Substance Name: 4-tert-butylphenol

EC Number: 202-679-0

CAS Number: 98-54-4

SUPPORT DOCUMENT TO THE OPINION

OF THE MEMBER STATE COMMITTEE

ON IDENTIFICATION OF

4-TERT-BUTYLPHENOL

AS A SUBSTANCE OF VERY HIGH CONCERN BECAUSE OF ITS ENDOCRINE DISRUPTING PROPERTIES WHICH CAUSE PROBABLE SERIOUS EFFECTS TO THE ENVIRONMENT WHICH GIVE RISE TO AN EQUIVALENT LEVEL OF CONCERN TO THOSE OF CMR[1]

AND PBT/VPVB[2] SUBSTANCES

Adopted on 15 December 2016

[1] CMR means carcinogenic, mutagenic or toxic for reproduction

[2] PBT means persistent, bioaccumulative and toxic; vPvB means very persistent and very bioaccumulative

CONTENTS

TABLES

FIGURES

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

Substance Name: 4-tert-butylphenol (PTBP)

EC Number: 202-679-0

CAS number: 98-54-4

- The substance should be identified as a substance of very high concern according to Article 57(f) of REACH Regulation 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 (a) to (e) of Article 57 of REACH.

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

4-tert-butylphenol should 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 (a) to (e) of Article 57 of REACH.

For 4-tert-butylphenol there is scientific 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-butylphenol acts as a ligand of estrogen receptor in fish and mammalian cells. Modulation of 4-tert-butylphenol-dependent and ER-mediated gene expression was observed on transcriptional, protein and cell physiological levels showing that 4-tert-butylphenol activates fish and mammal estrogen receptors.
- *In vivo* data substantiate the endocrine mode of action. Endpoints indicative for an estrogenic mode of action were affected in all fish species tested (3 species). Effects observed included Vitellogenin (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 one 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 are plausibly due to an estrogenic mode of action. But other modes of action cannot be entirely ruled out.

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 Guidance Document for endocrine disruptors (OECD, 2012) reveals that 4-tert-butylphenol needs 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-butylphenol can be considered to be an endocrine disruptor in the environment. This conclusion is supported by read-across from other alkylphenols (4-

nonylphenol and 4-tert-octylphenol) with regard to the environment.

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

- Exposure to 4-tert-butylphenol resulted in effects in fish on reproduction parameters (fecundity) as well as on sexual development (changes in sex ratio) and growth. Results for one fish species show that exposure to 4-tert-butylphenol may result in a complete sex reversal resulting in all female populations. This effect is considered a serious effect to the environment.
- Read-across of the effects observed for the structurally similar 4-nonylphenol and 4-tert-octylphenol in fish show that exposure during sensitive life stages may result in effects that remain during the entire life and even in following generations and even after exposure ceased. Thus local exposure of migratory species might not only affect population stability locally but also in other areas.
- On the basis of the available data for 4-tert-butylphenol itself and from read-across it appears difficult to derive a safe level although it may exist. 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.
    - It is not possible to clearly conclude that effects on other organisms such as invertebrates and amphibians are endocrine mediated, although steroids are known to play an important role in invertebrates (Kendall et al., 1998) and amphibians (Kortenkamp et al., 2012). Owing to the lack of in-depth knowledge of their endocrine system and the lack of test systems, it is currently difficult to estimate which species may be more sensitive than fish and which concentration can be regarded as safe for the environment.

Thus in summary, the endocrine mediated effects observed in fish after exposure to 4-tert-butylphenol and anticipated on the basis of read-across from other alkylphenols 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 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. No reliable information is available for 4-tert-butylphenol about whether it can cause ED-related adverse effects on taxa other than fish. 4-tert-octylphenol and 4-nonylphenol cause effects in amphibians and invertebrates that might be endocrine mediated, i.e. caused by an estrogen-like mode of action, although it is not possible to clearly conclude that they are endocrine mediated. Similar effects may be caused by 4-tert-butylphenol, but there are no confirmatory data. 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 (a) to (e) of Article 57 of REACH.

Registration dossiers submitted for the substance: Yes

Justification

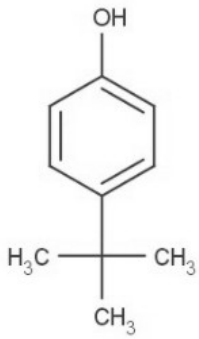
1. Identity of the substance and physical and chemical properties

1.1. Name and other identifiers of the substance

Table 1: Substance identity

EC number:	202-679-0
EC name:	4-tert-butylphenol
CAS number (in the EC inventory):	98-54-4
CAS number: **Deleted CAS numbers:**	98-54-4 1334243-56-9
CAS name:	Phenol, 4-(1,1-dimethylethyl)-
IUPAC name:	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 range:	150.2176 g/mol
Synonyms:	Phenol, p-tert-butyl- (8CI) 4-(1,1-Dimethylethyl)phenol 4-tert-Butylphenol Butylphenol NSC 3697; p-tert-butylphenol 4-t-BP

Structural formula:



1.2. Composition of the substance

Name: 4-tert-butylphenol

Description:

Substance type: mono-constituent

For further information please refer to the confidential annex.

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

4-tert-butylphenol can be considered as part of a group of alkylphenols with a linear or branched alkyl chain in para-position. The substances differ in the length of the alkyl chain 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:

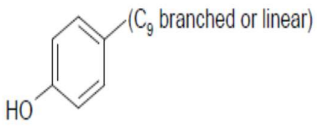


Table 3: Other Substance identifiers – 4-tert-octylphenol

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:

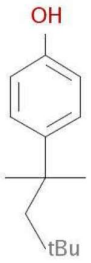
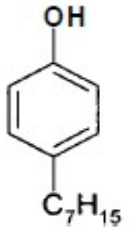


Table 4: Other Substance identifiers – 4-heptylphenol

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:



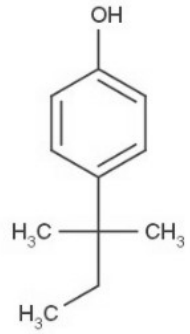
(branched and linear)

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

EC number:	201-280-9
EC name (public):	p-(1,1-dimethylpropyl)phenol
CAS number:	80-46-6
IUPAC name (public):	p-(1,1-dimethylpropyl)phenol
Index number in Annex VI of the CLP Regulation:	-
Molecular formula:	$C_{11}H_{16}O$
Molecular weight or molecular weight range:	164.24 g/mol

Substance type: mono-constituent

Structurally related substance(s) formula:



1.4. Physicochemical properties

Table 6: Overview of physicochemical properties

Property	Value [Unit]	Reference/source of information
Physical state at 20°C and 101.3 kPa	white flakes with a phenolic odour	visual and olfactory inspection.
Melting/freezing point	99.2 °C	Photocell detection (ISO1218)
Boiling point	238 °C at approx. 101 kPa	ASTM E 737-76; differential scanning calorimetry No data on decomposition and sublimation
Vapour pressure	0.5 Pa at 20 °C	ECB, 2008
Density	0.9 g/cm^3 at 110 °C	ICSC, NIOSH (US)
Water solubility	607.2 mg/L at 25 °C, pH = 6 - 7	ASTM E 1148 – 02; flask method
Partition coefficient n-octanol/water (log value)	3.0 at 23 °C, pH = 5.7	OECD Guideline 117, HPLC method

2. Harmonised classification and labelling

4-tert-butylphenol is covered by index number 604-090-00-8 in part 3 of Annex VI to the CLP Regulation as follows:

Table 7: Classification according to Annex VI, Table 3.1 (list of harmonised classification and labelling of hazardous substances) of Regulation (EC) No 1272/2008

Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Spec. Conc. Limits, M-factors	Notes
				Hazard Class and Category Code(s)	Hazard statement code(s)	Pictogram, Signal Word Code(s)	Hazard statement code(s)	Suppl. Hazard statement code(s)		
604-090-00-8	4-tert-butylphenol	202-679-0	98-54-4	Skin Irrit. 2 Eye Dam. 1 Repr. 2	H 315 H318 H361f	Health hazard, Corrosion, Danger	H 315 H318 H361f			

In addition, on 3 June 2016 RAC concluded that 4-tert-butylphenol shall be classified as Aquatic Chronic 1.

The conclusion was based on a study by Krueger at al., 2008 and NOEC of 9.6 µg/l (see chapter 5.2.2.3.1 for details).

3. Environmental fate properties

3.1. Degradation

3.1.1. Summary and discussion of degradation

Information on biodegradation 2016 is summarised in order to provide information about the fate of the substance.

According to the Registration dossier an OECD 301F study was performed resulting in the conclusion that the substance seems to be readily biodegradable, not fulfilling the 10 d window.

The following conclusion was made in the RAC conclusion on a harmonized classification and labelling of 4-tert-butylphenol, adopted in June 2016:

PPTBP is stable under visible light irradiation (Xiao *et al.*, 2014).

The DOC Die-Away Test (equivalent to OECD TG 301A) shows degradation of 98 % (DOC decrease) after 28 days and > 70 % within 10 days after the time at which the degradation reached 10 %. PTBP is readily biodegradable based on these results. RAC reviewed the available information for this test (see Supplemental information) and considered it reliable for the purposes of classification. PTBP was significantly degraded (60 % after 28 days) in two additional ready biodegradation tests (OECD TG 301B and OECD TG 301F) but failed to meet the 10-day window (i.e. there was a lag phase). As a result, ready biodegradability cannot be determined from those studies. Nevertheless, these studies indicate that PTBP has the potential to mineralise, with the more extensive degradation measured in the OECD TG 301A study (98% after 28 days) presumably reflecting the presence of competent degraders in this particular test (it is well known that the outcome of ready tests can be limited by compromised microbial diversity (see for example Kowalczyk *et al.*, 2015)).

The RAC opinion concludes: Taking into account all available data on degradability, 4-tert-butylphenol can be considered as a rapidly degradable substance in the environment.

3.2. Environmental distribution

3.2.1. Adsorption/desorption

For adsorption/desorption a QSAR predicted K_{oc} value of 1286 ($logK_{oc}$ = 3.1) is provided with the registration. The applicability of the QSAR used in the CSR was not demonstrated. In the EU RAR a calculated K_{oc}-value of 582 (EUSES; $logK_{oc}$ = 2.8) was used for risk assessment (ECB, 2008). In line with the EU RAR, a calculated $logK_{oc}$ value of 2.8 was used for distribution modelling within the scope of this dossier.

Based on the $logK_{ow}$ of 3.0, 4-tert-butylphenol is expected to partition to sediment and soil. A rapid decomposition of 4-tert-butylphenol is not expected, as the substance will not dissociate at environmentally relevant pH and hydrolysis will not occur due to the absence of hydrolysable functional groups. Partitioning to sediment and soil is driven by adsorption to organic matter. Therefore, the $logK_{oc}$ is a basic value for environmental distribution modelling.

There are uncertainties in calculating logKoc from logKow. 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 logKoc represents the worst case for assessment of the water compartment. Therefore, for the purpose of this assessment using the calculated logKoc of 2.8 is considered appropriate.

3.4 Bioaccumulation

A summary of bioaccumulation data based on a RAC opinion on harmonized classification of 4-tert-butylphenol, adopted in June 2016:

The measured octanol-water partition coefficient (log Kow) of 4-tert-butylphenol is in the range 2.4 – 3.3. Freitag et al. (1984) studied the bioaccumulation of 4-tert-butylphenol in golden orfe (*Leuciscus idus melanotus)* after three days of exposure. The measured bioconcentration factor from this study was 120 L/kg. No information is provided about the time to steady state or lipid content of the fish.

Based on the RAC opinion 4-tert-butylphenol has a low potential to bioaccumulate.

The tests to determine log Kow values might be unsuitable due to potential surface activity of the chemicals not least because of the tendency for surfactant molecules to accumulate at phase interfaces or form emulsions. Nevertheless, estimated log Kow values for 4-tert-butylphenol was 3.42 (EPISUITE v4.11).

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) of 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),* three 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 III.

5.1.2. Long term toxicity to aquatic invertebrates

Results are taken from the registration dossiers and were not further analysed.

The Japanese Ministry of Health and Welfare conducted 1996 a test on chronic toxicity to *Daphnia magna* with the duration 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.

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 (without specification) and 4-t-OP (Marcial et al., 2003). See table Table **8**.

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. Toxicokinetic data on fish

Information on toxicokinetics was included in order to support a read-across. It is described in some detail in this section. A review of the data with regard to its support for a read-across is provided in annex I.

Atlantic cod (*Gadus morhua*) was exposed in the laboratory to tritium labelled 4-tert-butylphenol, 4-n-pentylphenol, 4-n-hexylphenol, and 4-n-heptylphenol via seawater (8 ng/L) and via contaminated feed (5 µg/kg fish per day) (Klimisch 2). Measurements of different fish tissues during eight days of exposure and eight subsequent days of recovery revealed that alkylphenols (APs) administered via spiked seawater were readily taken up during the first two days of exposure. Steady state for spiked feed was also reached after day 2 but uptake was far less efficient when APs were administered in spiked feed. Approximately 10% of the AP administered (4-n-pentylphenol 8%, all other 12-14%) via spiked feed was accounted for in the tissues analysed (excluding the intestine) (Sundt et al. 2009). These values are comparable to results of feeding study in flounder (8% 4-tert-octylphenol residues in liver and muscle tissue) from Madson et al. (2003), however also lower values are reported for ^{3}H-4-n-nonylphenol (Cravedi and Zalko, 2005).

Elimination half-lives for ^{3}H-labelled 4-tert-butylphenol, 4-n-pentylphenol, 4-n-hexylphenol, and 4-n-heptylphenol independent of the exposure route (seawater or feed) range between 10 to 20 hours in cod (Sundt et al. 2009). This finding is consistent with the study (Klimisch 2) performed by Tollefsen et al. (1998). Steady state in Atlantic cod exposed via seawater to 4-[^{14}C]-heptylphenol (two para-substituted isomers: one branched and one linear) was reached by 58 hours with an elimination of 13 hours. Environment and Health Canada (2001) reported half-lives for nonylphenol in fish of 0.8 days in rainbow trout, 1.2 to 1.4 days in fathead minnow and 4 days in Atlantic salmon.

According to Sundt et al. (2009) tissue distribution of ^{3}H-labelled 4-tert-butylphenol, 4-n-pentylphenol, 4-n-hexylphenol, and 4-n-heptylphenol in large cod showed high residues particularly in the bile fluid as well as in the intestine, intestine content and stomach content. Also with spiked feed the bile fluid showed the highest concentrations. After 8 days of recovery, bile still had the highest residues. The liver and other tissues studied (muscle, pooled sample of spleen/heart/kidney/brain and gonads) contributed only little to the total radioactivity detected. Tollefsen et al. (1998) reported preferential distribution of 4-[^{14}C]-heptylphenol to bile, liver, intestines, kidney and heart compared to blood. Therefore, it can be assumed that excretion is primarily via bile and faeces. In addition, Cravedi and Zalko (2005) concluded in their review paper on the metabolic fate of nonylphenols and related alkylphenols in fish that excretion of nonylphenol and other alkylphenols occurred predominantly in faeces and bile. Tollefsen et al. (1998) identified high residues of 4-^{14}C-heptylphenol after 192 h seawater exposure in kidneys (comparable to concentrations in the liver) and indicated also excretion via urine. Excretion via gills was also suggested.

Metabolism in fish was investigated for nine individual APs including 4-tert-butylphenol, 4-n-pentylphenol and 4-n-heptylphenol after intermuscular injection in Atlantic cod (Jonsson et al. 2008). The glucuronic acid conjugate was the most abundant metabolite in cod bile (approx. 84%, 87% and 90%relative concentration for 4-tert-butylphenol, 4-n-pentylphenol and 4-n-heptylphenol, respectively). After 4-n-heptylphenol administration, also glucosides, sulfates and unchanged parent were detected in the bile with 6.1%, approx. 5% and approx. 4%, respectively (Jonsson et al. 2008). Biotransformation pathways were also investigated in different fish species and with different alkylphenols (4-n-NP, branched NPs, and 4-tert-octylphenol) according to Cravedi and Zalko (2005). Also here the predominant metabolic pathway for alkylphenols was the conjugation of the phenol group to glucuronic acid and secondly the oxidative biotransformation of the alkyl side-chain (subsequent or prior to glucuronidation). The terminal and sub-terminal oxidative biotransformation might be responsible for more hydroxylated metabolites form branched APs. AP sulfation is poorly demonstrated in fish compared to rat according to Cravedi and Zalko (2005). Linear side chain alkylphenols may enter the ß-oxidation pathway thereby producing shorter side-chain carboxylic acid metabolites. This pathway was

established and extensively characterized in-vivo for 4-n-nonylphenol. In addition, a ring-hydroxylated pathway was demonstrated for 4-tert-octylphenol yielding catechol metabolites and reactive intermediates (Cravedi and Zalko, 2005).

5.2.2. Endocrine Disruption in fish

5.2.2.1. 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, 2012). Although this document focuses on validated OECD test guidelines, general instructions on how to assess endocrine disrupting properties are provided. These are supplemented with information from other guidance documents (e. g. OECD 123 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)).

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 exerts 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 estrogenic or androgenic 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. Induction in females is also an indicator for an androgen antagonist mode of action (IPCS, 2002; Kendall et al., 1998; Knacker et al., 2010; OECD, 2004, OECD 2012).

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

- Male: increased proportion of spermatogonia (early sperm cells), presence of testis-ova (estrogenic response especially in juvenile and adult Japanese medaka, but also in other differentiated gonochorist species), increased testicular degeneration, interstitial (Leydig) 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).[3]

In addition, the following apical endpoints are considered to be indicators of an estrogen agonist or anti-androgen mode of action according to the OECD guidance document (OECD, 2012):

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

Decrease in *secondary sex characteristics* in males may indicate an estrogenic mode of action but should be interpreted with caution and based on weight of evidence according to (OECD, 2009). Induction of female secondary sex characteristics in males such as 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).

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 the life stage(s) under exposure need to be considered carefully while interpreting test results. Especially if effects on gonadal staging are analysed the reproductive cycle of a species should be considered. In particular 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

Alterations 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 or antiandrogen specific.

Other endpoints such as growth, sexual maturity, reproduction and behavior are known to be sensitive to estrogens (IPCS, 2002; OECD, 2004; OECD, 2012). 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). Table 9 summarizes endpoints that are considered indicators of estrogen activity and may be affected as a result of this activity *in vivo*.

Table 9: Summary of endpoints that are considered during analysis of fish data

Endpoints indicating an estrogen agonist (or antiandrogen) mode of action	**Apical endpoints considered to be sensitive to an estrogenic mode of action *in vivo***
• Vitellogenin induction in males and females • Increased proportion of spermatogonia (early sperm	• Female biased phenotypic sex ratio during sexual

[3] 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 recurs and, as their spawning is usually not synchronized, individual gonadal growth differs in time.

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.	development especially in medaka • Reproduction (fecundity, fertility, number of males or females with reproductive success) • Spawning behaviour • Growth of offspring

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.

5.2.2.2. *In vitro* information indicative of endocrine activity

In vitro data are evaluated with respect to the potential mode of action of 4-tert-butylphenol. *In vitro* estrogen activity of 4-tert-butylphenol was assessed in different assays including binding assays and three types of assays analyzing estrogen receptor activation, i.e. reporter gene assays (YES assay), assays analyzing vitellogenin (VTG) induction in primary hepatocytes of *Oncorhynchus mykiss* and MCF-7 cell proliferation assay.

Results are briefly summarized below and in Table 10. Results for selected reference substances (e. g. 4-tert-octylphenol) were described too. Results for other alkylphenols are summarized in Annex I - Additional information on read-across approach.

5.2.2.2.1. 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.

In two studies the authors assessed whether or not 4-tert-butylphenol is able to specifically bind to the estrogen receptor of fish (rainbow trout) (Olsen et al., 2005) (Tollefsen and Julie Nilsen, 2008):

In both studies, 4-tert-butylphenol was demonstrated to displace specifically bound 17ß-estradiol (E2) from the estrogen receptor (ER). The relative binding affinity (RBA[4]) of 4-tert-butylphenol (RBA = 4E-5 / 7.7E-5) was in the same range as the one of 4-*tert*-octylphenol (RBA approx. 6.9E-5 / 7.6E-5), a known endocrine disrupter to the environment.

Hornung et al. (Hornung et al., 2014) conducted competitive binding assays using liver cytosolic preparations (cyto rtERαß) from immature rainbow trout. The preparations contained all ER receptors found in trout liver. The RBA for 4-tert-butylphenol was 1.4E-5 and thus in the same order of magnitude compared to 4-tert-octylphenol.

Olsen et al. (2005) and Olsen et al. (2002) analysed the RBA to human estrogen receptor (hER) derived from human cells (MCF7). The RBA in the two studies was 2.1E-6 and 7.8E-6 respectively

[4] 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.

and thus one order of magnitude lower compared with results for 4-tert-octylphenol (Olsen et al. (Olsen et al., 2005) (RBA 1.4E-5).

Akahori et al. (Akahori et al., 2008) conducted a similar study: Recombinant human estrogen receptor α (hERα) ligand binding domain was expressed in *E. coli* and then purified. The RBA was 2.3E-5 (IC_{50} (E2)/IC_{50} (4-tert-butylphenol two orders of magnitude lower compared to 4-tert-octylphenol (RBA 0.00123).

Blair et al. (Blair et al., 2000) examined the competitive binding to the estrogen receptor in uterine cytosol preparation from ovariectomized rats. 4-t-BP displaced also in this case specifically bound 17ß-estradiol (E2) from the estrogen receptor. The RBA was 2.4E-6 and two orders of magnitude lower compared to 4-tert-octylphenol.

Another study conducted by Kwack et al. (Kwack et al., 2002) using MCF-7 cells gave no exact information regarding 4-tert-butylphenol. It was only stated that 4-tert-octylphenol and 4-nonylpheonl concentration-dependently inhibited the binding of [^{3}H]E2 to the ER of MCF-7 cells. Probably 4-tert-butylphenol had a less strong binding affinity.

In summary all available competent binding assays using fish receptors showed that 4-tert-butylphenol binds to the ER receptor. The relative binding affinity (RBA) was 1.4 – 7.7E-5 and in the same order of magnitude as observed for 4-tert-octylphenol. With regard to human and rat receptors three of four assays showed positive results with RBAs only slightly lower than those observed for fish (RBA 2.3E-5 – 7.8E-6) but one to two orders of magnitude lower compared to 4-tert-octylphenol. Results of one study are unclear.

