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Substances of very high concern under REACH – an evaluation of uncertainties in the environmental risk assessment of endocrine active substances

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Substances of very high concern under REACH – an evaluation of uncertainties in the environmental risk assessment of endocrine active substances

by

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Abstract

The aim of the present project was to identify the most relevant factors increasing the uncertainty of the environmental risk assessment (ERA) of endocrine active substances as compared to baseline toxicants. The evaluation was supported by data on endocrine effects of six model substances on fish and aquatic invertebrates. Two key factors were identified: (1) The current evaluation of endocrine effects only covers effects on the estrogen / androgen and thyroid axis, while other endocrine modes of action and, especially, effects on invertebrates are insufficiently covered. (2) At present, it is difficult to assess whether the results of tests with few standard test species are protective for all wildlife species. For fish, effect concentrations in species with similar metabolic capacities are often in the same order of magnitude, but larger differences are observed between species that differ in their metabolic capacities. For invertebrates, cross-species extrapolation is far more complex. This is due to the much higher diversity and heterogeneity of invertebrates and the often fragmentary knowledge on endocrine effects and the underlying processes. The uncertainty of the ERA of endocrine active substances is also increased by mixture effects. It may be increased if worst case exposure conditions coincide with sensitive developmental windows. Further factors (e.g. the irreversibility of effects, effects on the reproductive behaviour, and effects with uncertain population relevance and low-dose effects) and the specificity of the identified factors for endocrine active substances are discussed.

Kurzbeschreibung

Ziel des vorliegenden Projekts war die Identifizierung der wesentlichen Faktoren, die die Unsicherheit der Umweltrisikoabschätzung für endokrin aktive Substanzen im Vergleich zu Substanzen ohne spezifische Wirkmechanismen erhöhen. Die Auswertung wurde durch Daten zu endokrinen Effekten von 6 Beispielsubstanzen auf Fische und aquatische Invertebraten unterstützt. Zwei Hauptfaktoren wurden identifiziert: (1) Die zurzeit durchgeführte Bewertung endokriner Effekte deckt nur Effekte auf die östrogene / androgene und thyreoidale Achse ab, während andere endokrine Wirkungsweisen und vor allem endokrine Effekte auf Invertebraten nicht ausreichend abgedeckt werden. (2) Basierend auf dem aktuellen Wissensstand ist es schwer, zu beurteilen, ob die Ergebnisse von Tests mit wenigen Standardtestarten für alle Arten in der Umwelt protektiv sind. Vergleicht man Fischarten mit ähnlicher metabolischer Kapazität, liegen die Effektkonzentrationen oft in derselben Größenordnung. Vergleicht man jedoch Arten mit unterschiedlicher metabolischer Kapazität, treten größere Unterschiede auf. Für Invertebraten ist die Interspeziesextrapolation – bedingt durch die sehr viel höhere Diversität und Heterogenität und das oft fragmentarische Wissen über endokrine Effekte und zugrundeliegende Prozesse – deutlich schwieriger. Die Unsicherheit der Umweltrisiko-abschätzung für endokrin aktive Substanzen wird auch durch Mischungseffekte erhöht. Sie kann außerdem erhöht sein, wenn sensitive Entwicklungsfenster und worst case-Expositionsbedingungen zusammentreffen. Weitere Faktoren (Irreversibilität von Effekten, Effekte auf das Reproduktionsverhalten, Effekte mit unklarer Populationsrelevanz, low dose-Effekte u.a.) und die Spezifität der identifizierten Faktoren für endokrin aktive Substanzen werden diskutiert.

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List of Abbreviations

20E	20-Hydroxyecdysone
4-tert-OP	4-tert-octylphenol
ADME	Absorption, distribution, metabolism and excretion
AR	Androgen receptor
BCF	Bioconcentration factor
BPA	Bisphenol A
CF	Conceptual framework
CMR	Carcinogenic, mutagenic or toxic for reproduction
conc.	Concentrations(s)
CYP / cyp	Cytochrome P450 protein / gene
d	Day(s)
DMI	Demethylation inhibiting
DMSO	Dimethyl sulfoxide
DNEL	Derived no effect level
dpf	Days post fertilisation
dph	Days post hatch
dw	Dry weight
ED	Endocrine disruption / endocrine disrupting
EDC	Endocrine disruptive compound
EE_2	17α-Ethinylestradiol
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
ERA	Environmental risk assessment
F_0	Parental generation
F_1	Offspring (first filial generation)
GtH	Gonadotropin
GD	Guidance document
GLP	Good laboratory practice
GSI	Gonadosomatic index
HSI	Hepatosomatic index
LOD	Limit of detection
LOEC	Lowest observed effect concentration
LSC	Liquid scintillation counting

LSI	Liver-somatic index
^{m}	Measured concentration
mo	Month(s)
^{n}	Nominal concentration
n.d.	Not detected / below limit of detection
n.i.	Not indicated
NOEC	No observed effect concentration
NP	Nonylphenol
PBT	Persistent, bioaccumulative and toxic
PEC	Predicted environmental concentration
PNEC	Predicted no effect concentration
PTTH	Prothoracicotrophic hormone
QSAR	Quantitative structure-activity relationships
REACH	Registration, evaluation, authorisation of chemicals
RIA	Radioimmunoassay
sed.	Sediment
SIDS	Screening information dataset
SVHC	Substance of very high concern
TBT	Tributyltin
TG	Test guideline
TPT	Triphenyltin
TBT-Cl	Triphenyltin chloride
VDSI	Vas deferens sequence index (index for stage of development of imposex)
vPvB	Very persistent and very bioaccumulative
wk	Week(s)

Summary

Introduction:

According to Art 138(7) “the Commission shall carry out a review to assess whether or not, taking into account latest developments in scientific knowledge, to extend the scope of Article 60(3) to substances identified under Article 57 (f) as having endocrine disrupting properties.“

Art 60 (3) describes that for certain substances authorization “may only be granted if it is shown that socio-economic benefits outweigh the risk to human health or the environment arising from the use of the substance and if there are no suitable alternative substances or technologies.” Currently Art 60 (3) is restricted to substances of very high concern according to Article 57 (a), (b), (c) or (f) for which it is not possible to determine a threshold, and to substances meeting the criteria in Art 57 (d) or (e). Thus it focuses on substances for which, with regard to human health no threshold can be derived (CMR substances) or substances for which, with regard to the environment , it is not possible to derive a predicted no effect concentration (PNEC) with sufficient certainty (PBT or vPvB substances). In this context it is important to understand, that with regard to PBT or vPvB substances, inclusion into Art 60 (3) is not based on the fact that there is no threshold for these substances (i.e. that a single molecule may already cause an effect). PBT and vPvB substances are included because, due to the combination of different intrinsic properties, it is not possible to derive a „safe" concentration in the environment with sufficient reliability using traditional quantitative risk assessment methodologies (EC, 2007, ECHA 2008). Thus although a threshold may exist, it is currently not possible to determine where it may be.

In conclusion, with regard to Art 138 (7) and with regard to the environmental concern, the question arises whether or not it is possible to derive a „safe" concentration in the environment for Endocrine Disruptors with sufficient reliability using traditional quantitative risk assessment methodologies.

Results of the project are summarized followed by an UBA conclusion with regard to Art 138 (7).

Summary of project results:

Within the project, factors that may lead to an increased uncertainty of the assessment of environmental effects were identified, mainly on the basis of review publications and documents of international organisations (e.g. OECD). Specific examples for the identified factors were included. For these examples, the original literature was reviewed. The relevance of the identified factors, which might lead to an increased uncertainty of the environmental risk assessment (ERA) for EDCs, was evaluated.

The following factors were identified and further analysed:

- Availability and implementation of tests for assessing endocrine effects
- Possibility to extrapolate results for test species to other species in the environment
- Influence of sensitive time windows or delayed effects
- Influence of irreversibility of effects
- Importance of effects that might not be covered by traditional risk assessment methods (behavioural effects, other effect with uncertain relevance for the population, transgenerational / epigenic effects, immunotoxicological effects)
- Influence of potential unusual dose-concentration relationships (low dose effects, non-monotonic dose response curves)
- Mixture effects and exposure assessment

Results of the project are summarized in Table 1.

According to the project the following two key factors contribute most to an increased uncertainty of the environmental risk assessment of endocrine active substances as compared to baseline toxicants:

(1) the limited availability of test methods and

(2) the limited knowledge on the feasibility of cross-species extrapolation.

Both factors have highest relevance for aquatic invertebrates.

With regard to (1) the conclusion drawn was that for effects on the estrogen/androgen and thyroid axis of aquatic vertebrates the uncertainty is acceptable given that these effects are covered reasonably well by a tiered testing strategy. However, for other endocrine modes of action (e.g. effects on the corticosteroid system) in aquatic vertebrates resulting uncertainty of the environmental risk assessment is higher. Current test methods for fish are restricted to teleost fish, the most important fish taxa and thus it is not possible to assess whether an assessment based on teleosts is protective for these taxonomic groups. With regard to aquatic invertebrates only a few tests are available which do not cover endocrine specific endpoints. Further research is needed to systematically evaluate if test results obtained with these species are sufficiently protective for other invertebrate groups and consequently uncertainties for aquatic invertebrates are high.

With regard to (2) the conclusion is that, while for fish cross-species extrapolation is feasible with some restrictions, this does not hold true for invertebrates. For invertebrates, extrapolation between species is far more complex than for fish. This is due to the much higher diversity and heterogeneity of invertebrates and to the often fragmentary knowledge on endocrine effects and the underlying processes in invertebrate species. Consequently uncertainty for invertebrates is high. For fish some aspects need further consideration such as the finding that fish species exhibiting a high metabolic capacity (which are usually tested in long-term tests) may not be protective for species with slower metabolism such as rainbow trouts. In addition, potential risks to seasonally spawning fish species (e.g. brown trout) may be underestimated when the PNEC is derived based on effects on standard test species.

The following two factors also increase the uncertainty of the ERA of EDCs: Given that aquatic organisms are very likely to be exposed to complex mixtures of substances with endocrine activity, potential additive effects of EDCs are relevant. Worst case exposure situations coinciding with sensitive periods in the development of seasonally reproducing organisms may be an additional relevant factor.

Table 1: Relevance and specificity of the factors that may contribute to an increased the uncertainty of the ERA for substances with an endocrine mode of action.

Factor that may contribute to increased uncertainty		Relevance for environmental risk assessment	Specificity to EDCs	Feasibility to address this factor and to reduce the uncertainty that is causes
Limited availability and implementation of test methods	Fish	Low [1] / Medium [2]	Yes	High (but partly longer-term) [2]: further test development and standardisation / validation, implementation of tests
	Invertebrates	High	Yes	High (but partly longer-term) [4]: further test development, implementation of tests
Limited knowledge on feasibility of extrapolation between species	Fish	Low – medium	No	Medium – high (but longer-term): systematic evaluation, further studies
	Invertebrates	High	No	Medium (longer-term): systematic evaluation, further studies
Sensitive time windows for exposure, delayed effects	Fish	Low [1]	Yes	Not required: tiered testing framework with appropriate tests available [1]
	Invertebrates	Medium	Yes	Life-cycle testing in invertebrates
Irreversibility of effects		Low	No	Not required
Behavioural effects	Fish reproductive behaviour	Low [1]	Yes	Not required
	Other behavioural effects	(?) [5]	No	(Further investigations required)
Low-dose effects with non-monotonic dose-response relationship		Low	Yes	Not required
Effects with uncertain population relevance (secondary sexual characteristics in fish)		Low	Yes	High: triggering of further testing
Transgenerational / epigenetic effects		(?) [5]	No	(Further investigations required)
'Atypical' effects: immunotoxicity		(?) [5]	No	(Further investigations required)
Effects on the gene pool		(?) [5]	No	(Further investigations required)
Mixture effects		Medium – high	No	Medium to high (but partly longer-term)
Exposure assessment		Low – medium	No	High: worst case exposure estimates

(1) For estrogen receptor mediated effects, androgen receptor mediated effects and interference with steroidogenesis. (2) For other endocrine mechanisms of action. (3) Due to the lack of diagnostic endpoints in invertebrates. (4) For life-cycle tests without or with few specific diagnostic endpoints. (5) Further research is required to evaluate the relevance of these factors

As summarized in Table 1 some of the uncertainties are specific to Endocrine Disruptors. Other uncertainties are considered not to be specific for Endocrine Disruptors. For example uncertainties in extrapolating from a few test species to other wildlife species apply to environmental risk assessment in general. However, several publications show that for substances with specific modes of action such as Endocrine Disruptors uncertainties are higher compared to substances with no specific mode of actions (baseline toxicants). This is due to a higher toxicity and higher variation in toxicity between species. Thus the project concludes that the uncertainties associated with the extrapolation from a few test species to other wildlife species are very likely to be higher for Endocrine Disruptors than for substances with a narcotic mode of action but may be high for other specific modes of action, too. Similar holds true to endpoints not covered in traditional risk assessment methods such as behavioral effects.

For most of the uncertainties discussed, it is in principle feasible to reduce them. However, this may require further test development, systematic evaluations, further investigations and additional tests to be included in the traditional risk assessment. In most cases this implies that uncertainties can be reduced in the long-term only.

In conclusion, the study is suggesting that with respect to wildlife assessing a safe concentration for the environment is connected with higher uncertainties than for other substances and that it may require long-term actions to reduce these uncertainties.

UBA conclusion with regard to Art 138 (7) and the environment

With regard to the environment, the assessment of endocrine disruptors is influenced by the fact that the endocrine system, especially the hypothalymic-pituitary-gonadal axis which involves sex- steroids such as estradiol and testosterone is widely conserved in vertebrates. Several reviews show that these vertebrate type sex-steroids are also involved in reproduction in a range of invertebrate taxa including jellyfish, crustaceans, mollusks and echinodermata like sea urchins (see this project report and Kortenkamp et al, 2011 for details). Thus it is very likely that once released to the environment, such substances will cause effects in a variety of species including very different taxonomic groups.

Based on the analysis by this project report it seems to be possible to derive a "safe" concentration in the environment with sufficient reliability for sex steroids in gonochorist, frequently spawning teleost fishes with high metabolic acitivity using current test methods available. However, the analysis also indicates that this might not be true for all teleost fish species and that especially for seasonal spawners with low metabolic activity effects might be underestimated. With regard to invertebrates the analysis clearly shows that it is not possible to derive a „safe" concentration as it is currently unknown whether or not results obtained with the test methods available or under development are sufficient protective for other invertebrate groups. Results observed for some groups such as sea urchins indicate that they may not be protective enough. Although similar uncertainties might hold true for substances with other specific modes of action, they are higher than for substances with non-specific narcotic modes of action which account for at least 60% of all chemicals under the scope of REACH.

As indicated by this project report it might be possible to overcome these shortcomings on the long-term. However, this would require intensive research and probably would increase the testing requirements significantly.

Based on this analysis UBA draws the conclusion that for Endocrine Disruptors identified as SVHC according to Art 57 (f) due to their concern for the environment, it is currently not possible to predict a no effect concentration for the environment with sufficient certainty, and, hence, no risk quotient should be derived with regard to the environment. Thus, similar to PBT and vPvB substances, Endocrine Disruptors identified as SVHC according to Art 57 (f) due to an environmental concern should only be authorized, if it is shown that socio-economic benefits outweigh the risk arising from the use of the substance and if there are no suitable alternative substances or technologies. In conclusion the scope of Art 60 (3) should be extended to substances identified under Article 57 (f) as having endocrine disrupting properties causing serious effects for the environment. This conclusion is based on the following considerations:

- Due to the conservation of the endocrine system in various taxonomic groups during evolution it is very likely that once released to the environment, Endocrine Disruptors may cause adverse effects in a variety of species including very different taxa.
- Due to the differences in the endocrine response and the high variety of taxa involved, it is currently impossible to identify which species are sufficiently representatives for wildlife with regard to endocrine effects.
- Currently available test methods are very limited and especially with regard to invertebrates do not cover sensitive taxa and life stages.

Although it might be possible to overcome these shortcomings in future this is considered to be a long term activity and, based on the already available indications of harmful effects in the environment, it seems not to be adequate to await this progress.

References:

Kortenkamp A., et al, (2011), "State of The Art Assessment of Endocrine Disrupters, Final Report", Project Contract Number 070307/2009/550687/SER/D3, 23.12.2011. Page 27 http://ec.europa.eu/environment/endocrine/documents/4_SOTA%20EDC%20Final%20Report%20V3%206%20Feb%2012.pdf

1 Introduction

1.1 Background

Since the early 1990s, there has been growing concern about potential endocrine disruptive compounds (EDCs) in the environment (Stahl et al. 1999, Matthiessen 2000, IPCS 2002, Hotchkiss et al. 2008). Endocrine disrupters have been defined as substances that interfere with the functions of natural hormones and, consequently, cause adverse health effects in intact organisms or their progeny (Kavlock et al. 1996, EC 1997). Hormones have a critical organisational role during development, i.e. they are key factors for the progression and the timing of development and reproduction. Hormones are, for example, involved in the development of the central nervous system, skeletal growth and sexual differentiation (Ojeda & Griffin 1996).

Endocrine disrupters may affect the endocrine system by interacting with hormone receptors and either mimicking hormones or blocking their effects. They can also interfere with hormone synthesis (e.g. by interacting with enzymes), transport (e.g. by binding to transport proteins), catabolism or excretion (Matthiessen & Gibbs 1998, Van Der Kraak et al. 1998, LeBlanc et al. 1999, Lafont 2000, IPCS 2002, Schulte-Oehlmann et al. 2006a, ECHA 2007). Endocrine disruption (ED) is a mode of action[1] that may lead to adverse effects, e.g. effects on development and reproduction as well as neurotoxic, immunologic and carcinogenic responses (Matthiessen & Johnson 2007). ED is related to a variety of different mechanisms of toxicity. So far, most attention has focused on potential endocrine disruptive effects on development and reproduction in humans and other vertebrates, much less on invertebrates and on other endocrine processes that may be affected by endocrine disruptors (see e.g. Oehlmann & Schulte-Oehlmann 2003, Nichols et al. 2011). With regard to the underlying endocrine mechanisms of action (see footnote 1), estrogen and androgen receptor mediated effects and interference with the thyroid system have been most intensively studied (e.g. Schäfers 2003, Danish Ministry of the Environment 2011). Yet, other hormone systems may also be affected.

The extent to which adverse ecological effects are due to endocrine disruption is still unknown. So far, there have been relatively few clear cases of population declines that have been caused by endocrine disruption (Depledge & Billinghurst 1999, Matthiessen 2003), such as the effects of tributyltin on prosobranch molluscs (Matthiessen & Gibbs 1998). In fish, widespread endocrine effects, such as vitellogenin induction in male fish and intersex, have been observed in surface waters affected by sewage effluents (e.g. Purdom et al. 1994, Folmar et al. 1996, Harries et al. 1996, 1997, Larsson et al. 1999). However, it is not yet known if these effects result in adverse effects on fish populations (Campbell & Hutchinson 1998, Kidd et al. 2007).

[1] In analogy to the pharmacological definitions, the term 'mechanism of action' is used for the interaction(s) of a substance with specific target structures (e.g. enzymes or receptors). The term 'mode of action' is a less detailed description of the type of effect resulting from the mechanism(s) of action.

The essential elements in the abovementioned definition of endocrine disrupters are (1) an endocrine mode of action, (2) adverse effects and (3) a causal link between both. These elements are also mentioned in the REACH guidance document R.7b (ECHA 2008a). The assessment of potential endocrine disruption requires a weight of evidence approach considering information on mechanistic and apical endpoints (ECHA 2008a, OECD 2011a).

In standard ecotoxicity test methods, effects on endpoints such as survival, growth and reproduction are considered as adverse, population relevant effects (Traas & van Leeuwen 2007). However, more subtle effects such as changes in behaviour or an increased susceptibility to diseases may also be relevant (Lyons 2003). Concern about endocrine disrupters is also related to issues such as low-dose effects as well as delayed and, partly, irreversible effects following exposure during sensitive developmental phases. Due to the variety of endocrine systems and reproductive strategies in wildlife the feasibility of extrapolating between species has been questioned. In view of these concerns, the suitability of current environmental risk assessment procedures for the endocrine disrupting compound has been critically addressed.

