migration is a common pattern in species such as birds, amphibians, mammals and fishes. It includes long-distance migration of migratory birds or of fish species, such as salmonids and eel. Thus, exposure in one area might influence population stability in another area (e.g exposure during development of flatfish in coastal area may result in population changes in the open sea, or exposure of adult salmonids in estuarine areas during migration might influence sperm quality and fertilization success at the reproduction sites in rivers).

Due to the potentially long lasting effects, as well as the wide variety of species potentially affected it seems to be very difficult to estimate which species are most sensitive and which concentration should be regarded as safe for the environment. As described by Crane (2010), aspects such as influence on breeding behaviors and competition are not taken into account in laboratory tests and thus tests might underestimate effects in the environment.

In summary the type of effects observed and the variety of species potentially affected make it likely that, substances acting as (potent) estrogens may have an impact on community structure and function of ecosystems, which may prevail even after cessation of exposure. Furthermore, taking

account of the nature of the effects and the behavior of migratory species, there is some evidence that the area in which exposure takes place may not always be the same as the area in which effects occur. 4-tert-octylphenol is a potent endocrine disruptor at least in fish and thus it is expected that scenarios described above might hold true for 4-tert-octylphenol.

Further aspects, in addition to the ED properties might increase the concern.

4-tert-octylphenol degrades very slowly if the whole environment is considered (see chapter 3.1.2). The bioaccumulation potential of 4-tert-octylphenol in BCF studies is low to moderate (46 – 471, see chapter 3.3). However, results for *Z.viviparous* show that uptake and internal distribution in females could have a particular impact on intracorporal embryo development and thus increase the relative potencyof 4-tert-octylphenol in vivo compared to 17ß-estradiol.

6.2.1.3 Summary of ED properties and equivalence of concern

As described in chapter 5.1.2.3 to 5.1.2.5 and summarized in chapter 6.2.1.1, 4-tert-octylphenol is an endocrine disruptor eliciting adverse effects in fishes (low µg/L range). Transient exposure at sensitive life stages may already result in life-long adverse effects for example on reproduction. Among the effects observed those considered to be endocrine mediated, affect the most sensitive endpoints. The endocrine mediated effects are highly relevant for population stability and recruitment and may affect a variety of taxonomic groups (see summary in chapter 6.2.1.2). Thus it can be concluded that 4-tert-octylphenol is a potent endocrine disruptor that can exert serious effects on community structure and ecosystem function.

In addition, as described above, estrogen mediated effects are considered to be of similar concern to those exerted by substances with PBT/vPvB or CMR properties with respect to long-lasting of effects and difficulties to quantify their risks.

6.2.2 Human health

6.2.2.1 Endocrine disrupting properties

Summary on estrogenic activity of 4-tert-octylphenol

The potential of 4-tert-octylphenol to exert estrogenic activity was investigated in *in vitro* and *in vivo* screening tests. *In vitro* 4-tert-octylphenol stimulates cell proliferation in MCF-7 cells starting at concentrations of 10^{-7} mol/l in cell culture. Where possible, relative potencies of 4-tert-octylphenol were estimated based on concentrations causing a similar effect level in the particular tests than the positive controls (17ß-estradiol). The relative potencies in these assays ranged from 10^{-5} to10^{-3}.

Several reporter gene assays are available which were carried out in several mammalian cell lines as well as in yeast. These cells were transfected either with estrogen responsive elements containing reporter gene constructs alone (for green fluorescent protein, luciferase or β-galactosidase), or additionally with wildtype or mutant estrogen receptor gene constructs. It could be shown that 4-tert-octylphenol is capable of inducing gene expression which functions via estrogen receptor transactivation in these set-ups. Mutant ER were slightly less capable of ER transactivation. Relative potencies compared to the concurrent positive controls in these gene reporter assays ranged from 0.0001 to 0.008. In one test a far higher relative potency (0.5-0.7) of 4-tert-octylphenol

compared to the reference substance E2 was calculated for the gene reporter activity. However the dose of E2 was quite high and presumably resulted in saturation of the signal in this test.

