Annex XV dossier

PROPOSAL FOR IDENTIFICATION OF A SUBSTANCE AS A CMR 1A OR 1B, PBT, vPvB OR A SUBSTANCE OF AN EQUIVALENT LEVEL OF CONCERN

Substance Name(s): 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

EC Number(s): -

CAS Number(s): -

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EC Number(s): -

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- It is proposed to identify the substances identified as substances meeting the criteria of Article 57 (f) of Regulation (EC) 1997/2006 (REACH).

Summary of how the substances meet the criteria of Article 57 (f)

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 (short: 4-Nonylphenols) are proposed to be identified as substances of very high concern in accordance with Article 57 (f) of Regulation (EC) 1907/2006 (REACH) because they are substances with endocrine disrupting properties for which there is scientific evidence of probable serious effects to the environment which gives rise to an equivalent level of concern to those of other substances listed in points [(a) to (e)] of article 57 of REACH.

This conclusion is based on the fact that there is strong evidence from high quality studies of endocrine mediated adverse effects in fish species. Results for amphibians provide indication that effects in other taxa may be endocrine mediated i.e. caused by an estrogen-like mode of action, too.

According to the OECD (Organisation for Economic Co-operation and development) guidance document for endocrine disruptors (OECD, 2012) 4-nonylphenols need to be considered as endocrine disruptors based on these results. Moreover, based on the widely accepted IPCS definition for endocrine disruptors (WHO/IPCS, 2002; WHO: World Health Organization/IPCS: INSTITUTE OF PEACE & CONFLICT STUDIES) 4-nonylphenols are considered to be endocrine disruptors.

Based on the above conclusion, evidence that the substances are of an equivalent level of concern includes:

Evidence from several test data show that effects of the 4-nonylphenols on fish fit to those of other estrogen agonists which are considered serious for the environment due to the type of effects.

Effects remain manifest even after exposure has ceased and the fact that exposure during sensitive life stages may change the endocrine feedback system resulting in effects during the entire life:

- Exposure to nonylphenol resulted in effects in fish on reproduction parameters (fecundity) as well as on sexual development (including changes in sex-ratio) and growth. Results for at least 3 fish species show that exposure to nonylphenol may result in a complete sex reversal resulting in all female populations. Effects observed include behavioural effects that may influence the gene pool.

- Effects observed in several fish species show that transient exposure during sensitive life stages may result in effects that remain during the entire life and even in following generations. Thus exposure in one area might influence population stability in another area and effects persist even after exposure has ceased.

- In addition to the severity of effects, some results substantiate the hypothesis that it is difficult to quantify a safe level for 4-nonylphenols with regard to endocrine activity.

- Effects on non-traditional endpoints indicate that effects may start at much lower concentrations than those considered in OECD test guidelines.

- Exposure to 4-nonylphenols resulted in effects on reproduction and development in different invertebrates at concentrations below 1 µg/L (e.g. LOEC sex-ratio < 1 µg/l in mussels, LOEC development 0.09 µg/L in echinoderm species). Although it is not possible to clearly state that the effects are endocrine mediated, these effects fit to the knowledge that steroids are known to play an important role in invertebrates (Kendall et al., 1998). Owing to the lack of in depth knowledge of their endocrine system and the lack of test systems, it is currently nearly impossible to estimate which species are most sensitive and which concentration should be regarded as safe for the environment.

Thus in summary, effects observed after exposure to 4-nonylphenols are considered to impair population stability and recruitment. They may occur even after short term exposure and thus may result in adverse effects 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 or changes in the gene pool. Effects may influence a wide range of taxa and it is difficult to estimate a safe level. Consequently they are considered to be of an equivalent level of concern.

The concern is substantiated by an analysis of literature of current knowledge on endocrine disruptors which reveals strong evidence that exposure to endocrine disrupting chemicals is linked to reproductive disorder and disfunction in wildlife. Although this is mainly due to exposure to steroidal estrogens, at some sites xenoestrogens may significantly contribute to the effect.

Registration dossiers submitted for the substances? Yes

PART I

JUSTIFICATION

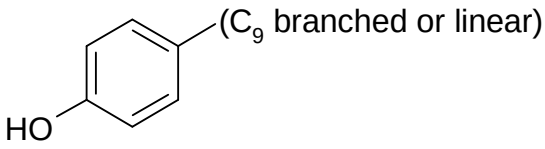
1 IDENTITY OF THE SUBSTANCE AND PHYSICAL AND CHEMICAL PROPERTIES

1.1 Name and other identifiers of the substance

Table 1: Substance identity

EC number:	-
EC name:	-
CAS number (in the EC inventory):	-
CAS number:	-
CAS name:	-
IUPAC name:	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 range:	220.35 g/mol
Synonyms:	-

Structural formula:



1.2 Composition of the substance

Name: 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

Description: group entry

Degree of purity: -

Table 2: Constituents

Constituents	Typical concentration	Concentration range	Remarks
n/a			

Table 3: Impurities

Impurities	Typical concentration	Concentration range	Remarks
n/a			

Table 4: Additives

Additives	Typical concentration	Concentration range	Remarks
n/a			

No detailed composition of the substance could be given. The given identity

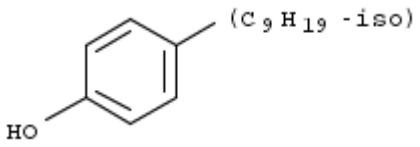
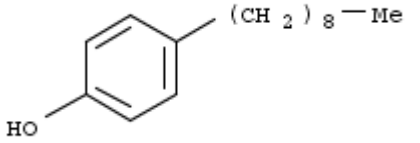
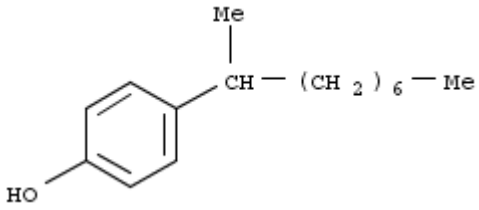
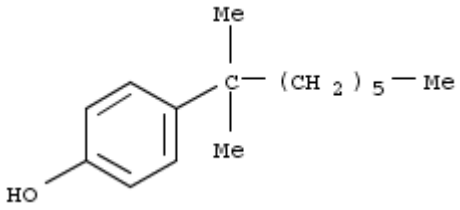
"4-Nonylphenol, branched and linear: substances with a linear and/or branched alkyl chain with a carbon number of 9 covalently bound in position 4 to phenol, covering also UVCB- and well-defined substances which include any of the individual isomers or a combination thereof"

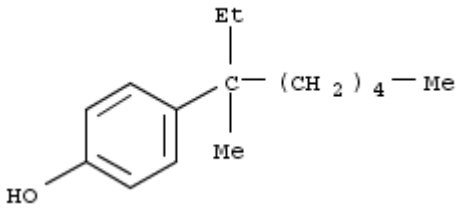
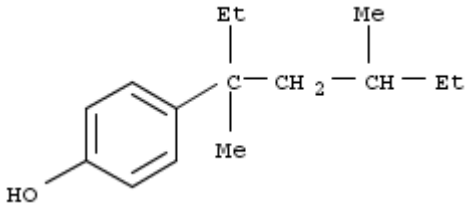
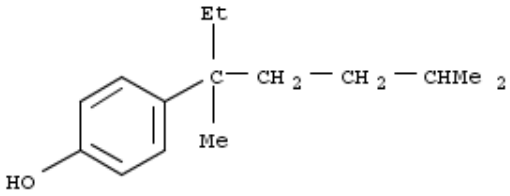
shall cover the group of p-Nonylphenols with linear or branched alkyl chain. In Table 5 a list of example substances are given which are covered by the group entry.

The substance Phenol, 4-nonyl-, branched (CAS-Nr. 84852-15-3) is registered and for p-nonylphenol (CAS-Nr. 104-40-5) C&L notifications are submitted. The other substances are listed as examples. None of them is registered or a C&L notification (in IUCLID format) is submitted at the time of the submission of this A.XV report.

Table 5 provides a non-exhaustive list of examples of substances covered by the group name.

Table 5: Non-exhaustive list of substances covered by the group entry*

EC Name **CAS Name:** **IUPAC Name:**	**EC – Nr.**	**CAS Nr.**	**Molecular formula**	**Structure**
EC Name: Phenol, 4-nonyl-, branched CAS Name: Phenol, 4-nonyl-, branched IUPAC Name: 4-Nonylphenol, branched	284-325-5	84852-15-3	$C_{15}H_{24}O$	-
EC Name: p-isononylphenol CAS Name: Phenol, 4-isononyl- IUPAC Name: p-isononylphenol	247-770-6	26543-97-5	$C_{15}H_{24}O$	
EC Name: p-nonylphenol CAS Name: Phenol, 4-nonyl- IUPAC Name: 4-Nonylphenol	203-199-4	104-40-5	$C_{15}H_{24}O$	
EC Name: p-(1-methyloctyl)phenol CAS Name: Phenol, 4-(1-methyloctyl)- IUPAC Name: 4-(1-Methyloctyl)phenol	241-427-4	17404-66-9	$C_{15}H_{24}O$	
EC Name: p-(1,1-dimethylheptyl)phenol CAS Name: Phenol, 4-(1,1-dimethylheptyl)- IUPAC Name: 4-(1,1-Dimethylheptyl)phenol	250-339-5	30784-30-6	$C_{15}H_{24}O$	

EC Name: 4-(1-ethyl-1-methylhexyl)phenol CAS Name: Phenol, 4-(1-ethyl-1-methylhexyl)- IUPAC Name: 4-(1-Ethyl-1-methylhexyl)phenol	257-907-1	52427-13-1	$C_{15}H_{24}O$	
EC Name: - CAS Name: Phenol, 4-(1-ethyl-1,3-dimethylpentyl)- IUPAC Name:4-(1-Ethyl-1,3-dimethylpentyl)phenol	-	186825-36-5	$C_{15}H_{24}O$	
EC Name: - CAS Name: Phenol, 4-(1-ethyl-1,4-dimethylpentyl)- IUPAC Name: 4-(1-Ethyl-1,4-dimethylpentyl)phenol	-	142731-63-3	$C_{15}H_{24}O$	

* This is a list of substances identified as covered by the generic substance description, however further substances not listed here may be covered as well.

1.3 Physico-chemical properties

For two of the example substances which are covered by the group entry, physico-chemical properties could be given. These data are given in table 6.

For the other example substances in chapter 1.2 no registration dossiers are available and no physical and chemical properties could be found in accepted databases.

Please attend that this values are specific for the example substances and do not cover all substances of the group entry.

Table 6: Overview of physicochemical properties

Property	Value	Remarks
Physical state at 20°C and 101.3 kPa	*Yellow liquid with phenolic odour*	*The result is valid for 4-nonylphenol (branched) (CAS-Nr. 84852-15-3) and nonylphenol (CAS-Nr. 25154-52-3)(see European Union Risk Assessment Report EUR 20387 EN)*
	Pale yellow viscous liquid	*4-Nonylphenol CAS-Nr.: 104-40-5*
Melting/freezing point	*< -7 °C at 1 atm*	*4-nonylphenol (branched) (CAS-Nr. 84852-15-3)*
Boiling point	*302 °C at ca.101 kPa*	*4-nonylphenol (branched) (CAS-Nr. 84852-15-3)* *Decomposition may occur according to European Union Risk Assessment Report*
Density	*0.95 g/cm³*	*4-nonylphenol (branched) (CAS-Nr. 84852-15-3)* *experimental result*
Vapour pressure	*0.3 Pa at 25 °C*	*The result is valid for 4-nonylphenol (branched) (CAS-Nr. 84852-15-3) and nonylphenol (CAS-Nr. 25154-52-3)(see European Union Risk Assessment Report EUR 20387 EN)*
	0.109 Pa at 25 °C	*4-Nonylphenol CAS-Nr.: 104-40-5*
Water solubility	*Ca. 5.7 mg/L at 25 °C, 6 < pH < 7*	*4-nonylphenol (branched) (CAS-Nr. 84852-15-3)* *experimental result*
	7 mg/L at 25 deg C	*4-Nonylphenol CAS-Nr.: 104-40-5*
Partition coefficient n-octanol/water (log value)	*5.4 at 23 °C, pH 5.7*	*4-nonylphenol (branched) (CAS-Nr. 84852-15-3)* *experimental result*
	Log Kow = 5.76	*4-Nonylphenol CAS-Nr.: 104-40-5*
Dissociation constant	*pKa ca. 10 (Value could be higher than this)*	*At this pKa value nonylphenol would be undissociated at environmental pHs*

2 HARMONISED CLASSIFICATION AND LABELLING

Nonylphenol is listed in Regulation (EC) No 1272/2008 as follows:

Table 7: Classification and labelling of nonylphenol according to part 3 of Annex VI, Table 3.1 of Regulation (EC) No 1272/2008

Index-No	**International Chemical Identification**	**EC No**	**CAS-No**	**Classification**		**Labelling**		**Specific concentration limits, M-factors**
				Hazard Class and Category Code(s)	**Hazard Statement Code(s)**	**Pictogram, Signal Word Code(s)**	**Hazard statement Code(s)**	
601-053-00-8	nonylphenol; [1] 4-nonylphenol, branched [2]	246-672-0 [1] 284-325-5 [2]	25154-52-3 [1] 84852-15-3 [2]	Repr. 2 Acute Tox. 4 * Skin Corr. 1B Aquatic Acute 1 Aquatic Chronic 1	H361fd H302 H314 H400 H410	GHS08 GHS05 GHS07 GHS09 Dgr	H361fd H302 H314 H410	

Table 8: Classification and labelling of nonylphenol according to part 3 of Annex VI, Table 3.2 of Regulation (EC) No 1272/2008

Index-No	**International Chemical Identification**	**EC No**	**CAS-No**	**Classification**	**Labelling**	**Concentration limits**
601-053-00-8	nonylphenol; [1] 4-nonylphenol, branched [2]	246-672-0 [1] 284-325-5 [2]	25154-52-3 [1] 84852-15-3 [2]	Repr. Cat. 3; R62-63 Xn; R22 C; R34 N; R50-53	C; N R: 22-34-62-63-50/53 S: (1/2-)26-36/37/39-45-46-60-61	

3 ENVIRONMENTAL FATE PROPERTIES

3.1 Degradation

3.1.1 Abiotic degradation

This chapter describes abiotic degradation test results according to the Risk Assessment Report (European Commission, 2002).

3.1.1.1 Hydrolysis

Due to the chemical structure of the 4-nonylphenols it is expected that hydrolysis normally will not occur under environmental conditions and therefore it is supposed hydrolysis is not a relevant path of abiotic degradation.

3.1.1.2 Phototransformation/photolysis

3.1.1.2.1 Phototransformation in air

4-Nonylphenols released to the atmosphere are likely to be rapidly degraded by reaction with hydroxyl radicals. The rate constant for the reaction with hydroxyl radicals has been estimated using the AOP program (v1.91, in EPISUITE, 2004) to be $5.17 \cdot 10^{-11}$ cm^3 s^{-1} $molecules^{-1}$. From this rate constant the estimated half-life for the reaction of 4-nonylphenols with hydroxyl radicals in the atmosphere is calculated as 2.48 hours (for 12 hour-day, $1.5 \cdot 10^6$ OH/cm^3). The fraction of chemical absorbed to aerosol particles is also regarded to be low. Therefore long-range transport would not occur because of previous degradation (European Commission, 2002). Also it is unlikely that 4-nonylphenols move from the troposphere to the stratosphere and contribute to ozone depletion. 4-Nonylphenols are not thought to contribute to low-level ozone formation.

3.1.1.2.2 Phototransformation in water

The rates of photochemical transformation of 4-nonylphenols (branched isomers) in natural waters were assessed by exposing their solutions in filtered lake water to sunlight (Ahel et al., 1994). Sunlight phototransformation was performed in 50 ml quartz tubes which were suspended in a shallow flat-bottomed container filled with tap water or in a creek at a depth of 20-25 cm. The first-order rate constant of sunlight photolysis was estimated at 0.09 m^2(kWh) with a corresponding half-life of 10-15 hours (surface layer). At depths of 20-25 cm the photolysis rate is approximately 1.5 times slower than at the surface.

When having in mind photochemical transformation in natural waters it has to be kept in mind that the environmental exposure occurs in the whole water column. Because of the substance's adsorption potential it will predominantly bind to suspended organic matter and sediment which is supposed to decrease the tendency for photodegradation. Therefore photodegradation of the 4-

nonylphenols are expected to be a relevant degradation process only in very shallow clear waters and in the first few centimetres layer of the water column. Therefore aquatic photodegradation is not considered to have relevant impact on the overall persistency of the 4-nonylphenols in the aquatic environment.

Summary

As already remarked according to the Risk Assessment Report (European Commission, 2002) abiotic degradation of nonylphenol resulting from hydrolysis or photolysis are thought to be negligible removal processes in the aquatic environment.

3.1.2 Biodegradation

Biodegradation studies are available for several nonylphenol isomers. The biodegradation behaviour of the linear isomer 4-n-nonylphenol is different to the other nonylphenols. Therefore, 4-n-nonylphenol is considered seperately.

3.1.2.1 Biodegradation in water

3.1.2.1.1 Estimated data

3.1.2.1.2 Screening tests

Table 9: Summary of screening tests

Test	Compound	Result	Reliability	Reference
OECD 301 C	Nonylphenol (CAS-No.:25154-52-3)	0% after 14 days	2	(National Institute of Technology and Evaluation, 2002)
OECD 301 B	4-Nonylphenol branched (CAS-No.:84852-15-3)	47.5 ± 7.2 % after 28 days	2	(Gledhill, 1999; Staples et al., 2001)
OECD 301 F	4-Nonylphenol branched (CAS-No.:84852-15-3)	57.4 – 68.4% after 28 days (average 62%; 10 day window was failed)	2	(Staples et al., 1999)
OECD 301 F	4-n-Nonylphenol (CAS-No.:104-40-5)	61.5 ± 6.0 % after 28 days (10 day window was fulfilled)	2	(Stasinakis et al., 2008)
OECD 301 B	4-Nonylphenol branched (CAS-No.:84852-15-	0 % after 32 days (with and without emulsifier) With adopted inoculum (adaption	3	(European Commission,

	3)	time 7 weeks): 0 % after 40 days (without emulsifier) 78 % after 40 days (with emulsifier)		2002)

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

Gledhill et al. determined the potential of biodegradation of nonylphenol following OECD Test Guideline 301B (Gledhill, 1999; Staples et al., 2001). Activated sludge from a Waste Water Treatment Plant, showing a high nonylphenol ethoxylate concentration, was used for this study. On day 28 47.5% $ThCO_2$ (48.2 % at day 35) was measured. 48.4% suspended organic carbon was determined on day 35. This suggests that nonylphenol incorporated into biomass or adsorbed to suspended material.

Staples et al. determined the extent of biodegradation of nonylphenol using the OECD 301F test (manometric respirometry) (Staples et al., 1999). After 28 days (62% $ThCO_2$) the pass level of ready biodegradability was fulfilled, but the 10 day window was failed (17 days). Based on assumed first order kinetics, the aerobic biodegradation half-life was 20 days.

In an OECD 301F test with 4-n-nonylphenol additional 10 mg/L allythiourea was added for preventing nitrification (Stasinakis et al., 2008). After a lag phase of 7.9±0.6 days, 4-n-nonylphenol was aerobically biodegraded with 61.5±6.0% at day 28.

The following information (italic) was copied from the Risk Assessment Report of 4-nonylphenol (branched) and nonylphenol (European Commission, 2002).

The biodegradability of nonylphenol has been determined in the modified Sturm test (EEC Directive 79/831 ENV/283/80). In the study nonylphenol at a concentration of 22.8 mg/l was added to a liquid mineral medium which was inoculated and aerated at a temperature of 21-23°C for 32 days. The inoculum used in the test was activated sludge from a municipal sewage plant and had a bacterial count of 18.105 CFU/ml (colony forming units/ml). The experiments were carried out both with and without an emulsifier (at a concentration of 20 mg C/l) present in the nonylphenol test solution. Control experiments were conducted using the emulsifier only and a control substance (sodium benzoate). Degradation was monitored by measuring the actual CO_2 evolution compared with the theoretical amount that would be evolved if the substance was completely oxidised. The control substance (sodium benzoate) achieved a degradation level of 102% within 20 days, reaching the threshold for ready biodegradability within 14 days. This indicated that the inoculum used had sufficient biological activity. Nonylphenol, with and without emulsifier, achieved a degradation level of 0% within a period of 32 days. When tested on its own the emulsifier achieved a degradation level of 0% within the 32-day period.

In a second study the biodegradability of nonylphenol was again studied in the modified Sturm test (EEC Directive 79/831 ENV/283/80) but adapted activated sludge was used as the inoculum. In this case the activated sludge was adapted prior to use in the test by incubation with nonylphenol at a concentration of 5 mg/l for 13 days and then 50 mg/l for a further 5 weeks. The test conditions were then the same as in the previous test with the exception that the duration of the test was 40 days. Nonylphenol (22.8 mg/l) was tested with and without an emulsifier (at a concentration of 20 mg C/l) and sodium benzoate was used as a control substance. Nonylphenol without emulsifier achieved a degradation level of 0% within the 40-day period. Nonylphenol and the emulsifier achieved a

degradation level of 78% within the 40-day period (the control with emulsifier alone showed 0% degradation).

3.1.2.1.3 Simulation tests

Table 10: Summary of simulation tests in water

Compound	Conditions	Result	Reliability	Reference
Sewage sludge				
Nonylphenol (not specified)	aerobic	Primary degradation: $DegT_{50}$ = 3.1 - 4.7 days (30 °C) $DegT_{50}$ = 7.5 days (20 °C)	2	(Chang et al., 2005b)
	anaerobic	Primary degradation: Sewage sludge: $DegT_{50}$ = 23.9 days (30 °C) DegT50 = 38.5 days (20 °C) Petrochemical sludge: DegT50 = 36.5 days (30 °C)	2	(Chang et al., 2005a)
Seawater				
4-n-Nonylphenol (CAS-No.:104-40-5)	aerobic	With bubbling: 98.8% dissipation after 1 week Without bubbling: $DisT_{50}$ = 5 days	2	(Ying and Kookana, 2003)
4-Nonylphenol (mixture of different branched isomers)	aerobic	$DegT_{50}$ ~ 56 days; lag phase 28 days	2	(European Commission, 2002; Ekelund et al., 1993)
Fresh water (stream and bond)				
Nonylphenol (mixture of monoalkyl phenols predominantly para substituted)	aerobic	Open flaks: $DisT_{50}$ = 2.5 days (volatilisation and co-distillaion) Closed flasks: $DisT_{50}$ ~ 16 days	2	(European Commission, 2002; Sundaram and Szeto, 1981)

Chang et al. investigated the degradation of nonylphenol in sewage sludge under aerobic conditions as well as under anaerobic conditions (Chang et al., 2005b; Chang et al., 2005a). Effects of various factors (initial concentration, temperature, pH, additives) were tested. Without additives, inital nonylphenol concentrations of 5 mg/L and a temperature of 30°C, the DegT50s (primary degradation) (the time when 50% of a substance degraded from a single medium) ranged from 3.1 days (bioreactor experiment) and 4.7 days (batch experiment) under aerobic conditions and 23.9 days (sewage sludge) or 36.5 days (petrochemical sludge) under anaerobic conditions.

Ying and Kookana studied the elimination of 4-n-nonylphenol in marine environment using seawater taken from a coastal area near Adelaide, Australia (Ying and Kookana, 2003). The initial concentration of 4-n-nonylphenol in water was 5 µg/L (incubation at 20 ± 3°C). The solutions were aerated by bubbling air through them. Rapid initial losses were seen in the non-sterile solutions and

the sterile control, indicating that these losses resulted from abiotic processes (volatilization, adsorption). After this initial removal, the concentration in the non-sterile solutions continued to decrease to 0.06 µg/L within 1 week. The concentration in the sterile control remained stable with little change. Experiments which were carried out without bubbling air through the solutions, showed a $DisT_{50}$ (the time when 50% of a substance dissipates from a single medium) of 5 days. Some loss may have been caused by adsorption, too.

The following information (italic) was copied from the Risk Assessment Report of 4-nonylphenol (branched) and nonylphenol (European Commission, 2002).

Ekelund et al. (1993) studied the biodegradation of 4-nonylphenol in seawater and sediment. In the experiments ^{14}C uniformly ring-labelled nonylphenol (synthesised using nonene containing a mixture of branched isomers) was used. The reaction flasks used contained seawater or seawater plus sieved soft bottom sediment. Formalin was added to four flasks containing seawater and half of the flasks containing seawater and sediment were bubbled with nitrogen gas prior to the start of the experiment. 11µg ^{14}C ring-labelled nonylphenol was dissolved in acetone and added to small glass plates, the solvent was then evaporated and the glass plates added to the reaction flasks. The flasks were incubated at 11 ± 2°C in the dark for 16 weeks. In flasks containing formalin no $^{14}CO_2$ was recovered, indicating that any $^{14}CO_2$ must come from the nonylphenol in the presence of living organisms. In the absence of sediment, degradation (as measured by $^{14}CO_2$ production) was very slow at 0.06% per day up to 28 days then 1% per day after 28 days, suggesting a period of adaptation is required. In the presence of sediment the degradation rate was faster at 1.2% per day. In the low oxygen experiments the reaction rate was slow. The increase in degradation rate in the sediment system was attributed to the higher number of microorganisms present. The overall recovery of ^{14}C from these experiments was around 64% (44% in the CO_2 fraction) in the flasks without sediment and 49% (46% in the CO_2 fraction) in the flasks with sediment. Thus around 45% of the ring-label was converted to CO_2 in 8 weeks, giving a mineralisation half-life of slightly longer than 56 days. However, the low overall recovery of ^{14}C-label in the experiments indicates that the actual extent of biodegradation may be higher (with a resulting shorter half-life) than implied by the $^{14}CO_2$ measurements (for example incorporation of the ^{14}C-label into biomass may have occurred (Ekelund et al., 1993).

The degradation of nonylphenol in stream and pond water has been studied under simulated field conditions (Sundaram and Szeto, 1981). The water and sediments used were taken from Northland Creek and Hargraft Lake, Ontario, Canada. The degradation experiments were carried out by incubating samples of the water or water plus sediment (100 g pond sediment in 200 ml pond water) with nonylphenol (1 mg/l) in either open or closed flasks at 16°C for up to 44 days, under artificial light (16 hours light and 8 hours dark per day). At various times during the study samples were analysed for the presence of nonylphenol by HPLC analysis. When incubated in either pond water (pH 7.3) or stream water (pH 6.9) in open flasks, nonylphenol was found to disappear from solution rapidly with a half-life of around 2.5 days for both systems. No degradation products were detected in the water during the experiment and it was thought that the removal from solution was due to volatilisation and co-distillation rather than degradation. When nonylphenol was incubated in either pond water or stream water in sealed flasks, the halflife was found to be 16.5 days in stream waterand 16.3 days in pond water. Unidentified transformation products (more polar than the parent nonylphenol) were also shown to be formed in the experiment and it was thought by the authors that these could be formed by microbial degradation or photo-oxidation. In incubations in pond water with sediment present, most of the nonylphenol initially adsorbed onto the sediment phase.
The sediment phase showed a maximum nonylphenol concentration after around 10 days which subsequently reduced, with only 20% of the added nonylphenol being present after 70 days. This

removal was thought to be due to microbial degradation as the concentration in autoclaved samples remained constant over the same time period (Sundaram and Szeto, 1981)

3.1.2.2 Biodegradation in sediments

Table 11: Summary of biodegradation tests in sediments and sewage sludge

Compound	Conditions	Result	Reliability	Reference
Fresh water sediment				
Nonylphenol (not specified)	aerobic	Primary degradation $DegT_{50}$ = 13.6 – 99.0 days (30 °C) $DegT_{50}$ = 40.8 days (20°)	2	(Yuan et al., 2004)
	anaerobic	Primary degradation $DegT_{50}$ = 46.2 – 69.3 days (30 °C) $DegT_{50}$ = 99 days (20 °C)	2	(Chang et al., 2004)
4-n-Nonylphenol (CAS-No.:104-40-5)	aerobic	Degradation > 90% after 32 days	2	(Bradley et al., 2008)
	anaerobic	$DegT_{50}$ > 154 days		
Nonylphenol (not specified)	aerobic	Original Sediment: dissipation 80-85% after 6 months Spiked Sediment (100 ppm nonylphenol): dissipation 50% after 6 months	2	(Dutka et al., 1998)
	anaerobic	25-30% increase after 6 months		
Nonylphenol technical mixture (tNP) (branched) and	anaerobic (methanogenic, sulphate reducing and nitrate reducing)	0% dissipation within 703 days	2	(De Weert et al., 2011)
4-n-NP (linear)	anaerobic (nitrate reducing)	100 % dissipation after 91-104 days		
	anaerobic (methanogenic and sulphate reducing)	0% dissipation within 703 days		
Nonylphenol (mixture of monoalkyl phenols predominantly para substituted	aerobic	Nonylphenol dissipated from water to sediment (0 % nonylphenol in water after 10 days) Sediment: 80 % dissipation after 71 days	2	(European Commission, 2002; Sundaram and Szeto, 1981)
Marine sediment				
4-n-Nonylphenol	aerobic	$DisT_{50}$ = 5.8 days	2	(Ying and Kookana,
	methanoge	$DisT_{50}$ > 70 days		

(CAS-No.:104-40-5)	nic			2003)
4-Nonylphenol (mixture of different branched isomers)	aerobic/ anaerobic	$DegT_{50}$ > 56 days	2	(European Commission, 2002; Ekelund et al., 1993)

Chang et al. studied the degradation of nonylphenol by anaerobic microorganisms from NP-acclimated river sediments (Chang et al., 2004). The $DegT_{50}$ (primary degradation) ranged from 46.2 to 69.3 days (30 °C). Degradation rates for nonylphenol were enhanced by increasing temperature and inhibited by the addition of acetate, pyruvate, lactate, manganese dioxide, ferric chloride, sodium chloride, heavy metals, and phthalic acid esters. Degradation was also measured under three anaerobic conditions. Results show the high-to-low order of degradation rates to be sulfate-reducing conditions > methanogenic conditions > nitrate-reducing conditions.

Yuan et al. sampled sediment from the same samples sites as Chang et al. 2004 and studied the aerobic degradation of nonylphenol (Yuan et al., 2004). The half-lives $DegT_{50}$ (primary degradation) ranged from 13.6 to 99.0 days. These results suggest that microorganisms adapt in a site specific manner, and therefore vary in terms of biodegrading capacity. If the sediment was additionally acclimated with nonylphenol, nonylphenol was completely dissipated after 28 days.

The potential for biodegradation of 4-nonylphenol (linear isomers) was investigated in three hydrologically distinct streams impacted by wastewater treatment plants (Bradley et al., 2008). Microcosms prepared with sediment collected upstream of the wastewater treatment plant outfalls and incubated under aerobic conditions (23 °C) showed rapid and complete mineralization of [U-ring-^{14}C]4-n-nonylphenol to CO_2 in all three systems. No mineralization of [U-ring-^{14}C]4-n-nonylphenol was observed under methanogenic conditions. The initial linear rate of [U-ring-14C]4-n-nonylphenol mineralization in sediments from upstream and downstream of the respective WWTP outfalls was inversely correlated with the biochemical oxygen demand (BOD) of the streambed sediments. These results suggest that the net supply of dissolved oxygen to streambed sediments is a key determinant of the rate and extent of 4-nonylphenol biodegradation in stream systems.

In a six month study the aerobic and anaerobic biodegradation (21°C) of nonylphenol in original (81 ppm nonylphenol) and spiked fresh water sediment (additionally 100 ppm nonylphenol) was observed (Dutka et al., 1998). Under aerobic conditions nonylphenol dissipated in the original sediment 80-85% within 6 months. In the spiked sediment the concentration of nonylphenol increased over the first 3 months followed by a 50% decrease after 6 months. Under anaerobic conditions increasing concentrations (25-30%) were observed. These results suggest that either nonylphenol is resistant to biodegradation under anaerobic conditions or that it is maintained by biodegradation of precursors. De Weert et al. got similar results (De Weert et al., 2011). The biodegradation of branched (t-nonylphenol) and linear (4-n-nonylphenol) nonylphenol was investigated under anaerobic conditions (methanogenic, sulphate reducing and nitrate reducing) in nonylphenol polluted sediment (mixture of t-nonylphenol; 4-n-nonylphenol was not present). The bottles were incubated in the dark at 30 °C for 703 days. Under denitrifying conditions 100 % dissipation of linear nonylphenol was observed within 91 days. No degradation was observed for branched and linear nonylphenol after 703 days under methanogenic and sulphate reducing conditions. Although the sediment was polluted with t-nonylphenol and the bacteria were potentially adapted, no degradation occurred under anaerobic conditions.

The studies of Ying and Kookana (see chapter 3.1.2.1.3 Simulation tests) were also carried out on marine sediments, collected from close to the same area. 5 g of marine sediment with 5 ml of seawater were used to make slurry during all experiments (incubation at 20 °C). In addition to 1µg/g 4-n-nonylphenol, four other substances (including 4-tert-octylphenol) were added. Under aerobic conditions complete degradation of 4-n-nonylphenol was seen within 70 days ($DisT_{50}$ = 5.8 days). Under anaerobic conditions no degradation was occurred.