The principle of current environmental risk assessment (ERA) procedures is to compare the predicted environmental concentration (PEC) of a substance with the substance's potential to cause harm (van Leeuwen 2007, Traas & van Leeuwen 2007). Information on the nature and severity of effects on wildlife species is investigated using surrogate test species. A predicted no effect concentration (PNEC) is derived from the effect concentration for the most sensitive species using assessment factors (see section 2.2). The risk quotient, i.e. the PEC/PNEC ratio, indicates the degree of risk expected to be caused by the substance in the respective environmental compartment. It is assumed that at a risk quotient below 1 risk is acceptable (Calow 1998, Hester & Harrison 2006).

1.1.1 Endocrine disrupting substances as substances of very high concern

The identification of substances of very high concern (SVHC) shall ensure a high level of protection for humans and for the environment (EC 2007). The underlying rationale for the identification of substances of very high concern is the precautionary principle (Article 1 of the REACH Regulation; EC 2007). Within REACH, substances with endocrine disrupting properties can be classified as SVHC, if there is evidence of probable serious effects on the environment and if effects are considered as of equivalent concern. In this case, the substances may be subject to authorisation (ECHA 2007). In Article 57 of the REACH Regulation (EC 2007), the criteria for identification of SVHC that are subject to authorization are described. Articles 57(a) to (c) refer to substances that are carcinogenic, mutagenic or toxic for reproduction (CMR). Article 57(d) and (e) refer to persistent, bioaccumulative and toxic (PBT), and very persistent and very bioaccumulative (vPvB) substances. According to Article 57(f) "substances – such as those having endocrine disrupting properties or those having persistent, bioaccumulative and toxic properties or very persistent and very bioaccumulative properties, which do not fulfil the criteria of points (d) or (e) – for which there is scientific evidence of probable serious effects to human health or the environment which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e)" can also be classified as substances of very high concern on a case-by-case basis.

SVHC substances are authorized, if the risk for humans and the environment can be adequately controlled. Adequate control of the risk is not possible for

1. substances meeting the criteria of Article 57(a) to (c) or (f) (i.e. CMR substances or substances of equivalent level of concern) for which thresholds, below which risks for humans and the environment are unlikely (i.e. risk quotients), cannot be derived;
2. substances meeting the criteria of Article 57(d) or (e) (i.e. PBT and vPvB substances);
3. substances identified under Article 57(f) having PBT or vPvB properties.

In case that adequate control of the risk is not possible, a substance is only authorised, if the socio-economic benefits outweigh the risk to human health or the environment and if no suitable technically and economically feasible alternative is available (Article 60 of EC 2007; see also Hennig & Thiemann 2011).

Until 1 June 2013 it has to be reviewed based on latest scientific knowledge whether the scope of Article 60 should be extended to substances of equivalent level of concern with endocrine properties identified (Article 138 of EC 2007).

When identifying a substance with endocrine disrupting properties as substance of very high concern, it should be "confirmed that a traditional hazard assessment could not be used or would be insufficiently protective". In addition to the mechanism(s) of action, the following issues should be considered: (a) the severity of the effects, and (b) the uncertainties with regard to possible low-dose effects and "whether the assessment factors used account sufficiently for the uncertainties in these" (ECHA 2007).

The rationale for identifying a substance with endocrine activity as substance causing an equivalent level of concern as CMR, PBT or vPvB substances is linked to the uncertainty in the assessment (i.e. to the difficulty of deriving a 'safe' concentration). For this reason, uncertainties in the environmental assessment of endocrine active substances were evaluated in the present project.

1.2 Objective and outline of the project

The overall aim of the present project is to contribute to the evaluation if a 'safe' concentration (i.e. a predicted no effect concentration, PNEC) can be derived for substances with an endocrine mode of action with an acceptable level of uncertainty. It is assumed that – based on their endocrine disrupting properties – the substances to be considered have already been classified as substances of very high concern.

For such substances, factors that may lead to an increased uncertainty of the environmental risk assessment (or, more specifically, in the assessment of environmental effects) as compared to baseline toxicants were identified mainly on the basis of review publications and documents of international organisations (e.g. OECD; see section 2). Concrete examples for the identified factors were included as far as possible within the available time frame. For these examples, the original literature was reviewed. The relevance of the identified factors, which might lead to an increased uncertainty of the ERA for EDCs, was evaluated (section 3).

Since the "equivalent level of concern" refers to CMR, PBT and vPvB substances, the rationales underlying the hazard based assessment of PBT, vPvB and CMR substances and the intrinsic properties of these substances are described in section 4.

In section 5, the uncertainties associated with the environmental risk assessment of endocrine active compounds are discussed in relation to the uncertainties related to the ERA of baseline

toxicants and – as far as possible within the present project – to the uncertainties related to the ERA of substances with other specific modes of action. The concern caused by EDCs is compared to the concern caused by PBT, vPvB and CMR substances.

Data on effect concentrations were compiled for the following model substances with endocrine activity and used to support the evaluation: the synthetic estrogen 17α-ethinyl-estradiol, the xenoestrogens bisphenol A and 4-tert-octylphenol, the aromatase inhibitor prochloraz, as well as the organotins tributyltin and triphenyltin. These data were used to provide concrete examples for the factors that might lead to an increased uncertainty of the ERA for EDCs. Accordingly, these examples were integrated in section 2 where appropriate.

It should be noted that due to the complexity of endocrine disruption, this report cannot address all issues related to the environmental risk assessment of EDCs. Instead, it is focusing on the most important topics of discussion.

The majority of examples of endocrine disruptive effects in wildlife – including most of the clearest examples – have been reported for aquatic species (see e.g. Ankley & Giesy 1998, Tyler et al. 1998, IPCS 2002, Kortenkamp et al. 2012). Less than 7% of the studies addressing endocrine effects in invertebrates, which were carried out until May 2011, were performed with terrestrial invertebrates (Oehlmann et al. 2011). The focus on aquatic organisms is partly due to the fact that the aquatic environment receives a considerable amount of discharges of many substances (e.g. via sewage effluents). In addition, it can be assumed that in many cases exposure of aquatic organisms is most intense, as substances that are dissolved in the surrounding water are taken up across the integument and, especially, via respiratory organs. Thus, it can be expected that aquatic organisms are most likely to be affected by endocrine disruptors (McKim & Erickson 1991, Kime 1999, Schäfers 2003, 2010, Crain et al. 2007). For this reason, the present report focuses on the aquatic environment.

It can be assumed that all (eco-) toxicological effects will in some way result in effects on the endocrine system, e.g. as part of a stress response to the toxicant (e.g. Schäfers 2003). However, in the present project only primary effects on the endocrine system (i.e. direct interactions with the endocrine system) will be considered. The main focus is on estrogen and androgen receptor agonistic and antagonistic effects and on interference with steroid synthesis. However, where relevant other endocrine mechanisms of action are also considered.

1.3 Model substances with different endocrine modes of action

Effect data were compiled for six model substances: 17α-ethinylestradiol, bisphenol A, 4-tert-octylphenol, prochloraz, tributyltin and triphenyltin. For 17α-ethinylestradiol and prochloraz, we focussed on studies on effects in fish, for triphenyltin on studies with molluscs. For the other three substances, we compiled data on effects on invertebrates and fish. Generally, we mainly included studies in which test organisms were subjected to aqueous exposure.

The following documents were used as starting point for the literature search: the OECD detailed review papers No. 47, 55 and 121 (OECD 2004a, 2006a, 2010a), a cross-species mode of action information assessment for bisphenol A (U.S. EPA 2005), the EU risk assessment report on bisphenol A (EC 2008a), the 'Annex XV report – identification of SVHC' for 4-tert-octylphenol (BAuA 2011), the case studies on 4-tert-octylphenol and prochloraz in OECD guidance document No. 150 (OECD 2011a) and reports on tributyltin (WHO 1990, 1999, U.S. EPA 2003). Where required, an additional literature search was performed based on reviews, Web of

Science and ScienceDirect using keywords such as the chemical name and e.g. endocrine disruption, invertebrate, mollusc, fish, *Daphnia* and / or the relevant endpoint (e.g. gonado-somatic index, vitellogenin or reproduction). As far as possible, the relevant original publications were checked with regard to criteria such as validity and employed methodology (based on Klimisch et al. 1997, EC 2003 and 2011a). Both results from standard and non-standard tests were considered when compiling data for the model compounds.

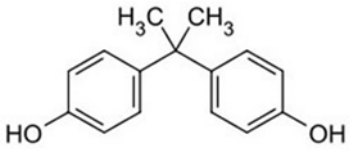
It should be noted that the data compilations (Tables 14 – 19 in the annex) are not exhaustive. In most cases, we have not included studies with less than two substance concentrations. In vitro studies were generally not considered; studies of gene expression were only included in a few cases. In addition, studies with mixtures were not considered.

1.3.1 Bisphenol A

Discovered in the 1930s by the biochemists E.C. Dodds and W. Lawson, bisphenol A (BPA, CAS No. 80-05-7; 4,4'-isopropylidenediphenol; $(CH_3)_2C(C_6H_4OH)_2$), was initially considered to be useful as synthetic estrogen for hormone replacement therapy, but was soon replaced by more potent substances (e.g. diethylstilbestrol, Dodds & Lawson 1938). In the 1960s, BPA was rediscovered for use in polycarbonate plastics, the field that subsequently became its primary commercial application. BPA is used in plastic production and epoxy resins (Staples et al. 1998, Fürhacker et al. 2000).

Because of its steric similarity to the steroid hormone 17ß-estradiol, BPA is able to elicit estrogenic effects and interfere with the action of endogenous endocrine pathways at different mechanistic levels. It is suspected to disrupt not only estrogen receptor pathways, but also progesterone receptor and thyroid receptor pathways (Moriyama et al. 2002, Scippo et al. 2004, Schreurs et al. 2005, Viswanath et al. 2008). Moreover, an anti-ecdysteroidal activity in daphnids has been discussed (Mu et al. 2005).

Fig. 1: Structural formula of bisphenol A.

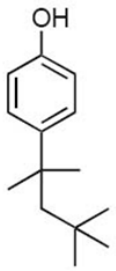


1.3.2 4-tert-Octylphenol

Octylphenols are a large number of isomeric compounds. The octyl group may be branched in a variety of ways or be a straight chain. It may be located at the 2-, 3- or 4-position of the benzene ring. Of these potential isomers, the phenolic surfactant 4-tert-octylphenol (4-tert-OP; CAS No. 140-66-9; 4-(1,1,3,3-tetramethylbutyl)phenol; $C_{14}H_{22}O$) is the commercially most important. 4-tert-Octylphenol is a high production volume chemical (Environment Agency 2005). The main areas of use are as intermediate in the production of phenol / formaldehyde resins and in the production of octylphenol ethoxylates, which are used in rubber, pesticides and paints. 4-tert-OP mainly reaches the aquatic environment in wastewaters from factories. In addition, it is a degradation product of alkylphenol ethoxylates. It also has been reported that octylphenols are present as an impurity in nonylphenol and that this may account to some extent for their detection in the environment (Environment Agency 2005).

4-tert-Octylphenol is a weak estrogen receptor agonist (Servos et al. 1999, Ackermann et al. 2002, OECD 2011a). In addition, it exhibits inhibitory effects on cytochrome P450 (CYP) activities and decreases testosterone hydroxylating CYP activities in rat liver. 4-tert-OP is considered to be a substance of equivalent level of concern due to its endocrine disrupting properties and consequent probable serious effects for the environment (BAuA 2011).

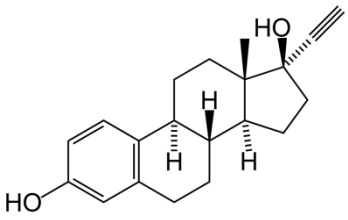
Fig. 2: Structural formula of 4-tert-octylphenol.



1.3.3 17α-Ethinylestradiol

17α-Ethinylestradiol (EE_2), the 17α-analogue of 17β-estradiol, is one of the first orally active semisynthetic steroidal estrogens. It was first synthesized in 1938 (Djerassi 2006). 17α-Ethinylestradiol (CAS No. 57-63-6, 19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol 3, $C_{20}H_{24}O_2$) is widely used as oral contraceptive. It mimics the effects of natural estrogens.

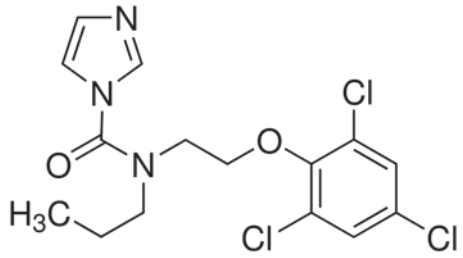
Fig. 3: Structural formula of 17α-ethinylestradiol.



1.3.4 Prochloraz

Prochloraz (CAS No. 67747-09-5, N-propyl-N-(2,4,6-trichlorophenoxy)ethyl-imidazole-1-carboxamide, $C_{15}H_{16}Cl_3N_3O_2$) is an imidazole fungicide widely used against a wide range of fungal diseases affecting field crops, fruit, flower production and vegetables.
Antifungal activity of imidazoles is based on inhibition of the enzyme sterol 14α-demethylase (Henry & Sisler 1984, Zarn et al. 2003). In fungi and yeast, this enzyme is involved in biosynthesis of ergosterol, an essential membrane component. Sterol 14α-demethylase is a cytochrome P450 enzyme, which does not only occur in fungi and yeast, but is found in many other species. In animals, it contributes to the biosynthesis of cholesterol that is a substrate for the production of other sterols, including sex steroids (Zarn et al. 2003). Prochloraz also affects other cytochrome P450 enzymes (Laignelet et al. 1989, Needham et al. 1992, Sturm et al. 2001), inhibits aromatase (Vinggaard et al. 2002), and has antagonistic effects on estrogen, androgen and aryl hydrocarbon receptors in vitro (Sturm et al. 2001, Andersen et al. 2002, Long et al. 2003, Vinggaard et al. 2006).

Fig. 4: Structural formula of prochloraz.



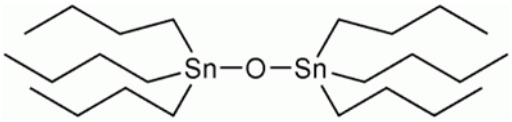
1.3.5 Tributyltin

Tributyltin compounds consist of tin covalently bound to three carbon atoms and a hetero-atom. It corresponds to the general formula $(CH_3CH_2CH_2CH_2)_3Sn\text{-}X$, where X is an anion or an anionic group covalently linked through the abovementioned hetero-atom (WHO 1990). Tributyltin compounds are paint additives used as molluscicides, antifoulants on boats and ships, wood preservatives, disinfectants, biocides for cooling systems, as well as in leather processing and textile mills. In most of the antifouling formulations, tributyltin is present as an organometallic copolymer. It is slowly released from the painted surface as the polymer is hydrolysed in seawater, providing protection against encrustations for as long as 4–5 years. In the environment, tributyltin compounds are expected to occur mainly as tributyltin hydroxide (CAS No. 80883-02-9), chloride (CAS No. 1461-22-9) and carbonate. Due to legal restrictions in the EU (a ban since 1989 for small boats and since 2003 for all uses) the use of tributyltin compounds in organotin antifouling paints has decreased in European coastal waters (see e.g. Rüdel et al. 2009). Since 2008, TBT it is banned internationally by the International Maritime Organisation.

TBT is a highly toxic compound with a complex toxicity profile (Sekizawa et al. 2001, OECD 2010a). It affects calcium homeostasis, inhibits oxidative phosphorylation and ion transport processes, and interacts with the cytochrome P450 dependent monooxygenase system (Fent 1998, Alzieu 2000). With regard to the endocrine effects of TBT, several mechanisms of action are under discussion. It is possible that TBT has different mechanisms of action in different species. Aromatase inhibition and interaction with the retinoid X receptor appear to be the

most likely mechanisms of action of TBT. Inhibition of cytochrome P450-dependent aromatase by TBT was shown to result in a dose- and time-dependent increase of testosterone levels, which in turn were correlated to imposex development (Spooner et al. 1991, Bettin et al. 1996). Interactions of TBT and other organotins with the retinoid X receptor might also contribute to the induction of imposex (Nishikawa et al. 2004, Horiguchi et al. 2007, Dmetrichuk et al. 2008). Other possible mechanisms of actions have also been discussed. A possible inhibition of testosterone excretion was mentioned by Ronis & Mason (1996), but not confirmed in other studies with environmentally relevant TBT concentrations. In addition, it was proposed that TBT might increase esterification of testosterone to a fatty acid ester and, thus, modulate the ratio of free testosterone to fatty acid bound testosterone (Gooding & LeBlanc 2001). Oberdörster & McClellan-Green (2000) suggested that effects of TBT on neuropeptides – together with effect on steroid hormones – might be involved in imposex induction.

Fig. 5: Structural formula of tributyltin-oxide.

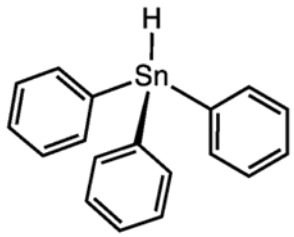


1.3.6 Triphenyltin

Triphenyltin (TPT) compounds are organotins with the general formula $(C_6H_5)_3Sn\text{-}X$ where X is an anion or anionic group hydride, hydroxide, chloride or acetate. Since the 1960s, TPT compounds are used as broad-spectrum agricultural fungicide (Keijzer & Loch 1995). Furthermore, TPT compounds have often been used as molluscicides in antifouling products, often in combination with TBT (Nakanishi 2008).

As for TBT, the mechanism of action of TPT might be due to interaction with the retinoid X receptor. This hypothesis is discussed especially with regard to invertebrates. Moreover, TPT might be an agonist of the peroxisome proliferator-activated receptor (PPAR) γ in mammals (Nakanishi 2008, OECD 2010a).

Fig.6: Structural formula of triphenyltin-hydride.



2 Factors that may increase the uncertainty of the ERA for substances with endocrine activity

2.1 Availability and implementation of tests for assessing endocrine effects

The type of effects observed and the effect concentrations derived for potential endocrine disruptive compounds depend on the availability of tests for the evaluation of possible effects and on the implementation of these tests in the respective environmental risk assessment procedures. The nature and the extent of effects that are detected is, for example, related to the selected test organism, the life stage that is exposed, test duration, test endpoints and, possibly, test conditions (see also section 2.2). The most sensitive effect will be missed when no appropriate test is available (Oehlmann & Schulte-Oehlmann 2003, Santillo & Johnston 2006).

In view of this fact, a brief overview of the tests for potential EDCs that are considered in the OECD Conceptual Framework is given in Table 1. More specific issues regarding the choice of the test organism and the test endpoints as well as the timing of exposure and test duration are discussed in sections 2.2 – 2.3.

The 'OECD guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption' (OECD 2011a) and the OECD Conceptual Framework (Appendix of OECD 2011a) cover the following endocrine modes of action:

a) estrogen receptor mediated effects;

b) androgen receptor mediated effects;

c) thyroid hormone mediated effects;

d) interference with steroidogenesis.

Relevant endpoints of ecotoxicity tests for endocrine disruption, which are included in OECD (2011a) and either validated or pending validation, are summarised in Table 11 in the annex to the present report.

Table 2: Overview of the in vitro tests and ecotoxicity tests (i.e. non-mammalian tests) included in the revised OECD conceptual framework as included in OECD (2011a). The framework includes standardised methods and methods that are being developed or standardised.