5.2.2.2.2. Binding to sex steroid binding proteins

The binding of alkylphenols to sex steroid binding proteins of rainbow trout (rtSBP) under competitive conditions was examined by Tollefsen (Tollefsen, 2007). Plasma samples of female rainbow trout were used and incubated with [^{3}H]E2 in combination with increasing concentrations of test compounds. The RBA was 6.1E-6, five times lower compared to 4-tert-octylphenol (RBA 1.3E-5).

Milligan et al. (Milligan et al., 1998) examined the competitive binding of 4-tert-butylphenol, 4-tert-octylphenol, 4-nonylphenol and other substances in rat amniotic fluid and to sex steroid binding proteins in human and rainbow trout (*Oncorhynchus mykiss*) plasma.
The concentration where 4-tert-butylphenol showed significant competition in rainbow trout plasma was about 1000–fold greater than that of estradiol. Displacement was less than 50% and the RBA was < 1E-3. 4-tert-butylphenol did not significantly displace [^{3}H]E2 in rat amniotic fluid and also not [^{3}H]DHT from human plasma. Compared to 4-tert-octylphenol (RBA < 0.0001) and 4-nonylphenol (RBA < 0.0001 or no displacement) 4-tert-butylphenol showed greater ability to displace [^{3}H]E2 and [^{3}H]DHT from their specific plasma binding sites. A reason could be the lower water solubility of the longer chained alkylphenols in this test. In rat amniotic fluid neither 4-tert-butylphenol nor 4-tert-octylphenol, 4-nonylphenol or Bisphenol A did significantly displace [^{3}H]E2.

In summary, the above results demonstrate that 4-tert-butylphenol and other alkylphenols not only bind to the estrogen receptor but also to sex steroid binding proteins (in plasma of rainbow trout). However the RBA to sex steroid binding proteins was only calculated in one test (with plasma from rainbow trout) and the RBA was 6.1E-6, about a factor of 10 lower than the RBA to ER from rainbow trout. [^{3}H]E2 was however not displaced by 4-tert-butylphenol in rat amniotic fluid.

Furthermore the androgen DHT (Dihydrotestosterone), a metabolite of testosterone can be displaced by 4-tert-butylphenol from sex steroid binding proteins in fish plasma and human plasma. However, the RBA for displacement of [^{3}H]DHT was about a factor of ten lower than the RBA for displacement of [^{3}H]E2.

5.2.2.2.3.Expression of estrogen-responsive genes

Vitellogenin expression:

This type of assay not only assesses binding to the ER but also activation and consequential induction of VTG in the cells.

Three *in vitro* studies (Jobling and Sumpter, 1993; Olsen et al., 2005; Tollefsen et al., 2008) investigated the effect of 4-tert-butylphenol on VTG expression in primary fish hepatocytes.

Primary hepatocytes were derived from male and/or immature rainbow trout (*Oncorhynchus mykiss*).

All studies demonstrated that exposure to 4-tert-butylphenol resulted in a dose-dependent increase in vitellogenin expression.

Jobling and Sumpter (Jobling and Sumpter, 1993) found that 4-tert-butylphenol enhanced VTG synthesis markedly. It increased the amount of VTG more than 100-fold above control value (REP 1.6E-4). In this test 4-tert-octylphenol was less potent compared to 4-tert-butylphenol. It caused at least a 90-fold increase in the amount of VTG (REP 3.2E-5).

In Olsen et al. (Olsen et al., 2005) the EC_{50} of 4-tert-octylphenol was about 10-fold higher compared to 4-tert-butylphenol but the potency was about an order of magnitude stronger in 4-tert-octylphenol (REP 3.2E-5) than in 4-tert-butylphenol (REP 5.6E-6).

Also the study by Tollefsen et al. (Tollefsen et al., 2008) showed the approx. 10-fold stronger relative endocrine potency (REP) of 4-tert-octylphenol ($1 \cdot 10^{-4}$) compared to 4-tert-butylphenol ($3.3 \cdot 10^{-5}$). However the LOEC were almost in the same range: 3E-6 M for 4-tert-butylphenol, compared to 1E-6 M for 4-tert-octylphenol.

In summary the relative estrogenic potency (REP) (compared to 17-ß-estradiol) was 5.6E-6 to 1.6E-4 and one magnitude lower or in the same range as 4-tert-octylphenol (3.3E-5 to 1E-4).

Regulation of the estrogen-sensitive protein pS2 (estrogen-regulated secretorial protein) and progesterone receptor (PgR):

Olsen et al. (Olsen et al., 2002) also investigated the regulation of the estrogen-sensitive protein pS2 (estrogen-regulated secretorial protein) and progesterone receptor (PgR). The relative induction of the protein pS2 by 4-tert-butylphenol was 0.39 compared to E2 (1). Much more pronounced was the effect on the regulation of the progesterone receptor: Both 17ß-E (at the concentration 30pM) and 4-tert-butylphenol (at 10 µM) upregulated the progesterone receptor 14-fold.

5.2.2.2.4.Reporter gene assays

Transcriptional activation in recombinant yeast (Yeast estrogen screen, YES):
The potential of 4-tert-butylphenol to act as agonist of the ER was also investigated by means of a reporter gene assay based on recombinant yeast cells. The DNA sequence of the human estrogen receptor was integrated into the yeast genome, which also contained expression plasmids carrying estrogen-responsive sequences controlling the expression of the reporter gene Lac-Z (encoding the enzyme b-galactosidase). Thus due to binding to the estrogen receptor ß-galactosidase is synthesized and causes a change of colour that is measurable. Not only binding but also activation of the receptor is measured.

Routledge and Sumpter (Routledge and Sumpter, 1997) determined the relative estrogenic potency (REP) of 4-tert-butylphenol to be 1.5E-6. EC_{50} values were not reported. In this assay 4-tert-butylphenol was 1500 times less potent compared to 4-*tert*-octylphenol. In a yeast two-hybrid assay the REC10 was 3×10^{-5} M (Nishihara et al., 2000). 4-tert-butylphenol was in this assay approx. 100 times less active than 4-tert-octylphenol.

5.2.2.2.5.MCF-7 cell proliferation assay (E-screen)

4-tert-butylphenol was further demonstrated to induce human breast cancer cell (MCF-7) proliferation in four studies and thus act as ER agonist in these cells.

Olsen et al. (Olsen et al., 2002) determined the relative proliferative potency (RPP) to be $3 \cdot 10^{-6}$. The RPP was calculated as: minimal concentration of E2 needed for maximal cell yield / minimal concentration of 4-tert-butylphenol needed for maximal cell yield. The maximal cell proliferation achieved by 4-tert-butylphenol was 78 % (compared to cell proliferation of E2 = 100 %).

When the cells were co-exposed with the ER antagonist 4-hydroxy-tamoxifen (100 nM), then the cell growth stimulated by 4-tert-butylphenol was reduced from 78% to 6%, confirming that 4-tert-butylphenol stimulates cell growth through the ER.

In order to examine anti-estrogenic properties, 4-tert-butylphenol was co-incubated with 30 pM 17ß-estradiol. At 10 µM, 4-tert-butylphenol reduced growth of MCF-7 cells by approx. 10 %. The reason is most probably that 4-tert-butylphenol also can bind to the ER without activation and therefore exhibit also anti-estrogenic properties. However, the estrogenic property of 4-tert-butylphenol is much stronger than the anti-estrogenic, by achieving 78 % stimulation of cell growth versus 10 % reduction of E2-induced cell growth.

Another study by Olsen et al. (Olsen et al., 2005) confirms the relative estrogen potency with a REP of 1.9E-7. REP was calculated as EC_{50}(E2)/EC_{50}(4-tert-butylphenol). 4-tert-butylphenol had a 10-fold lower REP than 4-tert-octylphenol. The proliferative effect concentration EC_{50} was 3.2E-5 M. In comparison to 4-tert-octylphenol, 4-tert-butylphenol was 10-fold less potent.

Also Soto et al. (Soto et al., 1995) examined proliferative effects on MCF-7 cells. The lowest concentration of 4-tert-butylphenol needed to obtain maximal cell yield was 10^{-5} M (1502 µg/L). The relative proliferative potency (RPP) was 3E-6, similar to the result obtained by Olsen et al. 2002. The relative proliferative effect (RPE) was 0.71 (calculated as PE-1 (4-tert-butylphenol) / PE-1 (E2)). In comparison with 4-NP: RPP was $3x10^{-5}$, RPE was 1, lowest concentration needed for maximal cell yield = 1E-6 M. Nonylphenol (technical grade) showed the same values like 4-tert-butylphenol for the lowest concentration needed for maximal cell yield =1E-5 M and for RPP=3E-6 , whereas RPE was 1.

Körner et al. (Korner et al., 1998) obtained a similar value like Soto et al. (1995) for the relative proliferative effect (RPE) of 0.78 for 4-tert-butylphenol, compared to 4-tert-octylphenol: 0.97 and 4NP: 1.05. Also the lowest concentration of 4-t-BP needed for maximal cell yield was the same: 10 µM (1502 µg/L), compared to 1E-6 M for both 4-tert-octylphenol and 4-nonylphenol.

The proliferative effect (PE) of 4-tert-butylphenol was 4.4, compared to 4-tert-octylphenol: 4.57 and 4-NP (technical grade): 6.13.

Kwack et al. (Kwack et al., 2002) exposed MCF-7 cells for 6 days to 4-tert-butylphenol. No exact data were given regarding results for 4-tert-butylphenol, but the information that 4-t-OP and 4-NP were considerably more potent than any other compound tested in the study.

Summarising all E-Screens it can be said, that 4-tert-butylphenol acts as an estrogen agonist in the MCF-7 cells and the effects are in most cases one order of magnitude lower or in the same range like 4-tert-octylphenol and 4-nonylphenol. No exact statement is possible regarding the result of the study by Kwack. The effects of 4-t-BP compared to 4-t-OP and 4-NP in this study are probably less strong.

5.2.2.2.6. Effect on steroidogenic activity of isolated immature rat ovarian follicles

In a study by Myllymäki et al. (Myllymaki et al., 2005) immature rat ovarian follicles (from 14-day-old rat) were exposed amongst other chemicals to 4-tert-butylphenol, 4-tert-octylphenol and DES (diethylstilbestrol). Effects on growth, survival, steroid hormone (estradiol and testosterone) production were measured. The duration of exposure was 3 or 5 days. Both alkylphenols did not interfere with growth or survival of the follicles. Diethylstilbestrol, 4-tert-butylphenol and 4-tert-octylphenol decreased estradiol and testosterone secretion in a dose-dependent manner. The concentration range used for all chemicals was E-8 to E-6 M. The estradiol production by the ovarian follicles was inhibited by DES, 4-tert-butylphenol and 4-tert-

octylphenol to about 50, 300 and 200 pg/follicle respectively after 3 days exposure (control values were 400, 500 and 550 pg/follicle, respectively). Also the testosterone production was decreased by DES, 4-tert-butylphenol and 4-tert-octylphenol to 20, 60 and 40 pg/follicle after 3 days. The control values were 70, 100 and 110 pg/follicle respectively.

5.2.2.2.7. Summary

The competitive ligand-binding studies clearly demonstrated that 4-tert-butylphenol is able to displace specifically bound E2 from the ER ligand-binding pocket. The RBA of 4-tert-butylphenol for ERs derived from human or rainbow trout ranged from 2.1E-6 to 7.7E-5.

Thus, 4-tert-butylphenol acts as a ligand of the ER. Binding was also examined not only to the ER but also to sex-steroid binding protein (SBP). In one test with plasma preparation of the rainbow trout the binding to the SBP was comparable to the binding to the ER. Another study showed that binding in plasma from trout has a higher RBA than binding in human plasma or rat amniotic fluid (where no binding appeared).

As described in the activation assays, binding of 4-tert-butylphenol to the ER leads to activation of the ER-mediated pathway and consequently to transcriptional activation of typically estrogen-responsive genes.

Modulation of ER-mediated gene expression was evidenced on the transcriptional, protein and cell physiological level.

The EC_{50} values in studies investigating the expression of the estrogen-dependent biomarker rainbow trout VTG ranged from = 2.06E-6 M (309 µg/L) to 1.8E-5 M (2700 µg/L). Also other estrogen-sensitive proteins (pS2 and progesterone receptor) were upregulated in MCF-7 cells. Progesterone was elevated 14-fold by 10 µM 4-tert-butylphenol, and also by E2 at 30 pM.

The relative estrogenic potency (REP) of 4-tert-butylphenol obtained in the transcriptional activation assay using recombinant yeast (yeast estrogen screen, YES) was 1.5E-6 (the potency was 1500-fold lower than that for 4-tert-octylphenol). The REC_{10} in a yeast two-hybrid assay was 3E-5 M (100-fold lower activity than 4-tert-octylphenol).

Exposure to 4-tert-butylphenol caused proliferation of MCF cells (measured by different parameters, see above).

A comparison of data summarized for other alkylphenols in Annex 1 shows that overall *in vitro* activity of 4-tert-butylphenol is in the same range (max. factor 10 difference in most cases) as observed for longer chain alkylphenols which are already identified as Substances of Very High Concern due to their endocrine disrupting properties for the environment:

- The potency for binding to the estrogen receptor and sex steroid-binding protein was in the same range with regard to rainbow trout receptors.
- Expression of estrogen responsive genes in mammal including human cell lines was two orders of magnitude lower with regard to the relative potency
- Vitellogenin induction in rainbow trout cells was in the same range compared to longer chain alkylphenols.
- Response in MCF cells was only slightly lower to those observed for 4-tert-octylphenol and 4-nonylphenol.

Based on the available mechanistic information it can be concluded that 4-tert-butylphenol has the potential to exert estrogen-like effects and disrupt endocrine homeostasis. Effects of 4-tert-butylphenol are occurring at the same dose/concentration ranges as for the longer chain alkylphenols in fish but in some tests are slightly lower in mammals.

Table 10: Summary of *in vitro* studies assessing the potential of 4-tert-butylphenol (4-t-BP) 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**	**Test conditions**	**Endocrine mediated measurement parameters**	**Relative binding affinity (RBA) compared to 17β-estradiol**	**Comment**
Oncorhynchus mykiss rainbow trout	(Olsen et al., 2005)	Cytosolic preparation of rainbow trout liver homogenates	Liver homogenates were incubated with [^{3}H]E2 for 16 h at 4°C in the absence or presence of different concentrations of 4-t-BP or E2 / Solvent: Methanol. c_{max} = 2 % (v/v) / n=3, i=2	IC_{50}(E2) = 6.6 x 10^{-9} M (1.79 µg/L) IC_{50}(4-t-BP)= 8.6 x 10^{-5} M (12.9 x 10^3 µg/L)	RBA (calculated from reported IC50 values) = 7.7 x 10^{-5} RBA was calculated as IC_{50}(E2)/IC_{50}(4-t-BP) RBA[1] = 4.6 x 10^{-5}	Note: The calculation of RBA[1] is not reproducible using the reported IC_{50} values. RBA comparable with 4-tertOP (7.6x10^{-5}), IC_{50} (4-tOP)= 8.4 x 10^{-4} M; Klimisch 2
Oncorhynchus mykiss rainbow trout	(Tollefsen and Julie Nilsen, 2008)	Cytosolic preparation of female trout liver homogenates	Pooled liver homogenates (2.5 mg/ml protein) was incubated with 2.5 nM [^{3}H]E2 for 16 h at 4 °C) in the absence or presence of different concentrations of 4-t-BP (0.25 x 10^{-6} M to 7.5 x 10^{-3} M) or E2 (75 x 10^{-12} M to 75 x 10^{-9} M) Solvent: Methanol, c_{max} =1.25 % (v/v) / n=3	IC_{50} (E2) = 3.5 x 10^{-9} M (0.95 µg/L) IC_{50} (4-t-BP) = 8.7 x 10^{-5} M (13.1 x 10^3 µg/L)	RBA = 4.0 x 10^{-5} RBA was calculated as IC_{50}(E2)/IC_{50}(4-t-BP)	RBA comparable with 4-tertOP (6.9x$10^{-5)}$ IC50 (4-tOP): 8.4x10^{-4} Klimisch 2
Oncorhynchus mykiss rainbow trout	(Hornung et al., 2014)	Cytosolic liver preparations (cyto rtERαß) from immature rainbow trout. Preparations contained the ER receptors (α1, α2, ß1, ß2).	Testing in duplicate at a minimum of six concentrations, together with [^{3}H]E2 Solvent: Ethanol	No IC_{50} value given	RBA (cyto rtERαß binding) = 1.4 x 10^{-5} RBA = IC_{50} (E2) / IC_{50} (4-t-BP)	Comparison with 4tOP: RBA 9.4 x 10^{-5} Klimisch 2

Human	(Olsen et al., 2002)	Cytosolic preparation of MCF-7 cells	Cytosol preparation was incubated with [^{3}H]E2 (2 nM) alone or in combination with 4-t-BP (10^{-7} to 10^{-3} M) for 2 h, solvent: DMSO (15 %)	IC_{50} (E2) = 2.98 x 10^{-9} M IC_{50} (4-t-BP) = 3.84 x 10^{-4} M (57.68 x 10^3 µg/L)	RBA = 7.76 x 10^{-6} RBA was calculated as IC_{50}(E2)/IC_{50}(4-t-BP) RBA[1] = 0.0001	Note: The calculation of RBA[1] is not reproducible using the reported IC_{50} values. KL. 2
Species	**Reference**	**Receptor origin and preparation**	**Test conditions**	**Endocrine mediated measurement parameters**	**Relative binding affinity (RBA) compared to 17β-estradiol**	**Comment**
Human	(Olsen et al., 2005)	Cytosolic fraction of lysed MCF-7 cells	Cell lysates were incubated with [^{3}H]E2 for 2h at 4°C in the absence or presence of unlabelled 4-t-BP or E2. Solvent: DMSO, c_{max} = 15 % (v/v) / n=3, i=2 Note: Solvent concentration appears to be very high.	IC_{50} (E2) = 1.8 x 10^{-9} M (0.49 µg/L) IC_{50} (4-t-BP)= 8.7 x 10^{-4} M (130.7 x 10^3 µg/L)	RBA = 2.1 x 10^{-6} RBA was calculated as IC_{50}(E2)/IC_{50}(4-t-BP)	In comparison with 4-t-Octylphenol the RBA of 4-t-BP is approx. 10-fold lower. RBA (4-tOP): 6.4x10^{-5} IC50 (4-tOP): 3.8x10^{-5},Klimisch 2
Rat	(Blair et al., 2000)	Cytosolic preparation of uteri from ovariectomized rats	Uterine cytosol preparation and [^{3}H]E2 (10^{-9} M) were incubated with increasing concentrations of 4-t-BP (1E-4 to 1E-9M) for 20 h at 4°C in duplicate.	IC50 (E2) = 8.99 x 10^{-10} M IC50 (4-t-BP) = 3.68 x 10^{-4} M (55.28 x 10^3 µg/L)	RBA = 2.4 x 10^{-6} RBA was calculated as IC_{50}(E2)/IC_{50}(4-t-BP)	Compared to 4-tertOP, 4-t-BP has a 62- fold lower RBA; Klimisch 2
Human	(Akahori et al., 2008)	Recombinant human estrogen receptor α (hERα) ligand binding domain was expressed in E.coli and then purified.	4-t-BP (concentrations: 1E-11 to 1E-4M) and [^{3}H]E2 (0.5nM) were incubated together with hERα for 1 h. The radioactivity of ligands bound to the receptor was measured. (Replicates: more than 3 per chemical)	See right (no IC_{50} values given)	RBA = 2.34 x 10^{-5} RBA= IC50 (E2)/ IC50 (4-t-BP) RBA calculated from the given log RBA value, based on 1 (not percent)	From the same study also data were obtained for 4-t-BP from an Immature rat uterotrophic assay: Lowest effective dosis (LED) for estrogenic effect: 660 µmol/kg/day (uterine weights was sign. increased) and antiestrogenic effect LED: 1995 µmol/kg/day (uterine weight sign. decreased); Klimisch 2

Human	(Kwack et al., 2002)	Diluted MCF-7 cells	Diluted MCF-7 cells were exposed to 4-t-BP (no concentration given) and ^{3}[H]estradiol for 45 min at 37 °C. After centrifugation at 4 °C the radioactivity was measured in the sediment.	Only 4-t-OP and 4-NP concentration-dependently inhibited the binding of [^{3}H]E2 to the ER of MCF-7 cells. No information regarding 4-t-BP given.		Klimisch 2
Species	**Reference**	**Receptor origin and preparation**	**Test conditions**	**Endocrine mediated measurement parameters**	**Relative binding affinity (RBA) compared to 17β-estradiol**	**Comment**
human ERα and ERß proteins	Kuiper et al. (1998)	Scintillating micro-titration plates, solid-phase binding system	Solid-phase (Scintistrip) competition experiments: ERα and ERß extract were diluted, Incubation: 18h.	IC_{50} not specified, only used for RBA calculation.	RBA < 1 x 10^{-4} RBA was calculated as IC_{50}(E2)/IC_{50} (4-t-BP) RBA (E2) = 1	The study was not used in evaluation above because no new information could be obtained. It is knwon that RBA < 0.0001.; Klimisch 2
Binding to sex steroid-binding protein						
Species	**Reference**	**Receptor origin and preparation**	**Test conditions**	**Endocrine mediated measurement parameters**	**Relative binding affinity (RBA) compared to 17β-estradiol (=1)**	**Comment**
Oncorhynchus mykiss rainbow trout	(Tollefsen, 2007)	Plasma preparation of female rainbow trout	Plasma samples were incubated with [^{3}H]E2 for 16h at 4 °C in the absence or presence of different concentrations of 4-t-BP or E2. Solvent: Methanol, c_{max} = 2.5 %	IC_{50} (E2) = 1.6 x 10^{-9} M (0.43 µg/L) IC_{50} (4-t-BP) = 2.5 10^{-4} M (37.5 x 10^{3} µg/L)	RBA = 6.1 x 10^{-6} RBA was calculated as IC_{50}(E2)/IC_{50}(4-t-BP)	Klimisch 2
Oncorhynchus mykiss, rainbow trout	(Milligan et al., 1998)	Rainbow trout plasma with [^{3}H]E2 or Rainbow trout plasma with [^{3}H]DHT	Plasma samples were incubated with [^{3}H]E2 or [^{3}H]DHT and 4-t-BP overnight at 4 °C in duplicate.		RBA < 0.001for displacement of [^{3}H]E2 RBA < 0.001for displacement of [^{3}H]DHT	50% Displacement of [^{3}H]E2 or [^{3}H]DHT with RBA 0.001: For [^{3}H]E2 4-t-BP similar to DES, NP1EC, Bisphenol-A. For [^{3}H]DHT 4-t-BP similar to NP1EC, Bisphenol A. Klimisch 2

