Annex XV report

PROPOSAL FOR IDENTIFICATION OF A SUBSTANCE OF VERY HIGH CONCERN ON THE BASIS OF THE CRITERIA SET OUT IN REACH ARTICLE 57

Substance Name(s):	**p-(1,1-dimethylpropyl)phenol**
EC Number(s):	**201-280-9**
CAS Number(s):	**80-46-6**
Submitted by:	**Germany**
Date:	**30 August 2016**

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PROPOSAL FOR IDENTIFICATION OF A SUBSTANCE OF VERY HIGH CONCERN ON THE BASIS OF THE CRITERIA SET OUT IN REACH ARTICLE 57

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- It is proposed to identify the substance as a substance of equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of Regulation (EC) No 1907/2006 (REACH) according to Article 57(f) of REACH Regulation.

Summary of how the substance meets the criteria set out in Article 57 of the REACH Regulation

p-(1,1-dimethylpropyl)phenol (4-tert-pentylphenol) is proposed to be 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 give rise to an equivalent level of concern to those of other substances listed in points (d) to (e) of Article 57 REACH.

For 4-tert-pentylphenol there is strong evidence from good quality studies that the substance causes endocrine mediated adverse effects in several fish species:

- *In vitro* data unambiguously show that 4-tert-pentylphenol acts as a ligand of fish estrogen receptors. Modulation of 4-tert-pentylphenol-dependent and ER-mediated gene expression was observed on transcriptional, protein and cell physiological levels.
- *In vivo* data substantiate the endocrine mode of action. Endpoints indicative for an estrogenic mode of action were affected in all fish species tested (6 species). Effects observed included VTG induction, feminization of gonadal ducts and other histological alterations and reduced male secondary sex characteristics.
- A sex ratio biased towards females was observed in 5 fish species. This endpoint is both diagnostic for an endocrine mode of action and an adverse effect.
- Other observed adverse effects (reduced reproduction, reduced growth) in fish fit to the mode of action. Data show no evidence that they are caused by systemic toxicity.

The analysis is based on a profound data basis including 8 fish sexual development tests (or comparable) and 6 reproduction assays. Effects observed are similar to those observed for 4-tert-octylphenol and 4-nonylphenol and occur at similar test concentrations (ECHA, 2011) and (ECHA, 2012). Effects observed are regarded as endpoints of particular relevance because they are likely to manifest themselves at the population level.

An analysis of results based on the OECD (Organisation for Economic Co-operation and development) guidance document for endocrine disruptors (OECD, 2012b) reveals that 4-tert-pentylphenol need to be considered as endocrine disruptor. It fulfills the WHO/IPCS definition of an endocrine disruptor and the recommendations from the European Commission's Endocrine Disrupters Expert Advisory Group (JRC, 2013) for a substance to

be identified as an endocrine disruptor.

In conclusion, 4-tert-pentylphenol can be considered to be an endocrine disruptor for the environment. This conclusion is supported by read-across from other alkylphenols (4-nonylphenol and 4-tert-octylphenol) with regard to the environment. Data provide indication that 4-tert-pentylphenol may not only cause effects in fish but also in other taxa of environmental organisms which may be endocrine mediated, also caused by an estrogen-like mode of action.

4-tert-pentylphenol is considered as a substance giving rise to an equivalent level of concern due to its estrogen agonist mode of action and the type of effects caused by this mode of action. Based on data for 4-tert-pentylphenol as well as other estrogen agonists, 4-tert-pentylphenol evidence that the substance is of an equivalent level of concern includes:

- Exposure to 4-tert-pentylphenol resulted in effects in fish on reproduction parameters (fecundity) as well as on sexual development (including changes in sex ratio) and growth. Results for at least 3 fish species show that exposure to 4-tert-pentylphenol may result in complete sex reversal of males resulting in all female populations.
- Effects observed for 4-tert-pentylphenol and the alkylphenols 4-nonylphenol and 4-tert-octylphenol show that transient exposure during sensitive life stages may result in effects that remain during the entire life and even in following generations. Thus local exposure of migratory species might not only locally affect population stability but also in other areas.
- On the basis of the available data for 4-tert-pentylphenol itself and from read-across it appears difficult to derive a safe level. Read-across from 4-tert-octylphenol and 4-nonylphenol with regard to organisms in the environment indicates that
    - Effects on non-traditional endpoints may start at much lower concentrations than those considered in OECD test guidelines.
    - Although it is not possible to clearly state that effects on other organisms such as invertebrates and amphibians are endocrine mediated, these effects fit to the knowledge that steroids are known to play an important role in invertebrates (Kendall et al., 1998). Owing to the lack of in-depth knowledge of their endocrine system and the lack of test systems, it is currently nearly impossible to estimate which species are most sensitive and which concentration should be regarded as safe for the environment.

Thus in summary, the endocrine mediated effects observed in fish after exposure to 4-tert-pentylphenol are considered to have the potential to adversely affect population stability and recruitment. These adverse effects not only persist after cease of exposure but also occur after transient short-term exposure at sensitive live stages. They thus may adversely affect populations in the longer-term and migratory species not only locally but also in regions where no exposure occurred. 4-tert-pentylphenol may affect taxa other than fish (e.g. invertebrates) too. Based on current data and knowledge, a safe level of exposure is difficult to derive although it may exist. Consequently, there is scientific evidence that 4-tert-butylphenol causes probable serious effects in the environment which give rise to an equivalent level of concern to those of other substances listed in points (d) to (e) of Article 57 REACH.

Registration dossiers submitted for the substance? Yes

PART I

Justification

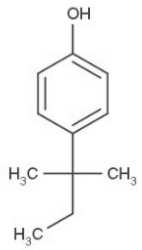
1 Identity of the substance and physical and chemical properties

1.1 Name and other identifiers of the substance

Table 1: Substance identity

EC number:	201-280-9
EC name:	p-(1,1-dimethylpropyl)phenol
CAS number (in the EC inventory):	
CAS number: **Deleted CAS numbers:**	80-46-6 -
CAS name:	Phenol, 4-(1,1-dimethylpropyl)-
IUPAC name:	4-(1,1-dimethylpropyl)phenol
Index number in Annex VI of the CLP Regulation	-
Molecular formula:	$C_{11}H_{16}O$
Molecular weight range:	164.24 g/mol
Synonyms:	Phenol, p-(1,1-dimethylpropyl)- (5CI); Phenol, p-tert-pentyl- (6CI,8CI); 4-(1,1-Dimethylpropyl)phenol; 4-t-Amylphenol; 4-t-Pentylphenol; 4-tert-Amylphenol; 4-t-pentylphenol; Amilfenol; BirexSE; NSC 403672; NSC 4965; p-(1,1-Dimethylpropyl)phenol; p-(α,α-Dimethylpropyl)phenol; p-tert-Amylphenol; p-tert-Pentylphenol; 4-t-PP

Structural formula:



1.2 Composition of the substance

Name: p-(1,1-dimethylpropyl)phenol

Description: mono-constituent

Substance type: organic

1.3 Identity and composition of structurally related substances (used in a grouping or read-across approach)

4-tert-pentylphenol can be considered as part of a group of alkylphenols with a linear or branched alkylchain in para-position. The substances differ in the length of the alkylchain and the degree of branching. The following substances can be considered as part of this group:

Table 2: Other Substance identifiers – 4-nonylphenol

EC number:	
EC name (public):	
CAS number:	
CAS name (public):	
IUPAC name (public):	4-Nonylphenol, branched and linear: substances with a linear and/or branched alkyl chain with a carbon number of 9 covalently bound in position 4 to phenol, covering also UVCB- and well-defined substances which include any of the individual isomers or a combination thereof
Index number in Annex VI of the CLP Regulation:	
Molecular formula:	$C_{15}H_{24}O$
Molecular weight or molecular weight range:	220.35 g/mol

Substance type: group entry

Structurally related substance(s) formula:

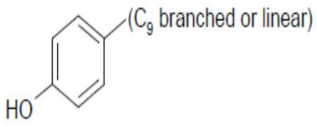


Table 3: Other Substance identifiers – 4-tert-octylphenol

EC number:	205-426-2
EC name (public):	4-(1,1,3,3-tetramethylbutyl)phenol
CAS number:	140-66-9
CAS name (public):	Phenol, 4-(1,1,3,3-tetramethylbutyl)-
IUPAC name (public):	4-(2,4,4-trimethylpentan-2-yl)phenol
Index number in Annex VI of the CLP Regulation:	601-053-00-8
Molecular formula:	$C_{14}H_{22}O$
Molecular weight or molecular weight range:	220.35 g/mol (for octylphenol)

Substance type: mono-constituent

Structurally related substance(s) formula:

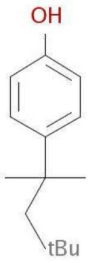
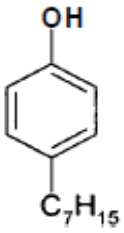


Table 4: Other Substance identifiers – 4-heptylphenol

EC number:	
EC name (public):	4-Heptylphenol, branched and linear
CAS number:	
CAS name (public):	
IUPAC name (public):	4-Heptylphenol, branched and linear *[substances with a linear and/or branched alkyl chain with a carbon number of 7 covalently bound predominantly in position 4 to phenol, covering also UVCB- and well-defined substances which include any of the individual isomers or a combination thereof]*
Index number in Annex VI of the CLP Regulation:	N/A
Molecular formula:	$C_{13}H_{20}O$
Molecular weight or molecular weight range:	192.2973 g/mol

Substance type: group entry

Structural formula:



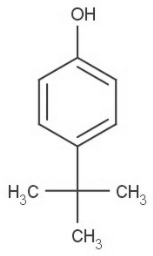
(branched and linear)

Table 5: Other Substance identifiers – 4-tert-butylphenol

EC number:	202-679-0
EC name (public):	4-tert-butylphenol
CAS number:	98-54-4
IUPAC name (public):	4-(1,1-dimethylethyl)-phenol
Index number in Annex VI of the CLP Regulation:	604-090-00-8
Molecular formula:	$C_{10}H_{14}O$
Molecular weight or molecular weight range:	150.2176 g/mol

Substance type: mono-constituent

Structurally related substance(s) formula:



1.4 Physicochemical properties

Table 6: Overview of physicochemical properties

Property	Description of key information	Value [Unit]	Reference/source of information
Physical state at 20°C and 101.3 kPa		slightly yellow solid (flakes) with a phenolic odour	Spilker 2012
Melting/freezing point	DSC method	94.7 °C at 101325 Pa	Spilker 2011
Boiling point	DSC method	255 °C at 101325 Pa	Spilker 2011
Vapour pressure	Method NFT 20-048, Isoteniscope	< 5 Pa at 20 °C	Conte T 2012
Density		0.962 g/cm^3	Scifinder; National Library of Medicine (US)
Water solubility	ASTM E 1148 – 02, flask method	193 mg/L at 21 °C, pH 6 – 7	Spilker 2011
Partition coefficient n-octanol/water (log value)	OECD 117, HPLC method	logP_{ow} 3.6 at 22 °C, pH 6 - 7	Spilker 2011

2 Harmonised classification and labelling

There is no harmonized classification for 4-tert-pentylphenol in Annex VI, Part 3, Table 3.1 (list of harmonised classification and labelling of hazardous substances) of Regulation (EC) No 1272/2008.

3 Environmental fate properties

Not relevant for the identification of the substance as SVHC in accordance with Article 57(f) REACH. However a core set of data is provided as background information.

3.1 Degradation

3.1.1 Screening tests

Table 7: Summary of available screening tests

Method	Result	Remarks	Reference
Test type: ready biodegradability Activated sludge, domestic, non-adapted OECD 301 B (Ready Biodegradability: CO_2 Evolution Test)	73 % degradation after 28 days 52 % degradation at end of 10-day window Readily biodegradable, but failing 10-day window	Reliability 1 (reliable without restriction) Key study 20 mg/L initial test substance concentration	Haener, A. (1999)
Test type: inherent biodegradability Activated sludge (adaptation not specified) OECD 302 C (Inherent Biodegradability: Modified MITI Test (II))	0 % degradation after 28 days Not inherently biodegradable	Reliability 2 (reliable with restrictions) Supporting study 30 mg/L initial test substance concentration	TL (1992a)

4-tert-pentylphenol was observed to be readily biodegradable, but failing the 10-day window in a ready biodegradability screening test. No degradation was observed in an inherent biodegradability screening test. However, the initial test substance concentration was 30 mg/L in the inherent test while only 20 mg/L have been used for the ready biodegradability test. The toxicity of 4-tert-pentylphenol is in the range of the test substance concentration. Therefore, the missing degradation in the inherent biodegradability screening test is most likely caused by toxicity of the test substance to the microorganisms.

3.1.2 Summary and discussion of degradation

Within the scope of this assessment 4-tert-pentylphenol was assumed to be readily biodegradable, but failing the 10-day window.

3.2 Environmental distribution

Not relevant for the identification of the substance as SVHC in accordance with Article 57(f) REACH. However a core set of data is provided as background information.

3.2.1 Adsorption/desorption

Based on the $logK_{ow}$ of 3.6, 4-tert-pentylphenol is expected to partition into sediment and soil. A rapid decomposition of 4-tert-pentylphenol is not expected as 4-tert-pentylphenol will not dissociate at environmentally relevant pH and hydrolysis will not occur due to the absence of hydrolysable functional groups. Partitioning into sediment and soil is driven by adsorption to soil and sediment constituents, mainly organic matter. Therefore, partition coefficients like K_{oc} are basic values for environmental distribution modelling.

A value of K_{oc} = 1470 was calculated based on a $logK_{ow}$ of 3.6 by the lead registrant with EUSES. The applicability of the QSAR used in the CSR was not demonstrated by the registrant. A K_{oc} of 2300 was calculated by the UK environment agency in a risk assessment report (2008) based on a $logK_{ow}$ of 4.0. The UK environment agency also calculated a K_{oc} of 3799 by using molecular connectivity indices using PCKOCWIN v1.66 and SMILES input. Depending on the method used for calculations and the assumptions made, the resulting $logK_{oc}$ differ from 3.17 to 3.58.

The $logK_{oc}$ is a basic value for modelling the environmental distribution. There are uncertainties in calculating $logK_{oc}$ from $logK_{ow}$. The difference between calculated and measured values is an indication for this. However, it cannot be excluded that other components than organic carbon influence the sorption and hence measured values. A low $logK_{oc}$ represents the worst case for the assessment of the water compartment. Therefore, for the purpose of this assessment the use of a calculated $logK_{oc}$ is considered appropriate.

4 Human health hazard assessment

Not relevant for the identification of the substance as SVHC with regard to the environment in accordance with Article 57(f) REACH.

5 Environmental hazard assessment

5.1 Aquatic compartment (including sediment)

5.1.1 Short term toxicity to aquatic organisms

Short term toxicity data are available for fish *(O.mykiss, P. promelas, C. carpio, O.latipes)* 3 invertebrate species and , one algae species. As they are not relevant for the assessment of the endocrine disrupting properties of the substance, they are not assessed for reliability and thus not discussed in this section. They are summarized in tabular form in Annex II.

5.1.2 Long term toxicity to aquatic invertebrates

In 1996 the Japan Ministry of health and welfare conducted a test on chronic toxicity to *Daphnia magna* with a duration of 21 days. The test was similar to Guideline OECD 211 and conducted according to OECD TG Part II (1984). The test was a semi-static test and no analytics were done. The tested concentrations were: nominal: 0.073, 0.23, 0.73, 2.3, 7.3 mg/L. The EC_{50} was 2 mg/L, the LOEC had a higher value of 2.3 mg/L and the NOEC was 0.73 mg/L. All values were based on reproduction. The test was assessed by the registrant with Klimisch 2.

Lee et al. (Lee et al., 2008) conducted a 14-d test with the harpacticoid copepod *Tigriopus japonicus. T.japonicus* seems to be sensitive to estrogenic compounds as the naupliar phase duration and development time was significantly affected by estrogenic compounds such as 4-NP and 4-t-OP (Marcial et al., 2003).

Table 8: Summary of the long-term toxicity to aquatic invertebrates

Test method	Results	Reliability acc. to Klimisch	Reference
OECD 211 or OECD 202 Part II (1984) *Daphnia magna*	21d-NOEC = 0.73 mg/L (nominal)	2	Ministry of Health and Welfare Japan (1996)
Harpacticoid copepod *Tigriopus japonicus*	14d-NOEC = 0.01 mg/L (F1 number of clutches reduced) (not affected in F0 generation)	2	(Lee et al., 2008)

5.2 Other effects

5.2.1 Endocrine Disruption in fish

5.2.1.1 General approach – environment

The evaluation, whether or not 4-tert-butylphenol is an endocrine disruptor in fish, is based on *in vitro* data and *in vivo* data. The assessment of *in vivo* data focuses on the question whether or not results are in accordance with the presumed mode of action based on *in vitro* tests or rather seem to be a consequence of systemic toxicity.

Assessment of *in vivo* data is mainly based on the OECD guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption (OECD, 2012b): Although it focuses on validated OECD test guidelines, general information on how

to assess endocrine disrupting properties can be extracted. 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 estrogenic mode of action.
One of the most common biomarkers indicating an estrogen or androgen endocrine mode of action is vitellogenin (VTG). Vitellogenin is naturally produced by female fish as a precursor of yolk proteins that are incorporated in eggs (IPCS, 2002). Induction of vitellogenin in female and (more pronounced) in male fish is a known indicator of an estrogen agonist mode of action (IPCS, 2002; Kendall et al., 1998; Knacker et al., 2010; OECD, 2004).

With respect to histological changes according to the OECD test guideline 229 for the fish short term reproduction assay (OECD, 2009b) 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, retained peritoneal attachments/gonadal duct feminization of the testis (estrogenic response in juvenile fathead minnow and zebrafish)
- 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 fish 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).[1]

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, 2012).

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

[1] The size of the gonads (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 reoccurs and, as their spawning is usually not synchronized, individual gonadal growth differs in time.

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, 2009b). Induction of female secondary sex characteristics in males such as urogenital 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 (IPCS, 2002; Kendall et al., 1998; OECD, 2004). 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).

Definition of sex ratio according to OECD guideline 234 (Fish Sexual Development Test): "The sex is defined as female, male, intersex (both oocytes and spermatogenetic cells in one gonad) or undifferentiated, determined in individual fish via histological examination of the gonads."

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.

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; Knacker et al., 2010; OECD, 2004).

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 9: Summary of endpoints that are considered indicators of estrogen activity in fish

Endpoints indicating an estrogen agonist mode of action	**Endpoints considered to be sensitive to an estrogenic 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, gonadal duct feminization of the testis/ retained peritoneal attachments 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

5.2.1.2 *In vitro* information indicative of endocrine activity

In vitro estrogen activity of 4-tert-pentylphenol was assessed in different assays including binding assays, reporter gene assays, YES assays and assays analyzing vitellogenin (VTG) induction in primary hepatocytes of *Oncorhynchus mykiss* and *Cyprinus carpio*.
Results are briefly summarized below and in Table 10. In addition, information for other similar alkylphenols (4-nonylphenol, 4-tert-octylphenol, heptylphenol, 4-tert-butylphenol were collected for read-across. Data are summarized in Annex 1 and briefly compared to those for 4-tert-pentylphenol in chapter 5.2.1.2.6 at the end of this section.

5.2.1.2.1 Competitive ligand-binding assays

Competitive ligand-binding assays are used to assess whether or not a test chemical is able to specifically bind to a given receptor or protein. Two tests using fish cytosol preparations are available, one using rat cytosol preparations and one using recombinant human estrogen receptor α in *E. coli*. Tollefsen and Nilsen (Tollefsen and Julie Nilsen, 2008) assessed whether or not 4-tert-pentylphenol is able to specifically bind to the rainbow trout estrogen receptor (rtER) a using cytosolic preparation of female trout liver. Pooled liver homogenates was incubated with E2 for 16 h in the absence or presence of different concentrations of 4-tert-pentylphenol or E2; three replicates were used and the solvent methanol (c_{max} =1.25 %). 4-tert-pentylphenol was demonstrated to displace specifically bound 17ß-estradiol (E2) from the rtER. The relative binding affinity (RBA[2]) of 4-tert-pentylphenol compared to E2 was 7E-5.

Hornung et al. conducted competitive binding assays using liver cytosolic preparations (cyto rtERαß) from immature rainbow trout (Hornung et al., 2014). The preparations contained all ER receptors found in trout liver (α1,α2, ß1, ß2). The test was conducted in triplicate with a minimum of six concentrations covering four to six log intervalls, together with [^{3}H]E2. The maximum achieved test concentration was 0.01 M. Ethanol was used as the solvent. The RBA for 4-tert-pentylphenol was 4E-5.

In a study conducted by Blair et al. (Blair et al., 2000) the ER-binding affinity was determined in a cytosolic preparation of uteri from ovariectomized rats. Uterine cytosol preparation and [^{3}H]E2 (E-9 M) were incubated with increasing concentrations of 4-tert-pentylphenol for 20 h in duplicate. The RBA of 4-tert-pentylphenol was 5E-6.

The study conducted by Akahori et al. (Akahori et al., 2008) was a special study, because

[2] RBA: calculated as IC50(E2)/IC50(4-t-BP). The IC50 in binding studies is the equilibrium inhibitory concentration, calculated as the concentration causing 50% inhibition of [3H]-E2 binding.

the recombinant estrogen receptor α (hERα) was expressed in *E. coli* and then purified. 4-tert-pentylphenol (concentrations: 1E-11 to 1E-4M) and [^{3}H]E2 (0.5 nM) were incubated together with hERα for 1 h. The radioactivity of ligands bound to the receptor was measured. (Replicates: more than three per chemical) 4-tert-pentylphenol displaced 17ß-estradiol from the receptor and the RBA was 1.7E-4. The RBA from this test is higher by a factor of 100 than the RBA from the study by Blair et al. (2000).

5.2.1.2.2 Binding to sex steroid binding proteins

Another study by Tollefsen (Tollefsen, 2007) evaluated the competitive binding to the rainbow trout plasma sex steroid-binding protein (rtSBP). Plasma samples from female rainbow trout were incubated with 100 µL of 2.5 nM [^{3}H]E2 in combination with increasing concentrations of 4-tert-pentylphenol (25 nM–250 µM), incubation: 16 h; the difference between total (2.5 % vehicle methanol alone) and non-specific binding (1000-fold excess of unlabeled steroid) represents the specific plasma steroid binding. 4-tert-pentylphenol bound to the rtSBP and the RBA obtained was 4.3E-5 which is in the same range like the binding affinity to the ER.

Summary:
All studies showed that 4-tert-pentylphenol binds to the estrogen receptor in fish, rat and human and binds to sex steroid binding proteins in fish. The relative binding affinity in the studies with rainbow trout estrogen receptor ranged from 4E-5 to 7E-5 and the RBA for the sex steroids was in a similar range. The RBA in the study with the rat ER (cytosolic preparation of uteri from ovariectomized rats) was slightly lower (RBA = 5E-6). Results from the study using recombinant human ERα are not directly comparable.

5.2.1.2.3 Expression of estrogen-responsive genes

Two *in vitro* studies investigated the effect of 4-tert-pentylphenol on vitellogenin (VTG) protein expression. In one study primary hepatocytes derived from male immature rainbow trout (*Oncorhynchus mykiss*) were used (Tollefsen et al., 2008). The duration of incubation was 4 d, renewal of medium after 2 d; (Solvent: DMSO, c_{max} < 0.3 % (v/v) / n = 3, i = 3). The relative estrogenic potency (REP) was 3.3E-5. Another study conducted by Smeets et al. (Smeets et al., 1999) used primary hepatocytes derived from genetically male carp. Here the REP was 1E-4. Cytotoxicity was determined to be significantly elevated at 100 µM, whereas the LOEC for VTG induction was at 50 µM.

One study conducted by Hornung et al. (Hornung et al., 2014) used a trout liver slice gene expression assay measuring induction of VTG mRNA. 4-tert-pentylphenol was determined to be an agonist to the ER and grouped in a slice efficacy of 0.1 to 1 (based on efficacy = 1 for E2). In comparison the slice efficacy of 4-t-OP was 1.