Level		Recommended test / method
1	Existing data and non-test information	Physical & chemical properties All available (eco)toxicological data from standardized or non-standardized tests Read across, chemical categories, QSARs and other in silico predictions, and ADME model predictions
2	In vitro assays providing data about selected endocrine mechanism(s) / pathways(s)	Estrogen or androgen receptor binding affinity Estrogen receptor transcriptional activation (TG 455, OECD 2009a) Androgen or thyroid transcriptional activation (if/when TGs are available) Steroidogenesis in vitro (draft TG, OECD 2010b) MCF-7 cell proliferation assays (ER ant/agonism) Other assays as appropriate
3	In vivo assays providing data about selected endocrine mechanism(s) / pathway(s)	*Xenopus* embryo thyroid signalling assay (when/if TG is available) Amphibian metamorphosis assay (TG 231, OECD 2009b) Fish short-term reproduction assay (TG 229, OECD 2009c) Fish screening assay (TG 230, OECD 2009d) Androgenized female stickleback screen (Katsiadaki et al. 2009)
4	In vivo assays providing data on adverse effects on endocrine relevant endpoints	Fish sexual development test (TG 234, OECD 2011b) Fish reproduction / partial life-cycle test (when/if TG is available) Larval amphibian growth and development assay (when TG is available) Avian reproduction assay (TG 206, OECD 1984) Mollusc partial life-cycle assays (when TG is available) Chironomid toxicity test (TG 218 and 219, OECD 2004b, c)
5	In vivo assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism	Fish life cycle toxicity test (when TG is available) Medaka multi-generation test (when TG is available) Avian 2-generation reproductive toxicity assay (when TG is available) Mysid life cycle toxicity test (when TG is available) Copepod reproduction and development test (when TG is available) Sediment-water chironomid life-cycle toxicity test (TG 233, OECD 2010c) Mollusc full life-cycle assays (when TG is available) *Daphnia* reproduction test (with male induction) (TG 211, OECD 2008a) *Daphnia* multi-generation assay (if/when TG is available)

2.1.1 Implementation of tests for endocrine effects in REACH

Despite the fact that substances with endocrine disrupting properties can be classified as substances of very high concern, if there is evidence of probable serious effects to the environment (see section 1.1) information on possible endocrine activity in aquatic organisms is not part of the standard information requirements according to REACH Annexes VII – X (EC 2007, ECHA 2008a). As stated in Appendix 7.8-5 of REACH guidance document R.7b (ECHA 2008a), no information on endocrine activity of a chemical or on its reproductive or specific developmental toxicity in aquatic organisms has to be provided for registration. Appendix 7.8-5 provides guidance on the assessment of "endocrine and other related effects". The initial assessment is solely based on the evaluation of available information (e.g. scientific literature). In cases where this evaluation provides evidence of a potential endocrine mode of action in aquatic organisms, this may lead to a concern requiring further investigation of possible "adverse effects on development and / or reproduction". Specific studies may then be requested

on a case-by-case basis by the competent authority in the context of the so-called substance evaluation. This may include specific studies for endocrine effects such as those mentioned in Appendix 7.8-5 (ECHA 2008a).

Appendix 7.8-5 covers the same endocrine mechanisms of action as OECD (2011a), i.e. effects on the estrogen / androgen axis, the thyroid system and "invertebrate systems" (without further specification). As explicitly mentioned, coverage of endocrine effects in invertebrates is sparse (ECHA 2008a).

2.1.2 Endocrine modes of action not covered

The ecotoxicity tests used for assessing potential environmental risks of a compound should allow identifying all adverse effects of the respective compound (Breitholtz et al. 2006). However, as outlined above the OECD 'Guidance document on standardized test guidelines for evaluating chemicals for endocrine disruption' (OECD 2011a) and the OECD Conceptual Framework only cover a limited part of all endocrine modes of action. Effects other than estrogen receptor, androgen receptor and thyroid hormone mediated effects, and interference with steroidogenesis are not addressed or only addressed to a limited extent. This means that other endocrine modes of action (e.g. progestin or retinoid effects, effects on the hypothalamus-pituitary-adrenal axis, the corticosteroid system, or the endocrine control of neural development) are likely to remain undetected when using only the available standardised tests.

Furthermore, no in vitro screening tests for thyroid effects are included in OECD (2011a). Most thyroid screening tests available to date require further development and validation. In addition and more importantly, it will most likely not be possible to cover all possible points of disruption of the thyroid system in a manageable battery of in vitro tests (OECD 2011a).

In addition, the in vivo screening tests ('level 3 screens') do not cover all possible effects on estrogen receptor, androgen receptor and thyroid system and interference with steroidogenesis (OECD 2011a). For example, OECD test guideline (TG) 230 (OECD 2009d) does not allow the detection of EDCs with an anti-androgenic effect, if it is not extended by further endpoints (e.g. measurement of 11-ketotestosterone; Knacker et al. 2010).

Consequently, current screening tests for endocrine disruption only cover a limited part of endocrine modes of action.

2.1.3 Taxa not considered

In the present section, taxa that are to our opinion not adequately covered in REACH R.7b, Appendix 7.8-5 and OECD (2011a) are briefly addressed. Interspecies differences and the resulting implications for the selection of representative test species are discussed in sections 2.2 and 2.2.9.

Most available studies on endocrine disruption in aquatic vertebrate species have been performed with fish and, to a lower extent, with amphibians. Very few studies have been carried out with reptiles, for which no standard tests are available and which have therefore not been included in OECD (2011a). As outlined by Talent et al. (2002) and Kortenkamp et al. (2012), it has been assumed that criteria for the protection of birds and mammals would be sufficient to also protect reptiles. Due to the limited amount of available data, this assumption cannot yet be verified.

With regard to invertebrates, Appendix 7.8-5 'Assessment of available information on endocrine and other related effects' of REACH guidance document R.7b (ECHA 2008a) includes effects on 'invertebrate systems' (see section 2.1.1), however information is very limited. The only test protocols that are mentioned are the revised *Daphnia magna* reproduction test (TG 211, OECD 2008a)[2] and a test guideline proposal for a development and reproduction test with marine copepods, which is currently in an interlaboratory validation phase (OECD 2011c). It is stated that adverse effects on invertebrate development or reproduction may be reported in non-standard tests and should be considered in the assessment. Yet, it is also noted that a causal link to a specific endocrine mode of action will in most cases not be found (ECHA 2008a). Effects on development, growth and reproduction of sediment-dwelling invertebrates that are evaluated in higher tier standard tests can be related to endocrine effects. However, the studied test endpoints are not specific to EDCs. In addition, these tests are only required at a relatively high tonnage (see Table 12 in the annex).

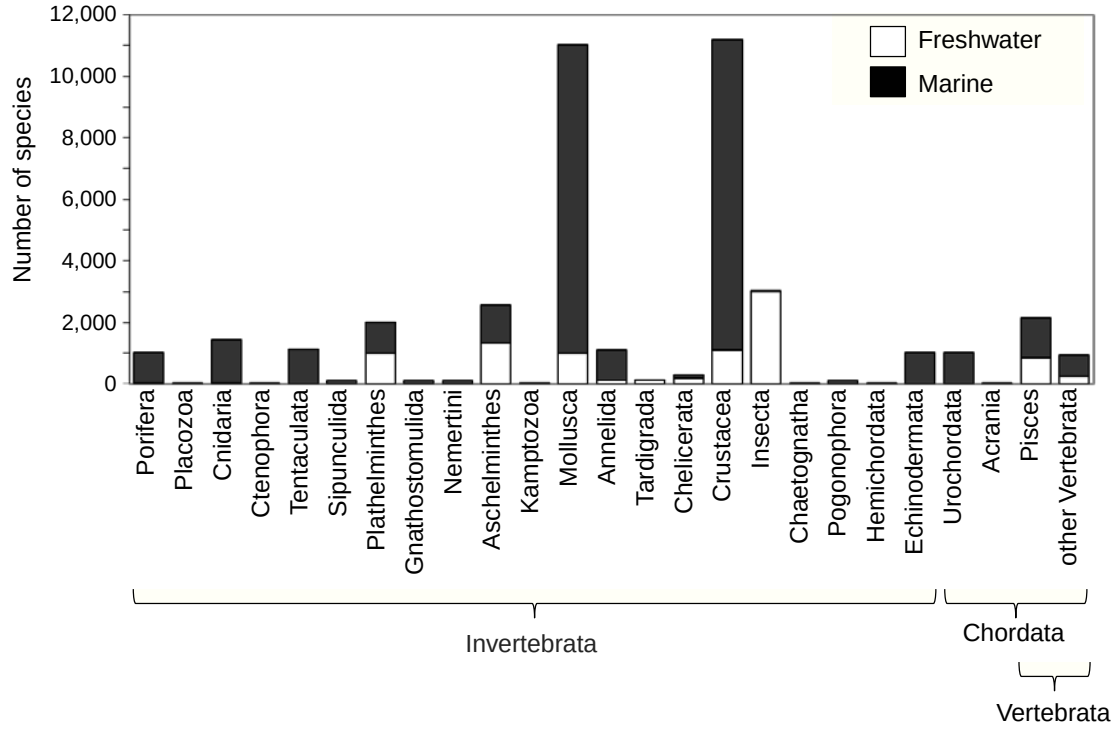
In the OECD conceptual framework, partial and full life-cycle tests with invertebrates are mentioned. However, OECD (2011a) does not provide guidance on interpretation of the results for tests with invertebrates, because (1) the present knowledge of invertebrate endocrinology is still very limited (see section 2.2.6), and (2) diagnostic endpoints for invertebrates are lacking (see section 2.1.5). Yet, invertebrates account for more than 95% of all animal species on earth (Wilson 1999) and are often key species for structure and function of ecosystems (Oehlmann & Schulte-Oehlmann 2003). Life cycles of invertebrates vary widely and include, for example, different larval stages, pupation, metamorphosis and diapause (DeFur et al. 1999a, LeBlanc et al. 1999, Oehlmann et al. 2011; see also section 2.2.5).

Fig. 7 gives an overview of the diversity of freshwater and marine organisms. As outlined by Floeter (2007), there are ca. 90,000 known freshwater invertebrate species and ca. 315,000 known marine invertebrate species. Several invertebrate phyla / subphyla exclusively consist of marine species. This is of importance with regard to estuarine regions, which often receive considerable amounts of potentially endocrine disrupting chemicals (OECD 2006a).

Based on the numbers of species, it is evident that invertebrate species (especially molluscs and crustaceans) deserve further attention (see also sections 2.1.5, 2.2.5 and 2.2.9).

[2] In the *D. magna* reproduction test (test guideline 211, OECD 2008a), sex ratio has been included as an optional endpoint, which has been shown to be sensitive (e.g. Dodson et al. 1999, LeBlanc 2007). Yet, under laboratory conditions daphnids reproduce parthenogenetically unless affected by stress. Thus, effects on sexual reproduction (including e.g. partner finding, sexual synchronisation and mating behaviour) would remain undetected when using *D. magna* as only representative test species for crustaceans (Breitholtz et al. 2006, OECD 2006a).

Fig. 7: Overview of the phyla and numbers of species per phylum for the freshwater and marine environment. From Floeter (2007), modified. Numbers of species (without parasitic species) based on Nelson (1984), May (1988), Storch & Welsch (1991), Barnes et al. (1993) and EC (2003).



2.1.4 Availability of test methods for fish

For fish, a comprehensive tiered testing framework has been developed. This framework includes screening tests – the fish screening assay (TG 230), the fish short-term reproduction assay (TG 229) and the androgenised female stickleback screen (Katsiadaki et al. 2009) – as well as the fish sexual development test (TG 234) and the full life-cycle test (see Table 1). The fish screening tests and the fish sexual development test have been developed based on information on the most sensitive life stages (see section 2.3) and critical events (e.g. reproduction). These tests allow the detection of three out of the four endocrine mechanisms of action that are mentioned in the 'OECD guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption' (OECD 2011a) and the OECD conceptual framework, namely estrogen and androgen receptor agonistic and antagonistic effects as well as effects on steroidogenesis. In order to detect thyroid effects, the amphibian metamorphosis assay is used (TG 231, OECD 2009b). In screening tests and the fish sexual development test, exposure of fish is limited to a certain part of their life cycle. Yet due to the evaluated diagnostic endpoints (see Table 11 in the annex) it was concluded that these assays appear sufficiently sensitive for screening purposes (OECD 2011a).

The full life-cycle test includes all life stages and a variety of endpoints and is, thus, the optimal method for detecting possible effects of EDCs on fish (Ankley & Johnson 2004). Effects on its apical endpoints allow the identification of adverse effects and are used in the environmental risk assessment. However, these apical endpoints (e.g. growth, time to first reproduction, fecundity, fertilisation rate; see Table 11 in the annex) per se do not provide causal evidence of an endocrine mode of action, since they can also be affected by non-endocrine modes of action

(Scholz & Klüver 2009). By including diagnostic endpoints such as vitellogenin or sex steroid concentrations, secondary sex characteristics or gonad histopathology indications of ED effects can be linked to population relevant effects (Crane et al. 2010, Knacker et al. 2010).

2.1.5 Availability of test methods for aquatic invertebrates

Protocols for life-cycle tests are available for a range of invertebrate species. A comprehensive list of relevant test organisms, for which test protocols are available or can be established with limited method development, was already compiled by Ingersoll et al. (1999). In the last few years, considerable advances in the development of test methods have been made (Hutchinson 2007). Test methods with invertebrates that are currently being developed include a mollusc partial life-cycle assay with the gastropod *Potamopyrgus antipodarum*, a mollusc full life-cycle assay with the gastropod *Lymnaea stagnalis* (OECD 2010a), a mysid two-generation reproductive and developmental toxicity test (OECD 2006a, Verslycke et al. 2007) and a copepod reproduction and development test (OECD 2011c). These protocols have already been included in the OECD conceptual framework (see Table 1). A test guideline for a sediment-water chironomid life-cycle test (TG 233, OECD 2010c) is available (see Table 13 in the annex).

The endpoints of such life-cycle tests include embryonic and larval development, hatching success, survival, growth, moulting time and success, time to emergence or pupation, sex ratio of adults and / or offspring, sexual maturation, time to first spawning, fecundity, fertilisation success, and viability of the offspring, i.e. endpoints that are sensitive to endocrine disruption. Additional endpoints such as mating behaviour, secondary sexual characteristics, ecdysteroid levels, vitellogenin levels and gonad histology may be included (Ingersoll et al. 1999, Vandenbergh et al. 2003, Geffard 2010, OECD 2010a). Relevant endpoints for the assessment of potential endocrine disruption have been compiled for instance by Ingersoll et al. (1999), OECD (2006a, 2010a), LeBlanc (2007) and Kortenkamp et al. (2012). Since different endpoints in the same test organism were shown to differ in their sensitivity (e.g. Watts et al. 2003; see also section 2.2), a range of endpoints should be evaluated in each test (OECD 2006a).

As already noted in section 2.1.4 for the fish tests, full life-cycle tests with invertebrates allow the identification of adverse effects in the respective test species. Yet, effects on the apical endpoints of such life-cycle tests do not provide causal evidence of an endocrine mode of action (Ingersoll et al. 1999, LeBlanc 2007, OECD 2010a). Impaired reproduction may, for example, also result from toxicity to organs such as the hepatopancreas. Likewise, systemic toxicity that results in reduced growth often leads to a reduced fecundity (Barata et al. 2004, OECD 2006a, Hutchinson 2007)

This also applies to some of the more specific endpoints (e.g. altered moulting frequencies, ecdysone levels, intersex) evaluated in such tests. These endpoints may also be affected by general toxicity (e.g. through effects on food intake and on the energy budget of the organism; Barata et al. 2004, OECD 2006a, LeBlanc 2007). In order to establish causal evidence for endocrine disruption, diagnostic endpoints and knowledge on the underlying hormonal processes and the mode of action of the respective compound in invertebrates is required. Yet as outlined above, few appropriate diagnostic endpoints are available for invertebrates.

In summary, a range of full life-cycle test methods for invertebrates is available or can be expected to be available in the near future. However, there is still a lack of appropriate diagnostic endpoints.

It should be noted that the experimental conditions for tests for endocrine disruption should be carefully selected, especially with regard to molluscs. Effects of bisphenol A on prosobranch reproduction were, for example masked during the main reproductive season or at elevated temperatures when the reproduction of the animals was maximal (Oehlmann et al. 2007, Crain et al. 2007, OECD 2010a, Sieratowicz et al. 2011).

2.2 Extrapolation between species

It is obviously not feasible to investigate the effects of a chemical on all relevant species in the relevant environmental compartment. In environmental risk assessment procedures, potential effects are thus evaluated using a few test species that have often been selected based on practical reasons (see section 2.2.9). It is a fundamental assumption of risk assessment that it is possible to extrapolate from effects observed in these test species under laboratory conditions to effects in all kinds of wildlife species exposed under the actual environmental conditions. In this extrapolation, assessment factors are applied when deriving the PNEC from results of laboratory tests. The selected assessment factors shall cover intra- and inter-laboratory variation in toxicity data, interspecies variations, the extrapolation from short-term to long-term toxicity where relevant, and the extrapolation from laboratory data to the field (EC 2003, ECHA 2008b, Celander et al. 2011). The magnitude of the assessment factor to be applied and the uncertainty that it can cover has been subject to intensive discussions (e.g. OECD 1995a, Hester & Harrison 2006).

Extrapolation between species is most feasible for those processes that are relatively conserved between taxa. Vice versa, it is most difficult to extrapolate between taxa where target structures of EDCs have not been conserved across species / taxa or where knowledge on target structures is lacking. In view of the observed interspecies differences in sensitivity to endocrine disrupting substances and the variety in endocrine systems and reproductive strategies, the feasibility of extrapolating between species has been questioned, especially for invertebrates (see e.g. Ingersoll et al. 1999, Hutchinson 2002, OECD 2006a).

In the present section, we will first briefly summarise the major factors contributing to interspecies differences in sensitivity / ecological vulnerability. Then, interspecies differences will be addressed for fish (sections 2.2.1 – 2.2.4) and aquatic invertebrates (sections 2.2.5 – 2.2.8).

The ecological vulnerability of a species, i.e. the extent to which the population of this species is affected in the field, is a result of (1) the extent of exposure to the toxicant, (2) the intrinsic sensitivity of the organism and (3) population sustainability, i.e. the population's potential to recover from a toxic effect (van Straalen 1994, De Lange et al. 2009, Rubach et al. 2011).

The extent of exposure mainly depends on the habitat and food choice of a species that may vary during different stages of the life cycle. In addition, life-cycle traits such as the lifespan of an organism, its home range or migration are also relevant (Rubach et al. 2011).

Intrinsic sensitivity is determined by (a) the uptake of the toxicant, its distribution in the body, its metabolic conversion and elimination (i.e. toxicokinetic processes) and (b) the interactions of the toxicant with the target site(s) and the consequences of these interactions at the suborganism and organism level (i.e. toxicodynamic processes) (Boelsterli 2003, Rubach et al. 2011).

The potential of a population to recover from a toxic effect is governed by (a) demographic traits such as life span, life stage specific survival rates, generation time, the number of reproductive events per year and the number of offspring, and (b) the recolonisation potential that is linked to the dispersal capacity, the presence of resistant stages (e.g. ephippia) and the mode of reproduction (Rubach et al. 2011). Equal levels of mortality or reduction in fecundity will have a higher impact on species with long generation times and low numbers of offspring. Short generation times and high numbers of offspring facilitate population recovery (Stark et al. 2004, De Lange et al. 2009, Rubach et al. 2011).

Vulnerable species are characterised by a high potential for exposure, a high intrinsic sensitivity and a low ability to recover from a toxic effect (van Straalen 1994).

In the context of the present project, main focus is on the questions

1. whether there are indications that certain species or taxa are especially sensitive to endocrine modes of action;
2. whether interspecies differences in sensitivity and the resulting uncertainty of the environmental risk assessment are expected to be higher for EDCs than for substances with non-endocrine modes of action.

The first question will be addressed in sections 2.2.3 and 2.2.7, while the second question will be addressed in section 5.2.