Rat	(Milligan et al., 1998)	Rat amniotic fluid with [^{3}H]E2	Amniotic fluid samples were incubated with [^{3}H]E2 and 4-t-BP overnight at 4 °C in duplicate.		No displacement	Klimisch 2
Human	(Milligan et al., 1998)	human plasma with [^{3}H]DHT	Plasma samples were incubated with [^{3}H]DHT and 4-t-BP overnight at 4 °C in duplicate.		RBA < 0.0001	
Endpoint: Expression of estrogen-sensitive genes						
Expression of vitellogenin						
Species	**Reference**	**Cell type and origin**	**Test conditions**	**Endocrine mediated measurement parameters**	**Potency (relative to 17 β-estradiol=1)**	**Comment**
Oncorhynchus mykiss, rainbow trout	(Jobling and Sumpter, 1993)	Primary hepatocytes derived from male, (mostly) immature fish	Cells were exposed to different concentrations of 4-t-BP or E2 for two days. Solvent: Ethanol, c_{max} = 0.3 % (v/v) / n = 4, i = 3 (It is not reported whether or not the hepatocytes used in the individual experiments were isolated from different fish.)	Expression of vitellogenin protein (rtVTG) ED_{50} (E2) = 1.81 x 10^{-9} M (0.49 µg/L) ED_{50} (4-t-BP) = 2.06 x 10^{-6} M (309 µg/L)	REP =1.6 x 10^{-4} REP was calculated as: ED_{50} (E2) / ED_{50} (4-t-BP)	ED_{50} similar to 4-tert octylphenol, relative estrogen potency of 4-t-BP is higher. 4t-OP: ED_{50}: 2.11 µM; REP: 3.7x10^{-5} ED50 was calculated and averaged for each compound over several experiments. Klimisch 2
Oncorhynchus mykiss rainbow trout	(Olsen et al., 2005)	Primary hepatocytes derived from immature fish	Hepatocyte monolayer cultures were exposed to different concentrations of 4-t-BP for 96 h. The exposure medium was renewed after two days. / Solvent: DMSO, c_{max} < 0.2 % (v/v)	Expression of vitellogenin protein (rtVTG) EC_{50} (E2) = 1 x 10^{-10} M (2.7 x 10^{-2} µg/L) EC_{50} (4-t-BP) = 1.8 x 10^{-5} M (2700 µg/L)	REP = 5.6 x 10^{-6} REP was calculated as: EC_{50} (E2) / concentration of 4-t-BP that resulted in equal induction levels as EC_{50} (E2).	4-t-OP approx. 10-fold stronger effect EC_{50} (4-t-OP): 3.1 µM REP (4-t-OP): 3.2 x 10^{-5} Klimisch 2

Oncorhynchus mykiss rainbow trout	(Tollefsen et al., 2008)	Primary hepatocytes derived from male, immature fish	Cells were exposed to serial dilutions of 4-t-BP for 96 h. The exposure medium was renewed after two days. Solvent: DMSO, c_{max} < 0.3 % (v/v) / n = 3, i = 3 (Cells from different isolations were used to perform replicates.)	Expression of vitellogenin protein (rtVTG) LOEC (E2) = 1 x 10^{-10} M (2.7 x 10^{-2} µg/L) LOEC (4-t-BP)= 3 x 10^{-6} M (450 µg/L)	REP = 3.3 x 10^{-5} REP was calculated as LOEC(E2) / LOEC(4-t-BP)	REP of 4-t-OP approx. 10-fold stronger REP (4-t-OP): 1x10^{-4} LOEC (4-t-OP): 1 µM ; Klimisch 2
Regulation of the estrogen-sensitive protein pS2 (estrogen-regulated secretorial protein) and progesterone receptor (PgR)						
Species	**Reference**	**Cell type**	**Test conditions**	**Endocrine mediated measurement parameters**	**Potency (relative to 17ß-estradiol=1)**	**Comment**
Human	(Olsen et al., 2002)	MCF 7	Cells were exposed to 4-t-BP or E2 for 3 days in cell culture medium.	PgR (in fmol/mg cyt prot): Control: 43 (± 30) E2 (30pM): 606 (± 164) 4-t-BP (10 µM): 604 (± 168), (p<0.01 different from control)	pS2 (estrogen-regulated secretorial protein): relative induction: E2 (30pM): 1 4-t-BP (10 µM): 0.39	Both 4-t-BP (10 µM) and 17ß-E (30pM) elevated PgR 14-fold; Klimisch 2
Endpoint: Transcriptional activation of reporter genes under the control of the ER						
Transcriptional activation assay using recombinant yeast						
Species	**Reference**	**Cell type**	**Test conditions**	**Endocrine mediated measurement parameters**	**Potency (relative to 17ß-estradiol=1)**	**Comment**
Human	(Routledge and Sumpter, 1997)	Yeast estrogen screen, YES Recombinant yeast expressing human ER (hER).	Yeast cells were exposed to increasing concentrations of 4-t-BP (diluted from 1 x 10^{-8} M) or E2 (5 x 10^{-12} – 10^{-9} M) for 84 h at 32 °C. Solvent: Ethanol	Induction of β-galactosidase activity No EC_{50} values reported	REP = 1.5 x 10^{-6}	1500-fold lower potency than 4-t-OP; Klimisch 2
Human	(Nishihara et al., 2000)	Yeast two-hybrid assay with estrogen receptor ERα	Yeast cells were exposed for 4 h at 30°C to 4-t-BP. Solvent: DMSO	REC10 : 3 x 10^{-5} M		REC10 (4-tert-Octylphenol): 2×10^{-7} M

				REC10: The concentration showing 10% activity of 10^{-7} M 17β-estradiol (relative activity).		100-fold lower activity than 4-t-OP; Klimisch 2
Endpoint: MCF-7 cell proliferation assay (E-Screen)						
Species	**Reference**	**Cell type**	**Test conditions**	**Endocrine mediated measurement parameters**	**Potency (relative to 17ß-estradiol=1)**	**Comment**
Human	(Olsen et al., 2002)	MCF-7	Cells were exposed for 6 d to 4-t-BP at concentrations (10^{-11} to 10^{-5} M). Solvent: Ethanol ($c_{max.}$ < 0.2%) In another experiment MCF-7 cells were co-incubated with 30pM 17ß-estradiol and 10 µM 4-t-BP to examine anti-estrogenic properties.	Lowest concentration of 4-t-BP needed for maximal proliferation: 10 µM (1502 µg/L) (E2: 30pM)	Relative proliferative potency (RPP) = 3 x 10^{-6} RPP was calculated as: minimal concentration of E2 needed for maximal cell yield / minimal concentration of 4-t-BP needed for maximal cell yield	Increase of cell growth to 78% by 10 µM 4-t-BP. Reduction of cell growth when cells were co-exposed with 4-hydroxy-tamoxifen (100 nM) to 6 %. Co-incubation with 17ß-E: at 10 µM 4-t-BP reduced growth by approx. 10% (slightly anti-estrogenic). Klimisch 2
Human	(Olsen et al., 2005)	MCF-7	Cells were exposed to for 6 d to 4-t-BP. Solvent: Ethanol, c_{max} < 0.2 % (v/v)	IC_{50} (E2) = 6.1 x 10^{-12} M (1.66 x 10^{-3} µg/L) IC_{50} (4-t-BP) = 3.2 x10^{-5} M (4807 µg/L)	Relative estrogen potency (REP) = 1.9 x 10^{-7} REP was calculated as EC_{50}(E2)/EC_{50}(4-t-BP)	In comparison with 4-t-OP, 4-t-BP has a 10-fold lower estrogenic potency. 4-t-OP: EC_{50} = 5 x10^{-6} M, REP = 1.2 x 10^{-6} Klimisch 2
Human	(Soto et al., 1995)	MCF-7	Cells were exposed to 4-t-BP in different concentrations for six days. Solvent: Ethanol?	Lowest concentration of 4-t-BP needed for maximal cell yield: 10 µM (1502 µg/L)	Relative proliferative potency (RPP): 3 x 10^{-6} Relative proliferative effect (RPE): 0.71	Comparison to 4-NP: Lowest concentration needed for maximal cell yield = 1 µM, RPE = 1, RPP = 3E-5;

					RPE was calculated as: PE-1 (4-t-BP) / PE-1 (E2)	Comparison to NP (technical grade): Lowest concentration needed for maximal cell yield =10 µM, RPE = 1, RPP = 3E-6; Klimisch 2
Human	(Korner et al., 1998)	MCF-7	Cells were exposed to 4-t-BP in different concentrations for six days. Testing in quadruplicate.	Lowest concentration of 4-t-BP needed for maximal cell yield: 10 µM (1502 µg/L) PE (proliferative effect): 4.4	Relative proliferative effect (RPE): 0.78 RPE was calculated as: PE-1 (4-t-BP) / PE-1 (E2)	Lowest concentration of 4-t-OP and 4-NP (techn.) needed for maximal cell yield: 1 µM PE (4-t-OP): 4.57 PE (4-NP (techn.)): 6.13 RPE (4-t-Octylphenol): 0.97 RPE (4-NP): 1.05 ; Klimisch 2
Human	(Kwack et al., 2002)	MCF-7	Cells were exposed to 4-t-BP for 6 days. Solvent: DMSO (0.2%)	No results for 4-t-PP given, only the information, that 4-t-OP and 4-NP were considerably more potent than any other compound.		Klimisch 2
Endpoint: Effect on steroidogenic activity of isolated immature rat ovarian follicles						
Species	**Reference**	**Cell type**	**Test conditions**	**Endocrine mediated measurement parameters**	**Potency (relative to 17ß-estradiol=1)**	**Comment**

Rat	(Myllymaki et al., 2005)	Immature rat ovarian follicles	Follicular cells were exposed to 4-t-BP at concentrations (10^{-8} to 10^{-6} M). On day 3 and 5 the amount of estradiol and testosterone produced from immature rat ovarian follicles was measured after exposure to 4-t-BP and 4-t-octylphenol.	4-t-BP caused disturbance of estradiol production after 3 d: Estradiol was significant decreased at 10^{-7} and 10^{-6} M 4-t-BP and significant increased at 10^{-8}. After 5 days estradiol was significant decreased at 10^{-7} and 10^{-6} M and at 10^{-8} M estradiol was decreased (not significant). Testosterone was significant decreased from 10^{-8} to 10^{-6} M 4-t-BP exposure after 5 days. (DES decreased estradiol significant at 10^{-8} to 10^{-6} M after 3 d and testosterone was sign. decreased at the same concentrations after 5 d)	n.a.	Exposure to 4-t-octylphenol caused significant decrease of estradiol on day 3 at 10^{-8} tp 10^{-6} M and on day 5 at 10^{-8} and 10^{-6} M. Testosterone was decreased on day 3 similar to 4-t-BP at 10^{-8} to 10^{-6} M and on day 5 at 10^{-8} and 10^{-6} M. Diethylstilbestrol (DES) also caused significant decrease of estradiol and testosterone. At 10^{-7} M approx. similar values for testosterone were reached for 4-t-BP, 4-t-OP and DES. Klimisch 2

* 4-tert-butylphenol might be surface active, which might potentially reduce the availability of the substance.
IC50 (in binding studies): equilibrium inhibitory concentration, calculated as the concentration causing 50% inhibition of [^{3}H]-E2 binding
ER = estrogen receptor, E2 = 17β-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-BP)
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 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-BP).
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-t-pentylphenol, 4-t-OP = 4-tert-octylphenol, 4-t-BP = 4-tert-butylphenol.

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

Available data are evaluated 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 4 fish species *in vivo* data at different levels (biomarker, histology and apical endpoints) are available:

- *Pimephales promelas,* Extended ELS, reliability 1
- *Sander lucioperca,* modified juvenile growth test, reliability 2
- *Cyprinus carpio,* adults, modified fish short term assay, reliability 2
- *Oryzias latipes,* modified reproduction assay 14 d, reliability 3 (because no solvent control used)

5.2.2.3.1.Pimephales promelas

Krueger et al. (Krueger et al., 2008) conducted a GLP extended fish early-life stage test according to OECD 234. Newly fertilized embryos were exposed (starting with a five-day hatching period) until 123 dph for a total of 128 days under flow-through conditions. Two incubation cups, each containing 25 embryos, were placed in each of five replicate test chambers (tanks) per treatment (50 embryos per tank, a total of 250 embryos per treatment). The control group had ten tanks with a total of 500 embryos. After hatching, 200 larvae per treatment (400 larvae in the control) were released from the incubation cups into larger test chambers (40 per tank) where exposure continued and observations of condition and mortality were conducted. On day 28 post-hatch (study day 33), the fish were thinned to 32 fish per tank, for a total of 160 fish per treatment group and 320 fish in the control group, and exposure to test concentrations continued for the duration of the study. The nominal and measured test concentrations were 10, 30, 100 and 300 µg/L (n) and 9.6, 27, 83, 255 µg/L (m). The reliability of the study is assessed to be 1.

Results:
VTG:
Male VTG levels in control fish were in the range described in OECD 2011 but female concentrations were much higher. In females VTG was significantly increased at the highest test concentration (255 µg/L (m)). The mean VTG concentrations in females in the negative control, 9.6, 27, 83 and 255 µg/L treatment groups were 317, 409, 344, 351 and 935 mg/mL respectively. As statistical calculation Dunnett's test was used. The **LOEC for increase of VTG in females is 255 µg/L**.
In males the mean plasma VTG values were increased at 255 µg/l, but this increase was not significant and mainly caused by an outlier in one 255 µg/L treatment group.

Histology:
Retained peritoneal attachments/gonadal duct feminization of the testis: At 255 µg/L almost all male fish (42 out of 45) exhibited feminization of gonadal ducts (classified in the study report minimal to mild). **The LOEC for gonadal duct feminization of the testis is therefore 255 µg/L.**

Stages testis development:
In general, it can be seen that at the end of the test the highest proportion of fish in all 4-tert-butylphenol-treatments are in entirely immature phase or even juvenile phase (54 to 69 %) compared to control fishes with 33 %. In contrast in the control the highest proportion of fishes (36 %) are in the next higher stage, in the stage 1 (early spermatogenic phase) or later stages. Results can be seen in Table 11 and are depicted in Figure 1.

Table 11: Testis staging results (Proportions of fish dedicated to the respective testis stage):

Gonad staging results for phenotypic male *P. promelas*					
Treatment 4-tert-butylphenol (µg/L, measured)	0	10	27	83	255
Number examined	83	41	48	46	45
Testis staging					
Stage Juvenile	5 (6)	1 (2)	14 (29)	6 (13)	5 (11)
Stage 0	22 (27)	23 (56)	19 (40)	19 (41)	25 (56)
Stage 1	30 (36)	7 (17)	11 (23)	16 (35)	8 (18)
Stage 2	19 (23)	9 (22)	2 (4)	2 (4)	6 (13)
Stage 3	7 (8)	1 (2)	2 (4)	3 (7)	1 (2)

Values in parentheses give percentage of totals examined

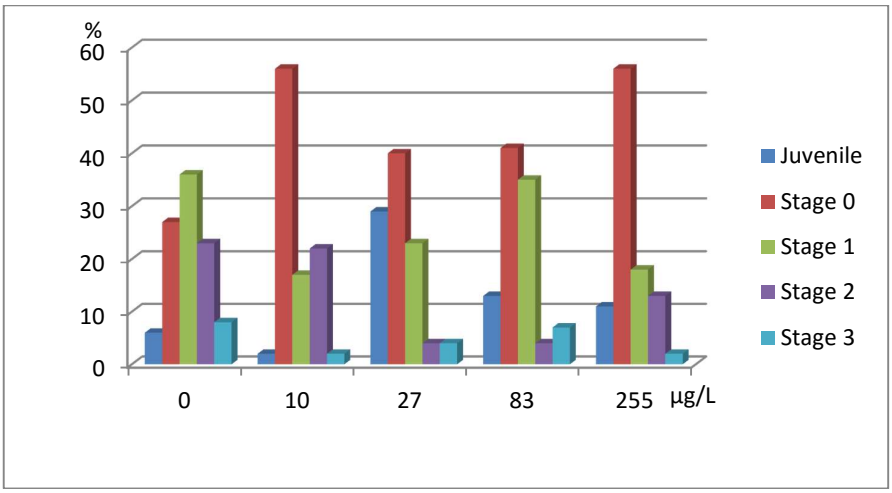


Figure 1: Testis staging results (percent fish at different stages)

Testis ova:
At 255 µg/L one fish with testis-ova was observed. This fish also had feminized gonadal ducts. In the pilot study the presence of testis-ova (minimal to mild) was observed.

Secondary sexual characteristics:
The effects were statistically significant at 27, 83 and 255 µg/L ($p \leq 0.05$).
The LOEC is 27 µg/L (Jonkheere-Terpstra trend test ($p \leq 0.05$)). See Table 12.
Five different attributes regarding secondary sexual characteristics (proportion of male fish with a pigmented spot on dorsal fin, with pigmentation on the nose/lip, with a fatpad present, fatpad score of male fish, proportion of male fish with one or more tubercles present) in male (gross internal sex) fish were significant decreased at the treatments 27, 83, 255 µg/L **(LOEC for all above mentioned secondary sex characteristics 27 µg/L)**[5]. The characteristics tubercle counts and score of male fish were significant decreased at the treatment at 255 µg/L.

Results from the pilot study were consistent with the current study, although decreases relative to the control were not as pronounced as in the final study.
It is important to note, that a significant number of male fish did not look like males

[5] A LOEC of 83 µg/L for the proportion of males with one or more tubercles present was described by Krueger et al, 2008, but calculations using the Step-down Rao-Scott-Cochran-Armitage Test Procedure revealed significance at 27 µg/L and above.

anymore at 27 µg/L and above. Success in reproduction is therefore more difficult and an adverse effect for the population is likely.

Table 12: Secondary sexual characteristics

Parameter	Control	9.6 µg/L	27 µg/L	83 µg/L	255 µg/L
Proportion of male with at least one secondary sex characteristic	0.97 ± 0.06	0.96 ± 0.07	0.91 ± 0.05 *	0.91 ± 0.06 *	0.85 ± 0.10 *†
Proportion of male fish with a pigmented spot on dorsal fin	0.92 ± 0.07	0.88 ± 0.10	0.76 ± 0.17 *†	0.80 ± 0.07 *	0.79 ± 0.14 *
Proportion of male fish with pigmentation on the nose/lip	0.91 ± 0.12	0.90 ± 0.12	0.77 ± 0.03 *	0.77 ± 0.10 *	0.65 ± 0.20 *†
Proportion of male fish with a fatpad present	0.13 ± 0.15	0.10 ± 0.12	0.02 ± 0.04 *	0.01 ± 0.02 *	0.05 ± 0.09 *
Fatpad score of male fish	0.14 ± 0.16	0.10 ± 0.12	0.02 ± 0.04 *	0.01 ± 0.02 *	0.05 ± 0.09 *
Proportion of male fish with one or more tubercles present	0.72 ± 0.16	0.75 ± 0.26	0.50 ± 0.05 **	0.56 ± 0.12 *	0.52 ± 0.29 *
Tubercle counts of male fish	7.34 ± 3.41	8.39 ± 3.22	5.25 ± 2.26	5.78 ± 1.14	5.05 ± 4.04 *
Tubercle score of male fish	7.46 ± 3.48	8.54 ± 3.30	5.30 ± 2.34	5.85 ± 1.23	5.27 ± 4.53 *

* Statistically significant decrease in comparison to the control using the Jonkheere-Terpstra trend test ($p \leq 0.05$).
† Statistically significant decrease in comparison to the control using Dunnett's test ($p \leq 0.05$).
** Statistically significant decrease in comparison to the control using Step-down Rao-Scott-Cochran-Armitage Test Procedure ($p \leq 0.05$).

Sex ratio:
The examination of the proportion of male by gross internal gonadal sex assessment showed no effects: 0.48 ± 0.09; 0.52 ± 0.05; 0.61 ± 0.09; 0.56 ± 0.10; 0.50 ± 0.08 at Control; 9.6, 27, 83, 255 µg/L (m) respectively.

However in the pilot study a shift of the sex ratio towards a lower number of males was observed at 500 µg/L.

There were altogether **24 fish whose sex could not be determined** by gross internal gonadal assessment. For these 24 fish, histological examination was used to determine their sex. One of these 24 fishes was histologically determined to be female (from treatment 30 µg/L). All other fishes were histologically determined to be male.
Distribution of the 23 male fishes over the treatments was as follows: Control, 9.6, 27, 83, 255 µg/L (m): 3, 1, 8, 6, 5 fishes, in percentage 0.9; 0.6; 5.1; 3.8; 3.1 % of all fishes in the respective treatment or control.

Growth:
Length and weight were slightly reduced at **27 µg/L (=LOEC)** and higher concentrations in males and females ($p<0.05$, Jonkheere-Terpstra trend test and Dunnett's test). The decreases in mean weight were 14 to 16 % relative to the control and did not follow a dose-response curve. The decrease in mean length was 4 to 5 % relative to the control. Similar, but not significant effects on weight and length were observed in the pilot study at 50 and 500 µg/L.
Time to hatch:
The endpoint was determined as time required for 50 % of eggs to hatch (T50).

In the control it was 4.45 days. In the treatment 255 µg/L the time to hatch was significantly increased to 4.79 days. Therefore the LOEC for time to hatch is 255 µg/L.

Survival:
Larvae/juvenile survival from post-hatch to thinning on day 33 of test at 255 µg/L was 90 % (significantly decreased). Survival rates in controls and lower test concentrations were very high. (Control: 95 %; 10 µg/L: 94 %; 30 µg/L: 96 %; 100 µg/L: 91 %; 300 µg/L: 90 %).

Summary:
Overall, study results by Krueger et al. (Krueger et al., 2008) show that 4-tert-butylphenol causes alterations in *Pimephales promelas* at 27 and 255 µg/L which are clearly diagnostic for an estrogenic mode of action:

VTG induction in females is a clear indicator of an estrogenic mode of action according to OECD 2012. VTG induction in males was not observed. Although this is a common reaction in other fish species, results obtained during OECD validation for the fish sexual development test indicate that VTG induction in males is not a sensitive parameter for *P. promelas* in fish sexual development tests. This was also observed for 4-tert-pentylphenol which did not cause VTG induction in the sexual development tests but did so in reproduction assays.