Further information for the studies of Ekelund et al. and also Sundaram and Szeto are listed in chapter 3.1.2.1.3 (Simulation tests)

3.1.2.3 Biodegradation in soil

Table 12: Summary of biodegradation tests in soil

Compound	Result	Reliability	Reference
Soil			
4-Nonylphenol	Biphasic kinetics 1. Inital phase: $DegT_{50}$ = 4.5 (±0.05) – 16.7 (±2.28) days 2. slower degradation phase ($DegT_{50}$ > 40 days)	2	(Topp and Starratt, 2000)
4-Nonylphenol (linear and branched)	Branched isomers: $DisT_{50}$ = 2.1-10.3 days Linear isomer: $DisT_{50}$ = 1.4 days	2	(Shan et al., 2011)
4-Nonylphenol (^{14}C-4-NP_{111})	After 58 days: bound residuals of ^{14}C-4-NP_{111}= 54.4% mineralization ($^{14}CO_2$) ~ 5%		
Soil + sludge			
Nonylphenol (not specified)	biphasic kinetic: 0-10 days: 55 % dissipation 10-110 days: > 90 % dissipation (NP < LOD (50µg/kg d.w.)) $DisT_{50}$ = 31 - 46 days	2	(Jacobsen et al., 2004)
Nonylphenol (not specified)	$DisT_{50}$ = 31 -51 days	2	(Dettenmaier and Doucette, 2007)
Nonylphenol (mixture of isomers, branched)	Biphasic kinetic $DisT_{50}$ = 11.5 days 26-35% of the initial concentration remained after 105 days	2	(Sjöström et al., 2008)
Linear 4-nonylphenol	Mineralization after 2 months: sludge-soil ratio 1:20 (40% water content) = 63.2 % sludge-soil ratio 1:20 (80% water content) = 56.0 % sludge-soil ratio 1:100 (40% water content) = 58.4 -63.7 %	2	(Gejlsbjerg et al., 2001)

	sludge-soil ratio 1:100 (80% water content) = 44.2 % sludge = 28.5 % soil = 56.3 %		
Technical 4-nonylphenol	ca. 90% dissipation after 322 days; triphasic kinetics: 1. Initital period (1-14 days): $DisT_{50}$ = 8 days 2. Transition time (30 – 90 days): $DisT_{50}$ = 90 days 3. Long-term persistence (> 150 days): $DisT_{50}$ > 360 days	2	(European Commission, 2002; Marcomini et al., 1989)
4-nonylphenol (mixture of uncharacterized isomers, branched)	89 % dissipation after 40 days (initial conc. 100 ppm) 62 % dissipation after 40 days (initial conc. 1000 ppm; 0.22% volatilization)	2	(European Commission, 2002; Trocmé et al., 1988)

The persistence of 4-nonylphenol in six various agricultural, noncultivated temperate, and Arctic soils was assessed in laboratory microcosm incubations (Topp and Starratt, 2000). At 30°C, mineralization of [ring-U-^{14}C]4-nonylphenol was biphasic, with a rapid initial phase lasting about 10 d, during which about 30% of the initial radioactivity was converted to carbon dioxide, followed by a much slower second phase with the final amount of carbon dioxide accumulated representing about 40% of that initially applied. The estimated half-lives (based on the inital mineralization rate) ranged from 4.5 to 16.7 days (without a lag phase). Furthermore, Topp and Starratt investigated the effect of sewage sludge on mineralization of [ring-U-^{14}C]4-nonylphenol in soil. Sludge solids did not inhibit 4-nonylphenol mineralization, although sewage sludge at high concentrations was inhibitory, apparently because of high biological oxygen demand.

Dissipation of five p-nonylphenol (4-NP) isomers including four branched (4-NP_{38}, 4-NP_{65}, 4-NP_{111}, and 4-NP_{112}) and one linear (4-NP_1) isomers in a rice paddy soil was studied under oxic conditions and 20±1°C (Shan et al., 2011). Dissipation followed availability-adjusted first-order kinetics with the decreasing order of dissipation half-life 4-NP_{111} (10.3 days) > 4-NP_{112} (8.4 days) > 4-NP_{65} (5.8 days) > 4-NP_{38} (2.1 days) > 4-NP_1 (1.4 days). Further tests with radiolabelled 4-NP_{111} exhibit, that at the end of incubation (54 days) bound residues of 4-NP_{111} amounted to 54% and only 5% of 4-NP_{111} mineralized to $^{14}CO_2$.

Degradation and mobility of nonylphenol was investigated in a lysimeter study (Jacobsen et al., 2004). In the top soil layer an initial rapid degradation of nonylphenol was observed (55% within 10 days), followed by slower but continuous degradation (nonylphenol content < 50 µg/kg d.w. (LOD) after 110 days). Assuming first-order degradation kinetics $DisT_{50}$ of 31 - 46 days were estimated. The lysimeter study showed no downward transport of nonylphenol, as it was not measured in the leachate samples or in soil layers below the top 15-cm soil layer with sludge incorporation at any time during the 110-d experimental period (first sampling after 10 days). Sjöström et al. observed also biphasic decay curves (Sjöström et al., 2008). They expected that the different degradation rates result from the presence of isomers with different side chain branching. Furthermore, a significant recalcitrant fraction (26-35%) of NP remained at the end of the tests (105 days).

The mineralization of linear nonylphenol was investigated in different sludge-soil mixtures and soils (Gejlsbjerg et al., 2001). Depending on the sludge-soil ratio (1:20 - initial concentration 55

mg/kg, 1: 100 - initial concentration 11.4 mg/kg) and oxygen demand 44.2 – 63.7 % nonylphenol were mineralized after two months. A higher content of sludge in the sludge-soil mixture should reduce the concentration of oxygen, which should result in a decrease of the mineralization rate. However, a large part of the NP was mineralized in all of the sludge-soil mixtures. Contrary to the typical situation for sludge-soil mixtures at 80% of content, the measurements of oxygen concentrations showed that the mixtures containing NP were dominated by aerobic conditions at the end of the experiment (Extension of aerobe conditions 68.8-91.6%% as compared to the experiment with nonylphenol-di-ethoxylate 16.3-37.5%). This indicates a loss of moisture by evaporation during the experiment. Only a minor mineralization of NP was seen in the core with sludge that was mainly anaerobic.

Dettenmaier and Doucette conducted microcosm experiments to evaluate the mineralization and plant uptake of nonylphenol (Dettenmaier and Doucette, 2007) in a soil/biosolids (99.5 : 0.5 w/w) environment planted with crested wheatgrass. The microcosms were located in a greenhouse with a 16:8-h light:dark photoperiod and a day/night temperature of 20±1/16±1 °C. The mineralization from ^{14}C-nonylphenol to $^{14}CO_2$ was 7 % within 150 days and was independent of the initial exposure concentration. The presence of crested wheatgrass did not enhance the percentage mineralization. Assuming first-order disappearance kinetics, half-lives for NP averaged from 31 to 51 d in the various planted, unplanted, and unplanted poisoned systems.

The following information (italic) was copied from the Risk Assessment Report of 4-nonylphenol (branched) and nonylphenol (European Commission, 2002):

Trocmé et al. (1988) studied the fate of nonylphenol in a simplified soil system and its effect on microbial activity. The soil system was made up of sewage sludge compost (1/3 dry matter) and sandstone (2/3 dry matter) and had the following characteristics: pH 6.8, total nitrogen 0.5%, organic carbon 11%, carbon:nitrogen ratio 20, total phosphorus 1%, cation exchange capacity 22.1 meq/100g, water holding capacity 51%. Nonylphenol was dissolved in ethanol (0.4 ml/g spiked compost) and mixed with part of the compost, the ethanol was then left to evaporate off. The spiked compost was mixed with the remaining compost to give a 60 g sample. Two concentrations of nonylphenol were applied (100 mg/kg and 1,000 mg/kg) plus a control sample spiked with ethanol. The cells were incubated at 60% field moisture capacity at 25°C in the dark for 40 days. Carbon dioxide was removed periodically by flushing the cells with carbon dioxide free air, the adsorbed carbon dioxide was determined by a conductivity method and volatilisation of nonylphenol measured by phenol traps. Nonylphenol persistence was also studied under aseptic conditions. Samples were sterilised by gamma irradiation, spiked with 100 mg/kg nonylphenol then incubated for 24 hours under the above conditions. The authors found that carbon dioxide evolution was significantly depressed by the 4th day in the 1,000 mg/kg spiked sample, and a decrease was noted in the adenosine triphosphate (ATP) content in the 1,000 mg/kg sample after 5 days. No significant changes in carbon dioxide evolution or ATP content were observed in the control and 100 mg/kg sample. After 40 days incubation 11% nonylphenol remained in the 100 mg/kg sample and 38% remained in the 1,000 mg/kg sample. In both samples volatilisation was insignificant with 0.22% volatilisation over 40 days in the 1,000 mg/kg sample. In both samples nonylphenol concentrations started decreasing after 5 days incubation; loss was rapid at first then slowed down. Nonylphenol was more persistent under the semi-sterile conditions with 76% nonylphenol recoverable after 24 days. The authors suggested that nonylphenol underwent microbial degradation after a period of induction of the microorganisms. The chromatographic profile for nonylphenol taken at various times during the test indicated that certain isomers of nonylphenol degraded more easily than others (Trocmé et al., 1988).

Marcomini et al. (1989) studied the fate of nonylphenol in sludge amended soil. Soil samples were collected from the upper 5 cm of planted grassland that had received anaerobically digested sludge at an average application rate of 13.5 tonnes/ha year (dry weight). The sludge was applied to the surface soil as a liquid spread, four to six times per year. Samples were dried at 60°C, pulverised to a particle size of <300 µm and stored in the dark at 4°C. Nonylphenol was analysed by extraction with hexane and quantified by HPLC with a UV-fluorescence detector. The initial concentration of nonylphenol in the soil was 4.7 mg/kg and this had dropped to 0.46 mg/kg dry weight after 322 days. The concentration of nonylphenol in a soil that did not have sludge applied was <0.02 mg/kg (dry weight). (Marcomini et al., 1989).

The primary biodegradation of nonylphenol in soil has been studied in field trials over a period of 1 year (Küchler et al., 1994). Areas of land (each 6·3 m) were treated with either sewage sludge or sanitary effluent which contained nonylphenol, along with nonylphenol ethoxylates. Initially it was found that the concentration of nonylphenol increased slightly, possibly due to formation from the degradation of nonylphenol ethoxylates present. The concentration of nonylphenol was then found to decrease rapidly in the soil, with no nonylphenol being detected in any sample (samples were collected at depths of 0-10 cm, 10-20 cm and 20-30 cm) after 20 days. Nonylphenol did not leach from the 0-10 cm depth layer into lower layers, indicating that biodegradation is the most likely removal mechanism.

Kirchman et al. (1991) studied the biodegradation of 4-n-nonylphenol in soil (the substance tested presumably has a straight alkyl chain rather than a branched chain as typically found in commercial products). In the test nonylphenol was added to soil at concentrations of 10 or 500 mg/kg and incubated in sealed flasks for 3 months. Degradation was monitored by analysis for the parent compound and also CO_2 evolution. Based on parent compound analysis, less than 10% of the added nonylphenol remained after 10 days incubation, and nonylphenol was not detected (<0.02 mg/kg) after 20 days incubation. At the higher concentration tested, CO_2 evolution was higher than that seen in controls and indicated that around 61% of the nonylphenol carbon was converted to CO_2 after 94 days incubation. However, at the 10 mg/kg concentration the CO_2 evolution was similar to controls and so it is not possible to infer anything about the rate of mineralisation at this concentration. A short-term (7 day) inhibition on nitrification was seen in the system exposed to 500 mg/kg nonylphenol

3.1.2.4 Summary and discussion on biodegradation

The linear isomer 4-n-nonylphenol:

For 4-n-nonylphenol only one test of ready biodegradability is available. This test suggests that 4-n-nonylphenol is ready biodegradable.

The results from simulation tests demonstrate that 4-n-nonylphenol dissipates and degrades in surface water ($DisT_{50}$ = 5 days, (Ying and Kookana, 2003)), fresh water sediment (90% degraded after 32 days, (Bradley et al., 2008)) and marine sediment ($DisT_{50}$ = 5.6 days, (Ying and Kookana, 2003)) under aerobic conditions. The dissipation in surface water is probably caused by adsorption to sediment. Under anaerobic conditions no dissipation was observed for marine sediment within 70 days and no degradation was observed for fresh water sediment until day 154 (Bradley et al., 2008; Ying and Kookana, 2003). Only limited data is available for dissipation of 4-n-nonylphenol in soil.

A dissipation half life of 1.4 days suggests that 4-n-nonylphenol dissipates fast in soil (Shan et al., 2011).

4-Nonylphenols:

The results of the ready biodegradability tests show that nonylphenol is not readily biodegradable. But after a time of adaption nonylphenol may undergo biodegradation. Hence, nonylphenol could be considered as inherently biodegradable.

Biodegradation of nonylphenol depends on several factors. Nonylphenol exists as linear and branched (differing degrees) isomers. Regarding this the following is applicable: the higher the branching, the lower the biodegradability. The different degrees of biodegradability were demonstrated in some soil simulation tests. Several authors observed a biphasic degradation or dissipation kinetic with a fast inital phase (for example degradation or dissipation of the linear isomer) and a following slower degradation phase (European Commission, 2002; Jacobsen et al., 2004; Marcomini et al., 1989; Sjöström et al., 2008; Topp and Starratt, 2000).

Further factors that affect the biodegradation of nonylphenol are that microorganisms need a period of adaption (Ekelund et al., 1993) and that the biodegradation is enhanced by increasing temperature (Chang et al., 2005a; Chang et al., 2005b; Chang et al., 2004; Yuan et al., 2004)

The results from simulation tests demonstrate that nonylphenol degrades and dissipates in surface water ($DisT_{50}$ = 2.5 days, $DegT_{50}$ ~ 56 days) and sediment ($DegT_{50}$ = 3.1- 99 days) under aerobic conditions. Dissipation from the surface water is probably caused by adsorption to sediment ((European Commission, 2002; Sundaram and Szeto, 1981). Different results exist for biodegradation in sediment under anaerobic conditions. The half-lives range from 23.9 days (primary degradation) to > 703 days. These differences under aerobic as well as anaerobic conditions results from the abovementioned factors. Additionally, in some studies the isomer is not indicated, so it is not known whether the isomer is linear or branched. The majority of the studies indicate a very slow or no biodegradation of nonylphenol in sediment under anaerobic conditions.

For biodegradation of nonylphenol in soil, most of the studies investigated dissipation or primary degradation ($DisT_{50}$ = 2.1-51 days). Like abovementioned, nonylphenol degrades biphasic with a fast initial phase ($DegT_{50}$ < 16.7 days) and a following slower degradation phase ($DegT_{50}$ > 40 days) (Topp and Starratt, 2000) with residual concentrations of nonylphenol after the end of the test (Sjöström et al., 2008). Only one study investigated mineralization (5% CO_2 after 58 days) (Shan et al., 2011).

3.1.3 Summary and discussion on degradation

Even if photodegradation (DT_{50}= 0.3 days) and photolysis in water (DT_{50}= 10-15 hours) may occur in the environment, overall the abiotic degradation is a negligible removal process.

The screening tests for ready biodegradability indicate that nonylphenol is inherently biodegradable after a time of adaptation. Nonylphenol is biodegradable in surface water, soil and sediment under aerobic conditions. But under anaerobic conditions nonylphenol biodegrades very slowly or not at all.

The screening test for ready biodegradability indicates that 4-n-nonylphenol is readily biodegradable. 4-n-nonylphenol dissipates in surface water, soil and sediment under aerobic conditions. But under anaerobic conditions no degradation was observed.

3.2 Environmental distribution

3.2.1 Adsorption/desorption

The Risk Assessment Report (European Commission, 2002) refers to several studies and calculated values for the adsorption behaviour which can be used for the assessment of the adsorption behaviour. The Risk Assessment cited a study conducted by Roy F. Weston Inc. (1990) which reported a wide range of log K_{OC} values ranging from log 4.35 to log 5.69. But these values deal with uncertainties because adsorption on the test vessels was detected. Ahel et al. (1994) measured the occurance of nonylphenol ethoxylates and their metabolites in surface waters and sediments in the Glatt River in Switzerland. The ratio of nonylphenol concentrations in sludge to nonylphenol concentrations in water ranged from 364 to 5100. In the RAR also a set of partitioning coefficients is presented:

K_{oc}	5,360 l/kg	Partition coefficient organic carbon-water
Kp_{susp}	536 l/kg	Partition coefficient solids-water in suspended matter
Kp_{sed}	268 l/kg	Partition coefficient solids-water in sediment
Kp_{soil}	107 l/kg	Partition coefficient solids-water in soil
$K_{soil\text{-}water}$	161 m³/m³	Soil-water partitioning coefficient
$K_{susp\text{-}water}$	135 m³/m³	Suspended matter-water partitioning coefficient
$K_{sed\text{-}water}$	135 m³/m³	Sediment-water partitioning coefficient

Finally experimental data and calculated partition coefficients lead to the conclusion that nonylphenol will strongly adsorb to soils, sludges and sediments.

3.2.2 Volatilisation

The volatilisation of 4-Nonylphenols from surface water to air may be estimated by the Henry's Law constant. This is calculated as 11.02 $Pa*m^3*mol^{-1}$ for substance properties of nonylphenol taken from the RAR (melting point: -8°C, Vapour pressure (@25°C): 0.3 Pa). The airwater partitioning coefficient ($K_{air\text{-}water}$) may be derived from the Henry's law constant and is calculated as $4.65*10^{-3}$ m^3/m^3. The Kair-water and Henry's law constant are low suggesting that volatilisation is unlikely to be a significant removal mechanism for nonylphenol from aquatic systems and it is unlikely that the substance will be transported very far in the atmosphere.

3.2.3 Distribution modelling

When released to the environment 4-nonylphenol (branched) will be distributed to the environmental compartments in different ratios. The table below shows the result of Fugacity Level III distribution modelling using EPI Suite v4.10 and the substance properties calculated within EPI Suite.

Table 13: Result of Fugacity Level III distribution modelling for p-nonylphenol (branched)

	Value (percent; EPI suite v4.10, standalone version)	Value (percent; EPI suite based calculation from www.chemspider.com)
Fraction to air	0.26	0.095
Fraction to water	12.2	4.47
Fraction to soil	66.0	36.9
Fraction to sediment	21.5	58.5

The results of distribution modelling indicate that the substance will strongly adsorb to soil and sediment when released to the environment. The reason for the different distribution figures is unclear because the EPI Suite modules of both show the same physical-chemical properties. Nevertheless both distributions show a similar result – the majority of 4-nonylphenol (branched) released to the environment will accumulate in soil and sediment.

Distribution in wastewater treatment plants

The dominant route of environmental releases for 4-nonylphenol (branched) is expected to be via wastewater which is treated in sewage treatment plants. Therefore different approaches – based on physical-chemical data retrieved from QSAR and measurements – have been used to estimate the distribution of the substance in sewage treatment plants.

Table 14: Fractions of emissions from sewage treatment plant into environmental compartments calculated with EPI Suite.

	Value (percent; EPI suite v4.10, standalone version)	Value (percent; EPI suite based calculation from www.chemspider.com)
Fraction to air (percent)	0.56	0.01
Fraction to water (percent)	69.47	8.22
Fraction via primary sludge (percent)	17.30	91.01
Fraction via surplus sludge (percent)	12.35	
Fraction degraded (percent)	0.32	0.77

As the modelling of the distribution of substances based on physical-chemical properties calculated with QSARs shows great differences in the results, additional calculations have been conducted with SimpleTreat and the measured physical-chemical data for nonylphenol from the Risk

Assessment Report (European Commission, 2002). As in the Risk Assessment Report for comparsion the calculation has been done assuming the substance being both inherently and not readily biodegradable (see table below).

Table 15: Fractions of emissions from sewage treatment plant into environmental compartments acc. To SimpleTreat 3.0 (debugged version 7 Feb 1997).

	Not readily biodegradable (k = 0/h)	**Inherently biodegradable (k = 0.1/h)**
Fraction to air (percent)	9.6	6.7
Fraction to water (percent)	52.7	35.0
Fraction via primary sludge (percent)	27.9	27.9
Fraction via surplus sludge (percent)	9.8	6.5
Fraction degraded (percent)	0.0	23.9

As well as mentioned in the Risk Assessment Report it can be seen that degradation predominantly effects the residual concentration of p-nonylphenol (branched) in the water phase while the predicted concentration in sludge and air nearly remains constant.

4 HUMAN HEALTH HAZARD ASSESSMENT

Not relevant for this dossier

5 ENVIRONMENTAL HAZARD ASSESSMENT

5.1 Aquatic compartment (including sediment)

5.1.1 Acute toxicity data

This chapter provides a short summary of acute toxicity test results in order to be able to compare between acute and chronic test results for the 4-nonylphenols. All available results are included in the IUCLID-file.

There are several acute data for fish, invertebrates and algae available, though some of them are use with care studies. For fish acute toxicity test results are in the range of 135 – 950 µg/L. For aquatic invertebrates the EC50 values for *D. magma* and *Hyalella azteca* (Saussure) were in the range between 85 and 150 µg/L. Other tests showed malformations and very low EC50 values: 43µg/L (*Americamysis bahia*) and 23.4µg/L (mollusk *Haliotis diversicolor suptertexta*, morphology embryo toxicity after 96h). After exposure of *Crassostrea gigas* larvae (48 – 72 h) D-shaped larvae were observed (NOECs between 0.1 and 10µg/L). Also *Arbacia lixula* (sea urchin) showed larval malformations 72h-LOEC 0.937µg/L after exposure from fertilization. The LC50 for the mollusk *Mulinia lateralis* was 37.9µg/L.

The acute values for algae were in the range of 27 to 1300µg/L.

5.1.2 Toxicity tests results concerning endocrine disruption

5.1.2.1 General approach

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

“An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations” (IPCS; cited in (European Commission, 1999)).

As it is assumed in this report, that a substance should fulfil at least this definition in order to be of equivalent concern (see chapter 8), information available is assessed based on the following questions:

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

As the 4-nonylphenols are described in the literature as showing estrogen agonist activity (estrogen receptor activation and other estrogen like activity), information is analysed with a focus on this mode of action. However, other modes of action are analysed too, where information is available.

Information is summarized by organism groups in the following chapters, starting with a summary of available *in vitro*-tests as supportive information.

5.1.2.2 *In vitro* data

In vitro estrogen activity of the 4-nonylphenols were assessed in different assays including binding assays, reporter gene assays, YES assays and assays analyzing vitellogenin (VTG) induction in primary hepatocytes of *Oncorhynchus mykiss* and *Xenopus laevis*. In addition some information on anti-androgen activity is available. Tests were either performed with the linear 4-nonylphenol isomer (4-n-NP) or the technical nonylphenol isomer mixture (4-NP) which is assumed to be a mixture of isomers with different branching. Results are briefly summarized below and in Table 16.

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. Typically, the estrogen receptor (ER) preparation is incubated with a radioactively labelled model ligand at a concentration that results in saturation of the receptors ligand-binding site. Then, unlabelled test chemical or unlabelled model ligand are added at increasing concentrations. Depending on the binding affinity of the test chemical to the receptor lower or higher concentrations are needed to displace a certain percentage of the radiolabelled ligand from the ligand-binding pocket. From the established binding curves the relative biding affinity of the test chemicals compared to model ligand can be compared

Various authors assessed whether or not 4-nonylphenols are able to specifically bind to the estrogen receptor. In total seven of such studies were analyzed for this dossier. In all studies, 4-nonylphenols were demonstrated to displace specifically bound 17β-estradiol (E_2) from the estrogen receptor (ER). The relative binding affinity (RBA) of 4-nonylphenols compared to E_2, however, varied between the individual studies. (Tollefsen et al., 2002), (Olsen et al., 2005) and (Tollefsen and Nilsen, 2008) found that NP had an approximately 100,000 lower affinity to the rainbow trout estrogen receptor (rtER) than the model ligand E_2 (RBA = 1×10^{-5}). In a study conducted by (Petit et al., 1997), a 270-fold excess of 4-nonylphenols was sufficient to displace 50 % of specifically bound E_2 (RBA = 0.37×10^{-2}).

The discrepancy between the RBA of 4-nonylphenol derived by (Petit et al., 1997) and the the RBA observed by the formerly quoted authors may be due to differences in the biochemical background of the ER preparation used. While (Tollefsen et al., 2002), (Olsen et al., 2005) and (Tollefsen and Nilsen, 2008) were using trout liver homogenates comprising various, in the native tissue coexisting ER isoforms, (Petit et al., 1997) was using a recombinant system in which only one concrete receptor isoform was (over)expressed.

However, the RBA reported by (Marlatt et al., 2006), who also performed ligand-binding studies in trout liver homogenates, was in the same order of magnitude as the RBA found by (Petit et al., 1997) (0.44 x 10-2). The difference might be due to the isomers tested. While both authors tested the technical nonylphenol, i.e. a mixture of branched isomers, all other authors tested the linear 4- nonylphenol isomer (4-n-NP).

In two of the analyzed studies (Tollefsen et al., 2002), (Olsen et al., 2005) ERs derived from different species were compared. No major difference could be found regarding the RBA of NP to the ER derived from rainbow trout (1×10^{-5}) and to the ER derived from atlantic salmon (1.2×10^{-4}) (Tollefsen et al., 2002). The RBA affinity of Nonylphenol to the human

estrogen receptor (hER) (RBA = 2.0 x 10^{-4}) was higher than the RBA for the rainbow trout estrogen receptor (rtER), which is generally about (RBA = 1 x 10^{-5}) (Olsen et al., 2005).

Reporter gene assays

Transcriptional activation assays in vertebrate cell lines

Transcriptional activation assays are used to assess whether or not a test chemical is able to activate the receptor. Activation of the receptor is demonstrated by means of reporter genes. These reporter genes are under the control of a promoter which contains specific sequences (responsive elements) to which the receptor binds upon its activation. The reporter genes typically encode for enzymes that convert an added substrate in a colored or fluorescent product. This enzyme activity correlates with the transcriptional activity of the receptor and thus is a measure of the agonistic potential of the test chemical.

(Ackermann et al., 2002a) determined the relative estrogenic potency (REP) of Nonylphenol (compared to E2) using RTG-2 cells (a cell line derived from rainbow trout gonad) transiently transfected with an rtER expressing vector and an estrogen-responsive luciferase reporter plasmid. NP was found to significantly induce luciferase activity at concentrations as little as 5 x 10-8 M (11 µg/L). The LOEC of E_2 in this test system was 5 x 10-11 M (0.014 µg/L). The REP of NP, here defined as ratio between the EC_{50} obtained for E2 and the concentration of NP that resulted in equal induction levels as EC_{50} (E_2), was 1 x 10-3.

Similar studies were conducted in human *in vitro* systems. (Lascombe et al., 2000) and (Van den Belt et al., 2004) used MCF-7 cells stably transfected with a plasmid bearing the sequence of the human estrogen response element (ERE) coupled to a luciferase reporter gene to investigate whether or not Nonylphenol has the ability to activate the human estrogen receptor (hER).

Both authors observed a concentration-dependent increase in luciferase activity. (Lascombe et al., 2000) tested only three different Nonylphenol concentrations and therefore was not able to establish a dose-response curve and/or calculate the potency of Nonylphenol compared to E_2. However, Nonylphenol was found to induce luciferase activity to a similar extent as E_2. Maximal induction levels were observed at Nonylphenol concentrations that were only 1 x 10^3-times higher than that of E_2 (EC_{max}(NP) = 1 x 10^{-5} M or 2203.5 µg/L, respectively and EC_{max}(E_2) = 1 x 10^{-8} M or 2.7 µg/L, respectively). Co-incubation of Nonylphenol with the ER-antagonist ICI 182,780 led to abolishment of NP-dependent luciferase activity giving evidence that the effect was mediated by the ER. However, it has to be taken into account that a decrease in luciferase activity (albeit to a lower extent) was also observed in the respective vehicle control. This observation suggests that the used culture medium was not completely devoid of estrogens (Lascombe et al., 2000). (Van den Belt et al., 2004) tested a broad range of Nonlyphenol concentrations. For both compounds, the reference estrogen E_2 and the test chemical NP, EC_{10} and EC_{50} values were estimated from dose-response curves that were fitted to the data. The EC_{10}-value of NP was with 1.28 x 10^{-7} M (=28.2 µg/L) about five order of magnitude higher than the EC_{10}-value derived for E_2 (3 x 10^{-12} M or 0.8 x 10^{-3} µg/L, respectively). The REP of NP (here calculated as the ratio of the EC_{50} values obtained for E_2 and Nonylphenol) was reported to be 3 x 10^{-5} (Van den Belt et al., 2004).

Thus, in the assay based on the human breast cancer cell line MCF-7 conducted by (Van den Belt et al., 2004) Nonylphenol appeared to be by a factor of 100 less potent than in the assay based on the piscine cell line used by (Ackermann et al., 2002a).

Whether this discrepancy in the REPs derived for Nonylphenol is due to differences in the experimental performance of the assay or due to species-dependent differences in the affinity of Nonylphenol to the respective receptor, remains to be elucidated.

Transcriptional activation in recombinant yeast (Yeast estrogen screen, YES)

The potential of 4-nonylphenols to act as agonist of the ER was also investigated by means of reporter gene assays based on recombinant yeast cells heterogously expressing the human or piscine (*O. mykiss*) ER. For this dossier ten of such studies were analyzed and evaluated. In two studies a yeast strain heterogously expressing the trout ER (rtER) was used (Madigou et al., 2001; Petit et al., 1997), in eight studies a yeast strain heterogously expressing the human ER (hER) was used (Coldham et al., 1997), (Gaido et al., 1997), (Lascombe et al., 2000), (Payne et al., 2000), (Folmar et al., 2002), (Rutishauser et al., 2004), (Van den Belt et al., 2004) and (Isidori et al., 2006).

In the two studies in which the activation of the trout ER was examined, the LOEC for NP was found to be 1 x 10^{-6} M (220.35 µg/L). The LOECs for the reference estrogen E_2 were similar in both studies (1 x 10^{-8} - 1 x 10^{-9} M or 0.027 – 0.272 µg/L, respectively). A lack of reporter gene induction in cells which were not transformed with the rtER (negative control). demonstrates that the NP-induced response was mediated by the receptor (Petit et al., 1997).

The REP of Nonylphenol (in both studies calculated as REP = LOEC(E_2)/LOEC(NP)) was 1 x 10^{-2} - 1 x 10^{-3}, respectively. However, Madigou et al. (2003) demonstrated that the relative transcriptional activity induced by Nonylphenol (compared to E_2) may depend on the reporter construct used. In total four reporter constructs differing in the number of estrogen responsive elements (EREs) and/or promoter were compared. While in one construct the ß-galoctosidase activity at 10^{-6} M (220 µg/L) NP was similar to the activity of E_2 at 10^{-9} M (0.272 µg/L), in three other assays the activity was less pronounced (60 %, 40 % or 15 % activity compared to E_2). No differences between the linear and branched nonylphenol isomers were observed (REP 4-NP: 1 x 10^{-3}; REP 4-n-NP: 1x 10^{-2} – 1 x 10^{-3})

These findings must be taken into consideration when quantitatively comparing the REPs obtained with different test systems.

In the studies which aimed to assess the interaction of 4-Nonylphenols with hER, the REPs of Nonylphenol (in all studies calculated as REP = $EC_{50}(E_2)$/ EC_{50}(NP)) ranged from 1 x 10^{-6} (Folmar et al., 2002) to 2 x 10^{-2} (Isidori et al., 2006). However, in six of the eight studies REPs of Nonylphenol were in the same order of magnitude (0.22 x 10^{-4} - 2 x 10^{-4}). No differences between the linear and branched nonylphenol isomers were observed (Coldham et al 1997: REP 4-NP: 0.5 x 10^{-4}; REP 4-n-NP: 0.22 x 10^{-4})

Expression of estrogen-responsive genes

Vitellogenin expression

Seven *in vitro* studies investigating the effect of 4-Nonylphenols on vitellogenin (Vtg) mRNA or protein expression were analyzed. In six of these studies primary hepatocytes derived from male and/or immature rainbow trout (*Oncorhynchus mykiss*) were used to assess if 4-Nonylphenols acts as agonists of the ER (Jobling and Sumpter, 1993), (Tollefsen and Nilsen, 2008), (Olsen et al., 2005), (Marlatt et al., 2006), (Petit et al., 1997) and (Madigou et al., 2001). In one of these studies primary hepatocytes derived from male African clawed frog (*Xenopus laevis*) were used to assess the potential of 4-Nonylphenols to activate the ER-

mediated pathway in frog (Kloas et al., 1999). All studies demonstrated that exposure to 4-Nonylphenols resulted in a dose-dependent increase in vitellogenin expression levels. In addition, co-exposure of 4-Nonylphenols and the model ER-antagonist tamoxifen led to a statistically significant decrease in Nonylphenol-dependent vitellogenin expression levels suggesting that the Nonylphenol-induced effect is mediated by the receptor (Jobling and Sumpter, 1993). A direct comparison of the studies however is difficult since not all authors calculated and/or reported EC_{50} values and/or LOECs. Moreover, different approaches were used to determine the REP of NP. Still, the REPs of NP determined by (Olsen et al., 2005), (Marlatt et al., 2006) and (Tollefsen and Nilsen, 2008), all measuring Vtg protein expression levels in trout hepatocytes, are in the same range (2.0×10^{-4}, 3.1×10^{-4} and 3.3×10^{-4}, respectively). No differences between the linear and branched nonylphenol isomers were observed (REP 4-NP: 3.1×10^{-4}; REP 4-n-NP: $2 \times 10^{-4} – 3.3 \times 10^{-4}$) The REP of NP reported by (Jobling and Sumpter, 1993) is with a value of 0.9×10^{-5} about one to two orders of magnitude lower. This difference might be explained by the shorter time the cells were exposed to NP and E2 in this study compared to those quoted above (48 h vs. 96 h).

The Vtg induction level is a function of the affinity of the test chemical to the receptor, its concentration and the exposure time. Data published by (Jobling and Sumpter, 1993) indicate that maximal induction levels were not reached after 24 h ("[…]Greater secretion of vitellogenin by the cells could be obtained by restimulation and incubation for a further 2 days […]"). Based on the assumption that exposure to E2 results in a steeper increase in Vtg protein levels than exposure to NP, the difference between the Vtg expression levels of both treatments can be expected to be higher after 48 h than after 24 h of incubation (especially if the exposure medium is renewed after the first two days as it was done by (Olsen et al., 2005) and (Tollefsen and Nilsen, 2008)).

In comparison, in the two studies in which rtVtg mRNA levels were measured, the REP of Nonylphenol was determined as being in the order of 0.25 (Petit et al., 1997), (Madigou et al., 2001). Again no differences between the linear and branched 4- nonylphenol isomers were observed.