When evaluating effect concentrations with regard to interspecies differences, it is only in rare cases (e.g. Routledge et al. 1998, Villeneuve et al. 2012) possible to compare results for different species that were tested under the same conditions, especially since most of the data compiled within the present project for the six model substances were obtained using non-standard tests. In cases where different studies are compared attention has to be paid to factors other than interspecies differences that might have affected the test result. As mentioned in section 2.1 effect concentrations depend on factors such as timing and duration of exposure in relation to the life cycle of the test species. In many cases, lowest effect concentrations are derived in tests that cover both the most sensitive life stages and the time window where the most sensitive effects manifest. For effects of sexual endocrine disrupting compounds on fish, the time of sexual development is the most sensitive time window for exposure, and the reproductive phase is the time window where the most sensitive effects often manifest (Knacker et al. 2010; see also section 2.3). In short-term tests, the age of the test organism in relation to its generation time may also influence the results. In addition, reproductive state is – especially for seasonally reproducing species – an important factor influencing the outcome of a test for potential endocrine disruption.

Methodological differences as for instance the type of enzyme-linked immunosorbent assay (ELISA) used for measuring vitellogenin (Mylchreest et al. 2003, Liao et al. 2006) may also affect test sensitivity, especially since methods often have been improved considerably in the last years. Likewise, the test design (e.g. replication, spacing factor between concentrations) contributes to differences in LOEC values. It should also be considered whether the effect concentrations are based on nominal or measured concentrations of the test substance and, in the case that nominal concentrations are used, whether the test substance can be assumed to be stable under the given experimental conditions or whether it is likely to degrade rapidly as e.g. bisphenol A. Exposure conditions such as temperature may also be crucial (see section

2.2.7). Even in cases were different species were tested under exactly the same conditions, some of the abovementioned factors (e.g. age of the test organism in relation to its generation time) may differ. Last but not least, different strains or clones of a test species may differ in their sensitivity to a toxicant. All these factors, which may result in significant variation between different tests with a single species, should be kept in mind when comparing the sensitivity of different species.

2.2.1 Extrapolation between fish species

Fundamental cellular mechanisms (e.g. signal transduction, key metabolising enzymes) are often conserved across taxa (Gunnarson et al. 2008). Vertebrate hormones and hormone receptors have, for example, been highly conserved through evolution (Van Der Kraak et al. 1998). Most (perhaps all) vertebrates are affected in a similar way by steroid hormones, such as 17β-estradiol, and by xenoestrogens such as nonylphenol (White et al. 1994, Sumpter & Johnson 2005, Matthiessen & Johnson 2007). Thus, information from tests with mammals may provide some information on potentially similar effects in non-mammalian vertebrates (Vos et al. 2000, OECD 2011a), i.e. information on endocrine disruption in mammals may indicate that ED can also be expected in fish (ECHA 2008a, c). In closely related species, binding affinities of an endocrine disrupting substance to the estrogen receptor are likely to be similar, but with larger evolutionary distance between species differences in binding affinity are likely to increase due to differences in the receptor's ligand binding region (see e.g. Tollefsen 2002, Olsen et al. 2005). Moreover, even in those cases where the same hormone receptor is present, the extent of the observed effect (i.e. the effect concentrations of an ED acting through this receptor) and the type of effect may differ, e.g. due to differences in metabolism, pharmacokinetics and hormone function in the respective species. For example prolactin, the hormone that regulates lactation in mammals, is involved in osmoregulation in fish (Sumpter & Johnson 2005, McCormick & Bradshaw 2006, Celander et al. 2011). As emphasised by Matthiessen & Johnson (2007), the extrapolation between fish species may be difficult as is, for example, the case for the endpoint ovotestis (see section 2.2.3).

Fish are a paraphyletic group of taxa including Agnatha (jawless fish; approx. 75 species including lampreys and hagfish), Chondrichthyes (cartilaginous fish, approx. 800 species including sharks and rays) and Osteichthyes (bony fish, more than 26,000 species). Teleostei (modern bony fish) are the largest group of bony fish (New World Encyclopedia 2008; see also Table 2) comprising more than 20,000 species (Kime 1998).

Most bony fish species (Osteichthyes) are gonochoristic, i.e. male and female sex are separated. However, there are also hermaphroditic fish species with both sexes in one individual, in most cases sequentially starting with males (protandry) or females (protogyny). In some species, sex is determined or can be influenced during early ontogenetic stages by environmental factors such as temperature (Lagler et al. 1977, Olsen et al. 1998, Baroiller et al. 1999, OECD 2008b). Sex determination in fish is more labile than in mammals. During critical windows of sensitivity, sex may be partially or fully reversed by administration of sex steroids. It has been assumed that this lability in sex determination might render fish (or at least some fish species) particularly sensitive to endocrine disruption (Devlin & Nagahama 2002, Scholz & Klüver 2009). Some fish species reproduce parthenogenetically (Lagler et al. 1977). Reproductive strategies in fish are very diverse. Fertilisation in most fish species is external, but internal fertilisation also occurs (e.g. in sharks). More than 95% of the fish species are oviparous (egg laying), but there

are also ovoviparous (livebearing, no maternal nourishment) and viviparous species (livebearing with maternal nourishment). In oviparous fish, the egg numbers and egg size range from large numbers of very small eggs to few very large eggs. Most fish species have a yearly reproductive cycle (i.e. exhibit seasonal iteroparity). Some fish species spawn more than once a year (e.g. guppies at about monthly intervals) or even more or less continuously (continuous iteroparity). Other species reproduce only once during their life (semelparity), e.g. Pacific salmons and freshwater eels. In seasonally reproducing fish, appropriate timing of reproduction ensures that conditions for the offspring are optimal (Lagler et al. 1977, IPCS 2002, OECD 2008b).

Table 3: Overview of fish groups with extant species (according to New World Encyclopedia 2008).

Agnatha (jawless vertebrates)
Myxinoidea (hagfish)
Cephalaspidomorphi
Petromyzontida (lampreys)
Gnathostomata (jawed vertebrates)
Chondrichthyes (cartilaginous fish)
Elasmobrachii (sharks, rays, skates)
Holocephali (chimaeras)
Osteichthyes (bony fish)
Actinopterygii (ray-finned fish)
Chondrostei (sturgeons, paddlefish)
Teleostei (modern bony fish)
Sarcopterygii (lobe-finned fish)
Actinistia (coelacanths)
Dipnoi (lungfish)

2.2.2 Overview of fish endocrinology

Most studies of fish endocrinology have been performed with teleost fish. The teleost endocrine system is relatively similar to the endocrine system of higher vertebrates. Reproduction is controlled by the hypothalamic-pituitary-gonadal axis, which is in its main aspects relatively conserved across vertebrates. The hypothalamic-pituitary-gonadal axis in fish is most similar to that of other egg laying vertebrates. Overall, there appear to be relatively few major differences between the reproductive endocrine systems in different teleost species (Kime 1998, Ankley & Johnson 2004). Releasing hormones from the hypothalamus (gonadotrophin releasing hormone, corticotrophin releasing hormone and thyrotrophin releasing hormone) trigger the release of hormones from the pituitary gland. These hormones (gonadotrophin, adrenocorticotrophin and thyrotrophin) stimulate hormone secretion in the gonad, adrenal and thyroid gland, respectively. For reproduction, gonadotrophin (GtH) is most relevant. In some species, two gonadotrophins have been found (GtH-I and GtH-II), with GtH-I being involved in vitellogenesis and spermatogenesis and GtH-II regulating final gamete maturation. In male fish, 11-ketotestosterone is the major hormone influencing secondary sexual

characteristics, sexual behaviour and gonadal development. In females, estradiol stimulates vitellogenesis. Several progestogens are also involved in final gamete maturation. Gonadotrophin and sex steroids are the major hormones controlling reproduction. However, their effects may be modulated by further factors such as the thyroid hormones thyroxine and triiodothyronine, which are also involved in larval development (Kime 1998).

2.2.3 Differences in sensitivity to EDCs between fish species

In the present project, it was not possible to systematically evaluate differences in sensitivity to endocrine disrupting substances between fish species. Such an evaluation would require a comprehensive review of all available data on the effects of EDCs on different fish species. To our knowledge such an evaluation has not yet been performed. Further studies on the sensitivity of additional fish species would most likely be necessary for complementing the available data, given that so far most investigations on endocrine disruption in fish have focused on a relatively low number of species. Many studies have been performed with the three fish species recommended in the fish screening tests for endocrine disruption (test guidelines 229 and 230; OECD 2009c, d): zebrafish (*Danio rerio*), fathead minnow (*Pimephales promelas*) and medaka (*Oryzias latipes*). Table 3 gives an overview of the taxonomic position, the habitat and the main characteristics of these three species. With regard to a number of aspects (e.g. external fertilisation, oviparity) these species are typical for the majority of teleost species. It should be noted that all three are relatively small, short-lived fish.

Table 4: Taxonomic position, habitat and main characteristics of the three teleost species that have been most frequently used in studies of endocrine disruption (based on Ankley & Johnson 2004, OECD 2008b and http://www.fishbase.us).

Species	**Zebrafish (*Danio rerio*)**	**Fathead minnow (*Pimephales promelas*)**	**Medaka (*Oryzias latipes*)**
Taxonomic position	Cyprinidae[1]	Cyprinidae[1]	Adrianichthyidae
Freshwater / seawater	Freshwater	Freshwater	Freshwater
Indigenous to	Asia (Pakistan, India, Bangladesh, Nepal and Myanmar)	North America	Asia (Japan, Korea, China and Vietnam)
Habitat	Streams, canals, ditches, ponds	Creeks, small rivers and ponds	Ponds, slow-flowing streams
Size	40–50 mm length	35–75 mm length	25–50 mm length
Generation time	Approx. 2–3 months	Approx. 4 months	Approx. 2–3 months
Gonochorism /hermaphroditism	Gonochoristic	Gonochoristic	Gonochoristic
Fertilisation	External	External	External[2]
Mode of reproduction	Oviparous	Oviparous	Oviparous
Type of spawning	Asynchronous spawning: successive spawning, approx. every second day	Asynchronous spawning: successive spawning, approx. every third day	Asynchronous spawning: successive spawning, approx. each day
Breeding time in the wild	All year round	May to August	April to September (in Japan)
Number of eggs per spawn	Approx. 25–150	Approx. 30–250	Approx. 10–30

Species	Zebrafish (*Danio rerio*)	Fathead minnow (*Pimephales promelas*)	Medaka (*Oryzias latipes*)
Degree of parental care	None	High: males are nest guarders	High: carries eggs for some time before deposition
Gonadal development	Juvenile hermaphroditism: all fish first develop a female-type gonad	Direct differentiation into ovaries and testes in early development	Direct differentiation into ovaries and testes in early development
Secondary sexual characteristics	Not distinct	Present	Present
Resilience	High, minimum population doubling time < 15 months	High, minimum population doubling time < 15 months	Low, minimum population doubling time 4.5 - 14 years

Remarks: (1) Cyprinids are the largest family of fish (OECD 2008b). (2) Very rarely internal fertilisation (Yamamoto 1975).

In addition, several other gonochoristic teleost species, in many cases commercially relevant species such as salmonids and cyprinids have been used to evaluate potential endocrine disruption (Kime 1998). Frequently studied species include e.g. rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus carpio*), goldfish (*Carassius auratus*), guppy (*Poecilia reticulata*) and sheepshead minnow (*Cyprinodon variegatus*). As stated by Kortenkamp et al. (2012), no data on endocrine disruption in hermaphroditic fish are available. Furthermore, data on endocrine disruption in minor taxonomic groups (e.g. lampreys, hagfish, and cartilaginous fish) are lacking.

The present section is mainly based on the effect concentrations that were compiled for the model substances 17α-ethinylestradiol, bisphenol A, 4-tert-octylphenol and prochloraz[3] . This includes data on effects on (a) apical endpoints, i.e. data that can be used in the environmental risk assessment, and (b) indicative (diagnostic) endpoints, i.e. data that may trigger further testing. While all endpoints of the short-term screening assays (OECD test guidelines 229 and 230 and similar test protocols) are considered as indicative[4], the fish sexual development test (OECD TG 234 and similar test protocols) and the fish life-cycle toxicity test include both indicative and apical endpoints (OECD 2011a; for details see Table 11 in the annex).

The comparative evaluation of the sensitivity of different fish species is complicated by the fact that in most cases tests with different species were performed according to different (often non-standard) protocols. The test results depend on factors such as exposed life stage(s), test duration, test endpoints, test design, test conditions and methodology (see sections 2.1 and 2.2).

[3] For tributyltin and triphenyltin the amount of available effect concentrations for fish is not sufficient for an interspecies comparison. In addition, these two organotins are most toxic to invertebrates and, hence, considered in section 2.2.7.

[4] Due to the high variability of fecundity, the relative short test duration and the fact that only three concentrations are tested no reliable NOEC or EC_X for fecundity can be derived in the fish short-term reproduction test (OECD TG 229; see also Table 11 in the annex).

The most relevant information on these factors is included in Tables 4 – 7 and is discussed in the following sections. Additional information is provided in Tables 14 – 17 in the annex.

In addition, we have evaluated review publications (e.g. Scholz & Klüver 2008) and the results of a whole lake study with 17α-ethinylestradiol in Canada, in which sensitivity of several fish species was compared. In the following, we will first address several factors, which are relevant for interspecies differences in fish. Afterwards, we will outline the major findings for the model substances.

Given that the hypothalamic-pituitary-gonadal axis is relatively conserved across vertebrates and does not differ much between teleost species (see section 2.2.1), the primary mode of action of sexual endocrine disrupting compounds should be comparable in different teleosts. Indeed, exposure of various fish species (including *D. rerio*, *P. promelas*, *O. latipes*, *O. mykiss*, *Rutilus rutilus*, *C. variegatus* and *Fundulus heteroclitus*) to estrogenic substances resulted in a relatively consistent pattern of effects although not all effects were observed in each species: sex reversal of males to females (i.e. sex ratios skewed towards females), intersex (ovotestis or feminised seminiferous ducts), reduced gonadosomatic indices and delayed gametogenesis in both sexes. In addition, a reduced number of primordial germ cells and an increased number of atretic oocytes (i.e. oocytes undergoing resorption) in females were often observed. Exposure to androgenic substances led to sex reversal of females to males (i.e. sex ratios skewed towards males), intersex, stimulation of spermatogenesis, delayed oocyte development, and an increased number of atretic oocytes. Furthermore, an increased number of Leydig cells and hypertrophy of Sertoli cells were often observed in the testes of exposed fish (Scholz & Klüver 2009).

Due to interspecies differences in sexual development the effects of EDCs may manifest in a different way. For instance, ovotestes have often been observed in a range of species including medaka (*O. latipes*), roach (*R. rutilus*), bream (*Abramis brama*) and flounder (*Platichthys flesus*) (Jobling et al. 1998, Balch et al. 2004, Kirby et al. 2004, Vethaak et al. 2005; see also section 2.3). By constrast, ovotestes are rarely found in other species such as zebrafish (*D. rerio*). This fact is related to the sexual development of zebrafish, which are protogynous juvenile hermaphrodites. Irrespective of the genetic sex, the gonads of all fish first develop into immature ovary-like tissues. In male fish, the oocytes then degenerate and the gonads transform into testes, which is probably triggered by the hormone titre. The process of sexual differentiation is completed when the fish are about 40 to 70 days old (Takahashi 1977, Maack & Segner 2003, Maack et al. 2003). In zebrafish, estrogenic compounds retard or arrest male development. In this case, male fish have immature female-like gonads (see e.g. Örn et al. 2003, Nash et al. 2004).

Interspecies differences in the compensation potential may also lead to a different manifestation of effects. Schäfers (2007) compared the sensitivity of different fish species to sterol demethylation inhibiting (DMI) fungicides. In full life-cycle tests, data for fathead minnow and zebrafish indicate comparable sensitivity of both species. It seems that there is a difference in the expression of the effects between zebrafish and fathead minnow due differences in sexual development. Probably due to hormone triggered conversion of the immature ovary-like gonads of male zebrafish into testes (see above), zebrafish seem to react more sensitive to aromatase inhibition than fathead minnows. Fathead minnows appear to possess a higher potential for compensation. Yet, due to their larger size and, thus, higher absolute growth

potential they allow a more accurate statistical discrimination of effects on growth, i.e. effects, which in turn may be a consequence of the compensatory action (Schäfers 2007).

17α-Ethinylestradiol

Effects of the estrogen receptor agonist 17α-ethinylestradiol (EE_2) have been studied in a number of fish species (see Table 4). In most cases, only short-term tests (short-term screening tests, in which vitellogenin is generally the main endpoint, and short-term reproduction tests) were performed, in some cases partial life-cycle tests. For *D. rerio* and *P. promelas*, full life-cycle tests are also available.

In the full life-cycle tests with zebrafish and fathead minnow, effects on population relevant endpoints were found at very similar concentration levels of 0.2 to approx. 1 ng/L. In *D. rerio*, growth of F_1 juveniles was most sensitive (LOEC: 0.3 ng/L), and effects on several reproductive endpoints (time to first reproduction, fertilisation rate and fecundity) were observed at a mean measured EE_2 concentration of 1.1 ng/L (Wenzel et al. 2001a, b). For *P. promelas*, fertilisation rate and sex ratio were the most sensitive endpoints (LOEC ≤ 0.32 ng/L) of the study by Parrott & Blunt (2005). In this study, the F_1 was only evaluated until hatch. In a second full life-cycle test with *P. promelas* that included a more detailed evaluation of the offspring (Länge et al. 2001), a reduction of growth of the F_1 was the most sensitive effect (i.e. a similar finding as described above for zebrafish). Length of the F_1 was significantly reduced at the lowest tested concentration of 0.2 ng/L, weight was reduced at 1.0 ng/L. Fertilisation rate was not evaluated; fecundity was not significantly affected at a nominal EE_2 concentration of ≤ 1.0 ng/L (corresponding to a mean measured concentration of 0.58–0.76 ng/L), but could not be assessed at ≥ 4 ng/L due to the lack of phenotypic males at these concentrations (Länge et al. 2001).

For a few other fish species, effects of EE_2 on apical endpoints were studied in fish sexual development or partial life-cycle tests. In a fish sexual development test, a feminisation of the gonads of *R. rutilus* was observed (but not statistically evaluated) at 0.3 ng/L (Katsu et al. 2007), i.e. at a the same concentration that affected apical endpoints of full life-cycle tests with *P. promelas* and *D. rerio*. For Japanese medaka (*O. latipes*) and sheepshead minnow (*Cyprinodon variegatus*), the lowest effect concentrations were higher. Following exposure of larval / juvenile medaka for 2 months and a subsequent recovery period of 6 weeks, fecundity was reduced at ≥ 10 ng/L and morphologic sex ratio was skewed towards females at 100 ng/L (Scholz & Gutzeit 2000). In a partial life-cycle test with the sheepshead minnow, effects on sex ratio and fecundity were observed at ≥ 20 ng/L (Zillioux et al. 2001). However, exposure duration was much shorter, and some sensitive endpoints (e.g. fertilisation rate) were not evaluated in these two partial life-cycle tests. In addition, effects in the study of Scholz & Gutzeit (2000) were only evaluated after a recovery period. Therefore, a comparison with the results of the full life-cycle tests is difficult.

Data on effects of EE_2 on diagnostic endpoints are available for a number of fish species. In short-term tests, vitellogenin was induced in male *D. rerio* and *P. promelas* at 1 ng/L or slightly higher concentrations (Rose et al. 2002, Duis & Knacker 2003, Örn et al. 2003, Pawlowski et al. 2004). Based on the available studies, *O. latipes* appears to be less sensitive to EE_2 than the two abovementioned species. In a short-term (28 d) screening test with adult males, vitellogenin was induced by EE_2 concentrations of 10 ng/L (Scholz et al. 2004). In studies of Seki et al. (2002) and Tilton et al. (2005), higher effect concentrations were reported for vitellogenin induction

and fecundity (see Table 4). In these two studies, adult fish were exposed for only 14 d (Tilton et al. 2005) or 21 d (Seki et al. 2002). Yet, stronger effects on fecundity can be expected when medaka are exposed during early development as in the study of Scholz & Gutzeit (2000). Vitellogenin should also be induced by short-term exposure. However, in this case the large spacing factor between concentrations in the study of Tilton et al. (2005) might have contributed to the observed difference (no concentration between 5 and 500 ng/L was tested; see Table 16).