The observed feminization of gonadal ducts is also described as double attachment to the mesentery, forming an ovarian-like cavity. The forming of an ovarian-like cavity is a diagnostic criteria for estrogen endocrine disrupting properties according to OECD Guidance Document 123 (OECD, 2010).

Effects observed on secondary sex characteristic at 27 µg/l and above show that 4-tert-butylphenol causes estrogen mediated effects at even lower concentrations. These effects which are indicative of an estrogenic mode of action are usually not considered as adverse effects. However, in this case a small but significant proportion of males did not show any secondary sex characteristics. These males were visually not distinguishable from females and thus it seems likely that their reproduction would be disturbed.

Adverse effects observed on growth (LOEC 27 µg/L) are not diagnostic for an endocrine mode of action. However this parameter is known to be sensitive to an estrogenic mode of action (Knacker et al., 2010).

Although no effects on sex ratio up to 255 µg/L were observed in this study, results from the pilot study indicate that 4-tert-butylphenol does cause such estrogen diagnostic adverse effects although at higher concentrations (500 µg/L).

According to Krueger et al. (Krueger et al., 2008) effects on growth and secondary sex characteristics are considered to be caused by a slight delayed development. Although this cannot be excluded, it should be noted that delayed development is a known response to estrogen acting chemicals.

The results are summarised in Table 13 below.

Table 13: Summary of effects in *P. promelas* for 4-tert-butylphenol (4-t-BP)

Life stage/ duration	Conc. / test condition / solvent	**Vitello-genin**	**Histology**	**Fertility/ Fecundity**	**Internal gonadal examination**	**Sec. sex characteristics**	**others**	**Positive control**	**Reference**	**reliability**
Pimephales promelas Extended ELS, Newly fertilized eggs, until 123 dph; Total 128 d	10, 30, 100, 300 µg/L (n), 9.6, 27, 83, 255 µg/L (m), Flow-through; at least 5 volume additions of test solution every 24 h, No solvent used	VTG females: LOEC 255 µg/L Males: > 255 µg/L	**LOEC 255 µg/L (m)**, 42 of 45 males: **feminization of gonadal ducts** (minimal to mild) **Staging testes** Highest proportion in all 4-t-BP treatments in **entirely immature phase**, control: highest proportion in **early spermatogenic phase** At 255 µg/L: Testis ova in one fish with feminized gonadal ducts Pilot study: At 500 µg/L testis-ova and feminisation of gonadal ducts (minimal to moderate)		**Skewed sex ratio towards females at 500 µg/L (from the pilot study)**	**LOEC 27 µg/L** (m) proportion of male fish with one or more tubercles present, LOEC 27 µg/L (m) proportion of males with at least one sec sex char. Pilot study: 500 µg/L	**Growth LOEC 27 µg/L (m)** length and weight in males and females; Time to hatch sign. increased at 255 µg/L Survival post hatch sign. decreased at 255 µg/L Inflammation at 255 µg/L	**no**	(Krueger et al., 2008)	**1** The sex of 24 fish could not be determined by necropsy (gross internal sex). 23 of them were histological determined to be males. One fish was determined to be female.

5.2.2.3.2.Sander lucioperca (Pikeperch, Sander).

Demska- Zakęś (Demska-Zakęś, 2005) exposed juvenile pikeperch to 4 tert-butylphenol from 60 dph to 88 dph. The fish were further reared without exposure until 144 dph. The testing condition was semi-static with renewal of half of the test solution every 24 hours. The concentrations tested were 1; 10; 100; 200 µg/L (n). A dilution water control and a solvent control (ethanol (10 µL/L)) existed as well as two positive controls (17ß-estradiol and 4',7-dihydroxyisoflavone). In addition the following substances were tested: 4-n-heptyloxyphenol, 4-n-nonylphenol, 4-n-butylphenol, 4-sec-butylphenol, 4-n-heptylhenol, phenol, 1,6-dihydroxynaphthalene and 1,5-dihydroxynaphthalene. The test was conducted using two replicates with 80 fish per tank. The fish were kept in tanks with a water volume of 80 L under semi-static conditions (approximately 50 % water exchange per 24 h) and permanent lighting (50-60 lux). The gonads of fish were histologically examined at day 59 (before exposure), day 88 (the end of exposure) and day 144 (after additional 56 days without exposure). Two kinds of intersex are reported ovotestis (testis-ova) and formation of an oviduct (with regressed spermatogenic lobules in the same fish). In the graphs the effects are described as bisex with no further discrimination. Furthermore growth (length and weight) and the condition factor were examined.

Assessment of the study regarding reliability can be found in Annex II.

Results:

Growth:

No effects on growth and condition factor appeared.

Sex ratio and intersex:

The histological examination of the gonads revealed the following results:

On day 59 all fish in controls and treatments were sexually undifferentiated.

Control fish had an approx. equal number of males and females at day 88 and 144 and no intersex fish were seen.

On day 88 the number of males was significantly decreased at 1 µg/L and above compared to solvent control and dilution water control (LOEC 1 µg/L). The percentage of females was significantly increased compared with SC (LOEC 1 µg/L). Testis-ova were seen, not significant increased compared to SC and DWC at 1 µg/L (about 10 %) but at 10 µg/L (17 %).

On day 144 roughly the same distribution appeared: the percentage of females was increased at 1 µg/L, but this time only significantly compared to DWC and not SC. The number of males was significantly decreased compared to both controls and the number of intersex fish was significantly increased, both significantly compared to SC and DWC.

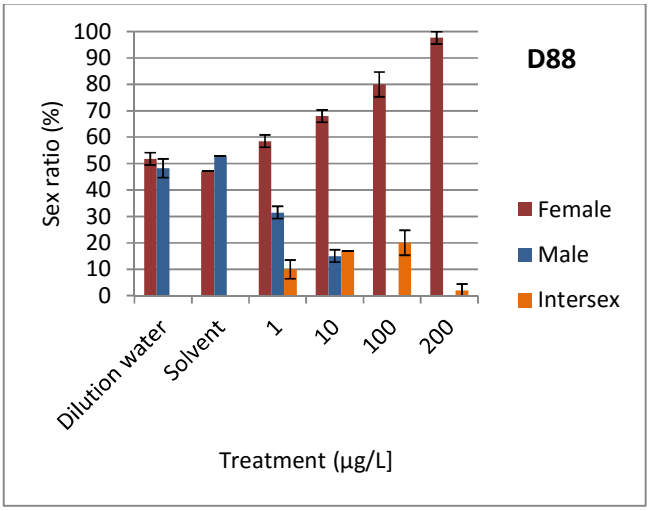
The **LOEC is 1 µg/L for sex ratio and intersex** respectively. For intersex the LOEC after 88 days is at 10 µg/L but after 144 d at 1 µg/L. At 100 µg/L and above no males were observable. See Table 14 below.

Table 14: Sex ratio and intersex in *Sander lucioperca* (values read from graph) after exposure to 4-tert-butylphenol and after a subsequent rearing of 56 days without test substance (D144). Values refer to mean numbers of fish in percent1 from a graph (Fig. 17 in Demska-Zakęś, 2005).

Treatment (µg/L)	Female	Male	Intersex	Sterile
D88				
Dilution water control	52^{ab}	48^{a}	0^{a}	0^{a}
Solvent control	47^{a}	53^{a}	0^{a}	0^{a}
1	58.5^{bc}	31.5^{b}	10^{ab}	0^{a}
10	68^{c}	15^{c}	17^{b}	0^{a}
100	80^{d}	0^{d}	20^{b}	0^{a}

200	98[e]	0[d]	2[a]	0[a]
D144				
Dilution water control	48[a]	52[a]	0[a]	0[a]
Solvent control	52[ab]	48[a]	0[a]	0[a]
1	57.5[b]	32[b]	10.5[b]	0[a]
10	68[c]	16.5[c]	15.5[b]	0[a]
100	78[d]	0[d]	22[c]	0[a]
200	100[e]	0[d]	0[a]	0[a]

Superscripts a – e: Values with the same superscript in the same column are not significantly different (P>0.05).
[1] For illustration purposes the values were estimated from the graphs of the study via manual measurement of the height of the bars and transformation via regression function to estimated values in percent. The superscripts indicating the statistical significance were taken from the original graphs.



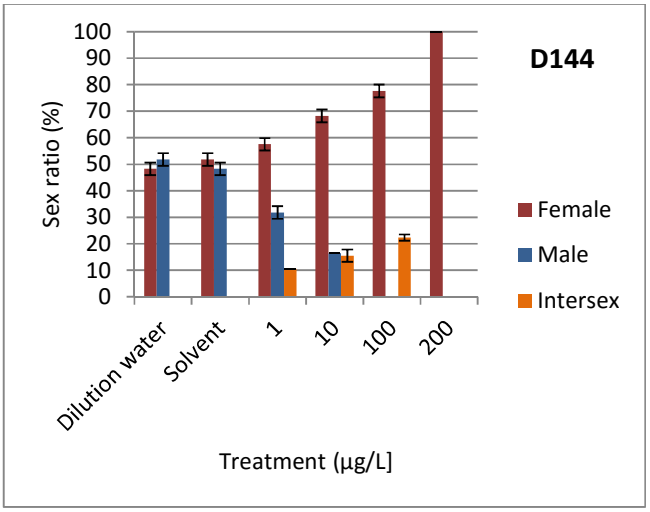


Figure 2 Sex ratio and intersex in *Sander lucioperca* after exposure to 4-tert-butylphenol (days 88 and 144). The values refer to mean numbers of fish in percent with indication of the standard deviation (n=2) and were extrapolated from a graph (Fig. 17 in Demska-Zakęś, 2005). If no error bar is indicated, the results of the particular replicates (n=2) were either equal or their variance too low for visualisation**.**

Summary:
In summary the study shows that 4-tert-butylphenol causes a sex ratio shift towards more

females and less males at 1 µg/L and above. No males were observed at the highest test concentrations (100 and 200 µg/L) Results at day 144 show that the effects on sex ratio persist even after exposure has ceased.

Incidence of testis-ova at lower concentrations but not at the highest concentration substantiate that the sex ratio shift is a result of sex-reversal. S*ander lucioperca* is a gonochoristic fish, like *Cyprinus carpio*. That means male and female gonads are developed separately and naturally intersex is rare.

All effects exerted by 4-tert-butylphenol are summarized in Table 15 and Table 22.

In order to compare results of this study for 4-tert-butylphenol with other compounds, the test results of 4-n-heptylphenol and 4-n-nonylphenol, as well as two positive controls (17ß-estradiol and dihydroxyisoflavone) are depicted in the tables 22-25 and in the figures 3 – 6 (see below). Effects on all substances examined in the study by Demska-Zakes are summarized in table 27.

Table 15: Summary of effects in *Sander lucioperca*

Life stage/ duration	Conc. / test condition / solvent	**Vitello-genin**	**Histology**	**Fertility/ Fecundity**	**Sex ratio / gonad histology**	**Sec. sex charact eristics**	**others**	**Positive control**	**Refere nce**	**reliab ility**
Sander lucioperca, Juveniles, exposure from 60 dph to 88 dph, further rearing without exposure until 144 dph	1; 10; 100; 200 µg/L (n); semi-static with renewal of the half of the test solution every 24 hours; Solvent: Ethanol (0.001 %), solvent control existed		**LOEC 1 µg/L (n) intersex**		**LOEC 1 µg/L (n)** **Sign. less males, sign. more females, sign. more intersex fish**		No effects on mortality, growth (lenght and weight), condition coefficient	**17ß-estradiol (1–200 µg/L);** at 1 µg/L sign. more females (approx. 78 %) based on gonad histology and almost 10 % intersex, at 10 µg/L only females, Significant mortality decreased body weight and lower condition coefficient at 100 and 200 µg/L, no effects on body length	(Demska-Zakęś, 2005)	**2**

5.2.2.3.3.Cyprinus carpio

A test with carp was conducted by Barse et al. (Barse et al., 2006). Adult fish were exposed for 28 d with three replicates in each treatment. Per treatment 36 fish were exposed. The test condition was semi-static, one-fourth of test solution was removed every 4 days, and a complete exchange was done once a week. Exposure concentrations ranged from 690 to 2300 µg/L. Acetone was used as solvent. A solvent control did exist, but no dilution water control. The lowest concentration used was 1/10 of the LC50 value. After exposure the fish were examined for muscle vitellogenin content. Testis, liver and kidney were examined histologically. The Klimisch reliability of the study is determined to be 2 because at least a solvent control did exist and acetone is not considered to cause endocrine effects.

Results:
VTG:
VTG was significantly induced in all exposed males but showed an inverse dose-response curve with increasing concentrations causing decreasing VTG induction. The **LOEC is 690 µg/L** and the **NOEC < 690 µg/L**.

Histomorphological observations of carp testis:
The histological architecture of testis was changed. The effects on the testis were reduction of testicular size (and thus the testiculosomatic index) necrosis of spermatozoa and the reduction in numbers of germ cells.

The **germinal epithelium cells were atrophied**. Atrophy of germinal epithelium is listed in OECD 123 (OECD, 2010) as an additional diagnostic criterion.

Others:
The testiculosomatic index (GSI) in males was reduced, the hepatosomatic index (HSI) elevated and enzyme activities disturbed (LOEC 690 µg/L). Liver degeneration, hyperplasia of connective tissue and increased vacuolization was observed.
No changes in behaviour were visible.

In summary the test shows that 4-tert-butylphenol alters the endocrine system of carp due to an estrogenic mode of action. Test concentrations were too high to conclude on the lowest observed effect concentration. Effects observed at the highest test concentration fit to the inverse VTG dose-response-curve indicating that systemic effects occurred at that concentration.
No apical endpoints were tested and thus no conclusion is possible if this alteration results in adverse effects.

The effects are summarised in table 16.

Table 16: Summary of effects in *Cyprinus carpio*

Life stage/ duration	Conc. / test condition / solvent	**Vitello-genin**	**Histology**	**Fertility/ Fecundity**	**Sex ratio / gonad histology**	**Sec. sex charact eristics**	**others**	**Positive control**	**Reference**	**reliability**
Cyprinus carpio, Modified short term screening assay, Adults, exposure 28 d,	690, 1380, 2300 µg/L (n), Semi-static, Solvent: acetone, solvent control existed, no dilution water control	VTG in males (muscle tissue homoge nate) increase d LOEC 690 µg/L, NOEC <690 µg/L	In Males: Reduction in number of germ cells, **atrophied germinal epithelium cells**, increased fibrous connective tissue				Reduced, GSI in males, Hepatosomatic index (HIS) elevated Enzyme activities disturbed LOEC 690 µg/L, NOEC <690 µg/L Liver: degeneration, hyperplasia of connective tissue, increased vacuolization No changes in behavior	**no**	(Barse et al., 2006)	**2**

5.2.2.3.4.Oryzias latipes

A reproduction assay was conducted by (Shioda and Wakabayashi, 2000) with medaka.

First reproduction trials were carried out without exposure. Then only male medaka were exposed for 14 days to 4-tert-butylphenol followed by a reproduction trial with the non-exposed females in the original spawning group. The numbers of eggs spawned were counted for one week. Eggs were transferred to dilution water for hatching. One spawning group consisted of 2 female and 1 male fish. Three of these groups were used in treatments and control. The statistical power of the test is not high, because less fish than normal were used. Furthermore, only male fish were exposed and the exposure duration is shorter compared to the short term reproduction guidelines (14 instead of 21 days).

A solvent control was not included. Therefore, the reliability is 3.

Results:

Reproduction:

At the lowest concentration of 151 µg/L (1 µmol/L), the number of hatchings was significantly decreased and eggs were unfertilized. At higher concentrations (453 and 1510 µg/L) the average number of hatchings was reduced too but this was not significant due to high replicate variances.

An effect was seen although only males were exposed and the exposure duration lasted only 14 days.

The effects are summarized in Table 17.

Effects are consistent with effects observed for 4-tert-octylphenol by Gronen et al. (Gronen et al., 1999) and although results are not reliable as such, they indicate a similar effect pattern – and thus mode of action - compared to 4-tert-octylphenol.

Table 17: Summary of effects in *O. latipes*

Life stage/ duration	Conc. / test condition / solvent	Vitello-genin	Histology	Fertility/ Fecundity	Sex ratio / gonad histology	Sec. sex charact eristics	others	Positive control	Reference	reliability
O. latipes, Reproduction assay, **only male medaka** were exposed for 14 days, then reproduction trial conducted without further exposure	151 , 453 , 1510 µg/L (n); Semistatic, half of the water changed every two days; Solvent: Acetone, <100 µL/L, no solvent control used, only dilution water control			**Sign. decreased number of hatchings due to high number of unfertilized eggs at 151 µg/L after exposure of males** (high variance at higher concentratio ns, no sign. effects)				17ß-estradiol, Exposure of females: significant decrease in the number of hatchings at 0.1 nmol/L (27 ng/L). At 1 nmol/L (270 ng/L) sign. reduced number of eggs both spawned and hatched. Exposure of males: from 3 nmol/L (817 ng/L): sign. reduced number of eggs both hatched and spawned.	(Shioda and Wakabayas hi, 2000)	**3**

5.2.2.4. Summary of the plausible causal link between adverse effects and endocrine mode of action

Analysing the plausible causal link requires information about the possible mode of action and an assessment whether or not adverse effects observed are caused by this mode of action. Information about the possible mode of action can be obtained from *in vitro* and *in vivo* studies.

5.2.2.4.1. Mode of action

For 4-tert-butylphenol the following information on an estrogenic mechanism and mode of action is available:

In vitro:

Results from *in vitro* tests are described in chapter 5.2.2.2. All *in vitro* studies showed that 4-tert-butylphenol binds to the fish estrogen receptor and activates it. It also binds to estrogen binding proteins. This finding similarly holds true for assays with mammalian receptors, although binding and activation was not as pronounced in some assays using mammalian receptors. Thus, *in vitro* studies show that 4-tert-butylphenol acts via an estrogenic mechanism of action.

In vivo:

Endpoints indicative for an estrogenic mode of action were assessed in three species (*P. promelas, S. lucioperca* and *C. carpio*). In all species all endpoints assessed (except VTG induction in males of *P. promelas*) were positive. This substantiates that 4-tert-butylphenol alters the function of the endocrine system in fish via an estrogenic mode of action.

5.2.2.4.2. Plausible causal link

A change in the sex ratio toward females is both indicative for an endocrine mode of action and adverse. Such an effect was observed in *S. lucioperca* and results indicate that it might hold true for *P. promelas* too.

Only few other adverse effects were observed. All of them are known to be sensitive for an estrogen mode of action. Effects are consistent with effects observed for other alkylphenols:

- Reduced growth and delayed development are comparable to effects observed for 4-tert-pentylphenol by Panter et al (Panter et al, 2006)
- Although results for medaka by Shioda and Wakabayashi (Shioda and Wakabayashi,2000) are considered not reliable as such, they indicate a similar effect pattern – and thus mode of action - compared to 4-tert-octylphenol as observed by Gronen et al (Gronen et al, 1999).

For these effects other modes of action cannot be entirely ruled out. However all other available information, including diagnostic effects at similar or lower concentrations, result in the conclusion that it is very plausible that they are caused by an estrogenic mode of action. The results are summarised in the Table 18 below.

Table 18: Summary of results in fish with regard to endocrine disruption. The conclusions are based on OECD GD 150 (OECD, 2012): Only studies with at least Klimisch reliability 2 are included

Species	Effects observed	Effect concentrations	Conclusion
P. promelas (extended ELS)	VTG induction in females, gonadal duct feminization, Visually no males based on secondary sex characteristics. Reduced growth	Indicative effects starting at 27 µg/L (secondary sex characteristics), adverse effects at 27 µg/L (growth, not diagnostic). Indication of diagnostic adverse effects (skewed sex ratio towards females) at 500 µg/L	Possible endocrine disruptor *in vivo* based on indicators such as VTG, gonadal duct feminisation and sec. sex-characteristics. Based on effects on secondary sex-characteristics the substance is "almost certainly an endocrine disruptor"[6]
S. lucioperca (comparable to FSDT)	Intersex, sex ratio shift towards females (no males at highest concentration),	LOEC 1 µg/L (sex ratio, indicative and adverse)	The substance is almost certainly an endocrine disruptor
C. carpio (modified short term sceening assay)	VTG induction in males, reduction of germ cells and other histological changes	LOEC ≤ 690 µg/L (lowest test concentration) (indicative effects)	Possible endocrine disruptor in vivo.

In summary, there is good evidence to conclude that 4-tert-butylphenol is an endocrine disruptor in fish.

5.2.2.5. Read-across from other alkylphenols

The conclusion is substantiated by a read across to other alkylphenols (4-tert-pentylphenol, 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 I.

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 alkyl chain, they show similar endocrine disrupting properties.
In vitro potency is comparable for all alkylphenols of the group with regard to fish and no systematic influence of the length of the alkyl-chain was observed. Similar toxico-kinetic patterns were observed for the whole group and again the length of the side chain did not influence results. In vivo data for other alkylphenols strengthen the reliability of results for 4-tert-butylphenol: Effects observed in pikeperch (*Sander lucioperca)* for 4-tert-butylphenol 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. While some data suggest that 4-tert-butylphenol might be less toxic compared to longer chain alkylphenols other data suggest the opposite and thus on balance it seems reasonable to assume that the substances are of similar toxicity to fish in long-term studies.

[6] Changes in secondary sex characteristics are usually not considered apical adverse effects. However in this case males did not look like males anymore and thus reproduction is likely to be effected. Based on this conclusion supported by results of the less reliable pilot study showing changes in sex-ratio, the conclusion is "almost certainly an endocrine disruptor"

Data for other alkyphenols support the conclusion that effects observed for 4-tert-butylphenol in fish are estrogen mediated. For example observed effects by 4-tert-butylphenol and 4-tert-pentylphenol on the gonads of *P. promelas* are very similar. For 4-tert-pentylphenol additional data are available which substantiate an endocrine mode of action in this species and thus strengthen the conclusion that the adverse effects observed 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 breaders, can be anticipated for 4-tert-butylphenol.

In summary the information available for the other alkylphenols substantiates that – with regard to fish - 4-tert butylphenol 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-butylphenol 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-butylphenol acts as an endocrine disruptor in other fish species too.

5.2.2.6 Environmental relevance

Effects observed in fish species after exposure to 4-tert-butylphenol are indicative and adverse and relevant with regard to the population level. They are considered to have the potential to impair population stability and recruitment. 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 show that 4-tert-butylphenol is an endocrine disruptor in fish. Both the types of effects observed as well as the concentrations at which effects are elicited 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) of REACH.