Estrogenic activity of 4-tert-octylphenol was also investigated in rodent bioassays such as the uterotrophic assay in the ovarectomised (ovex) as well as in the immature model. After two to three days of oral treatment a LOEL of 56 mg/kg bw was determined (immature, mouse). Subcutaneous treatment resulted in LOELs of 10, resp.50 mg/kg bw after 3 d (immature, resp. ovex, rat) or 25 mg/kg bw after 14 d of treatment (ovex, rat). From 4-tert-octylphenol doses that caused increased uterine weights, relative potencies can be estimated in comparision to estrogenic compounds (E2, ethinylestradiol or DES), which ranged from 0.00005-0.000005 in these screening assays. Further indication for some estrogenic potential of 4-tert-octylphenol may be inferred from investigations of a calcium binding protein, which is presumably regulated by ER transactivation. Relatively high doses of 4-tert-octylphenol (from 400mg/kg bw upwards) induced mRNA and protein expression in maternal and even fetal uteri. However, it should be noted that the positive controls (E2 and DES) in these investigations did not produce a positive result in all the settings.

On average 1000-fold higher doses were required *in vitro* and at least 2000-fold higher doses were required in the *in vivo* screenings to result in similar effect levels than exerted by the reference estrogens. Overall, the *in vitro* and in vivo screening tests indicate that 4-tert-octylphenol has some but low estrogenic potential in mammals.

Data from *in vitro* receptor binding assays revealed binding of 4-tert-octylphenol to progesterone or androgen receptors with similar weak affinity as binding to estrogen receptors. No treatment-related effect was seen regarding mRNA expression of these receptors in MCF-7 cells.

4-tert-octylphenol was investigated for possible impairment of gonadal steroidogenesis. The possible effect and mechanism of 4-tert-octylphenol on testicular steroidogenic competence was investigated *in vitro* in rat primary Leydig cells. With this test system, human chorionic gonadotropin (hCG)-stimulated testosterone production was determined. Testosterone production was inhibited by 4-tert-octylphenol whereas 17β-estradiol or an estrogen antagonist (ICI 182,780) had no effect. Studies on the conversion of various steroid substrates to testosterone in this *in vitro* system suggested no interference of 4-tert-octylphenol with 3 β HSD but inhibition of the P450C17 dependent step. Similar results were obtained from studies with a human adrenocortical cell line and a mouse Leydig tumour cell line. Cultivation of rat fetal testes (whole organ ex vivo – *in vitro* culture) with 4-tert-octylphenol revealed increases in basal and hCG-induced testosterone production. *In vitro* studies on ovarian steroid synthesis indicated some sensitivity to impaired synthesis due to 4-tert-octylphenol exposure for the immature ovary. Thus, there is some evidence for a potential of 4-tert-octylphenol to interfere with gonadal steroidogenesis and/or certain steps of steroidogenic pathways.

Summary on adverse effects of 4-tert-octylphenol and their possible relation to estrogenic/endocrine properties - effect pattern

Studies with repeat administration to 4-tert-octylphenol *via diet* showed toxic signs such as decrease in body weight gain at dietary concentrations of $\geq$ 300 ppm (calculated to daily exposures of 23 mg/kg bw/d) and $\geq$ 2000 ppm (resulting in daily exposures of 111-369 mg/kg bw/d). At higher dietary concentrations of 3000 ppm (calculated to a daily exposure of 230 mg/kg bw/d) additional effects, such as changes in hematocrit and thyroxin in females were observed.

Studies with repeat oral administration of 4-tert-octylphenol *via gavage* showed toxic signs such as decrease in body weight gain, increase in water consumption, decrease in food consumption, changes in kidney and liver weight and histopathology as well as decreases in blood cholesterol (in females) at daily doses of 250-300 mg/kg bw. Doses of 125 mg/kg bw/d were shown not to be

systemically toxic, whereas doses of 250 mg/kg bw/d were shown to be partially lethal for pregnant dams (g.d 0-8) and lethal to all animals (males/females) after application of doses of ≥ 500 mg/kg/d.