However, results of this type of assay (i.e. quantification of mRNA level) should be used with care if no thorough time course was performed prior to the actual experiment that was used to determine the REP. This seems to be the case in at least one of the two studies. (Petit et al., 1997) quantified Vtg mRNA expression levels for only one exposure concentration and at only one time point (after 48 h). Since induction at the mRNA level typically occurs relatively short after exposure to a respective agonist and most mRNAs have a relatively short half-life, at the time of measurement that was used in this study (after 48 h) an important amount of mRNA might have been already subject to degradation. The difference between E2-induced and NP-induced Vtg mRNA expression levels may therefore not be that pronounced as if Vtg mRNA expression levels had been quantified at an earlier time point (e.g. 8 h). The REP, which was calculated by both authors as the ratio of the maximal response provoked by NP and the maximal response provoked by E2, may therefore not accurately reflect the potential of NP to activate the ER-mediated pathway.

Vtg expression as response to 4-Nonylphenol exposure was also investigated in amphibian *in vitro* systems. In primary hepatocytes derived from *Xenopus laevis* Nonylphenol was observed to induce Vtg mRNA expression at much lower levels than in primary heptocytes derived from *Oncorhynchus mykiss*. The LOEC for NP in *Xenopus* hepatocytes was 10^{-8} M (2.2 µg/L) (Kloas et al., 1999), the LOECs for NP in trout heptocytes (as far as determined in the individual studies) were 3×10^{-5} M (=6610.5 µg/L) and 1×10^{-5} M (=2203.5 µg/L) (Tollefsen and Nilsen, 2008), (Marlatt et al., 2006). Comparing these results it must be borne in mind that Vtg expression in Xenopus was quantified at the mRNA level and Vtg

expression in trout was quantified at the protein level. Nonetheless, the LOECs obtained for E_2 were lower in the assays where rtVtg protein levels were measured than the LOEC obtained for E_2 in Xenopus suggesting that the higher sensitivity observed for *Xenopus* is not due to the measurement of different endpoints. (Mitsui et al., 2007) demonstrated NP to weakly induce Vtg protein expression in *Xenopus laevis* hepatocyte cultures. The lowest NP concentration at which a significantly higher Vtg protein induction compared to the control could be observed was 0.1×10^{-6} M (22.04 µg/L). Since, according to the author, LOECs were practically difficult to determine, another conceptual approach was used to calculate the estrogenic potency of NP compared to E_2. First, so called equivalently effective concentrations (EEC) of NP and E_2 were determined. In this study, the EECs were defined as those concentrations of NP and E_2, which are required to induce Vtg protein expression to certain level in the lower, linear part of the established dose-response curves (here: 2 ng/ml). The REP was then calculated on the basis of the EEC determined for E_2 (0.04×10^{-9} M) and the EEC determined for NP (0.74×10^{-6} M), resulting in a final REP of 5.4×10^{-5}. Moreover, in co-exposure experiments with E_2, NP was observed to show antagonistic effects on E_2-dependent Vtg induction. The relative inhibitory potency of NP compared to tamoxifen (a model inhibitor of the ER) was found to be 0.002. It was suggested that NP is not able to fully activate the receptor (near maximum induction levels obtained with NP were 1000-3000 fold lower than those obtained with E_2) and therefore may act as a competitive inhibitor.

Zona radiata protein expression

NP was demonstrated to induce zona radiata protein expression in primary hepatocytes derived from rainbow trout (Rutishauser et al., 2004). EC_{50} derived for NP was 14×10^{-6} M (3084.9 µg/L). The REP of NP in this study was 7.5×10^{-5}.

Expression of other estrogen-responsive genes (Microarray analysis)

(Terasaka et al., 2006) exposed MCF-7 cells to NP (1×10^{-9} M or 0.220 µg/L, respectively) and E_2 (1×10^{-6} M or 220 µg/L, respectively) and thereupon statistically compared the respective expression profiles of 120 estrogen-responsive genes (categorized into six groups: enzymes, signaling, proliferation, transcription, transport and other) using a custom cDNA microarray. NP was found to modulate most of the genes similarly as E_2. Statistical analysis revealed a significantly high ($p<0.01$) correlation between both expression profiles. The coefficient *R* describing the correlation between both profiles was 0.9.

MCF-7 cell proliferation assay

NP was further demonstrated to induce human breast cancer cell (MCF-7) proliferation (Olsen et al., 2005) and thus act as ER agonist in these cells. However, the proliferative effect induced by NP was relatively weak compared to that induced by E_2 (<50%).

Anti-androgenic effects

Besides the observed estrogenic effects, 4-nonylphenol was reported to also exert anti-androgenic effects. (Lee et al., 2003) tested NP for its anti-androgenic activity in a variety of *in vitro* assays yielding complementary information about the underlying molecular mechanisms. In a yeast two-hybrid assay system NP was observed to inhibit testosterone-dependent interaction of the AR and its co-activator protein ASC-1 in a concentration-dependent manner (IC_{50} = 2.6 M or 572.9 µg/L, respectively). Interestingly, NP was found to be a more potent AR antagonist than the model inhibitor cyproterone (CPA). In a competitive-ligand binding assay NP was found to be able to displace DHT from the AR. The inhibition reached a maximum of 30 % at a NP concentration of 5×10^{-9} M (1.1. µg/L). Note, the NP-dependent inhibition did not appear to follow a concentration-dependent relationship

suggesting that NP might not act as a competitive but rather non-competitive inhibitor. NP was also demonstrated to prevent testosterone-induced translocation of the AR into the nucleus as well as inhibit androgen-dependent expression of respective reporter genes.

In another study by (Jolly et al., 2009), primary kidney cells derived from female sticklebacks were treated with dihydrotestosterone (DHT) to yield high spiggin protein expression levels and then treated with either NP or E_2. At concentrations of 10^{-5} M (2204 µg/L) and 10^{-6} M (220 µg/L) NP caused a significant (p<0.01) inhibition of DHT-induced spiggin production. At 10^{-6} M (220 µg/L) a complete inhibition of DHT-induced spiggin production was observed, while E_2 induced a significant but incomplete inhibition of spiggin production only at the highest concentration applied (10^{-6} M or 272 µg/L, respectively). All together, these results corroborate the hypothesis that NP is an AR antagonist. Results are summarized in Table 16.

Summary

The competitive ligand-binding studies clearly demonstrated that 4-nonylphenols are able to displace specifically bound E2 from the ER ligand-binding pocket. The RBA of 4-nonylphenols for ERs derived from aquatic species ranged from 0.94 x 10^{-5} to 0.44 x 10^{-2}. Thus, 4-nonylphenols acts as a ligand of the ER. Furthermore, there is conclusive evidence that binding of 4-nonylphenols to the ER leads to activation of the ER-mediated pathway and consequently to transcriptional activation of typically estrogen-responsive genes. Modulation of 4-nonylphenols -dependent and ER-mediated gene expression was evidenced on the transcriptional, protein and cell physiological level. In frog hepatocytes concentrations as little as 10-8 M (2.2 µg/L) were sufficient to yield Vtg mRNA levels that are significantly higher than in the corresponding control. The EC50 values determined in the studies in which the expression of estrogen-dependent biomarkers (rtVtg, zona radiata protein) was quantified on the protein level ranged from 1.4 x 10-7 M (3084.9 µg/L) to 8 x 10-7 M (17,600 µg/L).The EC50 of 4-nonylphenols obtained in the transcriptional activation assay, which was based on transfected RTG-2 cells was even lower (1.21 x 10-11M or 26.66 µg/L, respectively).

Moreover, there is some indication that 4-nonylphenols may be able to interfere with other nuclear receptor-mediated pathways. Thus, 4-nonylphenols were demonstrated to inhibit androgen-mediated gene expression in vitro, with 50 % inhibition of hAR transactivation activity observed at concentrations as little as 1.97 x 10-6 M (536.59 µg/L).

Based on the available mechanistic information it can be concluded that 4-nonylphenols possesse the potential to exert estrogen-like effects and disrupt endocrine homeostasis *in vivo*. Based on the information for the different isomers, it can be concluded, that the estrogen receptor activation potency is comparable for linear and branched nonylphenols.

Table 16: Summary of *in vitro* studies assessing the potential of 4-nonylphenols to interact with the ER-mediated pathway.

ER =estrogen receptor, E2 = 17 β-estradiol, n = number of independent experiments, I = number of replicates within each experiment, ECmax = concentration, at which highest response was observed, LOEC = lowest observed effect concentration, cmax = maximal concentration of test chemical or solvent in the assay, REP = relative estrogen potency, RBA = relative binding affinity.

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-NP or E_2 for two day. 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 (rtVgt) EC_{50} (E_2) = 1.81 x 10^{-9} M (0.49 µg/L) EC_{50} (NP) = 16.15 x 10^{-6} M (3558.7 µg/L) LOEC (E_2) = 1 x 10^{-11} M (2.7 x 10^{-3} µg/L) EC_{max} (E_2) = 1 x 10^{-7} M (27.2 µg/L)	REP = 9 x 10^{-6} REP was calculated as: EC_{50} (E_2) / EC_{50} (4-NP)	A statistically significant ($p < 0.05$) decrease in NP-dependent vitellogenin production was observed in presence of the ER-antagonist tamoxifen.
Oncorhynchus mykiss rainbow trout	Tollefsen et al. (2008)	Primary hepatocytes derived from male, immature fish	Cells were exposed to serial dilutions of 4-*n*-NP 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 (rtVgt) LOEC (E_2) = 1 x 10^{-10} M (2.7 x 10-2 µg/L) LOEC (4-*n*-NP): 30 x 10^{-6} M (6610.5 µg/L) EC_{max} (E_2) = 1 x 10^{-7} M (27.2 µg/L) EC_{max} (4-*n*-NP): 30 x 10^{-6} M (6610.5 µg/L)	REP = 3.3 x 10^{-4} REP was calculated as LOEC(E_2) / LOEC(4-*n*-NP)	Note: 30 x 10^{-6} M 4-*n*-NP was the first concentration at which a slight, but significant cytotoxic effect was observed.
Oncorhynchus mykiss	Olsen et al. (2005)	Primary hepatocytes derived from immature fish	Hepatocyte monolayer cultures were exposed to different concentrations of 4-*n*-NP monolayers for 96 h whereas the exposure	Expression of vitellogenin protein (rtVgt)	REP = 2.0 x 10^{-4} REP was calculated as:	REP is not reproducible from reported EC_{50} values.

rainbow trout			medium was renewed after two days. / Solvent: DMSO, c_{max} < 0.2% (v/v)	EC_{50} (E_2) = 1 x 10^{-10} M (2.7 x 10-2 µg/L) EC_{50} (4-*n*-NP) = 80 x 10^{-6} M (17.6 x 10^{3} µg/L) EC_{max} (E_2) = 1 x 10^{-7} M (27.2 µg/L)	EC_{50} (E_2) / concentration of 4-*n*-NP that resulted in equal induction levels as EC_{50} (E_2).	
Oncorhynchus mykiss rainbow trout	Marlatt et al. (2006)	Primary hepatocytes derived from immature fish	Cells were exposed to different concentrations of NP or E_2 for 96 h. Solvent: DMSO, c_{max} = 0.2% (v/v) / n=1, i=4 (Note: Only one experiment was carried out.)	Expression of vitellogenin protein (rtVgt) The individual EC_{50} values are not reported in the publication.	REP = 3.1 x 10^{-4} REP was calculated as: EC_{50} (E_2) / EC_{50} (NP)	No full dose-response curves were established for NP and for E_2.
Oncorhynchus mykiss rainbow trout	Petit et al. (1997)	Primary hepatocytes derived from male fish	Hepatocyte aggregate cultures were exposed to a single concentration of 4-NP (10^{-5} M) or E_2 (10^{-6} M) for 48 h. Solvent: Ethanol, c_{max} = 0.1 % (v/v) / $n \leq 3$	Expression of vitellogenin mRNA (rtVgt mRNA)	REP = 0.259 REP was defined as (maximal) Vtg mRNA expression level induced by 4-NP relative to that induced by E_2.	Note: The rtVgt mRNA expression level was determined at only one time point and at only one given concentration. REP may be different under other experimental conditions.
Oncorhynchus mykiss rainbow trout	Madigou et al. (2001)	Primary hepatocytes derived from male fish	Hepatocyte aggregate cultures were exposed to increasing concentrations of 4-*n*-NP or E_2 (10^{-6} M) for 48 h. Solvent: Ethanol, $c_{max} \leq 0.1$ % (v/v) / $n \leq 3$	Expression of vitellogenin mRNA (rtVgt mRNA) EC_{max} (E_2) = 1 x 10^{-6} M (272.38 µg/L) EC_{max} (4-*n*-NP) = 1 x 10^{-5} M (2203.5 µg/L)	REP ≈ 0.25 REP was defined as (maximal) Vtg mRNA expression level induced by 4-NP relative to that induced by E_2. Note: The REP value stated in this table is not reported in the publication but was estimated from the published graph.	Dose-response curves for NP and E_2 are not presented in the publication. Note: There are some indications that the data reported in Madigou et al (2001). and Petit et al. (1997) are the same (co-authors). It was not tested if used concentrations may have cytotoxic effects.
Xenopus laevis	Kloas et al. (1999)	Primary hepatocytes derived from male, 2-3 years old *Xenopus laevis* animals	Hepatocytes were exposed to different concentrations of 4-NP (10^{-10} – 10^{-5} M) or E_2 (10^{-10} – 10^{-5} M) for 36 h. Solvent: Ethanol, c_{max} = 1 % (v/v) / n=3	Expression of vitellogenin mRNA (xlVgt mRNA) LOEC (E_2) = 10^{-9} M (0.27 µg/L) LOEC (4-NP) = 10^{-8} M	4-NP was about one order of magnitude less potent than E_2 in stimulating vitellogenin expression. (REP was not calculated by the author)	Note: No statistical analysis/ comparisons were performed to confirm that the LOECs are statistically significantly different from the control.

				(22.04 µg/L)		
Xenopus laevis	Mitsu et al. (2007)	Primary hepatocytes isolated from male *Xenopus laevis*	Cells were exposed to different concentrations of NP (0.04 x 10^{-6} M to 10 x 10^{-6} M) for six days, whereas after three days two thirds of the exposure medium volume was renewed. Solvent: DMSO, c_{max} = ≤ 0.1 % (v/v) / n=4 (same hepatocyte preparation)	Expression of vitellogenin protein (xlVgt) LOEC (E_2) = 0.02 nM (µg/L) LOEC (NP) = 100 nM (µg/L) EEC (E_2) = 0.04 n EEC (NP) =740 nM	REP = 0.00005	Note: REP was calculated as the ratio of equivalently effect concentrations determined for E_2 and NP. (for detailed information cf. to text)
Expression of zona radiata protein						
Oncorhynchus mykiss rainbow trout	Rutishauser et al. (2004)	Primary hepatocytes isolated from immature rainbow trout	Hepatocytes were exposed to different concentrations of NP (1 x 10^{-8} M – 0.1 x 10^{-3} M) or E_2 (1 x 10^{-11} M –1 x 10^{-6} M) for 72 h. Solvent: DMSO / n = 2-3 (independent preparations were used), i = 4	Expression of zona radiata protein: EC_{50} (E_2) = 1.05 x 10^{-9} M (0.29 µg/L) EC_{50} (NP) = 14 x 10^{-6} M (3084.9 µg/L)	REP = 7.5 x 10^{-5}	
Expression of other estrogen-responsive genes (DNA microarray assay)						
human	Terasaka et al. (2006)	MCF-7 cells	Cells exposed to 1 x 10^{-6} M NP (branched) or 1 x 10^{-9} M E_2 for three days. Afterwards the expression profiles of 120 estrogen-responsive genes were statistically compared. Solvent: Ethanol, c_{max} not stated.	Expression profile of 120 estrogen-responsive genes (custom cDNA microarray: EstrArray) Statistically significant correlation between the expression profiles induced by E_2 and NP	Correlation coefficient (*R*): *R* (E_2) = 0.91 (Average of nine assays for 120 genes (S.D. = 0.024)) *R* (NP) = 0.90	

Endpoint: Competitive ligand-binding (IC_{50} is the concentration displacing 50 % of $[^3H]E_2$ 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 (=100 %)**	**Comment**
Oncorhynchus mykiss rainbow trout	Petit et al. (1997)	Extract of yeast cells heterogously expressing rtER	Yeast extracts were incubated with 20 nM $[^3H]E_2$ for 16 h at 4°C in the absence or presence of increasing concentrations of 4-NP or E_2 / Solvent: Ethanol, $c_{max} \leq 1$ % (v/v) / n=2	270-fold excess of 4-NP displaced 50% of specifically bound $[^3H]E_2$.	$1/270 = 3.7 \times 10^{-3}$ RBA was calculated as $EC_{50}(E_2)$ / concentration of 4-NP resulting in equivalent effect levels as EC_{50} (E_2)	The data suggest that 4-NP is able to displace maximal 40 % of bound $[^3H]E_2$.
Oncorhynchus mykiss rainbow trout	Tollefsen et al. (2002)	Cytosolic preparation of female rainbow trout liver homogenates	Pooled female liver homogenates (~2.5 mg/ml protein) were incubated with 2.5 nM $[^3H]E_2$ for 16 h at 4°C in the absence or presence of different concentrations of 4-*n*-NP or E_2 / Solvent: Methanol, c_{max} = 2 % (v/v) / n=2-3	$IC_{50}(E_2) = 6.6 \times 10^{-9}$ M (1.79 µg/L) Note: IC_{50}(4-*n*-NP) could not be calculated since less than 50% inhibition of $[^3H]E_2$ binding occurred.	$RBA = 1 \times 10^{-5}$ RBA was calculated as $IC_{30}(E_2)$ /IC_{30}(4-*n*-NP) x 100.	Note: Not IC_{50} values, but IC_{30} values were used to calculate RBA.
Oncorhynchus mykiss rainbow trout	Olsen et al. (2005)	Cytosolic preparation of rainbow trout liver homogenates	Liver homogenates were incubated with $[^3H]E_2$ for 16 h at 4°C in the absence or presence of different concentrations of 4-*n*-NP or E_2 / Solvent: Methanol. c_{max} = 2 % (v/v) / n=3, i=2	$IC_{50}(E_2) = 6.6 \times 10^{-9}$ M (1.79 µg/L) IC_{50}(4-*n*-NP)= 3.6×10^{-4} M (79.33×10^3 µg/L) Note: IC_{50} was obtained by extrapolation	$RBA = 0.94 \times 10^{-5}$ RBA was calculated as $IC_{50}(E_2)/IC_{50}$(4-*n*-NP) x 100	Note: The calculation of RBA is not reproducible using the reported IC_{50} values. SD or SEM are not stated in the publication.
Oncorhynchus mykiss rainbow trout	Marlatt et al. (2006)	Cytosolic preparation of rainbow trout liver homogenates	Pooled liver homogenates were incubated with $[^3H]E_2$ in the absence or presence of different concentrations of NP or E_2. The incubation time and temperature is not stated/ Solvent: DMSO, c_{max} = 1% (v/v) / n=2, i = 3.	The IC_{50} values are not stated in the publication.	$RBA = = 4.4 \times 10^{-3}$ RBA was calculated as $IC_{50}(E_2)/IC_{50}$(NP) x 100	NP completely displaced specifically bound $[^3H]E_2$ when applied at a concentration of $1x10^6$ nM
Oncorhynchus mykiss rainbow trout	Tollefsen and Nilsen (2008)	Cytosolic preparation of female trout liver homogenates	Pooled liver homogenates (2.5 mg/ml protein) was incubated with 2.5nM $[^3H]E_2$ for 16 h at 4 °C) in the absence or presence of different concentrations of 4-*n*-NP (CAS 104-40-5) (0.25×10^{-6} M to 7.5×10^{-3} M) or E_2 (75×10^{-12} M to 75×10^{-9} M) Solvent: Methanol, c_{max} =1.25 % (v/v) / n=3	IC_{50} (E_2) = 3.5×10^{-9} M (0.95 µg/L) IC_{50} (4-*n*-NP) = 3.4×10^{-4} M (74.92×10^3 µg/L) Note: IC_{50} values for 4-*n*-NP were obtained by extrapolation.	$RBA = 1.0 \times 10^{-5}$ RBA was calculated as $IC_{50}(E_2)/IC_{50}$(4-*n*-NP) x 100	

Endpoint: Competitive ligand-binding (IC_{50} is the concentration displacing 50 % of [^{3}H]E_2 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 (=100 %)	Comment
Salmo salar atlantic salmon	Tollefsen et al. (2002)	Cytosolic preparation of female atlantic salmon liver homogenates	Pooled female liver homogenates (~2.5 mg/ml protein) were incubated with 2.5 nM [^{3}H]E_2 for 16 h at 4°C in the absence or presence of different concentrations of 4-*n*-P or E_2 / Solvent: Methanol, c_{max} = 2 % (v/v) / n=2-3	$IC_{50}(E_2)$=2.1 x 10^{-9} M (0.57 µg/L) IC_{50}(4-*n*-NP)=1.8 x 10^{-5} M (3966.3 µg/L)	RBA = 1.2 x 10^{-4} RBA was calculated as $IC_{50}(E_2)/IC_{50}$(4-*n*-NP) x 100	
Human	Olsen et al. (2005)	Cytosolic fraction of lysed MCF-7 cells	Cell lysates were incubated with [^{3}H]E_2 for 2h at 4°C in the absence or presence of unlabelled (4-*n*-NP) or E_2. Solvent: DMSO, c_{max} = 15% (v/v) / n=3, i=2 Note: Solvent concentration appears to be very high.	IC_{50} (E_2) = 1.8 x 10^{-9} M (0.49 µg/L) IC_{50} (4-*n*-NP)= 1.3 x 10^{-5} M (2864.5 µg/L)	RBA(4-*n*-NP) =2.0 x 10^{-4} RBA was calculated as $IC_{50}(E_2)/IC_{50}$(4-*n*-NP) x 100	Note: The calculation of RBA is not reproducible using the reported IC_{50} values. SD or SEM are not stated in the publication.

Endpoint: Transcriptional activation of reporter genes under the control of the ER

Transcriptional activation assay using recombinant yeast (yeast estrogen screen, YES)

Species	Reference	Yeast strain /receptor origin	Test condition	Endocrine mediated measurement parameters	Potency (relative to 17 β-estradiol=1)	Comment
Oncorhynchus mykiss rainbow trout	Petit et al. (1997)	Recombinant yeast heterogously expressing rtER.	Yeast cells were exposed to increasing concentrations of 4-NP (10^{-8} to 10^{-4} M) or E_2 (10^{-11} to 10^{-6} M) for 4 h at 30°C. Solvent: Ethanol, c_{max} = 1 % (v/v) / n ≥ 3	Induction of β-galactosidase activity: LOEC (E_2) = 10^{-9} M (0.27 µg/L) LOEC (4-NP) = 10^{-6} M (220.35 µg/L) EC_{max} (E_2) = 10^{-8} M (2.72 µg/L)	REP = 1 x 10^{-3} REP was calculated as: $LOEC(E_2)/LOEC$(4-NP) Note: No statistical comparison between LOEC and VC was performed.	No 4-NP-dependent lacZ induction was observed in the BJ-ECZ yeast strain, which does not express rtER giving evidence that the 4-NP induced response is mediated by the ER. Note: β-galactosidase activity in the absence of ligand = 14-18 %

				EC_{max} (4-NP) = 10^{-5} M (2203.5 µg/L) Note: The maximal β-galactosidase activity induced by 4-NP (10^{-5} M) was similar to that induced by E_2 (10^{-8} M) = 92.09 %		
Oncorhynchus mykiss rainbow trout	Madigou et al. (2003)	Recombinant yeast heterogously expressing rtER.	Yeast cells were exposed to increasing concentrations of 4-*n*-NP (10^{-8} to 10^{-4} M) or E_2 (10^{-10} to 10^{-4} M) for 4 h at 30°C Solvent: Ethanol, c_{max} = 0.1 % (v/v) / n=3	Induction of β-galactosidase activity: LOEC (E_2) = 10^{-9} to 10^{-8} M (0.27 - 2.72 µg/L) LOEC (4-NP) = 10^{-6} M (220.35 µg/L) Maximal response induced by 4-*n*-NP was around 90% of that induced by E_2.	REP = 1 x 10^{-2} - 1 x 10^{-3} Note: The REP was not reported by the authors, but can be estimated based on the reported LOECs (LOEC(E_2)/LOEC(4-*n*-NP))	Four different test systems were compared. The relative transcriptional activity induced by exposure to 4-*n*-NP (compared to E_2) was found to depend upon the reporter construct used (cf. text).
human	Coldham et al. (1997)	Recombinant yeast heterogously expressing hER	Yeast cells were exposed to increasing concentrations of 4-NP (1x10^{-11} - 1x10^{-5} M), 4-*n*-nonylphenol (1x10^{-11} - 1x10^{-5} M) or E_2 (1x10^{-13} - 1x10^{-11} M) for 18 h at 30°C. Solvent: Ethanol / n ≥ 2	Induction of β-galactosidase activity: EC_{50} values are not stated.	REP (4-NP) = 0.5 x 10^{-4} REP (4-*n*-NP) = 0.22 x 10^{-4} REP was calculated as: $EC_{50}(E_2)$ / EC_{50}(NP) RIE (4-NP) = 57 % RIE (4-*n*-NP) = 38.3 % The relative inductive efficiency (RIE) was calculated as the maximal response evoked by 4-NP expressed as percentage of the maximal response evoked by E_2.	The results for 4-n-NP were not presented together with the other test chemicals in the graph.
human	Gaido et al. (1997)	Recombinant yeast heterogously expressing hER.	Yeast cells were exposed to increasing concentrations of 4-NP (CAS No. 84852-15-3) (1x10^{-9} – 1x10^{-3} M) or E_2 (10^{-12} – 10^{-8} M) overnight at 30 °C. Solvent: Methanol, c_{max} = 0.1 % (v/v) / n = 3, i = 3	Induction of β-galactosidase activity: EC_{50} (E_2) = 2.25 x 10^{-10} M (0.06 µg/L) EC_{50} (4-NP) = 1.10 x 10^{-6} M	REP = 2 x 10^{-4} REP was calculated as: $EC_{50}(E_2)$ / EC_{50}(4-NP)	

				(242.39 µg/L)		
human	Lascombe et al. (2000)	Recombinant yeast heterogously expressing hER	Yeast cells were exposed to increasing concentrations of NP_m (mixture of ring and chain isomers) (10^{-8} – 10^{-5} M) or E_2 (10^{-11} – 10^{-6} M) overnight at 30 °C. Solvent: Ethanol, c_{max} = 0.1 % (v/v) / n = 2, i = 3	Induction of β-galactosidase activity: No EC_{50} values were calculated by the authors.	The maximal effect induced by NP_m (10^{-5} M) corresponded to 40 – 50 % of the maximal effect induced by E_2.	
human	Payne et al. (2000)	Recombinant yeast heterogously expressing hER	Yeast cells were exposed to increasing concentrations of 4-NP (0.1×10^{-6} - 100×10^{-6} M) or E_2 (concentration range not stated) for 72 h at 32 °C. Solvent: Ethanol, c_{max} not stated / n = 2, i = 2	Induction of β-galactosidase activity: EC_{50} (E_2) = 1.6×10^{-10} M (0.04 µg/L) EC_{50} (4-NP) = 1.94×10^{-6} M (427.48 µg/L) Note: 4-NP produced maximal responses similar to those obtained for E_2.	REP = 0.825×10^{-4} Note: The REP was not calculated by the authors, but was derived from the EC_{50} values as $EC_{50}(E_2)$ / EC_{50}(4-NP) based on the assumption that the Hill slope for both dose-response curves was similar.	
human	Folmar et al. (2002)	Recombinant yeast heterogously expressing hER	(concentration range not stated) overnight at 30 °C. Solvent: Triethylene glycol, c_{max} < 1% (v/v) / n=2, i=3	Induction of β-galactosidase activity: EC_{50} (E_2) = 0.21×10^{-9} M (0.05 µg/L) EC_{50} (4-NP) = 2.9×10^{-4} M (63.9×10^3 µg/L)	REP = = 1×10^{-6} REP was calculated as: EC_{50} (E_2) / EC_{50} (4-NP)	
human	Rutishauser et al. (2004)	Recombinant yeast heterogously expressing hERα	The assay was performed as described by Routledge and Sumpter (1996). Yeast cells were exposed to increasing concentrations of 4-NP (NP, 85% of branched *p*-isomers) or E_2.	Induction of β-galactosidase activity: EC_{50} (E_2) = 0.21×10^{-9} M (0.05 µg/L) EC_{50} (4-NP) = 8.4×10^{-6} M (1850.9 µg/L)	REP = 0.25×10^{-4}	
human	Van den Belt et al. (2004)	Recombinant yeast heterogously expressing hER	Yeast cells were exposed to increasing concentrations of 4-NP (8×10^{-6} – 6.25×10^{-3}M, dilution factor: 2) or E_2 (6×10^{-13} – 1×10^{-8}	Induction of β-galactosidase activity:	REP = 1×10^{-4} REP was calculated as:	

			M, dilution factor: 5) for three days at 32 °C / Solvent: E_2: Methanol, c_{max} = 0.5 % (v/v), 4-NP: DMSO, c_{max} = 0.5 % (v/v) / n=3, i=6	$EC_{10}(E_2)$ = 39 x 10^{-12}M (1.06 x 10^{-2} µg/L) $EC_{50}(E_2)$ = 153 x 10^{-12} M (4.16 x 10^{-2} µg/L) EC_{10}(4-NP) = 403 x 10^{-9}M (88.80 µg/L) EC_{50}(4-NP)=1276 x 10^{-9}M (281.17 µg/L)	EC_{50} (E_2) / EC_{50} (4-NP)	
human	Isidori et al. (2006)	Recombinant yeast heterogously expressing hERα	Yeast cells were exposed to increasing concentrations of 4-NP (1x10^{-9} – 1x10^{-5} M) or E_2 (1x10^{-11} – 1x10^{-5} M) overnight. Solvent: Ethanol, c_{max} = 0.1 % (v/v) / n=3	Induction of β-galactosidase activity: EC_{50} (E_2) = 1.03 x 10^{-7} M (28.05 µg/L) EC_{50} (4-NP) = 4.22 x 10^{-6} M (929.88 µg/L) Note: The EC_{50} for 4-NP was calculated as the concentration that causes 50% of the maximal response induced by E_2. LOEC (E_2) = 1x 10^{-11} M (2.7 x 10^{-3} µg/L)	REP = 0.03 REP was calculated as: $EC_{50}(E_2)$ / EC_{50}(4-NP) RIE = 72% The relative inductive efficiency (RIE) was calculated as the maximal response evoked by 4-NP expressed as percentage of the maximal response evoked by E_2.	For NP no full-response curve was obtained.
Transcriptional activation assay using vertebrate cell lines						
Species	**Reference**	**Cell type**	**Test condition**	**Endocrine mediated measurement parameters**	**Potency (relative to 17ß-estradiol=1)**	**Comment**
human	Lascombe et al. (2000)	MELN41 cells (derived from MCF-7 cells)	Cells were exposed to different concentrations of NP_m (mixture of ring and chain isomers) (10^{-7}– 10^{-5} M) or E_2 (10^{-12} – 10^{-6} M) for 12 h. Solvent: Ethanol, c_{max} = 0.1 % (v/v) / n=3	Luciferase activity: EC_{50} (E_2) = 1 x 10^{-10} M (27.2 x 10^{-3} µg/L) Note: No dose-response curve was established for Only three different concentrations of NP_m were tested.	EC_{max} (E_2) = 10^{-8} M EC_{max} (NP_m) = 10^{-5} M Note: NP_m (10^{-5} M) induced luciferase activity to an extent similar to E_2.	

human	Van den Belt et al. (2004)	MVLN-cell line (=MCF-7 cells stably transfected with a pVit-tk-Luc plasmide = human estrogen response element (ERE) coupled to a luciferase reporter gene).	Cells were exposed to different concentrations of 4-NP (3.2×10^{-7} – 1×10^{-6} M, dilution factor 2) or E_2 (3.2×10^{-13} – 1×10^{-9} M, dilution factor 5) for 19-20 h. Solvent: Methanol, c_{max} = 0.1 % (v/v) / n=3, i=4	Luciferase activity $EC_{10}(E_2) = 3 \times 10^{-12}$M ($0.82 \times 10^{-3}$ µg/L) $EC_{50}(E_2) = 15 \times 10^{-12}$ M (4.09×10^{-3} µg/L) EC_{10}(4-NP) = 128×10^{-9}M (28.21 µg/L) EC_{50}(4-NP) = 463×10^{-9}M (102.02 µg/L)	REP = 3×10^{-5} REP was calculated as: $EC_{50}(E_2)$ / EC_{50}(4-NP)	
Oncorhynchus mykiss rainbow trout	Ackermann et al. (2002)	Rainbow trout gonade cell line RTG-2 transiently transfected with rtERα expressing vector and a estrogen-responsive firefly luciferase reporter plasmid.	Cells were exposed to different concentrations of 4-NP ($1x10^{-10}$ - $7.5x10^{-6}$ M) or E_2 ($1x10^{-13}$ – $1x10^{-7}$ M) for 48 h. Solvent: DMSO, c_{max} = 0.05 % (v/v) / n=7, i=4	Luciferase activity $LOEC(E_2) = 0.05 \times 10^{-9}$ M (1.36×10^{-2} µg/L) LOEC(4-NP) = 50×10^{-9} M (11.02 µg/L) $EC_{50}(E_2) = 0.33 \times 10^{-9}$M ($9 \times 10^{-2}$ µg/L) EC_{50}(4-NP) = 121×10^{-9}M (26.66 µg/L)	REP = 1×10^{-3} REP was defined as ratio between the EC_{50} obtained for E_2 and the concentration of 4-NP that resulted in equal induction levels as EC_{50} (E_2). (In the study referred to as E_2 equivalent factor)	
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. (2005)	MCF-7	Cells were exposed to 4-NP for six days. Solvent: Ethanol, c_{max} < 0.2% (v/v) / n≥3, i=2	Cell proliferation	weak inducer (here defined as < 50% induction of cell growth).	SD or SEM are not reported.

Table 17: Summary of in vitro studies assessing the ED potential of 4-nonylphenols to interact with AR-mediated pathway.

AR = androgen receptor, CPA = cyproterone acetate, n = number of independent experiments, i= number of replicates within each experiment.