Summary:
Both studies analysing vitellogenin induction demonstrated that exposure to 4-tert-pentylphenol resulted in a dose-dependent increase in vitellogenin expression levels with a range of 3.3E-5 to 1E-4. Liver slice gene expression showed VTG mRNA induction.

5.2.1.2.4 Reporter gene assays

Transcriptional activation assays are used to assess whether or not a test chemical is able to activate the receptor. Activation of the receptor is demonstrated by means of reporter genes. Three yeast screening assays using recombinant human estrogen receptor are available and one assay analysing gene expression with the rainbow trout estrogen receptor (rtER).

Routledge and Sumpter (Routledge and Sumpter, 1997) conducted a yeast estrogen screen with recombinant yeast expressing human estrogen receptor (hER) measuring the firefly luciferase activity representing the expression of the E2-responsive reporter gene. Yeast cells were exposed to increasing concentrations of 4-tert-pentylphenol or E2. After 84 h

incubation (at 32 °C) the color change of the medium was measured. The relative potency of 4-tert-pentylphenol was approx. 1E-5 compared with E2 (read from graph). EC_{50} values were not given.

Another test with recombinant yeast expression human α-ER was conducted by Schultz et al. (Schultz et al., 2000). Yeast cells were exposed to increasing concentrations of 4-tert-pentylphenol or E2. After 5 d incubation, the color change of medium was measured. Evaluation in duplicate with a minimum of three replicates. Solvent: Ethanol. The REP value was not given, but by calculation from the EC_{50} values of E2 and 4-tert-pentylphenol the REP would be 8.2E-6. In comparison with the EC_{50} value of 4-tert-pentylphenol the EC_{50} of 4-t-OP is 27 times lower. In a yeast two-hybrid assay the REC10 was E-6 M (Nishihara et al., 2000). In this assay, 4-tert-pentylphenol was approx. 10 times less active than 4-t-OP.

One test is available conducted by Hornung et al. (Hornung et al., 2003) using rainbow trout hepatoma cells (RTH-149) transfected with the rainbow trout ER. Cells were exposed to different concentrations of 4-tert-pentylphenol or E2. (Incubation 72 h, three replicates). Firefly luciferase activity indicating the expression of the E2-responsive reporter gene was measured. 4-tert-pentylphenol was found to induce luciferase activity at concentrations EC_{50} 6.9E-7M (18 °C) and EC_{50} 7.6E-7M (11 °C). The relative estrogenic potency (REP) was 5.4E-4 and 3.6E-4 at 18 °C and 11 °C respectively.

Summary:
All tests described above indicate that 4-tert-pentylphenol acitivates human and fish estrogen receptors. The relative potency was higher for fish receptor than for human receptor.

5.2.1.2.5 MCF-7 cell proliferation assay

4-tert-pentylphenol was further demonstrated to induce human breast cancer cell (MCF-7) proliferation (Soto et al., 1995) and thus to act as ER agonist in these cells. MCF-7 cells were incubated in a range of 4-tert-pentylphenol concentrations for 6 days. Afterwards the nuclei were counted.

The relative proliferative potency (RPP) is the ratio between the minimal concentration of estradiol needed for maximal cell yield and the minimal concentration dose of 4-tert-pentylphenol needed to achieve a similar effect.

The RPE (relative proliferative effect) induced by 4-tert-pentylphenol was 1.05. If RPE is > 1 than is the substance determined to be a full agonist to E2. As the RPE is 1.05, 4-tert-pentylphenol is a full agonist to E2. The RPE is calculated by this equation: RPE = [(PE-1) of 4-tert-pentylphenol] / [(PE-1) of E2], in which the proliferative effect (PE) is calculated in this way: PE = highest cell yield obtained with the test chemical / highest cell yield obtained with the chemical-free control.

For comparison, 4-NP also exhibited a RPE of 1.

The RPP for 4-tert-pentylphenol was 3E-6 and for 4-NP 3E-5 and thus 4-NP had a magnitude stronger relative proliferative potency. The RPE indicates whether the tested compound induces a proliferative response quantitatively similar to E2.

5.2.1.2.6 Summary

The competitive ligand-binding studies clearly demonstrated that 4-tert-pentylphenol is able to displace specifically bound E2 from the ER ligand-binding pocket. The RBA of 4-tert-pentylphenol for ERs derived from rainbow trout ranged from 4E-5 to 7E-5. The RBA to the rat ER was 5E-6, approx. one order of magnitude lower than RBA to the rtER. In both cases 4-tert-pentylphenol acts as a ligand of the ER.

Furthermore, there is evidence that binding of 4-tert-pentylphenol to the ER leads to activation of the ER-mediated pathway and consequently to transcriptional activation of typically estrogen-responsive genes. Modulation of 4-tert-pentylphenol-dependent and ER-mediated gene expression was evidenced on the transcriptional, protein and cell physiological level.

The relative estrogenic potencies derived in the VTG assay were 3.3E-5 and 1E-4.
The relative potency obtained in the transcriptional activation assays (YES) was between 8.2E-6 and 5.4E-4, with the highest relative potency derived with the rainbow trout hepatoma cells.

In the MCF-7 cell proliferation assay the relative proliferative effect (RPE) obtained was 1.05 for 4-tert-pentylphenol, indicating that 4-tert-pentylphenol acts as a full agonist towards the ER. The relative proliferative potency (RPP) for 4-tert-pentylphenol was 3E-6.

A comparison of data summarised for other alkylphenols in Annex 1 shows that overall *in vitro* activity of 4-tert-pentylphenol is in the same range (max factor 10 difference) as observed for 4-nonylphenol and 4-tert-octylphenol which are already identified as substances of very high concern due to their endocrine disrupting properties for the environment.

Based on the available mechanistic information it can be concluded that 4-tert-pentylphenol has the potential to exert estrogen-like effects and disrupt endocrine homeostasis.

Table 10: Summary of *in vitro* studies assessing the potential of 4-tert-pentylphenol (4-t-PP) to interact with the ER-mediated pathway.

Endpoint: Competitive ligand binding (IC_{50} is the concentration displacing 50 % of [^{3}H]E2 from ER ligand binding pocket).					
Binding to ER					
Species	**Reference**	**Receptor origin and preparation**	**Endocrine mediated measurement parameters**	**Relative binding affinity (RBA) compared to 17β-estradiol (=1)**	**Comment**
Oncorhynchus mykiss rainbow trout	(Tollefsen and Julie Nilsen, 2008)	Cytosolic preparation of female trout liver homogenates	IC_{50}(E2) = 3.5E-9M (0.95 µg E2/L) IC_{50}(4-t-PP) = 5E-5M (8212 µg/L) (IC_{50}) was calculated as the concentration causing 50% inhibition of [^{3}H]E2 binding	RBA = 7 x 10^{-5} (RBA was calculated as IC_{50}(E2)/IC_{50}(4-t-PP))	Comparison with 4-t-OP: IC_{50} 5.1E-5 M; Klimisch 2
Oncorhynchus mykiss rainbow trout	(Hornung et al., 2014)	Cytosolic liver preparations from immature rainbow trout.	No IC_{50} value given	RBA (cyto rtERαß binding) = 4 x 10^{-5} (RB was calculated as IC_{50} (E2)/ IC_{50} (4-t-PP))	Comparison with 4-t-OP: RBA 9.4 x 10^{-5}; Klimisch 2
Rat	(Blair et al., 2000)	Cytosolic preparation of uteri from ovariectomized rats	IC_{50}(E2) = 8.99 x 10^{-10} IC_{50} (4-t-PP) = 1.65 x 10^{-4} M (55.28 x 10^3 µg/L)	RBA = 5 x 10^{-6} RBA was calculated as IC_{50}(E2)/IC_{50}(4-t-PP)	Comparison with 4-t-OP: 4-t-BP has a 30-fold lower RBA; Klimisch 2
Human	(Akahori et al., 2008)	Recombinant human estrogen receptor α (hERα) expressed in *E.coli*	See right (no IC_{50} values given)	RBA = 1.7 x 10^{-4} RBA = IC_{50}(E2)/ IC_{50} (4-t-PP) RBA calculated from the given log RBA value, based on 1 (not percent)	From the same study also data were obtained for 4-t-PP from an Immature rat uterotrophic assay: Lowest effective dosis: 1230 µmol/kg/day for both estrogenic (uterine weights were sign. increased in the estrogenic assay) and antiestrogenic (uterine weight sign. decreased in the antiestrogenic assay) effects respectively. ; Klimisch 2
Binding to the plasma sex steroid-binding protein					
Oncorhynchus mykiss rainbow trout	(Tollefsen , 2007)		IC50 values of the test compounds were calculated as the concentration that caused 50% inhibition of [^{3}H]-E2 binding.	Relative binding affinities (RBA) were determined by comparing the obtained IC_{50} values for the test compound	Comparison: 4tOctylphenol: IC50 1.2 x 10^{-4} M; Klimisch 2

			IC_{50} (4-t-PP): 4.6E-5 M (7500 µg/L) IC_{50} (E2): 1.6E-9 M (0.43 µg/L)	relative to that of E2. RBA: 4.3E-5 (3.5E-5)	
Endpoint: Expression of estrogen-sensitive genes					
Expression of vitellogenin					
Species	**Reference**	**Cell type and origin**	**Endocrine mediated measurement parameters**	**Potency (relative to 17 β-estradiol=1)**	**Comment**
Cyprinus carpio Carp	(Smeets et al., 1999)	Primary hepatocytes derived from genetically male carp	VTG induction LOEC (E2) = 0.002E-6 M LOEC (4-t-PP) = 50E-6 M (8272 µg/L)	Relative estrogenic potency (REP) = 1E-4 REP was calculated as Threshold concentration of E2 / Treshold concentration of 4-t-PP	Klimisch 2
Oncorhynchus mykiss rainbow trout	(Tollefsen et al., 2008)	Primary hepatocytes derived from male, immature fish	VTG induction LOEC (E_2) = 1E-10 M (2.7E-2 µg/L) LOEC (4-t-PP)= 3E-6 M (490 µg/L)	REP = 3.3E-5 REP was calculated as LOEC(E2) / LOEC(4-t-PP)	Comparison: 4-t-octylphenol: LOEC 1 µM REP: 1E-4; Klimisch 2
Oncorhynchus mykiss rainbow trout	(Hornung et al., 2014)	induction of VTG mRNA in trout liver slices	4-t-PP was determined as agonist to ER No values given	Slice efficacy was in the order of 0.1 to 1 (based on slice efficacy of E2 max = 1)	Slice efficacy of 4tOP was = 1; Klimisch 2
Endpoint: Transcriptional activation of reporter genes under the control of the ER					
Transcriptional activation assay using recombinant yeast (yeast estrogen screen, YES)					
human	(Routledge and Sumpter, 1997)	Recombinant yeast expressing hER	No EC_{50} values were given.	Relative potency of 4-t-PP is approx. 1E-5, compared with E2 (relative potency = 1)(read from graph)	Klimisch 2
human	(Schultz et al., 2000)	Recombinant yeast Expressing α-hER	EC_{50} (E2) $3.91x10^{-11}$ M EC_{50} (4-t-PP) $4.76x10^{-6}$ M	No value given, however by using the equation EC_{50}(E2)/EC_{50}(4-t-BP) the REP would be 8.2E-6	Comparison with 4-t-OP: EC_{50} $1.77x10^{-7}$ M The REP for 4-t-OP is $2.2x10^{-4}$ 4-t-OP is 27-fold more active than 4-t-PP; Klimisch 2
Human	(Nishihara et al., 2000)	Yeast two-hybrid assay with estrogen receptor ERα	REC10 : 1×10^{-6} M		REC10 (4-tert-Octylphenol): 2×10^{-7} M 4-t-PP has a 10-fold lower activity than

			REC10: The concentration showing 10% activity of 10^{-7} M 17β-estradiol (relative activity).		4-t-OP; Klimisch 2
Transcriptional activation assay using vertebrate cell lines					
Oncorhynchus mykiss rainbow trout	(Hornung et al., 2003)	Rainbow trout hepatoma cells (RTH-149) transfected with the rainbow trout ER and an estrogen-responsive firefly luciferase reporter gene.	Luziferase activity: EC_{50} (estradiol)= 3.7 x 10^{-10} M EC_{50} (4-t-PP)= 6.9 x 10^{-7} M (113.3 µg/L), 18°C EC_{50} (estradiol)= 2.7 x 10^{-10} M EC_{50} (4-t-PP)= 7.6 x 10^{-7} M (125 µg/L), 11°C	Relative potency: 5.4E-4 (18°C) 3.6E-4 (11°C)	Mean maximal efficacy for 4-t-PP: 69% of E2 (18 °C) 83% of E2 (11 °C) ; Klimisch 2
Endpoint: MCF-7 cell proliferation assay (E-Screen) E-screen uses the proliferative effect of estrogens on their target cells (cells of the female genital) as an end point.					
Species	**Reference**	**Cell type**	**Endocrine mediated measurement parameters**	**Potency (relative to 17ß-estradiol=1)**	**Comment**
Human	(Soto et al., 1995)	MCF-7 cells	The proliferative effect (PE) is measured as the ratio between the highest cell yield obtained with the test chemical and with the control. Lowest concentration needed for maximal cell yield: 4-t-PP: 10E-6M (1640 µg/L) E2: 30E-12M	Relative proliferative effect (RPE) (4-t-PP): 1.05 (full agonist) Relative proliferative potency (RPP) (4-t-PP): 3E-6 (Explanation RPE and RPP see below)	Comparison 4-nonylphenol RPE = 1 RPP = 3E-5; Klimisch 2

ER =estrogen receptor, E2 = 17 β-estradiol, n = number of independent experiments, I = number of replicates within each experiment, ECmax = concentration, at which highest response was observed, LOEC = lowest observed effect concentration, cmax = maximal concentration of test chemical or solvent in the assay
RBA = relative binding affinity.
REP = relative estrogen potency, calculated as EC_{50}(E2)/EC_{50}(4-t-PP)
RPE = Relative proliferative effect. RPE is calculated as (PE-1) of the test compound/(PE-1) of E2. Thus, the RPE indicates whether the compound being tested induces a proliferative response quantitatively similar to the one obtained with E2, that is, a full agonist (RPE = 1), or a proliferative yield significantly lower than the one obtained with E2, that is, a partial agonist.
PE: The proliferative effect is measured as the ratio between the highest cell yield obtained with the test chemical and with the hormone-free (chemical-free) control.
RPP: relative proliferative potency, which measures the ratio between the minimal concentration of estradiol needed for maximal cell yield and the minimal concentration of the test compound needed to achieve a similar effect. (E2 / 4-t-PP)
pS2 is a small secretorial peptide synthesized in the MCF-7 cells in presence of oestrogen (Olsen et al. 2003; Masiacowski et al. 1982).

4-t-PP = 4-tert-pentylphenol, 4-t-OP = 4-tert-octylphenol, 4-t-BP = 4-tert-butylphenol

5.2.1.3 *In vivo* effects with regard to an endocrine mode of action

Available data are assessed 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 are considered.

Overall for four fish species *in vivo* data at different levels (biomarker, histology and apical endpoints) are available.

- *Pimephales promelas,*
  Extended ELS (reliability 2);
  FSDT (results from validation report (Phase 1) for the FSDT, reliability 2);
  modified juvenile growth test (reliability 2);
  extended and modified 21-day reproduction study (reliability 2);
  Three Reproduction assay (results from 21-day-assay validation report, reliability 2)
- *Danio rerio,*
  Two FSDT (results from validation report (Phase 1) for the FSDT, reliability 2);
  Three Reproduction assay (results from 21-day-assay validation report, reliability 2 except one with reliability 3)
- *Oryzias latipes,*
  modified ELS 4 wk + 2 wk (reliability 2)
  Two FSDT (results from validation report (Phase 2) for the FSDT, reliability 2);
  Fish full life cycle test (reliability 2);
  Four reproduction assays (results from 21-day-assay validation report, reliability 2)
- *Cyprinus carpio,*
  Two modified Juvenile growth tests (one with reliability 2, the other reliability 3);
  one modified ELS (reliability 2);
  modified fish reproduction assay (reliability 2)
- *Cyprinodon variegates* (Sheepshead minnow)

5.2.1.3.1 Pimephales promelas

Extended early life stage test:

An extended early life stage test was conducted by Panter et al. (Panter et al., 2006). The study has been rated Klimisch 2.

Eggs were exposed from < 24 hours post fertilization (hpf) until 107 days post hatch (dph). 100 fish embryos per test concentration were used, in four replicates. The validity criteria were fulfilled.

Treatments and exposure duration:

Treatments	56	180A	180B	180	560
Measured concentration	56.6µg/L	194µg/L	202µg/L	188µg/L	599µg/L
Exposure duration	107dph	30dph	60dph	107dph	107dph

The fish from treatments with shorter exposure duration remained in dilution water until 107dph. Furthermore a solvent control (solvent triethylene glycol, 0.1 mL/L), a dilution water control and a positive control (EE2 0.01 µg/L) were included in the experiment until 107dph.

Results:

Mortality and growth: There were no significant differences in the hatching success (control 92%, between 81 and 92 % in treatments) and in survival (107 dph) between the treatments and the

solvent control.

Gonadal sex ratio:
At the end of the test (107 dph) the treatment 180A (exposure until 30dph) had 28 females, 16 males and 1 testis-ova. At the highest test concentration (599 µg/L) no males were observed. 93% of fish were females based on gonadal assessment. The others at 599µg/L were sexually undifferentiated. Effects were comparable to EE2.
The authors of the study used for statistical evaluation of the gonadal sex ratio the exact Fishers test. They calculated only statistical significance at 188µg/L after 60dph and at 599µg/L and EE2 after 60 dph and 107dph.
Own calculation using Fishers exact test showed significant less males at 202µg/L ($p<0.05$) by comparison of only males to females. (The following numbers of fish were used for statistical calculation: from solvent control 24 males against 19 females, from 202µg/L 7 males against 18 females.)
Also own calculations using the step-down Cochran-Armitage test procedure was used. This test procedure was selected by the UBA statistic program `Toxrat` for this case. It showed that the proportion of males in relation to the total number of fish was significantly decreased at 188 µg/L ($p < 0.05$), at 202 µg/L ($p < 0.01$) and 599 µg/L ($p < 0.001$). The **LOEC for histological sex ratio (less males) is therefore 188 µg/L**. It should be noted that the effect on sex ratio was also significant in the treatment 202 µg/L (180B) after 107 dph even though exposure ceased after 60 dph. See Table 11 and Figure 1 below.

Table 11: Ratio at 107 dph: male : female : testis-ova : sex. undifferentiated fish

Numbers in parenthesis are values in %. The numbers of fish were obtained from Fig. 5 of Panther et al. 2006.

	male	female	testis-ova	sex. undiff.	Total number of fish
Solvent control	24 (55.8)	19 (44.2)	0	0	43
56.6 µg/L	18 (58.1)	13 (41.9)	0	0	31
194µg/L (180A)	28 (35.6)	16 (62.2)	1 (2.2)	0	45
202µg/L (180B)	**7 (22.6)**	**18 (58.1)**	**6 (19.4)**	0	31
188 µg/L (180)	**10 (31.3)**	**17 (53.1)**	**5 (15.6)**	0	32
599 µg/L	0	26 (92.9)	0	2 (7.1)	28
EE2 10ng/L	0	37 (88.1)	0	5 (11.9)	42

Figure 1: Histological sex ratio at 107 dph:

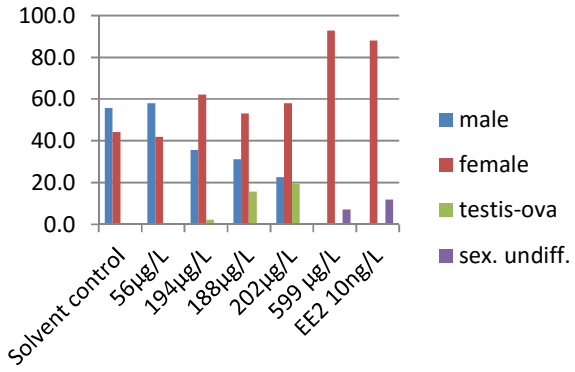


Chart derived from the table above, values based on %. Note, that treatments 194µg/L and 202µg/L were exposed until 30dph and 60dph respectively.

The skewed sex ratio and testis-ova in treatment 180B (202µg/L) show that effects persisted even after exposure ceased.
Manifested effects already observed at 60 dph where absence of testis at 599 µg/L and increase of number of undifferentiated fish. 50 % of these undifferentiated fish showed gonadal duct

feminization (double attachments of the testes to the mesentery) indicating that the lack of differentiation was not a delay of development due to systemic toxicity but due to a partial sex-change. The other 50% of undifferentiated fish were not assessable. In the treatments 180 and 180B the ratio females to males was shifted towards females (15 % males at 188 µg/L and 25 % males in 180B = 202 µg/L, both not significant). 30 % of fish at 202 µg/L and 5% at 188 µg/L were undifferentiated. All undifferentiated fish showed either gonadal duct feminisation or were not assessable (5 %).

Testis-ova:
At test end (**107 dph)** testis-ova were observed in the treatment 180B (202µg/L) and treatment 180 (188 µg/L). Results for 180B show that effects persisted after exposure ceased at 60dph. The 22 % of fish with testis-ova from treatment 180B had double attachment (gonadal duct feminisation), whereas the 3 % testis-ova from DWC had single attachment. The chart and the table in the publication do not show exactly the same results: For treatment 180B (202µg/L, exposure until 60dph) after 107dph: In the chart 19% (6 testis-ova) are depicted, whereas in the table 22% testis-ova are listed. At 188µg/L (exposure until 107dph): In the chart 15.6% (5 testis-ova) are depicted, whereas no testis-ova were listed in the table (probably a mistake). (See table 13 below, where parts from the table from the publication are depicted.)

Gonadal duct feminization of the testis (Gonadal attachment / Retained peritoneal attachments / gonadal duct feminization / formation of oviduct):

The positive control EE2 had significant more fish with double attachment in male and female fish compared to control at test end and effects were already observable at 60 dph.

At 56.6 µg/L (at 107 dph): There were double gonadal attachments in 8 of 66 males (12 %), whereas in both controls all males had single attachment. According to the authors this effect was not statistically significant (the authors compared the number of males with gonadal attachments to the collectivity of males and females). However according to our own statistical calculation using Fisher's exact test there were **significant more males with double attachment in the treatment 56.6 µg/L compared to solvent control.**[3] This was calculated by comparison of males with gonadal attachments to the total of males only. See Table 12 (excerpt from a table from Panter et al.).

At 180 µg/L (107 dph) the majority of males had an ovarian-like cavity: In treatment 180 (188 µg/L measured, exposure until 107 dph) 27 of 40 males had double attachment; in treatment 180B (202 µg/L measured, exposure until 60 dph) 14 of 21 males had double attachment. The double attachment remained regardless of length of exposure, indicating that this histological change was not reversible, even after a prolonged cease of exposure. The effect was significant for the collectivity of males and females.

At 560 µg/L (n) or in EE2 (107 dph) only gonads with double attachment occurred because no males existed and also the sexually undifferentiated fish had double attachment (in EE2 treatment some fish existed that were sexually undifferentiated or females that were not assessable). All females sampled at 30, 60 and 107 dph had a normal ovarian cavity.

There was one fish with testis ova in the dilution water control. This fish had a single point attachment to the mesentery, whereas those fish with testis-ova examined in the 180B µg/L 4-tert-pentylphenol treatment had double attachment.

[3] To confirm the calculation with the Fisher's exact test another statistical calculation was used additional. A LOEC of 56.6 µg/L ($p<0.01$) for gonadal attachment can be calculated by using for the statistical calculation the concentrations 56.6, 188 and 202 µg/L (only males). The same result is obtained by using not only the males, but also the sexually undifferentiated fish at 599 µg/L (because there is only a small distance between 188 and 202 µg/L and for calculation three concentrations are necessary). By using the concentrations 56.6, 188 and 599 µg/L (males and SU) also the LOEC 56.6 µg/L ($p < 0.01$) will be obtained. The Step-down Cochran-Armitage test procedure was used as statistical calculation method.