6.2. PBT and vPvB assessment

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

6.3. Assessment under Article 57(f)

4-tert-butylphenol 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-butylphenol is provided in chapter 5.2 Other effects.

Based on these data, 4-tert-butylphenol meets the World Health Organisation/IPCS definition of an endocrine disruptor:

- *In vitro* data unambiguously show that 4-tert-butylphenol acts as a ligand of estrogen receptors in fish and mammalian cells. Modulation of 4-tert-butylphenol-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-butylphenol has the potential to exert estrogen-like effects and disrupt endocrine homeostasis.
- The relative potency of 4-tert-butylphenol compared to 17ß estradiol ranged from 1.9E-7 to 1.6E-4 (binding and VTG induction in fish cell receptors) and was slightly lower for mammalian cell receptors.
- *In vivo* data substantiate the endocrine mode of action of 4-tert-butylphenol. Endpoints indicative for an estrogenic mode of action were affected in all fish species tested (3 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 one fish species. 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 are plausibly due to an estrogenic mode of action. But other modes of action cannot be entirely ruled out.
- Read-across from other alkylphenols to 4-tert-butylphenol substantiates these findings.

According to the OECD Guideline 150 (OECD, 2012) 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-butlphenol such observations are available for one fish species and read-across from other alkylphenols indicates that the substance can be expected to have the same effects in other species too. Thus it is concluded that 4-tert-butylphenol is an endocrine disruptor in fish.

A comparison with other alkylphenols shows that the overall *in vitro* activity of 4-tert-butylphenol

is comparable and occurring at the same concentration ranges 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-butylphenol elicits similar *in vivo* effects in *P. promelas*, *O. latipes* and *Sander lucioperca*. Some effects occurred at similar concentrations.

6.3.2. Equivalent level of concern assessment

6.3.2.1. Environment

The summary provided above shows that exposure of fish to 4-tert-butylphenol result in adverse effects such as female biased sex ratio, with halving the normal proportion of males already at 1 µg/l and complete suppression of development of males at exposure concentrations around 100 µg/l. Also other adverse effects like reduced reproduction and impaired growth have been observed. The effects seen are population relevant as they have the potential to adversely affect population structure and size and consequently ecosystem function and stability.

No data for 4-tert-butylphenol are available to assess whether or not exposure at particular sensitive life stages may result in delayed long-term effects or even intergenerational effects. However effects observed for 4-tert-butylphenol are similar to those observed for 4-tert-octylphenol and 4-nonylphenol and occur at comparable test concentrations. Therefore as described in Annex I, due to similarities in structure, physico-chemical and endocrine disrupting properties, it is possible to read across lacking information for 4-tert-butylphenol from studies available for 4-tert-octylphenol and 4-nonylphenol.

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 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-nonylphenol 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-butylphenol, 4-tert-octylphenol and 4-nonylphenol regarding to the endpoint endocrine disruption for the environment it seems very probable, that such effects could also occur after exposure to 4-tert-butylphenol.

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 (WHO/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 for alkylphenols with endocrine activity, although it may exist. 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. For example for 4-nonylphenol the most sensitive fully reliable LOEC for fish was 10 µg/L (sex-ratio *D. rerio* and growth *O.mykiss*) but effects on

semen volume in *O.mykiss* were observed at 0.75µg/l and although it is unclear whether or not this would result in adverse effects it indicates that other species and endpoints might be more sensitive.

No information is available on effects on other taxa than fish. No information about similar toxico-kinetics or binding affinity is available for other taxa such as invertebrates or amphibians. However, due to the high similarity of structures, it seems reasonable to assume that a similar pattern among the group of alkylphenols holds true and that thus read-across is possible too. For 4-tert-octylphenol and 4-nonylphenol effects on invertebrates and amphibians were observed, in some cases at concentrations lower than those causing effects in fish.

It is not possible to clearly conclude that effects on these other organisms are endocrine mediated, although 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 difficult to estimate which species may be more sensitive than fish and which concentration can be regarded as safe for the environment.

Thus, in summary, effects after exposure to 4-tert-butylphenol are considered to impair population stability and recruitment. They may occur even after exposure of sensitive life stages and thus may result in impairments in regions other than those where exposure occurred. Effects persist even after exposure has ceased and may influence population level on a long term basis e. g. due to transgenerational effects and/or changes in the gene pool. Effects may influence a wide range of taxa of environmental organisms. Consequently, for the observations and reasons listed above, the serious effects in the environment that 4-tert-butylphenol 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

4-tert-butylphenol should 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 (a) to (e) of Article 57 of REACH.

For 4-tert-butylphenol there is scientific 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-butylphenol acts as a ligand of estrogen receptor in fish and mammalian cells. Modulation of 4-tert-butylphenol-dependent and ER-mediated gene expression was observed on transcriptional, protein and cell physiological levels showing that 4-tert-butylphenol activates fish and mammal estrogen receptors.
- *In vivo* data substantiate the endocrine mode of action. Endpoints indicative for an estrogenic mode of action were affected in all fish species tested (3 species). Effects observed included Vitellogenin (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 one 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 are plausibly due to an estrogenic mode of action. But other modes of action cannot be entirely ruled out.

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 Guidance Document for endocrine disruptors (OECD, 2012) reveals that 4-tert-butylphenol needs 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-butylphenol can be considered to be an endocrine disruptor in the environment. This conclusion is supported by read-across from other alkylphenols (4-nonylphenol and 4-tert-octylphenol) with regard to the environment.

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

- Exposure to 4-tert-butylphenol resulted in effects in fish on reproduction parameters (fecundity) as well as on sexual development (changes in sex ratio) and growth. Results for one fish species show that exposure to 4-tert-butylphenol may result in a complete sex reversal resulting in all female populations. This effect is considered a serious effect to the environment.
- Read-across of the effects observed for the structurally similar 4-nonylphenol and 4-tert-octylphenol in fish show that exposure during sensitive life stages may result in effects that remain during the entire life and even in following generations and even after exposure ceased. Thus local exposure of migratory species might not only affect population stability locally but also in other areas.
- On the basis of the available data for 4-tert-butylphenol itself and from read-across it appears difficult to derive a safe level, although it may exist. 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.
    - It is not possible to clearly conclude that effects on other organisms such as invertebrates and amphibians are endocrine mediated, although steroids are known to play an important role in invertebrates (Kendall et al., 1998) and amphibians (Kortenkamp et al., 2012). Owing to the lack of in-depth knowledge of their endocrine system and the lack of test systems, it is currently difficult to estimate which species may be more sensitive than fish and which concentration can be regarded as safe for the environment.

Thus in summary, the endocrine mediated effects observed in fish after exposure to 4-tert-butylphenol and anticipated on the basis of read-across from other alkylphenols 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 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. No reliable information is available for 4-tert-butylphenol about whether it can cause ED-related adverse effects on taxa other than fish. 4-tert-octylphenol and 4-nonylphenol cause effects in amphibians and invertebrates that might be endocrine mediated, i.e. caused by an estrogen-like mode of action, although it is not possible to clearly conclude that they are endocrine mediated. Similar effects may be caused by 4-tert-butylphenol., but there are no confirmatory data. 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 (a) to (e) of Article 57 of REACH.

References

Akahori, Y., Nakai, M., Yamasaki, K., Takatsuki, M., Shimohigashi, Y., and Ohtaki, M. (2008). Relationship between the results of in vitro receptor binding assay to human estrogen receptor alpha and in vivo uterotrophic assay: comparative study with 65 selected chemicals. Toxicology in vitro : an international journal published in association with BIBRA 22, 225-231.

Baroiller, J.F., Guiguen, Y., Fostier, A. (1999). Endocrine and environmental aspects of sex differentiation. Cellular and Molecular Life Sciences 55:910-931.

Barse, A.V., Chakrabarti, T., Ghosh, T.K., Pal, A.K., and Jadhao, S.B. (2006). One-tenth dose of LC50 of 4-tert-butylphenol causes endocrine disruption and metabolic changes in Cyprinus carpio. Pesticide Biochemistry and Physiology 86, 172-179.

Blair, R.M., Fang, H., Branham, W.S., Hass, B.S., Dial, S.L., Moland, C.L., Tong, W., Shi, L., Perkins, R., and Sheehan, D.M. (2000). The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. Toxicological sciences : an official journal of the Society of Toxicology 54, 138-153.

Cravedi, J.P., Zalko, D. (2005). Chapter 5 Metabolic fate of nonylphenols and related phenolic compounds in fish. Biochemistry and Molecular Biology of Fishes, 6, 153-169

Demska-Zakęś, K. (2005). Wpływ wybranych ksenobiotyków na rozwój układu płciowego ryb. (Olsztyn, Uniwersytet Warminsko-Mazurski w Olsztynie - UWM Olsztyn), p. 61.

ECB (2008). P-Tert-Butylphenol CAS 98-54-4 Risk Assessment final approved version. In EU Risk Assessment Report (Oslo, Norwegian Pollution Control Authority), p. 245.

ECHA (2011). SVHC Support document- 4-(1,1,3,3-tetramethylbutyl)phenol, 4-tert-octylphenol. (ECHA).

ECHA (2012). SVHC Support document - 4-nonylphenol, branched and linear. (ECHA).

Environment and Health Canada (2001). Priority substances list assessment report, nonylphenol and its ethoxylates, Canadian Environmental Protection Act, 1999 http://www.hc-sc.gc.ca/ewh-semt/alt_formats/hecs-sesc/pdf/pubs/contaminants/psl2-lsp2/nonylphenol/nonylphenol-eng.pdf

Freitag, D., Lay, J.P., Korte, F. (1984). Environmental hazard profile – test results as related to structures and translation into the environment. Environmental Toxicol;111-36.

Gronen, S., Denslow, N.D., Manning, S., Barnes, S., Barnes, D., and Brouwer, M. (1999). Serum Vitellogenin Levels and Reproductive Impairment of Male Japanese Medaka (Oryzias latipes) Exposed to 4-tert-Octylphenol. Environ Health Perspect 107, 385-390.

Helfman, G., Collette, B.B., Facey, D.E. (1997). The Diversity of Fishes, 1st edn (Blackwell Science Ltd.).

Hornung, M.W., Tapper, M.A., Denny, J.S., Kolanczyk, R.C., Sheedy, B.R., Hartig, P.C., Aladjov, H., Henry, T.R., and Schmieder, P.K. (2014). Effects-based chemical category approach for prioritization of low affinity estrogenic chemicals. SAR and QSAR in environmental research 25, 289-323.

IPCS. 2002. Global assessment of the state-of-the-science of endocrine disruptors. Damstra T, Barlow S, Bergman A, Kavlock R, Van der Kraak G, editors, WHO.

Jobling, S., and Sumpter, J.P. (1993). Detergent components in sewage effluent are weakly oestrogenic to fish: An in vitro study using rainbow trout (Oncorhynchus mykiss) hepatocytes. Aquatic toxicology 27, 361-372.

Jonsson, G., Stokke, T.U., Cavcic, A., Jørgensen, K.B., Beyer, J. (2008): Characterization of alkylphenol metabolites in fish bile by enzymatic treatment and HPLC-fluorescence analysis. Chemosphere. Apr 71(7), 1392-400.

JRC (2013). Key scientific issues relevant to the identification and characterisation of endocrine disrupting substances. Report of the Endocrine Disrupters Expert Advisory Group. (European commission).

Kendall, R.J., Dickerson, R.L., Suk, W.A., and Giesy, J.P. (1998). Principles and Processes for Evaluating Endocrine Disruption in Wildlife (Society of Environmental Toxicology & Chemistry).

Knacker, T., Boettcher, M., Frische, T., Rufli, H., Stolzenberg, H.C., Teigeler, M., Zok, S., Braunbeck, T., and Schafers, C. (2010). Environmental effect assessment for sexual endocrine-disrupting chemicals: Fish testing strategy. Integrated environmental assessment and management 6, 653-662.

Korner, W., Hanf, V., Schuller, W., Bartsch, H., Zwirner, M., and Hagenmaier, H. (1998). Validation and application of a rapid in vitro assay for assessing the estrogenic potency of halogenated phenolic chemicals. Chemosphere 37, 2395-2407.

Kortenkamp, A., Evans, R., Martin, O., McKinlay, R., Orton, F., and Rosivatz, E. (2012). State of the art assessment of endocrine disrupters, Final Report Annex 1 Summary of the state of the science. pp. 1-486.

Kowalczyk, A., Martin, T J, Price, O.R., Snape, J.R., van Egmond, R.A., Finnegan, C.J., Schäfer, H., Davenport, R.J., Bending, G.D. (2015). Refinement of biodegradation tests methodologies and the proposed utility of new microbial ecology techniques. Ecotoxicology and Environmental Safety, 111, 9–22.

Krueger, H.O., Kendall, T.Z., Martin, K.H., and Nison, W.B. (2008). Final report para-tertiary butyl phenol (PTBP): A Partial Life Cycle Test with the Fathead Minnow (Pimephales promelas). (Wildlife Interantional, Ltd.), unpublished.

Kuiper, G.G.J.M, Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., Van Der Saag, P.T., Van Der Burg ,B., Gustafsson, J.-A. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology, 139 (19), 4252 -4263.

Kwack, S.J., Kwon, O., Kim, H.S., Kim, S.S., Kim, S.H., Sohn, K.H., Lee, R.D., Park, C.H., Jeung, E.B., An, B.S., and Park, K.L. (2002). Comparative evaluation of alkylphenolic compounds on estrogenic activity in vitro and in vivo. Journal of toxicology and environmental health. Part A 65, 419-431.

Lee, K.W., Raisuddin, S., Hwang, D.S., Park, H.G., Dahms, H.U., Ahn, I.Y., and Lee, J.S. (2008). Two-generation toxicity study on the copepod model species Tigriopus japonicus. Chemosphere 72, 1359-1365.

Madsen, L.L., Korsgaard, B., Bjerregaard, P. (2003): Estrogenic effects in flounder *Platichthys flesus* orally exposed to 4-*tert*-octylphenol. Aquat Toxicol. 2003 Sep 10;64(4):393-405.

Marcial, H.S., Hagiwara, A., and Snell, T.W. (2003). Estrogenic compounds affect development of harpacticoid copepod Tigriopus japonicus. Environmental Toxicology and Chemistry 22, 3025-3030.

Milligan, S.R., Khan, O., and Nash, M. (1998). Competitive binding of xenobiotic oestrogens to rat alpha-fetoprotein and to sex steroid binding proteins in human and rainbow trout (Oncorhynchus mykiss) plasma. General and comparative endocrinology 112, 89-95.

Ministry of Health and Welfare, Japan. (1996). Toxicity Testing Reports of Environmental Chemicals, Vol. 4, p-tert-Butylphenol. Ministry of Health and Welfare, Japan.

Myllymaki, S., Haavisto, T., Vainio, M., Toppari, J., and Paranko, J. (2005). In vitro effects of diethylstilbestrol, genistein, 4-tert-butylphenol, and 4-tert-octylphenol on steroidogenic activity of isolated immature rat ovarian follicles. Toxicology and applied pharmacology 204, 69-80.

Nakamura, M., Kobayashi, T., Chang, X.T., Nagahama, Y. (1998). Gonadal sex differentiation in teleost fish. J Exp Zool 281(5):362-372.

Nishihara, T., Nishikawa, J.I., Kanayama, T., Dakeyama, F., Saito, K., Imagawa, M., Takatori, S., Kitagawa, Y., Hori, S., and Utsumi, H. (2000). Estrogenic activities of 517 chemicals by yeast two-hybrid assay. Journal of Health Science 46, 282-298.

OECD (2004). Detailed review paper on fish csreening assays for the detection of endocrine active substances. 1-170.

OECD (2009). OECD guideline for the testing of chemicals- Fish Short Term Reproduction Assay (229). 1-40.

OECD (2010). Guidance document on the diagnosis of endocrine-related histopathology in fish gonads. pp. 1-114.

OECD (2012). Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption. No. 150. ENV/JM/MONO(2012)22. (Paris, France).

Olsen, C.M., Meussen-Elholm, E.T., Holme, J.A., and Hongslo, J.K. (2002). Brominated phenols: characterization of estrogen-like activity in the human breast cancer cell-line MCF-7. Toxicology letters 129, 55-63.

Olsen, C.M., Meussen-Elholm, E.T.M., Hongslo, J.K., Stenersen, J., and Tollefsen, K.E. (2005). Estrogenic effects of environmental chemicals: An interspecies comparison. Comparative Biochemistry and Physiology - C Toxicology and Pharmacology 141, 267-274.

Panter, G.H., Hutchinson, T.H., Hurd, K.S., Bamforth, J., Stanley, R.D., Duffell, S., Hargreaves, A., Gimeno, S., and Tyler, C.R. (2006). Development of chronic tests for endocrine active chemicals. Part 1. An extended fish early-life stage test for oestrogenic active chemicals in the fathead minnow (Pimephales promelas). Aquatic toxicology 77, 279-290.

Piferrer, F. 2001. Endocrine sex control strategies for the feminization of teleost fish. Aquaculture 197:229-281.

RAC (2016): Committee for Risk Assessment, RAC: Opinion proposing harmonised classification and labelling at EU level for 4-tert-butylphenol (CAS 98-54-4). 03.06.2016. https://www.echa.europa.eu/documents/10162/da9d846d-4c26-4a98-9106-8a42b0e459b7

Routledge, E.J., and Sumpter, J.P. (1997). Structural features of alkylphenolic chemicals associated with estrogenic activity. The Journal of biological chemistry 272, 3280-3288.

Shioda, T., and Wakabayashi, M. (2000). Evaluation of reproductivity of medaka (Oryzias latipes) exposed to chemicals using a 2-week reproduction test. In Water Science and Technology, pp. 53-60.

Soto, A.M., Sonnenschein, C., Chung, K.L., Fernandez, M.F., Olea, N., and Serrano, F.O. (1995). The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environmental health perspectives 103 Suppl 7, 113-122.

Sundt, R.C. (2009): Uptake and tissue distribution of C4-C7 alkylphenols in Atlantic cod (*Gadus morhua*): relevance for biomonitoring of produced water discharges from oil production. Mar Pollut Bull. 2009 Jan;58(1), 72-9.

Tollefsen, K.E. (2007). Binding of alkylphenols and alkylated non-phenolics to the rainbow trout (Oncorhynchus mykiss) plasma sex steroid-binding protein. Ecotoxicol Environ Saf 68, 40-48.

Tollefsen, K.E., Eikvar, S., Finne, E.F., Fogelberg, O., and Gregersen, I.K. (2008). Estrogenicity of alkylphenols and alkylated non-phenolics in a rainbow trout (Oncorhynchus mykiss) primary hepatocyte culture. Ecotoxicol Environ Saf 71, 370-383.

Tollefsen, K.E., and Julie Nilsen, A. (2008). Binding of alkylphenols and alkylated non-phenolics to rainbow trout (Oncorhynchus mykiss) hepatic estrogen receptors. Ecotoxicology and environmental safety 69, 163-172.

Tollefsen, K.E., Ingebrigtsen, K., Olsen, A.J., Zachariassen, K.E., Johnsen, S. (1998): Acute Toxicity and Toxicokinetic of 4-Heptylphenol in juvenile Atlantic Cod (*Gadus Morhua L.*). Environmental Toxicology, 17(4), 740–746.

WHO/IPCS (2002). Global assessment of the state-of-the-science of endocrine disruptors. WHO/PCS/EDC/02.2. (World Health Organization/International Programme on Chemical Safety), p. 180 p.

Xiao, X., Xing, C., He, G., Zuo, X., Nan, J., Wang, L. Solvethermal synthesis of novel hierarchical Bi4O5I2 nanoflakes with highly visible light photocatalytic performance for the degradation of 4-tert-butylphenol. Applied Catalisis B. Environmental Vol. 148-149, 27 April 2014 (14-163)

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
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
QSAR	Quantitative structure-activity relationship
PgR	Progesteron receptor
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 and supplement the findings for 4-tert-butylphenol, 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-pentylphenol (p-(1,1-dimethylpropyl)phenol)

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

These 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 alkyl chain 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-pentylphenol are proposed as SVHC due to their endocrine disruption properties for the environment in parallel with 4-tert-butylphenol.

The source substances above have a higher alkyl chain length than 4-tert-butylphenol. Data for source and target chemicals are summarised in order to analyse whether or not this influences the parameters relevant for read-across of endocrine disrupting properties with regard to the environment.

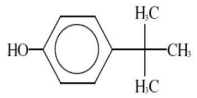
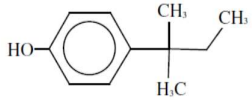
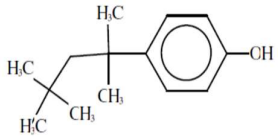
With regard to physico-chemical properties such as $\log K_{ow}$ and water solubility and bioaccumulation it is anticipated that they follow a linear trend within this group due to increasing lipophilicity with increasing alkyl chain length.

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 hydroxygroup 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 19: 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-tertoctyphenol and 4-nonylphenol branched and linear. Specific substance identities are included in cases were relevant (4-heptylphenol branched and linear and 4-nonylphenol branched and linear) or if differing from the substance under evaluation.