Endpoint: Interaction with the AR						
Competitive ligand-binding studies						
mouse	Lee et al. (2003)	HeLa cells transiently expressing mouse AR	HeLa cells were incubated incubated for 2 h at 37° with 5 nM radiolabelled dihydrotestosterone (DHT) in the presence and absence of increasing concentrations of unlabeled DHT ($0.005 \times 10^{-6} – 0.5 \times 10^{-6}$).or NP ($0.005 \times 10^{-6} – 5 \times 10^{-6}$).	AR binding: 5 nM (1.1 µg/L) NP displaced up to 30 % of labeled DHT specifically bound to the AR.		Note: the observed inhibition did not follow a dose-response relationship suggesting that NP may inhibit DHT-AR binding in a noncompetitive manner.
Inhibition of nuclear translocation of the AR						
human	Lee et al. (2003)	HeLa cells transfected with GFP-AR	HeLa cells overexpressing the GFP-AR fusion protein were incubated with NP (1×10^{-5} M) and/or testosterone (1×10^{-9} M). The subcellular distribution of GFP-AR was visualized by means of fluorescence microscopy.	Nuclear translocation of AR: NP inhibited the translocation of GFP-AR into the nucleus under the conditions used.		
Inhibition of AR-controlled gene expression						
human	Lee et al. (2003)	15p-1 cells (=sertoli cell line) and HepG2 cells (=human hepatoma cell lines) transiently transfected with AR expression vector.	Cells were treated with different concentrations of NP (1×10^{-8} M – 1×10^{-5} M) or CPA (1×10^{-8} M – 1×10^{-7}) in the presence of 10 nM testosterone. Solvent: Ethanol / n = 3	Transcriptional activity of AR NP was found to inhibit AR transactivation activity in a dose-dependent manner. IC_{50} (NP) = 1.97×10^{-6} M (536.59 µg/L) IC_{50} (CPA) = 520×10^{-9} M (216.81 g/L)		
Inhibition of androgen-dependent gene expression						
Gasterosteus aculeatus	Jolly et al. (2009)	Primary kidney cells derived from DHT-	Cells were pre-treated with $1x10^{-8}$ M (3 µg/L) DHT, and then incubated in presence of	Inhibition of spiggin protein expression (anti-androgenic	Note: 10^{-6}M of NP caused a complete inhibition of	

Three-spined stickleback		primed female sticklebacks	E_2 (2.7 pg/L to 270 µg/L or $1x10^{-14}$-$1x10^{-6}$ M, respceitvely) or NP (2.2 pg/L to 228 µg/L, or $1x10^{-14}$-$1x10^{-6}$ M, respectively for 48 h at 18°C. Solvent: Ethanol, c_{max}= 0.1% (v/v) / n=3	effect) The two highest concentrations of NP were able to significantly (p<0.01) inhibit DHT-induced spiggin production as compared to positive control.	DHT-induced spiggin production (= spiggin cell content similar to that of the vehicle control)	
Yeast two-hybrid assay						
human	Lee et al. (2003)	ARhLBD-ASCI Yeast Cells (Yeast two-hybrid assay system)	ARhLBD-ASCI yeast cells were grown in the absence or presence of 1 x 10^{-5} M testosterone and/or different concentrations of NP (1 x 10^{-7}–1 x 10^{-4} M) at 30°C for 3 h / n = 3, i = 2	Inhibition of β-galactosedas activity / inhibition of the interaction between AR and ASC-1. IC_{50} = 2.6 M (572.9 µg/L)		

5.1.2.3 Fishes

5.1.2.3.1 Approach used for assessing the endocrine activity in fish

The assessment of whether or not the 4-nonylphenols are actual endocrine disruptors in fish was mainly based on the OECD guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption (OECD, 2012). Although it focuses on validated OECD test guidelines, some general information on how to assess endocrine disrupting properties can be extracted. Information provided in this document is supplemented by information from other guidance documents (e.g. OECD guidance document on the diagnosis of endocrine related histopathology in fish gonads (OECD, 2010)) and information from literature (e.g. (IPCS, 2002; Kendall et al., 1998; Knacker et al., 2010; OECD, 2004)). In general two different types of effects are considered and analysed separately:

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

Indicators of endocrine mode of action:

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

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

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

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

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

Changes in the gonadosomatic index (GSI) may provide additional information about the gonad maturation and spawning readiness (OECD, 2004). It describes changes in the relation of gonad to whole body mass and thus may be an indicator of the reproductive effort of organisms (Helfman et

al., 1997). Although GSI might be influenced by other modes of action too, reduction of GSI in male fish is regarded as a sensitive parameter in reproductive studies with estrogenic substances (OECD, 2004). However, care must be taken as the GSI is highly dependent on the individual fish (frequent spawners) or seasonal gonadal stage (seasonal breeders) [1].

In addition, the following apical endpoints are considered to be indicators of an estrogen agonist mode of action according to the draft OECD guidance document (OECD, 2012).

- Depression of male secondary sex characteristics in fathead minnow or medaka

- Female biased phenotypic sex-ratio during sexual development

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

Change of sex-ratio towards females is a known result of estrogen exposure during sexual development (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 life stages under exposure need to be considered carefully while analysing test results. Especially if effects on gonadal staging are analysed the reproductive cycle of a species should be considered. Especially for total spawners having only one breeding season such as *O.mykiss* effects may be observed only during the process of maturing prior to spawning and may be missed at other times of the year.

Indicators that adverse effects are endocrine mediated

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

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

[1] The size of the sexual 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.

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

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

Table 18 summarizes endpoints that are considered indicators of estrogen activity and may be affected as a result of this activity *in vivo*.

Table 18: Summary of endpoints that are considered during analysis of fish data

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

5.1.2.3.2 Analysis of available data for fish species

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

Overall for 6 oviparous fish species and 3 viviparous fish species *in vivo* data at different levels (biomarker, histology and apical endpoints) are available. They are analyzed species by species.

In the studies analysed different isomers of 4-Nonylphenol were used. Owing to the fact that the *in vitro* data show no significant difference between linear and branched isomers all 4-Nonylphenols- are considered to exert estrogen mediated effects. Specific isomers tested are indicated in the summarizing tables.

Effects on O. latipes:

For the evaluation of *O. latipes* the following tests are available: Four fish sexual development tests (partly with considerable deviations), a full life-cycle test (1.5-generation) and 3 reproduction assays with some deviations. Two of the sexual development tests and two of the reproduction tests are scored as reliable 2 while the other tests are used as supportive information only. An overview of results derived from these tests is provided in Table 19. They are discussed in the following sections by comparing results from tests with similar test design followed by an overall conclusion.

Table 19: Summary of effects of 4-nonylphenols in *O. latipes*

Life stage/ duration	Concentration/ test condition/ tested substance / solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex-ratio / gonad histology	Sex –ratio / phenotypic	Sec. sex characteristics	others	Positive control	Reference	Reliability
FSDT, Exposure <12 h after fertilization until 60 d posthatch	3.30 - 6.08 - 11.6 - 23.5 - 44.7 µg/L (m) Flow-through, 4-nonylphenol (97.4% purity as a mixture of isomers) Solvent: DMSO (100µg/L)	hepatic VTG in males and females: LOEC: 11.6µg/L NOEC: 6.08µg/L	Induction of testis-ova LOEC: 11.6µg/L (9 testes: 4 testis-ova: 7 ovary)		At 44.7 µg/L 75% females (no statistics)	LOEC 23.5 µg/L (11 males: 47 females based on secondary sex char.) at 44.7µg/L only one phenotypic male out of 60 fish		LOEC: 23.5µg/L (body weight) and 44.7µg/L (length)	no	(Seki et al., 2003)	2
FSDT (with deviations) Exposure: after hatch for 100d	0.29 - 0.87 - 2.9 - 8.7 - 29 µg/L (m); Semi-static, renewal of test water every 48 h nonylphenol, HP-PNP, Lot No. 14081-001 Solvent: Acetone		LOEC: 29µg/L (18 of 22 phenotypic males had testis-ova)		No effects up to 29 µg/L		Mixed sec. sex char. (MSC): LOEC: 8.7µg/L (20%), Male sec sex char.: LOEC: 29µg/L (no fish with male papillary processes at the anal fin)		17ß-Estradiol (1µg/L nominal) Gonadal sex ratio: 48 females : 1 male 33% with MSC, no fish with papillary processes	(Balch and Metcalfe, 2006)	2
FSDT (with deviations) exposure from hatch for 3 months	first month: 5,48 - 27,4 - 54,8 µg/L second and third month: 6,61 - 33,1 - 66,1 µg/L (m), Semi-static, 4-nonylphenol, technical grade Solvent: Acetone		33µg/L: 50% testis-ova. 66µg/L: 86 % testis-ova.		LOEC 66 µg/L (male: female 7:13)				no	(Gray and Metcalfe, 1997)	3 (high control mortality of 30-40%)

Life stage/ duration	Concentration/ test condition/ tested substance / solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex-ratio / gonad histology	Sex –ratio / phenotypic	Sec. sex characteristics	others	Positive control	Reference	Reliability
Modified FSDT Exposure: from hatch for 30 d Subsequent reproduction trail without exposure	0.44 (vehicle control) - 0.54 - 0.77 - 1.93 µg/L (m) Flow-through, Nonylphenol, mixture of isomers, CAS-No.: 84852-15-3 Solvent: Acetone (0.1%)			No effects on fecundity.	At 0.77µg/L stat. sign. more males than females (101:75), no dose-response.			No effects on g growth and no mortality	Estradiol (E2): 0.1 – 1.66 µg/L (m) Sex-ratio:No males in all concentrations. Highly mortality at 1.66µg/L	(Nimrod and Benson, 1998)	3 (vehicle control contained Nonyl-phenol)
Full life-cycle 1,5-generation Exposure from ≤ 24 h postfertilisation for 104 d (F0 generation) and subsequent 60 d (F1 generation)	4.2 - 8.2 - 17.7 - 51.5 - 183 µg/L (m) Flow-through 4-nonylphenol, 97.4% purity, mixture of isomers Solvent: Ethanol (100µl/L), solvent control existed		Testis-ova: F0-generation (60d posthatch): 17.7µg/L: testis-ova in 20% of fish, (4 of 9 phenotypic males) 51.5µg/L: testis-ova in 40% of fish (no phenotypic males) F1-generation: (60d posthatch): 8.2µg/L: testis-ova were in 10% of all fish; 17.7µg/L testis-ova in 25% of all fish. (no statistics)	No significant effects up to 17.7µg/L	F0-Gener.: 60d posthatch LOEC: 51.5µg/L (no fish with normal testes) F1-Gener.: 60d posthatch LOEC: 17.7µg/L (5 out of 9 phenotypic male fish had testis-ova, 4 had testis), NOEC: 8.2µg/L	Based on secondary sex char: F0-generation: 51.5µg/L: (no phenotypic male out of 20 fishes) F1-generation: 17.7µg/L: ratio male to female 9:19) (no statistics)		F0-gener.: LOEC hatchability: 183µg/L (50% mortality, 100% swim-up failure) Post-swim-up cumulative mortality: LOEC: 17.7µg/L F1-gener.: No effects on mortality, growth and time to hatch	no	(Yokota et al., 2001)	2

Life stage/ duration	Concentration/ test condition/ tested substance / solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex-ratio / gonad histology	Sex –ratio / phenotypic	Sec. sex characteristics	others	Positive control	Reference	Reliability
Modified reproduction assay Sexually mature medaka pair wise exposed for 21 d, Eggs collected at the last 24 h and examined for 90 d without exposure	5.4, 16.5, 61.2µg/L (m) Semi static (renewal every 24 h), 4-nonylphenol, Solvent: DMSO, (100µL/L), solvent control existed	LOEC: 5.4µg/L, NOEC < 5.4µg/L Dose dependent increase		Fecundity and Fertility: LOEC: 61.2µg/L, NOEC: 16.5µg/L Declining fertility over testing period (no fertilized eggs during the last 24 h of exposure)	Not examined	F1-generation (without exposure): no effects		F0-generation: No effects on growth. Mortality:61.2µg/L: two of five males (abdomen was swollen). HSI: LOEC males 16.5µg/L, females 61.2µg/L F1-generation: increased time to hatch at 61.2µg/L. Hatchability sign. decreased at 61.2µg/L No effects on mortality after hatching and growth up to 16.5µg/L.	no	(Ishibashi et al., 2006)	2
Reproductive assay (with deviations) Sexually mature medaka pair wise exposed for 21 d	24.8 - 50.9 - 101 - 184 µg/L (m) Flow-through, 4-nonylphenol, 97.4% purity, mixture of isomers Solvent: DMSO (0.0001%), solvent control existed	Hepatic VTG in both males and females LOEC: 50.9µg/L NOEC: 24.8µg/L	Testis-ova: Some indication (at the three low conc. 1 out of 8 male fish, at the highest conc. 1 out of 3 male fish) spermatogenesis: sparse primary and secondary spermatocytes, LOEC: 184 µg/L NOEC: 101µg/L	Fertility LOEC: 184µg/L NOEC: 101µg/L, Fecundity LOEC: 101µg/L NOEC: 50.9µg/L,				GSI males:, LOEC 184µg/L NOEC 101µg/L HSI males: at 50.9µg/L increased to appr. 40% (not sign.), LOEC 101µg/L Mortality: at 184µg/L three male fish died (subcutaneous hemorrhage)	no	(Kang et al., 2003)	2

Life stage/ duration	Concentration/ test condition/ tested substance / solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex-ratio / gonad histology	Sex –ratio / phenotypic	Sec. sex characteristics	others	Positive control	Reference	Reliability
Modified reproduction assay: Exposure: male medaka for 2 weeks, afterwards reproduction of exposed males with unexposed females for one week.	(6.45µg/L - 21.5 - 64.5 µg/L) (n) Semi-static: water exchange every 2 days nonylphenol, contains 90% p- and 10% o-nonylphenol, Solvent: Acetone (<100µl/L)			High variation in the number of eggs. No sign. effects are visible.				Mean hatchability decreased slightly at 64.5µg/L (not significant)	17 ß-estradiol, At 3 and 10 nmol/L sign. decrease in the number of eggs. At 3nmol/L significant effect on hatching.	(Shioda and Wakabayashi, 2000)	3 No solvent control

(Seki et al., 2003) exposed fertilized eggs for 60 d. Although the exposure design did not exactly match the OECD draft guideline for the fish sexual development test in all aspects, the number of replicates (4), test concentrations (at least 3) and test duration matched the test guideline. The validity criteria were fulfilled: hatchability in controls ≥96%, post hatch mortality was less than 6%, weight and length in controls were 232 – 282 mg and 28.7 – 29.1mm (at termination of the test) respectively. Exposure began 12 hours post fertilization. The gonadal histology and secondary sexual characteristics were determined 60 days after hatch. Test results show that the hepatic VTG in males and females was increased at 11.6µg/L (LOEC) and above in a dose dependent manner. At the same concentration testis ova appeared in 4 of 20 fish (20%). The sex-ratio was significantly skewed toward females at 23.5 µg/L and above based on secondary sex characteristics, with only one male out of 60 fish remaining at 44.7 µg/L.

Comparable results were observed in the second reliable sexual development test which started one day after hatch. (Balch and Metcalfe, 2006) detected testis-ova after 100 d of exposure at 29 µg/L in 18 of 22 male fish. Similar to Seki et al, Balch and Metcalfe observed changes in the secondary sex characteristics. While Seki et al showed that the ratio of males based on secondary sex characteristics decreased down to 19% (11 out of 58 fish) at 23 µg/L, Balch and Metcalfe observed, that the number of true phenotypic males decreased at 29 µg/L (no fish had papillary process at the anal fin, which is a prominent secondary sex-characteristic of male medaka) and the percentage of fish with mixed secondary sex-characteristics increased (LOEC 8.7 µg/L). Based on the gonads, no change in the sex-ratio was observed by (Balch and Metcalfe, 2006). However, it should be kept in mind, that exposure started after hatch and thus after the critical window of female gonad differentiation in medaka.

Results by the – less reliable – sexual development test perfomed by (Gray and Metcalfe, 1997) support these findings. They exposed medaka for 3 months starting after hatch. Only a qualitative evaluation of the study is possible due to a control mortality of 30 to 40 %. The study was therefore assessed with Klimisch 3. At the middle and high concentration (33 and 66µg/L) testis-ova appeared in respectively 50% and 86% of histological male medaka. A change in the sex-ratio (13 females : 7 males) was seen at 66µg/L based on gonadal histology. Although this change might be due to high mortality which could be biased to males, effects are in line with those observed by Seki and Balch and Metcalfe.

Similar results with regard to sex-ratio were observed by (Yokota et al., 2001) in a 1.5-generation full-life-cycle test (exposure of F0 for 104 d until spawning and subsequent 60 d exposure of F1-generation). The first part of the test was similar to a fish sexual development test: The exposure of F0-generation began less than 24 hours after fertilization. Sixty embryos were exposed in each treatment divided in four replicates. The gonadal histology and secondary sexual characteristics were determined 60 days after hatch. The validity criteria of a fish sexual development test had been matched: The hatchability in the controls was 90 – 93 %, the swim-up failure 5.5 – 7 %. The weight and length at 60 days post hatch were 169 – 174 mg and 26 mm respectively. At 70d posthatch, six mating pairs were selected for the two lowest test concentrations and further exposed until 104 d posthatch. Eggs were collected and eggs spawned on the last two days were exposed until 60d posthatch.

In the F0-generation testis-ova were observed in 20% of all fish (4 of 9 phenotypic male fish had testis-ova) at 17.7 µg/L. At the next concentration (51.5µg/L) 8 of 20 fish showed testis-ova (40%) and no true male fish were observable (no fish had testis and none showed male secondary sex characteristics). Effects are coincident with increased post-swim-up mortality. At 51.5 µg/L approx. 35 % and at 17.7µg/l approx. 20% showed post swim-up mortality. However it seems unlikely, that the skewed sex-ratio is a secondary result of this increased mortality as effects on sex-ratio were

more pronounced than mortality (no male fish at 51.5 µg/L but only 35% mortality) and gonadal changes indicate that effects were endocrine mediated.

Similar, but even more pronounced effects were observed in the F1 generation: Occurrence of testis-ova started at 8.2 µg/L (2 of 20 fish (10 %)). Already at 17.7 µg/L 5 of 9 males had testis-ova (25% of all fish) and sex-ratio was significantly skewed to females (9:19 males:females). No significant mortality occurred at this concentration.

Effects on reproduction were observed in two of three modified fish reproduction assays:

(Ishibashi et al., 2006) exposed adult pairs of medaka for 21 d. The fertilized eggs from the last 24 h of exposure were collected. The eggs were maintained in clean water and the hatchability and time to hatch was determined for 90 d. Results show an endocrine biomarker response and provide evidence that nonylphenol impairs fecundity and fertility. The hepatic VTG concentration in males increased in a dose dependent manner (LOEC = 5.4 µg/L). At 61.2µg/L the total number of eggs per mating pair as well as the mean fertility was significantly decreased. There were no fertilized eggs during the last 24 h at this concentration.

In addition 2 of 5 male fish died at this concentration after their abdomen began to swell. According to the authors mortality could be due to the increased hepatic VTG production as male medaka are not able to excrete VTG. This fits to a significantly increased hepato-somatic index at 16.5 µg/l and above. Similar effects were observed by other authors for ethinylestradiol.

Results also show, that exposure of adults impairs the development of its offspring even if the offspring is not directly exposed: At 61.2µg/L embryos produced during the second and the last week of exposure showed a significantly increased time to hatch (12.3 days compared with control 8.7 – 9 days) and a significantly decreased hatchability. The phenotypic sex ratio in the – unexposed – F1 generation was - not surprisingly - not affected.

Similar results with regard to reproduction were observed by (Kang et al., 2003)in a modified fish reproduction assay:

They exposed sexually mature fish pair wise for 21 d. Appearance of testis-ova (one out of 8 males at 24.8µg/L to 101µg/L and 1 out of 3 males at 184µg/L) provides some indication of an endocrine mode of action. Furthermore abnormal spermatogenesis was observed (LOEC: 184µg/L) and the hepatic VTG in males and females was increased (LOEC: 50.9µg/L). Fecundity was affected at 101µg/L (LOEC) and fertility was affected at a LOEC of 184µg/L. Three male fish died in the highest concentration (184µg/L) with subcutaneous hemorrhage.

Results of the last reproduction assay (Shioda and Wakabayashi, 2000) are inconclusive due to high variations in fecundity.

Summary:

Overall, increased levels of VTG (a widely accepted biomarker for an estrogen mode of action) were determined in all studies analyzing this endpoint. The lowest LOEC was 5.4µg/L for hepatic VTG in males (Ishibashi et al., 2006). In addition, the occurrence of testis-ova, as an indicator of an estrogenic effect according to the OECD guidance document 123 (OECD, 2010), was observed in all sexual development tests if examined and in one full life-cycle study, some testis- ova were observed even after short term exposure of adult males. The most significant effects were determined if the exposure began within 24 h after fertilization which is not surprising as female gonad development starts before hatch. The lowest LOEC value was 11.6µg/L (Seki et al., 2003).

Apical effects observed fit to these indicators of an estrogen mediated effect: The sex- ratio was significantly skewed toward females in all sexual development tests which included exposure during sensitive life stages (before hatch). Based on secondary sex characteristics significant effects started at 51.5 µg/L (Yokota) and 23 µg/L (Seki) with no and only one male developed at 51 and 44 µg/L respectively. The effect concentration decreased to 17.5 µg/L when eggs from exposed parents were used (Yokota).

Results from reproduction assays indicate that, in addition to the sex-ratio, 4-nonylphenols influence reproduction in medaka by an estrogen mode of action after exposure of adults. In both reliable tests vitellogenin was increased at lower or similar concentrations compared to impaired fecundity and fertility, with some indication that the increased VTG level in males might have caused male specific mortality at high concentrations.

Table 20: Summary of evidence for endocrine disrupting effects for 4-nonylphenols in *O.latipes* based on the OECD Guidance document (OECD, 2012) and overall conclusion

Test system	Number of tests available	Indication of hormonal activity?	Apical endpoints positive?	Indication that apical endpoints fit to mode of action	OECD conclusion
Sexual development test	2 reliable, 1supporting	Yes LOEC = 11.6µg/L (VTG and testis-ova	Yes, if sensitive life stages are considered LOEC= 23.5µg/L (sex ratio)	Yes, testis-ova and changes in sex ratio are known effects of estrogens	Substance almost certain an actual endocrine disruptor
Reproduction assay	2 reliable,1 inconclusive	Yes LOEC=5.4µg/L (VTG) Inhibition of spermatogenesis at 184 µg/L	Yes LOEC=61.2µg/L (Fecundity and Fertility)	Yes, increase of VTG indicates and estrogen mode of action and reduction of fecundity and fertility are known responses to estrogens	Strong evidence that the substance is an actual endocrine disruptor
Fish full life cycle	1 Yokota	Yes Testis –ova at 17.7µg/L and above (no statistics)	YES LOEC=51.5µg/L (Sex ratio based on gonadal histology in F0)	Yes, testis-ova and a skewed sex ratio toward females are known	Substance is an actual endocrine disruptor

			LOEC=17.7µg/L (Sex ratio based on gonadal histology in F1)	responses to estrogens	
Overall conclusion		Yes, Testis-ova and VTG	Phenotypical and gonadal sex ratio, fecundity and fertility	Yes	Substance is an actual endocrine disruptor

Fathead minnow (Pimephales promelas)

With regard *to Pimephales promelas* two reproduction screeening assays, determining endpoints indicative for an endocrine disruption as well as apical endpoints are available (all with reliablity Klimisch 2). In addition, one behaviour study with two experiments was performed (including endpoints indicative for an endocrine mode of action as well as apical endpoints) and a normal early life stage test is available. The following table (Table 21) summarizes the effects oberserved.

Table 21: Summary of effects of 4-nonylphenols in *Pimephales promelas*

Life stage/ duration	Conc./ test condition/ tested substance/ solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex-ratio	Sec. sex charac-teristics	others	Positive control	Reference	reliability
Modified reproduction assay 3 wk exposure of sexual mature pairs. Age: 6 months (exp.1) and 4 months (exp. 2)	1.Exp: 71µg/L (m) 2.Exp: 0.65 - 8.1 - 57.7 µg/L (m) Flow through, 4-nonylphenol, technical grade, branched chain isomers, 99% purity; Solvent: methanol (0.2mL/L); Solvent control existed	Experiment 1: Plasma VTG was sign. elevated in males and females, LOEC: 71µg/L Experiment 2 (only in support): Plasma-VTG in males elevated: NOEC: 0.65µg/L, LOEC: 8.1µg/L In females: LOEC: 57.7µg/L, NOEC: 8.1µg/L		Number of eggs spawned: LOEC: 71µg/L; Number of spawnings: LOEC: 71µg/L; number of eggs per spawn: LOEC: 71µg/L (All values from experiment 1)		tubercles in males: Experiment 1: LOEC: 71µg/L; Experiment 2 (in support) NOEC: 8.1 µg/L, LOEC: 57.7µg/L	No effects on growth and survival (All values from experiment 1)	no	(Harries et al., 2000)	2 (only a few measurements were made, but additional measurements of estrogen activity were done by YEAST assay) Experiment 2 should be used with care due to low reproduction performance in controls
Reproduction assay sexually mature fish (12 – 18 months) paired for 42 d	Two experiments (data from experiment 2 not usable, egg production was totally inhibited by solvent control): First experiment was conducted July to August. Exp.1: 0,05 - 0,16 0,4 - 1,6 - 3,4 µg/L (m); Flow-through,4-p-nonylphenol, purity > 98%; Solvent: ethanol (0.0001%), solvent control existed		dose-dependent increase in severity scores in the testes of males at 1,6 and 3,4 µg/L LOEC: 1.6µg/L, NOEC: 0.4µg/L			Fat pad size and tubercles NOEC: >= 3.4µg/L		no	(Miles-Richardson et al., 1999) (the same experiment like (Giesy et al., 2000))	2

Life stage/ duration	Conc./ test condition/ tested substance/ solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex-ratio	Sec. sex charac-teristics	others	Positive control	Reference	reliability
Reproduction assay sexually mature fish (12 – 18 months) paired 42 d	Two experiments (data from experiment 2 are not usable, egg production was totally inhibited by solvent control): First experiment was conducted July to August. Exp.1: 0,05 - 0,16 0,4 - 1,6 - 3,4 µg/L (m) Flow-through, 4-p-nonylphenol, purity > 98% Solvent: ethanol (0.0001%), solvent control existed	Decrease in plasma VTG in females. No dose related changes of plasma VTG in males.		At 3.4µg/L: spawning of eggs almost completely inhibited			Survival: NOEC: >= 3.4µg/L, Plasma estradiol: significant elevation at 0.05 up to 1.6µg/L. At 3.4µg/L no difference was found compared to controls. The effects were the same in males and females.	no	(Giesy et al., 2000) (the same experiment like (Miles-Richardson et al., 1999))	2
Male fish, age 8 months. Exposure 28 day, followed by 7 day competitive spawning period in clear water (competition with control malesfor reproductive opportunities).	Experiment 1: 0.15, 0.25, 0.63, 3.2µg/L(m) Flow through, 4-nonylphenol, complex isomeric mixture of 4-NP with minor (<10%) amounts of 2-NP, 4-octylphenol, and dodecylphenol Solvent: ethanol, (≤1.8µg/L)		NOEC ≥ 3.2µg/L (7d after cessation of exposure)			NOEC ≥ 3.2µg/L (7d after cessation of exposure)	Competitive spawning assay: At 0.25µg/L (and higher) control males significantly out-competed exposed males by 6 – 8 % for access to nest sites. HSI and GSI: NOEC ≥ 3.2µg/L Mortality: at 0.15µg/L 42%, at 3.2 µg/L 16 %, other conc. and control ≤ 10 %.	no	(Schoenfuss et al., 2008)	2 Cannot be subsumed under a guideline.

Life stage/ duration	Conc./ test condition/ tested substance/ solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex-ratio	Sec. sex charac-teristics	others	Positive control	Reference	reliability
Male fish, age 9 months. Exposure 28 day, time series analysis of plasma VTG was examined and competitive spawning period as described above.	Experiment 2 0.3, 5, 11, 15µg/L (m) Otherwise the same as above.	Increased plasma VTG in males : LOEC: 15µg/L (after 7 and 14 days of exposure) NOEC: 11µg/L; No sign. effects 7d after cessation of exposure.	NOEC ≥ 15µg/L (7d after cessation of exposure)			NOEC ≥ 15µg/L (7d after cessation of exposure)	Competitive spawning assay: At 11µg/L (and higher) control males significantly out-competed exposed males by 5 - 10 % for access to nest sites. Mortality: at 0.3µg/L 20%, other concentrations and control ≤ 10 %.	no	(Schoenfuss et al., 2008)	2 Cannot be subsumed under a guideline.
ELS: Exposure: starting with eggs, 33 days	2.8, 4.5, 7.4, 14, 23 µg/L Flow through						Time to hatch: At 2.8, 4.5, 7.4µg/L and in the control began hatch on third day of exposure. At 14 and 23 µg/L hatch began on the fourth day. Growth: no significant effects Mortality LOEC = 14 µg/L, NOEC = 7.4µg/L	no	Ward and Boeri 1991 (from (U.S.EPA, 2005b))	4 Only summary available. Mortality in control: 13.3%.

(Harries et al., 2000) exposed sexual mature *Pimephales promelas* during a pair-breeding trial for 3 weeks. The test was performed similar to OECD 229. Experiment 1 was a limit test with 4 breeding pairs. In experiment 2 one male died, and 2 females were unable to release their eggs in the solvent control (hence 3 of 4 pairs stopped breeding). Therefore results of experiment 2 should be used with care with regard to effects on reproduction and may be used as supporting information only.

VTG and secondary sexual characteristics were determined as indicators for an endocrine mode of action. The VTG concentration in both males and females was significantly elevated at the single concentration 71µg/L in experiment 1. VTG was also increased in the supporting second experiment. The LOEC was 8.1µg/Lin males and in females the LOEC was 57.7µg/L. In both experiments induction of VTG in males was very pronounced: 4000-fold in the first experiment and 45,000-fold above controls in the second experiment. In addition, in both experiments a decrease in male secondary sex characteristics was observed: Male fish in experiment 1 had no tubercles (a prominent male secondary sex characteristic). And in the second experiment the LOEC for reduced tubercles (0-4) was comparable (57.7 µg/L).

Consistent with these findings of an estrogen mode of action, a pronounced decrease of fecundity was observed in experiment 1. The total number of eggs was reduced from approx. 1500 to 2300 eggs in controls and during pre-exposure to a total number of approx. 100 eggs at 71 µg/L. Similarly, the number (frequency) of spawning and the number of eggs per spawn (mean egg batch size spawned for three weeks) were significantly reduced at 71µg/L. Growth was not impaired in experiment 1 and no mortality occurred.

(Giesy et al., 2000) conducted two comparable reproduction experiments with sexually mature paired fish (age 12 – 18 months). 3 replicates with twhere males and two females each were exposed for 42 days. In the second experiment egg production in the solvent control was totally inhibited and thus results from this experiment are not valid. The first experiment was conducted from July to August and showed the following results: Plasma VTG concentrations in females decreased in a dose independent manner (LOEC 0.16 µg/L) while no dose dependent effects were observed in males up to 3.4 µg/L.

Reproduction was severely inhibited in the first experiment. While the egg production (per female) at the lowest concentration 0.05µg/L was considerably elevated (about 300 eggs per female compared to approx. 150 eggs in controls); the exposure to 3.4µg/l caused an almost complete inhibition of egg production. Although effects were very pronounced, they were not statistically significant according to the Kruskal-Wallis and Tukey test used by the authors.

Histological results from the same experiment (Giesy et al., 2000) are reported in (Miles-Richardson et al., 1999) . They determined a dose-dependent and significant increase in severity scores in the testes of males at 1,6 µg/L and above. The severity score was estimated on the basis of gonad scoring criteria for sertoli cell proliferation (see also FFLC detailed review paper, No. 95, ENV/JM/MONO(2008)22) (OECD, 2008a).

In addition to these reproduction assays (Schoenfuss et al., 2008) conducted two behavior experiments with 8 and 9 months old male *P. promelas*. A competitive spawning period in clear water was followed by exposure duration of 28 days. During exposure males had to compete with

control males for reproductive opportunities. In addition plasma VTG, histology and secondary sexual characteristics were determined.

Histology and secondary sexual characteristics revealed no pathological findings in both experiments up to the highest test concentrations (NOEC ≥ 3.2µg and NOEC ≥ 15µg/L). Valid VTG results are available from experiment two only, due to an abnormal VTG induction in control males in the first experiment.

VTG in males was significantly increased at the highest test concentration (15µg/L) after 7 and 14 days of exposure. No significant effects were seen 7 days after the end of exposure.

Furthermore the behavior in competitive spawning assays was affected: At 0.25µg/L and higher the exposed males were significantly out-competed by control males which had 6 – 8 % more access to nest sites. Experiment 2 showed similar effects at higher concentrations: at 11µg/l and higher the control males out-competed the exposed males by 5 – 10% for access to nest sites. In both experiments some treatments showed increased mortality, but not in a dose dependent manner (Experiment 1: 42% at 0.15 µg/L and 16 % at 3.2 µg/L; experiment 2: 20 % at 0.3µg/L, controls < 10 %). Due to the dose independency it is deemed to be of no relevance with regard to the other effects observed.

Ward and Boeri (1991) (from the report Aquatic Life Ambient Water Quality Criteria – Nonylphenol, (U.S.EPA, 2005b)) conducted an early-life-stage toxicity test with *Pimephales promelas*. Embryos and larvae were exposed under continuous-flow conditions for a total of 33 days to five concentrations of nonylphenol ranging from 2.8 to 23 µg/L. Larval hatch was delayed by one day at 14 µg/L and above and survival of the fish at the end of the test was significantly reduced at nonylphenol concentrations ≥ 14 µg/L. Growth (length or weight) of nonylphenol exposed fish was not significantly different from control organisms at any of the nonylphenol treatment concentrations. Survival of the fish at the end of the test was significantly reduced at nonylphenol concentrations ≥ 14 µg/L.