For the other fish species that were studied, effects of EE_2 were recorded in a similar concentration range. In the cyprinids *R. rutilus* and *C. carpio*, vitellogenin was induced by 4 and 10 ng/L of EE_2, respectively (Purdom et al. 1994, Katsu et al. 2007). In the salmonids *O. mykiss* and *Salvelinus namaycush* vitellogenin was induced at ≤ 0.1 to 1 ng/L and ≤ 15 ng/L of EE_2, respectively (Purdom et al. 1994, Sheahan et al. 1994, Werner et al. 2003). The few available data for the gobiid *Pomatoschistus minutus* point towards a similar sensitivity (effects at 6 ng/L, Robinson et al. 2003). With regard to the induction of vitellogenin *C. variegatus* and *F. heteroclitus* were less sensitive: effects were observed at about 100 ng/L (Folmar et al. 2000, Peters et al. 2007). This is at least partly due to the large spacing factors between test concentrations in these two studies (see Table 16). However, with effects at ≥ 20 ng/L, intersex was a more sensitive endpoint in *C. variegatus* (see above, Zillioux et al. 2001). Moreover effects on non-standard endpoints such as testes histology in *C. variegatus* (LOEC: 2 ng/L) and plasma levels of estradiol in *F. heteroclitus* (LOEC: 10 ng/L) were observed at lower EE_2 concentrations (Zillioux et al. 2001, Peters et al. 2007).

It is of note that the effect concentration for vitellogenin induction in male fathead minnows, which was obtained in a short-term test (1 ng/L; Pawlowski et al. 2004), is considerably lower than the effect concentration for the same endpoint and species obtained on day 172 of a full-life cycle test (16 ng/L; Länge et al. 2001). This might be due to a homeostatic response (Länge et al. 2001, Nash et al. 2004; see also section below on bisphenol A and 4-tert-octylphenol).

Effects of 17α-ethinylestradiol on several fish species, namely *P. promelas*, pearl dace (*Margariscus margarita*), lake trout (*Salvelinus namaycush*) and white sucker (*Catostomus commersonii*) were also studied in a whole lake study in Canada (Palace et al. 2002, 2006, 2009, Kidd et al. 2007). In this study, EE_2 was added to Lake 260 (Ontario) three times per week during the ice-free season for 3 consecutive years to obtain concentrations of approx. 5–6 ng/L. Vitellogenin levels, histopathology and population structure of the abovementioned species in Lake 260 and two reference lakes were studied for two years prior to the EE_2 additions, during the three years of EE_2 application, and during the two following years. Vitellogenin was induced in males of all four fish species. Vitellogenin induction was strongest in *P. promelas*, followed by *M. margarita* and *S. namaycush* and was less pronounced in *C. commersoni*. Vitellogenin levels were also increased in females. Histopathological effects (e.g. delayed spermatogenesis in males, delayed ovarian development in females) were only observed in *P. promelas* and *M. margarita*. Likewise, intersex was observed in these two species, but not in

S. namaycush and *C. commersoni* (Palace et al. 2006, 2009). After the second season of EE_2 additions, the population of *P. promelas* declined strongly until close to extinction. This was due to reproductive failure and, therefore, loss of young-of-the-year fish[5]. Such clear population level effects were not observed in the other three fish species. For *M. margarita* and *C. commersoni*, there was a trend towards a reduced abundance of young-of-the-year fish. In *S. namaycush*, a population decline to 2/3 of its previous size was observed in the third season of EE_2 additions (Palace et al. 2006, 2009, Kidd et al. 2007).

Differences in age at exposure may have contributed to the observed difference in sensitivity, given that only adult fish were used to evaluate vitellogenin levels and histopathology. While the studied *P. promelas* and *M. margarita* were 1- to 2-year old, the studied *S. namaycush* and *C. commersoni* were approximately 5- to 10-year old. In addition, this whole lake study exemplifies how – in addition to the intrinsic sensitivity of each species / life stage – differences in life histories (e.g. life span and generation time), habitat preferences, feeding and seasonality of reproduction may contribute to differences in population level effects. The most affected species in Lake 260, *P. promelas* is small and has a short life span. In Lake 260, few fathead minnows are older than 2 years. As *P. promelas* reaches maturity at the age of one year, each fish typically only spawns during a single season in its life time. Two successive years of reproductive failure can thus be expected to lead to a massive population decline as was observed in Lake 260 (Kidd et al. 2007). In the longer-lived species *M. margarita*, *S. namaycush* and *C. commersoni*, such population declines were not observed (Palace et al. 2006). Yet, chronic exposure of longer-lived species can be expected to result in similar population declines, although the response of these species is slower (Kidd et al. 2007, Palace et al. 2009). In addition to life span, habitat preference is also a relevant factor. In the warmer season, Lake 260 is thermally stratified. In the whole lake study, EE_2 was added to the the upper water layer (i.e. the epilimnion). This resulted in higher EE_2 concentrations in the epilimnion (approx. 4–6 ng/L) than in the deeper water layers, the meta- and hypolimnion (approx. 1–2 ng/L; Palace et al. 2006, 2009). *P. promelas* and *M. margarita*, which mainly inhabit the shallow littoral zones of the epilimnion, were therefore probably exposed to higher EE_2 concentrations than *S. namaycush*, which mainly inhabits the metalimnion and upper hypolimnion, and *C. commersoni*, which mainly inhabits the hypolimnion. In the latter two species, exposure can be expected to increase temporarily during their feeding migrations to the epilimnion as well as in autumn, when the water layers mix. Timing / seasonality of reproduction is another important factor. For example, in *S. namaycush*, maturation of the gonad from its post-breeding quiescent form and spawning occur in winter, when Lake 260 was ice-covered and no EE_2 was added. By contrast, gonadal maturation and spawning of *P. promelas* occur in late spring and summer, i.e. during the period of EE_2 additions (Palace et al. 2009).

[5] Fish that have not yet reached an age of one year.

Table 5: Comparison of the sensitivities of different fish species to the estrogen receptor agonist 17α-ethinylestradiol. For more detailed information on the tests and additional studies with the included fish species see Table 16 in the annex. Grey shading indicates apical endpoints (mainly based on OECD 2011a; see Table 11).

Species	Type of test, test duration		Endpoint		LOEC	Reference
Danio rerio	Short-term screening test	8 – 10 d	Vitellogenin in ♂		1.1 [m] – 3.58 ng/L [m]	Rose et al. 2002, Duis & Knacker 2003
	Fish sexual development test (shorter than TG 234)	40 d	Vitellogenin in ♂		1.5 ng/L [m]	Örn et al. 2003
			Delayed sexual differentiation in ♂		1 ng/L [n] (< 0.6 ng/L [m])	
	Partial life-cycle test	3 mo	Inhibition of gonad development [1]		0.1 ng/L [n]	Van den Belt et al. 2003
	Two-generation test	315 d	F_0: Growth of juveniles, d 42–78		1.1 ng/L [m, 10]	Wenzel et al. 2001a, b
			F_0: Time to first reproduction			
			F_0: Fecundity (number of eggs / ♀ and d)			
			F_0: Fertilisation rate			
			F_1: Growth of juveniles, d 35–75		0.3 ng/L [m]	
			F_1: Fecundity (no eggs/♀/day)		2.0 ng/L [m]	
			F_1: Fertilisation rate$_1$		2.0 ng/L [m]	
Pimephales promelas	Short-term reproduction test (gonadal recrudescence assay) [2] Reproduction evaluated in subsequent 3-week period in control water	21 d exposure	Vitellogenin in ♂ and ♀		1 ng/L [n]	Pawlowski et al. 2004
			Secondary sexual characteristics in ♂ (nuptial tubercles)		1 ng/L [n]	
			Ultrastructure of testes		1 ng/L [n]	
			Fecundity (number of eggs / spawning pair) [11]	Increase	0.1 ng/L [n, 3]	
				Reduction	100 ng/L [n]	
	Full life-cycle test starting < 24 hpf	301 d	F_0: Ovotestes, d 56 and d 172		4.0 ng/L [n]	Länge et al. 2001
			F_0: Vitellogenin, d 172		16 ng/L [n]	
			F_0: Egg production		> 1.0 ng/L [n, 4]	
			F_1: Length d 28		≤ 0.2 ng/L [n]	
			F_1: Weight, d 28		1.0 ng/L [n]	
	Life-cycle test starting 48–60 hpf, F_1 only evaluated until hatch	155 d	F_0: Fertilisation rate		≤ 0.32 ng/L [n, 10]	Parrott & Blunt 2005
			F_0: Sex ratio			
			F_0: Ovipositor index in ♀		3.2 ng/L	
			F_0: Secondary sexual characteristics in ♂		0.96 ng/L [n]	
Oryzias latipes	Short-term screening test	28 d	Vitellogenin in ♂		≤ 10 ng/L [n]	Scholz et al. 2004
	Short-term reproduction test	14 d	Vitellogenin in ♂ and ♀		500 ng/L [n]	Tilton et al. 2005
			Fecundity (number of eggs / spawning pair and day)	Increase	0.2 ng/L [n, 5]	
				Reduction	500 ng/L [n]	

Species	Type of test, test duration		Endpoint	LOEC	Reference
			Fertilisation rate	500 ng/L [n]	
			Ovarian estradiol release	0.2 ng/L [n]	
O. latipes (continued)	Short-term reproduction test	21 d	Vitellogenin in ♂	63 ng/L [m]	Seki et al. 2002
			Fecundity (number of eggs / spawning pair and day)	488 ng/L [m]	
	Partial life cycle test (2 mo exposure starting with newly-hatched fish, followed by 6 wk recovery period; effects determined at the end of the recovery period)	2 mo + 6 wk	Sex ratio	100 ng/L [n]	Scholz & Gutzeit 2000
			Gonadosomatic index in ♀	10 ng/L [n]	
			Fecundity (number of eggs / ♀ and day)	10 ng/L [n]	
Rutilus rutilus	Fish sexual development test starting with freshly fertilised eggs	84 d	Vitellogenin	4 ng/L [m]	Katsu et al. 2007
			Morphological sex ratio (feminisation)	Effect at 0.3 ng/L [m, 6]	
Cyprinus carpio	Short-term screening test with juveniles (9.5°C)	10 d	Vitellogenin	10 ng/L [n]	Purdom et al. 1994
Onco-rhynchus mykiss	Short-term screening test with males (16.5°C)	10 d	Vitellogenin	≤ 0.1 ng/L [n]	
	Test for vitellogenin induction with juveniles at 11.4 and 17.4°C	28 wk	Vitellogenin in ♂ (11.4°C)	0.3 ng/L [n]	Sheahan et al. 1994
			Vitellogenin in ♂ (17.4°C)	1.0 ng/L [n]	
Salvelinus namaycush	Short-term screening test with juveniles	21 d	Vitellogenin in ♂ and ♀	≤ 15 ng/L [m, 7]	Werner et al. 2003
			Gonadosomatic index in ♂ and ♀		
Cyprinodon variegatus	Short-term screening test with adult males	16 d	Vitellogenin in males	Effect at 109 ng/L [m, 6]	Folmar et al. 2000
	Partial life-cycle test starting with juveniles	73 d	Intersex	Effect at ≥ 20 ng/L [n, 6]	Zillioux et al. 2001
			Testes histology: fibrosis	Effect at ≥ 2 ng/L [n, 6]	
			Fecundity (number of eggs / ♀ and day)	Effect at ≥ 20 ng/L [n, 6]	
Pomatoschistus minutus	Short-term screening test with adult males	16 d	Vitellogenin in ♂	6 ng/L [n, 6, 8]	Robinson et al. 2003
			Secondary sexual characteristics in ♂	6 ng/L [n, 8, 10]	
			Fecundity (number of fertile eggs / ♀)		
			Fertilisation rate		
Fundulus heteroclitus	Short-term reproduction test [8]	21 + 7 d [9]	Vitellogenin in ♂ (d 21 and 28)	100 ng/L [n, 10]	Peters et al. 2007
			Gonadosomatic index in ♂ (d 28)		
			Plasma estradiol levels in ♀ (d 28)	10 ng/L [n]	
			Total number of eggs / ♀	100 ng/L [n]	

(1) Based on macroscopic evaluation. (2) In gonadal recrudescence assays, mature *P. promelas*, which have been maintained under simulated winter conditions (short day length, low temperatures) and therefore exhibit regressed secondary sex characteristics and gonad maturation, are subjected to increasing photoperiod and temperature regime and exposed to a test substance to determine potential effects on gonadal

recrudescence, i.e. maturation of the gonad from its regressed form (Pawlowski et al. 2004). (3) At 0.1 and 1 ng/L, the number of eggs was significantly increased compared to the control. (4) Reproduction not evaluated at $\geq$ 4 ng/L due to lack of phenotypic males at these concentrations. (5) At the lowest test concentration (0.2 ng/L), the number of eggs was significantly increased compared to the control. (6) No statistical evaluation. (7) Lowest tested concentration. (8) 17α-Ethinylestradiol was used as positive control in a test with sewage effluent. Therefore, only a single EE_2-concentration was used. (9) Males and females were separately exposed for 21 d. Subsequently, half of the fish were sampled to determine effects on vitellogenin levels. The remaining fish were further exposed for 7 d. During this period, reproduction was evaluated. (10) The same effect concentration was obtained for several endpoints (see left). (11) Due to the high variability of fecundity, the relative short test duration and the fact that usually few concentrations are tested in short-term reproductive assays no reliable NOEC or EC_x for fecundity can be derived.

Bisphenol A and 4-tert-octylphenol

Effects of bisphenol A on apical endpoints were studied in a full life-cycle test with *D. rerio*, a long-term (164 d) reproductive study with *P. promelas* and a sexual development test with *O. latipes* (see Table 5). In the full-life cycle test with zebrafish, a LOEC of 157 µg/L (mean measured concentration; 1500 µg/L nominal) was obtained based on the apical endpoints growth of the F_0 (75 dpf), time to first spawn, fecundity and fertilisation success (Schäfers & Wenzel 2000, Segner et al. 2003a,b, Wenzel et al. 2001b, Teigeler et al. 2007). In the long-term reproductive study with fathead minnow, a LOEC of 1280 µg/L was derived for cumulative fecundity and a LOEC of 640 µg/L for hatching of the F_1 (nominal values, measured concentrations ranged from 70 to 96% of the nominals; Sohoni et al. 2001). In the sexual development test with medaka, a LOEC of 1820 µg/L (measured; 2000 µg/L nominal) was obtained for the endpoints growth and sex ratio (Yokota et al. 2000). Great care has to be taken when comparing effect concentrations given that the test methods are very different. For example, the long-term study of Sohoni et al. (2001) does not include the most sensitive life stages. Yet, based on these three studies effect concentrations for apical endpoints in *D. rerio*, *P. promelas* and *O. latipes* were roughly in the same order of magnitude[6].

In both, the full life-cycle test with *D. rerio* and the long-term reproductive study with *P. promelas*, effects on indicative endpoints were observed at lower concentrations than effects on apical endpoints. In *D. rerio*, the LOEC for vitellogenin induction in males and gonad histology was 40 µg/L, a concentration that is by a factor of 4 lower than the most sensitive effect on apical endpoints. In *P. promelas*, effects on gonad histology were observed at ≥ 16 µg/L (Table 5).

The two available LOEC values for the induction of vitellogenin in male zebrafish illustrate the variation that may occur between different tests with a single species. While a LOEC of 7.5 µg/L[7] was derived in a very recently published short-term screening test (Villeneuve et al. 2012), a LOEC of 40 µg/L was determined for adult fish at the end of a full life-cycle test (see above). The higher sensitivity of the ELISA used in the short-term screening test and the fact that three replicates were used in screening test but only two in the full life-cycle test may have contributed to the difference between the LOEC values. Moreover – as already mentioned in the previous section for effects on EE_2 on vitellogenin levels in *P. promelas* – a homeostatic response (mediated by a decrease in steroid production) might also have contributed to the

[6] One order of magnitude corresponds to the factor of 10.

[7] Based on mean measured concentrations. Based on nominal concentrations, the difference between these two LOEC values is even higher. In the short-term study that was performed using a flow-through system, the LOEC based on nominal concentrations is 10 ng/L (Villeneuve et al. 2012). In the life-cycle test that was performed under semi-static conditions, mean measured concentrations were considerably below nominals (Teigeler et al. 2007). Based on nominal concentrations, the LOEC for vitellogenin is 375 µg/L.

reduced sensitivity of zebrafish following longer-term exposure as suggested by Villeneuve et al. (2012). Similarly, the LOEC for vitellogenin induction in male fathead minnow was lower in a short short-term screening test (81 µg/L: Villeneuve et al. 2012) than in the long-term reproduction test (160 µg/L; Sohoni et al. 2001). LOEC values for vitellogenin induction in other fish species range from 100 µg/L in goldfish (*Carassius auratus*; Ishibashi et al. 2001) to 3120 µg/L in medaka (Kang et al. 2002).

Based on the compiled data (Table 5) there are strong indications for a particularly high sensitivity of effects on spermatogenesis. In the above-mentioned long-term reproduction test with fathead minnows, an inhibition of spermatogenesis occurred at a bisphenol A concentration of ≥ 16 µg/L (Sohoni et al. 2001). In a long-term (2 month) test with brown trout (*Salmo trutta* f. *fario*) density and motility of sperm were affected at the lowest tested concentration of 1.75 µg/L (Lahnsteiner et al. 2005). Notably, these effects were only observed at the beginning and, for motility, in the middle of the spawning season. Since motility is an indicator of sperm maturity, Lahnsteiner et al. (2005) concluded that bisphenol A is causing a delay of approx. four weeks in sperm maturation in brown trout. In female brown trout, gamete maturation was even affected more strongly: ovulation was delayed by approx. 2–3 weeks at bisphenol A concentrations of 1.75 and 2.4 µg/L, and completely suppressed at 5.0 µg/L (Lahnsteiner et al. 2005)[8].

Delays in male sexual development, reproductive behaviour and reproduction have often been observed upon exposure to estrogen receptor agonists (Schäfers 2003, Nash et al. 2004, Scholz & Klüver 2009). They are particularly relevant for seasonal spawners, for which timing of reproduction is crucial. A delay in sexual development as a consequence of direct inhibition by an EDC or of energy lack due to compensatory processes can prolong the duration of a sensitive life stage and cause the loss of an age class (Crain et al. 2007). Such a delay should be detected in a full life-cycle test, in which time to first spawning is an endpoint (OECD 2004a, 2008b, 2011a; see also Table 11 in the annex). However, in the available full life cycle test with the zebrafish, time to first spawn was only increased at a mean measured bisphenol A concentration of 157 µg/L (Table 5, Table 14 in the annex). Thus zebrafish, which are continuous spawners (see Table 3), appear to be less sensitive to bisphenol A than the seasonal spawner brown trout. Slower metabolism of bisphenol A in salmonid species as was observed for rainbow trout in comparison to zebrafish might contribute to a higher sensitivity of salmonids (Lindholst et al. 2003). The suppression of ovulation in brown trout females exposed to 5.0 µg/L of BPA is a clear indicator of the higher sensitivity of this species as compared to zebrafish and fathead minnow.

For 4-tert-octylphenol, effects on apical endpoints were evaluated in full life-cycle tests with *D. rerio* and *O. latipes*. The NOEC values obtained for both species are very similar: 12 µg/L for

[8] It should be noted that in the short-term screening test of Villeneuve et al. (2012) an only slightly higher LOEC of 7.5 µg/L was derived for effects on vitellogenin levels in male zebrafish and female fathead minnows (see Table 5).

zebrafish (Wenzel et al. 2001a) and 9.9 µg/L for medaka (Japanese Ministry of the Environment 2006 as cited in OECD 2011a; see also Table 6). In addition, effects on the apical endpoint sex ratio were studied in fish sexual development tests with *D. rerio*, *O. latipes* and *Gasterosteus aculeatus* within the validation of OECD test guideline 234. LOEC values determined in three laboratories ranged from ≤ 13.8 to 26.0 µg/L for zebrafish and from ≤ 11.2 to 50.4 µg/L for medaka. Thus, it was concluded that these two species are equally sensitive to 4-tert-octylphenol (OECD 2011d). In stickleback (*G. aculeatus*), no significant effects on sex ratio were observed at concentrations up to 66.9 and 41.9 µg/L in two laboratories. As systemic toxicity was already observed at at 66.9 and 130.6 µg/L, respectively, higher concentrations could not be evaluated with regard to effects on sex ratio (OECD 2011d).