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear (4-HPbl)	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 [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]	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	$C_{10}H_{14}O$	$C_{11}H_{16}O$	$C_{13}H_{20}O$	$C_{14}H_{22}O$	$C_{15}H_{24}O$

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear (4-HPbl)	4-tert-octylphenol	4-nonylphenol
Molecular weight (g/mol)	150.2176	164.244	192.3 (mono-subst.) 290.5 (di-subst.)	206.32	220.35
PHYSICO-CHEMICAL PROPERTIES					
Physical state at 20°C and 101.3 kPa	Solid (flakes)	Solid (flakes)	liquid at 20°C and 101.3 kPa (phenol, heptyl derivs. (EC No. 276-743-1))	Solid	pale yellow viscous liquid (4-nonylphenol, CAS-No: 104-40-5)
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 (phenol, heptyl derivs. (EC No. 276-743-1))	19 mg/L at 22 °C	Ca. 5.7 mg/L at 25°C (4-nonylphenol (branched), CAS-No: 84852-15-3)
Partition coefficient n-octanol/water (log Kow)	3.0 at 23 °C, pH = 5.7 3.42 (EPISUITE v4.11)	3.6 at 22 °C, pH 6 – 7 3.91 (EPISUITE v4.11)	4.78 – 5.01 (EPISUITE v4.11) for 117 heptylphenol isomers	4.12 at 20.5°C (OECD 107, shake flask method) 3.7, temperature not indicated 5.28 (EPISUITE v4.11)	5.4 at 23°C, pH 5.7 (4-nonylphenol (branched), CAS-No: 84852-15-3) 5.99 for 4-n-nonylphenol (EPISUITE v4.11) 5.77 for a branched 4-tert-nonylphenol (C(C)(C)(c1ccc(O)cc1)CCC(C)(C)C)
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}	Hornung et al. 2014: RBA =9.4 x10^{-5}	Hornung et al. 2014: 5 different isomers tested (1 linear, 4 branched, containing a variety of

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear (4-HPbl)	4-tert-octylphenol	4-nonylphenol
			RBA for 4-tert-Heptylphenol: 1.4 $x10^{-4}$		secondary, but mostly tertiary branched isomers): 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} (4-n-heptylphenol)	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 (substance identity not specified in more detail) 10^4-fold > those of the maxium displacement achieved was E2 required to produce similar amounts of displacement of specifically bound - [^{3}H]E2 Maximum displacement achieved: ca. 60%	Knudsen and Pottinger, 1999: Concentrations of alkylphenols including octylphenol 10^4-fold > those of the maxium displacement achieved was E_2 required to produce similar amounts of displacement of specifically bound [^{3}H]E2 Maximum displacement achieved: ca. 45%	Knudsen and Pottinger, 1999: Concentrations of alkylphenols including nonylphenol (substance identity not specified in more detail) 10^4-fold > those of the 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 (4-n-heptylphenol);: ERα-RBA =0.00163; ERß no binding	Satoh and Nagai, 2002: ERα-RBA =0.008; ERß-RBA =0.00708;	Satoh and Nagai, 2002 (4-n-nonylphenol): ERα-RBA = 0.0222; ERß-RBA = 0.0213
	Akahori et al., 2008: RBA = 2.3 x 10^{-5}	Akahori et al., 2008: RBA = 1.7 x 10^{-4}			

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear (4-HPbl)	4-tert-octylphenol	4-nonylphenol
	Olsen et al, 2005: RBA 2.1 x 10^{-6}			Olsen et al, 2005: RBA 6.4 x 10^{-5}	
Rat	Blair et al, 2000: RBA = 2.4x 10^{-6}	Blair et al, 2000: RBA = 5 x 10^{-6}	Laws et al., 2006: RBA (4-n-heptylphenol)= 1.24 x 10^{-5}	Blair et al, 2000: RBA 1.4 x 10^{-4}	Blair et al, 2000: RBA = 3.7 – 1.9 x 10^{-} (different 4-nonylphenols either technical or 85-96% purity 4-n-nonylphenol RBA = 3.2 x 10^{-5}
Binding to sex steroid-binding protein					
Rainbow trout	Tollefsen, 2007: RBA = 6.1 x 10^{-6}	Tollefsen, 2007: RBA = 4.3 x 10^{-5}	Tollefsen, 2007: RBA (4-n-heptylphenol)= 6.6 x 10^{-6}	Tollefsen, 2007: RBA = 1.3 x 10^{-5}	Tollefsen, 2007: 4-n-Nonylphenol 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 (4-n-heptylphenol)	Tollefsen et al, 2008: LOEC = 1 µM	Tollefsen et al, 2008: LOEC = 30 µM (4-n-nonylphenol)
	Jobling and Sumpter, 1993: REP 1.6 x 10^{-4}			Jobling and Sumpter, 1993: REP 3.7 x 10^{-5} 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 (4-n-heptylphenol)	Terasaka et al., 2006: R-value (statistical correlation) for EstrArray: 0.75	Terasaka et al., 2006: R-value (statistical correlation) for EstrArray: 0.9

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear (4-HPbl)	4-tert-octylphenol	4-nonylphenol
					(4-nonylphenol, identity not further specified)
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 = $3E^{-4}$ (4-nonylphenol (95%))
		Schultz et al , 2000: EC_{50} = 4.76 µM		Schultz et al , 2000: EC_{50} = 0.177 µM	
	Nishihara et al., 2000: REC10 3 $x10^{-5}$		Nishihara et al., 2000: negative (4-n-heptylphenol)	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}			4-nonylphenol (not further specified): 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 (technical nonylphenols (85% para- isomers)
TOXICOKINETICS AND BIOACCUMULATION IN FISH					
Toxico-kinetics in fish Absorption	Rapid uptake via seawater (co-exposure) in Atlantic cod, steady state reached within 24 h	Rapid uptake of 4-n-pentylphenol via seawater (co-exposure) in Atlantic cod, steady state reached within 24	Rapid uptake via seawater or spiked feed (co-exposure) in Atlantic cod, steady	Steady state conditions in the whole fish (*Oncorhynchus mykiss*) were reached after 4	Steady state reached within 12 h in rainbow trout (Lewis and Lech, 1996) ([^{14}C]-ring uniformly labelled

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear (4-HPbl)	4-tert-octylphenol	4-nonylphenol
	(or 48 h exposure via spiked feed) (Sundt et al, 2009).	h (or 48 h exposure via spiked feed) (Sundt et al, 2009).	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). (^{3}H-4-n-heptylphenol (CAS no 1987-50-4)	days in a flow-through system (ECHA, 2011).	nonylphenol (purity >99%).)
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). (^{3}H-4-n-heptylphenol (CAS no 1987-50-4)	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]-nonylphenol residues were highest in bile after 14 h waterborne exposure in rainbow trout. (Lewis and Lech, 1996) ([^{14}C]-ring uniformly labelled nonylphenol (purity >99%)). [^{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) (4-tert-butylphenol (purity 99.5%)	Predominant metabolic pathway: conjugation to glucuronic acid. (Jonsson et al, 2008) (4-n-heptylphenol (purity 98.5%)	Predominant metabolic pathway: conjugation to glucuronic acid. (Jonsson et al, 2008)	Metabolic pathway:hydroxylation and/or conjugation to glucuronic acid (Ferreira-Leach and Hill cited in Cravedi and Zalko, 2005).	Predominant metabolic pathways for 4-n-nonylphenol and branched nonylphenols: conjugation to glucuronic acid and

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear (4-HPbl)	4-tert-octylphenol	4-nonylphenol
					oxidative biotransformation of the alkyl side-chain (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) (4-[^{14}C]heptylphenol (1 Ci/mol), containing 2 para-substituted isomers of heptylphenol: a branched and a n-heptylphenol Half-life 10 – 20 hours (Atlantic cod), rapid excretion via bile and feces (Sundt et al. 2009) (^{3}H-4-n-heptylphenol (CAS no 1987-50-4)	Excretion via bile and feces. Half-live 7.7 h in medaka (Cravedi and Zalko, 2005).	Half-live of 0.8 d in rainbow trout ([^{14}C]-ring uniformly labelled nonylphenol; purity >99%), (Lewis and Lech, 1996) 1.2 to 1.4 days in fathead minnow and 4 days in Atlantic salmon (test substance: technical nonylphenol) (Environment and Health Canada 2001). Excretion via bile and feces. Half-life 9.9 h in medaka, (Tsuda et al. cited in 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	The bioaccumulation potential in aquatic organisms is low to moderate. The experimentally determined BCF ranges between 12 and 471	No data in SVHC dossier

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear (4-HPbl)	4-tert-octylphenol	4-nonylphenol
			potential is moderate to low. (4-[14C]heptylphenol (1 Ci/mol), containing 2 para-substituted isomers of heptylphenol: a branched heptylphenol and n-heptylphenol.		
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 (Identity not further specified)
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 (Identity not further specified)
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 (Identity not further specified)
ENDOCRINE EFFECTS IN FISH (NOECs/LOECs in mg/L if not stated otherwise)					
Sander lucioperca					
Effects on sex ratio (histological)					

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear (4-HPbl)	4-tert-octylphenol	4-nonylphenol
Decrease of male fish	28d-NOEC: <0.001(nom.) 28d-LOEC: 0.001 (nom.) Demska-Zakęś (2005)		4-n-heptylphenol: 28d-NOEC: <0.001(nom.) 28d-LOEC: 0.001 (nom.) Demska-Zakęś (2005)		4-n-nonylphenol: 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)		4-n-heptylphenol: 28d-NOEC: 0.001 (nom.) 28d-LOEC: 0.010 (nom.) Demska-Zakęś (2005)		4-n-nonylphenol: 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)		4-n-heptylphenol: 28d-NOEC: <0.001 (nom.) 28d-LOEC: 0.001 (nom.) Demska-Zakęś (2005)		4-n-nonylphenol: 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)		4-n-heptylphenol: 28d-NOEC: >0.2 (nom.) 28d-LOEC: >0.2 (nom.) *S. lucioperca* (Demska-Zakęś, 2005)		4-n-nonylphenol: 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 testis stages 0.5 sex ratio (increase	0.18 VTG (increase females) (Panter et al, 2006) 0.093 VTG (decrease females) (OECD, 2011a) 0.056 feminisation gonadal duct (Panter et al, 2006)			

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear (4-HPbl)	4-tert-octylphenol	4-nonylphenol
	females)[7] 0.027 SSC 0.027 growth (m + f)´ 0.255 time to hatch, survival post hatch (Krueger et al, 2008)	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)>			4-nonylphenol, technical grade,branched chain isomers: 0.071 fecundity (4-nonylphenol, complex isomeric mixture of 4-NP with minor (<10%) amounts of 2-NP, 4-octylphenol, and dodecylphenol) 0.00025 behaviour 0.015 VTG 4-nonylphenol, technical grade,

[7] From pilot study

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear (4-HPbl)	4-tert-octylphenol	4-nonylphenol
		0.560 survival, hatchability (Panter et al, 2010)			branched chain isomers: 0.071 secondary sexual characteristics
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)			4-nonylphempol, technical grade: 0.01 skewed sex ratio 0.1 Gametogenesis females 0.01 Gametogenesis males 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, 2012) 0.094 testis-ova (OECD, 2012) 0.010 - 0.318 sex ratio [(less males) Hagino et al, 2001, OECD, 2012) 0.100 SCC (Hagino et al, 2001) > 0.317 hatch, survival		0.011 VTG 0.023 testis-ova 0.0481 sex ratio	4-nonylphenol, 97.4% purity as a mixture of isomers (not further specified): 0.012 VTG and testis-ova 0.024 sex ratio
Reproduction Assay				0.02 VTG $\leq$ 0.02 fertility	4-nonylphenol, Identity not further specified: 0.005 VTG

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear (4-HPbl)	4-tert-octylphenol	4-nonylphenol
					4-nonylphenol, 97.4% purity, mixture of isomers (Identity not further specified: 0.184 Inhibition of spermatogenesis 0.0061 fecundity and fertility
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 $\leq$ 0.01 fertility	4-nonylphenol, 97.4% purity, mixture of isomers: 0.018 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					4-nonylphenol, 98% purity, technical

Parameters	4-tert-butylphenol	4-tert-pentylphenol	4-heptylphenol, branched and linear (4-HPbl)	4-tert-octylphenol	4-nonylphenol
					grade; Identity not further specified 0.00105 VTG 4-tertiary-nonylphenol, mixture of different isomers and oligomers 0.01 Growth
Reproduction Assay & other				0.039 VTG ≤ 0.039 increased percentage of early sperm stages (spermatogonia), reduced GSI in initial experiment	technical nonylphenol, which consisted of 98% nonylphenol isomers (90% 4-nonylphenol, 10% 2-nonylphenol: 0.001 VTG 0.01 VTG (F1 without exposure) 4-nonylphenol, 95% 4-substituted isomers) 0.037 Inhibition of spermatogenesis 4-nonylphenol, 99% purity, mixture of isomers; Identity not further specified: 0.086 non developed ovaries technical nonylphenol, which consisted of 98% nonylphenol isomers (90% 4-nonylphenol, 10% 2-nonylphenol) and 2% dinonylphenol: 0.01 sexual steroids in F1

Analogue approach justification

Overall, data collected in Table 19 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 $\log K_{ow}$ (3.0 for 4-*tert*-butylphenol and 5.4 for 4-nonylphenol, according to calculated EPISUITE results) 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 at very similar ranges (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 studies no correlation with the length of the alkyl chain was observed.

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 Blairs 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 magnitude.
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 gave positive results. No trend was obsered 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 transcriptional activation assays positive results were obtained for all alkylphenols, though not in every assay. While some results indicate a linear trend others do not.

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. Similar results were observed for 4-tert-octylphenol and 4-nonylphenol. 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. This was observed for both linear and nonlinear alkylphenols. With regard to non-linear alkylphenols subsequent or prior to glucoronidation, also oxidative biotransformation of the alkyl side-chain occurs. Linear side chain alkylphenols may enter the ß-oxidation pathway thereby producing shorter side-chain carboxylic acid metabolites. Branched APs are not expected to undergo a complete breakdown of the alkyl side chain by ß-oxidation. Thus these isomers are expected to produce more hydroxylated metabolites."

Alkyphenols were mainly excreted via bile and feces with similar half-lives that range from 10 to 20 hours (for water or feed exposure).

In summary, comparison is hampered by the fact that in some cases only the linear alkylphenols were tested. However, data available for 4-tert-butylphenol, 4-tert-octylphenol and 4-tert-nonylphenol indicate similar uptake, distribution, metabolism and elimination patterns across the group of non-linear alkylphenols with no systematic influence of the length of the alkyl-chain.

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) to 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 0.027 mg/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 0.195 mg/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 0.225 mg/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-butylphenol 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-butylphenol and

can be used to substantiate the effects observed for 4-tert-butylphenol 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-butylphenol and can be used to substantiate the conclusions made for 4-tert-butylphenol 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-butylphenol. 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 - Detailed description of long term study with *Sander lucioperca* (Demska-Zakęś, 2005)

In one available long term study with fish (pikeperch, *Sander lucioperca*) the effects of 4-tert-butylphenol and other substances on mortality, development (weight, length, condition factor[8], gonads) and sex ratio (based on histological examination) were investigated (Demska-Zakęś, 2005). Sexually undifferentiated fish from artificial spawning were collected after being held in recirculation system. A preliminary fish selection was performed by excluding either too slow or too fast growing individuals. The fish were exposed to 4-tert-butylphenol from 60 days post hatch (dph) until 88 dph. These 28 days of exposure were followed by 56 days of rearing without test substance (until 144 dph). The test included a dilution water control (dwc), a solvent control (sc) (ethanol, 10 µL/L) and four treatment concentrations of 1, 10, 100, 200 µg/L (nominal) for 4-tert-butylphenol and as well for the positive controls (17ß-estradiol and 4',7-dihydroxyisoflavone) and the other tested substances: 4-n-heptylphenol, 4-n-heptyloxyphenol, 4-n-nonylphenol, 4-n-butylphenol, 4-*sec*-butylphenol, phenol, 1,6-dihydroxynaphthalene and 1,5-dihydroxynaphthalene(for each of the -in total three - sequential test series a selection of the test substances was tested in parallel[9]). The purity of the test compound 4-tert-butylphenol was about 97% (Demska-Zakes, 2016, personal communication). Per treatment 80 fish per tank were tested in two replicates (overall 160 fish per treatment, dwc and solvent control respectively). The fish were kept in tanks with a water volume of 80 L under semi-static conditions (approximately 50% water exchange per 24h) and permanent lighting (50-60 lux). Each tank was separately filtered by a biological filter (filter performance was 4 L/min corresponding to the 3-fold tank volume per hour). The test temperature was 22.0±0.5°C.

The stocking density was about 2 and 7 kg biomass/m³ at d 59 and d 88 with fish body weights of 2 and 9 g respectively and thus below the range propose for aquaculture (10-15 kg³ for fish weights up to 10 g. At d 144 the density was 38 kg biomass/m³ and thus only slightly above the recommended value of 30 kg/m³ (Schmidt and Kühn, 2013). The latter is not deemed crucial as the water quality parameter were still fulfilled.

The test is rated with Klimisch 2, as the study is well conducted although the study is not an OECD Guideline study, and raw data are not available: The number of fish in treatments and controls was high and the results show high consistency. All physico-chemical parameters regarding testing conditions like temperature, pH, oxygen concentration (table 29) were measured and remained constant during test duration. Fish data regarding length, weight and mortality (table 27 and table 28) were measured, as well were male, female and intersex fishes histologically determined. The dose response curves of treatments and positive controls are stringent and give a consistent picture of the effects. For the alkylphenols tested, estrogenic mediated effects were seen in the absence of mortality. No systemic or endocrine mediated effects were observed in the negative controls.

No measurements of the test concentrations were made and this includes some uncertainties with regard to the actual test concentration. Nevertheless, as nominal concentrations in semi-static conditions are considered worst case assumptions of real test concentrations due to possible degradation and adsorption and surface activity during the test, this study is considered valid in assuming that the actual test concentrations were not likely higher than the nominal, but probably lower. The latter is not affecting the results for clear endocrine effects as the NOEC for decrease in male fish and appearance of intersex species is below the lowest nominal test concentration and therefore probably also below the assumed actual test concentration. Cross

[8] Condition factor =100*bodyweight*length-³

[9] Test series I: 17 ß-estradiol, 4',7-dihydroxyisoflavone, 1,5- and1,6-dihydroxynaphthalene Test series II: 4-n-heptylphenol, 4-n-heptyloxyphenol, 4-n-nonylphenol and phenol Test series III: 4-n-butylphenol, 4-sec-butylphenol and 4-*tert*-butylphenol

contaminations seems unlikely this would probably result in inconsistent effects which was not the case.

The solvent control was adjusted to an equal value in every test concentration (Demska-Zakes, 2016, personal communication). The solvent (ethanol) used is a recommended solvent according to OECD (OECD Guidance 23) and its concentration is below the maximum solvent concentration recommended by OECD (0.1 ml/L). The test design of the study is shown in table 20. The study is compared to OECD Guideline 234 in table 21. In spite of some differences to OECD Guideline 234 it can be stated, that overall the test design of the study by Demska-Zakes is well elaborated and fit to reliably assess estrogenic mediated adverse effects. The study is in Polish, but most relevant parts are either available in English or were discussed with the author. Due to the fact that the overall study is not available in English, a detailed robust study summary is provided. The study was subject to a review process which included three reviewers and a habilitation colloquium[10].

For the statistical analyses of data, the software STATISTICA© was used. To compare differences between the groups, one-way ANOVA was performed. Fish were categorized in four groups (females, males, bisex individuals and sterile individuals). For each of these categories the difference between each test concentration versus other test concentrations, dilution water control and solvent control was tested (Demska-Zakes, personal communication). If the differences between the groups were significant ($P \leq 0,05$), the post-hoc Tukey HSD (Honestly Significant Difference) test was conducted. The percent values of mortality and sex ratio were arcsin transformed before the statistical analysis.

Sex ratio and general information on *Sander lucioperca*:

Although the pikeperch is not a validated OECD species, it is a very important fresh water species for agriculture and therefore well investigated also by the study author indicated by several publications (e. g. Zakęś, Z. and Demska-Zakęś, K. (1998), Wlasow et al. (2010), Kowalska et al. (2012) and Jarmolowicz et al. (2014)).
Furthermore historical data about the normal sex ratio and factors influencing this parameter are summarized:

In a publication from Raikova-Petrova and Zivkov (Raikova-Petrova and Živkov, 1998) the sex ratio of *Sander lucioperca* (here called *Stizostedion lucioperca)* was determined from two different Bulgarian lakes. Examined were fish beginning with 1 up to 6 years.
The first lake (Ovcharitsa Dam (South Bulgaria), cooling reservoir of a thermo-electric power station) has higher temperatures (mean): annual = 16.2 °C, January = 6.3 °C, August = 25.6 °C. The second lake (Batak Dam (in the Rhodopes)) has lower temperatures (mean): annual = 10.3 °C, January = 1 °C, August = 19.8 °C.
The sex ratio for the population in the Batak Dam was close to 1:1, and differences (44.8 %: 55.2 %) were not significant (P = 0.05), except in the age group 5 years where more female fish were found.

In the cooling reservoir (Ovcharitsa Dam) male pikeperch dominated in all but the fifth age group. However, the number of fish in the entire sample was too low for a proper assessment. The sex ratios found for the second, third and fifth age groups were not significantly different (p = 0.05). The overall sex ratio was 68.3 %: 31.7 % (m:f) for the entire population, and was statistically significant (p = 0.001).
From this study it is indicated that the sex ratio is 1:1 (male : female). In a lake with higher temperature were more males than females seen, however with poor statistics. The fish were

[10] Art 20 of the polish Act on academic titles and on awarding the academic titles in art and science" from 14 March 2003 states: "For the doctoral degree, at least two reviewers are selected, and for the habilitation degree, at least three reviewers are selected. Not more than one of the reviewers can be from the same University or from the same research facility as the person applying for the habilitation."(Ustawa, 2003)

older than in the study by Demska-Zakes. However it can be reasonably assumed that there is no significant difference in the sex ratio between 2 months and 1 or 2 years old fish.

In a study by Lappalainen et al. (Lappalainen et al., 2003) it is written that the sex ratio is 1:1 and the fish have paired spawning with nest guarding by the male.

The authors Ablak and Yilmaz (Ablak, 2004) examined 326 pikeperch of different age. The sex ratio in the first year was 1.2 : 1 (female : male).

Overall data from wildlife indicate that the normal sex ratio is close to 1:1 with some indication that at higher temperature, males may prevail.