Summary:

In summary, results of the three reproduction assays are conclusive. They show, that 4-nonylphenols act via an estrogen mode of action in *P. promelas*:

The indicative endpoint VTG was examined in three assays (Harries 2000 and Giesy 2000 and Schoenfuss 2008). In two cases exposed mature males showed VTG induction at a LOEC of 71 µg/L (Harries, limit test) and 15 µg/L (Schoenfuss et al., 2008) while no effects in males up to 3.4°µg/L were observed in the third study (Giesy et al., 2000). Results from the second – less valid - test by Harries showed that vitellogenin induction might occur at lower concentrations (LOEC 8.1°µg/L).

In addition to this indicator for an estrogen mode of action, reduced male secondary sexual characteristics were observed in one test: Male fish at 71µg/L (limit test) had no tubercles and this result was supported by results from the –less valid - second experiment of this study (Harries,

LOEC 57.7 µg/L). No such effects were observed at lower concentrations ((Miles-Richardson et al., 1999) and (Schoenfuss et al., 2008)) indicating that the LOEC is between 15 and 71 µg/L.

Results observed by Harries and Giesy, show that nonylphenol also impairs reproduction: (LOEC fecundity 71 µg/L (Harries, limit test, with some indication that effects may start at 3.4 µg/L (Giesy et al., 2000). Although apical effects started at similar or even lower concentration compared to biomarker responses, it seems very likely that they are estrogen mediated. Effects observed fit the endocrine mode of action and to effects observed in other species.

The endpoints for behavior determined by Schoenfuss during two competitive spawning assays are in line with the values for VTG and support the estrogen mode of action. Exposed males were out-competed in two experiments with regard to access to nest-sites at 0.25 µg/L and 11µg/L for about 5 – 10%. Similar results with other endocrine disrupting substances support the hypothesis.

Results from the fish early life stage test by Ward and Boeri (1991) (LOEC mortality and time to hatch = 14 µg/L) fit to these findings. It is well known, that estrogens may induce mortality and delays in development.

Table 22: Summary of evidence for endocrine disrupting effects of 4- nonylphenols in *P.promelas* based on the OECD Guidance document (OECD 2011) and overall conclusion

Test sytem	Number of tests available	Indication of hormonal activity?	Apical endpoints positive?	Indication that apical endpoints fit to mode of action	OECD conclusion
ELS	1	Not considered	Yes, LOEC mortality 14µg/L	no conclusion possible	-
Reproduction screen	4	Yes, LOEC VTG 15 µg/L, LOEC Secondary sexual characteristics 71µg/L	Yes, LOEC Fecundity between 3.4 and 71µg/L, LOEC Behaviour 0.25µg/L (only in competitive spawning assays)	Yes, Biomarker response and apical endpoints fit to the expected mode of action	Strong evidence that the substance is an actual endocrine disruptor
Overall		Yes, VTG and secondary	Yes, effects on fecundity,	Yes	Substance is an actual

conclusion		sexual characteristics are sensitive endpoints of estrogen activity.	behaviour		endocrine disruptor

Danio rerio:

With respect to *Danio rerio*, one modified reproduction screening assay (reliability 3) and two prolonged fish sexual development tests are available (reliability 2 and 3). All Studies include endocrine specific biomarkers as well as apical endpoints.

Table 23: Summary of effects of 4-nonylphenols in *Danio rerio*

Life stage/ duration	Conc. / test condition / tested substance / solvent	Vitello-genin	Histology	Fertility/ Fecundity	Sex ratio	others	Positive control	Reference	reliability
Reproduction screening assay (modified) Exposure: adults for 3 weeks Reproduction period without exposure: one week (cross-wise as well as parallel experiments). Following monitoring until hatch.	0.1 - 1 - 10 - 50 - 100 - 500µg/L (n), Reproduction trial: fishes formerly exposed to 50 µg/L Semi static, half of the water was renewed every other day Nonylphenol, technical grade Solvent: DMSO (0.1‰), solvent control probably did not exist	VTG in males (whole body mass) increased NOEC: 50µg/L LOEC: 100µg/L	At 50µg/L significant decrease (23.6%) of eggshell thickness	Fecundity (in formerly exposed females) was lowered, but effects were not significant (50µg/L)		Malformation in embryos of exposed females: NOEC: 10µg/L LOEC: 50µg/L Results should be used with care due to high mortality in the offspring in control.	no	(Yang et al., 2006)	3 (nominal, no vehicle control)

Life stage/ duration	Conc. / test condition / tested substance / solvent	Vitello-genin	Histology	Fertility/ Fecundity	Sex ratio	others	Positive control	Reference	reliability
FSDT with following depuration period and afterwards reproduction Exposure: 2 to 60 dph[1]. Reproduction starting at 240 dph (without exposure).	10; 100 µg/L (n) Semistatic, (100% water exchange every 48 h) 4-nonylphenol, technical grade Solvent: Acetone (0.01%), solvent control, but no normal water control	NOEC: 10µg/L, LOEC: 100µg/L, (60dph, whole body VTG was increased from male and female fish combined)	Females (60dph): Ovarian oogenesis staging skewed to earlier stages: NOEC: 10µg/L, LOEC: 100µg/L Males (60dph): At 10µg/L testicular development was skewed to earlier stages; At 100µg/l no histological males, but only females existed.	Reduced fecundity (cumulative egg production:, at 100µg/L: 6705 eggs; in the control: 10752 eggs) (not significant). Successful breeding trials: at both 10 and 100µg/L: 88.9%; in the control 95.6% (not sign.)	sex-ratio skewed to females based on gonadal histology: NOEC < 10µg/L, LOEC: 10µg/L (60dph); At 100µg/L only females	F1-Generation (240dph): NOEC swim-up succes: 10µg/L, LOEC: 100µg/L No significant effects on mortality, growth (F0 generation), egg viability and hatchability (F1).	Yes 17 α Estradiol: LOEC VTG: 10 ng/L LOEC sex ratio and changes in female gametogenesis 1 ng/L (89.4% females, 5.3% males, 60.5 % oogonia in females);	(Lin and Janz, 2006)	2 (nominal, only two concentrations)

[1] dph: days post hatch

Life stage/ duration	Conc. / test condition / tested substance / solvent	Vitello-genin	Histology	Fertility/ Fecundity	Sex ratio	others	Positive control	Reference	reliability
FSDT with following depuration period and afterwards reproduction Exposure: 2 to 60 dph Reproduction period: starting at 120 dph until 300 dph (no exposure)	10 – 30 – 100µg/L (n), Semi static, renewal every 48 h, 4-nonylphenol, technical grade Solvent: Acetone (0.2% v/v), solvent control existed, but no normal water control		60dph: Female gametogenesis: At 100µg/L only earliest stages of cells (oogonia and previtellogenic) existed. 60 dph: Male gametogenesis: At 100µg/L: no mature sperm, but higher proportions of spermatogonia. (in both cases no statistical analysis was conducted) Females (300dph): sign. increased ovarian follicle atresia rate (from primary exposition to 100µg/L and afterwards clean water)				EE (1 and 10 ng/L), Female gonads: only earliest stages (oogonia and previtellogenic) and atretic cells at 10 µg/L Male gonads: LOEC spermatogenesis = 1ng/L (lack of mature sperm) 300dph: atretic ovarian follicles sign. increased.	(Weber et al., 2003) (the same experiment like (Hill and Janz, 2003))	3 (nominal, high control mortality)

Life stage/ duration	Conc. / test condition / tested substance / solvent	Vitello-genin	Histology	Fertility/ Fecundity	Sex ratio	others	Positive control	Reference	reliability
FSDT with following depuration period and afterwards reproduction Exposure: 2 to 60 dph Reproduction period: starting at 120 dph until 300 dph (no exposure)	10 – 30 – 100µg/L (n), Semi static, renewal every 48 h, 4-nonylphenol, technical grade Solvent: Acetone (0.2% v/v), solvent control existed, but no normal water control	VTG from hearts: At 30 and 100µg/L VTG detection in males (no statistics), no measurement possible in 10 µg/L	Ovo-testes: At 30µg/L: in 1 of 20 fish At 100µg/L: in 2 of 20 fish (previtellogenic ovarian follicles embedded within testicular tissue).		At 100µg/L =LOEC sex ratio was sign. skewed to females based on gonadal histology. NOEC= 30µg/L.	Significant decrease of hatchability and swim-up success at 100µg/L (some effects at 30 µg/L but not significant) No evaluation in F0 possible due to high control mortality.	17α-ethinylestradiol (EE) 1 or 10ng/L, F0 (60dph): Sex ratio (histological) skewed to females: 1ng/L: 75% females, 20% males, 5% undifferentiated 10 ng/L: 20 % females and 80% undifferentiated VTG in males at 10 ng/L (no statistics) F1: significant decrease of hatchability, swim-up success and egg viability at 10ng/L	(Hill and Janz, 2003) (the same experiment like (Weber et al., 2003))	3 (nominal, high control mortality)

(Yang et al., 2006) exposed mature males and females for 3 weeks and examined the endpoints VTG and GSI. After the exposure period fish exposed to 50µg/L were paired for one week without exposure. The experiment included cross-wise breeding pairs (only males or females exposed) and parallel breeding pairs (both exposed or both control fish). Development of the F1 generation was observed until hatch.

The increase of the VTG concentration in males after three weeks exposure was significant and very pronounced at 100µg/L and higher (nominal concentration). The GSI was reduced in females at 500µg/L while no effects occurred in males.

Fecundity in groups with formerly exposed females was lowered, but effects were not significant (mean number of eggs of exposed females: 1919, control females: 2676). All reproduction trials using formerly exposed females (either paired with exposed males or with unexposed males) resulted in a significant increase of malformations (spinal column flexure) in offspring after hatch (18.2%) compared to the groups with control females (2.6 %). However, results observed with regard to the development of offspring should be used with care as the hatching success in controls was below OECD guideline validity standards (55% instead of 80%).

(Lin and Janz, 2006) conducted an experiment comparable to the OECD fish sexual development test guideline (FSDT ;exposure until 60d posthatch) but with a following depuration period for 6 months in clean water. Reproduction was assessed after these 6 months (at 240 dph (days post hatch)).

After 60d of exposure (60 dhp) Vitellogenin (whole body VTG, females and males combined) was significantly elevated at the highest concentration (100µg/L, nominal value). Histological changes in gametogenesis indicating estrogen mediated effects were observed in both females and males starting at 10 µg/L. In the control 81.7 +- 2.3% of female tissue consisted of previtellogenic oocytes surrounded by oogonia and early oocyte stages. At 10 µg/l fewer provitellogenic oocytes and more oogonia were found (not significant). After exposure to 100 µg/L only 38.8% of the ovarian tissue consisted of previtellogenic oocytes and 61.2% were yet oogonia. While control males showed in majority a full spectrum of sperm cell differentiation stages, histologically differentiated males exposed to 10µg/L 40% had only early stages of differentiation.

In addition to the observed changes in gametogenesis, the sex-ratio (based on histological observations) was significantly skewed towards females at 10 µg/L (58% females compared to 30% in control) with no histological males observed at 100 µg/L.

There were also effects on fecundity, and viability of the F1 generation: The egg production at 100µg/l was reduced (not significant; the number of cumulative egg production in the control: 10752 eggs, at 100µg/L: 6705 eggs). This effect and also the effect on fecundity revealed by Yang et al (2006) match with the other endocrine effects of 4-nonylphenols. The swim-up success of the F1 generation was significantly impaired at 100µg/L (NOEC= 10µg/L). No significant differences were observed in egg viability and hatchability.

Similar effects were observed by (Hill and Janz, 2003)and (Weber et al., 2003). They exposed *D. rerio* from 2 dph up to 60 dph. At 60 dph 20 fish per group were randomly chosen, weighed, measured and histological examined. The remaining fish were maintained in clean water until 120 dph and then used for breeding trails. A total of five breeding trials were conducted for each treatment with a resting period of 7 -8 days before beginning another trial until 160 dph. A random subsample was maintained in clean water until 300dph and examined histological. The publication by Hill and Janz (2003) concentrates on the effects on sex -ratio and breeding success, whereas the publication by Weber et al (2003) describes the histological evaluation of the same experiment.

The following effects were observed after exposure to 4-nonylphenols:

VTG could be detected in males at 30 and 100µg/L after 60d of exposure (VTG in heart homogenates by western blot). VTG at 10 µg/L was not determined due to low protein yield. By histological evaluation ovo-testes (previtellogenic ovarian follicles embedded within testicular tissue) were seen at 30µg/L in 1 of 20 fishes and at 100µg/L in 2 of 20 fishes (Hill and Janz, 2003). Both endpoints indicate an estrogen mode of action.

The female gametogenesis as well as the male gametogenesis was considerably affected after 60dph. At 100 µg/L in females only the two earliest stages (oogonia and previtellogenic cells) were visible (no statistical analysis) and no mature sperms were visible in males, but a high proportion of the young cell stage spermatogonia. At 10 and 30µg/L all stages were seen (Weber et al., 2003). While no significant change in ovarian follicle atresia was observed after 60 d of exposure, at day 300 (240 days after the end of exposure), female fish exposed to 100 µg/L showed a significant increase in ovarian follicle atresia. Such effects are considered as indicators for an estrogen agonist mode of action and similar effects were observed after exposure to 10 ng/L EE2.

The sex-ratio at 100µg/L was significantly skewed to females, while aside from the ovo-testes no abnormalities at 30µg/L were observed. With the endpoint sex- ratio an apical endpoint was influenced.

The mortality in the F1-generation in the control was not affected; the values for viability of eggs, hatchability and swim-up success were between 70 and 90 %. The progeny of parents exposed to 30µg/L had slightly lower values for hatchability and swim-up success; at 100µg/L the values were significantly decreased in comparison to the positive control 17α-ethinylestradiol at 10ng/L.

Due to high initial mortality in the control, both publications can only be used as supporting information with regard to endpoints that might be influenced by an overall reduced fitness. As the observed increase in VTG, histological changes as well as the sex-ratio are considered endocrine specific it is assumed, that the high mortality does not influence the validity of these results.

Summary:

Tests available clearly prove an estrogen mode of action. All experiments showed an elevated concentration of VTG in males. The lowest concentration for VTG revealed was at 30µg/L in the fish sexual development test ((Hill and Janz, 2003), by western blot, no statistical analysis). In all other tests elevated VTG was observed at 100µg/L.

Other endpoints indicating the endocrine mode of action are:

- The impaired gametogenesis described in the two FSDT studies by Lin and Janz (2006) and Weber (2003). In both studies the gametogenesis was shifted to younger stages of cells in males and females at 100µg/L. Weber conducted no statistical evaluation for that endpoint. But Lin and Janz showed that the effect on the oogenesis in females at 100µg/L was significant. In males at 10µg/L initial effects on testicular development were observed; at 100µg/l only histological females existed. In addition in one study ovo-testes were observed by Hill and Janz: At 30µg/l 1 of 20 fish and at 100µg/l 2 of 20 fish had ovo-testes.
- The increase of ovarian follicle atresia being significant at 100 µg/L even 180 days after the end of exposure (Weber et al, 2003).

In addition, the endpoint sex ratio was significantly impaired in two sexual development tests (Hill and Janz, 2003; Lin and Janz, 2006), with significant effects at 10 µg/L in one study (Lin and Janz,

2006) and no or only few males being observed in both studies after exposure to 100 µg/L nonylphenol.

Effects observed on fecundity by (Lin and Janz, 2006; Yang et al., 2006) at 50 and 100 µ/L even after exposure has been ceased, fit to the endocrine mode of action.

In summary, adult exposure as well as exposure during sexual development resulted in clearly endocrine mediated changes on the biomarker and the histological level. Changes in sex-ratio (a clear indicator of an estrogen agonist mode of action) as well as changes in fecundity fit to these changes. Fecundity was lowered (but not significantly) at 50µg/L, while the sex ratio was significantly impaired at 10µg/L. Thus, with regard to *D.rerio,* exposure to nonylphenol results in clearly endocrine mediated adverse effects, which are considered relevant for the population. In comparison to the OECD Guideline 2011 this results in the conclusion summarized in Table 24.

Table 24: Summary of evidence for endocrine disrupting effects of 4- nonylphenols in *D.rerio* based on the OECD Guidance document (OECD 2011) and overall conclusion

Test system	Number of tests available	Indication of hormonal activity?	Apical endpoints positive?	Indication that apical endpoints fit to mode of action	OECD conclusion
Reproduction assay, with reproductive period in clean water	1 (Reliability 3)	Yes LOEC 100µg/L (VTG)	(Some indication of reduced fecundity at the only concentration tested (50µg/L), but not significant)	Reduction of fecundity is a known response to estrogens however results are not conclusive as effects were not significant	Strong evidence that the substance is a possible endocrine disruptor (in vivo estrogen activity) some evidence that this may result in adverse effects

Prolonged sexual development test	2 (one test with reliability 3)	Yes Gametogenesis: LOEC 100µg/L (in females) , effect at 10µg/L (in males, no statistic) Indication of ovo-testes: at 30µg/L and above Ovarian follicle atresia: LOEC 100µg/L VTG induction: LOEC 100µg/L (pooled males and females), indication of effects at 30µg/L (no statistics)	Skewed sex ratio: LOEC 10µg/L	Yes, change in sex ratio is an indicator for estrogen mediated effects	Substance is almost certain an endocrine disruptor
Overall conclusion		Yes, Testis-ova, gametogenesis, ovarian follicle atresia and VTG	Gonadal sex ratio, (fecundity).	Yes	Substance is an actual endocrine disruptor

Rainbow trout: Oncorhynchus mykiss

For the evaluation of the effects on *O. mykiss* several tests are available. Three tests can roughly be classified as short term screening assays (Harris et al., 2001; Jobling et al., 1996; Lahnsteiner et al., 2005; Schwaiger et al., 2002) and two tests are similar to the OECD fish sexual development test (Ackermann et al., 2002b; Ashfield et al., 1998); in addition one extended early life stage test is available (Brooke 1993 from the report (U.S.EPA, 2005b)).

Table 25: Summary of effects of 4-nonylphenols in *Oncorhynchus mykiss*

Life stage/ duration	Conc. / test condition / tested substance / solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex-ratio / gonad histology	Sex –ratio / phenotypic	Sec. sex charac-teristics	others	Positive control	Reference	Reliability
Modified sexual development test, Exposure: from hatch for 35 d, post-exposure period: 431 d, all-female population,	1 - 10 - 30 µg/L (n) Flow-through, 4-tertiary-nonylphenol, mixture of different isomers and oligomers Solvent: Methanol (0.0005%)							Growth: length, weight: NOEC 1µg/L, LOEC 10µg/L; Increased Ovosomatic index, beginning effect: 10µg/L, LOEC: 30µg/L	no	(Ashfield et al., 1998)	2 Only nominal
Extended sexual development test Exposure: fertilized eggs for 1 year	1.05 - 10.17 µg/L (m) Flow-through, 4-nonylphenol, 98% purity, technical grade Solvent: DMSO (0.001%)	Hepatic VTG in males and females: NOEC: <1.05µg/L; LOEC: 1.05µg/L	No effect on maturity stages of gonads. No testis-ova like also in the positive control.			No effect		Zona radiata protein in males and females: NOEC: 1.05µg/L LOEC: 10.17µg/L Hatching rate: no effect Mortality: no effect Body weight: no effects after 1 year exposure	EE2, Monthly injections (5µgEE2 pro 10g bw., from 6 to 12 months); no effects on maturity stages of gonads; no testis-ova, no alteration of sex ratio, but VTG was increased.	(Ackermann et al., 2002b)	2

Life stage/ duration	Conc. / test condition / tested substance / solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex-ratio / gonad histology	Sex –ratio / phenotypic	Sec. sex charac-teristics	others	Positive control	Reference	Reliability
Early life stage, 91 day	6, 10.3, 23.1, 53, 114µg/L flow through							High level of larval abnormalities at ≥53µg/L). Reduced growth (weight and length): LOEC: 10.3µg/L, NOEC 6µg/L Reduced survival at the end of the test LOEC: 23.1µg/L, NOEC: 10.3µg/L No effects on time to hatch and survival at hatch.	n.a.	(Brooke 1993 from the report (U.S.EPA , 2005b))	4 (only summary available)
Modified screening assay 2-year old male rainbow trouts, Exposure: 3 weeks in may	36.81µg/L (m) (Limittest) Flow-through 4-nonylphenol, 95% 4-substituted isomers Solvent: Methanol	LOEC: 36.81µg/L	LOEC: 36.8µg/L Spermatogenesis: cell type Spermatogonia A was significantly elevated					GSI: LOEC: ≤ 36.8 µg/L	17α-ethynyl-estradiol 2ng/L: Effects on spermato-genesis, VTG, GSI	(Jobling et al., 1996)	2

Life stage/ duration	Conc. / test condition / tested substance / solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex-ratio / gonad histology	Sex –ratio / phenotypic	Sec. sex charac-teristics	others	Positive control	Reference	Reliability
Modified screening assay 2-year old male rainbow trouts, Exposure: 3 weeks in August	0.24 - 1.06 - 1.85 - 5.02 - 20.3 - 54.3 µg/L (m) Flow-through, 4-nonylphenol, 95% 4-substituted isomers Solvent: Methanol	NOEC: 5.02µg/L LOEC: 20.3µg/L at 54.3µg/L VTG increased more than 10'000-fold						GSI in males: LOEC: 54.3µg/L NOEC: 20.3µg/L (completely inhibited testicular growth at 54.3µg/L; inconstancy in dose-relationship, but overall effect was significant)	no	(Jobling et al., 1996)	2
Modified screening assay: Exposure of adult females: 18wk during early ovarian development (March - July)	0.7 - 8.3 - 85.6µg/L (m) Flow-through, 4-nonylphenol, 99% purity, mixture of isomers Solvent: methanol (0.002%)	Increased plasma VTG: NOEC: 0.7µg/L, LOEC: 8.3µg/L						Sign. decreased GSI: At 85.6µg/L = LOEC ovaries of fish had not developed. NOEC = 8.3µg/L Plasma estradiol was significantly decreased: NOEC: 8.3µg/L LOEC: 85.6µg/L At 85.6µg/L: Increasing mortality over the duration of the test.	No	(Harris et al., 2001)	2

Life stage/ duration	Conc. / test condition / tested substance / solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex-ratio / gonad histology	Sex –ratio / phenotypic	Sec. sex charac-teristics	others	Positive control	Reference	Reliability
Modified reproduction assay: Exposure of adult males: 60d during spermiation period (Dec. – end of Jan.), subsequent exposure of eggs for 60d	0.13 – 0.28 – 0.75 µg/L (calculated by water flow and injection rate of NP), Flow-through, 4-nonylphenol Solvent: DMSO			No effect on fertility of semen in vitro				Semen volume after 30 and 60d: LOEC: 0.13µg/L, NOEC: <0.13µg/L At 0.75µg/L no semen available after 60d of exposure (third stripping) No significant effects on sperm density and motility up to 0.28 µg/l Mortality: Eyed stage embryos, hatched larvae, yolk sac stage larvae: LOEC: 0.28µg/L, NOEC: 0.13µg/L	no	(Lahnsteiner et al., 2005)	2 Concentrations were only calculated, not measured. Eggs were disinfected with formaldehyde.

Life stage/ duration	Conc. / test condition / tested substance / solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex-ratio / gonad histology	Sex –ratio / phenotypic	Sec. sex charac-teristics	others	Positive control	Reference	Reliability
Modified screening assay with subsequent maintenance of eggs in clear water. F0-generation: Intermittently exposure of adults (10 day per month from July to October) prior to spawning. Subsequent maintainance of Eggs and sperms (not exposed) until sexual maturity	1.2, 10.4 µg/L (m), Flow-through technical NP, which consisted of 98% NP isomers (90% 4-NP, 10% 2-NP) and 2% dinonylphenol solvent: ethanol	F0 -generation: VTG increased in males LOEC: 1µg/L NOEC: < 1µg/L, F1 -generation: female offspring, age 3 years, VTG sign. increased: LOEC: 10µg/L NOEC: 1µg/L,			F1 generation (without exposure): 23 out of 217 offspring (all males from their gross morphology) from parents exposed at 10µg/L revealed histologically to be females. 6 of these 23 fish had spermatogenic activity. (no data about significance) No effects on 1 and 10µg/L without determination of gross morphology before examination			F1-generation: Viability of eggs before the eyed-egg stage LOEC:1µg/L, NOEC:<1µg/L No effects on mortality between eyed-egg stage and hatching Hatching rate: LOEC: 10µg/L, NOEC: 1µg/L Sexual steroids were significantly increased at the age of 3 years of the progeny: Estradiol in males: LOEC:10µg/L Testosterone in females: LOEC:10µg/L	no	(Schwaiger et al., 2002)	2
Juvenile female fish 5 d	40µg/L, 80µg/L flow-through, 4-nonylphenol, mixture of several isomers							Behaviour: Significant effects: Distance to other fishes increased; more aggression directed to exposed fish; fish obtained fewer food pellets. Effects increased with increased dose beginning at 40µg/L. No effects on swimming speed and escape response to predator attack.	17ß-estradiol 0.1µg/L No effects were seen.	(Ward et al., 2006)	2

Fish sexual development and early life stage tests:

Ashfield et al. (Ashfield et al., 1998) examined the effects on growth and ovosomatic index (OSI) during a modified sexual development test with an all-female population of rainbow trout. The exposure began at hatch and lasted 35 d; afterwards the fish were kept for 431 d in clean water. Significant effects were on growth: The length and weight were reduced at 10µg/L on day 55 and on day 84 respectively. At 10µg/L the effect on weight was reversed until day 466 so that fish were significant heavier than controls. The weight at 30µg/L remained significantly lower until day 466. The OSI was significantly increased at 30µg/L = LOEC, a NOEC of 10µg/L was determined (a dose response relationship existed with a beginning effect at 10µg/L).

Ackermann et al. (Ackermann et al., 2002b) exposed rainbow trout for one year starting with fertilized eggs (extended sexual development test). Only two concentrations were used: 1.05 and 10.17µg/L. Hepatic VTG in males and females was elevated at 1.05µg/L = LOEC, the NOEC was < 1.05µg/L. There were no effects on histology and sex- ratio. However, at this age the development of fish gonads is in an early stage. Control female and male gonads were in the early gonadal growth (females: only early stage oocytes, males: only primary and secondary spermatogonia). Effects of estrogen substances usually involve the delay of germatogenesis (i.e. reduced percentage of late stages) and such an effect may not be observable if control fish had not developed until late stages. This is supported by the positive control 17α-ethinylestradiol (monthly injections of 5µg EE2 per 10g b.w.) which also showed no effects on histology like testis-ova, maturity stages of gonads and sex -ratio. But VTG was significantly increased like in the nonylphenol treatment. There were no effects on hatchability or body weight.

A 91-day early life-stage test was conducted with embryos and fry of the rainbow trout, *Oncorhynchus mykiss* (Brooke 1993 from the report (U.S.EPA, 2005b)). For this test only a well described summary from a publication from the U.S. EPA is available. Therefore it can be assessed with reliability 4 only. Five nonylphenol exposure concentrations were tested, ranging from 6.0 to 114 µg/L in the flow-through test. Time to hatch and percent survival at hatch were not affected by the nonylphenol concentrations tested; however, nearly all of the larvae were abnormal at the two highest exposure concentrations (≥ 53.0 µg/L). At the end of the test, survival was significantly reduced at concentrations ≥ 23.1 µg/L. Growth (both weight and length) was a more sensitive chronic endpoint than survival. At the end of the test, the fish were significantly shorter (14 %) and weighed less (30 %, dry weight) than control fish at nonylphenol concentrations ≥ 10.3 µg/L. Based on growth, the NOEC and LOEC determined in this study were 6.0 and ≥ 10.3 µg/L, respectively.

Short term reproduction tests:

(Jobling et al., 1996) conducted two modified screening assays with two year old male rainbow trout. Exposure lasted in each case for 3 weeks. The first test (a limit test) was conducted in May and the second (a dose response study) in August. In the first test the distribution of cell types in gonads was determined and revealed effects on spermatogenesis. While in most cells spermatogenesis was at the stage of spermatocyte A (a middle stage), gonads of nonylphenol exposed fish at 36.8µg/L were mainly at the stage of Spermatogonia A and B (a very early stage) and significantly with regard to Spermatogonia A (LOEC 36.8µg/L). Rainbow trout reproduce annually. Usually in late spring (May) the testes of fish beginn to grow, spermatogenesis starts and the GSI is normally rising. In the test in May nonylphenol inhibited growth of testes significantly resulting in a significant reduction of the GSI at the end of the test compared to normally grown control testes. (LOEC 36.8µg/L). VTG was significantly elevated at 36.8µg/L (LOEC).

The second test was conducted in August when “testicular growth is well underway” (cited from Jobling) and again testicular growth was affected. At 54.3µg/L = LOEC the testicular growth was

completely inhibited, basically indicating that no spermatogenesis occured. A dose response relationship was observable (only exception 20.3 µg/L).VTG was affected at 20.3µg/L and above (NOEC 5.02µg/L). At 54.3µg/L VTG was increased more than 10'000-fold.

Harris et al (Harris et al., 2001) conducted a modified screening assay with two year old female rainbow trout and exposed them for 18 weeks beginning at the time of early ovarian development (May-June) . The concentration of VTG in plasma showed a dose related increase during the whole experiment: (LOEC 8.3µg/L, NOEC: 0.7µg/L) VTG concentration in the highest test concentration (85.6 µg/L) at the end was even higher than usually found in female rainbow trouts during ovarian development. According to the authors this could be the reason for an increased mortality at 85.6µg/L (about 60% at the end of the test) due to an impairment of organ functions. Also other authors observed this effect (e.g. Zha et al. 2008, Seki et al. 2002).

Similar to spermatogenesis, oogenesis in rainbow trout usually occurs during summer prior to the spawning season and results in a growth of ovaries. Such growth was observed in control females (GSI increased from 0.16 to 0.57). However, the ovaries of fish exposed to 85.6µg/L (= LOEC) had not developed at all since the start of the experiment and no increase in GSI was observed.

As the level of estradiol in the plasma is regulated by the oocytes it is not surprising that almost no plasma estradiol was formed at 85.6µg/L (=LOEC, NOEC 8.3µg/L).

Lahnsteiner et al. (Lahnsteiner et al., 2005) exposed 2 year old male rainbow trout for 60 days (modified reproduction assay) during the spermiation period (the release of mature spermatozoa begins in December, lasts until end of January). Effects on semen volume, semen fertility, motility and density were determined.

No effects on sperm density, sperm motility and sperm fertility were observed up to 0.75 µg/L (fertility up to 0.28 µg/L as no determination was possible at 0.75µg/L).

However, effects on the semen volume were observed: The semen volume was determined three times by stripping: The first time on the onset of the experiment, the second time after 30 weeks, and the last time after 60 weeks. In the control the semen volume had at each time approximately the same volume. The semen volume was significantly lower after 30d in all test concentrations (LOEC $\leq$ 0.13 µg/L in a dose- dependent manner. The reduction was even more pronounced after 60d. At 0.75µg/L after 30 days the sperm volume was decreased to about half of the beginning value and after 60 days no semen at all could be stripped (semen volume = zero).

In addition to effects on sperm quality and quantity, Lahnsteiner and co-workers (Lahnsteiner et al., 2005) examined the influence of 4-nonylphenol on egg and larvae. Eggs were fertilized *in vitro* and exposed for 60d. There is no explicit information available if the parent animals were exposed or not, therefore this part of experiment can be seen as a fish early life stage test. The percentage of eyed stage embryos at 0.28 and 0.75µg/L after 30 d were slightly but significantly lower than in the controls (LOEC 0.28µg/L, NOEC 0.13µg/L). Also the number of hatched larvae and yolk sac stage larvae after 45 and 60d respectively were significantly lower at 0.28µg/L = LOEC, NOEC = 0.13µg/L. However, results should be used with care as egg incubators were supplied with water from the fish tanks. The eggs were regularly disinfected with 4% formaldehyde, as the risk of infection with fungus under such conditions is high.

(Schwaiger et al., 2002) exposed adult rainbow trout at the age of 3 years intermittently (10 days per month) over 4 months (July to October) by use of a modified screening assay. The concentrations were 1 and 10µg/L. The last exposure was finished just prior to spawning. Eggs and sperm of exposed fish were collected, fertilized *in vitro* and grown up until sexual maturity without

exposure. Some individuals were maintained until the age of 3 years and blood samples were taken at spawning time to determine VTG and sex steroid levels.

Results: In the F0-generation VTG was significantly increased (approx. tenfold) at 1 and 10µg/L (NOEC: <1µg/L, LOEC: 1µg/L, $p<0.001$). In the F1-generation the male offspring from parents exposed to 10µg/L did not differ significantly from the control regarding the plasma VTG level, but the female offspring showed significantly higher levels of VTG (NOEC: 1µg/L, LOEC: 10µg/L, $p<0.05$). The whole hormone system in the offspring seemed to be confused as the females showed a 13-fold higher value of testosterone content than controls and the estradiol in males was 2.3-fold higher than in controls. Both values were significant (LOEC: 10µg/L, $p<0.01$, no measurements at 1µg/L).

A group of 217 offspring of exposed parents (10µg/L) which from their gross morphology appeared to be male were checked for the occurrence of intersex gonads. Thereof 23 fish (11%) proved histologically to be females. 6 of these 23 'females' revealed spermatogenic activity as indicated by the presence of spermatocysts containing either spermatocytes or spermatids within otherwise normal ovarian tissue. The occurrence of masculinisation may be seen in connection with the confused hormone system in the offspring described above. This effect was observed in this group only (with determination of gross morphology before histological examination). In another group of offspring with males and females from parents exposed to 10µg/L (and 1 group from parents exposed at 1µg/L) no effects on sex-ratio and maturation stage of germ cells were determined.

(Ward et al., 2006) exposed juvenile female rainbow trout for 5 days to 40 and 80µg/L nonylphenol. Behaviour of fish was examined. Effects started at 40µg/L (e.g. increased distance to other fish, reduced capability to obtain food pellets). Swimming speed and escape response were not affected. The positive control 17ß-estradiol did not show effects. According to the authors effects do not appear to be related to the estrogenic potential of nonylphenol but rather due to olfactory cues that underlie social behaviour in fishes.

Summary:

The studies revealed effects of 4-nonylphenols on endpoints indicative for an estrogen mode of action as well as on apical endpoints.