In the fish sexual development test, effects on the indicative endpoint vitellogenin were also investigated. LOEC values in all three species were in the same order of magnitude: 26.0–42.5 µg/L in zebrafish, ≤ 12.1 to 105 µg/L in medaka and > 41.9 and 66.9 µg/L in stickleback (OECD 2011d). LOEC values for vitellogenin induction in males obtained in a short-term reproduction test with *P. promelas* were in the same range (0.8–37 µg/L, Biever et al. 2007). Similarly, LOEC values for vitellogenin induction in other fish species range from 10 µg/L in *O. mykiss* to 100 µg/L in *R. rutilus* (Routledge et al. 1998; see Table 6). For *Cyprinodon variegatus* significant effects were already observed at the lowest tested concentration of 11.5 µg/L (Karels et al. 2003). Based on the available data, there are no indications of a particularly sensitive species, but an indication for a relatively low sensitivity of the guppy (*Poecilia reticulata*). This is probably related to the fact that the metabolic capacity of guppies is relatively high (Schäfers 1998).

It is of note that 4-tert-octylphenol concentrations that were shown to have endocrine effects are relatively close to concentrations causing systemic toxicity. This was observed in the fish sexual development test with stickleback (see above) and – in one of three participating laboratories – in the fish sexual development test with zebrafish (OECD 2011d) and the short-term reproduction test with fathead minnow (Biever et al. 2007). In a fish early life stage test with *O. mykiss*, effects on growth were observed at 11 µg/L, i.e. at the same concentration as vitellogenin induction (Analytical Bio-Chemistry Laboratories Inc. 1986 as cited in OECD 1995b).

Table 6: Comparison of the sensitivities of different fish species to the estrogen receptor agonist bisphenol A. For more detailed information on the tests see Table 14 in the annex. Grey shading indicates apical endpoints (mainly based on OECD 2011a; see Table 11).

Species	Type of test, test duration		Endpoint	LOEC [1]	Reference
Danio rerio	Short-term screening test with adults	4 d	Vitellogenin in ♂	7.5 ug/L [m]	Villeneuve et al. 2012
			Vitellogenin in ♀	> 81 ug/L [m]	
	Full life-cycle test starting with freshly fertilised eggs, semi-static	205 d	Vitellogenin in ♂	40 ug/L [m]	Schäfers & Wenzel 2000, Segner et al. 2003a, b, Wenzel et al. 2001b, Teigeler et al. 2007
			Histologic alterations in gonads		
			Growth, 75 dpf	157 ug/L [m, 5]	
			Time to first spawn		
			Fecundity (number of eggs / ♀)		
			Fertilisation success		
			Hatching success of F_1 and survival of F_1 until 35 dpf	> 157 ug/L [m, 5]	
Pimephales promelas	Short-term screening test with adults	4 d	Vitellogenin in ♂	81 ug/L [m]	Villeneuve et al. 2012
			Vitellogenin in ♀	7.5 ug/L [m]	
	Long-term reproductive test starting with adults fish (F_0), flow-through (164 d), F_1 only evaluated until hatch	164 d	Vitellogenin in ♂, F_0, d 71 and 164	160 ug/L [n]	Sohoni et al. 2001
			Gonadosomatic index in ♀, d 164	640 ug/L [n]	
			Reduced proportion of spermatozoa in testes	16 ug/L [n]	
			Cumulative fecundity (total number of eggs / ♀)	1280 ug/L [n]	
			Hatching of F_1	640 ug/L [n]	
Oryzias latipes	Short-term reproduction test	21 d	Vitellogenin in ♂	3120 ug/L [m]	Kang et al. 2002
			Gonadosomatic index in ♂ and ♀	> 3120 ug/L [m, 5]	
			Cumulative fecundity (total number of eggs / pair)		
			Fertilisation rate		
	Fish sexual development test starting with freshly fertilised eggs	approx. 70 d	Sex ratio	1820 ug/L [m, 5]	Yokota et al. 2000
			Growth (length, weight)		
Xiphophorus helleri	Short-term screening test starting with 30 d-old fish	60 d	Reduced sword length in ♂	2 ug/L [n]	Kwak et al. 2001
Carassius auratus	Short-term screening test with adults	28 d	Vitellogenin in ♂	100 ug/L [n]	Ishibashi et al. 2001

Species	Type of test, test duration		Endpoint	LOEC [1]	Reference
Oncorhynchus mykiss	Short-term screening test with juveniles	12 d	Vitellogenin	556 ug/L [m, 2] EC_{50}: 95 ug/L [m]	Lindholst et al. 2000, 2003
Salmo trutta f. *fario*	Chronic test with late prespawning and spawning adults	2 mo	Reduction of sperm density [3]	≤ 1.75 ug/L [n, 5]	Lahnsteiner et al. 2005
			Reduction of sperm motility [4]		
			Reduction of swimming velocity of sperm [3]		
			Reduction of male semen mass	5 ug/L [n, 5]	
			Suppression of ovulation in females		

(1) If not indicated otherwise. (2) Very clear effects were already observed at 70 ug/L. Due to the small number of fish used effects observed at 70 and 100 ug/L were not significant. (3) Significant reduction at the beginning and in the middle of the spawning period, but not at the end of the spawning period. (4) Significant reduction at the beginning of the spawning period, in the middle of the spawning period significant effect only at next higher concentration (2.4 ug/L), at the end of the spawning period no significant effect. (5) The same effect concentration was obtained for several endpoints (see left).

Table 7: Comparison of the sensitivities of the different fish species to the estrogen receptor agonist 4-tert-octylphenol. For more detailed information on the tests see Table 15 in the annex. Grey shading indicates apical endpoints (mainly based on OECD 2011a; see Table 11).

Species	Type of test, test duration		Endpoint	LOEC [1]	Reference
Danio rerio	Fish sexual development test	approx. 65 d	Vitellogenin	Lab 1: 40.6 ug/L [m] Lab 2: 42.5 ug/L [m] Lab 3: 26.0 ug/L [m]	OECD 2011d
			Sex ratio	Lab 1: ≤ 13.8 ug/L [m] Lab 2: 17.6 ug/L [m] Lab 3: 26.0 ug/L [m]	
	Full life-cycle test starting with fertilised eggs	185 d	F_0: growth (d 78)	35 ug/L [m, 2] (NOEC: 12 ug/L [m, 2])	Wenzel et al. 2001a
			F_0: time to first spawning		
			F_0: number of eggs / ♀ and d		
			F_0: fertilisation rate		
			F_0: sex ratio	> 35 ug/L [m]	
Pimephales promelas	Short-term screening test for endocrine effects	14 d	Vitellogenin in ♂	EC_{50}: 48.2 ug/L	Brian et al. 2005
	Fish short-term reproduction test	21 d	Vitellogenin in ♂	Lab A: 37 ug/L [m] Lab B: 31 ug/L [m] Lab C: 0.8 ug/L [m]	Biever et al. 2007
			Secondary sexual characteristics in ♂ (tubercle score)	Lab A: 37 ug/L [m] Lab B: 98 ug/L [m] Lab C: 42 ug/L [m]	
			Fecundity (eggs per ♀ and day)	Lab A: 120 ug/L [m] Lab B: 98 ug/L [m] Lab C: > 120 ug/L [m]	
			Fertisation rate (%)	Lab A: 120 ug/L [m] Lab B: 98 ug/L [m] Lab C: > 120 ug/L [m]	
Oryzias latipes	Fish sexual development test	approx. 65 d	Vitellogenin	Lab 4: 105 ug/L [m] Lab 5: ≤ 12.1 ug/L [m] Lab 9: 12.3 ug/L [m]	OECD 2011d
			Sex ratio	Lab 4: ≤ 11.2 ug/L [m] Lab 5: 30.6 ug/L [m] Lab 9: 50.4 ug/L [m]	

Species	Type of test, test duration		Endpoint	LOEC [1]	Reference
	Full life-cycle test (no details available)	n.i.	Vitellogenin in ♂	NOEC: 4.3 ug/L	Japanese Ministry of the Environment 2006 as cited in OECD 2011a
			Ovotestis	NOEC: 9.9 ug/L	
Gasterosteus aculeatus	Fish sexual development test	approx. 65 d	Vitellogenin	Lab 6: 66.9 ug/L [m] Lab 8: > 41.9 ug/L [m]	OECD 2011d
			Sex ratio	Lab 6: > 66.9 ug/L [m] Lab 8: > 41.9 ug/L [m]	
Poecilia reticulata			Growth of ♂	200 ug/L [n]	Toft & Baatrup 2003
			Sex ratio	> 200 ug/L [n]	
			Secondary sexual characteristics in ♂ (coloration index)	200 ug/L [n]	
Zoarces viviparus	Short-term screening test with adult ♂ for endocrine effects	21 d	Vitellogenin in ♂	35 ug/L [m]	Rasmussen et al. 2005
			Gonadosomatic index in ♂	35 ug/L [m]	
Oncorhynchus mykiss	Fish early life stage test (started post-hatch)	60 d	Growth	11 ug/L [m]	Analytical Bio-Chemistry Laboratories Inc. 1986 as cited in OECD 1995b
	Short-term screening test for endocrine effects with adult ♂	21 d	Vitellogenin in ♂	10 ug/L [m]	Routledge et al. 1998
Rutilus rutilus	Short-term screening test for endocrine effects with adults	21 d	Vitellogenin in ♂	100 ug/L [m]	
Cyprinodon variegatus	Short-term screening test for endocrine effects with adults	24 d	Vitellogenin in ♂	≤ 11.5 ug/L [m]	Karels et al. 2003

(1) If not indicated otherwise. (2) The same effect concentration was obtained for several endpoints (see left).

Prochloraz

Celander et al. (2011) used the effects of prochloraz on *D. rerio*, *P. promelas* and *O. latipes* as reported in the OECD ring test (OECD 2006b) and the studies of Kinnberg et al. (2007) and Zhang et al. (2008) as case study for evaluating a mechanism of action based framework for interspecies extrapolation. For prochloraz, the mechanism of action in all three studied fish species is the same (Celander et al. 2011). Prochloraz inhibits the enzyme aromatase (CYP19) and thus the conversion of androstendione to estrone and of testosterone to estradiol (Zarn et al. 2003, Sanderson 2006). Unlike mammals that have a single cyp19 gene, most teleost species have two isoforms: cyp19a1 (aromatase A), which is mainly expressed in the gonads and cyp19a2 (aromatase B), which is mainly expressed in the brain[9]. An assessment of homology of cyp19a1 and cyp19a2 in *D. rerio*, *P. promelas* and *O. latipes* indicated that the protein sequences and, therefore, the three-dimensional structures of the two enzymes in the three fish species were very similar. Accordingly, the three model fish species had a very similar sensitivity to prochloraz (Celander et al. 2011).

Within the present project, additional studies were included in the comparative evaluation of the sensitivity of *D. rerio*, *P. promelas* and *O. latipes* to prochloraz (see Table 7). Effects on apical endpoints were studied in fish sexual development tests with zebrafish and fathead minnow. In these two species, LOEC values for sex ratio were comparable: ≤ 60 to >434 µg/L in zebrafish and 284 to 301 µg/L for fathead minnow (Kinnberg et al. 2007, Thorpe et al. 2011, Holbech et al. 2012). In male fish, a significant reduction of growth was observed at similar concentrations (at 297 µg/L in zebrafish and at ≥ 88 µg/L in fathead minnow; Thorpe et al. 2011).

In both species, effects on indicative endpoints were investigated in screening tests and in sexual development tests. With regard to a reduction of the vitellogenin levels in female fish, lowest effect concentrations for both species were comparable: for zebrafish 67 to >217 µg/L in screening tests (OECD 2006b) and 48 to 202 µg/L in sexual development tests (Kinnberg et al. 2007, Thorpe et al. 2011, Holbech et al. 2012), and for fathead minnow 121 to 299 µg/L in screening tests (OECD 2006b) and ≤ 29 to 106 µg/L in sexual development tests (Thorpe et al. 2011, Holbech et al. 2012). Due to much lower background levels reductions in the vitellogenin content of male fish are more difficult to detect and the resulting effect concentrations are more variable (see Table 7). In their evaluation of the validation study for the fish sexual development test, Holbech et al. (2012) concluded that with mean LOECs of 134 µg/L (*D. rerio*) and 293 µg/L (*P. promelas*) for effects on sex ratio, and mean LOECs of 110 µg/L (*D. rerio*) and 68 µg/L (*P. promelas*) for reduced vitellogenin level in females, both species were similarly sensitive to prochloraz.

[9] In addition, prochloraz is an agonist of the aryl hydrocarbon receptor (AhR; Sturm et al. 2001), i.e. it induces other cyp genes and can therefore affect catabolism of steroid hormones (Celander et al. 2011; see also section 1.3.4.

For *O. latipes*, a LOEC of 30 µg/L was derived for cumulative fecundity in a short-term reproduction test with a test duration of only 7 d (Zhang et al. 2008). This value is lower but in the same order of magnitude than the LOEC of 116 µg/L for cumulative fecundity derived in a short-term reproduction test with *P. promelas* and a test duration of 21 d (Ankley et al. 2005). Since effects on vitellogenin levels were evaluated on the gene expression level in the study of Zhang et al. (2008) and on the protein level in all other studies, a comparison of effect concentrations is difficult.

Overall, variation between different tests with the same species appears to be higher than variations between species (see Table 7 and Table 17 in the annex).

Table 8: Comparison of the sensitivities of different fish species to the aromatase inhibitor prochloraz. For more detailed information on the tests see Table 17 in the annex. Grey shading indicates apical endpoints (mainly based on OECD 2011a; see Table 11).

Species	Type of test, test duration		Endpoint	LOEC	Reference
Danio rerio	Fish screening test with adult ♂ and ♀	21 d	Vitellogenin ↓ in ♀	67 [m] – >217 ug/L [m]	OECD 2006b [1]
			Vitellogenin ↓ in ♂	No effect	
	Fish sexual development test	60 d	Vitellogenin ↓ in ♀	48 [m] – 202 ug/L [m]	Kinnberg et al. 2007, Thorpe et al. 2011, Holbech et al. 2012 (see also OECD 2011e) [1]
			Vitellogenin ↓ in ♂	44 [m] – >320 ug/L [m]	
			Sex ratio	≤ 60 – >434 ug/L [m]	
		60 d	Total length of ♂	297 ug/L [m]	Thorpe et al. 2011
Pimephales promelas	Fish screening test with adult ♂ and ♀	21 d	Vitellogenin ↓ in ♀	121 [m] – 299 ug/L [m]	OECD 2006b [1]
			Vitellogenin ↓ in ♂	No effect	
	Short-term reproduction test	21 d	Vitellogenin ↓ in ♀	≤ 23 [m] – >220 ug/L [m]	Ankley et al. 2005, Biever et al. 2007 [1]
			Vitellogenin ↓ in ♂	No effect	
			Cumulative fecundity (eggs / ♀ and d)	116 ug/L [m]	Ankley et al. 2005
	Fish sexual development test	60 d	Vitellogenin in ♀	≤ 29 ug/L [m]	Holbech et al. 2012 [1] (see also OECD 2011e)
			Sex ratio	284 ug/L [m]	
		102 d	Vitellogenin in ♀	106 ug/L [m]	
			Sex ratio	301 ug/L [m]	
		125 d	Total length of ♂	88 ug/L [m]	Thorpe et al. 2011
			Vitellogenin ↓ in ♀	88 ug/L [m, 2]	
			Vitellogenin ↓ in ♂		
			Sex ratio	294 ug/L [m]	
Oryzias latipes	Short-term reproduction test	7 d	Cumulative fecundity (total number of eggs / ♀)	30 ug/L [n]	Zhang et al. 2008
			Expression of gene for vitellogenin I in liver of ♀ ↓	300 ug/L [n]	
			Expression of gene for vitellogenin II in liver of ♀ ↓	≤ 3 ug/L [n]	

(1) Ring test with several participating laboratories. (2) The same effect concentration was obtained for several endpoints (see left).

2.2.4 Summary: extrapolation between fish species

Most studies on endocrinology and endocrine disruption with fish have been carried out with teleosts. Within teleost species, there seem to be few major differences in the reproductive endocrine system. Consequently, the primary effects of sexual endocrine disrupting substances on different fish species are comparable, although single endpoints may vary in their sensitivity (e.g. ovotestis). If a suite of endpoints is studied as is the case in the fish screening tests for endocrine effects (test guidelines 229 and 230, OECD 2009c, d), the fish sexual development test (TG 234, OECD 2011c) and the fish full life cycle test (OECD 2008b), and results of similar tests are compared, effect concentrations in different fish species with similar metabolic capacities are often in the same order of magnitude. This applies especially to effects on apical test endpoints. In most cases, effects on indicative endpoints are also observed at comparable concentrations.

However, data for the evaluated estrogen agonists show that there appears to be a tendency towards a lower sensitivity of medaka (*O. latipes*) and guppy (*P. reticulata*) and a higher sensitivity of salmonids, which is linked to higher metabolic capacities of medaka and guppy and slower metabolism in salmonids.

In addition, gamete maturation seems to be a particularly sensitive endpoint for estrogen agonists, especially in salmonids. In the seasonal spawner brown trout (*S. trutta* f. *fario*), effects of bisphenol A on sperm density and motility were observed at concentrations that were by a factor of 9 lower than the bisphenol A concentration affecting spermatogenesis in *P. promelas*. As timing of reproduction is crucial for seasonal spawners, this issue deserves further study.

It should also be noted that there is a lack of knowledge on endocrine disruption in minor taxonomic groups.

2.2.5 Extrapolation between aquatic invertebrate species

Extrapolation from vertebrates to invertebrates and vice versa is very difficult (IPCS 2002, Matthiessen & Johnson 2007). While natural and synthetic estrogens and androgens have, for instance, very strong effects on fish, they have in many cases little or no effect on arthropods (Segner et al. 2003a, b, Young et al. 2004, Sumpter & Johnson 2005, Breitholtz et al. 2006). In cases where effects are observed, the type of effect is often different from the type of effect observed in vertebrates (see sections 2.2.6 and 2.2.7). This is obviously related to the substantial differences between the endocrine systems of vertebrates and invertebrates. Endocrine disrupting effects as well as the underlying endocrine processes and receptor homologies have been thoroughly studied in fish (see e.g. review by Tyler et al. 1998) and in other vertebrates, but to a much lower extent in invertebrates (Stahl et al. 1999, Oehlmann & Schulte-Oehlmann 2003, OECD 2006a, Oehlmann et al. 2011).

The present evaluation of the feasibility to extrapolate between invertebrate species is mainly based on (1) reviews resulting from the 'Workshop on endocrine disruption in invertebrates: endocrinology, testing, and assessment (EDIETA)' (deFur et al. 1999a, b, Ingersoll et al. 1999, LeBlanc et al. 1999, Stahl et al. 1999), (2) reviews from a special issue of the journal 'Ecotoxicology', in which the progress in research on endocrine disruption in aquatic invertebrates since the EDIETA workshop was described (Duft et al. 2007, Hutchinson 2007, Lagadic et al. 2007, LeBlanc 2007, Oehlmann et al. 2007, Soin & Smagghe 2007, Tarrant 2007,

Verslycke et al. 2007, Weltje & Schulte-Oehlmann 2007) and (3) additional reviews by Oehlmann & Schulte-Oehlmann (2003), OECD (2006a, 2010a) and Kortenkamp et al. (2012).