Many of the available studies with *subcutaneous injection* do not provide information on the possible side effects of this route of application, respectively whether concomitant local or systemic effects were induced. Few studies mention that effects on body weight, body weight gain, or on terminal body weights were seen after s.c. administration of doses of 20, 80 or 100 mg/kg bw. In a study on females with repeat s.c. administration over 28 days dose- and duration-dependent local effects and skin lesions (scab or abscess formation) were seen (≥ 12.5 mg/kg bw) as well as increases in spleen organ weight (due to extramedullary hematopoiesis) and in relative liver and kidney organ weights. Similar conditions were probably on hand or should be assumed for the other studies with this route of administration and in particular for those, where neonatal pups were treated with 4-tert-octylphenol via subcutaneous injection.

The observed effects are not considered to be due to an estrogenic mode of action.

Oral (gavage) administration of 4-tert-octylphenol to adult *female* rats at dosages of 100 and 125 mg/kg bw/d did not reveal any adverse effects on reproductive organs (ovarian and uterine organ weights/histopathology) or changes in serum estradiol. However, changes in oestrous cyclicity (showing a lower number of cycles with change to dioestrous) were observed at an oral dose of 200 mg/kg bw/d in one study, whereas no impairment with cycling at the same dose regimen was observed in a second study. In studies with subcutaneous administration abnormal cycling and disruption of cyclicity were observed after application of doses of ≥ 20 mg/kg bw/d. At s.c. dosages high enough to completely disrupt oestrous cycling (100 mg/kg bw/d), also reductions in female plasma estrogen levels were observed. In contrast to these findings on interference with oestrous cycling, no effects were revealed on the numbers of cycling females, the numbers of females with abnormal cycles, the oestrous cycle length or on the pattern of the oestrous cycle stage at necropsy in a comprehensive guideline compliant two-generation reproduction toxicity study in the females of the parental F0 generation (monitored for 30 females/group), or in the females of the F1 parental generation (also monitored for 30 females/group) at continuous daily exposures of 111-369 mg 4-tert-octylphenol/kg bw.

Overall, there are conflicting results with regard to 4-tert-octylphenol interfering with the oestrous cycle in sexually mature females. In addition manifestation of these effects may significantly depend on the route of application. Taken on their own, the observed changes in normal oestrous cycle and disruption of cycling are considered adverse effects, predictive for possible impairment of female fertility, for which an estrogen mode of action may be assumed.

Oral (gavage) administration of 4-tert-octylphenol to juvenile/adult *male* rats did not reveal any morphological abnormalities in testes and germinal epithelium at daily doses of approximately 150 mg/kg bw. Whereas, when administered at high doses (≥ 400 mg/kg bw/d) with systemic effects such as reduced body weight gain, adverse effects were revealed also for male reproductive organs (decreased organ weights of testes, epididymis and prostate, morphological defects in testes), testicular sperm (reduced sperm count) and serum prolactin levels (increase). In studies using subcutaneous injection, doses of ≥ 20 mg/kg bw revealed histopathological changes in testes (with reductions in number of germ cells and in testicular sperm count, changes in sperm morphology), decreases in epididymal and in seminal vesicle organ weight, and decreases in serum testosterone and increases in serum LH levels, a pattern of effects identical to that seen with concomitant s.c. administration of the estrogen 17β-estradiol. In contrast to this, in a Two-Generation Reproduction Toxicity Study (870.3800, USEPA, 1996) with several enhancements, such as higher numbers of animals and assessment of certain ED related endpoints included in the revised OECD TG 416 using oral administration no effects were revealed on reproductive organ weights (testes, epididymides, prostate, seminal vesicles with coagulating glands) in males of the F0 parental

generation (evaluated for 30 males/group), or in males of the F1 parental generation (evaluated for 30 males /dose group) or in F2 male offspring retained until and beyond acquisition of sexual maturation at continuous daily exposures to 111-369 mg 4-tert-octylphenol/kg bw. Further, no treatment-related effects were found on gross or histological examinations of the reproductive organs, and no effects were seen on epididymal sperm count and motility or on testicular sperm count and morphology or on efficiency of daily sperm production in this study.