An indicative endpoint is the increasing concentration of VTG. This was examined in 4 tests (3 screening or reproduction assays and 1 development test). Vitellogenin induction was observed in all tests with LOEC values in the range from 1 µg/L to 36.81µg/L. Results by Schwaiger et al (Schwaiger et al., 2002) showed that the vitellogenin level is increased even in adult fish if only their parents were exposed (LOEC 10 µg/L). Similarly, this holds true with regard to changes of estradiol and testosterone level observed in that test. A further endpoint substantiating an estrogen mode of action is the effect on spermatogenesis observed in a screening assay by Jobling et al. (Jobling et al., 1996) at 36.8 µg/L and the inhibition of testicular growth (measured as GSI) at 54 µg/L. Thus study results clearly indicate 4-nonylphenols induced endocrine activity in *O.mykiss.*

No apical endpoints which are clearly endocrine mediated (i.e. effects on sex-ratio) were examined. Results by Ackermann et al. (Ackermann et al., 2002b) which found induced vitellogenin but no effects on gonads and sex-ratio should not be considered as an indicator that estrogen activity does not result in adverse effects as the lack of effects could be due to the fact that fish were not mature enough at the end of the test to detect such effects.

However, apical effects observed by Ashfield et al. (Ashfield et al., 1998) and Brooke et al. 1993 such as reduced growth and impaired development- with an high level of larval abnormalities

(LOEC 10 and 53 µg/L respectively) fit to the endocrine mode of action. Reproduction was not assessed in any of these tests. But effects observed by Jobling et al (Jobling et al., 1996) with regard to testicular growth should be considered as strong evidence for an impaired reproduction. Testicular growth during the annual sperm production period (August) was totally inhibited at 54 µg/L indicating, that males did not produce sperms. This is in line with a delayed spermatogenesis observed early in the year by the same author and results by Lahnsteiner and co-workers. (Lahnsteiner et al., 2005) who found a reduction in the total sperm number. Similar holds true for effects observed by Harris and colleagues. (Harris et al., 2001) who found ovaries did not develop at all after exposure to 85.6 µg/L based on GSI during oocyte production (March-July). Again, total inhibition of oocyte germination is considered to be a strong evidence for impaired reproduction. Induction of vitellogenin and estradiol provide some evidence that the effect is endocrine mediated. However, due to high mortality at this concentration it can not be excluded that reduced ovarian growth was a result of a reduced overall fitness. Results observed by Lahnsteiner and co-workers (Lahnsteiner et al., 2005) provide some indication that effects on reproduction may occur at much lower concentration. The semen volume was significantly reduced after exposure to 0.75 µg/L with the effect that no semen was available for a third stripping. The biological relevance of such effects is unclear. However, as no real reproduction data are available, effects should be considered in the overall assessment.

In summary the observed elevated concentration of VTG is an indicative endpoint, that is unambiguously caused by an endocrine mode of action and the apical endpoints semen production and development of ovaries are relevant for the viability of the population. Effects observed on growth and development are known to be estrogen sensitive (growth and abnormal development). Results by two tests show that adverse effects which are considered endocrine sensitive start at 10 µg/L (LOEC). All in all the results give clear indications for an endocrine mediated mode of action of the 4-nonylphenols and subsequent adverse effects.

Table 26: Summary of evidence of endocrine disrupting effects of 4 -nonylphenols in *O.mykiss*

Test system	Number of tests available	Indication of hormonal activity?	Apical endpoints positive?	Indication that apical endpoints fit to mode of action	OECD conclusion
Sexual development test	3 (one test with reliability 4)	Yes LOEC = 1.05µg/L (VTG) VTG only in one test examined	Yes, Growth LOEC 10µg/L in two tests	Yes, growth is a known endpoint of an endocrine mode of action	Strong evidence that the substance is a possible endocrine disruptor (in vivo endocrine activity)
Reproduction assay	5	Yes	Yes,	Yes, development	Strong evidence

		LOEC= 1µg/L (VTG) VTG (F1-generation) LOEC 10µg/L (without exposure) Inhibition of Spermatogenesis LOEC= 36.81µg/L	Non developed ovaries LOEC 85.6µg/L Sexual steroids in F1-generation LOEC 10µg/L	of ovaries is under influence of an endocrine MoA The hormone system in the F1-generation can be impaired by endocrine MoA.	that the substance is an endocrine disruptor.
Overall conclusion		Yes, VTG Inhibition of Spermatogenesis	Yes Growth, developing of ovaries, sexual steroids in the F1-generation	Yes	Strong evidence that the substance is an endocrine disruptor.

Viviparous fish species

Tests are available for three viviparous fish of the family Poeciliidae (*Poecilia reticulata*, *Xiphophorus helleri* and *Gambusia holbrooki)*:

Two assays for *Poecilia reticulata* (adult males and a sexual development test with following reproduction period; (Cardinali et al., 2004; Li and Wang, 2005)), one for *Gambusia holbrooki* (sexual development, (Drèze et al., 2000)) and two for *Xiphophorus helleri* (juvenile growth test and short term test (Kwak et al., 2001)). Tests include endocrine biomarkers as well as apical endpoints.

Table 27: Summary of effects of 4-nonylphenols in viviparous fish

Life stage/ duration	Concentration / test condition / tested substance / solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex-ratio / gonad histology	Sex –ratio / phenotypic	Sec. sex charac-teristics	others	Positive control	Reference	reliability
Poecilia reticulata Exposure from 5 days after birth for 90d. Reproduction period without exposure for 5 months. Crosswise experiments (control fish with treated fish).	100µg/L (n), Limit test, semi-static, 100% water weekly changed Nonylphenol; mixture of isomers with differently branched nonyl side chains, contains approx. 85% p-isomers. Major impurities are 2-nonylphenol (o-isomers), dodecylphenol, dinonylphenol, which together comprise approx. 10 % of NP-mixture. Solvent: ethanol (<0.1mL/L) Solvent control existed, but no normal water control.	Hepatic VTG expression was significant elevated in both females and males LOEC: 100µg/L				Sex-ratio skewed to females (based on secondary sexual characteristic): 29: 71 (m:f) compared to 46:54 (m:f) in controls (no statistics)	See sex ratio.	Delayed appearance of offspring after exposure of males (at month 8 compared to month 5 in controls) Delay in appearance of sexual behavior (approach to females and sigmoid display). (No statistic). GSI significant decreased in males and increased in females. No effects on mortality.	No	(Cardinali et al., 2004)	2 (nominal, only one concentration, semi-static, 100% water weekly changed)
Poecilia reticulata Adult males exposed 21 d	10, 60, 150µg/L (n) Semi static (renewal of water three times a week) 4-nonylphenol Solvent: Acetone (30ppm), Solvent control existed but no normal water control	LOEC increased Plasma VTG = 10µg/L						GSI was decreased in all treatments but only at 60µg/L after 14 d sign. Mortality: At 60µg/L: 29% after 21 d, at 150µg/L: 33% after 14 d, otherwise < 20%	17β-estradiol (1µg/L) Sign. induction of VTG GSI sign. decreased after 14 d Mortality: 29% after 7 d	(Li and Wang, 2005)	2

Life stage/ duration	Concentration / test condition / tested substance / solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex-ratio / gonad histology	Sex –ratio / phenotypic	Sec. sex charac-teristics	others	Positive control	Reference	reliability
Mosquito-fish (Gambusia holbrooki) 3 days old 75 d exposure	0.5; 5; 50µg/L (n) Semi-static 4-nonylphenol, consisted of 85% p-isomers Solvent: ethanol (10 ppm) Solvent control existed		Oocytes: 50µg/L: only females with oocytes in the late perinucleolus stage; no other stages existed (no statistics). Number of fish with male gonads decreased with increasing concentration; Fish with atrophied gonads increased with increasing concentration; 0.5µg/L: 5% 5µg/L: 18% 50µg/L: 31% (no fish with male gonads).		50µg/L: no histological males, females with small gonads and individuals with small undifferentiated gonads	Sex-ratio on the basis of male anal fin: NOEC 5µg/L, LOEC 50µg/L; at 50µg/L: only females (no fish with gonopodium)	Partially developed gonopodium: At 0.5µg/L: 5% At 5µg/L: 18% At 50µg/L no fish with gonopodium existed.	Sign. effects on length and weight LOEC females 5 µg/L, LOEC males 0.5 µg/L individuals with atrophied gonad and partially developed gonopodium were smaller than fully developed males from the same group	no	(Drèze et al., 2000)	2 (nominal concentrations)

Life stage/ duration	Concentration/ test condition/ tested substance/ solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex-ratio / gonad histology	Sex –ratio / phenotypic	Sec. sex charac-teristics	others	Positive control	Reference	reliability
Xiphophorus helleri 30 day-old juvenile male fish 60 d	1.experiment: 0.2 ; 2 ; 20 µg/l (n) Static, Nonylphenol, Solvent: ethanol						Sword growth LOEC 0.2µg/L			(Kwak et al., 2001)	3 (static and nominal)
Xiphophorus helleri adult male fish 3 d	2. experiment 4; 20; 100µg/L Semistatic, Nonylphenol, Solvent: ethanol	Increasing hepatic VTG production in all concentrations; no statistics)	Increasing apoptotic changes in the testes at 4µg/L ; many apoptotic cells at 100µg/L (no statistics) At 100µg/l: testicular degeneration with multinucleated cells.							(Kwak et al., 2001)	3 (miss-leading information on concentration, nominal)

Poecilia reticulata

(Li and Wang, 2005) exposed adult male *Poecilia reticulata* for 21 days. At 10µg/L plasma VTG was significantly elevated. Mortality occurred at 60 and 150µg/L (29% after 21 days and 33% after 14 days respectively), and also in the positive control at 1µg/L 17ß-estradiol (29% after 7 days). During the fish sexual development test conducted by (Cardinali et al., 2004) *Poecilia reticulata* were exposed after birth for 90 day to a limit concentration of 100µg/L (nominal). Afterwards a reproduction period without exposure with cross wise paired fish (control fish with treated fish) was performed for 5 months.

The VTG expression in the liver determined after 90 days of exposure was significantly elevated in both sexes compared with controls. At the end of the exposure period, when fish attained sexual maturity sex- ratio was determined based on the secondary sexual characteristics male anal fin (gonopodium) and pigmentation. In the control group a balanced sex ratio of 46 male to 54 females existed while the sex-ratio in the treatment group was skewed to females: 29 males to 71 females (no statistics performed). In the following reproduction period behaviour and reproduction were monitored. Exposed male fish showed significant effects as these males did not approach females and did not assume sigmoid display. The display of normal courtship behavior was delayed for 3 months compared with controls. This result matches with the time of first appearance of progeny. In the control group as well as in the group with exposed females and control males progeny appeared in the 5th month. In the group with exposed males progeny did not appear until the 8th month.

In summary, induction of vitellogenin as well as the sex-ratio skewed to females clearly indicates an estrogen mode of action for *P. reticulata*. Significant apical population relevant effects fit to this mode of action (sex-ratio, behavior, first appearance of progeny).

- *Gambusia holbrooki*

(Drèze et al., 2000) exposed three days old Mosquitofish (*Gambusia holbrooki*) for 75 days. Treatment was stopped when the modified anal fin of males (gonopodium) appeared in control males. Fish were measured; sex- ratio was analyzed by secondary sexual characteristics (appearance of gonopodium) and gonads (primary sexual characteristic). Oocytes were classified in five developmental stages (according the modified method described by (Koya et al., 1998)

The determination of secondary sexual characteristics revealed a significant impact on the sex ratio at 50µg/L: No fish with gonopodium and thus no phenotypic males existed at this concentration. At 0.5 and 5µg/l fish with only partially developed gonopodium were observed (5 and 18% respectively). These results match with the results of the primary sexual characteristics. At 50µg/L no males were observed. Females had small ovaries; and individuals with small undifferentiated gonads (atrophied gonads) were identified (statistically not significant). The same fish with partially developed gonopodium showed also atrophied gonads.

No spermatozoa were found in individuals with atrophied gonad and partially developed gonopodium at 0.5 and 5 µg/L.

The oocyte development stages at 0.5 and 5 µg/L were similar to control females where all stages described by Koya were found. But at 50µg/L the late perinucleolus stage (a very early oocyte development stage) was the only stage found. Such changes in oocytes development stages are indicative for an estrogen mode of action according to OECD 123 (2010). Growth was impaired too: At 0.5 and 5µg/l the length and weight of males was significantly decreased with the individuals with atrophied gonads being smaller than fully developed males. At 5 and 50µg/L length and weight of females was significantly decreased. Although such effects cannot be

considered as clearly endocrine mediated effects, in connection with the other effects it seems likely that they are influenced by the endocrine mode of action.

In summary, for *Gambusia holbrooki* indications are available that proves an endocrine mode of action (gonadal histology in males and females) and population relevant effects (skewed phenotypic sex-ratio, only females at 50µg/L).

- *Xiphophorus hellerie (swordtail)*

A short-term test conducted by (Kwak et al., 2001) is described. He exposed adult male Xiphophorus *helleri* for 3 days up to 20 µg/L. Increasing hepatic VTG production was detected in all concentrations. Furthermore histological changes were determined: Apoptotic changes in the testes began at 4 µg/L and at 100µg/l an increase of apoptotic cells was seen. Also at 100µg/L testicular degeneration with multinucleated cells (a sign of degeneration in testes according to OECD 123) was described.

In a long-term test over 60 days Kwak et al. (2001) exposed juvenile (30 day old) *Xiphophorus hellerie.* Normally male swordtails show a long fin - the "sword" as secondary sexual characteristic. The sword growth was inhibited in a concentration-dependent manner beginning at 0.2µg/L. At 20µg/L almost no sword growth was seen.

In summary, increased vitellogenin level as well as changes in secondary sex-characteristics clearly indicates an endocrine mode of action in *X. hellerie*. Based on available tests, apical effect concentrations are not available.

Table 28: Summary of effects of 4- nonylphenols in the viviparous fish *Poecilia reticulata, Gambusia holbrooki, Xiphophorus helleri*

Test sytem	Number of tests available	Indication of hormonal activity?	Apical endpoints positive?	Indication that apical endpoints fit to mode of action	OECD conclusion
Sexual development test (one test with juvenile male fish)	2	Yes, LOEC = 50µg/L (change in gonadal staging in females) Depression of secondary sexual characteristic in males affected at 0.2 – 20µg/L	Yes, LOEC 50µg/L (Sex ratio)	Yes, changes in sex ratio as known effect of estrogens	Substance is almost certain an actual endocrine disruptor

Sexual development test with following reproduction period	1	LOEC = 100µg/L (VTG, limit test)	Yes, Sex ratio affected at 100µg/L Display of normal courtship behavior and time to hatch delayed for 3 months at 100µg/L	Yes, changes in sex ratio as known effect of estrogens, as well as courtship behavior and time to hatch	Substance is almost certain an actual endocrine disruptor
Modified screening assay with adult males	1	Yes, LOEC = 10µg/L (VTG)			Strong evidence that the substance is a possible endocrine disruptor (in vivo endocrine activity)
Overall conclusion		Yes, VTG and change in gonadal staging in females	Yes, effects on sex-ratio, courtship behavior, time to hatch	Yes	Substance is almost certain an actual endocrine disruptor

Other fish species

The effects of 4-nonylphenols in two different fish species (Chinese rare minnows – *Gobiocypris rarus* and Silver Carp – *Carassius auratus*) are described in the following:

For *Gobiocypris rarus* a reproduction assay and for *Carrassius auratus* an experiment with adult male fish is available. Both studies are assessed with Klimisch 2. The experiments give indications for an estrogen mode of action.

Table 29: Summary of effects of 4-nonylphenols in other species

Life stage/ duration	Concentration / test condition / tested substance / solvent	Vitellogenin	Histology	Fertility/ Fecundity	Sex-ratio / gonad histology	Sex – ratio / phenotypic	Sec. sex charac-teristics	others	Positive control	Reference	reliability
Rare minnows (*Gobiocypris rarus*) Reproduction assay Adults, age 9 months, pairwise exposure for 21 d,	4.52 ; 9.13 ; 18.53 µg/L (m), Flow-through, 4-nonylphenol, technical grade, consisted of 98% NP isomers (90% 4-NP, 10% 2-NP) and 2% dinonylphenol. Solvent: acetone (<0.01%), solvent control existed	Plasma-VTG increased in males: NOEC <5µg/L, LOEC 5µg/L	Testis-ova: LOEC: 18µg/L, Lesions in liver: LOEC 9µg/L, Lesions in kidneys: LOEC 5µg/L, Males: In liver and kidney tissues eosinophilic material accumulated at 18µg/L NP (probably VTG produced under estrogenic stimulation).	No significant effects					17α-estradiol, EE2, 4ng/L, Effects: increased VTG, Fertilisation rate, number of eggs, Histology: Lesions in liver and kidneys. In males: Eosinophilic material accumulated in hepatic tissue.	(Zha et al., 2008)	2
Carassius auratus (silver carp) Sex: male Age: 2 – 3 years 21 d	1, 10, 100µg/L (n), Values immediately after spiking 60 – 80% of nominal; 1.39, 8.57, 64.5µg/L (initial, per water exchange); (NP in controls: 0.21 – 0.31 µg/L) Semistatic, water exchange every 48 h Nonylphenol, technical grade, t-NP Solvent: methanol, (0.01%) Solvent control existed.		Hypertrophic leydig cells Broken spermatogenic cysts, Increased number of sertoli cells,					Serum steroid hormones Estrone and 17ß-estradiol significant elevated, LOEC 10µg/L Testosterone significant decreased LOEC 10µg/L	Diethylstilbestrol (10µg/L) Estrone and 17ß-estradiol significant elevated, Testosterone significant decreased Hypertrophic leydig cells Broken spermatogenic cysts, Increased number of sertoli cells	(Yang et al., 2008)	2

Gobiocypris rarus

(Zha et al., 2008) exposed adult fish (age 9 months) pair wise for 21 days. At the end of exposure plasma VTG in males was considerably increased at the lowest concentration (LOEC =5µg/L). No effects were seen in females. Further indications for an endocrine mode of action are testis-ova. At 9µg/L one of 8 male fish had testis-ova. At 18µg/L three out of 8 male fish had testis-ova (LOEC = 18µg/L). No significant effects on reproduction (fertility) were observed up to 18µg/L.

Carassius auratus

(Yang et al., 2008) conducted an assay with male silver carp of the age 2 – 3 years. In the controls were also measurable concentrations of nonylphenol. As this was very low (0.21 – 0.31µg/L), the serum steroid hormones showed a notable difference between controls and treatments and as there appeared to be no effects on histology it is deemed to be of no relevance. Histological examination was conducted after exposure for 21 d. The structure of testes was severely degenerated when exposed to 100µg/L NP. Effects observed were similar to those observed after exposure to 10µg/L Diethylstilbestrol. Leydig cells were slightly hypertrophic. According to the OECD Guidance on the diagnosis of endocrine related histopathology in fish gonads hypertrophic leydig cells are diagnostic for histopathological changes following estrogen exposure in fathead minnow, medaka and zebrafish (OECD 123).

Furthermore serum steroid hormones were affected: Estrone and 17ß-estradiol were significantly elevated and testosterone was significantly decreased after 3 weeks exposure. All effects were dose dependent and the LOEC was 10 µg/L in all cases. The positive control diethylstilbestrol (10µg/L) induced the same significant effects.

Summary:

Tests for both fish species show that exposure to 4-nonylphenols results in *in vivo* endocrine activity in these species in the low µg/L range. The observed induction of vitellogenin as well as the induction of testis-ova in male *Gobiocypris rarus,* are clear indicators for an estrogen mode of action. The induction of hypertrophic leydig cells in *C. auratus* provide evidence for an endocrine mode of action. Due to the lack of information about apical endpoints it is not possible to conclude about adverse effects as a result of this endocrine activity. With regard to *Gobiocypris rarus*, no change in fertility was observed up to 18 µg/L. However,this does not exclude effects at higher concentrations. Based on experience with other fish species, it seems likely that 4-nonylphenols will impair reproduction in these two species.

Table 30: Summary of evidence for endocrine disrupting effects of 4- nonylphenols in *G.rarus and C. auratus* based on the OECD Guidance document (OECD 2011) and overall conclusion

Test system	Number of tests available	Indication of hormonal activity?	Apical endpoints positive?	Indication that apical endpoints fit to mode of action	OECD conclusion
Reproduction assay	1	Yes LOEC=5µg/L	No (low concentration)		Strong evidence that the

(Gobiocypris rarus)		(VTG) Testis-ova LOEC = 18µg/L			substance is a possible endocrine disruptor (in vivo endocrine activity)
Assay with male adult fish (*Carrassius auratus)*		Hypertrophic leydig cells (no statistics) Sexual hormones (17ß-estradiol and estrone elevated; testosterone decreased)			Strong evidence that the substance is a possible endocrine disruptor (in vivo endocrine activity based on histological findings)
Overall conclusion		Yes, Testis-ova and VTG			Strong evidence that the substance is a possible endocrine disruptor

Overall summary for fishes

Overall indication of estrogen activity was observed in all fish species tested. Estrogen activity started at the concentration of 1µg/L (*O.mykiss*) with respect to increased vitellogenin and between 11.6µg/L (*O.latipes*, testis-ova) and 36.8µg/L (*O.mykiss*, sperm stages) with respect to histological changes.

In three species (*O.latipes, P.reticulata, D.rerio*) observed effects on apical endpoints are very likely to be estrogen mediated. In one another species (*O.mykiss*) and the viviparous fish there is strong evidence for endocrine mediated apical endpoints.

In summary results show that 4-nonylphenols act as endocrine disruptors in all fish species tested. Clearly endocrine mediated effects start between 1.05µg/L (*O.mykiss)* and 15µg/L (*P.promelas*).

Table 31: Summary over all fish species tested

Test sytem	Indication of hormonal activity?	Apical endpoints positive?	Indication that apical endpoints fit to mode of action	Conclusion
O. latipes	Yes, Testis-ova LOEC 11.6µ/L VTG LOEC 5.4µg/L	Yes, Phenotypic sex ratio LOEC 23.5µg/L, Sex ratio based on gonadal histology in F1 generation 17.7µg/L fecundity and fertility LOEC 61.2µg/L	Yes	Substance is an actual endocrine disruptor
P.promelas	Yes, VTG LOEC 15µg/L; Secondary sexual characteristics 71µ/L	Yes, effects on fecundity (between 3.4 and 71µg/L), behavior (LOEC 0.25µg/L)	Yes	Substance is an actual endocrine disruptor
D. rerio	Yes, Testis-ova 30µg/L, gametogenesis 100µg/L, ovarian follicle atresia and VTG 100µg/L	Yes, Gonadal sex ratio LOEC 10µg/L, (fecundity).	Yes	Substance is an actual endocrine disruptor
O.mykiss	Yes, VTG LOEC 1.05µg/L Inhibition of Spermatogenesis LOEC 36.81µg	Yes, Growth LOEC 10µg/L, developing of ovaries LOEC 85.6µ/L, sexual steroids in the F1-generation LOEC 10µg/L	Yes	Strong evidence that the substance is an endocrine disruptor.
Viviparous fish	Yes, VTG LOEC 10µg/L change in	Yes, effects on sex-ratio LOEC 50µg/L,	Yes	Substance is almost certain an actual endocrine

	gonadal staging in females 50µg/L	courtship behavior 100µg/L, time to hatch delayed for 3 months 100µg/L		disruptor

5.1.2.4 Amphibians

In this chapter information about the potential endocrine mode(s) of action of 4-nonylphenols in amphibians (only anurans) is summarized, as far as available.

While in fishes estrogen-, and/or androgen-mediated effects are the most commonly assessed modes of action, in amphibians impact on the thyroid activity is a known potent endocrine mode of action which is linked to the thyroid-dependent process of amphibian metamorphosis..

According to the OECD guideline (231) for the amphibian metamorphosis assay (OECD, 2009a), the following effects indicate a thyroid mode of action:

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

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

In order to identify whether or not 4-nonylphenols induce also estrogen-like effects in amphibians, the effects observed are compared to effects observed after exposure to 17ß-estradiol (E2).

Although, no specific guidance is available on how to identify estrogen-mediated effects and knowledge of vertebrate steroid hormones and their role in normal development and reproduction in non-mammalians is scarce (OECD, 2008b; U.S.EPA, 2005a) effects of E2 and/or 17 α-ethinylestradiol (EE2) on larval gonadal sex differentiation and sex -ratio of several frog and toad species were shown in a number of studies summarized by (Kortenkamp et al., 2012).

To illustrate estrogen- mediated effects relevant studies with the African clawed frog *Xenopus laevis* are summarized as examples: In a study by (Hu et al., 2008) fertilized eggs embryos were exposed 1-100 µgE2/L through metamorphosis and could develop in untreated media for 2 months after metamorphosis. Compared to controls, intersex gonads (11%) occurred and sex ratio skewed towards females at the lowest concentration (1 µg E2/L with an increase to 99% females at 10 µg E2/l, (Sharma and Patino, 2010) used a similar design to study the effects of E2 on gonad structure and metamorphosis. Compared to controls 1 ug E2/l (nominal) enhanced intersex gonads (10%) and sex- ratio significantly skewed towards females. This confirms the findings of (Hu et al., 2008) that about half of would-be genetic males appeared to be partially or fully sex reversed by exposure to 1 ug/L E2.

(Gyllenhammar et al., 2009) examined the effects of EE2 on the related frog species *Silurana (Xenopus) tropicalis*. Exposure of tadpoles to 1,8 ng EE2/L was shown to lead to a significant increase in females (72%) compared to controls.

Further "feminizing effects" of these estrogens on larval reproductive development in other anuran species were summarized by Kortenkamp et al. (2010).

While estrogens are not considered to lead to direct effects on the thyroid axes in amphibians there is some evidence for hormone system cross-talk between sex steroids and thyroid axes as stated in a recent review by (Pickford, 2010). Also a delay in metamorphosis due to exposure to E2 was reported by (Hu et al., 2008; Pickford et al., 2003; Sharma and Patino, 2010).

One Amphibian metamorphosis Assay with E2 was performed during the validation process during guideline development for the OECD 231 guideline. In this study E2 did not affect developmental stage or thyroid histopathology. Nevertheless, a small but significant reduction in hind-limb-length relative to controls was found starting at concentrations of 2 ug/L (OECD, 2008b). Even though the actual mechanism exerted by E2 is still unclear there is a possibility of an amphibian specific hormone system cross-talk between sex steroids and thyroid axes.

Overall, 8 studies with 5 frog and 2 toad species are available assessing possible endocrine modulated effects on larval (sexual) development and metamorphosis. Results are summarized in Table 32. As age and developmental stages differed among studies and were examined according to different criteria (by (Gosner, 1960; Nieuwkoop and Faber, 1994)) information about duration, development stage and, criteria used for determination are included. None of the summarized studies was performed according to the OECD Guideline for the amphibian metamorphosis assay (assay (OECD, 2009b)) or is reliable without restriction according to (Klimisch et al., 1997).

In the studies analysed different isomers of 4-Nonylphenol were used. Owing to the fact that the *in vitro* data show no significant difference between linear and branched isomers all 4-Nonylphenols are considered to exert estrogen mediated effects. Specific isomers tested are indicated in the summarizing tables.

Table 32: Summary of effects on amphibians after exposure to 4-nonylphenols. E2 = 17ß-estradiol. Substance identity/ specific isomer tested indicated in the last column.

Species	Life stage tested/ test duration	test conditions/ test concentration	Relevant parameter(s)	Effect concentration(s)	Reference	Reliability (Klimisch)
Xenopus laevis African clawed frog	Embryos (Gosner stage 10.5 up to stage 37)/ 2 days	Static/ 2 – 20 – 100 – 200 – 1000 - 2000 µg/L (nominal) + DMSO	apoptosis, melanocyte differentiation	LOEC $_{\text{melanocyte differentiation}}$ = 20 µg/L Mortality starting at 200 µg/L (no statistics)	(Bevan et al., 2003)	2 - nominal conc., well documented but no guideline used (4-nonylphenol)
Bombina orientalis Oriental fire-bellied toad	2 h p.f. (fertilized eggs)/ 10 d	10 - 40 embryos; glass water bath; 300 mL tap water; 18°C, / ethanol < 0.05% - 0.1 - 1 - 10 - 100 µM (nominal)	pigmentation of tadpoles (melanophore size decreased) pigmentation of tadpoles (number of melanophore increased)	NOEC $_{\text{melanophore size}}$ = 22 µg/L NOEC $_{\text{number of melanophore}}$ = 22 µg/L	(Park et al., 2012)	2 – nominal concentration, well documented but no guideline used (nonylphenol)
Xenopus laevis African clawed frog	Males/ 28 days	single dose of 100 µg/g/week; injected intraperitoneally (IP) with the specific treatment chemical on days 1, 7, and 14 of the experiment	Breeding (nuptial) gland activity, body condition, GSI, HIS, plasma VTG, plasma testosterone	no variation (lowering) in the mean epithelium heights of nuptial glands (in contrast to OP) no significant effects on the other endpoints E2 (10 µg/g/week): sign increased conc. of plasma VTG E2 (10 µg/g/week): sign lower conc. of plasma testosterone	(van Wyk et al., 2003)	2 – unusual exposure route, single dose (4-t-nonylphenol hydroxyl)

Species	Life stage tested/ test duration	test conditions/ test concentration	Relevant parameter(s)	Effect concentration(s)	Reference	Reliability (Klimisch)
Xenopus laevis African clawed frog	2-3 days post hatch larvae/ 12 weeks up to stage 38/40 (Nieuwkoop and Faber 1975)	semistatic, controls: 60% males/ 10-7 - 10-8 M (nominal)	Sex ratio	NOEC = 10^{-8} M (= 22.035 µg/L) LOEC = 10^{-7} M: changes in sex ratio with increased proportion of females E2: 2.7 µg/L sex ratio 70% w	(Kloas et al., 1999)	3 - nominal conc., reproducibility questionable (only a method developing study, not optimally designed to establish a NOEC) (nonylphenol)
Rana pipiens Northern leopard frog	Gosner stage 25-28 (start of independent feeding) - 46 (end of metamorphosis and complete tail resorption)/ ca. 47 d	semistatic, 50 L/Lacetone, glass aquaria, 19-23°C, 16hlight/d, density: 1g tadpole/5L water, 1 Replicates, 37 tadpoles/ 10 - 100 µg/L (nominal), WR 9 - 41% (real)	Gonad histology; Sex-ratio	NOEC < 10 µg/L (nominal) LOEC $_{\text{different sex ratio, increased incidence of intersex gonads}}$ = 10 µg/L (nominal)	(Mackenzie et al., 2003)	2 - no Guideline but well documented study; nominal conc. (4-nonylphenol technical grade)
Rana sylvatica Wood frog	Gosner stage 25 (start of independent feeding) - 46 (end of metamorphosis and complete tail resorption)/ ca. 124 d	semistatic, 50 L/L acetone, glass aquaria, 19-23°C, 16hlight/d, density: 1g tadpole/5L water, 2 Replicates, 30 tadpoles/ 10 - 100 µg/L (nominal), WR 9 - 41% (real)	Gonad histology; Sex-ratio	NOEC < 10 µg/L (nominal) LOEC $_{\text{sex ratios}}$ = 10 µg/L (nominal) LOEC $_{\text{intersex gonads}}$ = 10 µg/L (nominal) (sex ratio F:M:I at 10µg/L = 1:0.9:0.3 and at 100 µg/L = 1:1:0.3 in contrast to control 1:1.1:0.1) (at 10 µg/L: 16 from 35 *R.sylvatica* were females; at 100 µg/L: 14 from 32) (% intersex: at 10 µg/L: 14% or 5 frogs of 35; at 100µg/L: 13% or 4 frogs of 32; control: 3% or 1 frog of 36)	(Mackenzie et al., 2003)	2 - no Guideline but well documented study; nominal conc. (4-nonylphenol technical grade)

Species	Life stage tested/ test duration	test conditions/ test concentration	Relevant parameter(s)	Effect concentration(s)	Reference	Reliability (Klimisch)
Rana pipiens Northern leopard frog	Gosner stage 25 (start of independent feeding) - 46 (end of metamorphosis and complete tail resorption)/ ca. 124 d	semistatic, 50 L/Lacetone, glass aquaria, 19-23°C, 16hlight/d, densitiy: 1g tadpole/5L water, 2 Replicates, 30 tadpoles/ 10 - 100 µg/L (nominal), WR 9 - 41% (real)	morphometrics (time to metamorphosis, weight, length)	NOEC = 100 µg/L (nominal) → no significant effect Relatively high mortality (40-58%)	(Mackenzie et al., 2003)	2 - no Guideline but well documented study (4-nonylphenol technical grade)
Rana sylvatica Wood frog	Gosner stage 25-28 (start of independent feeding) - 46 (end of metamorphosis and complete tail resorption)/ ca. 47 d	semistatic, 50 L/Lacetone, glass aquaria, 19-23°C, 16hlight/d, densitiy: 1g tadpole/5L water, 1 Replicates, 37 tadpoles/ 10 - 100 µg/L (nominal), WR 9 - 41% (real)	morphometrics (time to metamorphosis, weight, length)	NOEC = 100 µg/L (nominal) → no significant effect Low mortality (0-19%)	(Mackenzie et al., 2003)	2 - ; no Guideline but well documented study (4-nonylphenol technical grade)
Rana nigromaculata Dark-spotted frog	Tadpoles/ 60 d	semistatic/ 2 - 20 - 200 µg/L (nominal) + DMSO, also combined treatments with BPA	malformations of tail flexure	NOEC (45 d) = 20 µg/L LOEC (45 d) = 200 µg/L (16.7% malformations)	(Yang et al., 2005)	2 – no Guideline but well documented study; nominal conc. (nonylphenol technical)

Species	Life stage tested/ test duration	test conditions/ test concentration	Relevant parameter(s)	Effect concentration(s)	Reference	Reliability (Klimisch)
Rana catesbeiana American Bullfrog	Stage 35 – 37 (premetaphoric tadpoles)/ 7 d	semistatic, 21°C, 16 h light/d,3 Replicates / 234-468-936 µg/L (nominal) with or without addition of 10^{-7}M 3,3',5-triiodothyronine (T3), solvent: 40 µL/L methanol + 100 µL/L DMSO	tail length	NOEC = 468 µg/L Significant dose-response curve	(Christensen et al., 2005)	2 – no Guideline but well documented study, nominal conc., (Nonylphenol)
Bombina orientalis Oriental fire-bellied toad	2 h p.f. (fertilized eggs)/ 10 d	10 - 40 embryos; glass dishes; ANOVA/ 3 groups: 1 treated with 50 nM T3 for 1d + 6d without chemical; group 2: treated with T3 and NP (0.1 or 1 µM) for 1 d + 6d NP (0.1 or 1 µM); group 3: 7d treated with NP (0.1 or 1 µM) without T3	tail and body length	$NOEC_{tail\ length}$ = 22 µg/L $NOEC_{shorter\ body\ length}$ < 22 µg/L T3-exposed toads showed significant shorter tadpoles than the control.	(Park et al., 2012)	2 – nominal concentration, well documented but no guideline used (nonylphenol)
Xenopus laevis African clawed frog	Embryos (Gosner stage 10.5 up to stage 37)/ 2 days	Static/ 2 – 20 – 100 – 200 – 1000 - 2000 µg/L (nominal) + DMSO	body shape, length	$LOEC_{decreased\ body\ length}$ = 100 µg/L	(Bevan et al., 2003)	2 - nominal conc. (4-nonylphenol)
Bufo raddei Radde's Toad	period of amplexus and fertilization/ 3d	50 - 200 - 400µg/L (nominal) - 0,04‰ Alcohol	sperm motility and fertilization rate	NOEC = 50 µg/L LOEC = 200 µg/L (direct exposure of sperm) (reduction of sperm motility and fertilization rate)	(Feng et al., 2011)	2 - Nominal conc. (4-nonylphenol)

Two studies were carried out investigating the impact of 4-nonylphenols on the pigmentation of tadpoles. With the oriental fire-bellied toad *Bombina orientalis* the test conducted started with fertilized eggs. Here a decreased size and increased number of melanophore could be observed with a NOEC of 22 µg/L and a LOEC of 220 µg/L. The melanocyte differentiation is altered by exposure to a number of estrogenic chemicals. So maybe the inhibition of the melanophore growth in tadpoles was mediated by the estrogenic effects of nonylphenol. The increased number of melanophore could be a result of a compensatory mechanism for the insufficient growth of melanophores or result from the absence of a contact inhibition between developing melanophores. A result from the effect of nonylphenol on the pigmentation of the tadpoles could be that light coloured tadpoles may be more vulnerable to predation, increasing the predation pressure (Park et al., 2012). The other study is conducted with *Xenopus laevis* and resulted in a LOEC of 20 µg/L. Here Co-incubation of embryos with the pure antiestrogen ICI 182,780 blocked the ability of nonylphenol to induce abnormalities in body shape and in melanocyte differentiation, emphasising the assumption that the inhibition of melanophore growth was mediated by the estrogenic effects of nonylphenol (Bevan et al., 2003).