As mentioned in section 2.1.3, invertebrate species are extremely diverse and heterogeneous in their biology and physiology. Overall, there are more than 30 different invertebrate phyla compared to only one vertebrate phylum (LeBlanc et al. 1999, Oehlmann & Schulte-Oehlmann 2003; for an overview of freshwater and marine invertebrate phyla see Fig. 7 in section 2.1.3). Diversity within the different invertebrate phyla is also high. There are, for example, more than 66,000 known crustacean species that differ in their physiology and life strategies (Breitholtz et al. 2006, LeBlanc 2007), and more than 130,000 mollusc species that differ in their mode of reproduction and life-cycle strategies (Oehlmann et al. 2007, OECD 2010a).

Life cycles of invertebrates include a number of specific hormone controlled processes that are not present in most vertebrate species, such as moulting, metamorphosis (with a huge diversity of larval forms), pupation, polyphenism (the occurrence of various phenotypes in a population, which are not based on genetic differences), diapause or other resting stages, pheromone production, and limb regeneration (McHugh & Rouse 1998, DeFur et al. 1999a, LeBlanc et al. 1999, IPCS 2002, Oehlmann & Schulte-Oehlmann 2003, Soin & Smagghe 2007, Jacobs & Podolsky 2010, Oehlmann et al. 2011). Invertebrates vary in their mode of reproduction. For example, species can reproduce asexually, sexually or both. In case of sexual reproduction, fertilisation can be external or internal (McHugh & Rouse 1998, Ingersoll et al. 1999). Some species reproduce continuously or almost continuously (continuous iteroparity), while other reproduce cyclically (seasonal iteroparity) or only once during lifetime (semelparity). Many invertebrates have highly complex reproductive cycles (Stahl et al. 1999, IPCS 2002). Consequently, the endocrine systems of invertebrates are very diverse (LeBlanc et al. 1999, Stahl et al. 1999, Oehlmann et al. 2011).

2.2.6 Overview of aquatic invertebrate endocrinology

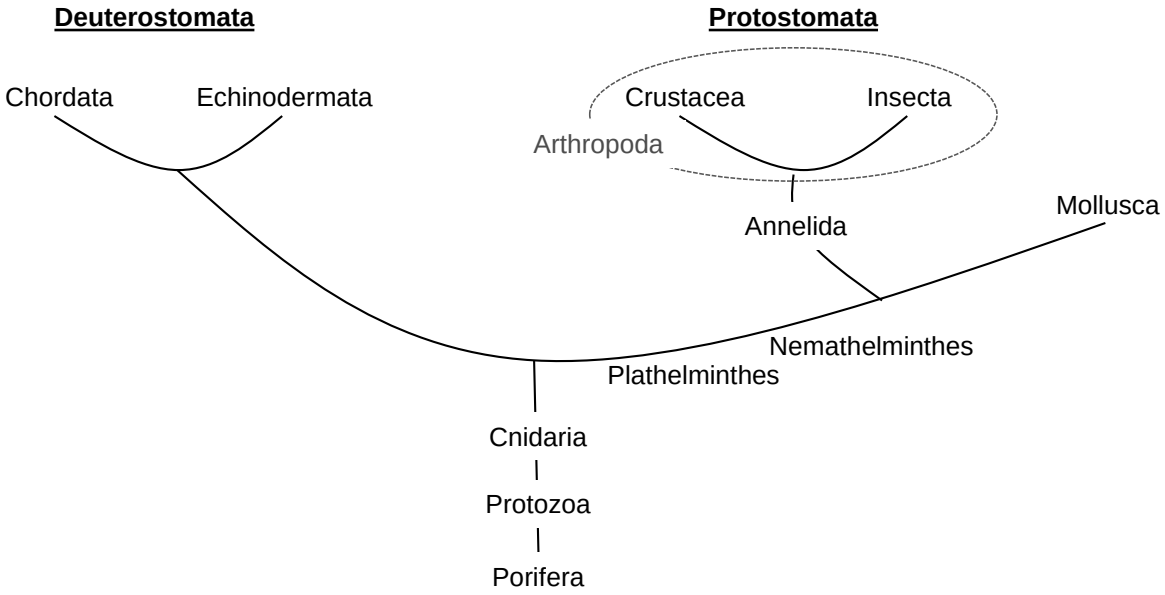
Knowledge on the underlying endocrine processes is a crucial requirement in order to identify if observed adverse effects on apical endpoints are caused by endocrine disruption or by secondary effects on the endocrine system (DeFur et al. 1999b, Ingersoll et al. 1999, Stahl et al. 1999, Soin & Smagghe 2007, Weltje & Schulte-Oehlmann 2007). Therefore, a brief overview of the current knowledge on endocrinology of the major groups of aquatic invertebrates is given in the present section.

The most detailed knowledge is available on the endocrine system of insects, especially on those insect species and hormones that are targeted by insecticides (LeBlanc et al. 1999, Stahl et al. 1999, Soin & Smagghe 2007, Oehlmann et al. 2011). Thus, more information is available for terrestrial insect species than for aquatic insects (Soin & Smagghe 2007). There is also a considerable amount of information on endocrinology of some crustacean species, mainly decapods that are relevant for commercial and recreational fisheries (Ingersoll et al. 1999, LeBlanc et al. 1999, Stahl et al. 1999, OECD 2006a). By contrast, limited information is available on the endocrine system of most other taxonomic groups (LeBlanc et al. 1999, Breitholtz et al. 2006, Matthiessen & Johnson 2007). In many cases, knowledge is fragmentary. Only single or few species of an invertebrate group have been investigated, and knowledge is restricted to relatively few hormonal processes (DeFur et al. 1999b, Oehlmann & Schulte-Oehlmann 2003, Oehlmann et al. 2011).

Invertebrates rely on steroid, terpenoid and peptide hormones. Their hormone-secreting structures are often of neuronal origin (neurosecretory cells or organs) and the endocrine system is closely linked to the nervous system (DeFur et al. 1999b, LeBlanc et al. 1999). Invertebrates possess (a) vertebrate-type hormones, i.e. hormones that have developed from common ancestral molecules (e.g. the neurotransmitter / neurohormone serotonin) and (b) hormones that are specific to invertebrates (e.g. ecdysteroids; Lafont 2000, Tarrant 2007, Kortenkamp et al. 2012).

Most of the main invertebrate phyla belong to the protostomes that diverged early in evolution from the deuterostomes, which include the vertebrates (see Fig. 8). This evolutionary divergence corresponds to major differences in endocrinology (LeBlanc et al. 1999, OECD 2006a). While reproduction in deuterostome invertebrates is, for example, regulated by vertebrate-type sex steroids, protostome invertebrates rely to a much lower extent on vertebrate-type steroids. Instead, reproduction in lower protostomes is regulated by neuropeptides and reproduction in insects and crustaceans is regulated by ecdysteroids and terpenoids (LeBlanc et al. 1999).

Fig. 8: Phylogeny of metazoans including the main invertebrate groups based on Storch & Welsch (1991) and LeBlanc et al. (1999).



Vertebrate-type sex steroids have been detected in a range of invertebrate taxa. While there is evidence for a functional role of these hormones in echinoderms and molluscs, their possible role in other invertebrate groups is in most cases still unclear (DeFur et al. 1999a, OECD 2006a). In addition, there are still substantial gaps with regard to our knowledge on sex steroids receptors in many invertebrate phyla. As emphasized by OECD (2006), structurally related molecules may have other functions in invertebrates than in vertebrates. For instance, in the rotifer *Brachionus manjavacas* progesterone appears to induce the transition from asexual to sexual reproduction. Hence, this hormone seems to be conserved over a wide range of phyla, yet with a changed function (Stout et al. 2010).

Various hormone groups are specific to invertebrates (i.e. not found in vertebrates), for example ecdysteroids that regulate moulting, embryonic development, metamorphosis and

reproduction in arthropods. Terpenoids (e.g. juvenile hormones in insects and methyl farnesoate in crustaceans) are also specific to arthropods. They contribute to the regulation of embryogenesis, development and reproduction (deFur et al. 1999a, OECD 2006a; see also Table 8). These specificities in the endocrine system of invertebrates result in specific susceptibilities to endocrine disrupting chemicals (IPCS 2002).

In addition, the neuroendocrine system in invertebrates is more diverse than in vertebrates. Neuropeptide hormones acting as endocrine regulators in invertebrates include for example moult-stimulating and moult-inhibiting-hormones in arthropods, egg-laying hormones in molluscs, regeneration-stimulating hormones in annelids and crustaceans and metamorphosis-stimulating hormones in cnidarians. These neurohormones either regulate the production or secretion of a terminal hormone (e.g. an ecdysteroid or a terpenoid) or directly regulate endocrine processes (DeFur et al. 1999a, LeBlanc et al. 1999, OECD 2006a).

In the following, a very brief overview of important features of the endocrinology of the main groups of aquatic invertebrates is given. For an overview of hormones playing important roles in the major invertebrate taxa see Table 8. Different species within larger taxonomic groups exhibit important similarities in their endocrine system (e.g. the use of ecdysteroids as moulting hormones by arthropods). However, it should also be noted that differences in hormonal processes, which are related to differences in physiology and life history, can be found between different species or taxonomic groups within a single class or phylum (Oehlmann et al. 2007, see also below).

Cnidarians, which are positioned at the stem of the invertebrate phyla (Fig. 8) do not possess defined endocrine glands. Instead, regulatory substances are mainly secreted by neurons. They include neuropeptides, such as LW-amides and RF-amides, and retinoids as well as vertebrate-type sex steroids such as 17β-estradiol (OECD 2006a, Tarrant 2007). There are still large gaps in the current knowledge on endocrine systems in cnidarians (Tarrant 2007).

In annelids, neurosecretory cells synthesise neuropeptides such as FRMFamide. Ecdysteroids have also been found in some annelids, but their function has not been elucidated. Methyl farnesoate, juvenile hormone, fatty acids and eicosatrienoic acid are involved in metamorphosis and reproduction (LeBlanc et al. 1999, OECD 2006a).

The endocrine system of insects consists of neurosecretory cells in the central nervous system, the gonads and three endocrine glands (LeBlanc et al. 1999). Neuropeptides, ecdysteroids and terpenoids are the most important hormones (Soin & Smagghe 2007). Neuropeptides, which are secreted from the neurosecretory cells into the hemolymph, regulate growth, moulting and reproduction (LeBlanc et al. 1999, Lafont 2000). Moulting (ecdysis) is, for instance, controlled by prothoracicotropic hormone (PTTH) that is released from neurosecretory cells in the brain. PTTH leads to the synthesis and secretion of ecdysone, a prohormone that is converted to 20-hydroxyecdysone (20E), which induces moulting (Soin & Smagghe 2007). Embryonic development, metamorphosis and reproduction are regulated by ecdysteroids and juvenile hormones. The latter are terpenoids that modulate the effects of ecdysteroids. Four slightly different juvenile hormones have been identified so far (juvenile hormones 0, I, II and III, see also Table 8) with juvenile hormone III being the most widespread form. The related terpenoid methyl farnesoate has been identified in dipterans. So far, there is no information on juvenile hormone receptors (Soin & Smagghe 2007). PTTH, juvenile hormones and diapause hormone are involved in the regulation of diapause. Most of these hormones are unique to insects and

related arthropods (LeBlanc et al. 1999, Lafont 2000, Soin & Smagghe 2007). The majority of agricultural insecticides interact with ecdysteroids or juvenile hormones (OECD 2006a).

Crustaceans possess a complex endocrine system, in which neuropeptides regulate the production of hormones by the endocrine organs, such as the Y-organ, the mandibular organ, the androgenic gland and the sinus gland. The peptide hormones include moult-inhibiting hormone, which inhibits production of ecdysteroids by the Y-organ (LeBlanc et al. 1999), and androgenic hormone, which stimulates sexual differentiation in males (DeFur et al. 1999a). Apart from the peptide hormones, ecdysteroids and terpenoids are the most important hormones in crustaceans (OECD 2006a, LeBlanc 2007). Ecdysteroids (ecdysone that is converted to 20E, 3-dehydroecdysone and 25-deoxyecdysone) are secreted by the Y-organ, and regulate moulting (LeBlanc et al. 1999). Ecdysteroids are also involved in embryogenesis and reproduction (OECD 2006a). The fact that ecdysteroids are structurally similar to steroid estrogens explains that the latter may affect moulting in crustaceans (Zou & Fingerman 1997a, OECD 2006a). Testosterone and a number of known estrogen receptor agonists (e.g. bisphenol A and 4-nonylphenol) appear to function as anti-ecdysteroid in crustaceans (Mu & LeBlanc 2002, LeBlanc 2007). Methyl farnesoate (the unepoxidated form of the insect juvenile hormone III) is produced by the mandibular organ, and is involved in regulation of ecdysteroid synthesis (Lafont 2000, LeBlanc 2007). Methyl farnesoate is the most important terpenoid hormone of crustaceans. It is involved in metamorphosis, gonad maturation and reproduction. In daphnids, high levels of methyl farnesoate lead to the production of male offspring. So far only limited information on the methyl farnesoate signalling pathway is available (LeBlanc 2007). Diapause is assumed to be under neuro-endocrine control (LeBlanc et al. 1999). Vertebrate-type steroids that have been detected in some crustaceans might be involved in reproduction (OECD 2006a). Some aspects of the endocrine system differ between different crustacean classes. Larval development in decapod crustaceans is, for example, inhibited by methyl farnesoate, while the same hormone has a stimulatory effect on larval development in barnacles (Cirripedia; LeBlanc 2007).

The endocrine system of molluscs consists of neurosecretory centres in the cerebral, pleural, pedal and abdominal ganglia of the central nervous system, which produce neuropeptides (LeBlanc et al. 1999). FMRFamide that regulates various physiological processes (including heartbeat) is one of the most widespread neuropeptides. Other neuropeptides are involved in the regulation of reproduction (e.g. egg-laying homone), growth and development (OECD 2006a). Vertebrate-type sex steroids (e.g. testosterone, progesterone) are produced in the gonads. Ecdysteroids and juvenoids have been detected in some mollusc species, but their function is unknown (OECD 2006a). It should be pointed out that the hormone system of molluscs is very diverse. Differences are found between different classes and also within a single class as is the case for the gastropods with their three subclasses (prosobranchs, pulmonates, opisthobranchs; Oehlmann et al. 2007, OECD 2010a). For example, differences in metabolism of the androgen precursor androstendione were found between different species of the Muricidae, a prosobranch family (Lyssimachou et al. 2009). In addition, knowledge on endocrinology of some mollusc groups (e.g. aquatic pulmonate snails) is still relatively limited (Lagadic et al. 2007).

Due to their relative close evolutionary relationship to vertebrates, the endocrine system of echinoderms shares more similarities with vertebrates (both echinoderms and vertebrates are deuterostomata) than with the abovementioned protostomata. For example, echinoderms

produce vertebrate-type sex steroids (progesterone and testosterone) and possess an estradiol receptor. Apart from steroids, neuropeptides are involved in the control of reproduction (LeBlanc et al. 1999, OECD 2006a).

Table 9: Examples of important hormones reported in major invertebrate taxa based on LeBlanc et al. (1999), Oehlmann & Schulte-Oehlmann (2003), OECD (2006a), Lagadic et al. (2007), LeBlanc (2007), Soin & Smagghe (2007) and Tarrant (2007). Please note that some of these hormones may occur only in selected species or groups and not in the whole taxon.

Taxon	Hormone type	Example	Controlled process
Porifera	Unknown	Unknown	Unknown
Cnidaria	Neuropeptides	LW-amides	Metamorphosis, muscle contraction
		RF-amides	Release of gametes, feeding, muscle contraction
	Thyroids	Thyroxine	Strobilation
	Retinoids	9-cis-Retinoic acid	Strobilation
	Steroids	17β-Estradiol	Reproduction
Nematoda	Ecdysteroids	Unknown	Unknown
	Terpenoids	Juvenile hormone-like hormones	Growth
	Neuropeptides	FMRFamide	Neuromodulation
Annelida	Ecdysteroids	Ecdysone	Unknown
	Neuropeptides	FMRFamide	Neuromodulation
		Gonadotropin	Vitellogenesis
	Terpenoids	Eicosatrienoic acid	Metamorphosis
		Aracidonic acid	Unknown
Insecta	Ecdysteroids	Ecdysone (a prohormone that is converted to 20-hydroxyecdysone (20E))	Growth and development, moulting, gonad maturation, reproduction (egg maturation, vitellogenesis)
	Neuropeptides	Prothoracicotropic hormone (PTTH)	Control of ecdysteroid production
		Allostatin	Inhibition of juvenile hormone production
		Allatotropin	Stimulation of juvenile hormone production
		FMRFamides	Neuromodulation
		Diapause hormone	Initiation of diapause
	Terpenoids	Juvenile hormones 0, I, II and III, methyl farnesoate	Modulation of ecdysteroid action (moulting / metamorphosis and reproduction)
Crustacea	Ecdysteroids	Ecdysone	Moulting, embryogenesis, reproduction (vitellogenesis)
	Steroids	Testosterone	Uncertain
		17β-Estradiol	Uncertain
		Progesterone	Uncertain
	Terpenoids	Methyl farnesoate	Ecdysteroid production (metamorphosis, gonad maturation, reproduction)

Taxon	Hormone type	Example	Controlled process
Crustacea (continued)	Neuropeptides	Androgenic gland hormone (androgenic hormone)	Sexual differentiation in males, vitellogenesis inhibition
		Crustacean hyperglycemic hormones	Energy metabolism
		Moult-inhibiting hormone	Inhibition of ecdysteroid production
		Mandibular organ-inhibiting hormone	Inhibition of methyl farnesoate production
		Gonad inhibiting hormone (vitellogenesis-inhibiting hormone)	Inhibition of gonad maturation and vitellogenesis
Mollusca	Ecdysteroids	Unknown	Unknown
	Steroids	Testosterone	Sexual differentiation, reproduction
		17β-Estradiol	Sexual differentiation, reproduction
		Progesterone	Sexual differentiation, reproduction
	Terpenoids	Juvenile hormone	Questionable
	Neuropeptides	APGWamide	Sexual differentiation, gonad maturation, spawning
		Dorsal body hormone	Sexual differentiation in females, vitellogenesis, oocyte maturation
		Egg-laying hormone	Spawning
		FMRFamide	Various physiological processes (incl. regulation of heartbeat), egg laying
		Molluscan insulin-like peptides	Growth, development, energy metabolism
Echinodermata	Steroids	Progesterone	Reproduction (vitellogenesis, oogenesis, spermatogenesis, spawning)
		Testosterone	
		17β-Estradiol	
		Estrone	
	Neuropeptides	Gonad-stimulating substance	Spawning
		Maturation-promoting factor	Fertilisation
Tunicata	Steroids	Testosterone	Oogenesis, spermatogenesis, spawning
		17β-Estradiol	
	Neuropeptides	Gonadotropin releasing hormone analogue	Gonad development
	Thyroids	Thyroxine	Probably tunic formation

2.2.7 Differences in sensitivity to EDCs between aquatic invertebrate species

Within the present project, a comprehensive evaluation of interspecies differences in sensitivity of aquatic invertebrates to endocrine disrupting substances was not feasible. Such an evaluation would require a detailed review of all available data on the effects of EDCs on invertebrates. Moreover, further systematic studies of the sensitivity of different invertebrate species / taxa to substances with different endocrine mechanisms of action are required in order to fill gaps in the available data. As outlined by Oehlmann et al. (2011), 37% of the available studies on endocrine disruption in (aquatic and terrestrial) invertebrates have been performed with crustaceans, 36% with molluscs, 11% with insects, 7% with echinoderms, 5% with annelids, 2% with cnidarians, 1% with rotifer and less than 1% with nematodes, tunicates and sponges, respectively. This also means that the approximately 20 other invertebrate phyla have not been studied at all. Consequently, there are a number of invertebrate groups, for which the available information on endocrine disruption is too sparse to systematically evaluate interspecies differences (OECD 2006a). In addition, most of the available studies have focused on effects on reproductive endpoints and on moulting (Kortenkamp et al. 2012). Effects on other endocrine endpoints / pathways have been addressed to a much lower extent.