Overall, there are conflicting results with regard to 4-tert-octylphenol interfering with the male reproductive system including spermatogenesis in juvenile or adult males, with their manifestation significantly dependent on the route of administration. Taken on their own, the observed changes after subcutaneous administration in male reproductive organs and in serum hormone levels are considered adverse effects that fit to the pattern of effects induced in males by application of natural estrogens. Although the mechanisms for inducing these effects are not yet clear, the involvement of an estrogen mode of action should not be dismissed.

Effects on fertility resulting from oral treatment with 4-tert-octylphenol were investigated in several guideline compliant reproduction toxicity studies. In one reproduction toxicity screening study some evidence of fertility impairment such as a decrease in weights of specific reproductive organs and minor microscopic changes in the testes and epididymis were observed. However these effects were only seen in the dosing group where also severe systemic toxicity including death occurred. Lower doses resulted also in systemic effects but not in effects related to fertility such as specific organ toxicity. A second reproduction toxicity screening test resulted in an equivocal increase in corpora lutea at a dose that also affected food consumption and body weight gain. In a two generation study on rats, no effects on any parameters of mating, fertility, pregnancy or parturition in the F0 or F1 generation were observed up to doses of 369 mg/kg bw per d. Treatment related effects were observed at the highest treatment dose in terms of systemic toxicity which resulted in reductions of body weights and body weight gains in the animals of the F0, F1 and F2 generation. Further, pup body weights were reduced in both F1 and F2 offspring during the later period of lactation when the pups were self-feeding and exposed to high doses of 4-tert-octylphenol. Minor delays in vaginal patency in females and preputial separation in males were observed, effects which can be contributed to the lower pup body weight. No effects on reproductive parameters, testes weights or morphology, epididymal sperm counts, or morphology, daily sperm production, efficiency of daily sperm production, or prostate or dorsal prostate weights or histopathology were observed. Furthermore, no oestrogen-like effects on males or females and no low dose effects were evident. The findings of this study are supported by the results from a repeated dose toxicity study, where effects on the reproductive system were observed only at a dose level, which also caused systemic toxicity. Overall, the effects observed in fertility studies may represent systemic toxic effects or be related to general systemic toxicity instead of being due to substance related target specific effects.

From the available studies with exposure of 4-tert-octylphenol to pregnant dams there is no evidence for any treatment-related impairment of postnatal testosterone surge in male offspring or for an impairment of oocytes and ovarian morphology in female offspring.

Direct treatment of male newborns (s.c. application) with 4-tert-octylphenol approximating exposures of 150 mg/kg bw/d did not provide evidence for an impairment of testicular development or for any hormonal changes. These findings are supported from the results of the two-generation reproduction toxicity study, which did not find any indications for an impairment of male reproductive system development. At very high doses applied to newborn males (8 x 100 mg/kg bw, s.c., postnatal day 1-15), serum testosterone levels were found to be decreased and the pattern of pre-/postpubertal serum FSH levels was changed. These latter findings may indicate some direct effect of 4-tert-octylphenol on postnatal development and function of the testis or modulation of the hypothalamic-pituitary hormonal regulation and feedback system. Thus, 4-tert-octylphenol applied

at very high doses postnatally may have the potential to interfere with development and maturation of the male reproductive system, the mechanism for which is so far unknown.

Direct treatment of female newborns (s.c. application) with 4-tert-octylphenol with exposures to 4 x 50 mg/kg bw/d did not provide evidence for adverse effects on sexual maturation, onset of and regularity of oestrous cycling or impairment of pre- and postpubertal serum pituitary and gonadal hormone levels. These findings are supported from the results of the two-generation reproduction toxicity study, which did not reveal effects (up to and including daily exposures of 111-369 mg 4-tert-octylphenol/kg bw) on female sexual maturation (vaginal patency) in offspring of the F1 generation or in offspring of the F2 generation, or in their cycling competence, and with all females exposed pre- and postnatally finally fertile. However, higher s.c. dosages applied to newborn females (of 4 x 100, respectively 10 x 50 mg/kg bw or even more), were found to accelerate sexual maturation, prevent or disrupt cycling (persistent oestrous), severely affect reproductive organs (ovary, uterus), interfere with the pre- and postpubertal hormonal status and prevent spontaneous and estrogen-induced preovulatory LH-surge. These latter findings indicate some direct effect of 4-tert-octylphenol on female sexual development, for which an estrogenic mode of action may be considered responsible.