For *Xenopus laevis* four studies examining different development stages and endpoints are available. None of the studies provides evidence about a thyroidal mode of action, in fact no changes in development were observed by (Kloas et al., 1999). Unfortunately this endpoint was not examined in the two other studies. Results of the above mentioned study indicate that nonylphenol might influence sexual development of amphibians by a similar mode of action as 17ß-estradiol. Also a shift of sex-ratio towards females was observed and fits to effects observed in fish species after exposure to the natural estrogen. The effect is consistent with effects observed for 17ß-estradiol at 2.7 µg/L (70% female) in this study and is also consistent with effects observed for 17ß-estradiol in a guideline conforming study performed as part of the validation of the OECD amphibian metamorphosis assay (OECD, 2008b). In this test between 2 and 10 µg E2/L an almost complete feminization was observed. Indication of an estrogen like mode of action is supported by *in vitro* results showing reporter gene binding in *X. laevis* cells (see chapter 5.1.2.2). However, results provided by the study by (Kloas et al., 1999) should be used with care as it was not a reliable method development study. Results are not in line with results by (van Wyk et al., 2003) who found no vitellogenin induction in male *X. laevis* after intraperitoneal injection in high dosage. Nevertheless, as described above several other studies substantiate the sex-reversal found in the one single test with E2 during the validation of the guideline for the amphibian metamorphosis assay.

For *Rana sp.* two studies with 3 different species are shown in **Table 32**. For *Rana pipens* and Rana *sylvatica* (Mackenzie et al., 2003) the effects of nonylphenol were examined on the gonadal histology. As for *X. laevis* in the (Kloas et al. 1999)-Study the examination from (Mackenzie et al., 2003) revealed a change in sex- ratio but already at concentrations of 10 µg nonylphenol/L. There was an increased incidence of intersex gonads and for *Rana sylvatica* the sex- ratio was female-biased. So *Rana sp*. seems to be more sensitive for influences of nonylphenol on the gonadal endpoints. As there were changes in sex-ratio, an estrogen like mode of action is expected for this species. (Chang and Wischi, 1955) exposed *Rana sp.* to E2. In low concentrations of 0.07 to 0.37 µM (corresponds to 19 to 100 µg/L) this resulted in a change in sex -ratio with 100% female. In (Mackenzie et al., 2003) there was no effect on morphometrics such as the time to metamorphosis, weight and length described up to a concentration of 100 µg nonylphenol/L. For *Rana nigromaculata* there was a test conducted by (Yang et al., 2005) showing malformations of the tail flexure under the influence of 200 µg NP/L.

Due to exposure to nonylphenol there were also other effects e.g. on the body and tail length visible at *Bombina orientalis, Rana catesbeiana*, *Rana nigromaculata* and *Xenopus laevis*. The body and/or

tail was significantly shorter than in the control groups at concentrations above 20 µg/L (NOEC) for *B. orientalis* (Park et al., 2012) and *X. laevis* (Bevan et al., 2003) and 468 µg/L for *R. catesbeiana* (Christensen et al., 2005). (Christensen et al., 2005) revealed also malformations of the tail flexure of *R. catesbeiana* above 20 µg/L (NOEC). Also the limb development was accelerated by 936 µg nonylphenol/L. When the American Bullfrog was exposed to NP and T3 together the effect of nonylphenol on tail length was not significant.

In summary, although all studies should be used with care, the overall weight of evidence suggests that organism groups other than fish may be adversely affected by exposure to 4-nonylphenols at low concentrations (low µg/L range and below). Comparison with effects observed for 17ß-estradiol is suggestive of being estrogene like with respect to *X. laevis* and *Rana sp.* For *R. sylvatica* and *R. pipiens* the effects of 4-nonylphenols exerted on gonadal sexual differentiation and changes in sex-ratio for these 3 species fit to an estrogen-like mode of action.

Thus, in summary some information indicates that 4-nonylphenols might be estrogen like endocrine disruptors for additional taxonomic groups other than fishes whereas no definite conclusion can be drawn on direct or indirect effects of 4-nonylphenols of a thyroid mode of action owing to lack of guideline-conforming metamorphosis studies and/ or lack of knowledge on cross-talk feedback of sex-steroid and thyroid axes.

5.1.2.5 Aquatic invertebrates

Invertebrate endocrine systems are highly diverse. Although hormones that can be examined in vertebrate species often also occur in invertebrate species the functions of these hormones differ greatly between the phyla since their action depends on which cell and tissue types express receptors for them, and at what time in an organism's development these receptors are expressed. We have only limited knowledge about invertebrate endocrinology with some focused research on special areas like the juvenile and moulting hormones of insects and some of the mollusc and arthropod neurohormones. There is only limited information available about endocrine disrupting effects of 4-nonylphenols on (aquatic) invertebrates. Even though this phylum is very large and diverse the knowledge on how exogenous substances influence invertebrate endocrine systems is up till now scarce (U.S.EPA, 2005a). OECD development of test methods for the detection of adverse effects on development and reproduction for several groups of invertebrates is still underway (Gourmelon and Ahtiainen, 2007). Owing to our lack of knowledge on hormonal systems of most invertebrates, no biochemical endpoints are available. Therefore no specific mode of action can be ascertained and no conclusion can be drawn if a substance is an actual endocrine disruptor on invertebrate species alone.

In the studies analysed different isomers of 4-Nonylphenol were used. Owing to the fact that the *in vitro* data show no significant difference between linear and branched isomers all 4-Nonylphenols are considered to exert estrogen mediated effects. Specific isomers tested are indicated in the summarizing tables.

Table 33 summarizes adverse effects on development and reproduction in invertebrates. Where possible, the observed effects are assessed in relation to knowledge on endocrine effects and effects observed for natural and synthetic estrogens.

Table 33: Summary of most sensitive apical effects on aquatic invertebrates after exposure to 4-nonylphenols. Substance identity/ specific isomer tested indicated in the last column.

Species	Life stage tested/ test duration	Test conditions/ test concentration	relevant parameters	Effect concentrations [µg/L)	Reference	Reliablity
Crustacea						
Americamysis bahia	< 24h/ 14 d	EPA/600/4-91/003; semi static; 25°C; 16 h light/d; 8 replicates; 40 organisms; pH 8.0; salinity 2.5% / DMSO 0.01 mL/L - 0.3 - 1 - 3 - 10 - 30 µg/L (nominal)	Body length, total number of molts, number of immature mysids	NOEC $_{\text{body length}}$ = 0.3 NOEC $_{\text{total number molts}}$ = 3 NOEC $_{\text{number immature mysids}}$ = 10	(Hirano et al., 2009)	1 – guideline study (Nonylphenol technical grade – mixture of ring and chain isomers)
Americamysis bahia	< 24 h/ 28d	static, 23.3-26.4°C, pH7.5 - 8.2	Length, survival, reproduction	NOEC $_{\text{length}}$ = 3.9 NOEC $_{\text{survival and reproduction}}$ = 6.7	(Ward and Boeri, 1991) In: (U.S.EPA, 2005b)	4 – secondary source
Ceriodaphnia dubia	<24h/ 7d	ISO/CD 20665; semi static, 25°C/ (real)	Reproduction	EC_{50} = 8	(Isidori et al., 2006)	1 – guideline study with measured concentrations (4-nonylphenol)
Daphnia magna	4-21h/ 22 d	OECD 202 (1984) mod. For chronic study; semi static, 20.2 +/- 0.29 °C, pH 7.68 - 8.81, O2 99.8 - 77.4 %, GLP, 10 daphnia individually held/ GC-FID 1.55 - 1.34 - 3.45 - 10.70 - 47.81 µg/L (real)	Reproduction: offspring per survivor or total no offspring d9 – intrinsic rate r	NOEC $_{\text{offspring/survivor}}$ = 3.45 NOEC $_{\text{d9: total no offspring}}$ = 10.7	(Fliedner, 1993) UBA-002/4-22 Test report	1 – guideline study + GLP (93.1 Gew. - % p – Nonylphenol / 6.7 Gew. - % o – Nonylphenol)

Species	**Life stage tested/ test duration**	**Test conditions/ test concentration**	**relevant parameters**	**Effect concentrations [µg/L)**	**Reference**	**Reliablity**
Daphnia galeata mandotae	30 d	semi static/ 10-50-100 µg/L; solvent c. + c. (different!)	(number of female offspring, number of male offspring, number of ephippia) number of developmentally abnormal male and female offspring (11% of live young), fecundity	$NOEC_{abnormal\ offspring} = 10$ $LOEC_{abnormal\ offspring} = 50$ only animals that were prenatally exposed to NP exhibited this deformity NOEC fecundity = 10 Questionable result, because also increased fecundity in acetone control	(Shurin and Dodson, 1997)	3 – nominal concentration, no guideline, increased fecundity in acetone control
Daphnia magna	6-24h/ 21 d	semi static; 3 replicates; 3 daphnia per replicate; feeding with Chlorella; ANOVA; Dunnett's Procedure/ nominal: 13 – 25 – 50 – 100 – 200 µg/L)	Reproduction, survival, molting process	$NOEC_{Reproduction} = 13$ $NOEC_{survival} = 25$ $NOEC_{molting\ process} = 25$	(Sun and Gu, 2005)	2 – comparable to guideline study, nominal concentrations (Nnonylphenol)
Daphnia magna	21 d	ISO guidelines [ISO 10706, 2000. Water quality— Determination of long term toxicity of substances to Daphnia magna Straus (Cladocera, Crustacea). British Standards Institute, London.]; semistatic;10 replicates with 1 org; fed with Algae and fish food suspension; 16h light/d; 19-21 °C; ANOVA; Dunnett's multiple t-test/ solvent (0.1 mL/L) - 20 - 40 - 60 - 80 - 100 µg/L (nominal)	Mortality Cumulative offspring/female (2^{nd} generation)	$NOEC_{mort., Cumulative\ offspring/female\ (2nd\ generation)} = 20$ $LOEC_{mort., Cumulative\ offspring/female\ (2nd\ generation)} = 40$ $LC_{50,\ 1st\ generation}$ (21d) = 58 $NOEC_{Cumulative\ offspring/\ female\ (1st\ generation)} = 60$ $LOEC_{Cumulative\ offspring/\ female\ (1st\ generation)} = 80$	(Brennan et al., 2006)	2 – nominal concentration (4-nonylphenol)

Species	**Life stage tested/ test duration**	**Test conditions/ test concentration**	**relevant parameters**	**Effect concentrations [µg/L)**	**Reference**	**Reliablity**
Daphnia magna	<24h, 21 d	OECD 202 (1984) mod. for chronic study; semi static, 20+/-1°C, ph 8.25+/-0.25; GLP;10 daphnia individually held/ HPLC WR 70 - 78% 0-14-24-39-71-130-250 µg/L (real)	survival offspring, fecundity (mean no live offspring/ surviving parent), length	$NOEC_{survival\ offspring}$= 24 LC50 (21d) = 100 (real) $NOEC_{length}$ = 39	(Comber et al., 1993)	1 – guideline study with measured concentrations (91.8% nonylphenol, 86.1% 4-nonylphenol)
Daphnia magna	<24h, 21 d	semi static; 18 - 22 °C; 16 h light/d; 10 replicates with 1 daphnia; HPLC-UV analysis/ 8 µg/L Ethanol - 12.5 - 25 - 50 µg/L (nominal). WR 89-95%	neonate deformities (esp. late-stage neonate deformities like curved or unextended shell spines and undeveloped second antennae); mortality, molting, fecundity, sex ratio	$NOEC_{neonate\ deformities}$ = 25 $NOEC_{mortality,\ molting,\ fecundity,\ sex\ ratio}$ = 50	(Zhang et al., 2003)	2 – well documented study, nominal concentrations (4-nonylphenol (85% based on p-isomers))
Daphnia magna	<24h/ 21 d	semi static; 20°C; 16 h light/d; 50mL-beakers with 40mL culture medium; 10 replicates; 1 Daphnia/repl.; Student's t-test; Dunnett's t-test/ 0.001% vehicle solvent (ethanol); 0.2 - 0.46 - 0.91 µM (corresponds to 44 - 101.36 - 200.52 µg/L)	Embryotoxicity - developmental abnormalities (curved or unextended shell spines and underdeveloped first antenna) (result of exposure of gravid females); mortality of maternal organisms	$NOEC_{embryotoxicity}$ = 44 $LOEC_{embryotoxicity}$ = 101.36 (23% abnormal embryos) $LOEC_{embryotoxicity\ testosterone}$ = 1154 $LC50_{mortality\ maternal\ org}$ (21d) = 200.52	(LeBlanc et al., 2000)	2 – well documented study, nominal concentrations (4-nonylphenol)
Daphnia magna	<24h/ 21 d	semi static; Statistics: one-way ANOVA, Dunnett's multiple comparison test (sign =< 0.05)/ 6.2 - 12 - 25 - 50 - 100 µg/L (nominal)	Reproduction	NOEC = 50	(Baldwin et al., 1997)	2 – nominal concentrations (4-nonylphenol Cas-No.:104-40-5)
Daphnia magna	10 d old females/ 21 d	semi static, 48h NP then 16h C14-testosterone; Statistics: one-way ANOVA, Dunnett's multiple comparison test (sign =< 0.05)/ 6.2 - 12 - 25 - 50 - 100 µg/L (nominal)	higher acc. of C14-testosterone (reduced prod. of major testosterone elimination product t.-glucose and higher prod. of reduced/ hydrogenated metabolites)	NOEC = 50 Effect is concentration related	(Baldwin et al., 1997)	2 – nominal concentrations (4-nonylphenol Cas-No.:104-40-5)

Species	**Life stage tested/ test duration**	**Test conditions/ test concentration**	**relevant parameters**	**Effect concentrations [µg/L)**	**Reference**	**Reliablity**
Daphnia galeata	24 h/ 25 d	semi static; 20°C; 16 h light/d; pH 6.9-7.2; DO 6.8-7.0 mg/L; CaCO3 30 mg/L; Chlorella vulgaris; 10 neonates/ DMSO - 3 - 10 - 30 - 50 - 70 - 100 µg/L (nominal)	Survival	$NOEC_{survival}$ = 70	(Tanaka and Nakanishi, 2002)	2 – nominal concentrations (p-nonylphenol)
Ceriodaphnia dubia	1st instar/ 7d	static; 24-25°C; 6.4 - 7.9 mg O_2/L; pH 8.3 - 8.6/ real	Reproduction	$NOEC_{reproduction}$ = 88.7	(England, 1995) in: (U.S.EPA, 2005b)	4 – secondary source (there: reliability 1) (Cas-No.:84852-15-3)
Daphnia magna	21 d	semi static; nominal	Reproduction	$NOEC_{reproduction}$ = 100	(Hüls 1992b)	4 – secondary source (there: reliability 2 because of nominal conc.) (Cas-No.:25154-52-3)
Daphnia magna	<24h females/ 14d	18-22°C; 10 replicates; 1 daphnid each;16 h light/d or 8 h light/d; 300 - 450 lux/ 100 µg/L WR 89-95%	survival, total number of molts, total number of live neonates, total number of deformed neonates	LOEC = 100 (5.5% increased total number of deformed neonates - curved or unextended shell spines and undeveloped second swimming antennae)	(Gibble and Baer, 2003)	2 - well documented but no guideline used (4-nonylphenol)
Daphnia magna	21 d	ASTM; flow through; 16h light/d; 20 organisms; 2 replicates; measured test conditions	(mean number of young/starting adult;) mean length (mm) of surviving adults; mean number of young/surviving adult	$NOEC_{growth}$ = 116 $NOEC_{number\ young/surviving\ adult}$ = 215	(Spehar et al., 2010)	1 – guideline study with measured conc. (4-nonylphenol)

Species	**Life stage tested/ test duration**	**Test conditions/ test concentration**	**relevant parameters**	**Effect concentrations [µg/L)**	**Reference**	**Reliablity**
Echinodermata						
Arbacia lixula (sea urchin)	Sperm and eggs/ 3 days	static; saltwater; 17-21°C/ 0.937 - 1.874 - 2.811 - 3.748 - 4.685 - 9.37 - 18.74 µg/L (nominal) + solvent (DMSO)	developmental anomalies [normal plutei (N), retarded) plutei, pathologic malformed plutei (P1), pathologic embryos (P2) unable to differentiate up to the pluteus larval stages and dead (D) embryos/larvae]	LOEC $_{\text{larval malformations}}$ = 0.937 clear dose-response relationship	(Arslan and Parlak, 2007)	2 - - nominal conc. (NP $C_{15}H_{24}O$; Aldrich)
Paracentrotus lividus (sea urchin)	Sperm and eggs/ 3 days	static; saltwater/ 0.937 - 1.874 - 2.811 - 3.748 - 4.685 - 9.37 - 18.74 µg/L (nominal)	sperm fertilization success, quantitative and morphologic changes in mitotic activity, larval malformations, developmental arrest, embryonic/larval mortality	LOEC $_{\text{larval malformations}}$ = 1.874 LOEC $_{\text{larval malformations P1 and arrest of differentiation at the gastrula stage P2}}$ = 4.685	(Arslan et al., 2007)	2 – nominal conc. $C_9H_{19}C_6H_4(OCH_2CH_2)_nOH$
Molluscs						
snails						
Lymnaea stagnalis L.	Adult/ 49 d	semistatic; 22°C; 16 h light/d; 15-20 snails per treatment/ 1 - 10 - 100 µg/L (nominal)	mortality, shell growth, fecundity, F1 hatching success and fecundity after 84 d	NOEC = 100	(Czech et al., 2001)	2 – nominal conc. (4-nonylphenol)

Species	**Life stage tested/ test duration**	**Test conditions/ test concentration**	**relevant parameters**	**Effect concentrations [µg/L)**	**Reference**	**Reliablity**
Lymnaea stagnalis L. freshwater pulmonate snail	life-cycle/ 20 d	static; 18-20°C; glass aquarium with 80L water and 3-4cm sediment depth; 12h light/d; pH=8; 6.7mg O2/L / 105 µg/L (real)	embryonic growth, embryo mortality, hatching success	NOEC < 105	(Lalah et al., 2007)	2 –well documented study; no guidline available (4(3',6',-dimethyl-3'-heptyl) phenol constitute 10% of technical p-NP, which is a mixture of > 20 differently branched- and linear-chain isomers)
Haliotis diversicolor supertexta species of sea snail, a marine gastropod mollusk in the family Haliotidae, the abalone	from fertilization to the end of metamorphosis/ 96 h	100mL petri dishes containing 50mL seawater; 24-26°C; 5.8-6.2mg O2/L; salinity 29-31ppm; pH 8.0-8.2; methanol as solvent; 3 replicates; 4 embryos per mL; after 20h: living style from swimming to settlement -> 500mL glass beakers containing 400 mL culture media and 8 glass slides ccoated with Navicula incerta in euch beacker as the settlement substrate ! pre-exposed to the target chemical for 96h rather than cultured in F/2medium !; GC-2010 gas chromatography; real: WR -3 u +8%	embryo toxicity (no un-metamorphosed larvae could survive after 96-h culture -> completion of larval metamorphosis were used to indicate the suvival of abalone embryos)	EC_5 = 0.99 EC_{50} = 11.65	(Liu et al., 2011)	2 - not according to a guideline but very well documented study! (4-n-nonylphenol)

mussels						
Dreissena polymorpha Zebramuschel	112 d	semistatic; collected at the beginning of January (water temp. 5-6°C); size 15-30 mm; 185 mussels per tank; 5-8°C; 12 h light per day; triplicate/ ethyl acetate - 5 and 500 µg/L (nominal)	Vn-like proteins and alkali-lagile phosphates	NOEC = 5	(Quinn et al., 2006)	2 – well-documented study, nominal conc. (4-n-nonylphenol)
Mulinia lateralis marine mussel	embryo/ 48 h	static; unmeasured conc.; pH 7.8 - 8.2; salinity 32 g/kg; 18.3°C; 3 replicates; 26 embryos per mL// 20% acetone and 80% triethylene glycol - 2.4 - 8 - 24 - 80 - 240 - 800 µg/L (nominal)	Mortality	LC_{50} = 37.9	(Lussier et al., 2000)	1 - guideline study (ASTM); with measured conc. (4-nonylphenol (or para-nonylphenol) (CAS-No.:84852-15-3))
Mytilus edulis L. common mussel	30 d	semsitatic; 16-18°C; 30-34 ‰/ acetone - 18 - 56 - 100 - 200 µg/L (nominal)	Byssus strength energy budget (scope of growth … SFG) fertilization and early developmental success	NOEC $_{\text{byssus strength + energy budget}}$ = 18 (decrease) NOEC $_{\text{fertilization}}$ = 200	(Granmo et al., 1989)	2 – nominal conc. (4-nonylphenol (CAS-No.: 25154-52-3))
Mytilus edulis L. common mussel	30 d	flow-through; 8-12°C; 30-34 ‰/ acetone - 18 - 32 - 56 - 100 - 200 µg/L (nominal)	Byssus strength energy budget (scope of growth … SFG)	NOEC $_{\text{byssus strength + energy budget}}$ = 32 (decrease)	(Granmo et al., 1989)	2 – nominal conc. (4-nonylphenol (CAS-No.: 25154-52-3))
Crassostrea gigas pacific oyster	3 months p.f. (gametogenesis)/ 72 h	18-22°C; 6 replicates; 5 oysters/replicate; seawater (35ppt); fed with 50000 cells per mL; ANOVA with linear regression, Tukey test/ methanol - 1 - 100 µg/L (nominal)	Sperm motility shell length, fresh body weight, sex ratio	NOEC $_{\text{sperm motility}}$ < 1 NOEC $_{\text{shell length + sex ratio}}$ = 100	(Nice, 2005)	2 - nominal conc.; well documented publication which meets basic scientific principles (nonylphenol)

Crassostrea gigas pacific oyster	post fertilization/ 72 h	35 ‰; 28mL glass test vessels (27.5mL volume); 3 replicates; 19-21°C; Kruskal-Wallis one-way ANOVA; Tukey-HSD/ methanol - 0.1 - 1 - 10 - 100 - 1000 - 10000 µg/L (nominal)	deformities (D-shaped larvae with a "convex-hinge")	NOEC = 10 (48h); 0.1 (56h); 10 (64h, 72h) (delay in the embryonic development)	(Nice et al., 2000)	2 - acceptable, well-documented publication which meets basic scientific principles (4-nonylphenol)
Crassostrea gigas pacific oyster	eggs 7 days p.f./ 48 h (+ 10 months without NP)	2L-test vessels; 3 replicates; 10000 eggs/mL; after 48h larvae put into clear seawater - after 10 mo pf oysters sexed - test-crosses - resulting embryos adjusted to a density of 200/mL - after 48h dev of embryos and larvae was arrested; filtered seawater (35ppt); 20 - 24 °C; pH 7.8 - 8.1; O2= 95 - 100 %; food: mixture of algae species/ GC-MS; 100 µg methanol/L - 1 - 100 µg/L (nominal); <1 - 4 µg/L (real)	sex ratio of resulting oysters (skewed towards females) and fully functional hermaphrodites; transgenerational effects - gamete viability	LOEC < 1 (17% hermaphrodites) LOEC < 1 (offspring from control parents had a significantly higher survival rate than offspring where at least one parent had been exposed to nonylphenol during larval development)	(Nice et al., 2003)	2 – acceptable, well documented publication (nonylphenol Lot No. 74430 Sigma-Aldrich Chemcial Company Ltd)

In summary, effects on three different phyla (crustaceans, echinoderms and molluscs) were examined.

Within the group of crustaceans two species were tested (*Daphnia magna* and *Americamysis bahia)*. No effect on reproduction was observed on *D. magna* at concentrations lower than 3.45 µg/L (Fliedner, 1993). A 7d-guideline study with *Ceriodaphnia dubia* revealed an EC50 of 8µg/L for reproduction (Isidori et al., 2006). Ceriodaphnia is morphologically very similar to Daphnia but is smaller and has a shorter generation time (U.S.EPA, 2002). (Baldwin et al., 1997) investigated the effects on the testosterone metabolism of *Daphnia magna.* It was shown that concentrations < 25 µg /L can significantly affect the androgen metabolism and therefore may contribute to the overall effects on reproduction. (LeBlanc et al., 2000) describe embryotoxic effects which include developmental abnormalities such as curved or unextended shell spines and underdeveloped first antenna as a result of an exposure of gravid females. At 100 µg 4-nonylphenol/L, 23% of the embryos showed these developmental abnormalities with a NOEC of 44 µg/L. Also prenatally exposed animals were under examination in a study by (Shurin and Dodson, 1997) with similar results beginning at 50 µg/L and a NOEC of 10 µg/L. This examination revealed that prenatally exposed *Daphnia galeata mandotae* showed abnormalities like curled tail spines and lacked or had severely reduced terminal setae on their second antennae, which is characteristic for *Daphnia* in embryonic stages. Similar neotade deformities were found in *Daphnia magna* in the study of (Zhang et al., 2003) with a NOEC of 25 µg/L. (Brennan et al., 2006) describes in a guideline-conform study an effect on *Daphnia magna* that seems to become more sensitive from the first generation to second. This effect applies to the mortality and cumulative number of offspring per female. In the second generation the NOEC (20 µg/L) is one third lower than in the first generation. A similar NOEC value for the mortality of the offspring resulted from the guideline-conforming study conducted by (Comber et al., 1993).

For the mysid *A. bahia* the NOEC on reproduction was in the same range as for the daphnids (NOEC = 6.7 µg/L) (Ward and Boeri, 1991). In both species the reproduction was reduced starting from 10 µg/L. No developmental effects (moulting) were observed in *A. bahia* up to 3 µg/L (Hirano et al., 2009). In the treatment groups from 10 µg/L the total number of moults was significantly lower than in the control groups (Hirano et al., 2009). Molting characterizes the crustacean growth and is under the immediate control of moult-promoting steroid hormones, the ecdysteroids (Verslycke et al., 2007). It should be noted that growth effects in mysids are likely to have important implications for development, metamorphosis, and reproductive success since fecundity is related directly to female body size (Winkler and Greve, 2002). As the endpoints assessed did not include indicative parameters for endocrine mediated effects e.g. biomarkers, it cannot be concluded that it is endocrine mediated but it fits to the assumption of an endocrine activity. However, for *A. bahia* also the effect of 4-nonylphenol on production of 20-hydroxyecdysone (20E) was compared with the control during a moult cycle. In contrast to the normal pattern of ecdysteroid cycling during the moult cycles of *A. bahia,* in mysids exposed to 30 µg NP /L a significant suppression in 20E levels was observed (Hirano et al., 2009).

Effects on echinoderms were assessed with two sea urchin species. In the two tested species (*P.lividus* and *A. lixula*) larval malformation after exposure of sperms and eggs were observed, starting to occur at concentrations of respectively 1.9 and 0.9 µg/L (Arslan and Parlak, 2007; Arslan et al., 2007). Echinoderms are relatively closely related to vertebrates. Therefore, their endocrine systems may have some similarities. Vertebrate sex steroids may play a role in echinoderm reproduction. (OECD Series on Testing and Assessment No.50 (Kropp et al., 2005)) Pentachlorophenol, an anti-estrogen and thyroid active substance, tested on *P.lividus* resulted in a

similar effect which means an alteration in embryonic development and differentiation (Ozretic and Krajnovic-Ozretic, 1985).

Effects on molluscs are summarized separately for mussels and snails. For mussels there are six tests with four different species available.

Up to the highest concentration tested (100 or 200 µg/L) there is no effect on sex-ratio or fertilization for *Crassostrea gigas* (Nice, 2005) and *Mytilus edulis* (Granmo et al., 1989). Other endpoints like energy budget or sperm motility were more sensitive with a NOEC at concentrations of 18 or lower than 1 µg/L for *M. edulis* (Granmo et al., 1989) or *C. gigas* (Nice, 2005). Egg production in oysters requires 50% more energy than sperm production. So it is not surprising that the energy budget of the common mussel was a sensitive endpoint and affected in the study conducted by (Granmo et al., 1989). (Nice et al., 2003) describes an increased incidence of hermaphroditism (17%) and sex ratio skewed towards females resulting from an exposure of *C. gigas* to nonyphenol (<1 and 4 µg/L - real) at a key stage of sexual differentiation. The global incidence of hermaphroditism in oviparous oysters is generally very low in a range between 0 and 1.1% for *C. gigas*. In this test there was no significant difference between the sex-ratios in the control and expected from historical data deduced sex ratios. Although *Crassostrea gigas* has the capability to change sex between seasons, usually there is a clear period during which the gonad remains undifferentiated between reproductive seasons; and once gametogenesis has been initiated the oyster loses the ability to change sex for that season (Kennedy et al., 1996). Eggs usually only begin to develop after the sperm have been extruded – usually with a winter (period of sexual undifferentiation) between the two sexual phases. So it is extremely unusual to find evidence of both male and female gametes in the same individual simultaneously. Several studies describe that estrogens are involved in sexual maturation following an undifferentiated phase in older (2 to 3 years) *C. gigas* (Matsumoto et al., 1997; Mori, 1968a; Mori, 1968b). In studies where E2 was administered to adult (2 to 3 yr) *Crassostrea gigas*, sex reversal from male to female was induced when administration began at early stages of sexual maturation between reproductive seasons (Mori et al., 1969). However, at a later stage, i.e. once gonad development had begun, the addition of E2 had no effect on sex- ratio (Mori et al., 1969). Exposure to E2 was also found to accelerate sexual maturation in female *C. gigas* (Mori, 1969). There is evidence to suggest that the reproductive physiology of an oyster can be affected by water-borne pheromones from another oyster (Kennedy, 1983). Therefore, it follows that this system may also be sensitive to other chemicals, hormonal or otherwise, present in the local environment during particular stages of development. Oestrogens are known to be involved in the development of *Crassostrea gigas* ovaries and gametes (Matsumoto et al. 1997). Another effect of the 4-Nonylphenol described in the test of (Nice et al., 2003) is a transgenerational one. The examination indicates that 4-Nonylphenol had an influence on the quality of the developed gametes so that they are of poor quality resulting in a reduced survival rate of the offspring from parents where at least one had been exposed to 4-Nonylphenol during the larval development.

A test for the snail species *Lymnaea stagnalis* shows a NOEC for fecundity and F1 hatching success of 100µg/L (Czech et al., 2001). Adults were exposed and reduction effects on egg production and hatching rate after 6-12 weeks of exposure were seen. (Czech et al., 2001) also reported transfer of the endocrine effect, from maternal exposure to the next generation, by analysis of symptoms in F1 generation snails. According to (Segner et al., 2003) the ovulation and egg-laying behavior in *L. stagnalis* are regulated by a neurosecretory peptide, the egg-laying hormone. Another test with *L. stagnalis* (Lalah et al., 2007) with a limit concentration of 105 µg/L Nonylphenol caused significant delay in all stages of growth and an increase in embryo mortality. Also the hatching success of embryos was significantly reduced.

6 CONCLUSIONS ON THE SVHC PROPERTIES

6.1 PBT, vPvB assessment

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

6.2 CMR assessment

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

6.3 Substances of equivalent level of concern assessment

6.3.1 Environment

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

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

a) Are 4- nonylphenols substances having endocrine disrupting properties?

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

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

6.3.1.1 Endocrine disrupting properties

Endocrine disrupting properties are one example of inherent properties that might, if scientific evidence of probable serious effects is available, give rise to an equivalent level of concern as exerted by CMR and/or PBT/vPvB substances. Although the term "endocrine disrupting properties" is not equivalent to the term "endocrine disruptor" the definition of an endocrine disruptor provided by WHO/IPCS (WHO/IPCS, 2002) is used as starting point to analyse the endocrine disrupting properties of nonylphenol:

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

In chapter 5.1.2 available in vitro data as well as data for fish, amphibians, and invertebrates are examined in order to analyze whether 4-nonylphenols should be regarded as endocrine disruptors according to this definition. This examination is based on the criteria set out in the OECD guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption (OECD, 2012).