Table 12: Gonadal attachment, shown as a percentage of males, fish with mixed gonad, females or sexually undifferentiated fish at 107 dph.

	Males		**IS**		**Females**		**SU**	
	S	**D**	**S**	**D**	**D**	**NA**	**D**	**NA**
DWC	57	0	3	0	37	3	0	0
SC	58	0	0	0	42	0	0	0
56.6 µg/L	58	**8**	0	0	33	0	0	0
202 µg/L (180B)	7	**14**	0	22	57	0	0	0
188 µg/L (180)	13	**27**	0	0	60	0	0	0
599 µg/L	0	0	0	0	82	0	**18**	**0**
EE2	0	0	0	0	73	9	**14**	**4**

Number of examined fish: DWC: 30; SC: 24; 56.6 µg/L: 12; 202 µg/L: 14; 188 µg/L: 15; 599µg/L: 11; EE2: 22
S: single attachment
D: double attachment
IS: fish with mixed gonad
SU: sexually undifferentiated
NA: not assessable

Discussion on undifferentiated fish (potential causal link between undifferentiated fish and gonadal duct feminization):

Controls: In the controls no fish existed that were sexually undifferentiated at 60 or 107 dph. The sexually undifferentiated fish in the solvent control at 30 dph had normal formation of vas deferens (single attachment of the testes) or were not assessable and 35 % females and 5 % males. Only the dilution water control (DWC) had some undifferentiated fish with double attachment (5 %) and 30 % undifferentiated fish which were not assessable and furthermore 65 % females (however there was a problem with the DWC, for example after 107 d, 3 % of fish showed testis-ova in the DWC and the VTG examination had a very high error bar).
At **60 dph** all sexually undifferentiated fish at the three treatments 180 µg/L had a double attachment of the testes to the mesentery (gonadal duct feminization). 5% of all fish at 180B were not assessable.

There was a big part of sexually undifferentiated fish at 560 µg/L and EE2 treatments. 40 % of fish at 560 µg/L were sexually undifferentiated whereof 20 % had double attachment, the other 20 % were not assessable; furthermore there were 60 % females and no males. Also in the EE2 treatment no males existed, instead 30 % sexually undifferentiated fish, whereof two third had double attachment, one third of fish were not assessable.

At 107 dph all sexually undifferentiated fish at 560 µg/L had double attachment and in EE2 treatment almost all sexually undifferentiated fish had double attachment. At this time point no sexually undifferentiated fish were seen at the lower concentrations.

The conclusion is that the appearance of sexually undifferentiated fish is treatment dependent.

Appearance of gonadal duct is not reversible, as has been shown in the treatment 180B, the fish of which had been exposed only until 60 dph, and gonadal duct feminization was observed at 107 dph. In the treatments 180 µg/L, 560 µg/L and EE2 sexually undifferentiated fish showed double attachment, as far as it was possible to assess them. So, because undifferentiated fish have double attachment it does not just appear to be a delay in sexual development, but an endocrine mediated effect, which is irreversible.

VTG:
The examination of plasma VTG showed no effects up to 180 µg/L in males, at 560 µg/L no males existed anymore. For the treatment 180B (exposure up to 60 dph at measured 202 µg/L) the value was significantly increased for males, but an effect was almost not visible from chart.

The VTG in females was slightly but significantly increased at 180 µg/L (= LOEC), but slightly

decreased again at 560 µg/L to control value. The effects seen were not very distinctive.
At 560 µg/L all males were converted to females. An explanation for the decreasing VTG content in females could be, that the phenotypically sex reversed new females produce less VTG (see also the validation report Phase 1 for the FSDT) (OECD, 2011).

High VTG variance in males of the dilution water control and male fish from treatment 56 µg/L was observed. However high variation did not result in higher mean VTG levels compared to solvent control. Treatment related results were compared to solvent control.

Secondary sexual characteristics (SSC), nuptial tubercles:
For this endpoint the number and prominence of nuptial tubercles on male and female fish were recorded after 107 dph.

At **560 µg/L (= LOEC) no fishes with nuptial tubercles** were seen. At 180 µg/L fewer male fish with nuptial tubercles were seen (33 % compared with 54 % in solvent control and 64 % in dilution water control) and the number of tubercles was reduced, but the effect was not significant.
Also the nuptial tubercle prominence was lower (but not statistical significant) at 180 µg/L than in solvent control.

Prolongation of exposure in the three treatments 180A (until 30 dph), 180B (until 60 dph) and 180 (until 107 dph) resulted in less male fishes with tubercles. It appears that there a time-response as well as a dose-response relationship is existing.

Time to hatch
LOEC 560 µg/L (4.6 days, compared to solvent control 4 days).

Growth
Sexually undifferentiated fish at the highest test concentration had a lower weight and length after 30 dph only. Length of male fish was significantly lower in the 180A and 180B treatments after 107 days, but no effects were observed in the continuous exposure concentrations. At 107 days no effect of EE2 on growth could be observed.

Fish sexual development test:

4-tert-pentylphenol was used as test substance for the Validation report (Phase 1) for the fish sexual development test for the detection of endocrine active substances Series on Testing and Assessment No. 141 (OECD, 2011). *P. promelas* was exposed from 0 to 60 dph at concentrations of 32, 100, 320 µg/L (nominal), (36.1, 93, 295.6 µg/L measured). There were four replicate aquaria per concentration and 45 individuals per aquarium. VTG was determined as concentration in head/tail homogenate. The sex ratio (phenotypic) was determined by gonadal examination. One Lab tested *P. promelas* in the FSDT. The reliability was assessed with Klimisch 2.

Results:

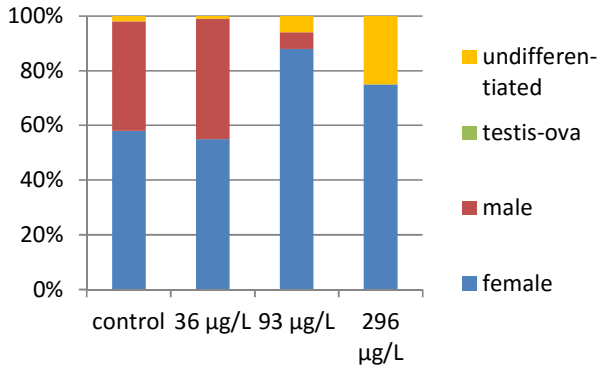
VTG:
In females decreased VTG LOEC 93 µg/L; no significant effect in males up to 93 µg/L.

Sex ratio:
The sex ratio was histologically assessed by gonadal examination (phenotypic): No effect was visible at 36 µg/L. At the treatment 93 µg/L and 296 µg/L the number of females was significantly increased (at 93 µg/L were about 88% females) → LOEC 93 µg/L. At 296 µg/L were approx. 75% female and 25% undifferentiated fish. See Table 13 below and Figure 2 (numbers are estimated because they are read from a chart).

Table 13: Sex ratio from FSDT with *P. promelas* shown as a percentage, Lab 5

	female	male	testis-ova	undifferen-tiated
control	58	40	0	2
36 µg/L	55	44	0	1
93 µg/L	88	6	0	6
296 µg/L	75	0	0	25

Figure 2: Sex ratio from FSDT with P.promelas, Lab 5



Modified juvenile growth test:

Another test by Panter et al. (Panter et al., 2002) on *Pimephales promelas* was conducted as a modified juvenile growth test. The fishes were in the age between 45 and 100 dph at the beginning. The exposure duration was 21 d. Test concentrations were 10; 29.6; 56 µg/L (m). There were 4 replicates, with 8 fish per concentration. Growth and VTG were determined. VTG (whole body homogenate) was measured after 7, 14 and 21 d.

The test was assessed with Klimisch 2 as the test was in general well described and conducted. The only limitation was the unclear information on age of the fish.

VTG: At the lowest test concentration (10 µg/L) and above **VTG was significantly increased (LOEC 10 µg/L)**. VTG was also increased after exposure to EE2 at concentrations of 0.005 µg/L and above.

Growth: For both ethinylestradiol and 4-tert-pentylphenol no consistent or dose-related effects on somatic growth (wet weight, total length, and condition factor) were observed.

Table 14 summarises the effects seen in extended ELS, FSDT and modified juvenile growth tests in *P. promelas*.

Table 14: Tests with *Pimephales promelas* (extended ELS, FSDT and modified juvenile growth test)

Life stage/ duration	Conc. / test condition / solvent	**Vitello-genin**	**Histology**	**Fertility/ Fecundity**	**Sex ratio / gonad histology**	**Sec. sex characte ristics**	**others**	**Positive control**	**Referen ce**	**reliability**
P.promelas Extended ELS, <24 hpf, 107 dph, Fish in the groups 180A and 180B were only exposed until 30 and 60 dph respectively and then remained in dilution water until 107 dph.	56, 180A, 180B, 180, 560 µg/L (n), 56.6; 194; 202; 188; 599µg/L (m); Exposure durations: 180A: 30 dph, 180B: 60 dph, 180: 107 dph; Flow-through, Solvent: Triethylene glycol (0.1 mL/L), Solvent control (SC) and dilution water control (DWC) existed	Plasma VTG, no effect in males up to 180 µg/L, at 560 µg/L no males existed Females: increased VTG: **LOEC 180 µg/L**; decreased VTG at 560 µg/L	**At 56.6 µg/L (m) Gonadal duct feminization of the testis** significant increased(12 % of male fish, control 0 %) **Testis-ova appeared at 202 µg/L (m) = 180 µg/L (n)** (on 107 dph 22 testis-ova compared with 3 testis-ova in DWC)		LOEC 188 µg/L	LOEC 599 µg/L (m) no fishes with nuptial tubercles; at 180 µg/L fewer, but not sign.	Growth sexually undifferentiated fish (weight and length) after 30 dph LOEC 599 µg/L (m) Time to hatch LOEC 599 µg/L (m)	EE2 0.01 µg/L, Delay in hatching time VTG in females significantly elevated; no males existed, no testis, Gonadal attachment in undifferentiated fish No effect on growth after 107 d	(Panter et al., 2006)	**2**

Life stage/ duration	Conc. / test condition / solvent	**Vitellogenin**	**Histology**	**Fertility / Fecundity**	**Sex ratio / gonadal histology**	**Sec. sex characteristics**	**others**	**Positive control**	**Reference**	**reliability**
P. promelas FSDT 0 - 60 dph Starting with newly fertilized eggs	32, 100, 320 µg/L (n); 36.1, 93, 295.6 µg/L (m); Flow through	In females decreased **VTG LOEC 93 µg/L**; no effect in males			**LOEC 93 µg/L,** more females than males		Mortality in % (Deviation): Control: 9 (10.5) 36 µg/L: 5 (6.4) 93 µg/L: 10 (5.6) 295 µg/L: 18 (9.2)		Validation report (Phase 1) for the FSDT (OECD, 2011)	**2**
P. promelas modified juvenile growth test Juvenile fathead minnows, age of fish between 45 and 100 dph; duration 21 d	10; 29.6; 56 µg/L (m); Flow through, at least five volume changes in 24 h; no solvent	After 7, 14 and 21 d **VTG increased LOEC 10 µg/L** , no differentiation between male and female					No consistent or dose-related effects on somatic growth (wet weight, total length, and condition factor)	EE2 0.002 – 0.02 µg/L VTG increased from 0.005 (day 4 and then continuously) No consistent or dose-related effects on somatic growth (wet weight, total length, and condition factor)	(Panter et al., 2002)	**2** (age of fishes is not very exactly specified)

Reproduction assays:

Panter et al. (2010):
An extended and modified 21-day reproduction study was conducted by Panter et al. (Panter et al., 2010) (assessed with Klimisch 2). Test concentrations were 56, 180 and 560 µg/L. The study design used here was pair-breeding and not (like in the OECD 229 Short term reproduction assay guideline) a group breeding design of two males and four females with four replicates per treatment. The number of eggs spawned per fish pair and the number of eggs per pair was determined. At the end of the exposure F0 phase fish were weighed and measured, secondary sexual characteristics (SSC) were examined and plasma VTG determined. A minimum of two hatching assessments, were performed per breeding pair. Eggs from F1 generation were further examined following the ELS protocol but without exposure. At day 90 the fish were weighed and measured, SSC and plasma VTG were examined. The gonads were examined microscopically to determine, where possible, fish sex and stage of testicular or ovarian development. All effects were compared with the solvent control.

Results:

Fecundity:
The number of eggs spawned per fish pair, the number of eggs per batch and the number of spawns was decreased. The LOEC in all cases was 56 µg/L. No effects were caused by the positive control 17α-Ethinylestradiol (EE2) at 10 ng/L.

Secondary sexual characteristics:
F0: Number of nuptial tubercles was reduced in 560 µg/L and EE2, but not significant. (11.8±2.0 and 11.7±1.0 versus 15.8±0.9). However, in the 560 µg/L treatment, the nuptial tubercle count ranged from 3 to 19, whereas in the solvent control the count ranged between 12 and 20, suggesting suppression in some males. Nuptial tubercle prominence was significantly reduced ($p < 0.05$) in males exposed to 560 µg/L (1.9±0.4 versus 3.6±0.3, respectively). The LOEC is 560 µg/L.

F1 (not exposed): No significant differences were observed in the number of nuptial tubercles. Tubercle prominence was significantly reduced in the 56 µg/L treatment (1.3±0.1 versus 2.0±0.2, respectively).

VTG:
F0: Plasma VTG was increased at 180 µg/L, but not significant. At 560 µg/L it was in the same range as the positive control (almost 100000 µg VTG/ml Plasma) → LOEC 560 µg/L (in males). VTG was not significantly increased in females in both treatments and EE2.

F1 (not exposed): VTG in males was statistically significantly elevated at 180 µg/L and above but induction at 180 µg/L was very low. No effect appeared in females in both EE2 and the treatments. The VTG in males exposed to EE2 was elevated but not significant due to high variance.

Survival and growth:
F0: There were no effects on survival and growth. F1: No treatment related effects on growth and condition factor were observed in all treatments. There were no treatment related effects on hatching success and survival.

OECD 21-day Fish Screening Assay Validation Report
4-tert-pentylphenol was used for validation of the 21-day reproduction assay with *P. promelas* (OECD, 2006). The age of fishes was between 18 and 24 wk. Three nominal concentrations were tested: 100, 320, 1000 µg/L. Two replicates per treatment were used. Each vessel contained 5 males and 5 females. Three laboratories (Labs 7, 8 and 9) tested *P. promelas* in the 21-day assay. The reliability of the studies is assessed with 2. From Lab

9 VTG values cannot be used due to technical problems (overdose in the last day of the experiment in the mid-dose and a lack of test substance delivery in the high dose group in the last 15 hours of the study).
Three core endpoints as indicators of endocrine activity of the test substances were observed during the course of the test or measured at termination of the test, namely:

i) gross morphology (e.g. secondary sexual characteristics such as nuptial tubercles on the head in fathead minnow),

ii) vitellogenin levels,

iii) gonadal histology.

As apical endpoint fecundity was examined.

Results:

Fecundity:
Spawning was reduced in a concentration-dependent manner. No spawning was observed in the three labs at the highest concentrations. At 320 µg/L the number of spawns was reduced in all labs but no statistics were applied and spawning was low in controls too (the group-spawning conditions used here were sub-optimal for fathead minnow). Spawning was also reduced after exposure to the positive control (17β-estradiol, 100 ng/L) in two laboratories (Lab 8 and 9). No clear effect was observed in the positive control of Lab 7.

VTG:
LOECs for increased VTG were 270 to 300 µg/L for males in the two reliable tests from laboratories 7 and 8. VTG in females was increased in one test at the high concentration of 962 µg/L.

Secondary sexual characteristics:
In one study (Lab 7) the number of nuptial tubercles was significantly affected at **270 µg/L (= LOEC)** and at 962 µg/l no nuptial tubercles existed anymore. In the other study (Lab 8) at the LOEC 887 µg/L no nuptial tubercles existed anymore. A dose depended decrease was observed in both studies. Results from Lab 9 showed that no nuptial tubercles were seen at 81 and 277 µg/L; at 820 µg/L nuptial tubercles were seen, but in significantly reduced number (no dose response dependent); in control nuptial tubercles were existing; the positive control E2 (100 ng/L (n)) did not cause significant effects on nuptial tubercles. Regarding the positive control it could be a false negative finding (from validation report). The concentration of the positive control was not measured.

Gonadal histology:
Increased number of spermatogonia compared to other spermatogenic cells were observed at the highest and partly at the middle concentration in all three labs.
Testicular degeneration was found by one lab in male fish at the highest concentration but not in the positive control. Another lab found testicular degeneration only in the positive control

Table 15 summarises the reproduction assays in *P. promelas.*

Table 15: *P. promelas* Reproduction assays with 4-tert-pentylphenol (4-t-PP)

Life stage/ duration	Conc. / test condition / solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex ratio	Sec. sex characteristics	others	Positive control	Reference	reliability
P. promelas, Sexually mature male and female fish, Exposure 21 d, concurrent ELS (F1 without exposure until 90 dph)	56, 180, 560 µg/L (n), measured values within +- 20% of nominal values Flow-through, Solvent: triethylene glycol, (0.002 mL/L) Solvent control existed	Plasma-VTG F0: LOEC 560 µg/L (increased in males); not sign. increased in females F1 males: stat. sign. elevated from 180 µg/L (but not visible by eye from chart), visible from 560 µg/L, LOEC 560 µg/L no effect in females		LOEC 56 µg/L Number of eggs per pair decreased; LOEC 56 µg/L, Number of eggs per batch decreased; LOEC 56 µg/L, Number of spawns NOEC <56 µg/L in all cases		F0: Nuptial tubercles prominence LOEC 560 µg/L, Number of tubercles no effect F1: Only at 56 µg/L tubercle prominence reduced, no significant effect in the number of tubercles, No effect on gonadal staging	No effects on survival in F0 and F1 No effect on hatchability	EE2 0.01 µg/L F0: VTG males: Increase in the same range like at 560 µg/L (4-t-PP), no effect in females, No sign. effect on number of nuptial tubercles, No sign. effects on fecundity. F1: elevated VTG level, but very high variance, not sign. No effect on sex ratio in F1. No sign. effect on number of tubercles No effect on gonadal staging Prominence of tubercles not sign. in F0 and F1	(Panter et al., 2010)	**2**

Life stage/ duration	Conc. / test condition / solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex ratio	Sec. sex characteristics	others	Positive control	Reference	reliability
P. promelas 21-day assay Age of fish approx. 18 wk	100, 320 and 1000µg/l (n), 80.1, 270; 962 µg/L (m) flow-through	Plasma VTG in males increased LOEC 270 µg/L, dose related effect Plasma VTG increased in females LOEC 962 µg/L	Spermatogonia increased at 962 µg/L Spermatocytes and Spermatids decreased at 962 µg/L Spermatozoa increased at 962 µg/L Testicular degeneration at 962 µg/L	Spawning reduced in concentration -dependent manner, No eggs produced at 962 µg/L Page 41		Nuptial tubercles, LOEC 270 µg/L at 962 µg/L no nuptial tubercles observed, dose depended decrease	Mortality 5% at 962 µg/L, other treatments and control 0%	17ß-estradiol 90.6ng/L Mortality 5% Sign. less nuptial tubercles in males, Plasma VTG sign. increased in males and females Gonadal staging affected like by 4-tert-PP No clear effect on spawning	Validation report for 21-d assay; Lab 7 (OECD, 2006)	2

Life stage/ duration	Conc. / test condition / solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex ratio	Sec. sex characteristics	others	Positive control	Reference	reliability
P. promelas 21-day assay Age of fish 18 wk	100, 320 and 1000 µg/l (n), 85.7; 298; 887 µg/L (m) flow-through	Plasma VTG in males increased LOEC 298 µg/L, dose related effect No effect in females	Spermatogonia increased at 887 µg/L Spermatocytes decreased at 887 µg/L Spermatids decreased at 298 and 887 µg/L Spermatozoa decreased at 298 and 887 µg/L	Spawning reduced in concentration -dependent manner, No eggs produced at 887 µg/L		Nuptial tubercles (males) at LOEC 887 µg/L no nuptial tubercles observed dose depended decrease	Mortality 15% at 320 µg/L and 45% at 1000 µg/L	17ß-estradiol 100ng/L (n) No mortality, Sign. less nuptial tubercles in males, Plasma VTG sign. increased in males and females Gonadal staging affected like by 4-tert-PP Testicular degeneration Reduced spawning	Validation report for 21-d assay; Lab 8 (OECD, 2006)	2
P. promelas 21-day assay Age of fish 24 wk	100, 320 and 1000 µg/l (n), 81; 277; 820 µg/L (m) flow-through	Due to a technical problem and resulting mortalities no VTG data available.	Spermatogonia increased at 820 µg/L Spermatocytes and Spermatids decreased at 820 µg/L	Spawning reduced in concentration -dependent manner, No eggs produced at 820 µg/L		Nuptial tubercles were decreased or not existing, but no dose response	Mortality 10%, 100%, 30% at 100, 320, 1000 µg/L respectively (technical error: over-dosage on the last day)	17ß-estradiol 100ng/L (n) Mortality5% No sign. effect on nuptial tubercles in males (concentration of E2 not measured) Reduced spawning	Validation report for 21-d assay; Lab 9 (OECD, 2006)	2 Technical problem at the last day

Summary for *P. promelas*

In summary results of two fish sexual development studies using *P. promelas* are available. Both tests showed significant effects on sex ratio at gonadal level at 188 and 93 µg/L. Sex ratio was biased towards females or less males were observed. Female biased sex ratio is a clear indicator of an endocrine mode of action according to OECD 2012 and an apical adverse effect.

As can be seen in the test by Panter et al. (Panter et al., 2006) the positive control EE2 and the highest concentration of 4-tert-pentylphenol caused also the appearance of undifferentiated fish in considerable numbers. All assessable undifferentiated fish had double attachment of the testis (= gonadal duct feminisation). This is strong evidence that the appearance of undifferentiated fish is causally linked to the endocrine properties of the test substance.

Additional parameters investigated in the study by Panter et al. (Panter et al., 2006) substantiate an estrogenic mode of action: feminization of male gonadal ducts was observed, male fish showed reduced secondary sex characteristics and vitellogenin induction in females was observed. No systemic toxicity (mortality, growth) was observed at test concentrations causing both diagnostic as well as apical effects.

Effects observed in the reproduction assays in the Validation report for 21-d assay (OECD, 2006) further substantiate the estrogenic mode of action. VTG was induced in male fish in both tests measuring the parameter and estrogen diagnostic effects were observed: Increased proportion of spermatogonia compared to other spermatogenic cell types and reduced male secondary sex characteristics. In all reproduction tests spawning was reduced in a dose dependent manner and ceased at high test concentrations (820-962 µg/L). In one test effects on reproduction were significant at 56 µg/L and above while no statistical evaluation was performed in the other tests. Except for one test, mortality was not increased compared to controls. Reduced fecundity is not an endocrine specific endpoint and may have been caused by systemic toxicity too. But it fits to the estrogenic mode of action as reduced reproduction is an endpoint known to be sensitive for estrogen agonist (Knacker et al., 2010). Some aspects don't seem to fit into this clear picture at a first glance but do so if analysed in detail:

- A decrease of VTG level in females was observed in both fish sexual development tests at high concentrations. This effect does not fit to the mode of action anticipated at first glance. However effects occurred at concentrations where the sex ratio was shifted towards females and it is likely that this caused the VTG decrease. As described in the OECD validation report (OECD, 2011), "new" females have less developed gonads and also less VTG than "normal" females and thus reduce the mean VTG concentration.
- VTG induction in males was not observed in the fish sexual development test and unfortunately no information on this parameter for the positive control is available. However, VTG induction in male fish was observed in a juvenile growth test at low concentrations (10µg/L) and VTG was also induced in male fish in the 2 reproduction assays available.
- Fecundity was not reduced in the positive control (EE2) group of one reproduction test. However, if compared to other tests it seems possible that the concentration chosen for E2 was not suitable to induce this effect. This was also observed by (Dammann et al., 2011) who described that at low level of exposure to potent estrogens (E2 (2-7 ng/L) egg production was stimulated and at higher concentrations (26-35 ng/L) inhibited. Exposure to values between these concentration ranges did not result in consistent effects on fecundity in two experiments.