Based on currently available information there is evidence that 4-tert-octylphenol features some inherent potential for being toxic for reproduction, probably in relation to female sexual maturation and female fertility and the integrity of male the reproductive organ system after oral (gavage) administration of high doses causing systemic toxicity and) or adminsitration routes other than oral dosing.

Discussion of Endocrine disrupting properties

4-tert-octylphenol already came into consideration for serious health effects and was proposed for classification as toxic to reproduction CAT 2 (CLP). However, in a weight of evidence evaluation of the available data, including data on estrogenic action, it was concluded that classification as “toxic for reproduction” was considered to be not justified (ECBI/60/05 Rev. 3, Technical Committee for Classification and Labelling, 14-17 November 2005)

For the evaluation of adverse health effects of 4-tert-octylphenol with relevance to the human situation, during this SVHC identification proposal data from studies are taken into consideration utilizing routes of application that are of relevance according to presumable human exposure scenarios. Data from experimental studies with non oral exposure conditions, such as the data on 4-tert-octylphenol effects after subcutaneous injection, may be informative for a more comprehensive inherent effects pattern, however, results might not be directly comparable to effects with routes of application that are of relevance for common human exposure scenarios. Thus, the data from the available studies with the oral route of administration are taken into consideration for the identification of adverse health effects resulting from exposure to 4-tert-octylphenol. After repeat oral application (via diet or gavage), decrease in body weight gain, increase in water consumption, decrease in food consumption, changes in kidney and liver weight and histopathology in male and female rats as well as decreases in blood cholesterol, changes in hematocrit and in thyroxin in the females were observed at and above dose levels of 23 mg/kg bw/d. Dosages of 250 mg/kg bw/d were partially lethal in pregnant dams, doses of 500 mg/kg bw/d were completely lethal. Dosages of 200 mg/kg bw/d resulted in changes in oestrous cyclicity in one study, which were not reproduced in another study. In a guideline compliant two generation study with rats no effects were seen with respect to the numbers of cycling females, the numbers of females with abnormal cycles, the oestrous cycle length or the pattern of the oestrous cycle stage in the females of the parental F0, or in the females of the F1 parental generation at continuous daily exposures ranging from 111 to 369 mg 4-tert-octylphenol/kg bw. In this study also no effects were revealed on reproductive organ

weights (testes, epididymides, prostate, seminal vesicles with coagulating glands) in males of the F0 parental generation, or in males of the F1 parental generation or in F2 male offspring retained until and beyond acquisition of sexual maturation. Adverse effects on reproductive organs (decreased organ weights of testes, epididymis and prostate, morphological defects in testes), testicular sperm (reduced sperm count) and hormonal status (serum prolactin levels increased) of male rats only occurred together with systemic toxicity at doses of 400 mg/kg bw/d.

In summary, it is concluded that 4-tert-octylphenol causes adverse systemic effects, such as decrease in body weight gain, altered food and water consumption, changes in organ weights and blood parameters and in addition effects to cyclicity and to the male reproductive system, generally at higher doses than those causing systemic effects. It is recognized that no information is available for 4-tert-octylphenol from an extended one-generation study according to OECD testing guideline 443 which currently is considered the most informative standard guideline test to provide information on endocrine sensitive endpoints in rodents.

In vitro and in vivo screening studies provide evidence, that 4-tert-octylphenol has some but a low estrogenic potential in comparison to reference estrogens. The relative potencies in cell proliferation assays, receptor binding assays, gene reporter assays and uterotrophic assays ranged from 10^{-5} - 0.008. The adverse systemic toxic effects of 4-tert-octylphenol are not considered to be specifically due to an endocrine mode of action. However, effects such as those observed on oestrous cycling in females may not necessarily be secondary to general systemic toxicity and according to stress. Rather, the impact of an estrogenic mode of action as revealed from the mechanistic studies needs to be considered. Therefore, for the cycle irregularities observed in females after treatment with 4-tert-octylphenol an ED-related mode of action is assumed. The mechanisms of effects on male reproductive organ system and spermatogenesis as observed at high systemically toxic exposure levels are less plausible, but the pattern of the effects was similar to that obtained from the reference estrogen. After subcutaneous application, further adverse effects (on organs of the reproductive system and on female/male development) were observed, which may be attributed to an endocrine mode of action. However, these effects are restricted to the s.c. route of application and did not occur after oral administration.