In vitro results described in chapter 5.1.2.2 demonstrate that 4-nonylphenols act as ligands of the estrogen receptor. Gene activation as well as vitellogenin expression as a result of this activation were observed for different vertebrate estrogen receptors including mammals, fishes (*O.mykiss* and *S. Salar*) and frogs (*X. laevis*) in isolated receptors as well as primary hepatocytes. Furthermore, data available show that 4-nonylphenols is able to interfere with other nuclear receptor-mediated pathways as nonylphenol inhibited androgen-mediated gene expression *in vitro*. Thus in summary, the available mechanistic information provides clear evidence that 4-nonylphenols act as estrogen receptor agonists and may have anti-androgenic properties as well. The relative potency of 4-nonylphenols compared to 17ß estradiol ranged from $0.94\ 10^{-5}$ to $0.44\ 10^{-2}$.

As described in chapter 5.1.2.3 data available for fish substantiate the endocrine mechanism of action of 4-nonylphenols *in vivo* and provide strong evidence that it results in population relevant adverse effects as a consequence of the endocrine mode of action:

- In four fish species (*O.latipes, D.rerio* and the two vivivarous species *P. reticulata, G. holbrooki*) reliable sexual development tests and a full life cycle test (*O.latipes*) clearly showed that nonylphenol is endocrine active in fish and causes population relevant adverse effects (change in sex-ratio) which are clearly linked to the endocrine activity:Vitellogenin was induced, testis-ova or other indicative histological changes occurred and the sex-ratio was skewed towards females.

- For two other species (*P.promelas nd O.mykiss*) results clearly show that 4-nonylphenols are endocrine active *in vivo*: Vitellogenin was induced and either changes in secondary- sex characteristics (*P.promelas*) or testis-ova and inhibition of spermatogenesis (*O.mykiss*) were observed. Observed adverse effects fit to the endocrine mode of action (reduced growth during early life stages and reduced fecundity) providing strong evidence that the substance is an endocrine disruptor also in these species.

- In all other species (*Gobiocypris rarus, Crassius auratus*) endpoints indicating population relevant effects were not analyzed. But changes in the vitellogenin level and the estradiol level as well as histological changes (testis-ova and hypertrophic leydig cells respectively) give clear hints that 4-nonylphenols are endocrine active in these species, too.

Thus, there is strong evidence from high quality data that 4-nonylphenols actually act as endocrine disruptors in fishes i.e. that the substance alters the function of the endocrine system and consequently causes adverse, population relevant effects. Data for several fish species show that this holds true for a variety of different species.

For amphibians study results, as well as our knowledge about estrogen mediated effects in these taxa, do not allow for a definitive conclusion as to whether the adverse effects observed are estrogen endocrine mediated. However, *in vitro* results for one species *(X.laevis*) indicate that 4-nonyphenols may induce vitellogenin expression by binding to the estrogen receptor. Adverse effects in a low quality study for X. *laevis* (change in sex-ratio) are comparable to effects observed for 17ß-estradiol providing some indication that effects may be caused by an estrogen-like mode of action. This estrogen-like effect was also found in two other frog species (*R. sylvatica* and *R.pipens*) where sex reversal towards females was shown to start at low µg/L concentrations in reliable studies.

With regard to invertebrates, no clear conclusion can be drawn. Effects observed for crustaceans, echinoderms, and mollusks provide some indication of endocrine activity and apical effects observed in two mussel species fit to those observed for the natural estrogen 17ß-estradiol.

Overall summary of endocrine disrupting effects of 4-nonyphenols in taxonomic groups analysed

In summary, available information shows that 4-nonylphenols act as endocrine disruptors in fish and there is some evidence for estrogen-like disruption in anuran amphibians. Some data indicate that 4-nonylphenols may be endocrine active in invertebrate species too, but no clear conclusion can be drawn due to the lack of knowledge about the exact endocrine mechanism in invertebrates and the lack of test systems which include endocrine biomarkers diagnostic of endocrine mechanisms.

These concluding aspects are summarized in Table 34.

Table 34: Endocrine disrupting effects of 4-nonylphenols in different taxonomic groups

Taxonomic group	Number of species	Indication of hormonal activity?	Apical adverse effects observed?	Indication that apical endpoints fit to mode of action
Fishes	9	Yes, in all species observed (increased vitellogenin level in males and females, changes in female gonadal staging, changes in sperm stages in males, testis-ova, secondary sex-characteristics, elevated estradiol levels)	Yes, effects in all species with tested apical endpoints (6 species). Most sensitive adverse endpoints: Sex-ratio (*O.latipes, D.rerio, P,reticulata, G. holbrooki*), Fecundity (*P.promelas),* growth (*O.mykiss*) Most sensitive fully reliable LOEC = 3.4 µg/L (fecundity, *P.promelas*) with some indication that effects may start at 0.75µg/L (semen volume *O.mykiss)*	Yes, based on studies with nonylphenol clear link for four fishes Effects observed in all species substantiate the endocrine mode of action and are known to be estrogen sensitive
Amphibians	7	Yes, in vitro receptor binding for one species. Some hints that effects might be endocrine mediated in another species but not conclusive.	Yes, in 3 species (change in sex – ratio, occurrence of intersex gonads, changes in development) Most sensitive LOEC≤ 10 µg/L (sex-ratio in *R. sylvatica*,and *R. pipiens* Klimisch 2)	Effects observed on sex-ratio in *X.laevis* in low quality study and changes in sex-ratio in *R. sylvatica and R. pipelines* in a Klimisch 2-study point to an estrogen mediated mode of action
Invertebrates	2 crustacean species	Yes, effects on androgen metabolism in *D.magna*	Yes (reproduction,development, moulting) Most sensitive fully	Some indication but no clear conclusion possible due to lack of knowledge

		Depression of 20-hydroxyecdysone production during amolt cycle	reliable EC_{50} = 8 µg/L (reproduction in *C. Dubia*)	
	2 echinoderm species	Effects observed are similar to those observed for a known anti-estrogen and thyroid active substance (pentachlorphenol)	Yes (larval malformations)) Most sensitive reliable LOEC = 0.9 µg/L (larval malformation in *A. Lixula*	Some indication but no conclusion possible due to lack of knowledge
	4 mussel species	Induced hermaphrodism Effects fit to those observed for 17ß estradiol and our knowledge about the influence of estrogens on female sexual maturation	Yes (sex ratio skewed to females in one study, survival ofspring) Most sensitive reliable LOEC ≤ 1 µg/L (survival, sex-ratio in *C:gigas*	Some indication but no clear conclusion possible
	1 snail species	-	Yes (fecundity, hatching success F1 generation, growth) Most sensitive reliable LOEC 1 µg/L (embryonic toxicity in H. diversicolor	No conclusion possible

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

As described in article 57 (f), an endocrine disruptor should be regarded as of very high concern if the probable serious effects to the environment are of equal concern compared to CMR and/or PBT/vPvB substances (REACH, Art. 57 f). The seriousness of effects and the equivalency of concern needs to be analysed case by case employing a weight of evidence approach.

The effects observed as a result of exposure of several species including fish, and amphibians to nonylphenol are considered to be of equivalent concern due to the severity of the effects and the difficulties to quantify a safe level of risk for estrogene-like endocrine disruptors.

Effects are severe with regard to the type of effects and the concentrations causing the effects. As described in chapter 5.1.2 nonylphenol results in endocrine mediated adverse effects on apical endpoints in several fish species at very low test concentrations (low µg/L range). Sensitive endpoints observed in fish are clearly endocrine mediated. Compared to 17ß-estradiol in vivo potency ranged from 10^{-4} to 10^{-5} (sex-ratio and histological changes in *D.rerio*) with some indication that the relative potency might be higher in other fish species (factor ≤ 0.11 for *O.latipes* with regard to male secondary sex characteristics; factor ≤ 0.1 for *P.reticulata* with regard to vitellogenin induction).

Effects are considered serious for the environment as they impair population stability or recruitment. Exposure to 4-nonylphenols resulted in effects in fish on reproduction parameters (fecundity) as well as on sexual development (including changes in sex-ratio) and growth. Results for at least 3 fish species and three frog species show that exposure to nonylphenol may result in a complete sex-reversal and all female populations.

Several effects observed after exposure to 4-nonylphenols indicate that they may cause long lasting effects which persist even after exposure has ceased.

Exposure in one generation resulted in effects in the next generation even if that generation was not exposed: In *O.mykiss* VTG induction and increased level of sexual steroids were observable in 3 year old unexposed progeny of parents exposed as adults. Viability of eggs and hatching rate of unexposed progenity was reduced (Schwaiger et al., 2002). In *D.rerio* exposure of female adults resulted in malformation of embryo although embryos were not exposed and reproduction started after the end of exposure (Yang et al., 2006). Exposure during sexual development resulted in a reduced fecundity and a reduced swim-up success of progeny even if adults were not exposed any longer (Lin and Janz, 2006).

Effects observed in two fish species provide evidence, that exposure to 4-nonylphenols reduces the reproductive capacity of exposed male fish. In *P.promelas* exposed males were outcompeted during spawning by unexposed males (Schoenfuss et al., 2008) and in *P. reticulata* reproduction was three months delayed due to the fact that males did not show courtship behavior. Such changes in male reproduction capacity might influence the genetic variability of populations in the long-term, as only a part of the males may be capable to reproduce (Sumpter and Johnson, 2008)

Effects observed in *O.latipes* provide indication that a continous exposure may result in more pronounced effects not covered in one generation tests as effects on the sex-ratio in the F1 generation of *O.latipes* were more pronounced than in the F0 generation (Yokota et al., 2001).

These observations are in line with our knowledge about the endocrine system. Endocrine modulation is a very complex feedback process that is set up during critical life stages. As summarized in (IPCS, 2002) disturbance of this set up may result in effects during the entire life-time.

Results show that a transient short term exposure during sensitive life stages may result in life long effects and even in following generations. This must be considered as serious effects as migration is a common pattern in species such as birds, amphibians, mammals and fishes. It includes long-distance migration of migratory birds or of fish species, such as salmonids and eel. Thus a short term exposure in one area may result in effects that impair population stability in another area (e.g exposure during development of flatfish in coastal area may result in population changes in the open sea, or exposure of adult salmonids in estuarine areas during migration might influence sperm quality and fertilization success at the reproduction sites in rivers).

In addition to the severity of these effects, some results substantiate the hypothesis that it is difficult to quantify a safe level of exposure to 4-nonylphenols owing to their endocrine activity.

Effects in *O.mykiss* indicate that effects on reproduction (which was not analyzed in available tests) may start at much lower concentrations than the most sensitive adverse endpoint observed in the tests (growth at 10 µg/L) as exposure to 0.75 µg/L 4-nonylphenol resulted in a reduced sperm volume after exposure of males.

Effects on reproductive behavior as observed in *P.promelas* and *P.reticulata* indicate that effects on endpoints not covered in OECD guideline tests may influence reproduction. As described by Crane (2010), aspects such as influence on breeding behaviors and competition are not taken into account in laboratory tests. Thus it seems possible that effects on reproduction may occur at concentrations lower than those observed in the fish reproduction and screening tests.

Effects observed in *X.helleri* (reduced sword tail length, relevant secondary sex characteristic in that species at 0.2 µg/L) indicate that species other than those normally tested might be more sensitive to exposure to 4-nonylphenols.

Although it is difficult to estimate whether or not such effects might result in a population change in ecosystems they indicate that exposure to 4-nonylphenols might result in effects at concentrations below those observed. This is substantiated by the fact that exposure to4- nonylphenosl resulted in effects on reproduction and development in different invertebrates at concentrations below 1 µg/L (e.g. LOEC sex-ratio < 1 µg/l in mussels, LOEC development 0.09 µg/L in echinoderm species). Although it is not possible to clearly state that the effects are endocrine mediated, these effects fit to the knowledge that steroids are known to play an important role e.g. in vertebrates other than fishes (Baroiller et al., 1999) and in invertebrates (Kendall et al., 1998). Due to highly conserved estrogen receptors it is very likely that a wide range of species with different functions in ecosystems could be affected. Owing to the lack of in depth knowledge of their endocrine system and the lack of test systems it is currently nearly impossible to estimate which species are most sensitive and which concentration should be regarded as safe for the environment.

Thus, in summary, effects observed after exposure to 4-nonylphenols are considered to impair population stability and recruitment. They may occur even after short term exposure 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 and it is difficult to estimate a safe level of exposure to 4-nonylphenols. Consequently they are considered to be of an equivalent level of concern.

PART II

INFORMATION ON USE, EXPOSURE, ALTERNATIVES AND RISKS

INFORMATION ON MANUFACTURE, IMPORT/EXPORT AND USES –CONCLUSIONS ON EXPOSURE

MANUFACTURE, IMPORT/EXPORT

All companies with the intention still to produce or import the substance in quantities above 100 tonnes per year after the end of the first registration phase had to register until 1 December 2010 because 4-nonylphenol is classified as "R50/53 - very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment" according to the Dangerous Preparations Directive (1999/45/EC).

Several companies registered 4-nonylphenols during the first registration phase ending on 01 December 2010. In fact, from the substances listed in Table 5 (Part I) only the substance Phenol, 4-nonyl-, branched (CAS-Nr. 84852-15-3) was registered, with a total tonnage band of 10,000 - 100,000 t/y. Further information from registration dossiers on produced and imported tonnages is provided in the confidential annex. For common CAS-numbers of nonylphenol (104-40-5, 136-83-4, 139-84-4, 11066-49-2, 17404-66-9, 25154-52-3, 26543-97-5, 27938-31-4, 30784-30-6, 52427-13-1) no registration dossiers (full or intermediate) were available and they also are not intended to be registered in the second registration phase[2]. There is no information available on quantities of 4-nonylphenol exported to countries which are not members of the European Union.

The Risk Assessment Report for Nonylphenol (European Commission, 2002) gives some additional indication for quantities manufactured inside or imported into the EU. Data for the year 1997 showed that 73500 tonnes per year were produced by four companies inside the EU, 3500 tonnes were exported and 8500 tonnes were imported giving a total volume for use within the EU of 78500 tonnes. The volume of 4-nonylphenol placed on the European market actually decreased in total in the last 15 years.

[2] "Substances identified by industry to be registered by 31 May 2013", European Chemical Agency, 2012, http://echa.europa.eu/web/guest/information-on-chemicals/registered-substances/identified-substances-for-registration-in-2013

USES

A list of the uses identified by the registrants of Phenol, 4-nonyl-, branched (CAS-Nr. 84852-15-3) is available at ECHA's dissemination site: http://echa.europa.eu/information-on-chemicals .

Further information on uses from registration dossiers is provided in the confidential annex.

A risk assessment report for 4-nonylphenol (branched) and nonylphenol was prepared in 2002 by UK (European Commission, 2002). As several marketing and use restrictions were introduced within the EU in 2005, uses described in this report have to be considered as outdated. However, in the final report of Workpackage 4 of the COHIBA Project (COHIBA Project Consortium, 2012) uses of nonylphenol ethoxylates have been identified, that have not been considered in the original RAR (2002) but might be relevant due to the environmental degradation of the ethoxylates. These uses are:

- Washing of imported textiles: Although the use of nonylphenol and nonylphenol ethoxylates for textile production is widely restricted within the EU it is or may be still contained in imported textiles, textiles produced before the restriction was introduced and products produced in compliance with the restriction (using closed processes). The same might be relevant for the use of the ethoxylates as auxiliary in the leather processing industry.
- Pulp, paper and board industry: NP is used in paper production as a component of phenolic resins used in coatings, e.g. for carbonless copy paper, and other NP-resins used for printing inks.

Emissions of 4-nonylphenol as degradation product from uses and environmental releases of nonylphenol ethoxylates

It is known that 4-nonylphenol and accordingly the ethoxylates are still used in textile production outside the EU as detergent and auxiliaries, such as dispersing agents for dyeing, emulsifiers and spinning lubricants. Imported textiles therefore contain nonylphenol ethoxylates as contamination which was detected in 52 out of 78 textile samples with concentrations from 1 mg/kg up to a maximum concentration of 1100 mg/kg (Greenpeace, 2011). One sample with a plastisol print contained 27000 mg/kg ethoxylates. Textiles containing nonylphenol ethoxylates were bought in 17 countries. Likewise, in other studies high concentrations of those ethoxylates were found in towels, t-shirts, overalls and underwear. In general concentrations between 1.6 and 1300 mg/kg were reported, one outlier even reached 10600 mg/kg (OECD, 2011). During washing the nonylphenol ethoxylate is released to wastewater. As an emission factor the average value (excluding the outlier) of 0.25 kg/t was suggested (OECD, 2011). It was estimated by the OECD that 90% of the nonylphenol ethoxylates used for dry cleaning, car care products, cleaning agents etc. are released to wastewater (OECD, 2011).

In paints and printing inks nonylphenol ethoxylates are typically used in concentrations of 0.6 to 3%. Nonylphenols or their ethoxylates are mainly used in paints used to wet room floors, water-based paints and varnishes for indoor use, printer's ink, concrete floor paint, metal coating and anti-corrosive paints (please see OECD, 2011 for further details). In 2006 a total of 11 tonnes of nonylphenols and 15.1 tonnes of nonylphenol ethoxylates were registered as used in paints, varnishes, colouring agents and adhesives in Sweden (OECD, 2011). In the same year 3 tonnes of nonylphenol and 130 tonnes of ethoxylates were registered for use in paints, lacquers and varnishes in Finland. 1 tonne of nonylphenol and 3 tonnes of ethoxylates were registered in Norway for the

same types of products, respectively (OECD, 2011). The most important emission pathways are leaching from painted and varnished surfaces and from cleaning of painting equipment. It was assumed that <1% of nonylphenol and the related ethoxylates are released to wastewater treatment plants and an emission factor of 0.5% to wastewater is suggested (please see table A1.3.4 in OECD, 2011 for further details).

Nonylphenol ethoxylates are used in water-based adhesives in the textile industry, pulp and paper industry, printing-works, construction industry, and in the plastic products industry. In Sweden 1 tonne of nonylphenol ethoxylates and 0.46 tonnes of nonylphenole were used in water based adhesives in 2006. Emissions may occur during cleaning of equipment and it is assumed that 1 % of all adhesives used may enter the wastewater. Altogether, adhesives are not considered to be a major emission source of nonylphenole or their ethoxylates to the environment (OECD, 2011).

In the agricultural sector nonylphenol ethoxylates were used as wetting agents in pesticide formulation to improve the efficiency of spraying and reduce the amount of active ingredient and as additive in veterinary medical products like teat dips. In 2006 0.31 tonnes of those ethoxylates were used in biocide products and 1.5 tonnes were used in plant protection products in Sweden and 1.7 tonnes and 15 tonnes were used in Denmark in biocides and plant protection products, respectively (OECD, 2011). However, these uses are restricted under the REACH regulation.

Nonylphenol ethoxylates are also used in metal working fluids and lubricating oils. According to a case study by Norway (OECD, 2011) these products are applied in the metal extraction, refining and processing industries in the formulation and usage of cutting and drilling oils and in the metal working industry as emulsifiers, in metal cleaning aids and in anti-mist additives. 4-nonylphenol is used in the preparation of lubricating oil additives. According to the OECD, 2011 document, in Sweden the following products were registered for 2006: Cutting fluids, Base oils, surface treatment of metals, galvano-technical agents, motor oils, other lubricants, rust removers, gear oils, hydraulic fluids and hydraulic oils, corrosion inhibitors, anti-corrosion materials, slip agents for modeling metal. The following uses were reported from Denmark for 2006: >1.8 tonnes as cooling agents for metal processing, approximately 80 tonnes as lubricants and approximately 93 tonnes as metal surface treatment remedies. During normal usage spills of these products are not intended to reach wastewater treatment plants. However, it is estimated that about 5% of the total usage is released directly to the waste water system by incorrect uses.

Nonylphenols and their ethoxylates may be used as antioxidants and plasticizers in plastic products. Nonylphenol is also used as an accelerator or cutting agent in the hardening components in some epoxy resins and it has been detected in plastic products used in the building industry, in food contact plastics and baby toys, too. According to the Swedish products register 0.14 tonnes of nonylphenol ethoxylates and 0.41 tonnes of nonylphenol were used in floor covering materials, 0.33 tonnes of nonylphenol were used in joint-less floors and 0.01 tonnes were used as curing agents in plastics in 2006. Norway registered 0.3 tonnes of nonylphenol ethoxylates and 0.8 tonnes of nonylphenol in joint-less floors and Finland reported the use of 0.1 tonnes of nonylphenol as fillers. It is assumed that 60% of all plastic in the building industry contains nonylphenols or their ethoxylates. Due to very slow release of these substances from plastic material into the environment and the very low amounts added to the material there is only a very small input to the environment (further details provided in OECD, 2011). Nevertheless these uses might be relevant sources for emissions into the environment because of the wide dispersiveness.

Several uses of nonylphenols and their ethoxylates have strongly decreased and are less/not relevant today. However due to long-time uses of such products nonylphenol or nonylphenol ethoxylates might still be released. This includes the use as concrete modifiers, where release still occurs in spite of industry agreements in the EU to reduce the use of nonylphenol ethoxylates in construction

and building products such as paints, concrete and plastics. The estimated emission of nonylphenol ethoxylates from concrete to urban storm water is 0.2 mg/m^2 per year (calculation from OECD, 2011). Also the historical use of nonylphenols and their ethoxylates in paints and adhesives might contribute to today's emissions.

Emission from products made of phenol-formaldehyde-resins might be an additional source of 4-nonylphenol. Although most of the 4-nonylphenol is chemically bound and cannot be released even on subsequent chemical or biological degradation the resins may contain a small proportion (~3-4%) of unreacted 4-nonylphenol. This may be the case in a wide range of products made of phenol-formaldehyde-resins, like rubber products, printing inks, paints, adhesives and others. This assumption is supported by the Risk Reduction Strategy on 4-tert-octylphenol which is similar in its chemical structure and uses (please see (DEFRA, 2008) for further details).

Additional information on uses from product registers

In the product register of Switzerland[3] several products are listed that contain nonylphenol (CAS 25154-52-3), its branched isomer (CAS 84852-15-3) and ethoxylates (CAS 9016-45-9, 68412-54-4, 127087-87-0). Most products with nonylphenol are registered as hardening agents and activators (48 of 145), as adhesive, putty and filler (20 of 145) and as paints and lacquers (18 of 145). Products containing nonylphenol ethoxylates are mainly registered as paints and lacquers (101 of 341) and as laundry detergents and cleaning agents (56 of 341). Most products contain nonylphenol or the releated ethoxylates in concentrations below 10%. However, for some applications higher amounts are used, e.g. up to 40% nonylphenol for paints and lacquers, up to 60% nonylphenol for adhesives and sealants and up to 30% nonylphenol ethoxylates in laundry detergents and cleaning agents. In hardening agents and activators most products contain up to 40%, but one product contains between 80 and 90%.

The SPIN-database[4] shows for nonylphenol with CAS-number 25154-52-3 the following entries

	number of preparations				total amount (tonnes)				
Year	S	DK	N	FIN	S	DK	N	FIN	Total
1999	116	-	-	-	19.0	-	-	-	
2000	121	298	54	47	35.0	128.1	9.9	0	173.0
2001	116	312	54	46	12.0	113.0	8.5	12.7	146.2
2002	131	350	43	55	46.0	117.9	5.0	14.6	183.5
2003	139	299	37	50	26.0	52.0	18.6	7.5	104.1
2004	131	242	39	42	10.0	33.6	21.6	4.9	70.1
2005	113	284	28	45	10.0	471.0	23.9	9.7	514.6
2006	116	267	42	53	10.0	36.0	3.6	4.6	54.2
2007	130	265	38	55	14.0	28.2	6.8	5.9	54.9
2008	131	283	43	57	8.0	66.4	3.3	9.6	87.3
2009	141	273	32	63	7.0	26.4	2.3	13.1	48.8
2010	152	242	39	63	8.0	30.9	2.5	16.4	57.8

[3] Information received from Swiss Federal Office of Public Health by personal communication; dated September 2011

[4] SPIN – Substances in Preparations in Nordic Countries (http://www.spin2000.net/); accessed 15 July 2012

129 of all preparations listed in the reporting year 2010 refer to the subcategory “paints, lacquers, varnishes” and contribute to 40 percent of the total amount. Other relevant preparations are hardeners (7.0 tonnes) and filling agents (5.0 tonnes).

The SPIN-database[5] showed for “4-nonylphenol, branched” with CAS-number 84852-15-3 the following entries

	number of preparations				total amount (tonnes)				
Year	S	DK	N	FIN	S	DK	N	FIN	Total
1999	9	-	-	-	21.0	-	-	-	21.0
2000	8	11	4	5	1.0	3.1	0.0	0.0	4.1
2001	10	14	5	12	0.0	3.3	0.1	0.5	3.9
2002	13	18	7	12	1.0	3.5	0.3	1.3	6.1
2003	16	15	10	12	3.0	3.7	0.2	1.2	8.1
2004	15	14	6	4	4.0	2.2	0.3	0.4	6.9
2005	30	24	7	7	9.0	2.7	1.0	0.2	12.9
2006	36	25	7	7	133.0	2.9	0.9	0.2	137.0
2007	26	19	7	8	12.0	1.2	1.0	0.2	14.4
2008	26	21	8	-	6.0	1.3	1.4	-	8.7
2009	31	19	6	4	6.0	5.4	0.5	0.8	12.7
2010	35	21	-	-	5.0	5.4	-	-	10.4

11 of all preparations listed in the reporting year 2010 refer to the subcategory “paints, lacquers, varnishes” and contribute to 3.0 tonnes of the total amount. The other relevant use of the substance seems to be as constituents of stabilizers (2.0 tonnes). But there is a lack on data from Finland and Norway because the information for 2010 is not publically available due to flagging it as confidential.

EXPOSURE

Releases to the environment

According to the registrants CSRs the following uses are the most important emission sources[6] of 4-nonylphenol on local scale:

- Manufacture of 4-nonylphenol,
- use as monomer in the production of polymers,
- use as intermediate in the production of ethoxylates and phenolic oximes,
- manufacture of coatings and inks (powder product) and
- industrial end use of coatings and inks in spray coating or coil coating.

Emissions of 4-nonylphenol ethoxylates also might be a relevant source of 4-nonylphenol for the environment because of degradation processes. In the registrants CSRs several uses of 4-

[5] SPIN – Substances in Preparations in Nordic Countries (http://www.spin2000.net/); accessed 15 July 2012

[6] Information retrieved by comparing the PECs of the single Exposure Scenarios from the CSR

nonylphenol ethoxylates are considered as the most relevant emission sources of 4-nonylphenol on local scale:

- The use of ethoxylates in the formulation of paints,
- The industrial end use of paints containing NPEO in spray coating and
- the use of ethoxylates in emulsion polymerisation.

According to the RAR for nonylphenol (European Commission, 2002) on the regional scale the major releases of nonylphenol to the environment (here surface water) result from:

- Industrial and institutional cleaning (142 kg / day)
- Other applications (75.2 kg / day)
- Textile processing (46.6 kg /day)
- Leather processing (19.4 kg / day)
- production of nonylphenol ethoxylates (15.3 kg/day).

On continental scale relevant life cycle steps with high contribution to emission of nonylphenol to the environment are:

- production of nonylphenol ethoxylates (152 kg/day)
- use of ethoxylates for industrial and institutional cleaning (1276 kg / day)
- use of ethoxylates for other applications (677 kg / day)
- use of ethoxylates for textile processing (419 kg /day)
- use of ethoxylates for leather processing (174 kg / day).

It has to be kept in mind, that nearly all emissions from the uses named above in the RAR have decreased in the last years because of the restriction of several uses. Nevertheless the contribution of washing textiles containing 4-nonylphenol or its ethoxylates is still unclear but when having in mind the measured concentrations in textiles this might be a relevant pathway for emissions to the environment.

Summary

Emissions of nonylphenol ethoxylates might be a relevant source of 4-nonylphenol for the environment. In the registrants CSRs several uses of nonylphenol ethoxylates are considered as sources of 4-nonylphenol. The use of ethoxylates in the formulation of paints, industrial spray coating, end use of paints containing those ethoxylates and the use of ethoxylates in emulsion polymerisation are among the most relevant emission sources of 4-nonylphenol on a local scale. According to the Resource Compendium of PRTR Release estimation techniques (Part 1 of (OECD, 2011)) on releases of nonylphenol and its ethoxylates from the use phase of end-products in the Nordic countries, the major releases originate from textiles and cleaning agents. Also paints, adhesives, pesticides and veterinary medical products, concrete, metal working products and plastics are sources of nonylphenols or their ethoxylates (OECD, 2011; Part 1). There might be also releases from leather, paper, aircraft deicer, photo chemicals and electronic components. While textiles and cleaning agents are considered as the most important sources of nonylphenols or their ethoxylates in urban areas, pesticides are a significant source in rural areas. The main release pathway is to wastewater. Emissions to air are of minor importance (OECD, 2011; Part 1).

Measured releases

Data provided in the European Pollutant Release and Transfer Register (PRTR) according to Regulation EC 166/2006 for Germany and EU27 indicate that wide dispersive uses resulting in emission to wastewater might be important:

Table 35: Total German emission rates into aquatic compartment (German PRTR[7], Umweltbundesamt)

Reporting year	Number of installations	Total amount of nonylphenol and ethoxylates emitted (kg/a)
2007	13	1044.93
2008	16	870.04
2009	13	634.76
2010	11	695.19

Only in 4 of 11 cases (reporting year 2010) the origin of the emitted nonylphenol and its ethoxylates are industrial wastewater treatment plants but their contribution to the total emission in Germany is 555.7 kg.

Table 36: Total European emission rates into aquatic compartment (EU-PRTR[8], European Environment Agency, 2012)

Reporting year	Number of installations	Total amount of nonylphenol and ethoxylates emitted (kg/a)
2007	196	115000
2008	262	114000
2009	203	30800
2010	264	63600

For the year 2010 in 221 of 264 cases the origin of the emitted nonylphenols are urban wastewater treatment plants which contribute to the emission of 60700 kg nonylphenol and its ethoxylates.

It should be noted, that the values above only allow a rough estimation on total releases of nonylphenol or its ethoxylates to the environment, because there might be waste water treatment

[7] http://www.prtr.bund.de/; accessed 2 May 2012

[8] http://prtr.ec.europa.eu/PollutantReleases.aspx; accessed 2 May 2012

plants which are below the reporting threshold of Regulation EC 166/2006 which is set to be above 1 kg per year. Also it is unclear if the affected wastewater treatment plants receive wastewater from industrial sites in a relevant proportion.

Nonylphenol (CAS 104-40-5) was detected in several monitoring programs since 2006[9] in 7 out of 7 samples from Swiss municipal wastewater treatment plants with an average concentration of 267 ng/L (Kase et al., 2011). The 90% percentile was 353 ng/L, respectively.

Summary

Even with uses and emissions decreased since the restriction on uses of 4-nonylphenols and their ethoxylates coming into force, the amounts emitted to the environment are still significant and occur all over Europe. The majority of emission sources appear to be urban wastewater treatment plants, but it is unclear if the emissions are related to wastewater emitted from industrial sites into the public sewage system or emissions from wide dispersive application of products (e.g. paints) or washing of (imported) textiles where 4-nonylphenols and their ethoxylates remain as contaminants from the production process.

Measured concentrations in the environment

Nonylphenol (CAS 104-40-5) was detected in several monitoring programs since 2006[8] in 15 out of 25 swiss surface water samples with an average concentration of 441 ng/L (Kase et al., 2011). The 90% percentile was 1100 ng/L.

Although the use of nonylphenol is regulated since 2005 it has been detected in freshwaters (up to 270 ng/L), marine waters (up to 12 ng/L) and freshwater sediments (up to 120 µg/kg dw mean) within the EU in several studies conducted in 2005 or later ((Boitsov et al., 2007), (Loos et al., 2007), (Vigano et al., 2006), (SWECO VIAK, 2007)).

Monitoring data were collected between 2007 and 2009 in the context of the water framework directive (DGEnv, 2009). In whole water samples mean concentrations of 270 ng/L (maximum 28800 ng/L, median 30 ng/L) were detected and in the water dissolved fraction mean concentrations of 40 ng/L (maximum 460 ng/L, median 30 ng/L) were observed. Concentrations in the sediment (fraction below 2 mm) were 1469.28 µg/kg dw on average (maximum 7500 µg/kg dw, median 500 µg/kg dw).

Numerous studies in several EU countries found about 0.1 to 4.1 µg/L nonylphenol in several effluent water samples of waste water treatment plants and 0.1 to 40 µg/g dw nonylphenol were reported from sludges (Aquateam – Norsk vannteknologisk senter A/S 2006[10], (Baugros et al., 2008), (Butwell et al., 2008), (Clara et al., 2007), (Hansen and Lassen, 2008)). Much higher concentrations of 9.6 to 1385 µg/g dw were detected in sludges of two waste water treatment plants in southern Spain (Santos et al., 2007).

[9] For nonylphenol only values since 2006 have been considered because of the ban of certain products with nonylphenol from 01.08.2006 (regulation ChemrrV of Switzerland)

[10] Source: http://test.aquateam.no/index.php/en/; (accessed: 17. January 2012)

At the moment these measured data can not be associated with information on the presence of industrial installations or specific uses of 4-nonylphenols or their ethoxylates.

Summary of information on uses, emissions and exposure

Information on uses, emission and exposure show that, besides emission from the washing of textiles, other uses contribute to the emission of 4-nonylphenols and their ethoxylates. Most relevant additional sources are considered to be the manufacture of 4-nonylphenols and their ethoxylates and manufacture of resins as well as the manufacture and formulation of paints and end use of these paints. But as 4-nonylphenols, ethoxylates and resins are used in a variety of applications, other sources may be important, too. According to the COHIBA report (COHIBA, 2012) restricted uses such as use for industrial and private cleaning may still significantly contribute. Based on the available information, emission is mainly via waste water treatment plants with municipal treatment plants being most important. Articles other than textiles and leather articles, containing 4-nonylphenols and their ethoxylates (e.g. painted or printed articles, plastics) may also contribute to the overall emission.

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