In summary, it can be concluded that results from the fish sexual development tests are clearly indicative for an estrogenic mode of action which is resulting in adverse effects at

population level. Effects observed in other studies support this conclusion. Thus, 4-tert-pentylphenol is an endocrine disruptor due to indicative effects on feminization of gonadal ducts and sex ratio and adverse effects on sex ratio and fecundity. Indicative effects occurred at 10 µg/L and above and adverse effects were observed at 56 µg/L (fecundity, not diagnostic) and 93-188 µg/L (sex ratio, diagnostic).

5.2.1.3.2 Danio rerio

Fish sexual development test

4-tert-pentylphenol was used as test substance for the Validation report (Phase 1) for the fish sexual development test for the detection of endocrine active substances Series on Testing and Assessment No. 141 (OECD, 2011).

Three Labs (numbers 2, 3 and 4) evaluated 4-tert-pentylphenol with *D. rerio*. The results of Lab 3 cannot be used due to a failed exposure system. Five test concentrations were used by Lab 2, with two replicate aquaria per concentration. There were 45 individuals per aquarium. Lab 4 used three test concentrations with four replicate aquaria and 45 individuals per aquarium. The exposure lasted from 0-60 dph. The sex ratio (phenotypic) was determined by gonadal examination and defined as female, male, intersex or undifferentiated. Mortality in controls was high in one test (Lab 4) but below the validation criteria (70% post hatch survival) and thus both tests are considered valid. The studies were assessed with Klimisch 2.

Results:

Mortality:
In both tests mortality was observed in treatments but not in a dose dependent manner. In Lab 2 mortality occurred at 34 µg/L (29 %), slightly decreased at 62 µg/L to 24.4 % and increased at 97 µg/L to 52%. In the negative control mortality was 13.9 %. Mortality in the positive control (E2, 63 ng/L) was 35.6 %. (For standard deviation see Table 18 below). The values for mortality from Lab 4 at 100 µg/L (36 %, standard deviation 11.5 %) and 320 µg/L (36 %, standard deviation 4.9 %) are probably not statistically different compared to control (27.3 %, standard deviation 14.5 %) because of high standard deviation. A positive control did not exist in that test.
Mortalities at 97 µg/L in Lab 2 and at 100 µg/l and above in Lab 4 are above the validation limit but only effects at 97 µg/l in Lab 2 are considerably higher than in the control. No indication of sex specific mortality is available, but cannot be excluded with regard to effects at 97 µg/L in Lab 2. However significant effects on sex ratio already occurred at 62 µg/l in Lab 2. Even if sex-specific mortality has occurred, it would be likely that this is due to a disturbance of the endocrine system.

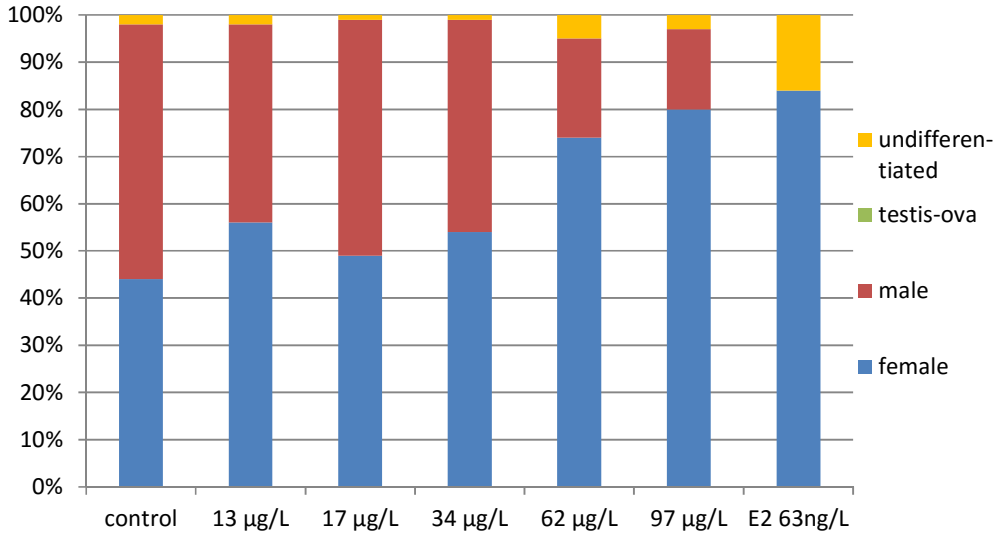
VTG:
Lab 4 determined a LOEC for increased VTG in males of 100 µg/L nominal. No effect in females was seen. Lab 2 did not determine an effect up to 97 µg/L (m).

Sex Ratio:
Lab 2 determined significantly more females and fewer males at the **LOEC 62 µg/L** (measured). The ratio female : male : undifferentiated fish was approx. 74 : 21 : 5 (in %). At 97 µg/L the effect was even more pronounced. At 97 µg/L the proportion of females was 80 %, and there were about 17 % males and 3 % undifferentiated fish. The positive control (E2 73 ng/L) had about 84 % females and 16 % undifferentiated fish (See Table 16 and Figure 3). Percentages are estimated because they are read from a chart in the validation report.

Table 16: Sex ratio (%) from FSDT with *D. rerio*, Lab 2

	female	male	testis-ova	undifferen-tiated
control	44	54	0	2
13 µg/L	56	42	0	2
17 µg/L	49	50	0	1
34 µg/L	54	45	0	1
62 µg/L	74	21	0	5
97 µg/L	80	17	0	3
E2 63ng/L	84	0	0	16

Figure 3: Sex ratio from FSDT with *D. rerio*, Lab 2

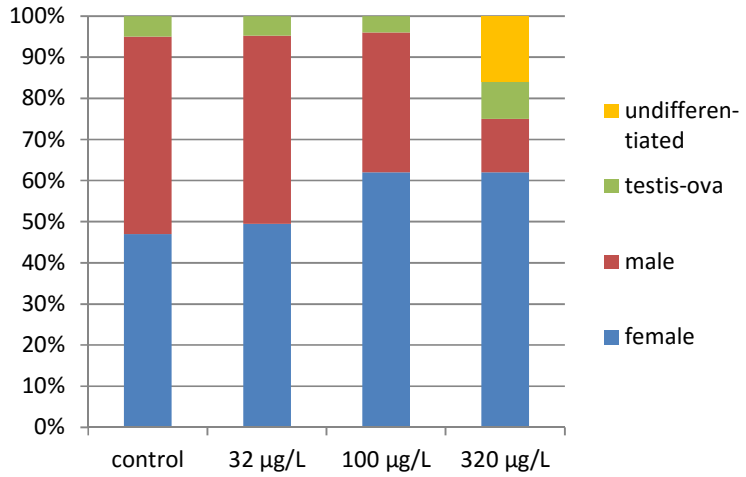


In Lab 4 at 100 µg/L (nominal), significantly more females were observed (about 62 % females, 34 % males and 4 % testis-ova; 158 fish were examined, the error bar was very small). At 320 µg/L both increase of females as well as decrease of males was significant (62 % females, 13 % males, 9 % testis-ova (named as intersex), 16 % undifferentiated fish). The ratio in the control group was approx. 47 % females, 48 % males, 5 % testis-ova, 160 fish were examined. See Table 17 and Figure 4. Percentages are estimated because they are read from a chart in the validation report.

Table 17: Sex ratio from FSDT with *D. rerio*, Lab 4

	female	male	testis-ova	undifferen-tiated
control	47	48	5	0
32 µg/L	52	48	5	0
100 µg/L	62	34	4	0
320 µg/L	62	13	9	16

Figure 4: Sex ratio from FSDT with *D.rerio*, Lab 4



In Table 18, the effects on *D. rerio* are summarised.

Table 18: *D. rerio* - FSDT

Life stage/ duration	Conc. / test condition / solvent	Vitello-genin	Histology	Fertility/ Fecundity	Sex ratio / gonad histology	Sex –ratio / phenotypic (gonadal examination)	Sec. sex characteristics	others	Positive control	Reference	reliability
D. rerio FSDT 0 – 60 dph	38, 75, 150, 300, 600 µg/L (n); 12.9; 17.3; 34; 62.2; 96.8 µg/L (m) flow-through	**No effect in females and males up to 96.8 µg/L,** at 96.8 µg/L increased , but not sign.				**LOEC 62 µg/L more females**		Mortality (standard deviation): Control: 13.9 (4.9)%, 12.9 µg/L: 20 (3.1)% 17.3 µg/L: 13.3 (6.3)% 34 µg/L: 28.9 (9.4)% 62.2 µg/L: 24.4 (12.6)% 96.8 µg/L: 52.2 (11)% E2 (63 ng/L): 35.6 (3.1)% Hatchability 80 – 90 %	E2 63ng/L: VTG sign. increased; sex ratio approx. 80 % females and 20 % undifferentiated fish total mortality: 35.6%	VALIDATION REPORT (PHASE 1) for the FSDT (LAB 2) (OECD, 2011)	**2**
D. rerio FSDT 0 – 60 dph	32, 100, 320 µg/L (n); (2, 0.9, 1.8 µg/L (m) Due to preservation problems with the samples; measured values are not reliable and the nominal concentrations are used. See page 29 of the validation report.) flow-through	**VTG increased in males LOEC 100 µg/L (n) ($p < 0.05$) dose response existed No effect in females**				**LOEC 100 µg/L (n) more females**		Mortality (standard deviation): Control: 27.3 (14.5)% 32 µg/L: 23.3 (12.6)% 75 µg/L: 17 (15.6)% 100 µg/L: 36 (11.5)% 320 µg/L: 39 (4.9)% Hatchability always > 90 % No data for E2		VALIDATION REPORT (PHASE 1) for the FSDT (LAB 4) (OECD, 2011)	**2**

Reproduction assays:

4-tert-pentylphenol was used for validation of the 21-day reproduction assay (OECD, 2006) with *D. rerio* and tested in three laboratories (Labs 12, 13 and 14). The age of fishes was between 8 and 24 wk. Three exposure concentrations and two replicates per treatment were used. Each vessel contained 5 males and 5 females. The studies were assessed with Klimisch 2 except the study from Lab 14 (Klimisch 3). Results of Lab 14 are reported in Table 19 but not further analysed as there were high VTG values in control males, which indicate that controls might have been contaminated.

Two endpoints as indicators of endocrine activity of the test substances were observed during the course of the test or measured at termination of the test, namely:

i) vitellogenin levels,

ii) gonadal histology.

As apical endpoints fecundity and fertility were observed. The fecundity was examined by counting of the number of eggs of zebrafish per day and tank. Fertility was assessed by counting the numbers of fertilised and unfertilised eggs.

Results:

Fecundity and fertility:
Fecundity and fertility were significantly reduced at 721 µg/L (= LOEC) in Lab13 but no effects up to 787 µg/L were observed in the other lab. The statistical method to calculate the significance for fecundity was one-way ANOVA, Dunnett´s post-hoc test.
In Lab 13 at the highest concentration (721µg/L) the number of fertilised eggs was significantly decreased compared to the control ($p<0.05$, one-way ANOVA, Dunnett´s post-hoc test; information from a chart in the validation report). At this concentration the number of unfertilised eggs was higher than the number of fertilised eggs.

VTG:
Significantly elevated VTG was determined in male fish in both tests. LOECs were **229 µg/L (m)** and **294 µg/L (m)** respectively.
Females: Significantly elevated in one lab **(LOEC 294 µg/L (m))** but not in the other up to 721 µg/L.

Histopathological findings:
Several histological changes were observed, some of which are diagnostic for an endocrine mode of action according to OECD Guidance Document No. 123:
Males: Testis-ova were observed in the highest concentration (2 of 7 males) and in positive control E2 100 ng/L (1 of 7 males) in one lab (Lab 12). From Lab 13 no information is given in this case, therefore it is not possible to say, if the effect was evaluated or not.

In males increased number of spermatogonia were seen, treatment related starting at 229 µg/L in one lab (Lab 13) but not in the other Lab 12.

Oocyte atresia in females had treatment related the highest severity grade in the highest concentration in one lab (Lab 12). The same severity grade was also observed in the positive control, but fewer cases were seen. The lower concentrations had lower severity grade and/or less cases. The control had the lowest severity grade and fewer cases. It is not obvious, if the effect was examined by Lab 13.

In addition the following histological changes were observed which are secondary diagnostic criteria for endocrine disruptors:

- Interstitial fibrosis at low (77 µg/L), middle (229 µg/L) and high (721 µg/L) concentrations in females (Lab 13).
- interstitial proteinaceous fluid in males (in one of the three labs) in the middle and high concentration treatment related (Lab 12).

The effects on *D. rerio* in the reproduction assays are summarised in Table 19.

Table 19: *D. rerio* - Reproduction assays

Life stage/ duration	Conc. / test condition / solvent	**Vitello-genin**	**Histology**	**Fertility/ Fecundity**	**Sex ratio / gonadal histology**	**Sec. sex characteristics**	**others**	**Positive control**	**Reference**	**reliability**
D. rerio 21-day assay Age of fish 12 wk	100, 320 and 1000 µg/l (n), 90; 294; 787 µg/L (m) flow-through Solvent: acetone, solvent control existed	Plasma VTG in males increased LOEC 294 µg/L, Plasma VTG in females increased LOEC 294 µg/L	**Testis-ova at 787 µg/L (2 of 7)** **Females: Oocyte atresia at 787 µg/L** **Proteinaceous fluid, intravascular in males from 294 µg/L**	No clear decrease of spawning could be observed, no effect on fertility			Mortality ≤ 10 %	17ß-estradiol 100ng/L (n) Mortality ≤ 10% No effect on cumulative number of eggs, no effect on fertility, Plasma VTG in males sign. increased, no effect in females Testis-ova (1 of 7) Proteinaceous fluid, intravascular in males Oocyte atresia (lower number, not relevant)	Lab 12 (OECD 2006)	2 cumulative number of eggs is also decreased in solvent control
D. rerio 21-day assay Age of fish 20-24 wk	100, 320 and 1000 µg/l (n), 76.8; 229; 721 µg/L (m) flow-through	Plasma VTG in males increased LOEC 229µg/L, no effect in females	**Spermatogonia increased from 229µg/L,** **Spermatozoa decreased from 229µg/L** **Interstitial fibrosis from 77 µg/L in females**	LOEC fecundity 721 µg/L, LOEC fertility 721 µg/L, dose related trend in decreasing fertility			Mortality ≤ 10 %	17ß-estradiol 100ng/L (n) Mortality ≤ 10% High variability in numbers of produced eggs VTG in males sign. increased but not in females Spermatogonia increased Spermatozoa decreased	Lab 13 (OECD 2006)	2
D. rerio 21-day assay	100, 320 and 1000 µg/l (n), 22.6, 69.5, 473 µg/L (m)	Plasma VTG in males decreaed at 22.6 µg/Lcompared to high control value; increased at 473 µg/L; No effect in females	Increased spermatogonia at 473 µg/L	No clear effect on spawning				17ß-estradiol 100 ng/L (n) No effect on VTG in males and females Increased spermatogonia	Lab 14 (OECD 2006)	3 Very high value for VTG in the control males, therefore not used for evaluation

Summary *Danio rerio*:

In summary, two fish sexual development tests show that 4-tert-pentylphenol causes adverse effects in *D. rerio* which are clearly endocrine mediated (increased proportion of females). VTG induction was observed in only one of the tests. However this is consistent with findings during the validation phase of the fish sexual development test which revealed that VTG is a less sensitive parameter for weak estrogens.

Results observed in the fish reproduction assays support the estrogenic mode of action. 4-tert-pentylphenol caused several histological changes which are diagnostic for an endocrine mode of action such as increased proportion of spermatogonia, testis-ova and oocyte atresia. Effects on fecundity and fertility occurred in one of the tests but not in the other and thus results do not unambiguously show that 4-tert-pentylphenol causes adverse effects in adult zebrafish. However such adverse, estrogen mediated effects of 4-tert pentylphenol were observed in both fish sexual development tests with zebrafish.

In summary it can be concluded that results from the fish sexual development tests with zebrafish are clearly indicative for an estrogenic mode of action of 4-tert-pentylphenol which results in adverse effects. Effects observed in other studies support this conclusion. Thus according to the OECD guidance document 150 (OECD, 2012a) 4-tert-pentylphenol is almost certainly an endocrine disruptor for *D. rerio* due to indicative histological findings and changes in sex ratio. Indicative effects occurred at 100 µg/L (VTG induction) and above and adverse effects were observed at 62-100 µg/L (sex ratio, diagnostic).

5.2.1.3.3 Oryzias latipes

Modified ELS and FSDT

Hagino et al (2001):
A modified ELS study is available from Hagino et al. (Hagino et al., 2001). Newly hatched larvae were exposed for 4 weeks and afterwards reared for 2 weeks in fresh water. The treatments were 1, 10, 100, 1000µg/L. EE2 (Ethinylestradiol) and DES (Diethylstilbestrol) were both tested at the concentrations 0.001, 0.003, 0.01, 0.032 and 0.1µg/L. E2 was tested at 0.01 and 0.1µg/L. In the beginning 80 fish were exposed. Only one replicate per treatment was used. The test was assessed as reliable (Klimisch 2) with regard to qualitative observations (type of effects observed), because it was well conducted and documented. However, due to statistical insufficiencies (only on replicate) the quantitative examination of effects should be used as supporting information only. S-rR strain medaka were used. The body colour in this strain is inheritable and unchanged even on sex reversal because the body colour is encoded in genes linked to sex chromosome X or Y. Hence females are always white and males orange-red. Sex reversal induced by a chemical is detected by morphological changes of the gonads and secondary sexual characteristics on dorsal and anal fins.

The following secondary sexual characteristics (SSC) were examined:

- the maximum length of dorsal fin (dm)
- the cleft depth between the last ray and preceeding one of the dorsal fin (dc)
- maximum length of the anal fin (am)
- length of second ray from the last one of anal fin (a2)
- presence of small papillary processes on the posterior region of anal fin (app)
- total length (tl) (no SSC)

Results:

Secondary sexual characteristics (SSC):
The SSC on the dorsal fin (males only) decreased considerably at 100µg/L and 1000µg/L (no statistics). Effects were similar to those observed for E2 and EE2.
The SSC on the anal fin of males also decreased significantly with increasing from 10 to 1000µg/L (significant at 100µg/L). Again effects appeared to be similar to EE2 and E2 (of course at different concentrations).
Papillary processes of the posterior region of the anal fin were decreased in males at 10 µg/L and above (no statistics).

Sex reversal:
Sex reversal was observed as gonads of genotypic males exposed to 4-tert-pentylphenol differentiated into ovaries at 10 µg/L. The effect was dose related. At 100 µg/L most genotypic males had ovaries and at 1000 µg/L, all examined males had ovaries. The gonads of females differentiated into ovaries.
Also in the positive controls sex reversal of the gonads of genotypic males into ovaries was observed after exposure to 0.032 µg/L EE2, 0.032 µg/L DES and 0.1 µg/L E2.

Fish sexual development test

4-tert-pentylphenol was also used as test substance for the Validation report (Phase 2) for the fish sexual development test for the detection of endocrine active substances in *O. latipes* (Updated 2012), Series on Testing and Assessment No. 142. (OECD, 2012c):
Two labs (nos. 9 and 10) evaluated 4-tert-pentylphenol. Newly fertilized eggs were exposed, 40 per replicate, four replicates per treatment level. The nominal concentrations used were 32, 100 and 320 µg/L. Lab 9 exposed the fish for 70 d, Lab 10 exposed them for 60 d. Reliability of the tests was assessed Klimisch 2.

Results:

VTG:
Lab 9 determined significant increases at 104 µg/L in males → **LOEC 104 µg/L**, dose response existed. At the highest test concentration (318 µg/L) VTG in females was decreased.

Lab 10 determined significantly increased VTG in males at 94 µg/L too → **LOEC 94 µg/L (males)**. A very pronounced increase was observed at 294 µg/L (125-fold of control). The VTG concentration in females was increased at 27 µg/L. However the difference to the control was low, but a dose response existed with higher VTG levels at higher concentrations. LOEC for females: 294.3 µg/L.

Sex ratio:
Lab 9 determined **significantly less males at 318 µg/L (=LOEC).**
Proportions males : females:
Control 0.551 : 0.448
31.5 µg/L 0.411 : 0.588
104.2 µg/L 0.403 : 0.596
318 µg/L 0.304 : 0.521*
* = significant

Lab 10 determined a similar **LOEC (294 µg/L, less males)**
Proportions males : females : testis-ova:
Control 0.45 : 0.55 : 0
27 µg/L 0.40 : 0.60 : 0
94 µg/L 0.35 : 0.50 : 0.15 (sign. more testis-ova)
294 µg/L 0.20 : 0.70 : 0.10 (sign. less males)

The **LOEC for sex ratio (testis-ova) is 94 µg/L**.

Yokota et al.:
4-tert-pentylphenol was also used in a study by Yokota et al. (Yokota et al., 2005) who examined the effects on the gene expression of P450 11ß-hydroxylase ($P450_{11ß}$). It is a key steroidogenic enzyme in the synthesis of 11-ketosterone. The gene expression of $P450_{11ß}$ was investigated in the testes of genetically male medaka exposed to 4-tert-pentylphenol. The fish were exposed from the egg stage up to 60 dph (FSDT, or partial life cycle test) at concentrations from 62.2 to 783 µg/L (m). Regarding testing conditions the author refers to the study done by Seki et al. (2003), which is also included in the dossier (see below). Seki used four replicates with 15 fish embryos in each test chamber, which matches to the total amount of 60 fish used by Yokota.
Three fishes displaying externally male characteristics were taken for further assessment from each of the concentrations 62.2 and 121 µg/L and the controls at 60 dph. At 238 µg/L only one phenotypical male was found due to biased sex ratio towards females and no males existed at 413 and 783 µg/L. Instead females were used.
Caudal fins and gonads of the fish were used for sex genotyping and for analysis of $P450_{11ß}$.

Results:

The genotypic sex of several fishes exposed to 4-tert-pentylphenol did not concur with their phenotypic sex according to secondary sex characteristics (SSC). "In the three highest treatments (238, 413 and 783 µg/L), one or two out of three fishes displaying female characteristics were genetical males. For the two lower treatments (62.2 and 121 µg/L) and controls, the genotypic sex of all fishes coincided with the morphological phenotype."
Hence it can be said that for 33 to 66% of fish exposed to the concentrations 238, 413 and 783 µg/L the secondary sex characteristics and the genetic sex were not concurring.

Analysis of $P450_{11ß}$ mRNA by RT-PCR (Reverse transcription polymerase chain reaction): The long-term exposure with 4-tert-pentylphenol completely inhibited expression of $P450_{11ß}$ mRNA in the gonads of XY sex-reversed female medaka. The expression of $P450_{11ß}$ mRNA was detected in the controls and in the three lowest treatment groups (62.2, 121 and 238 µg/L), but was totally absent in XY fish in the two highest treatments (413 and 783 µg/L).

Normally P450 11ß-hydroxylase exists in the testis of male fish. $P450_{11ß}$ is an important enzyme in production of 11-ketosterone. As $P450_{11ß}$ is not produced anymore in the XY sex-reversed female medaka the exposure of 4-tert-pentylphenol did not only disturb the formation of secondary sexual characteristics, but also had an adverse impact on the steroidogenesis.

Effects on *O. latipes* in modified ELS and FSDT are summarised in Table 20.