As indicated, the adverse effects of 4-tert-octylphenol on female/male reproduction most probably related to its estrogen-active properties, were not considered appropriate to justify classification as toxic for reproduction and/or carcinogenic Cat 1A or Cat 1B (ECBI/60/05 Rev. 3, Technical Committee for Classification and Labelling, 14-17 November 2005).

In addition, the adverse effects of 4-tert-octylphenol such as oestrous cycle disruption, damage of male reproductive organs and spermatotoxicity, were induced at relatively high dosages with concurrent systemically toxic effects only, indicating that other modes of action and effects may be more sensitive to 4-tert-octylphenol action than the estrogenic mode of action.

A similar conclusion was drawn, when 4-tert-octylphenol was evaluated within the Community Strategy for Endocrine Disrupters [COM(2001)262] during the course of the "Study on the scientific evaluation of 12 substances in the context of endocrine disrupter priority list of actions". There, it was concluded that "The available data from in vivo studies in laboratory animals (using oral or dermal exposure routes) indicates that 4-tert octylphenol does not cause adverse effects on reproductive and developmental endpoints (which may be endocrine mediated) at exposure levels where general systemic toxic effects are observed. … As a result it appears that endocrine mediated responses via oestrogen-like effects may be among a number of mechanisms responsible for the most toxic effects observed." (European Commission, 2002).

6.3 Conclusion of PBT and vPvB or equivalent level of concern assessment

As described in the chapters above, 4-tert-octylphenol is considered to be endocrine active in aquatic organisms through an estrogen mediated mode of action. It results in adverse effects as a consequence of the alteration of the endocrine system.

Observed effects on different levels (biomarker, histological changes as well as apical endpoints) in different organisms demonstrate that 4-tert-octylphenol is an endocrine disruptor for fish according to the OECD draft guidance document on the assessment of chemicals for endocrine disruptors (OECD, 2010c) and the WHO/IPCS definition (WHO/IPCS, 2002). As summarized in chapter 6.2.1.1 there is strong evidence from high quality studies of adverse effects in two fish species, which are estrogen mediated. Similar evidence is available for a third fish species but it is based on tests without statistics. In all other tested fish species the endpoints affected are known to be influenced by estrogen activity and, with one exception, an estrogen mediated mode of action was observed. Exposure to 4-tert-octylphenol may result in effects that relevantly influence ecosystems with respect to the community structure and function. Comparable to other estrogens, 4-tert-octylphenol influences reproduction parameters as well as sexual development (including changes in sex-ratio) and growth and thus endpoints are affected that may impair population stability and recruitment. Impairment of reproduction due to the endocrine disrupting properties of 4-tert-octylphenol may already occur after a transient short term exposure as observed in two fish species.

Exposure to estrogens such as 4-tert-octylphenol may cause long lasting effects. A change in the endocrine feedback system during sensitive life stages may result in effects during the entire life. The evidence presented indicates that 4-tert-octylphenol may have the potential to cause adverse effects in a range of species across different taxonomic groups.

The type of effects observed (e.g. short exposure of adults to 4-tert-octylphenol affects fertilization success) provide some evidence that exposure in one area might influence population stability in another area (e.g. for migratory fish). With respect to 4-tert-octylphenol there is evidence of internal distribution toward embryos in viviparous fish species.

All criteria suggested so far in order to analyze the level of concern are fulfilled by 4-tert-octylphenol.

As described above, estrogen mediated effects are considered as of similar concern to certain other substances of very high concern with respect to long-lasting effects and the difficulties to quantify risks.

Thus, in summary, *4-(1,1,3,3-tetramethylbutyl)phenol, (4-tert-octylphenol)* is identified as a substance of very high concern in accordance with Article 57 (f) of Regulation (EC) 1907/2006 (REACH) because it is a substance with endocrine disrupting properties for which there is scientific evidence of probable serious effects to the environment which gives rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of REACH.

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