Table 20: Test design of the study by Demska-Zakes (2005):

Parameter	Value	Unit	Remark
Tank volume (nominal)	100	L	
Tank volume (actual)	80	L	
Loading	80	fish/tank	
Selection of fish from whole batch	7	days before test begin	too small or too large fish were excluded from testing
Determination of total length	± 0.1	cm	
Determination of body weight	0.01	g	
Range of body weight before test begin	1.6 - 2.1	g	
Narcotic treatment before manipulation of fish	1.5	ml/L	Propiscin solution (for 5 min), Propiscin contains a 0.2% stabilized solution of etomidate ((Demska-Zakęś, 2016, personal communication)
Distribution of fish to test concentrations			randomized
No. of test concentrations per substance	4	-	1, 10, 100 and 200 µg/L, dilution water control, solvent control
No. of replicates per test concentration	2	-	
Solvent			Ethanol
Stock solution	100	mg/L	
Solvent content in the stock solution	5	ml/L	96% ethanol
Dilution medium in stock solution	995	ml/L	Aqua destillata
Dilution medium for preparation of test concentration			Tap water
Solvent content in the highest test concentration	0.01	ml/L	Dilution factor of stock solution (100 mg test substance /L) to highest test concentration (0.2 mg/L) = 500
Adjustment of solvent concentration in all test conc.	0.01	ml/L	Solvent concentration was adjusted to an equal concentration in every test concentration (1, 10, 100, 200 mg/L) (Demska-Zakęś, 2016, personal communication)
Semi-static conditions			
Volume exchange of the test system	50	%/day	
Treatment of test medium			each tank obtained each own recirculation system including a

			biological filter (filter mats/foam blocks)
Flow rate in the recirculation system	4	L/min	Filter performance was 4 L/min corresponding to the 3-fold tank volume per hour.
Measurement abiotic parameter	Temp, pH, dissolved O_2		Daily
	TAN (NH_4-N + , NH_3-N), NO_2-N		Measurement every second day Total ammonia nitrogen (TAN) with salicylate-hypochlorite method (Bower, Holm-Hansen 1980), nitrite (NO_2-N) with α-naphthylamine method (Hermanowicz et al. 1999)
	Total hardness ($CaCO_3$), Fe		Test day 59; 80; 100
Control of fish	1	per day	
Feeding amount	6	% of fish biomass per day	
Food for the first three weeks of age	*Artemia salina* naupliae and commercial trout starter		
Food after day 25 dph	Only commercial trout diet, NUTRA (TROUTFIT, Nutreco Aquaculture, France), the pellet size was increased during the test in relation to fish size		
Food application	Feeding automate 4305 FIAP (Fishtechnik GmbH, Germany)		
Fish origin	Experimental Fish Stocking Centre Dgał, Institute for Inland Fishery, Olsztyn		
Fish age at moving to the recirculation system	4	dph	
Temperature adjustment during rearing	Gradually increased from 15 ± 0.5 to 22.0 ± 0.5°C		
Test temperature	22.0 ± 0.5	°C	
$CaCO_3$	200 ± 10	mg/L	
Fe	0.025 ± 0.005	mg/L	
Histological examination	3011	Fish	Randomized, at day 59 (before exposure) and each treatment/control at d88 and d144
	Gonads were collected and afterwards fixed in Bouin's fluid. After dehydration in graded ethanol (increasing concentration from 70 to 100%), the samples were embedded in paraffin wax and serial cross-sections were cut at 5 μm with rotary microtome LEICA 125 RM. The samples were mounted on glass slides and stained with two different methods: hematoxylin and eosin and Mayer's method (trichrome stain) (ZAWISTOWSKI 1986, modified by DEMSKA-ZAKĘŚ, not published.		
	For the sex determination 4 criteria were used: Male = normal male gonad (presence of only testicular tissue) Female = normal female gonad presence of only ovarian tissue) Intersex = comprising sex characteristics from both sexes		

[11] Before test begin (day 59) in total: 30 fish, at day 88 and day 144: 30 fish per treatment, dilution water control and solvent control. The examined fish were randomly chosen out of the two replicates. So in total 390 fish (30 fish at D59 + 6x30=180 fish at D88 + 6x30=180 fish at D144) were examined.

	(simultaneous presence of ovarian and testicular tissue) e.g. testis-ova/ ovo-testis, formation of an oviduct, with regressed spermatogenic lobules in the same fish, ovo-testes without or with incomplete oviduct. Sterile= absence of germ cells
Statistical calculations	Program: STATISTICA®, : To compare differences between the groups, one-way ANOVA was performed. If the differences between the groups were significant (P ≤ 0,05), the post-hoc Tukey HSD (Honestly Significant Difference) test was conducted. The percent values of mortality and sex ratio were arcsin transformed before the statistical analysis. In general results were provided as mean and standard deviation (as indicated for the respective results)

Table 21: Comparison of the OECD Guideline 234 with the study by Demska-Zakes (2005) (x = criteria fulfilled)

Validity criteria	TG 234	Demska-Zakes (2005)
Dissolved oxygen (% air saturation value)	≥ 60	fullfilled
Water temperature differences (°C)	± 1.5	fullfilled
Analysis method (LOD<<lowest test concentration)		n.a.
Test concentrations ± 20% mean measured values		n.a.
Hatching success (%)	> 80	n.a.
Post hatch survival (%)	≥ 70	fulfilled, after 60 dph
No effects of the solvent on survival		fullfilled
No endocrine disrupting effects of the solvent		fullfilled
Test design		
Test substance exposure start (dph)	newly fertilized eggs (before cleavage of the blastodisc)	60 dph
Test substance exposure duration (dph)	60	28 d (60dph-88dph)
Flow-through or semi-static		
Flow-through: volume exchange (per day)	≥ 5	n.a.
Semi-static: volume exchange (per day)	≥ 66%	50%
Photoperiod (light h / dark h)	12-16 / 8-12	24 / 0
Light intensity (lux)	540-1080	50-60
No. of treatments	≥ 3	4
No. of replicates per treatment	≥ 4	3
No. of animals per treatment	≥ 120 eggs	160 (2*80)
Solvent max. final concentration	100 µl/L	10 µl/L
Abiotic monitoring	Temperature[1], dissolved oxygen, salinity (if relevant) as a minimum weekly	Temperature, dissolved oxygen daily
	pH, total hardness, conductivity as a minimum at beginning and end of the test	pH daily, total hardness ($CaCO_3$) at test day 59, 80 and 100.
	Conductivity as a minimum at beginning and end of the test	-

	-	NH_4-N, NH_3-N and NO_2-N every second day
	-	Fe at test day 59, 80 and 100.
Validated test species	*Oryzias latipes*	*Sander lucioperca*
	Danio rerio	
	Gasterosteus aculeatus	
	(Pimephales promelas)	
Endpoints	Sex ratio	Sex ratio
	VTG level	
	Mortality	Mortality
	Standard length	Total length
	Body weight	Body weight
		Condition factor
	Time to start/end of hatching	
	Observed abnormalities (deformation, behaviour)	
	(Genetic sex)	
	(Histopathology)	Histopathology

n.a. not applicable, [1] should preferably be monitored continuously in at least one test chamber, [2] please see historical data regarding sex ratio of *Sander lucioperca*.

Table 22: Sex ratio and intersex in *Sander lucioperca* (values read from graph) after exposure to 4-tert-butylphenol and after a subsequent rearing of 56 days without test substance (D144). Values refer to mean numbers of fish in percent[1] from a graph (Fig. 17 in Demska-Zakęś, 2005).

Treatment (µg/L)	Female	Male	Intersex	Sterile
D88				
Dilution water control	52^{ab}	48^{a}	0^{a}	0^{a}
Solvent control	47^{a}	53^{a}	0^{a}	0^{a}
1	58.5^{bc}	31.5^{b}	10^{ab}	0^{a}
10	68^{c}	15^{c}	17^{b}	0^{a}
100	80^{d}	0^{d}	20^{b}	0^{a}
200	98^{e}	0^{d}	2^{a}	0^{a}
D144				
Dilution water control	48^{a}	52^{a}	0^{a}	0^{a}
Solvent control	52^{ab}	48^{a}	0^{a}	0^{a}
1	57.5^{b}	32^{b}	10.5^{b}	0^{a}
10	68^{c}	16.5^{c}	15.5^{b}	0^{a}
100	78^{d}	0^{d}	22^{c}	0^{a}
200	100^{e}	0^{d}	0^{a}	0^{a}

Values with the same superscript in the same column are not significantly different (P>0.05).
[1] For illustration purposes the values were estimated form the graphs of the study via manual measurement of the height of the bars and transformation via regression function to estimated values in percent. The superscripts indicating the statistical significance were taken from the original graphs.

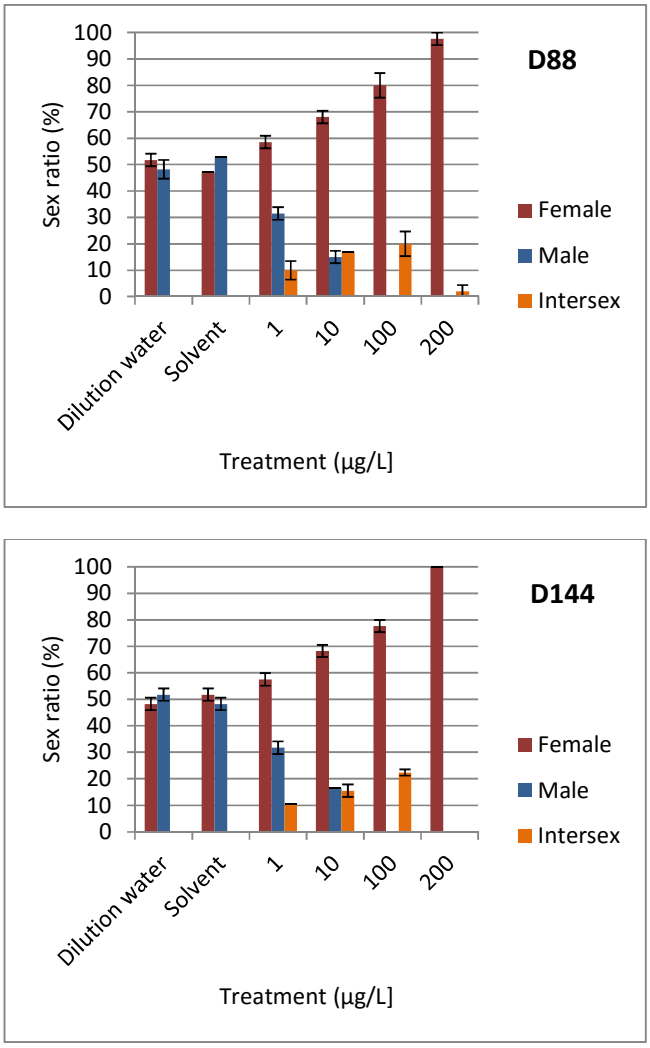


Figure 3 Sex ratio and intersex in *Sander lucioperca* after exposure to 4-tert-butylphenol (days 88 and 144). The values refer to mean numbers of fish in percent with indication of the standard deviation (n=2) and were extrapolated from a graph (Fig. 17 in Demska-Zakęś, 2005). If no error bar is indicated, the results of the particular replicates (n=2) were either equal or their variance too low for visualisation**.**

Summary:

In summary the study shows that 4-tert-butylphenol causes a sex ratio shift towards more females and less males at 1 µg/L and above. No males were observed at the highest test concentrations (100 and 200 µg/L) Results at day 144 show that the effects on sex ratio persist even after exposure has ceased.

Incidence of testis-ova at lower concentrations but not at the highest concentration substantiate that the sex ratio shift is a result of sex-reversal. Sa*nder lucioperca* is a gonochoristic fish, like *Cyprinus carpio*. That means male and female gonads are developed separately and naturally intersex is rare.

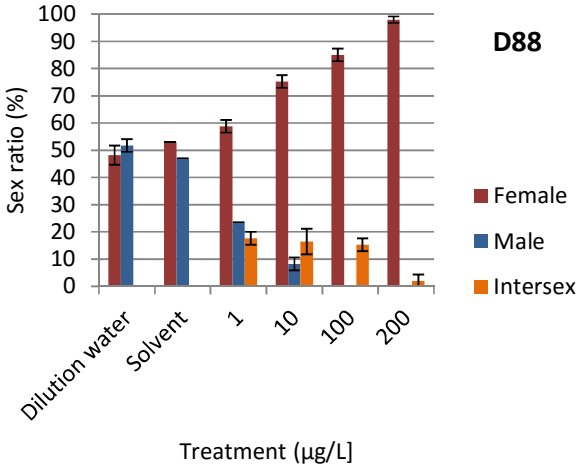
All effects exerted by 4-tert-butylphenol are summarized in table 22.

In order to compare results of this study for 4-tert-butylphenol with other compounds, the test results of 4-n-heptylphenol and 4-n-nonylphenol, as well as two positive controls (17ß-estradiol

and dihydroxyisoflavone) are depicted in the tables 23-26 and in the figures 3 – 6 (see below). Effects on all substances examined in the study by Demska-Zakes are summarized in table 27.

Table 23: Sex ratio and intersex in *Sander lucioperca* (values read from graph) after exposure to 4-n-heptylphenol

Treatment (µg/L)	Female	Male	Intersex	Sterile
D88				
Dilution water control	48[a]	52[a]	0[a]	0[a]
Solvent control	53[ab]	47[a]	0[a]	0[a]
1	59[b]	24[b]	18[b]	0[a]
10	75[c]	8[c]	16[b]	0[a]
100	85[d]	0[d]	15[b]	0[a]
200	98[e]	0[d]	2[a]	0[a]
D144				
Dilution water control	50[a]	50[a]	0[a]	0[a]
Solvent control	50[a]	50[a]	0[a]	0[a]
1	59[a]	20[b]	21[b]	0[a]
10	75[b]	5[c]	20[b]	0[a]
100	87[bc]	0[c]	13[ab]	0[a]
200	100[c]	0[c]	0[a]	0[a]



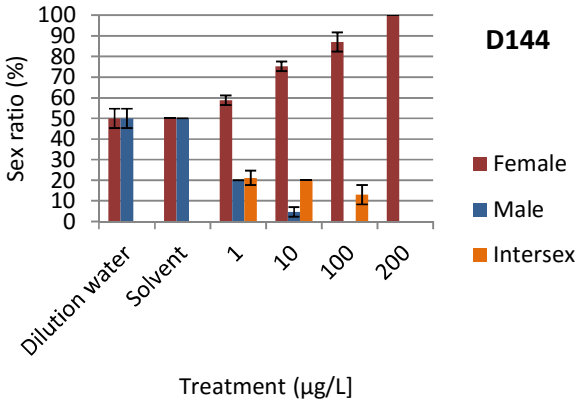
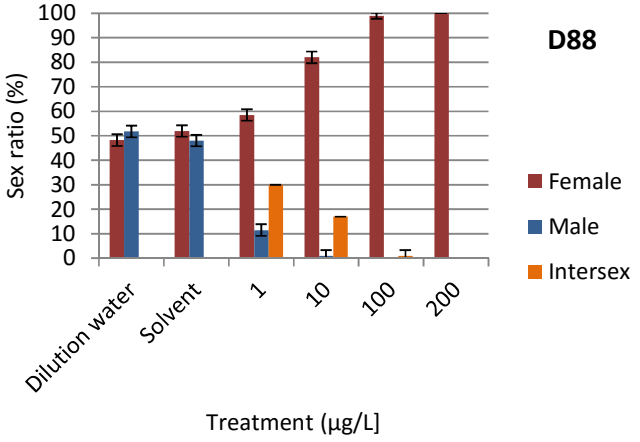


Figure 4: Sex ratio and intersex in *Sander lucioperca* after exposure to 4-n-heptylphenol (days 88 and 144). These values refer to mean numbers of fish in percent with indication of the standard deviation (n=2) and were extrapolated from a graph (Fig. 17 in Demska-Zakęś, 2005). If no error bar is indicated, the results of the particular replicates (n=2) were either equal or their variance too low for visualisation**.**

Table 24: Sex ratio and intersex in Sander *lucioperca* (values read from graph) after exposure to 4-n-nonylphenol

Treatment (µg/L)	Female	Male	Intersex	Sterile
D88				
Dilution water control	48[a]	52[a]	0[a]	0[a]
Solvent control	52[ab]	48[a]	0[a]	0[a]
1	58.5[b]	11.5[b]	30[b]	0[a]
10	82[c]	1[c]	17[c]	0[a]
100	99[d]	0[c]	1[a]	0[a]
200	100[d]	0[c]	0[a]	0[a]
D144				
Dilution water control	50[a]	50[a]	0[a]	0[a]
Solvent control	52[a]	48[a]	0[a]	0[a]
1	56[a]	12[b]	32[b]	0[a]
10	82.5[b]	2.5[bc]	15[c]	0[a]
100	100[c]	0[c]	0[a]	0[a]
200	100[c]	0[c]	0[a]	0[a]



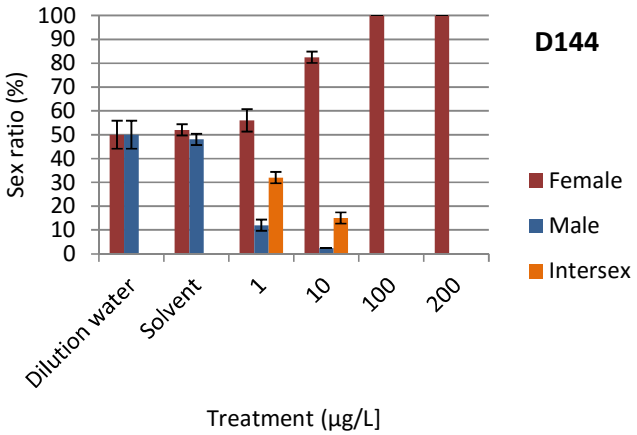
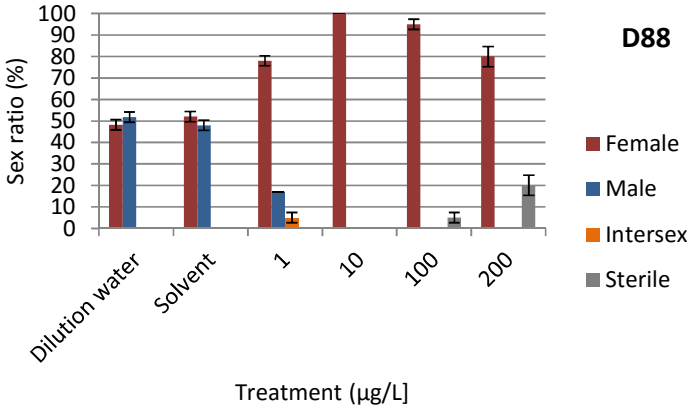


Figure 5: Sex ratio and intersex in *Sander lucioperca* after exposure to 4-n-nonylphenol (days 88 and 144)

Table 25: Sex ratio and intersex in *Sander lucioperca* after 28 days of exposure to 17ß-estradiol (D88) and after a subsequent rearing of 56 days without test substance (D144). These values refer to mean numbers of fish in percent[1] (Fig. 3 in Demska-Zakęś, 2005).

Treatment (µg/L)	Female	Male	Intersex	Sterile
D88				
Dilution water control	48^{a}	52^{a}	0^{a}	0^{a}
Solvent control	52^{a}	48^{a}	0^{a}	0^{a}
1	78^{b}	17^{b}	5^{b}	0^{a}
10	100^{c}	0^{c}	0^{a}	0^{a}
100	95^{c}	0^{c}	0^{a}	5^{a}
200	80^{b}	0^{c}	0^{a}	20^{b}
D144				
Dilution water control	51^{a}	49^{a}	0^{a}	0^{a}
Solvent control	52^{a}	48^{a}	0^{a}	0^{a}
1	77^{b}	14.5^{b}	8.5^{b}	0^{a}
10	100^{c}	0^{c}	0^{a}	0^{a}
100	93^{c}	0^{c}	0^{a}	7^{b}
200	78^{c}	0^{c}	0^{a}	22^{c}

Values with the same superscript in the same column are not significantly different (P>0.05).
[1] For illustration purposes the values were estimated form the graphs of the study via manual measurement of the height of the bars and transformation via regression function to estimated values in percent. The superscripts indicating the statistical significance were taken from the original graphs.



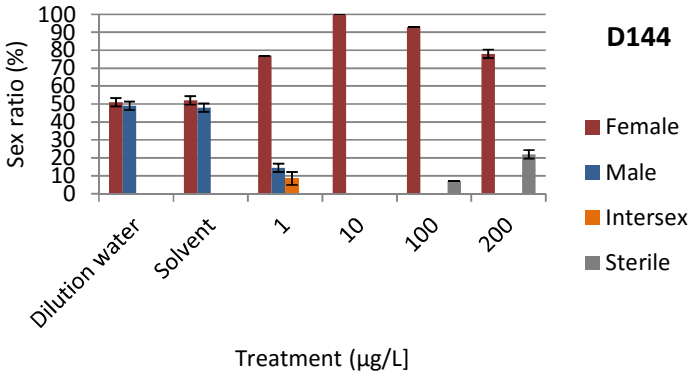


Figure 6: Sex ratio and intersex in *Sander lucioperca* after exposure to 17ß-estradiol (days 88 and 144)

Table 26: Sex ratio and intersex in *Sander lucioperca* after 28 days of exposure to 4',7-dihydroxyisoflavone (D88) and after a subsequent rearing of 56 days without test substance (D144). These values refer to mean numbers of fish in percent1 (Fig. 4 in Demska-Zakęś, 2005).

Treatment (µg/L)	Female	Male	Intersex	Sterile
D88				
Dilution water control	48^{a}	52^{a}	0^{a}	0^{a}
Solvent control	50^{a}	50^{a}	0^{a}	0^{a}
1	63^{b}	32^{b}	5^{b}	0^{a}
10	72^{bc}	20^{c}	8^{b}	0^{a}
100	81.5^{c}	3.5^{d}	15^{c}	0^{a}
200	100^{d}	0^{e}	0^{a}	0^{a}
D144				
Dilution water control	52^{a}	48^{a}	0^{a}	0^{a}
Solvent control	52^{a}	48^{a}	0^{a}	0^{a}
1	64^{b}	28^{b}	8^{b}	0^{a}
10	71^{bc}	17^{c}	12^{bc}	0^{a}
100	84^{c}	1.5^{d}	14.5^{c}	0^{a}
200	100^{d}	0^{d}	0^{a}	0^{a}

Values with the same superscript in the same column are not significantly different (P>0.05).
[1] For illustration purposes the values were estimated form the graphs of the study via manual measurement of the height of the bars and transformation via regression function to estimated values in percent. The superscripts indicating the statistical significance were taken from the original graphs.

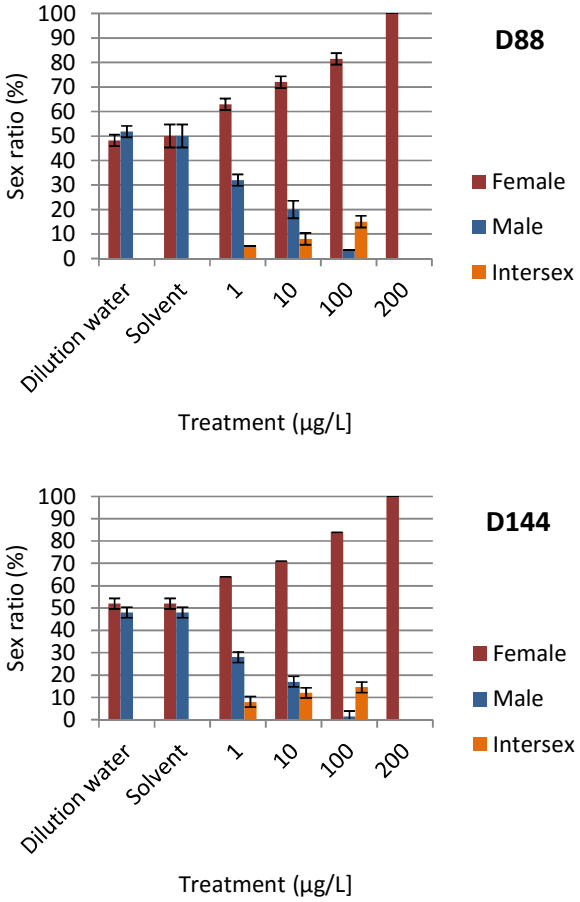


Figure 7: Sex ratio and intersex in *Sander lucioperca* after exposure to dihydroxyisoflavone (days 88 and 144)

An overview of the results (NOECs and LOECs) of all substances is provided in table 27.