Table 20: *Oryzias latipes* - Modified ELS and FSDT

Life stage/ duration	Conc. / test condition / solvent	Vitello-genin	Histology	Fertility/ Fecundity	Sex ratio / gonadal histology	Sec. sex characteristics	others	Positive control	Reference	reliability
O. latipes, modified ELS, beginning with newly hatched larvae, exposed for 4 wk afterwards 2 wk in fresh water S-rR strain; genotypic sexes are identified by the body color	1; 10; 100; 1000 µg/L (n) Measured concentration at 1 µg/L was in the range of 70 to 80% of nominal concentration Flow-through / solvent DMF, Solvent control existed				**Gonadal sex reversal in males at 10 µg/L**	**SSC decreased: Dorsal fin of males at 100 µg/L ;** **Anal fin of males at 100 µg/L,** **papillary processes on the anal fin decreased from 10 µg/L in males** **Females: no effect**		EE2: 0.001 – 0.1 µg/L DES: 0.001-0.1 µg/L E2: 0.01 and 0.1 µg/L In males: SSC decreased on dorsal fin and on anal fin. Papillary processes were decreased. Gonadal sex reversal.	(Hagino et al., 2001)	**2** **Only one replicate used, therefore quantitative examination unreliable and only supporting**
O. latipes, FSDT Starting with newly fertilized eggs 0 – 70 dph	32; 100; 320 µg/L (n) 31.5; 104.2; 317.7 µg/L (m) Flow-through, no solvent used	**LOEC 104 µg/L increase in males** At 318 µg/L sign. decrease in females			**LOEC 318 µg/L less males**		No effects on hatching and survival		VALIDATION REPORT (PHASE 2) FOR THE FSDT (Updated 2012), Lab 9	**2**

O. latipes, FSDT Starting with newly fertilized eggs 0 – 60 dph	32; 100; 320 µg/L (n) 27; 93.6; 294.3 µg /L (m) Flow-through, no solvent used	**LOEC 94 µg/L increase in males;** in females increased from 27 µg/l, **LOEC 294 µg/L,** dose response existed	**LOEC 94 µg/L testis-ova**		**LOEC 94 µg/L testis-ova (referred to as intersex) LOEC 294 µg/L less males**		No effects on hatching and survival		VALIDATION REPORT (PHASE 2) FOR THE FSDT (Updated 2012), Lab 10	**2**
O. latipes, Partial life cycle (FSDT) From egg stage to 60 dph	62.2; 121, 238, 413, 783 µg/L (m) Flow-through				**Sex reversal at 238 µg/L and higher;** Genotyping using a sex determining gene (DMY)		Expression of $P450_{11\beta}$ mRNA (in XY-fish = genetically male) was detected in the controls and at 62.2, 121 and 238 µg/L, but was totally absent at 413 and 783 µg/L		Yokota et al. 2005	**2**

Fish full life cycle tests

A fish full life cycle test with 4-tert-pentylphenol was conducted by Seki et al. 2003 (Seki et al., 2003). The concentrations were 51.1, 100, 224, 402, 931 µg/L (measured). There were four replicates with 15 fish embryos in each test chamber.

The exposure of the F0 generation began <12 hours post fertilization. At day 70 after hatch six mating pairs from each of the three lowest treatment groups (51.1, 100, and 224 µg/L) and from the control group were selected for the examination of fecundity and fertility. Each pair was assigned to a test chamber and exposed until 101 d after hatch. No pairs from the 402- and 931-µg/L treatment groups could be selected, because of the female biased sex ratio in these groups.

F1 generation: The newly hatched larvae were transferred to four test chambers per treatment until hatching. Then 15 larvae were selected (60 larvae per treatment group) and exposed until 61 dph. Reliability of the test was assessed Klimisch 2.

Results:

VTG (hepatic):
F0: VTG was elevated in all test concentrations but not in a clear dose dependent manner (Values read from graph (approximated): Control, 51.1, 100, 224 µg/L: 60, 1100, 1000, 1300 µg/L VTG in ng/mg liver respectively). The VTG levels at 51.1 and 224 µg/L were significantly elevated.

F1: The hepatic VTG levels of F1 male medaka at 61 d after hatch were positively correlated with the 4-tert-pentylphenol treatment concentrations ≤224 µg/L, however they were not significant elevated.

Secondary sexual characteristics (SSC):
The number of papillary processes and the number of fin rays with papillary processes were counted.

F0 (101 dph): A significant correlation between increasing 4-tert-pentylphenol concentration and decreasing number of papillary processes on the anal fin was observed, as well as a significant correlation between increasing 4-t-PP concentration and decreasing number of fin rays with papillary processes.

Only the control and the treatments from 51 to 224 µg/L could be evaluated because **no fish with male secondary sex characteristics existed at 402 µg/L and at 931 µg/L.** At 224 µg/L the SSC were not affected. **LOEC 402 µg/L.**
F1: At 224 µg/L significantly more females than males were visible. The ratio males : females was 12 : 47 (in control 27 : 32) Therefore the **LOEC is 224 µg/L** (p = 0.003).

Sex ratio (histology) and testis-ova:
F0: At 100 µg/L and lower no testis-ova were seen. At 224 µg/L five testis-ova (in 5 of 20 fish) were seen **(LOEC sex ratio (testis-ova) = 224 µg/L).** At 402 µg/L only one testis existed and the proportion testis : ovary : testis-ova was **1 : 14 : 5**. At 931 µg/L no males were observed. LOEC 402 µg/L (male : females).

F1: **Testis-ova appeared already at 51 µg/L** (1 of 20gonads) **and at 100 µg/L** (3 of 20 gonads). A dose dependent increase of number of testis-ova was determined. At 100 µg/L the proportion testis:ovary:testis-ova was **5:12:3** (not significantly biased). At

224 µg/L the proportion testis : ovary : testis-ova was **0 : 12 : 8 .** That means that no males existed anymore **(LOEC sex ratio 224 µg/L in F1)**. See Table 21 for F0 and F1 generation below.

Table 21: Sex ratio of *O. latipes*

	testis	ovary	testis-ova
F0 Control	6	14	0
F0 at 51 µg/L	8	12	0
F0 at 100 µg/L	11	9	0
F0 at 224 µg/L	6	9	**5**
F0 at 402 µg/L	1	14	5
F0 at 931 µg/L	0	9	3
F1 Control	9	11	0
F1 at 51.1 µg/L	10	9	**1**
F1 at 100 µg/L	5	12	**3**
F1 at 224 µg/L	**0**	**12**	**8**

Fertility (mean fertility per pair):
F0-Generation: **LOEC 224 µg/L**. The mean fertility of two of six pairs was reduced to 7 and 53 %, respectively, whereas that of the other four pairs ranged from 80 to 96 %. Thus, the mean fertility of the treatment was reduced to 72 % of that of the control pairs, resulting in a significant difference ($p = 0.027$) with the large error bar.

Fecundity (total number of eggs per pair):
F0: Linear regression analysis indicated that the fecundity of paired medaka during the reproductive phase from 71 to 101 d after hatch decreased significantly with increasing 4-t-PP concentration ($r^2 = 0.93$).

Growth:
F0: No significant differences were observed in either mean total length or body weight at 60 dph up to 402 µg/L. However, the growth of fish in the 931 µg/L group was significantly reduced compared with the controls ($p < 0.001$ for length; $p = 0.003$ for weight).

F1: The body weights of F1 juveniles at 61 d after hatch were not affected in any 4-tert-pentylphenol treatment groups. However, the mean total length was significantly ($p < 0.001$) lower in the 224 µg/L group than in the control.

Mortality:
At 224 µg/L approx. 10% mortality appeared (from hatch to 60 dph). At 402 µg/L approx. 20 % mortality occurred.

All results are summarised in Table 22 below.

Table 22: *Oryzias latipes* – FFLC with 4-tert-pentylphenol (4-t-PP)

Life stage/ duration	Conc. / test condition / solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex ratio / gonad histology	Sec. sex characteristics	others	Positive control	Reference	reliability
O. latipes **FFLC** F0: Exposure began < 12 hours post fertilization, Duration 101 d; F1: until 61 d after hatch	51.1, 100, 224, 402, 931 µg/L (m), Flow-through, renewal 17 times a day, Solvent: no (Testing in quadruplicate, 15 embryos per chamber)	F0 Hepatic VTG in males: **LOEC 51.1 µg/L,** also significant at 224 µg/L, at 100 µg/L elevated but not significant (p = 0.052); Females: no effect until ≤224 µg/l (not higher tested) F1 hepatic VTG concentrations positively correlated with 4-t-PP concentration, no single value sign. up to 224 µg/L	F0 at 100 µg/L no testis-ova; at 224 and at 402 µg/L 5 testis-ova of 20 gonads, F1: at 51 µg/L 1 testis-ova of 20 gonads; at 100 µg/L 3 testis-ova of 20 gonads; at 224 µg/L 8 testis-ova of 20 gonads (no testis); dose dependent increase of number of testis-ova;	**F0 Fertility LOEC 224 µg/L** (in two of six pairs the fertility declined from the beginning of the assay) **Fecundity:** Sign. corr. between increasing 4-t-PP and decreasing fecundity	**F0 LOEC (testis-ova) 224 µg/L**, **at 402 µg/L only one testis (1 of 20)** **F1 LOEC 224 µg/L (no males and 8 testis-ova of 20 fish)**,	F0 LOEC 402 µg/L (at 402 and 931 µg/L no males detected) F1 LOEC 224 µg/L (males : females 12:47) Significant correlation between increasing concentration and decreasing number of papillary processes and fin rays with papillary processes.	F0: LOEC growth (reduced length and weight) 931 µg/L; F0: LOEC cumulative mortility 931 µg/L, F1: no effects on hatchability and time to hatch, Total length: LOEC 224 µg/L (highest concentration in F1) Cumulative mortality until 60 dph (F0): at 931 µg/L 60%; at 402 µg/L 20%; at 224 µg/L 12%, others lower like control (around 8%) (approx. values, read from chart)	no	(Seki et al., 2003)	**2**

Reproduction assay

Four labs participated in the validation of the 21-day reproduction assay (OECD, 2006): Three exposure concentrations were tested: 100, 320 and 1000 µg/L nominal. Two vessels per treatment with each vessel containing 5 males and 5 females were used. The exposure lasted 21 d, at the end the fish were sampled. Reliability of the studies was assessed Klimisch 2.

Three core endpoints as indicators of endocrine activity of the test substances were monitored during the course of the test or measured at termination of the test, namely:

i) gross morphology (e.g. secondary sexual characteristics (papillary processes on the anal fin in medaka were counted according to standard operating procedures))
ii) vitellogenin levels
iii) gonadal histology

Results:
VTG:
Hepatic VTG was induced in a concentration-dependent manner in all studies. Significant VTG increases compared to control were found in all exposed groups in both sexes, except at LAB 2 in females at 100 µg/l, at LAB 5 in females at all treatment levels and at LAB 5 in males at 100 µg/L. In LAB 5 in females, statistical significance was not achieved despite a dose-dependent increase of VTG, probably due to high variability of measurements within each group, combined with a reduced sample size because of mortality. In Lab 5 in males only one male had a high VTG level compared to the others and therefore the VTG is not significant elevated.

Secondary sexual characteristics (SSC):
One lab (Lab 3) observed at the highest concentration significantly less fish with male SSC. All other labs and also all positive controls did not indicate any changes regarding SSC.

Histopathological findings in males
Primary diagnostic criteria for ED:
- Increased spermatogonia (treatment related) connected with decreased spermatocytes and spermatids at the highest concentration (Lab 3).
- Testis-ova: low level occurrences in the high dose groups (Lab 1 and 3) and dose dependent increase in positive controls
- Testicular degeneration: Three of four labs observed treatment related testicular degeneration at the highest concentrations and two labs observed it also at lower concentrations to a lesser degree.

Secondary diagnostic criteria:
- Proteinaceous fluid (intravascular) treatment related at the highest concentration observed in Lab 3
- Proteinaceous fluid (interstitial) treatment related at the highest concentration observed from Lab 3

Histopathological findings in females:
Primary diagnosis:
- Oocyte atresia, treatment related increased in two labs: Lab 3 at the highest concentration and Lab 5 at the middle and high concentration. The effect appeared also in the positive control, but to a lesser extent.

Spawning:
In two of four labs effects on fecundity appeared: In one lab at concentrations of 272 and 857 µg/L and in the other lab at 937 µg/L markedly less eggs were spawned. No statistics were used.
See Table 23 below.

Table 23: *O. latipes*, Reproduction assay

Life stage/ duration	**Conc. / test condition / solvent**	**Vitellogenin**	**Histology**	**Fertility/ Fecundity**	**Sex ratio / gonad histology**	**Sec. sex characteristics**	**others**	**Positive control**	**Reference**	**reliability**
O. latipes 21-day assay Age of fish 16 wk	100, 320 and 1000 µg/l (n); 91; 284; 882 µg/L (m) flow-through, no solvent	Hepatic VTG increased in males, **LOEC 91 µg/L,** NOEC < 91 µg/L VTG increased in females **LOEC 91 µg/L,** NOEC < 91 µg/L	**Testicular degeneration treatment relevant at 882 µg/L**	No effects		No effect on papillary processes	No Mortality	17ß-estradiol 102 ng/L Mortality: no effect, VTG sign. increased Testis-ova Testicular degeneration, no effect on spawning	OECD_61_Validation_21-day_assay_ENV-JM-MONO(2006)29[1] LAB 1 (OECD, 2006)	2
O. latipes 21-day assay Age of fish 16 wk	100, 320 and 1000µg/l (n) , 86.8; 287; 959 µg/L (m) flow-through, no solvent	Hepatic VTG increased in males**, LOEC 87 µg/L,** NOEC < 87 µg/L VTG increased in females LOEC 287 µg/L, NOEC 87 µg/L		No effects		No effect on papillary processes	Mortality 5 % at 1000 µg/L, other treatments and control 0 %	17ß-estradiol 105 ng/L Mortality: no effect, VTG sign. increased	OECD_61_Validation_21-day_assay_ENV-JM-MONO(2006)29[1] LAB 2 (OECD, 2006)	2

O. latipes 21-day assay Age of fish 17 wk	100, 320 and 1000µg/l (n), 98.6; 314; 937 µg/L (m); flow-through, no solvent	Hepatic VTG increased in males, **LOEC 100 µg/L,** NOEC < 100 µg/L, VTG increased in females **LOEC 100 µg/L,** NOEC < 100 µg/L	**Spermatogonia treatment relevant increased at 937 µg/L** Spermatocytes and spermatids treatment relevant decreased at937 µg/l **Testicular degeneration treatment relevant at 937 µg/L** Proteinaceous fluid(intravascular and interstitial) treatment relevant at 937 µg/L	At 937 µg/L markedly fewer eggs		No effect on papillary processes, except at 937 µg/L (may be considered false positive)	No Mortality	17ß-estradiol 103ng/L Mortality: no effect Spawning: no effect, VTG sign. increased Spermatogonia increased Spermatocytes, spermatids and spermatozoa decreased Testis-ova Testicular degeneration Proteinaceous fluid(intravascular and interstitial)	OECD_61_Validation_21-day_assay_ENV-JM-MONO(2006)29[1] LAB 3 (OECD, 2006)	2
O. latipes 21-day assay Age of fish 16 wk	100, 320 and 1000µg/l (n), 85.5; 272; 857 µg/L (m) flow-through	Hepatic VTG increased in males, LOEC 272 µg/L, NOEC 85.5 µg/L; No effect on females	Spermatozoa treatment relevant increased at 857 µg/L **Testicular degeneration treatment relevant at 857 µg/L**	Markedly fewer eggs at 272 and 857 µg/L		No effect on papillary processes	Mortality: 20% at 272 µg/L and 857 µg/L resp. In control and at 85.5 µg/L no mortality, see also right positive control	17ß-estradiol, 78ng/L Mortality 35% Spawning decreased, VTG sign. increased Spermatozoa increased Testicular degeneration	OECD_61_Validation_21-day_assay_ENV-JM-MONO(2006)29[1] LAB 5 (OECD, 2006)	2

Summary for *O. latipes*

Three extended early life stage tests or fish sexual development tests with *O. latipes* are available. All tests showed sex reversal of genetic males to phenotypic females or a significant change in sex ratio towards less males based on gonadal histology and thus adverse effects diagnostic for an estrogenic mode of action. Sex reversal of genetic males was observed at 10 µg/L and above (insuffient statistics) while changes in sex ratio were observed at higher test concentrations only (LOEC 294- 318 µg/L). Similar effects were observed in an FFLC where a clear effect on sex ratio was observed at 402 µg/L in the F0 generation (less males) and at 224 µg/L in the F1 generation.

The described effects were accompanied by other estrogen related effects such as decrease in male secondary sex characteristics, testis-ova or vitellogenin induction and reduced fertility.
The estrogenic mode of action is substantiated by histological findings in four reproduction assays which are diagnostic for an endocrine mode of action and vitellogenin induction in males in all tests.

In summary it can be concluded that results from the fish sexual development tests and the full life cycle test are clearly indicative of an estrogenic mode of action resulting in adverse effects. Effects observed in other studies (reproduction assays, modified ELS) support this conclusion. Thus according to the OECD guidance document 150 (OECD, 2012a) 4-tert-pentylphenol is an actual endocrine disruptor for *O. latipes* due to indicative histological findings, vitellogenin induction and changes in sex ratio.

Indicative effects occurred at 51 – 100 µg/L (VTG induction, secondary sex characteristics) and above and effects both adverse and indicative were observed at 224–318 µg/L (sex ratio, diagnostic) with some indication that effects might occur already at 10 µg/L (insuffient statistic).

In addition, results observed in the FLC indicate that effects are even more distinct in the second generation as both effects on secondary sex characteristics and on sex ratio occurred in F1 at lower exposure concentrations compared to F0.

5.2.1.3.4 Cyprinus carpio

Modified juvenile growth tests and modified ELS

Two modified juvenile growth tests and one modified early life stage test were performed by Gimeno and co-authors (Gimeno et al., 1998, Gimeno et al., 1996, Gimeno et al., 1997).

Gimeno etal (1996):
Gimeno et al. (Gimeno et al., 1996) conducted a test with juvenile *Cyprinus carpio* (genetic males). Like in the test conducted in 1998 by Gimeno et al. the fish had at the beginning an age of 50 d (sexually undifferentiated). The fish were exposed for 90 d (intermittend flow-through), 20 days less than in the study by Gimeno et al. 1998). Most probably, only one replicate per treatment was used. The test was assessed Klimisch 3 because information is missing about the number of tested animals and replicates.

Results:

Formation of an oviduct (Gonadal attachment / Retained peritoneal attachments / gonadal duct feminization of the testis):
The LOEC for formation of the oviduct in the test from 1996 was 320 µg/L (nominal) with 100 % oviduct formation. At the lower concentration of 100 µg/L (nominal) approx. 30 % of the fish had an oviduct (read from chart).

Primordial germ cells (PGC):

The number of primordial germ cells was significantly elevated at 1000 µg/L (nominal) after 40 d exposure.

Gimeno et al. (1998):
The second modified juvenile growth test with *Cyprinus carpio* (genetic males) was conducted by Gimeno et al. (Gimeno et al., 1998a). The fish were exposed from 50 dph until 160 dph at flow-through conditions. At the beginning 120 individuals (weighing between 1.3 and 1.7 g) were exposed in one 25 L aquarium per treatment. Only one replicate per treatment was used. The test was assessed as reliable (Klimisch 2) with regard to qualitative observations (type of effects observed), because it was well conducted and documented. However, due to statistical insufficiencies (only on replicate) the quantitative examination of effects should be used as supporting information only.
At 50 dph the fish were still sexually undifferentiated. Exposure to 4-tert-pentylphenol comprised the labile period of sex differentiation in common carp. The gonads were histologically examined with regard to Primordial germ cells (PGC) at days 20 up to 80 (every ten days) and oviduct formation. Plasma VTG was measured. Test concentrations were 36, 90 and 256 µg/L (m).
The nominal concentrations of the positive control E2 were 10 and 100 µg/L (highest E2 dosage was measured 23 µg/L).

Results:

Primordial germ cells (PGC):
The number of PGC was counted per gonad section. At test end at 36 µg/L and above PGC numbers were significantly reduced ($p < 0.001$). The effects started at day 40 with significant reduction in the highest test concentration ($p < 0.001$). The significance was calculated using the Student t-test by the authors.
Reduced number of primordial germ cells is an indicator of inhibition of spermatogenesis as PGC are the first germ cell population established during development and are immediate precursors for both the oocytes and spermatogonia.

Spermatogenesis was inhibited in some of the fish at higher test concentrations (90 and 256 µg/L) and totally inhibited after exposure to E2.

Formation of an oviduct (Gonadal attachment / Retained peritoneal attachments / gonadal duct feminization of the testis):
At 36 µg/L after 90 days exposure 50 % of the fish developed an oviduct ($p < 0.01$), while the other 50% differentiated into normal males. At this time all control males had developed a normal reproductive tract with a vas deferens. All fish exposed at 90 and 256 µg/L and to E2 had feminized testis with oviduct. E2 induced oviduct formation already after 20 d exposure, whereas this occurred after 40 days in 4-tert-pentylphenol-exposed carp (both effects significant). As statistical calculation was used the Chi-squared test.

Ovo-testis:
Oocytes were seen in testes of carp exposed to 90 and 256 µg/L, which had developed an oviduct.

VTG in plasma:
The VTG concentration in plasma at 256 µg/L was significantly elevated (127300 ± 95500 ng/mL, compared with control 317 ± 260 ng/mL, $p < 0.05$) but not as high as in the positive controls. No significant effects were observed at lower concentrations. As statistical calculation was used one-way analysis of variance (ANOVA $P < 0.05$) followed by a Student t-test.

Gimeno et al. (1997):
Gimeno et al. (Gimeno et al., 1997) conducted another test (different ages of fish) with

genetic male common carp in 1997. One concentration was tested (nominal concentration 140 µg/L) and one replicate for each exposure duration was used. The test was assessed as reliable (Klimisch 2) with regard to qualitative observations (type of effects observed), because it was well conducted and documented. However, due to statistical insufficiencies (only on replicate) the quantitative examination of effects should be used as supporting information only. In the beginning 300 to 400 eggs were fertilized and then allocated to the different exposure groups. The number of examined fish can be seen in Table 24 below. The aim of the study was to examine the labile period for endocrine disruption. The exposures of the carp to 4-tert-pentylphenol had variable durations and started at two different stages of development:

(a) As embryos, yolk sac larvae, or larvae, i. e. at-3, 0, or 3 dph (further referred as to ±3 (because no differences within this group were observed) until the fish were 0, 3, 24, 51, or 110 dph.
(b) As fingerlings, i. e. at 24 dph until the fish were 51 or 110 dph.
Sampling took place at 51 dph: just prior to sexual differentiation and at the start of multiplication of the PGCs; at 110 dph where PGCs are known to have entered spermatogenesis. Fish that were only exposed until 0, 3, 24, and 51 dph were placed in clean water until the sampling took place either at 51 or at 110 dph.

Results:

Formation of an oviduct (Gonadal attachment / Retained peritoneal attachments / gonadal duct feminization of the testis), vas deferens:
A short exposure (from ±3 dph or 24 dph up to 51 dph) and sampling at 51 dph induced in all fish an oviduct. In some fish the oviduct was incomplete.

After longer exposure (from ±3 dph or 24 dph up to 110 dph) all fish showed a complete oviduct.

In none of the fish exposed from ±3 dph or 24 dph up to 51 dph or 110 dph at the sampling time 110 dph a vas deferens (normal reproductive system for males) was seen.
The absence of the vas deferens and the formation of a complete oviduct in fish exposed from ±3 to 110 or 24 to 51 or 24 to 110 dph could be seen as indication for abnormal sex reversal (see Table 24).

The period sensitive to endocrine disruption in carp seems to be the time before sexual differentiation (24-51 dph), since exposure before this period (3-24 dph) did not cause the formation of oviducts and the percentage of fish forming a vas deferens was similar to the control. In the control 78 % of fish had a vas deferens at 110 dph and 0 % had an oviduct.

Table 24: Formation of oviduct and vas deferens at 51 and 110 dph at 140 µg/L (Excerpt from a table of the study by Gimeno et al. 1997)

Timing of exposure (dph)	At 51 dph	At 110 dph	
	Oviduct in % *	Oviduct in % *	Vas deferens in %
Controls	(16) 0	(14) 0	78
3-3	(8) 0	(13) 0	80
±3-24	(11) 0	(12) 0	80
±3-51	(12) 100 12/16 complete	(12) 100 20/23 complete	0
±3-110		(16) 100 all complete	0
24-51	(6) 100 6/11 incomplete	(4) 100 all complete	0
24-110		(4) 100 all complete	0

±3 is a grouping of exposures starting at the embryo, yolk sac larva, or larva stage (-3, 0, or 3, respectively), not relevant because no differences at that different time points.
* The number of examined individuals is specified in parenthesis.

Primordial germ cells (PGC) and arrested spermatogenesis:

The proliferation of PGCs could only be examined in fish aged 110 dph. An increased duration of exposure reduced the number of PGCs counted per section.
Exposures from ±3 to 51 or from ±3 to 110 dph and from 24 to 110 dph significantly reduced the number of PGCs. At the only concentration of 140 µg/L the gonads did not exhibit any sign of spermatogenesis and were reduced to an epithelium and the germ cell development was inhibited. No such effects were observed if exposure stopped at 24 dph and earlier.
Results show that the decrease in PGC persists even after the end of exposure if fish are exposed during the critical development stage (effects were observed at day 110 dph for fish exposed until 51 dph but not for fish exposed until 24 dph).

Growth:
In contrast to the other studies above, growth was significantly reduced at the only tested concentration 140 µg/L (exposure from 3 dph to 51 dph ($p < 0.001$) or 24 dph to 51 dph ($p < 0.05$)). The effect was stronger in the group with exposure starting at earlier life stages.

The effects on *Cyprinus carpio* in modified Juvenile growth tests and modified ELS are summarised in Table 25.

Table 25: *Cyprinus carpio* - modified Juvenile growth tests and modified ELS

Life stage/ duration	Conc. / test condition / solvent	Vitello-genin	Histology	Fertility/ Fecundity	Sex ratio / gonad histology	Sex ratio / phenotypic	Sec. sex characteristics	others	Positive control	Reference	reliability
***Cyprinus carpio* (genetic males)** Modified juvenile growth test Exposure from 50 dph until 160 dph	36, 90, 256 µg/L (m), 100, 320, 1000 µg/L (n); Flow-through, solvent: tertiary butyl alcohol only for positive control (maximum 0.1 mL/L)	Elevated VTG: 256 µg/L (p< 0.05)	**Formation of oviduct (feminisation gonadal ducts) at 36 µg/L in 50% of males; at all higher concentrations in 100% of fish (90d exposure)** **Number of primordial germ cells decreased at 36 µg/L, partly inhibition of spermatogenesis;** **Ovo-testis at 90 µg/L**					No effects on growth	E2 10 and 100 µg/L (n), All fish: feminized testis with oviduct at 10 and 100 µg/L growth reduced (low and high conc., no statistics). VTG elevated: (p< 0.001), Primordial germ cells did not multiply at all, no spermatogenesis At 10 µg/L Oogenesis (prophase oocytes), at 100 µg/L pre-vitellogen. oocytes	Gimeno et al. 1998a (Gimeno et al., 1998a)	**2 Only one replicate used, therefore quantitative examination unreliable and only supporting**

Life stage/ duration	Conc. / test condition / solvent	**Vitellogenin**	**Histology**	**Fertility/ Fecundity**	**Sex ratio / gonad histology**	**Sex –ratio / phenotypic**	**Sec. sex characteristics**	**others**	**Positive control**	**Reference**	**reliability**
***Cyprinus carpio* (genetic males)** Modified juvenile growth test Age 50 d, sexually undifferentiated, exposed for 90 day	100, 320, 1000 µg/L (n), Intermittent flow-through		Ovi-duct was formed, after 60 d at 320 µg/L, Testis-ova and severely inhibited spermatogenesis at 1000 µg/L (2 of 6 fish), Number of primordial germ cells decreased at 1000 µg/L (after 60 d) , very poorly developed testis (1000 µg/L)						17ß-estradiol, 10 µg/L, Oviduct and oocytes, no testicular tissue, phenotypic females PGC significantly decreased	(Gimeno et al., 1996)	**3 Probably only one replicate used, no information about number of examined animals.**
***Cyprinus carpio* (genetic males)** Different exposure times and different ages Exposure from ±3 dph to 51 dph or ±3 dph to 110 or 24 dph to 51 dph or 24 dph to 110 dph	140 (n), measured concentration approx. 50% of nominal, semi static At the beginning of the experiment the test solutions were renewed three times a week and then daily from 40 dph onwards. Without solvent		All effects at 140 µg/L: **Ovi-duct was formed in all fishes** exposed from 3 dph to 51 dph/ 110 dph or 24 dph to 51 dph / 110 dph. A vas deferens was absent in all these fishes. Number of primordial germ cells reduced LOEC 140 µg/L No signs of any spermatogenesis. Gonads were reduced to a thin epithelium (exposure from 3 dph to 51 dph/ 110 dph or 24 dph to 110 dph).		At 110 dph indication for abnormal sex-reversal: Absence of vas deferens (0 %) and formation of a complete oviduct (100 %) in genetic male fish			Growth reduced 140 µg/L at 51 dph: (exposure from 3 dph to 51 dph ($p < 0.001$) or 24 dph to 51 dph ($p < 0.05$)), effect stronger in the earlier exposed group	17ß-estradiol Formation of oviduct No information to effect on growth and PGC	(Gimeno et al., 1997)	**2 Only one replicate used, therefore quantitative examination unreliable and only supporting**

Modified reproduction assay

Also by Gimeno et al. (Gimeno et al., 1998b) a modified assay with mature genetic male common carp, without reproduction was conducted. There were 13 fishes per aquaria and treatment, two positive controls (E2 0.1 and 1 µg/L) and two negative controls (solvent control and dilution water control). Only one replicate per treatment was used. The test was assessed as reliable (Klimisch 2) with regard to qualitative observations (type of effects observed), because it was well conducted and documented. However, due to statistical insufficiencies (only on replicate) the quantitative examination of effects should be used as supporting information only. The fish were exposed during spermatogenesis for 3 months, i. e. from 210 to 300 dph at a concentration range of 32–1000 µg/L (n). Effects on the process of spermatogenesis and on VTG were examined.

Results:

Histology
At the beginning of the exposure, the testes were fully mature. All spermatogenic stages were present, including spermatozoa in the center of the lobules (lumen).

After 2 months exposure: At the three higher 4-tert-pentylphenol concentrations (from 100 µg/L (n)) and the lower E2 treatment in 60 % of fishes the lobules of testes were disorganized. **Atrophy of germinal epithelium, absence of spermatozoa** and necrosis were seen. Exposure to the higher E2 concentration (1 µg/L) resulted in testicular atrophy and inhibition of spermatogenesis in all exposed fish.
After three months exposure: Figures from the publication show that the exposed fish (100 µg/L nominal) have considerably less spermatozoa and increased spermatogonia A.

VTG:
Plasma VTG was significantly elevated at 1000 µg/L (nominal) after 2 and 3 months of exposure.

The effects are summarised in Table 26.

Table 26: *Cyprinus carpio*, modified reproduction assay

Life stage/ duration	Conc. / test condition / solvent	Vitello-genin	Histology	Fertility/ Fecundity	Sex ratio / gonad histology	Sex ratio / phenotypic	Sec. sex characteristics	others	Positive control	Reference	reliability
***Cyprinus carpio* (genetic males)** Sexually mature, age 210 dph; Exposure 3 months	32, 100, 320, 1000 µg/L (n), Flow-through, Solvent: tertiary butyl alcohol (0.1mL/L) only for E2, solvent control existed	**Increased at 1000 µg/L (n)**	**Atrophy of germinal epithelium from 100 µg/L (n)** **Absence of spermatozoa from 100 µg/L (n)** **At 1000 µg/L (n) no sign of spermatogenesis**					**No effect on weight**	E2: 0.1 and 1 µg/L; disorganization of the lobules, atrophy of germinal epithelium, absence of spermatozoa and necrosis Elevated VTG from 0.1 µg/L Ovo-testis at 1 µg/L	Gimeno et al., 1998b	**2**

Summary for *Cyprinus carpio*

In an extended early life stage test with genetic carp males sex reversal (formation of oviducts and absence of vas deferens) was seen after exposure to 4-tert-pentylphenol as well as reduced numbers of primordial germ cells and inhibited spermatogenesis. Growth as an apical endpoint was reduced too. Although this is not an endpoint diagnostic for an endocrine mode of action it is well known that growth is a sensitive endpoint for endocrine disruptors.

Also in two juvenile growth tests feminising effects were seen. Gonads of genetic male carp were feminised after exposure to 4-tert-pentylphenol even though the tests did not encompass life stages sensitive for gonadal development (which is considered to be between 24 and 51 d post-hatch).

One modified 21 d screening assay provides further evidence for an estrogenic mode of action as VTG induction and histological changes diagnostic for endocrine disruption were observed.

In summary the tests corresponding to OECD level 3 indicate an estrogenic mode of action also in carp. Although the tests do not follow an OECD guideline protocol the effect of sex-reversal observed in male fish in combination with an inhibition of germ cell development could be considered as apical endpoint. Thus 4-tert-pentylphenol is almost certainly an endocrine disruptor for Cyprinus carpio due to the indicative findings in combination with sex-reversal in male fish.

5.2.1.3.5 Cyprinodon variegatus

Screening of the fish plasma for estrogen-responsive biomarkers

Salinas et al (2008):
Fish were exposed by Salinas et al. (Salinas et al., 2008) to 100 µg/L 4-tert-pentylphenol or different concentrations of 17ß-estradiol for 7 days in an intermittent flow system. Afterwards the fish were examined for estrogen-responsive biomarkers. The authors demonstrated in the study the performance of a mass spectral method for screening of estrogen-responsive biomarkers in fish plasma using matrix-assisted laser desorption/ionization (MALDI) time of flight mass spectrometry coupled with a short-term fish assay (exposure of *Cyprinodon variegates* for 7 d). 15 fish were exposed and the plasma of 13 fish was pooled to make one pooled plasma sample.

The mass spectra of plasma from fish treated with 4-tert-pentylphenol at 100 µg/L (n) or E2 at 0.2 µg/L and from solvent control fish were processed and compared to discover protein masses that were differentially expressed between the groups. The mass spectrum is the response curve of relative intensity of signal against m/z (mass-to-charge ratio).

Results:

The pattern of mass spectra from 4-t-PP treated fish and E2 treated fish looked very similar. That means the same biomarkers existed in the plasma of 4-t-PP treated fish and E2 treated fish. The negative control has not shown any peaks.

Walker et al (2007):
A related test was conducted by Walker et al. (Walker et al., 2007): Also there male *Cyprinodon variegatus* were exposed for 7 days (15 fish in one vessel at 100 µg/L) and the plasma of fish were then analyzed for estrogen-responsive biomarkes. The plasma of 12 to 15 fish was pooled.

Results:

Again the pattern of the mass spectra of the plasma probe of 4-tert-pentylphenol (100 µg/L)

treated and 17ß-estradiol (0.2 µg/l) treated fish looked almost similar. A discriminatory peak was identified as a fragment of zona radiata (ZR) protein (an egg shell protein). Estrogen agonists induce the biosynthesis of ZR proteins in fish and other oviparous species, and ZR proteins appear in plasma earlier than VTG. Levels of ZR proteins in plasma of male or juvenile fish are usually low or not detectable. Elevated levels of these proteins in males or juveniles indicate exposure to chemical(s) with estrogenic activity.

The plasma mass spectra of fish exposed to 4-tert-pentylphenol showed similar peaks like the spectra of fish exposed to E2.

Results of both the Walker et. al. 2007 and the Salinas et. al. 2008 studies are provided in Table 27.

Table 27: *Cyprinodon variegatus*, screening of fish plasma for estrogen-responsive biomarkers

Life stage/ duration	Conc. / test condition / solvent	Mass spectra of biomarkers	Positive control	Reference	reliability
Sheepshead minnow (Cyprinodon variegatus) Adult male fish, exposure: 7 days	100 µg/L (n) Intermittent flow (20 L/h), Solvent: triethylene glycol 12 µL/L, solvent control existed Matrix-assisted laser desorption/ionization (MALDI) time of flight mass spectrometry was used to screen the fish plasma for estrogen-responsive biomarkes.	Almost similar mass spectra of plasma of fish exposed to 17ß-Estradiol (0.2 µg/L) or 4-tert-pentylphenol (100 µg/L) (Expression of estrogenic biomarkers in plasma compared with positive control.)	17ß-Estradiol (0.2 µg/L)	(Salinas et al., 2008)	2
Sheepshead minnow (Cyprinodon variegatus) Adult male fish, exposure: 7 days	100 µg/L (n) Solvent: triethylene glycol (12 µL/L), solvent control existed Surface enhanced laser desorption and ionization time-of-flight mass spectrometry (SELDI)	Almost similar mass spectra of plasma of fish exposed to 17ß-Estradiol (0.2 µg/L) or 4-tert-pentylphenol (100 µg/L) (Expression of estrogenic biomarkers in plasma compared with positive control.)	17ß-Estradiol 0.05 – 1.0 µg/L (n); 0.055 – 0.91 µg/L (m)	(Walker et al., 2007)	2

In summary, evidence from analysis of plasma of *C. variegatus* indicates an estrogenic mode of action of 4-tert-pentylphenol in this species. No information available as to whether this biomarker response correlates with the occurrence of adverse effects.

5.2.1.4 Summary of the plausible link between adverse effects and endocrine mode of action

In summary there is good evidence allowing to conclude that 4-tert-pentylphenol is acting as an endocrine disruptor in all species for which both effects indicative of an endocrine mode of action and adversity were assessed. Changes of sex ratios towards lower percentages of males, increased proportions of females or increased numbers of undifferentiated fish or sex-reversal of genetic males was observed for *P. promelas, D. rerio* and *O. latipes* and – less comparable - for *C. carpio*. Other effects observed for these species substantiate the estrogenic mode of action of 4-tert-pentylphenol. Indication of an estrogenic mode of action is available for one additional species (*C. variegates)* for which no apical endpoints were assessed. The effects are summarised in Table 28.

Table 28: Summary of *in vivo* effects in fishes

Species	Effects observed	Effect concentrations	Conclusion based on OECD GD 150
P. promelas	Modified sex ratio, feminization of gonadal ducts	Indicative effects starting at 10 µg/L, adverse effects at 56 µg/L (not diagnostic) and 93- 188 µg/L (sex ratio, diagnostic)	Almost certain an endocrine disruptor
D. rerio	Modified sex ratio, histological findings, VTG induction	Indicative effects occurred at 100 µg/L (VTG induction) and above and adverse effects were observed at 62 – 100 µg/L (sex ratio, diagnostic).	Almost certain an endocrine disruptor
O. latipes	Modified sex ratio, VTG induction, reduced male secondary sex characteristics	Indicative effects occurred at 51 – 104 µg/L (VTG induction, secondary sex characteristics) and above and adverse effects were observed at 224 µg/L – 318 µg/L (sex ratio, diagnostic). It is indicated that effects on sex reversal occurred at 10 µg/L (insufficient statistic).	Actual endocrine disruptor
C. carpio	Sex-reversal in genetic males (feminization of gonadal ducts), inhibition of germ cell development, testis-ova	Indicative effects started at 36 µg/L and were very distinct indicting adverse effects (sex-reversal) at 140 µg/L (insufficient statistic and no guideline followed).	Almost certain an endocrine disruptor based on sex-reversal, but based on insufficient statistic
C. variegatus	Biomarker response after exposure to 4-tert-pentylphenol similar to response on exposure to E2	100 µg/L (indicative)	Indication of an estrogenic mode of action

In addition, results observed in the FLC with *O.latipes* indicate that effects are even more distinct in the second generation as both effects on secondary sex characteristics as well as on sex ratio occurred in the test with F1 at lower exposure concentrations than in the test with F0.

5.2.1.5 Read-across from other alkylphenols

The conclusion is substantiated by a read across to other alkylphenols (4-tert-butylphenol, 4-heptylphenol, 4-tert-octylphenol, 4-nonylphenol) with regard to the endocrine disrupting properties for the environment. A detailed justification document for read-across is provided in Annex 1.

The read-across is based on the hypothesis that all these alkylphenols share the same structural moieties responsible for an estrogenic mode of action (phenol with alkyl chain in para-position).

Available *in vitro* and *in vivo* studies for fish show that, although substances differ in the length and branching of the alkylchain, they show similar endocrine disrupting properties and thus results from these other alkylphenols can be used to substantiate the effects observed for 4-tert-pentylphenol with regard to the environment in a weight of evidence approach. Data for other alkylphenols strengthen the reliability of results for 4-tert-pentylphenol. Effects observed

for 4-tert-pentylphenol and other alkylphenols, including 4-nonylphenol are very similar. They are in line with results observed for other fish species with other alkylphenols, which supports that the observations of effects are reliable. Data for other alkyphenols support the conclusion that effects observed for 4-tert-pentylphenol in fish are estrogen mediated.

For other alkylphenols a much broader variety of fish was tested. Available data clearly show that the alkylphenols act as endocrine disruptors for these fish species too. Applying read-across similar effects in a variety of fish species, including seasonal breeders, can be anticipated for 4-tert-pentylphenol.

In summary the information available for the other alkylphenols substantiates that – with regard to fish - 4-tert pentylphenol and all these other alkylphenols share the same mode of action and cause endocrine mediated adverse effects at similar exposure levels. Thus the available data substantiate that 4-tert-pentylphenol is an endocrine disruptor comparable to 4-tert-octylphenol and 4-nonylphenol.
Based on information available for other alkylphenols it is very plausible, that 4-tert-pentylphenol acts as an endocrine disruptor in other fish species too.

5.2.1.6 Environmental relevance

Effects observed in fish species after exposure to 4-tert-pentylphenol are indicative and adverse and relevant with regard to the population level. Effects are considered to have the potential to impair population stability and recruitment. Reproduction was inhibited, the sex ratio was biased towards females and growth was suppressed. These effects may impair population stability and thus effects must be considered environmentally relevant.

5.3 Summary and discussion of the environmental hazard assessment

In summary, *in vitro* data and *in vivo* data demonstrate that 4-tert-pentylphenol is an endocrine disruptor in fish. Both the types of effects observed as well as the concentrations at which effects occurred are similar to those observed for 4-tert-octylphenol and 4-nonylphenol.

6 Conclusions on the SVHC Properties

6.1 CMR assessment

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

6.2 PBT and vPvB assessment

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

6.3 Assessment under Article 57(f)

4-tert-pentylphenol is assessed in order to identify if it is a "substance 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" (Art 57 (f).

6.3.1 Summary of the data on the hazardous properties

A detailed description of the hazardous properties (endocrine disruption for the environment) of 4-tert-pentylphenol is provided in chapter Endocrine Disruption
Based on these data, 4-tert-pentylphenol meets the World Health Organisation/IPCS definition of an endocrine disruptor:

- *In vitro* data unambiguously show that 4-tert-pentylphenol acts as a ligand of estrogen receptors in fish and mammalian cells. Modulation of 4-tert-pentylphenol-dependent and ER-mediated gene expression was observed on the transcriptional, protein and cell physiological level. Thus based on the available mechanistic (*in vitro*) information it can be concluded that 4-tert-pentylphenol has the potential to exert estrogen-like effects and disrupt endocrine homeostasis. The relative potency of 4-tert-pentylphenol compared to 17ß estradiol in fish ranged from 3.3E-5 to 1E-4 (binding and VTG induction) and was slightly lower for mammalian receptors.
- *In vivo* data substantiate the endocrine mode of action of 4-tert-pentylphenol. Endpoints indicative for an estrogenic mode of action were affected in all fish species tested (6 species). Effects observed included VTG induction, feminization of gonadal ducts and other histological alterations and reduced male secondary sex characteristics.
- A sex ratio biased towards females was observed in 5 fish species (and not assessed in a sixth one). This endpoint is both diagnostic for an estrogenic mode of action and an adverse effect.
- Other observed adverse effects (reduced reproduction, reduced growth) in fish fit to the mode of action. Data show no evidence that the observed adverse effects are caused by systemic toxicity.

According to the OECD Guideline 150 (OECD, 2012b) a substance is almost certain an endocrine disruptor, causing estrogen mediated adverse effects, if the sex ratio is biased towards females and effects observed at other levels (*in vitro*, histological changes) fit to this observation.
As summarized above, for 4-tert-pentylphenol such observations are available for 5 fish species and thus 4-tert-pentylphenol is an endocrine disruptor in fish. The analysis is based on a profound data basis including 8 fish sexual development tests (or comparable) and 6 reproduction assays.
A comparison with other alkylphenols shows that the overall *in vitro* activity of 4-tert-pentylphenol is occurring at similar concentration ranges (general factor 10 difference) as observed for 4-nonylphenol and 4-tert-octylphenol, which are already identified as substances

of very high concern due to their endocrine disrupting properties for the environment.
Compared to 4-nonylphenol and 4-tert-octylphenol, 4-tert-pentylphenol elicits similar *in vivo* effects in *P. promelas*, *D. rerio*, and *O. latipes*. Some effects occurred at similar concentrations while for some endpoints concentrations causing effects were up to factor 100 higher.

6.3.2 Equivalent level of concern assessment

6.3.2.2 Environment

The summary provided above shows that exposure of fish to 4-tert-pentylphenol is likely to result in adverse effects such as female biased sex ratio with significant shifts seen at exposure to around 60 ug/L and complete suppression of males at approx. 220µg/L (F1-generation) or 300µg/L. Also impairment of reproduction up to complete cessation of spawning has been seen in studies with 4-tert-pentylphenol. The effects seen are population relevant as they have the potential to adversely affect population structure and size and consequently ecosystem function and stability.

Results in one full life cycle test with *O. latipes* indicate that exposure to 4-tert-pentlyphenol may result in inter-generation effects. Effects on the sex ratio were more pronounced in the F1 generation and occurred at lower exposure concentrations than in the F0 generation.

Effects observed for *P. promelas* in an extended early life stage test (Panter at al., 2006) indicate the occurrence of long term effects if sensitive life stages are exposed. Inter-sex effects such as testis-ova and change of sex ratio were obsevred at the end of study (day 107) although exposure had already been discontinued at day 60. Apart from this study there are no data for 4-tert-pentylphenol available indicating long-term or delayed effects after short-term exposure. However, respective information can be read across from studies with 4-tert-octylphenol and 4-nonylphenol, which are similar to 4-tert-pentylphenol, as set out in Annex 1.

For 4-nonylphenol and 4-tert-octylphenol several studies show that these substances may cause long lasting effects which persist after cease of exposure:

- Effects observed in several fish species show that transient exposure during sensitive life stages may cause effects that not only remain irreversible during the entire life of the exposed individuals but also in following generations. Thus effects persist after exposure has ceased and exposure of migrating fish might not only adversely affect population stability locally but also in other areas (see SVHC dossiers on 4-nonylphenol and 4-tert-octylphenol, (ECHA, 2012) and (ECHA, 2011) for details).

- Exposure of male fish to 4-nonylphenols results in reduced reproduction even if females are not exposed (see (ECHA, 2012) for details).

- Continuous exposure may result in more pronounced effects in fish not covered in one generation tests (4-nonylphenol, (ECHA, 2012)).

Due to the similar properties of 4-tert-pentylphenol, 4-tert-octylphenol and 4-nonylphenol regarding endocrine disruption for the environment it seems very probable that such effects could also occur after exposure to 4-tert-pentylphenol.

These observations are in line with our knowledge about the endocrine system. 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-time.

In addition, results for 4-nonylphenol and 4-tert-octylphenol indicate that it is difficult to quantify a safe level of exposure with regard to their endocrine activity. And results indicate that other species might be affected too:

- Effects on non-traditional endpoints indicate that effects may start at much lower concentrations than those considered in OECD test guidelines.
- Although it is not possible to clearly state that effects on other organisms such as invertebrates and amphibians are endocrine mediated, these effects fit to the knowledge that steroids are known to play an important role in invertebrates (Kendall et al., 1998). Owing to the lack of in depth knowledge of their endocrine system and the lack of test systems, it is currently nearly impossible to estimate which species are most sensitive and which concentration should be regarded as safe for the environment.