Table 27: Overview of the NOEC and LOEC results of all substances in the study by Demska-Zakes (2005)

NOEC (µg/L)	Mortality	TL	BW	CF	Female ↑	Male ↓	Intersex	Sterile
4-n-heptylphenol	>200	>200	>200	>200	1	<1	<1	>200
17 ß-estradiol	10	>200	10	10	<1	<1	<1	100 / 10
4',7-dihydroxyisoflavone	100	>200	>200	100	<1	<1	<1	>200
1,6-dihydroxynaphthalene	>200	>200	>200	>200	10	10	10	>200
1,5-dihydroxynaphthalene	>200	>200	>200	>200	>200	>200	>200	>200
Phenol	>200	>200	>200	>200	>200	>200	100	>200
4-n-heptyloxyphenol	>200	>200	>200	>200	10	<1	<1 / 1	>200
4-n-nonylphenol	>200	>200	>200	>200	1	<1	<1	>200
4-n-butylphenol	>200	>200	>200	>200	1	1	1	>200
4-sec-butylphenol	>200	>200	>200	>200	1	1	1	>200

4-tert-butylphenol	>200	>200	>200	>200	<1 / 1	<1	1 /<1	>200
LOEC (µg/L)	**Mortality**	**TL**	**BW**	**CF**	**Female ↑**	**Male ↓**	**Intersex**	**Sterile**
4-n-heptylphenol	>200	>200	>200	>200	10	1	1	>200
17 ß-estradiol	100	>200	100	100	1	1	1	200 / 100
4',7-dihydroxyisoflavone	200	>200	>200	200	1	1	1	>200
1,6-dihydroxynaphthalene	>200	>200	>200	>200	100	100	100	>200
1,5-dihydroxynaphthalene	>200	>200	>200	>200	>200	>200	>200	>200
Phenol	>200	>200	>200	>200	>200	>200	200	>200
4-n-heptyloxyphenol	>200	>200	>200	>200	100	1	1 / 10	>200
4-n-nonylphenol	>200	>200	>200	>200	10	1	1	>200
4-n-butylphenol	>200	>200	>200	>200	10	10	10	>200
4-sec-butylphenol	>200	>200	>200	>200	10	10	10	>200
4-tert-butylphenol	>200	>200	>200	>200	1 / 10	1	10 / 1	>200

The difference between D88 and D144 is indicated as D88 / D144.

Effects on mortality, growth (length, weight) and condition coefficient:

No effects on mortality, growth and condition coefficient were observed for 4-tert-butylphenol, 4-n-heptylphenol and 4-n-nonylphenol. In the positive controls these endpoints were affected, however at higher concentrations compared to those causing changes in sex ratio. Results are summarized in table 28. For substances where effects appeared; the effects are presented for all concentrations. For substances without effects only the range over all concentrations is given.

Table 28: Effects on mortality, growth (length, weight) and condition coefficient

Substances causing significant effects:

	Treatment (µg/L)	Mortality (%)		Length (cm)		Weight (g)		Condition coefficient K	
E2		D 88	D 144	D 88	D 144	D 88	D 144	D 88	D 144
	DWC	3.75 A	4.38 A	9.27 A	16.51 A	8.31 A	46.23 A	1.21 A	1.00 A
	SC	3.13 A	3.13 A	9.08 A	16.49 A	8.35 A	45.85 A	1.21 A	1.06 A
	1	2.50 A	3.13 A	9.06 A	16.39 A	8.35 A	45.92 A	1.24 A	1.01 A
	10	3.75 A	3.75 A	9.05 A	16.38 A	8.18 A	45.91 A	1.23 A	0.99 A
	100	14.38 B	17.50 B	9.11 A	16.21 A	4.97 B	38.70 B	0.96 B	0.81 B
	200	20.00 C	25.00 C	8.94 A	15.96 A	4.48 B	34.10 B	0.85 B	0.74 B
4',7-DHI	0	3.13 A	3.13 A	9.19 A	16.52 A	8.23 A	46.05 A	1.26 A	1.07 A
	0*	4.38 A	4.38 A	9.27 A	16.59 A	8.22 A	45.95 A	1.20 A	1.00 A
	1	2.50 A	2.50 A	9.11 A	16.45 A	8.29 A	46.03 A	1.21 A	1.04 A
	10	3.13 A	3.13 A	9.06 A	16.33 A	8.25 A	45.60 A	1.23 A	1.04 A
	100	2.50 A	3.13 A	9.18 A	16.41 A	8.27 A	46.00 A	1.23 A	1.05 A
	200	10.63 B	14.38 B	9.09 A	16.20 A	5.56 A	44.05 A	0.99 B	0.86 B

Substances without effects:

	Mortality (%)		Length (cm)		Weight (g)		Condition coefficient K	
	D 88	D 144	D 88	D 144	D 88	D 144	D 88	D 144
4-t-BP	2.50 - 4.38	2.50 - 4.38	9.31 - 9.34	16.73 - 16.80	8.20 - 8.30	45.92 - 46.34	1.18 - 1.26	0.99 - 1.10
4-n-HP	0.63 - 4.38	2.50 - 4.38	9.20 - 9.35	16.58 - 16.70	8.30 - 8.40	46.80 - 47.11	1.19 - 1.25	1.00 - 1.05
4-n-HOP	2.50 - 4.38	2.50 - 4.38	9.19 - 9.30	16.59 - 16.73	8.28 - 8.45	46.79 - 47.05	1.20 - 1.26	0.98 - 1.05
4-n-NP	2.50 - 4.38	2.50 - 4.38	9.20 - 9.34	16.60 - 16.72	8.28 - 8.41	46.81 - 47.10	1.20 - 1.25	1.00 - 1.07

E2 = 17ß-estradiol
4',7-DHI = 4',7-dihydroxyisoflavone
SC = Solvent control
DWC dilution water control,
4-n-HP 4-n-heptylphenol, 4-n-HOP 4-n-heptyloxyphenol, 4nNP 4-n-nonylphenol, 4-t-BP 4-tert-butylphenol.
Values with the same superscript in the same column are not significantly different (P>0.05).

For the same substances also the physico-chemical parameters temperature, pH, oxygen concentration are presented. As there are only very small deviations over the test duration only the range from the different concentrations are given. See table 29.

Table 29 Temperature, pH, oxygen concentration;

	Temperature [°C]	pH	Oxygen concentration [mg/L]	Ammonia concentration [mg/L]	Nitrite concentration [mg/L]
E2	21.7 - 22.2	7.52 - 7.96	7.74 - 7.90	0.092 – 0.104	0.009 -0.020
4',7-DHI	21.7 - 22.3	7.54 - 7.99	7.83 - 7.95	0.092 – 0.102	0.011 – 0.019
4-t-BP	21.7 - 22.2	7.55 - 7.95	7.73 - 7.79	0.088 -0.106	0.013 -0.019
4nHP	21.7 - 22.2	7.65 - 7.96	7.75 - 7.89	0.089 -0.100	0.013 -0.020
4nNP	21.8 - 22.3	7.61 - 7.98	7.78 - 8.01	0.082 – 0.099	0.008 -0.015

Ammonia and nitrite concentrations were in the range recommended for aquaculture of Sander (Schmidt & Kühn, 2013) which should be < 0.5 mg/L NH_4 and < 2 mg/L nitrite.

Annex III – Short-term toxicity to fish

Table 30: Summary of short-term toxicity to fish of 4-tert-butylphenol

Test method	Results	Reliability acc. to Klimisch	Reference
OECD 203 *Oncorhynchus mykiss* Limit-Test	96h-LC_{50} > 1 mg/L (nominal)	2 – no analytical confirmation; number of fish; vehicle used	(Sewell, 1991)
EPA-660/3-75-009 *Pimephales promelas*	96h-LC_{50} = 5.14 mg/L (real); Deformities at 5.44 mg/L	2	(Holcombe et al., 1984)
ASTM + EPA-600/4-85/013 *Cyprinus carpio*	96 h-LC_{50} = 6.9 mg/L (nominal)	2	(Barse et al., 2006)

References for Annexes I, II and III

Ablak, Ö., Yilmaz, M. (2004). Growth Properties of Pikeperch (Sander lucioperca (L., 1758)) Living in Hirfanlı Dam Lake Turk J Vet Anim Sci, 455-463.

Akahori, Y., Nakai, M., Yamasaki, K., Takatsuki, M., Shimohigashi, Y., and Ohtaki, M. (2008). Relationship between the results of in vitro receptor binding assay to human estrogen receptor alpha and in vivo uterotrophic assay: comparative study with 65 selected chemicals. Toxicology in vitro : an international journal published in association with BIBRA 22, 225-231.

Barse, A.V., Chakrabarti, T., Ghosh, T.K., Pal, A.K., and Jadhao, S.B. (2006). One-tenth dose of LC50 of 4-tert-butylphenol causes endocrine disruption and metabolic changes in Cyprinus carpio. Pesticide Biochemistry and Physiology 86, 172-179.

Blair, R.M., Fang, H., Branham, W.S., Hass, B.S., Dial, S.L., Moland, C.L., Tong, W., Shi, L., Perkins, R., and Sheehan, D.M. (2000). The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. Toxicological sciences : an official journal of the Society of Toxicology 54, 138-153.

Crane, M., Fisk, P., Maycock, D., Watts, C., Wildey, R., Jordinson, H., Ridgwas., P. (2008): Environmental risk evaluation report: 4-tert-pentylphenol (CAS no. 80-46-6). UK Environment Agency.

Cravedi, J.P., Zalko, D. (2005). Chapter 5 Metabolic fate of nonylphenols and related phenolic compounds in fish. Biochemistry and Molecular Biology of Fishes, 6, 153-169Demska-Zakęś, K. (2005). Wpływ wybranych ksenobiotyków na rozwój układu płciowego ryb. (Olsztyn, Uniwersytet Warminsko-Mazurski w Olsztynie - UWM Olsztyn), p. 61.

ECHA (2011). SVHC Support document- 4-(1,1,3,3-tetramethylbutyl)phenol, 4-tert-octylphenol. (ECHA).

ECHA (2012). SVHC Support document - 4-nonylphenol, branched and linear. (ECHA).

Environment and Health Canada (2001). Priority substances list assessment report, Nonylphenol and its Ethoxylates, Canadian Environmental Protection Act, 1999 http://www.hc-sc.gc.ca/ewh-semt/alt_formats/hecs-sesc/pdf/pubs/contaminants/psl2-lsp2/nonylphenol/nonylphenol-eng.pdf

Gimeno, S., Gerritsen, A., Bowmer, T., and Komen, H. (1996). Feminization of male carp. Nature *384*, 221-222.

Gimeno, S., Komen, H., Venderbosch, P.W.M., and Bowmer, T. (1997). Disruption of sexual differentiation in genetic male common carp (Cyprinus carpio) exposed to an alkylphenol during different life stages. Environmental Science and Technology *31*, 2884-2890.

Gimeno, S., Komen, H., Gerritsen, A.G.M., and Bowmer, T. (1998a). Feminisation of young males of the common carp, Cyprinus carpio, exposed to 4-tert-pentylphenol during sexual differentiation. Aquatic toxicology *43*, 77-92.

Gimeno, S., Komen, H., Jobling, S., Sumpter, J., and Bowmer, T. (1998b). Demasculinisation of sexually mature male common carp, Cyprinus carpio, exposed to 4-tert-pentylphenol during spermatogenesis. Aquatic toxicology *43*, 93-109.

Hermanowicz, W., Dojlido, J., Dozanska, W., Koziorowski, B., Zerbe, J. (1999). Fizyczno-chemiczne badanie wody i sciekow. Wydawnictwo ARKADY. Warszawa (in Polish).

Holcombe, G.W., Phipps, G.L., Knuth, M.L., and Felhaber, T. (1984). The acute toxicity of selected substituted phenols, benzenes and benzoic acid esters to fathead minnows Pimephales promelas. Environmental Pollution Series A, Ecological and Biological 35, 367-381.

Hornung, M.W., Tapper, M.A., Denny, J.S., Kolanczyk, R.C., Sheedy, B.R., Hartig, P.C., Aladjov, H., Henry, T.R., and Schmieder, P.K. (2014). Effects-based chemical category approach for prioritization of low affinity estrogenic chemicals. SAR and QSAR in environmental research 25, 289-323.

Jarmolowicz, S., Demska-Zakęś, K. and Zakęś, Z (2014). Impact of butyl benzyl phthalate on development of the reproductive system of the reproductive system of European pikeperch, *Sander lucioperca* (L.). Acta Veterinaria Hungarica 62 (3): 397–407.

Jobling, S., and Sumpter, J.P. (1993). Detergent components in sewage effluent are weakly oestrogenic to fish: An in vitro study using rainbow trout (Oncorhynchus mykiss) hepatocytes. Aquatic toxicology 27, 361-372.

Jonsson, G., Stokke, T.U., Cavcic, A., Jørgensen, K.B., Beyer, J. (2008): Characterization of alkylphenol metabolites in fish bile by enzymatic treatment and HPLC-fluorescence analysis. Chemosphere. Apr;71(7):1392-400.

Knudsen, F.R., Pottinger, T.G. (1999). Interaction of endocrine disrupting chemical, singly and combination, with estrogen-, androgen-, and corticosteroid-binding sites in rainbow trout (*Oncorhynchus mykiss*). Aquat Toxicology 44 (1999) 159-170.

Körner, W., Hanf, V., Schuller, W., Bartsch, H., Zwirner, M., and Hagenmaier, H. (1998). Validation and application of a rapid in vitro assay for assessing the estrogenic potency of halogenated phenolic chemicals. Chemosphere 37, 2395-2407.

Kowalska, A., Zakęś, Z, Siwicki, A.K., Jankowska, B., Jarmołowicz, S and Demska-Zakęś, K. (2012). Impact of diets with different proportions of linseed and sunflower oils on the growth, liver histology, immunological and chemical blood parameters, and proximate composition of pikeperch Sander lucioperca (L.). Fish Physiol Biochem, 38: 375-388.

Krueger, H.O., Kendall, T.Z., Martin, K.H., and Nison, W.B. (2008). FINAL REPORT PARA-TERTIARY BUTYL PHENOL (PTBP): A Partial Life Cycle Test with the Fathead Minnow (Pimephales promelas). (Wildlife Interantional, Ltd.), p. 311.

Lappalainen, J., Dörner, H., and Wysujack, K. (2003). Reproduction biology of pikeperch (Sander lucioperca (L.)) – a review. Ecology of Freshwater Fish 12, 95-106.

Lewis, S.K., Lech, J.J. (1996): Uptake, disposition, and persistence of nonylphenol from water in rainbow trout (*Oncorhynchus mykiss*). Xenobiotica. 1996 Aug;26(8):813-9.

Madsen, L.L., Korsgaard, B., Bjerregaard P. (2003): Estrogenic effects in flounder *Platichthys flesus* orally exposed to 4-*tert*-octylphenol. Aquat Toxicol. 2003 Sep 10;64(4):393-405.

Nishihara, T., Nishikawa, J.I., Kanayama, T., Dakeyama, F., Saito, K., Imagawa, M., Takatori, S., Kitagawa, Y., Hori, S., and Utsumi, H. (2000). Estrogenic activities of 517 chemicals by yeast two-hybrid assay. Journal of Health Science 46, 282-298.

OECD (2000): Guidance Document on Aquatic Toxicity Testing of difficult substances and Mixtures. OECD Series on Testing and Assessment No. 23. ENV/JM/MONO(2000)6.

OECD (2006). Report of the validation of the 21-day fish screening assay for the detection of endocrine substances (Phase 1B). In Series on testing and assessment (Paris, OECD Organisation for Economic Co-operation and Development), p. 140.

OECD (2009). Report of the expert consultation to evaluate an estrogen receptor binding affinity model for hazard identification

OECD (2010). Guidance document on the diagnosis of endocrine-related histopathology in fish gonads. pp. 1-114.

OECD (2011). OECD guideline for the testing of chemicals Test No. 234 Fish Sexual Development Test.

OECD (2011a). Validation report (phase 1) for the fish sexual development test for the detection of endocrine active substances, Series on Testing and Assessment. OECD Organisation for Economic Co-operation and Development, Paris, pp. 91.

OECD (2012). Validation report (phase 2) for the fish sexual development test for the detection of endocrine active substances (updated 2012), Series on Testing and Assessment. OECD Organisation for Economic Co-operation and Development, Paris, pp. 95.

Olsen, C.M., Meussen-Elholm, E.T.M., Hongslo, J.K., Stenersen, J., and Tollefsen, K.E. (2005). Estrogenic effects of environmental chemicals: An interspecies comparison. Comparative Biochemistry and Physiology - C Toxicology and Pharmacology 141, 267-274.

Panter, G.H., Hutchinson, T.H., Hurd, K.S., Bamforth, J., Stanley, R.D., Duffell, S., Hargreaves, A., Gimeno, S., and Tyler, C.R. (2006). Development of chronic tests for endocrine active chemicals. Part 1. An extended fish early-life stage test for oestrogenic active chemicals in the fathead minnow (Pimephales promelas). Aquatic toxicology 77, 279-290.

Panter, G.H., Hutchinson, T.H., Hurd, K.S., Bamforth, J., Stanley, R.D., Wheeler, J.R., and Tyler, C.R. (2010). Effects of a weak oestrogenic active chemical (4-tert-pentylphenol) on pair-breeding and F1 development in the fathead minnow (Pimephales promelas). Aquatic toxicology *97*, 314-323.

Raikova-Petrova, G., and Živkov, M. (1998). Maturity, spawning and sex ratio of pike perch, Stizostedion luciopevca (L.), in two Bulgarian reservoirs as compared to other European habitats. Journal of Applied Ichthyology 14, 31-35.

Routledge, E.J., and Sumpter, J.P. (1997). Structural features of alkylphenolic chemicals associated with estrogenic activity. The Journal of biological chemistry 272, 3280-3288.

Satoh, K., Nagai, F., (2002). Competitive binding of alkylphenols to human estrogen and androgen receptors. Ann. Rep. Tokyo Metr. Res. Lab. P.H. 53, 265–267.

Seki, M., Yokota, H., Matsubara, H., Maeda, M., Tadokoro, H., and Kobayashi, K. (2003). Fish full life-cycle testing for the weak estrogen 4-tert-pentylphenol on medaka (Oryzias latipes). Environmental toxicology and chemistry / SETAC *22*, 1487-1496.

Schmidt, G, Kühn, C: Entwicklung einer Zanderaquakultor in Mecklenburg-Vorpommern: Die Warmwasser-Pilotanlage Hohen Wangelin. AUF 2/2013 (in German)

Schultz, T.W., Sinks, G.D., and Cronin, M.T.D. (2000). Effect of substituent size and dimensionality on potency of phenolic xenoestrogens evaluated with a recombinant yeast assay. Environmental Toxicology and Chemistry *19*, 2637-2642.

Sewell (1991). Acute toxicity to rainbow trout. (Safepharm Laboratories, Derby , UK).

Soto, A.M., Sonnenschein, C., Chung, K.L., Fernandez, M.F., Olea, N., and Serrano, F.O. (1995). The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environmental health perspectives 103 Suppl 7, 113-122.

Sundt, R.C. (2009): Uptake and tissue distribution of C4-C7 alkylphenols in Atlantic cod (*Gadus morhua*): relevance for biomonitoring of produced water discharges from oil production. Mar Pollut Bull. 2009 Jan;58(1), 72-9.

Terasaka, S., Inoue, A., Tanji, M., Kiyamaa, R. (2006). Expression profiling of estrogen-responsive genes in breast cancer cells treated with alkylphenols, chlorinated phenols, parabens, or bis- and benzoylphenols for evaluation of estrogenic activity. Toxicology Letters 163 (2006) 130–141.

Tollefsen, K.E. (2007). Binding of alkylphenols and alkylated non-phenolics to the rainbow trout (Oncorhynchus mykiss) plasma sex steroid-binding protein. Ecotoxicol Environ Saf 68, 40-48.

Tollefsen, K.E., Eikvar, S., Finne, E.F., Fogelberg, O., and Gregersen, I.K. (2008). Estrogenicity of alkylphenols and alkylated non-phenolics in a rainbow trout (Oncorhynchus mykiss) primary hepatocyte culture. Ecotoxicol Environ Saf 71, 370-383.

Tollefsen, K.E., and Julie Nilsen, A. (2008). Binding of alkylphenols and alkylated non-phenolics to rainbow trout (Oncorhynchus mykiss) hepatic estrogen receptors. Ecotoxicology and environmental safety 69, 163-172.

Tollefsen, K.E., Ingebrigtsen, K., Olsen, A.J., Zachariassen, K.E., Johnsen, S. (1998). Acute Toxicity and Toxicokinetic of 4-Heptylphenol in juvenile Atlantic Cod (*Gadus Morhua L.*). Environmental Toxicology, 17(4), 740–746

Tsuda, T., A. Takino, K. Muraki, H. Harada and M. Kojima (2001). Evaluation of 4-nonylphenols and 4-tert-octylphenol contamination of fish in rivers by laboratory accumulation and excretion experiments. Water Res. 35: 1786-1792.

Ustawa, 2003: USTAWA z dnia 14 marca 2003 r. o stopniach naukowych i tytule naukowym oraz o stopniach i tytule w zakresie sztuki

WHO/IPCS (2002). Global assessment of the state-of-the-science of endocrine disruptors. WHO/PCS/EDC/02.2. (World Health Organization/International Programme on Chemical Safety), p. 180 p.

Wlasow, T., Demska-Zakes, K., Gomulka, P., Jarmolowicz, S. (2010): Various aspects of piscine toxicology. Interdiscip Toxicol, 3 (3), 100-4

Zakęś, Z., Demska-Zakęś, K. (1998). Intensive rearing of juvenile Stizostedion lucioperca (Percidae) fed natural and artificial diets. Italian Journal of Zoology, 65:S1: 507-509.

Zawistowski, S. (1986): Technika histologiczna, histologia oraz podstawy histopatologii. PZWL, Warszawa (in Polish).