Consequently, for the observations and reasons listed above, the serious effects in the environment that 4-tert-pentylphenol has the potential to cause, are considered to be of an equivalent level of concern to those of other substances listed in points (d) to (e) of Article 57 REACH.

6.3.3 Conclusion on the hazard properties and equivalent level of concern assessment

p-(1,1-dimethylpropyl)phenol (4-tert-pentylphenol) is proposed to be 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 (d) to (e) of Article 57 REACH.

For 4-tert-pentylphenol there is strong evidence from good quality studies that the substance causes endocrine mediated adverse effects in several fish species:

- *In vitro* data unambiguously show that 4-tert-pentylphenol acts as a ligand of fish estrogen receptors. Modulation of 4-tert-pentylphenol-dependent and ER-mediated gene expression was observed on transcriptional, protein and cell physiological levels.
- *In vivo* data substantiate the endocrine mode of action. Endpoints indicative for an estrogenic mode of action were affected in all fish species tested (6 species). Effects observed included VTG induction, feminization of gonadal ducts and other histological alterations and reduced male secondary sex characteristics.
- A sex ratio biased towards females was observed in 5 fish species. This endpoint is both diagnostic for an endocrine mode of action and an adverse effect.
- Other observed adverse effects (reduced reproduction, reduced growth) in fish fit to the mode of action. Data show no evidence that they are caused by systemic toxicity.

The analysis is based on a profound data basis including 8 fish sexual development tests (or comparable) and 6 reproduction assays. Effects observed are similar to those observed for 4-tert-octylphenol and 4-nonylphenol and occur at similar test concentrations (ECHA, 2011) and (ECHA, 2012). Effects observed are regarded as endpoints of particular relevance because they are likely to manifest themselves at the population level.

An analysis of results based on the OECD (Organisation for Economic Co-operation and development) guidance document for endocrine disruptors (OECD, 2012b) reveals that 4-tert-pentylphenol need to be considered as endocrine disruptor. It fulfills the WHO/IPCS definition of an endocrine disruptor and the recommendations from the European Commission's Endocrine Disrupters Expert Advisory Group (JRC, 2013) for a substance to be identified as an endocrine disruptor.

In conclusion, 4-tert-pentylphenol can be considered to be an endocrine disruptor for the environment. This conclusion is supported by read-across from other alkylphenols (4-nonylphenol and 4-tert-octylphenol) with regard to the environment. Data provide indication that 4-tert-pentylphenol may not only cause effects in fish but also in other taxa of

environmental organisms which may be endocrine mediated, also caused by an estrogen-like mode of action.

4-tert-pentylphenol is considered as a substance giving rise to an equivalent level of concern due to its estrogen agonist mode of action and the type of effects caused by this mode of action. Based on data for 4-tert-pentylphenol as well as other estrogen agonists, 4-tert-pentylphenol evidence that the substance is of an equivalent level of concern includes:

- Exposure to 4-tert-pentylphenol resulted in effects in fish on reproduction parameters (fecundity) as well as on sexual development (including changes in sex ratio) and growth. Results for at least 3 fish species show that exposure to 4-tert-pentylphenol may result in complete sex reversal of males resulting in all female populations.
- Effects observed for 4-tert-pentylphenol and the alkylphenols 4-nonylphenol and 4-tert-octylphenol show that transient exposure during sensitive life stages may result in effects that remain during the entire life and even in following generations. Thus local exposure of migratory species might not only locally affect population stability but also in other areas.
- On the basis of the available data for 4-tert-pentylphenol itself and from read-across it appears difficult to derive a safe level. Read-across from 4-tert-octylphenol and 4-nonylphenol with regard to organisms in the environment indicates that
    - Effects on non-traditional endpoints may start at much lower concentrations than those considered in OECD test guidelines.
    - Although it is not possible to clearly state that effects on other organisms such as invertebrates and amphibians are endocrine mediated, these effects fit to the knowledge that steroids are known to play an important role in invertebrates (Kendall et al., 1998). Owing to the lack of in-depth knowledge of their endocrine system and the lack of test systems, it is currently nearly impossible to estimate which species are most sensitive and which concentration should be regarded as safe for the environment.

Thus in summary, the endocrine mediated effects observed in fish after exposure to 4-tert-pentylphenol are considered to have the potential to adversely affect population stability and recruitment. These adverse effects not only persist after cease of exposure but also occur after transient short-term exposure at sensitive live stages. They thus may adversely affect populations in the longer-term and migratory species not only locally but also in regions where no exposure occurred. 4-tert-pentylphenol may affect taxa other than fish (e.g. invertebrates) too. Based on current data and knowledge, a safe level of exposure is difficult to derive although it may exist. Consequently, there is scientific evidence that 4-tert-butylphenol causes probable serious effects in the environment which give rise to an equivalent level of concern to those of other substances listed in points (d) to (e) of Article 57 REACH.

Part II

7 Registration and C&L notification status

7.1 Registration status

Table 29 Registration status

From the ECHA dissemination site[4]	
Registrations	☒ Full registration(s) (Art. 10)

7.1 CLP notification status

Table 30: CLP notifications

	CLP Notifications[5]
Number of aggregated notifications	16
Total number of notifiers	≥ 355

8 Total tonnage of the substance

Table 31: Tonnage status

Total tonnage band for the registered substance (excluding the volume registered under Art 17 or Art 18)[6]	100 - 1000 t/pa

9 Information on uses of the substance

Information on uses of 4-tert-pentylphenol is available from the registration dossiers, a risk evaluation report (RER) from 2008 (Crane et al, 2008), a survey among relevant downstream user associations by a consultant at the beginning of 2015 (Moch et al., 2015) and a consultation of producers and downstream users in summer 2015 (DE PACT 2015).

According to the information provided by the registrant 4-tert-pentylphenol is used at industrial sites for the production of perfumes and fragrances and as a monomer in the production of polymers (phenolic resins). There are no downstream uses of 4-tert-pentylphenol itself or in preparations within the EU. Uses of the polymers are not considered in the latest versions of the registration dossiers as, according to the registrants, there is no obligation to cover downstream uses of polymers in the monomer registration dossier. Uses described in Table 32 include uses of the substance as such (registered uses) as well as uses of the polymers (non-registered uses no longer included in the registration dossiers).

[4] Accessed in July 2016.
[5] Accessed in July 2016.
[6] Accessed in July 2016.

Table 32: Uses

	Use(s)[7]	Registered use *(If not, specify the source of the information)*	Use in the scope of Authorisation
Uses as intermediate	industrial use as intermediate in the production of perfumes and fragrances	Yes	No
Formulation or repacking	- industrial formulation of adhesives - industrial formulation of coatings, printing inks, paints	No	No
Uses at industrial sites	- as monomer in production of polymers (phenolic resins) - end use of adhesives - industrial application of coatings or inks	Yes No no	No Yes/No
Uses by professional workers	- end use of adhesives indoor - end use of adhesives outdoor - application of coatings and inks	No	No
Consumer uses	- end use of adhesives indoor - application of coatings indoor	No	No
Article service life	- all named end use	No	No

The following uses are described in the RER by Crane et al (2008): 4-tert-pentylphenol is used in the phenolic resin industry as an intermediate to produce the phenolic resins resoles and novolac. Resoles are used as part of paints, varnishes and printing inks. Novolac phenolic resins are ethoxylated to form specific surfactants which are used for crude oil separation both offshore and onshore. Residue concentrations are between 1-3% in the surfactant. Ethoxylated phenolic resins are used as oil field demulsifiers and the disulphide derivative of 4-tert-pentylphenol are used as vulcanising agent for rubber curing as indicated in the RER (Crane et al., 2008). This is in line with the information provided in the non-updated registration.

10 Information on structure of the supply chain

A full description of the supply chain is not available. However national consultations revealed that the supply chain is complex and includes several steps of distributors and downstream users. It includes SME at several steps, professional users and in some cases consumers. A wide spread use is assumed.

With regard to resin industry it probably includes:

- Distribution of the substance as such
- Production and distribution of different types of phenolic resins
- Further processing of the phenolic resins (e.g. ethoxylation) and distribution of the products
- Formulation and distribution of coatings, adhesives

[7] Based on a previous version of the registration dossier.

The supply chain is not fully known by registrants and the content of formulations are often not known by downstream users.

11 Additional information

11.1 Substances with similar hazard and use profiles on the Candidate List

The substance has a similar hazard profile compared to 4-tert-octylphenol and 4-nonylphenol which are substances of very high concern due to their endocrine disrupting properties for the environment.

Both substances are also used in resin industry. Downstream uses of the resins may be similar to 4-tert-pentylphenol but this was not assessed in detail.

11.2 Alternatives

A national survey revealed that 4-tert-pentylphenol based resins are used in a variety of specific uses. Alternatives might become available but based on industries answers development and implementation to the market would be both time consuming and expensive.

11.3 Existing EU legislation

No existing other EU legislations applies to the substance.

11.4 Previous assessments by other authorities

The UK Environment Agency released an environmental risk evaluation report for 4-tert-pentylphenol in 2008 (Crane et al., 2008).

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Abbreviations

4-(t-)NP	4-(tert-)Nonylphenol
4-(t-)OP	4-(tert-)Octylphenol
4-t-BP	4-tert-Butylphenol
4-t-PP	4-tert-Pentylphenol
AP	Alkylphenol
CSR	Chemical safety report
DES	Diethylstilbestrol
DHT	Dihydrotestosterone
dPH	days post-hatch
DHI	4',7-Dihydroxiisoflavone
DNA	Desoxyribonucleic acid
DWC	Dilution water control
E2	17ß-estradiol
EC	Effective concentration
EE2	Ethinylestradiol
ELS	Early life stage
ER	Estrogen receptor
ErC	EC in terms of reduction of growth rate
ERC	Environmental Release Category
FLC	Fish full life cycle
FSDT	Fish sexual development teyt
GSI	Gonadosomatic index
hER	Human estrogen receptor
HSI	Hepatosomatic index
IC	Inhibitory concentration
IPCS	International Programme on Chemical Safety
JRC	Joint Research Centre
OECD	Organisation for Economic Cooperation and Development
LC	Lethal concentration
LOEC	Lowest observed effect concentration
NOEC	No observed effect concentration
NOErC	NOEC in terms of reduction of growth rate
PGC	Primordial germ cell
PgR	Progesteron receptor
QSAR	Quantitative structure-activity relationship
RAR	Risk assessment report
RBA	Relative binding affinity
REC	Relative effective concentration
REP	Relative endocrine potency
RPE	Relative proliferative effect
RPP	Relative proliferative potency
SBP	Steroid binding protein
SC	Solvent control
STP	Sewage treatment plant
VTG	Vitellogenin
TG	Test guideline
WHO	World Health Organisation
WWTP	Waste water treatment plant
YES	Yeast estrogen screen

Annex I - Additional information on read across approach

Hypothesis for the analogue approach

To substantiate the findings for 4-tert-pentylphenol, a read across approach is applied using the following source alkylphenols:

- 4-nonylphenol, branched and linear:
- 4-tert-octylphenol (4-(1,1,3,3-tetramethylbutyl)phenol)
- 4-heptylphenol, branched and linear
- 4-tert-butylphenol

The read across is made for hazard identification of the estrogenic mediated endocrine disrupting properties with regard to the environment.

This substances are a group of alkylphenols with a carbon chain substituent in para position at the phenolic part of the molecule. The length of the carbon chain ranges from C4 to C9. The substances can be considered to be of a similar structure – they have an aromatic ring and a sterically unhindered hydroxyl-group (-OH), which is considered relevant for interaction with the estrogen receptors. They differ in chain length and branching of the alkylchain only.

4-Nonylphenol, branched and linear as well as 4-tert-octylphenol are already on the candidate list due to their endocrine disrupting properties for the environment. 4-heptylphenol branched and linear and 4-t-butylphenol are proposed as SVHC due to their endocrine disruption properties for the environment in parallel.

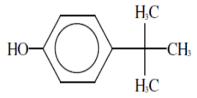
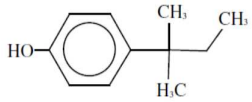
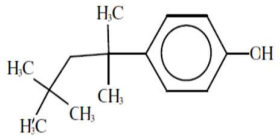
Except 4-tert-butylphenol, the source substances above have a higher alkylchain length than 4-tert-pentyllphenol. Data for source and target chemicals are summarised in order to analyse whether or not this influences the endocrine disrupting properties with regard to the environment.

With regard to physico-chemical properties such as $\log K_{ow}$ and water solubility and bioaccumulation it is anticipated that they follow a linear trend within this group due to increasing lipophilicity with increasing alkylchain length.
With regard to endocrine disruption it is anticipated that all substances of the group activate the estrogen receptor as they all share structural moieties responsible for binding (i. e. a sterically unhindered hydroxygroup attached to an aromatic ring. Binding of the hydroxyl group to the A site of the receptor pocket can be increased through hydrophobic forces in the center of the ER subpocket (OECD, 2009). Thus it could be anticipated that estrogen binding affinity increases with increasing chain length. However, *in vitro* data are not consistent (see below).

Information on endpoints regarding identification, physical and chemical properties, toxiokinetics/bioconcentration in fish and environmental toxicity data (including *in vitro* and *in vivo* data) of 4-tert-butylphenol, 4-tert-pentylphenol, 4-heptylphenol, branched and linear, 4-tert-octyphenol and 4-nonylphenol branched and linear are summarized in the table below. For 4-tert-octylphenol and 4-nonylphenol, only a selection of fish data is provided due to abundancy.

For 4-nonylphenol and 4-tert-octylphenol data are taken from the relevant SVHC dossiers (see ECHA, 2011 and ECHA, 2012) with the exception of nonylphenol toxicokinetics data (also other sources used) and additional nonylphenol data on *Sander lucioperca* from Demska-Zakęś (2005). Only data from studies rated Klimisch 1 or 2 are included in the section for *in vivo* data for endocrine disruption in fish.

Table 33: Summary data on identification, physical and chemical properties, environmental fate/behaviour and environmental toxicity data of 4-tert butyl phenol, 4-tert-pentylphenol, 4-heptylphenol, branched and linear, 4-tert-octyphenol and 4-nonylphenol branched and linear

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear	4-tert-octylphenol	4-nonylphenol
Identity					
Chemical name	4-tert-butylphenol IUPAC4-(1,1-dimethylethyl) phenol	p-(1,1-dimethylpropyl)phenol	4-heptylphenol, branched and linear	4-(1,1,3,3-tetramethylbutyl)phenol, 4-tert-octylphenol	4-nonylphenol, branched and linear: substances with a linear and/or branched alkyl chain with a carbon number of 9 covalently bound in position 4 to phenol, covering also UVCB- and well-defined substances which include any of the individual isomers or a combination thereof
CAS no.	98-54-4	80-46-6	-	140-66-9	-
EC no.	202-679-0	201-280-9	-	205-426-2	-
Chemical structure			UVCB		UVCB
SMILES	CC(C)(C)c1ccc(O)cc1	CCC(C)(C)c1ccc(O)cc1	UVCB-substance	Oc(ccc(c1)C(CC(C)(C)C)(C)C)c1	Covers UVCB as well as well-defined substances
Molecular formula	$C_{10}H_{14}O$	$C_{11}H_{16}O$	$C_{13}H_{20}O$ (mono-subst.) $C_{20}H_{34}O$ (di-subst.)	$C_{14}H_{22}O$	$C_{15}H_{24}O$
Molecular weight (g/mol)	150.2176	164.244	192.3 (mono-subst.) 290.5 (di-subst.)	206.32	220.35

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear	4-tert-octylphenol	4-nonylphenol
PHYSICO-CHEMICAL PROPERTIES					
Physical state at 20°C and 101.3 kPa	Solid (flakes)	Solid (flakes)	liquid at 20°C and 101.3 kPa	Solid	pale yellow viscous liquid
Water solubility (mg/L, 20 °C)	610 mg/L at 25 °C, pH = 6 – 7	190 mg/L at 21 °C, pH 6 - 7	42.1 mg/L at 20 °C	19 mg/L at 22 °C	Ca. 5.7 mg/L at 25°C
Partition coefficient n-octanol/water (log Kow)	3.0 at 23 °C, pH = 5.7	3.6 at 22 °C, pH 6 – 7	4.5 at 20 °C	4.12 at 20.5°C (OECD 107, shake flask method) 3.7, temperature not indicated	5.4 at 23°C, pH 5.7
Dissociation constant (pKa)	10.13 - 10.23 at 25 °C	10.4 (Crane et al., 2008)	---	pKa 10.33 at 25 °C (calculated)	pK ca. 10
***IN VITRO* DATA FOR ESTROGEN RECEPTOR MEDIATED PATHWAY**					
Binding to Estrogen Receptors					
Rainbow trout	Hornung et al. 2014: RBA = 1.4 x10^{-5}	Hornung et al. 2014: RBA = 4 x10^{-5} RBA for 4-n-Pentylphenol = 5.3 x10^{-5}	Hornung et al. 2014: RBA for 4-n-Heptylphenol 2.1 x10^{-4} RBA for 4-tert-Heptylphenol: 1.4 x10^{-4}	Hornung et al. 2014: RBA =9.4 x10^{-5}	Hornung et al. 2014: 5 different isomers tested (1 linear, 4 branched): RBA ranges from 1.6 x 10^{-4} to 4.6 x 10^{-4}
	Tollefsen and Nilsen 2008: RBA = 4 x 10^{-5}	Tollefsen and Nilsen 2008: RBA = 7 x 10^{-5}	Tollefsen and Nilsen 2008: RBA = 3.2 x 10^{-5}	Tollefsen and Nilsen 2008: RBA = 6.9 x 10^{-5}	Tollefsen and Nilsen 2008: RBA = 1 x 10^{-5}
	Olsen et al 2005: RBA =7.7 x 10^{-5}			Olsen et al. 2005: RBA = 7.6 x 10^{-5}	
			Knudsen and Pottinger, 1999: Concentrations of alkylphenols including heptylphenol 10^4-fold > those of the	Knudsen and Pottinger, 1999: Concentrations of alkylphenols including octylphenol 10^4-fold > those of the maxium displacement achieved	Knudsen and Pottinger, 1999: Concentrations of alkylphenols including nonylphenol 10^4-fold > those of the

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear	4-tert-octylphenol	4-nonylphenol
			maxium displacement achieved was E2 required to produce similar amounts of displacement of specifically bound - [^{3}H]E2 Maximum displacement achieved: ca. 60%	was E_2 required to produce similar amounts of displacement of specifically bound [^{3}H]E2 Maximum displacement achieved: ca. 45%	maxium displacement achieved was E2 required to produce similar amounts of displacement of specifically bound - [^{3}H]E2 Maximum displacement achieved: ca. 50%
Human			Satoh and Nagai, 2002: ERα-RBA =0.00163; ERß no binding	Satoh and Nagai, 2002: ERα-RBA =0.008; ERß-RBA =0.00708;	Satoh and Nagai, 2002: ERα-RBA = 0.0222; ERß-RBA = 0.0213
	Akahori et al., 2005: RBA = 2.3×10^{-5}	Akahori et al., 2005: RBA = 1.7×10^{-4}	Akahori et al., 2005: RBA = 8.5×10^{-6}	Akahori et al., 2005: RBA = 0.00123	
	Olsen et al, 2005: RBA 2.1 x 10-^{6}			Olsen et al, 2005: RBA 6.4 x 10-^{5}	
Rat	Blairs et al, 2000: RBA = 2.4×10^{-6}	Blairs et al, 2000: RBA = 5×10^{-6}	Laws et al., 2006: RBA = 1.24×10^{-5}	Blairs et al, 2000: RBA 1.4×10^{-4}	Blairs et al, 2000: RBA = 3.7 – 1.9 x 10^{-4} 4-n-Nonylphenol RBA = 3.2×10^{-5}
Binding to sex steroid-binding protein					
Rainbow trout	Tollefsen, 2007: RBA = 6.1×10^{-6}	Tollefsen, 2007: RBA = 4.3×10^{-5}	Tollefsen, 2007: RBA = 6.6×10^{-6}	Tollefsen, 2007: RBA = 1.3×10^{-5}	Tollefsen, 2007: 4-n-Nonylohenol was here only a weak binder
Expression of vitellogenin					
Rainbow trout	Tollefsen et al, 2008: LOEC = 3 µM	Tollefsen et al, 2008: LOEC 3 µM	Tollefsen et al, 2008: no effect under condition employed	Tollefsen et al, 2008: LOEC = 1 µM	Tollefsen et al, 2008: LOEC = 30 µM
	Jobling and Sumpter, 1993: REP 1.6×10^{-4}			Jobling and Sumpter, 1993: REP 3.7×10^{-5}	

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear	4-tert-octylphenol	4-nonylphenol
				Olsen et al, 2005: REP 3.2 x 10^{-5}	
	Olsen et al, 2005: REP 5.6 x 10^{-6}			Olsen et al, 2005: REP 3.2 x 10^{-5}	
Expression profiling of estrogen-responsive genes					
Human			Terasaka et al., 2006: R-value (statistical correlation) for EstrArray: 0.82	Terasaka et al., 2006: R-value (statistical correlation) for EstrArray: 0.75	Terasaka et al., 2006: R-value (statistical correlation) for EstrArray: 0.9
Transcriptional activation assay using recombinant yeast (yeast estrogen screen, YES)					
Human	Routledge and Sumpter,1997: REP = 1.5 E^{-6}	Routledge and Sumpter,1997: REP = $1E^{-5}$	Routledge and Sumpter,1997: 4-*tert*-heptylphenols: REP = $3E^{-3}$ 4-n-heptylphenol: REP = $7.5E^{-4}$ = 25-fold less potent than 4-*tert*-heptylphenol	Routledge and Sumpter,1997: REP = $1E^{-3}$	Routledge and Sumpter,1997: REP = $3E^{-4}$
		Schultz et al , 2000: EC_{50} = 4.67 µM			Schultz et al , 2000: EC_{50} = 0.177 µM
	Nishihara et al., 2000: REC10 3 x10^{-5}		Nishihara et al., 2000: negative	Nishihara et al., 2000: REC10 2 x10^{-7}(= positive)	Nishihara et al., 2000: negative for 4-n-Nonylphenol
MCF cell proliferation assays (E-Screen)					
Human	Soto et al, 1995: RPE = 0.71 RPP = 3 x 10^{-6}	Soto et al, 1995: RPE = 1.05 RPP = 3 x 10^{-6}			Soto et al, 1995: RPE = 1 RPP = 3 x 10^{-5}
	Körner et al, 1998: RPE= 0.78			Körner et al, 1998: RPE=0.97	Körner et al, 1998: RPE= 1.05
TOXICOKINETICS AND BIOACCUMULATION IN FISH					

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear	4-tert-octylphenol	4-nonylphenol
Toxico-kinetics in fish Absorption	Rapid uptake via seawater (co-exposure) in Atlantic cod, steady state reached within 24 h (or 48 h exposure via spiked feed) (Sundt et al, 2009).	Rapid uptake of 4-n-pentylphenol via seawater (co-exposure) in Atlantic cod, steady state reached within 24 h (or 48 h exposure via spiked feed) (Sundt et al, 2009).	Rapid uptake via seawater or spiked feed (co-exposure) in Atlantic cod, steady state reached within 48 h. Higher body burden compared to 4-tert-butylphenol and 4-n-pentylphenol (related to higher logK_{ow} value) (Sundt et al, 2009).	Steady state conditions in the whole fish (*Oncorhynchus mykiss*) were reached after 4 days in a flow-through system (ECHA, 2011).	Steady state reached within 12 h in rainbow trout (Lewis and Lech, 1996)
Distribution	Highest residues after 8 day co-exposure in bile and to a lesser extent in the intestine, intestine/stomach content (12-14% residues of spiked administered feed were recovered in tissue) (Sundt et al, 2009)	Highest residues after 8 day co-exposure in bile and to a lesser extent in the intestine, intestine/stomach content. (8% residues of spiked administered feed were recovered in tissue) (Sundt et al, 2009).	Highest residues after 8 day co-exposure in bile and to a lesser extent in the intestine, intestine/stomach content (12-14% residues of spiked administered feed were recovered in tissue) (Sundt et al, 2009).	In rainbow trout highest residues after 10 days waterborne exposure in bile, followed by feces, pyloric caeca, liver and intestine, in rudd highest concentrations were in bile and liver (cited in Cravedi and Zalko, 2005). 8% 4-*tert*-octylphenol residues in liver and muscle tissue after 10 day exposure in flounder (Madsen et al. 2003).	[^{14}C]NP residues were highest in bile after 14 h waterborne exposure in rainbow trout. [^{3}H]4-n-NP residues in Atlantic salmon showed wide tissue distribution with high levels in bile, viscera, liver, fat and kidney (cited in Cravedi and Zalko, 2005).
Metabolism	Predominant metabolic pathway: conjugation to glucuronic acid. (Jonsson et al, 2008)	Predominant metabolic pathway: conjugation to glucuronic acid. (Jonsson et al, 2008)	Predominant metabolic pathway: conjugation to glucuronic acid. (Jonsson et al, 2008)	Predominant metabolic pathway: conjugation to glucuronic acid (Cravedi and Zalko, 2005).	Predominant metabolic pathway: conjugation to glucuronic acid (Cravedi and Zalko, 2005).
Elimination	Half-life 10 hours, rapid excretion via bile and feces (Sundt et al. 2009).	Half-life 10 – 20 hours, rapid excretion via bile and feces (Sundt et al. 2009).	Half-life 13 hours (Atlantic cod) (Tollefsen et al. 1998)	Excretion via bile and feces. Half-live 7.7 h in medaka (Cravedi and Zalko, 2005).	Half-live of 18.6 and 19.6 h in rainbow trout in muscle and

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear	4-tert-octylphenol	4-nonylphenol
			Half-life 10 – 20 hours (Atlantic cod), rapid excretion via bile and feces (Sundt et al. 2009)		fat (Lewis and Lech, 1996) Excretion via bile and feces. Half-life 9.9 h in medaka, but higher half-lives after i. v. in Atlantic salmon (clearance half-live 4 days) (Cravedi and Zalko, 2005).
Bio-concentration factor (BCF)	125 (calculated based on TGD method) *C. carpio*: 48 -88 *Chlorella. fusca* 34 measured *Lecicus idus*: 120	No experimental data, fish BCF of 501 L/kg may be estimated from the logK_{ow} (4.0) using the QSAR recommended in the TGD11	The bioaccumulation cannot be fully excluded, as the study is not state-of the art, but based on the BCF values < 2000 and the elimination half-live of 0.052 / hour (Tollefsen et. al, 1998) the bioaccumulation potential is moderate to low.	The bioaccumulation potential in aquatic organisms is low to moderate. The experimentally determined BCF ranges between 12 and 471	No data in SVHC dossier
ACUTE AQUATIC TOXICITY [mg/L]					
Acute toxicity to fish:	96h-LC_{50}: 5.14 mg/L (meas.)	96h-LC_{50}: 1 (nom)	Phenol, heptyl derivs. 96h-LC_{50}: 2.4 (nom.) 96h-LC_{50}: 0.41 (meas.) 96h-LC_0: 1.8 (nom.) 96h-LC_0: 0.066 (meas.) O.mykiss 4-n-heptylphenol 96h-LC_{50}: 0.56 (nom.) Gadus morhua	LC_{50}: 0.17	LC_{50}: 0.135 mg/L
Acute toxicity to invertebrates	96-LC_{50}: 1.9 (meas.)	96h-EC_{50}: 1.7 (meas.)	Phenol, heptyl derivs. 48h-EC_{50}: 0.38 (meas.)	EC_{50}: 0.013	EC_{50}: 0.085

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear	4-tert-octylphenol	4-nonylphenol
Acute toxicity to algae	72h-ErC_{50}: 14 (nom.)	72h-EC_{50}: 4.2 (nom.)	Phenol, heptyl derivs. 72h-ErC_{50}: 1.2 (nom.)	EC_{50}: 0.300	ErC_{50}: 0.027
ENDOCRINE EFFECTS IN FISH (NOECs/LOECs in mg/L if not stated otherwise)					
Sander lucioperca					
Effects on sex ratio (histological)					
Decrease of male fish	28d-NOEC: <0.001(nom.) 28d-LOEC: 0.001 (nom.) Demska-Zakęś (2005)		28d-NOEC: <0.001(nom.) 28d-LOEC: 0.001 (nom.) Demska-Zakęś (2005)		28d-NOEC: <0.001(nom.) 28d-LOEC: 0.001 (nom.) Demska-Zakęś (2005)
Increase of female fish	28d-NOEC: <0.001(nom.) 28d-LOEC: 0.001 (nom.) Demska-Zakęś (2005)		28d-NOEC: 0.001 (nom.) 28d-LOEC: 0.010 (nom.) Demska-Zakęś (2005)		28d-NOEC: 0.001 (nom.) 28d-LOEC: 0.010 (nom.) Demska-Zakęś (2005)
Intersex (histological)	28d-NOEC: <0.001(nom.) 28d-LOEC: 0.001 (nom.) Demska-Zakęś (2005)		28d-NOEC: <0.001 (nom.) 28d-LOEC: 0.001 (nom.) Demska-Zakęś (2005)		28d-NOEC: <0.001 (nom.) 28d-LOEC: 0.001 (nom.) Demska-Zakęś (2005)
Chronic toxicity to fish Mortality/length/weight/condition factor	28d-NOEC: >0.2 (nom.) 28d-LOEC: >0.2 (nom.) *S. lucioperca* (Demska-Zakęś, 2005)		28d-NOEC: >0.2 (nom.) 28d-LOEC: >0.2 (nom.) *S. lucioperca* (Demska-Zakęś, 2005)		28d-NOEC: >0.2 (nom.) 28d-LOEC: >0.2 (nom.) *S. lucioperca* (Demska-Zakęś, 2005)
Pimephales promelas					
FSDT or comparable tests	0.225 VTG (increase females) 0.225 feminisation gonadal ducts, higher proportion immature	0.18 VTG (increase females) (Panter et al, 2006) 0.093 VTG (decrease females) (OECD,			

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear	4-tert-octylphenol	4-nonylphenol
	testis stages 0.5 sex ratio (increase females)[8] 0.027 SSC 0.027 growth (m + f)´ 0.255 time to hatch, survival post hatch (Krueger et al, 2008)	2011a) 0.056 feminisation gonadal duct (Panter et al, 2006) 0.180 testis ova (Panter et al, 2006) 0.093 - 0.195 sex ratio (increase females/decrease males) (OECD, 2011a, Panter et al, 2006) 0.599 SSC (no statistics) (Panter et al, 2006) 0.599 growth, time to hatch (Panter et al, 2006) > 0.320 mortality (OECD, 2011a)			
Reproduction assay or comparable		0.270 - 0.560 VTG (increase males) (OECD, 2006, Panter et al, 2010 0.820 - 0.962 higher proportion immature testis stages (OECD, 2006) 0.270 – 0.997 SCC (OECD, 2006) 0.056 Fertility (Panter et al, 2010) (no spawning at 1 mg/L) (OECD, 2006)>			0.071 fecundity 0.00025 behaviour 0.015 VTG 0.071 secondary sexual characteristics

[8] From pilot study

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear	4-tert-octylphenol	4-nonylphenol
		0.560 survival, hatchability (Panter et al, 2010)			
Danio rerio					
FSDT or comparable tests		> 0.096 – 0.100 VTG increase males (OECD, 2011a) 0.062 – 0.100 sex ratio (increase females/decrease males) (OECD, 2011a)			0.01 skewed sex ratio 0.1 Gametogenesis females 0.01 Gametogenesis males 0.03 testis-ova 0.1 Ovarian follicle atresia 0.1 VTG
Reproduction Assay		0.022 VTG (increase males) 0.229 higher proportion immature testis stages 0.787 testis-ova 0.721 - > 787 Fertility All (OECD, 2011a)			
FLC				0.035 fertility, time to first spawn, body length	
Oryzias latipes					
FSDT		0.094 – 0.104 VTG (OECD, 2012a) 0.094 testis-ova (OECD, 2012a) 0.010 - 0.318 sex ratio [(less males) Hagino et al, 2001, OECD, 2012a) 0.100 SCC (Hagino et al, 2001) > 0.317 hatch, survival		0.011 VTG 0.023 testis-ova 0.0481 sex ratio	0.0012 VTG and testis-ova 0.024 sex ratio
Reproduction Assay				0.02 VTG $\leq$ 0.02 fertility	0.005 (VTG) 0.184 Inhibition of spermatogenesis 0.0061 fecundity and fertility

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear	4-tert-octylphenol	4-nonylphenol
FLC		0.051 VTG 0.224 testis ova, 0.224 sex ratio 0.224 Fertility 0.224 SSC 0.224 length F1 0.931 growth, mortality (Seki et al, 2003)		0.0099 VTG 0.03 testis-ova ≤ 0.01 fertility	0.0018 testis- ova 0.052 sex ratio based on gonadal histology in F0 0.018 sex ratio based on gonadal histology in F1
Cyprinus carpio					
Reproduktion Assay	0.690 VTG (up males) 0.690 GSI, HIS, liver degeneration (Barse et al, 2006)	1.00 VTG > 1.00 weight (Gimeno_et al_1998b)			
other		0.036 feminisation gonadal ducts (Gimeno et al, 1998a)0.090 – 1.00 testis-ova (Gimeno et al, 1998a, Gimeno et al 1996) 0.140 - > 256 growth (Gimeno et al, 1997, Gimeno et al, 1998a			
Oncorhynchus mykiss					
FSDT					0.00105 VTG 0.01 Growth
Reproduction Assay & other				0.039 VTG ≤ 0.039 increased percentage of early sperm stages (spermatogonia), reduced GSI in initial experiment	0.01 VTG 0.001 VTG (F1 without exposure) 0.037 Inhibition of spermatogenesis 0.086 non developed ovaries 0.01 sexual steroids in F1

Analogue approach justification

Overall data collected in Table 33 justify the analogue approach.

Physico-chemical data:
The substances in this group (4-tert-butylphenol, 4-tert-pentylphenol, 4-heptylphenol, branched and linear*, 4-tert-octylphenol* and 4-nonylphenol, branched and linear) do have similar physical chemical properties or have expected trends due to the differing molecular weight and the growing length of carbon chain (e.g. regarding water solubility).
With growing molecular weight the partition coefficient $logK_{ow}$ (3.0 for 4-tert-butylphenol and 5.4 for 4-nonylphenol) is rising. Water solubility is declining with the molecular weight from 607.2 mg/L for 4-*tert*-butylphenol to ca. 5.7 mg/L for 4-nonylphenol.

Mechanistic *in vitro* data:
Numerous *in vitro* data show that all members of this group of substances exhibits interaction with estrogen receptors and act as estrogen agonists. Data with regard to fish (receptor binding, binding to sex steroid binding protein, VTG expression) show no linear trend with increasing chain length. With regard to human receptors, data are ambiguous with some showing a linear trend and others not.

Binding to rainbow trout estrogen receptors was shown in several studies for all 5 alkylphenols in a very similar range (1.4×10^{-4} - 7.7×10^{-5}) : 1.4×10^{-5} to 7.7×10^{-5} for 4-tert-butylphenol, 4×10^{-5} – 7×10^{-5} for 4-tert-pentylphenol, 1.4×10^{-4} to 3.2×10^{-5} for 4-n-heptylphenol, 6.9×10^{-5} to 9.4×10^{-5} for 4-tert-octylphenol and 4.6×10^{-4} to 1 for 4-nonylphenol. No linear trend with increasing chain length was observable. This becomes even more obvious if data for different alkylphenols obtained in the same study are compared: Values for all five substances are available from Hornung et al. (2014) and Tollefsen and Nilsen (2008): In the Tollefsen and Nilsen (2008) study the values are very similar ranging from 1×10^{-5} to 7×10^{-5}. In Hornung et al. (2014) the values vary from 1.4×10^{-5} to 4.6×10^{-4}. In both tests no correlation with the length of the alkylchain was observable.

Binding to human and rat estrogen receptors was seen for all alkylphenols. For the human estrogen receptors varying results are obtained from different studies. Some indicate a linear trend while others do not: Satoh and Nagai, 2002 report rather high values for 4-nonylphenol (0.0213-0.222), 4-*tert*-octylphenol (0.00708 to 0.008) and 4-n-heptylphenol (0.00163). Generally lower values are reported by Olsen et al, 2005: 6.4×10^{-6} and 2.1×10^{-6} for 4-ter-octylphenol and 4-*tert*-butylphenol. Akahori et al. reported a high value for 4-tert-octylphenol (0.00123), a "medium" value for 4-tert-pentylphenol (1.7×10^{-4}) and rather low values for 4-n-heptylphenol and 4-tert-butylphenol (8.5×10^{-6} and 2.3×10^{-5}). With regard to rat estrogen receptors the study by Blair et al (2000) tested all alkylphenols of this group. Results indicate that all bind to the receptor but affinity increases with increasing chain length by two orders of magnitudes. There, is also a study available evaluating the binding affinity to sex steroid-binding protein of rainbow trout: Here binding affinity was observed for all alkylphenols in a very similar range (2.4×10^{-6} - 4.3×10^{-5} (no linear trend). 4-n-Nonylphenol was only a weak binder in this assay.

In test systems examining the expression of vitellogenin (rainbow trout) all alkylphenols but 4-n-heptylphenol provided positive results. No trend was observable and binding affinity of the different alkylphenols was in a very narrow range (e.g. LOEC 1 – 30 µM observed by Tollefsen et al, 2008).

Regarding expression profiling of estrogen-responsive genes (human) data are available for the longer chain alkylphenols (heptyl to nonyl): all three tested substances showed high correlation coefficients to the profiles of E2: The R-value for 4-n-heptylphenol is 0.82, which is in the range as 4-*tert*-octylphenol (R-value = 0.75) and 4-nonylphenol (R-value =0.90). In a transcriptional

activation assays positive results were obtained for all alkylphenols, though not in every assay. While some results indicate a linear trend others don't.
Two E-Screen assays (MCF cell proliferation assays) are available comparing 4 of the 5 alkylphenols. While relative proliferative effects (RPE) were similar for 4-tert-pentylphenol, 4-tert-octylphenol and 4-nonylphenol (0.97 – 1.05 with no specific trend), RPE values for 4-tert-butylphenol were slightly lower (0.71- 0.78); (Soto et al., 1995) and Körner et al. (1998).

Toxicokinetic data in fish
Uptake and tissue distribution of 4-*tert*-butylphenol, 4-n-pentylphenol, and 4-n-heptylphenol in Atlantic cod (*Gadus morhua*) followed a similar pattern: uptake was rapid via seawater. For exposure via feed, the time to reach steady state was similar for 4-t-butylphenol, 4-n-pentylphenol and 4-n-heptylphenol. Slightly higher body burdens were found for 4-n-heptylphenol compared to 4-tert-butylphenol and 4-n-pentylphenol. This correlates well with the increasing $\log K_{ow}$ value within the alkylphenol group.

Distribution in Atlantic cod of 4-*tert*-butylphenol, 4-n-pentylphenol, and 4-n-heptylphenol residues was also similar irrespective whether fish were exposed via seawater or feed. Highest alkylphenol residue concentrations after 8 day co-exposure were detected in bile and to a lesser extent in the intestine, intestine content and stomach content (Sundt et al., 2009). Also, for 4-tert-octylphenol and nonylphenols highest residues were detected in bile.

The predominant metabolic pathway for alkylphenols is the conjugation of the phenol group to glucuronic acid. Alkyphenols were mainly excreted via bile and faeces with similar half-lives that range from 10 to 20 hours (for water or feed exposure).

Acute aquatic toxicity
Acute fish toxicity data show also that all five alkylphenols have very similar values: The range of the lowest acute toxicity values for each substance for fish was 0.135 to 5.14 mg/L.

For acute algae and acute aquatic invertebrate data, there seem to be tendencies of higher toxicity with a higher chain length. For aquatic invertebrates the acute toxicity values range from 0.013 to 1.9 mg/L (invertebrate data like sea urchin which are available for 4-nonylphenol are not included here), for algae the acute toxicity values range is 0.027 (4-nonylphenol) – 4 mg/L.

Endocrine disrupting properties in fish
Alkylphenols in this group all exert similar endocrine disrupting effects. A number of indicative as well as adverse effects were seen in several fish species: Female biased sex ratio was observed for all alkylphenols, which is indicative for an endocrine mode of action and is an adverse effect. Moreover, several indicative effects like feminisation of gonadal ducts, ovo-testes and effects on secondary sex characteristics were demonstrated. Effect concentrations for all alkylphenols are in a similar range or in most cases not differing in more than factor 10 based on comparable studies with regard to the most relevant adverse endpoints.

In the study from Demska-Zakęś, 2005, using *Sander lucioperca* 3 of the 5 alkylphenols were tested (4-*tert*-butylphenol, 4-n-heptylphenol and 4-n-nonylphenol): They all show a sex ratio biased towards females in very similar test concentrations. The LOEC for a decrease of male fish (histologically determined) was 0.001 mg/L for all three substances – no NOEC could be established. The LOEC for the increase of female fish (histologically determined) was slightly different due to very small but statistical significant divergences: after 28 days of exposure the LOEC for 4-tert-butylphenol was 0.001 mg/L and for 4-n-heptylphenol and 4-n-nonylphenol 0.01 mg/L, resulting in NOECs of 0.001 mg/L for 4-n-heptylphenol and 4-n-nonylphenol. But after subsequent 56 days of rearing without exposure to the test substances the LOEC and NOEC for all three substances were the same (0.01 mg/L and 0.001 mg/L, respectively). The LOEC for Intersex (also histologically determined) was again 0.001 mg/L for all three substances with no established NOECs. No effects were seen on mortality, length, weight or condition factor at any concentration tested (highest concentration tested 0.2 mg/L) for all three substances.

Furthermore, effects are seen for 4-tert-butylphenol, 4-tert-pentylphenol and 4-nonylphenol in *Pimephales promelas* in several studies. Different endpoints are available. For vitellogenin induction the effect values range from 0.015 to 0.56 mg/L (LOEC), secondary sex characteristics vary from 0.027mg/L for 4-tert-butylphenol to 0.071 mg/L in 4-nonylphenol and 0.599 mg/L in 4-tert-pentylphenol. Effects on sex ratio were observed at 0.5 mg/L for 4-tert-butylphenol in a pilot study of one FSDT. For 4-tert-pentylphenol effects on sex ratio were observed in the range 0.093 to 0.195mg/L.
Very low values were also found for fecundity and behaviour (0.071 mg/L and 0.00025 mg/L respectively; values only available for 4-nonylphenol).

For *Danio rerio* data are available for 4-tert-pentylphenol, 4-tert-octylphenol and 4-nonylphenol: Values for vitellogenin induction vary from 0.022 to 0.1 mg/L, for testis-ova from 0.03 to 0.787mg/L (4-tert-pentylphenol and 4-nonylphenol). Fertility was affected for 4-tert-pentylphenol and 4-tert-octylphenol. Effects on sex ratio are available for 4-tert-pentylphenol and 4-nonylphenol with values between 0.062 and 0.1 mg/L for 4-tert-pentylphenol and 0.01 for 4-nonylphenol. For 4-nonylphenol also effects on gametogenesis and ovarian follicle atresia were observed.

For *Oryzias latipes* there are also data for 4-tert-pentylphenol, 4-tert-octylphenol and 4-nonylphenol available: Values for vitellogenin induction vary from 0.0012 to 0.104 mg/L, for testis-ova from 0.0012 to 0.225mg/L and effects on sex ratio from 0.01 to 0.318 mg/L for the three substances.

For *Cyprinus carpio* data are available for 4-tert-butylphenol and 4-tert-pentylphenol: Similar values were gained for vitellogenin induction 0.69 mg/L for 4-tert-butylphenol and 1 mg/L for 4-*tert*-pentylphenol. Moreover, additional data are available for 4-tert-pentylphenol showing effects at 0.036 for feminisation of gonadal ducts and 0.09 -1 mg/L for testis-ova.

For *Oncorhynchus mykiss* data are available for 4-tert-octylphenol and 4-nonylphenol: Values for vitellogenin induction vary from 0.001 to 0.039 mg/L. For 4-nonylphenol data on unexposed F1 generation are also available: 0.001 for vitellogenin induction and 0.01 for sexual steroids seen in F1 generation. Effects on sperm stages and spermatogenesis were observed at $\leq$0.039 to 0.37 mg/L for the two substances.

Conclusion on read across for environmental hazard assessment
In vitro data as well as *in vivo* data show that a read-across for the target chemical 4-tert-pentylphenol from alkylphenols with longer chain length is justified with regard to identification of endocrine disrupting properties for the environment:

- All *in vitro* data for fish estrogen receptors unambiguously show estrogen receptor binding without systematic differences in binding affinity among the group. Activation was seen in most studies. All *in vitro* data for rat and human estrogen receptors unambiguously show estrogen receptor binding and activation. Some of the tests indicate a correlation of the binding affinity with the length of the chain length but differences were low (maximum two orders of magnitude) and others did not find such pattern. Thus, data obtained for other alkylphenols substantiate the data found for 4-tert-pentylphenol and can be used to substantiate the effects observed for 4-tert-pentylphenol in a weight of evidence approach.
- Only few data from comparable test with fish for more than one alkylphenol are available. However an analysis of all available data show that all alkylphenols show very common effects (histological changes, changes in sex ratio and secondary sex characteristics, VTG induction) which fit to the anticipated mode of action. Test concentrations differ among tests, but no systematic pattern was observable.

Thus the data on the structurally related alkylphenols support the findings for 4-tert-pentylphenol and can be used to substantiate the conclusions made for 4-tert-pentyllphenol in a weight of evidence approach.

It can be concluded that although the carbon chains of these 5 alkylphenols differ, endocrine disrupting properties for the environment are induced by all the four source substances as well as the target substance 4-tert-pentylphenol. Data are available for the *in vitro* endocrine mode of action (from fish, rats, humans) as well as for *in vivo* endocrine effects in several fish species (*Sander lucioperca*, *Pimephales promelas*, *Danio rerio*, *Oryzias latipes*, *Cyprinus carpio*, *Oncorhynchus mykiss*). These include effect data such as a female biased sex ratio (shown for all 5 alkylphenols), which is considered to be adverse as well as indicative for an estrogen mode of action. Also numerous other effects were seen in different studies with different fish species regarding indicative effects such as feminisation of gonadal ducts, testis-ova and changes in secondary sex characteristics.

Annex II – Short-term toxicity to fish

Table 34: Summary of short-term toxicity to fish

Test method ***Species***	**Results**	**Reliability acc. to Klimisch**	**Reference**
EPA-660/3-75-009 *Pimephales promelas*	96h-LC_{50} = 2.5 mg/L (real)	2 key study	(Holcombe et al., 1984)
Cyprinus carpio	96h-LC_{50} = 1.6 mg/L (real)	2 – compared to OECD 203: 5 instead of 7 animals; 25 °C instead of 20 °C water temperature Supporting study	(Gimeno et al., 1998a)
Oncorhynchus mykiss	96h-LC_{50} > 1 mg/L (real)	3 – Very few information concerning test conditions; no analytics; vehicle used	(TL, 1992)
OECD 203 *Oncorhynchus mykiss*	96h-LC_{50} = 1.0 mg/L	2 – vehicle used; no analytical monitoring; test substance: sodium 4-(1,1-dimethylpropyl) phenolate (CAS 31366-95-7)	(Davoren and Fogarty, 2005)
Oryzias latipes	96h-LC_{50} = 2.6 mg/L	2 – no replicates but comparable with other values	(Hagino et al., 2001)
OECD 203 *Pimephales promelas*	96h-LC_{50} = 2.59 mg/L	4 (1 acc. to registrant)	(Broderius et al., 1995)