The development of a database on susceptibility of invertebrates to endocrine disruptive chemicals with special focus on those endocrine processes, which are specific to invertebrates, was already suggested by DeFur et al. (1999b). Yet to our knowledge, such a database is not yet available.

In the present section, we have instead compiled information on interspecies differences in sensitivity of aquatic invertebrates to EDCs and on factors that contribute to such differences. The present evaluation is mainly based on the reviews mentioned in section 2.2.5 and on the data compiled for the model substances bisphenol A, 4-tert-octylphenol, tributyltin and triphenyltin[10]. In the following, we will first address some factors that are relevant for interspecies differences in invertebrates. Then, we will outline the major findings for the model substances.

Sensitivity of the same hormone or hormonal pathway to endocrine disruption can vary between species (OECD 2006a). For receptor-mediated effects, differences in the structure of the ligand-binding domain (for instance between different insect orders) result in different binding affinity of an EDC to the receptor, as observed e.g. for 20E analogues (LeBlanc et al. 1999).

As mentioned above interspecies differences in sensitivity to pollutants can also be caused by differences in metabolic capacities. This is, for example, the case for molluscs, which have a limited capacity to metabolise and excrete organic chemicals. The consequences are a higher bioaccumulation as compared to other species and, consequently, a high sensitivity to organic pollutants including – but not restricted to – endocrine disrupting substances (Lee 1986, Oehlmann et al. 2007). This is outlined in further detail below, for effects of TBT on molluscs.

The type of effect may vary between species, given that in different invertebrate taxa structurally similar hormones may have very different functions (Lafont 2000, OECD 2006a). This is e.g. the case for methyl farnesoate in decapods and cirripeds (see section 2.2.6). Accordingly, insect growth regulators that act as methyl farnesoate mimics in crustaceans have contrary effects on these two groups of crustaceans: they delay metamorphosis in decapods, but stimulate metamorphosis in cirripeds (LeBlanc 2007). An unexpected type of effect was also observed by Hahn et al. (2001) for tebufenozide. In the target species (Lepidoptera), this insecticide stimulates precocious moulting, which leads to death (Dhadialla et al. 1998). However, in *Chironomus riparius* exposed to tebufenozide concentrations of 10 – 30 µg/L no effects on larval moults and pupation were observed. Yet, emergence was significantly reduced at ≥ 17.4 µg/L of tebufenozide. Thus, an inhibitory effect was observed on the final moult from pupae to adults (Hahn et al. 2001).

Within a single phylum, the pattern of species sensitivity may even vary for substances with a similar endocrine mechanism of action. As reviewed by Ingersoll et al. (1999) and Hutchinson (2002), different crustacean taxa differ in their sensitivity to endocrine disruption. Such

[10] As mentioned in section 1.3 data compilation for 17α-ethinylestradiol and prochloraz focused on studies on endocrine disruption in fish.

differences were e.g. found between two estuarine crustaceans, the grass shrimp *Palaemonetes pugio* (Decapoda, Palaemonidae) and the estuarine mud crab *Rhithropanopeus harrisii* (Decapoda, Panopeidae). In most cases, *R. harrisii* was more sensitive to juvenile hormone analogues than *P. pugio*. For example, when exposed to (S)-methoprene during complete larval development, metamorphic success in *R. harrisii* was by a factor of 10 more sensitive than in *P. pugio*. By contrast, *P. pugio* was by a factor of 5 more sensitive to fenoxycarb (reviewed by McKenney 2005).

Consequences at population level depend on the life-cycle of the species. Mobile species can to a certain extent avoid exposure, while this is not possible for sessile species as barnacles (LeBlanc 2007). As noted below, the extent to which prosobranch populations are affected by imposex depends on the existence of planktonic larvae (Matthiessen & Gibbs 1998, Oehlmann et al. 2007; see also next section). While sessile invertebrate species can be expected to be most vulnerable, aquatic insects with flying adult stages have a high potential for recolonisation (Soin & Smagghe 2007).

The organotins: tributyltin and triphenyltin

The masculinisation of female gastropods by tributyltin (TBT) is one of the clearest examples of endocrine disruption in invertebrates (Matthiessen & Gibbs 1998, OECD 2010a). At the same time, it is a clear example of interspecies differences and – even more importantly – of gaps in the current testing framework for endocrine disrupting substances (OECD 2010a). Masculinised snails – female dogwhelk *Nucella lapillus* that had developed a penis – were first reported in 1970 in Plymouth harbour (UK; Blaber 1970). Since male characteristics (penis and / or sperm duct) were superimposed onto females, this condition was termed imposex (Smith 1971). A similar effect, termed intersex response (the transformation of the oviduct into a non-functional prostate) was observed later in *Littorina littorea* (Bauer et al. 1995). Levels of imposex and intersex were shown to be associated with TBT leaching from antifouling paints and with declines of the population of many affected species (see e.g. Gibbs & Bryan 1996, Matthiessen & Gibbs 1998, OECD 2010a).

Imposex apparently only occurs in prosobranch snails, where is has been documented for more than 180 species (Oehlmann et al. 2011). Given that prosobranchs are not yet part of the current set of tests for potential EDCs, this effect would have been missed in an ERA (Schulte-Oehlmann et al. 1996, Matthiessen & Gibbs 1998, Sumpter & Johnson 2005). In *N. lapillus*, TBT concentrations of ≤ 1.1 ng Sn/L[11] induced imposex (Davies et al. 1997), in the Eastern mudsnail (*Ilyanassa obsoleta*) TBT concentrations of ≥ 1.0 ng Sn/L (Gooding et al. 2003). In other prosobranch snails, LOECs for the induction of imposex were higher, e.g. 20.5 ng Sn/L for

[11] There are no indications that the different forms of tributyltin (mainly tributyltin chloride and tributyltin oxide) differ in their toxicity. In order to allow a comparison of tests, in which organisms were exposed to different forms of tributyltin, effect concentrations were converted to the concentration of Sn where possible, i.e. where information is provided on the form of TBT that was used in the respective test.

Hexaplex trunculus and *Bolinus brandaris* (Abidli et al. 2012; see Table 18 in the annex). In addition, there are prosobranch species, in which exposure to TBT does not lead to the development of imposex (Gibbs et al. 1997, Schulte-Oehlmann et al. 1997).

The extent to which reproduction is affected by imposex also varies strongly between different gastropod species. In some species (e.g. *I. obsoleta* and *Nassarius reticulatus*), imposex seems to have little effect on reproduction. By constrast, in other species (e.g. *N. lapillus* and *Ocenebra erinacea*) imposex in its final stages results in sterility (Matthiessen & Gibbs 1998, Schulte-Oehlmann et al. 1996). The extent of population decline in the field also depends on the life cycle of the respective snail species. For species with planktonic larvae (e.g. *L. littorea*) a recolonisation of affected areas is much easier than for species lacking a planktonic larval phase (e.g. *N. lapillus*; Matthiessen & Gibbs 1998, Oehlmann et al. 2007, OECD 2010a).

Bivalve molluscs also proved to be highly sensitive to TBT. Effects on growth and survival of Pacific oyster (*Crassostrea gigas*) were found at concentrations of ≥ 20 ng Sn/L (His & Robert 1983, His 1991). TBT has probably contributed to the decline of populations of European flat oyster (*Ostrea edulis*), e.g. in the U.K. However, this has not been unequivocally proven given that the effects of TBT on bivalves have been studied to a much lower extent than those on prosobranch gastropods (OECD 2010a).

The overall high sensitivity of molluscs to TBT is at least partly due to their limited metabolic capacity (Oehlmann et al. 2007). In the liver of vertebrates and the hepatopancreas of invertebrates, TBT is metabolised by cytochrome P450 dependent monooxygenases (Lee 1986, Schulte-Oehlmann et al. 1996, Fent 1998). As molluscs possess less cytochrome P450 dependent monooxygenases than crustaceans and vertebrates, they have a much lower capacity to metabolise and, thus, detoxify, TBT (Lee 1986). Consequently, they accumulate TBT to a greater extent than crustaceans and vertebrates (Schulte-Oehlmann et al. 1996).

Overall, TBT is one of the substances with highest toxicity to aquatic organisms (OECD 2010a). However, effect concentrations of TBT in other organisms are generally higher than in molluscs. Chronic toxicity to *Daphnia magna* was observed at 0.91 µg Sn/L (Oberdörster et al. 1998). In a fish full life-cycle test with *Cyprinodon variegatus*, a LOEC of 0.27 µg Sn/L was obtained (Manning et al. 1999). Yet, in a sexual development test with *Danio rerio*, effects on sex ratio were observed at TBT concentrations ≥ 0.041 ng Sn/L (McAllister & Kime 2003), i.e. in a similar or even lower order of magnitude than effects on molluscs. Clear evidence of population declines is, however, restricted to molluscs.

The situation appears to be similar for triphenyltin (TPT), although much less data are available for this compound than for TBT. Triphenyltin was shown to induce imposex in some but not all studied prosobranch gastropods (Schulte-Oehlmann et al. 2000), an observation which illustrates that even within certain classes of invertebrates cross-species extrapolation might not be easy (Oehlmann & Schulte-Oehlmann 2003).

Effects of TPT were studied in *Marisa cornuarietis*, *Nucella lapillus*, and *Potamopyrgus antipodarum* using water-only test systems, and in *Nassarius reticulatus* and *P. antipodarum* using water-sediment tests with exposure via spiked sediment (see Table 19). There were considerable differences in the type of observed effect between the studied prosobranch species. In *M. cornuarietis*, imposex – indicated by a concentration-dependent increase of the vas deference sequence index (VDSI) and the penis sheath length in females – already occurred at the lowest tested concentration (75 ng Sn/L[12]). Additionally, fecundity was reduced at all tested concentrations, and penis length in males was reduced at ≥ 250 ng Sn/L (Schulte-Oehlmann et al. 2000). By contrast, exposure of *N. lapillus*, *N. reticulatus* and *P. antipodarum* did not lead to imposex development (Schulte-Oehlmann et al. 2000, Duft et al. 2003a, 2007, Albanis et al. 2006). Yet, other effects were observed in these species, in most cases already at the lowest tested concentration. In *N. lapillus*, exposure to TPT led for instance to a strong increase in the incidence of tissue excrescences, e.g. on gills and pallial sexual organs, with a LOEC of ≤ 5 ng Sn/L (Schulte-Oehlmann et al. 2000). In *P. antipodarum*, fecundity was reduced with LOEC values of ≤ 30 ng Sn/L in the water-only system (Albanis et al. 2006, Duft et al. 2007). Hence, the LOECs for the most sensitive effect in these two species were in the same order of magnitude as the LOEC derived for imposex in *M. cornuarietis*. In water-sediment tests, effects were also observed at the lowest tested concentrations. A LOEC of ≤ 10 µg/kg sediment dry weight (dw) was derived for *P. antipodarum* based on fecundity (Duft et al. 2003a), and a LOEC of ≤ 50 µg/kg sediment dw for *N. reticulatus* based on an increased incidence of atrophy in both female and male gonads (Schulte-Oehlmann et al. 2000).

The xenoestrogens: bisphenol A and 4-tert-octylphenol

A range of laboratory studies have shown that molluscs (more specifically prosobranch gastropods) are also extremely sensitive to bisphenol A (BPA). BPA has a strong estrogenic effect on prosobranch gastropods: it increases fecundity. In the freshwater snail *Marisa cornuarietis*, an EC10-value of 14 ng/L was derived for an increase in egg production (Schulte-Oehlmann et al. 2001). Affected *M. cornuarietis* developed highly enlarged reproductive tracts including extra female organs, enlarged sex glands and gross malformations of the pallial oviduct section, a condition referred to as 'superfemales'. At bisphenol A concentrations of about 1 µg/L and above, these malformations and the overstimulation of oogenesis and spawning lead to an increased mortality in the affected snails (Oehlmann et al. 2000). The initial studies demonstrating effects of environmental relevant BPA concentrations on *M. cornuarietis* have caused considerable controversy and several follow-up experiments. Based on the latter it was concluded that the superfemale response can be observed before and after but not during the main spawning season. The effect is visible at lower temperatures (20°C, 22°C), but it is at least in part masked at a temperature of 27°C (Oehlmann et al. 2006a, b, Crain et al. 2007). These findings underline the importance of the test conditions in tests for endocrine disruption.

[12] Effect concentrations were converted to the concentration of Sn where possible.

With an increased embryo production at concentrations above 1 – 5 µg/L the freshwater snail *Potamopyrgus antipodarum* was also very sensitive to BPA (Schulte-Oehlmann et al. 2001, Jobling et al. 2004). Interestingly, concentrations of BPA and 17α-ethinylestradiol that caused an increased embryo production in *P. antipodarum* were identical (Jobling et al. 2004). A similar increase in egg production was also observed in the marine prosobranch *Nucella lapillus* with effects at the lowest tested BPA concentration (1 µg/L, Oehlmann et al. 2000).

Some crustacean species exhibit a similar sensitivity to bisphenol A than molluscs, while others are much less sensitive. For instance, in a two-generation test with the harpacticoid copepod *Tigriopus japonicus* naupliar development was significantly delayed at BPA concentrations ≥ 0.1 µg/L in the parental generation (F_0). In the offspring (F_1), such a delay was already observed at 0.01 µg/L. Moreover, time to sexual maturity was increased at the highest tested concentration (1 µg/L) in the F_0 and at all tested concentrations (0.01 – 10 µg/L) in the F_1. However, effects on fecundity and sex ratio were neither observed in the F_0 nor in the F_1 (Marcial et al. 2003). In the calanoid copepod *Acartia tonsa* exposed to 20 µg/L of BPA, a stimulation of egg production was observed on day 10 of the experiment, but not on days 9 and 11 (Andersen et al. 1999). Thus, further studies are needed to evaluate potential effects of bisphenol A on copepod reproduction.

By contrast, cladocerans appear to exhibit a much lower sensitivity to BPA. Reproduction of *Ceriodaphnia dubia* was reduced at ≥ 1.88 mg/L (Tatarazako et al. 2002). In *D. magna*, naupliar development was only delayed at concentrations ≥ 8 mg/L, and reproduction was reduced at approx. 7–10 mg/L (Mu et al. 2005).

Similarly low toxicity with effect concentrations in the low mg/L range was also reported for the rotifer *Brachionus calyciflorus* (Springborn-Smither Laboratories 2006a cited in Wright-Walters et al. 2011), the sponge *Heteromyenia* sp. (Hill et al. 2002) and the hydrozoans *Hydra vulgaris* and *Hydra oligactis* (Pascoe et al. 2002, Fukuhori et al. 2005).

For 4-tert-octylphenol, the situation is similar, but differences between the various taxa are less distinct than for bisphenol A. Again, prosobranchs (*M. cornuarietis*, *N. lapillus*, *P. antipodarum*) are highly sensitive to 4-tert-octylphenol with LOEC values in the low µg/L-range (Oehlmann et al. 2000, Duft et al. 2003b, Jobling et al. 2004). Copepods (*T. japonicas*, *A. tonsa*) exhibit a similar sensitivity than the prosobranchs (Andersen et al. 2001, Marcial et al. 2003), while *D. magna* is less sensitive (Analytical Bio-Chemistry Laboratories Inc. 1988, cited in OECD 1995b and in IUCLID 2000, Zou & Fingermann 1997b). In addition, there are indications of a high sensitivity of echinoderms to 4-tert-octylphenol: embryonic development of the sea urchin *Strongylocentrotus purpuratus* was delayed by 4-tert-octylphenol with an EC_{50} of 0.174 µg/L (Roepke et al. 2005).

2.2.8 Summary: extrapolation between invertebrates

Endocrine systems of invertebrates differ substantially from those of vertebrates. In addition – given that invertebrate species are extremely diverse in their biology and physiology – there are also considerable differences between the endocrine systems of various invertebrate taxa. For example, neuroendocrine systems in invertebrates are very diverse. Invertebrates also differ in the type of endocrine glands and in the chemical structure and function of the main hormone groups.

Invertebrate hormones can be distinguished in (a) vertebrate-type hormones and (b) hormones that are specific to invertebrates. The former are more common in deuterostome invertebrates than in protostomes. Vertebrate-type sex steroids are involved in the control of reproduction in echinoderms and molluscs. They have also been detected in other invertebrate groups, but their function in these groups is in most cases not clear.

Hormones that are specific to invertebrates include neuropeptides (e.g. LW- and RF-amides in cnidarians, FMRFamides in nematodes, annelids, insects and molluscs and moult-inhibiting hormone in crustaceans) and ecdysteroids (e.g. ecdysone in annelids, insects and crustaceans) and terpenoids (e.g. juvenile hormones in insects and crustaceans). These hormones are involved in the control of a variety of physiological processes including growth, development, and reproduction as well as processes such as moulting, which are specific to invertebrates. The specificities in the endocrine systems of invertebrates lead to specific susceptibilities of invertebrate species to endocrine active substances.

Only fragmentary information is available on endocrinology of many taxonomic groups. Likewise, studies on endocrine effects on invertebrates have focussed on few invertebrate groups. For this reason, a systematic evaluation of interspecies differences in the sensitivity of aquatic invertebrates to EDCs is not possible. Yet, some conclusions can be drawn from the evaluation of the data compiled for the model substances bisphenol A, 4-tert-octylphenol, tributyltin and triphenyltin.

Both organotins were highly toxic to prosobranch molluscs, i.e. species that have only recently been included in the OECD testing framework for endocrine disrupters and for which standard tests are still being developed (see section 2.1). For TPT, the type of effect varies strongly between different prosobranch species. However, LOECs for the most sensitive effect in the studied prosobranch species are in the same order of magnitude.

Effects of the xenoestrogens bisphenol A and 4-tert-octylphenol on invertebrates were observed at similar or even lower concentrations than effects on fish. Highest toxicity was observed in molluscs, copepods and echinoderms, i.e. species that are not yet part of the OECD testing framework for endocrine disrupters (echinoderms) or that have only recently been included (copepods, molluscs).

2.2.9 Feasibility to select representative test species

Due to specificities in their endocrine systems or, more generally, their physiology it can be assumed that specific groups of wildlife species will be selectively affected by certain EDCs (Sumpter & Johnson 2005; see sections 2.2.1 – 2.2.8). For instance, invertebrate groups with unique characteristics in their endocrinology may be highly sensitive to certain types of endocrine disruption (DeFur et al. 1999b, LeBlanc et al. 1999). In view of the substantial gaps in our current knowledge on endocrine disruption and the underlying endocrine processes in invertebrates, it is difficult to predict which invertebrate taxa or species will be most strongly affected by which endocrine mechanism of action (Ingersoll et al. 1999, Breitholtz et al. 2006, OECD 2006a). This is similar for minor taxonomic groups of fishes, for which knowledge on endocrine disruption is scarce.

This difficulty to predict the most sensitive taxa applies to all endocrine active substances, i.e. to substances interacting with endocrine processes that are specific to invertebrates as well as to substances interacting with vertebrate-type endocrine processes. As detailed in sections 2.2.7

and 2.2.8 invertebrate taxa possessing vertebrate-type hormones (especially molluscs) have been shown to be highly sensitive towards substances affecting vertebrate-type endocrine processes.

2.3 Sensitive time windows for exposure, delayed effects

Effects on endocrine systems can be latent for a substantial amount of time. This is for example the case when effects are induced by short exposure periods during sensitive time windows, but only become apparent when the organisms reproduce (OECD 2006a). In vertebrates, the early life stages, i.e. embryos, foetuses, larvae and juveniles, are often most sensitive to endocrine disruptors. During these stages, endocrine disrupters may interfere with developmental and organisational processes such as sexual differentiation (see e.g. Arcand-Hoy & Benson 1997, Jobling et al. 1998, Piferrer 2001, van Aerle et al. 2002, Ankley & Johnson 2004, Maack & Segner 2004, Knacker et al. 2010, Danish Ministry of the Environment 2011). Exposure during this critical window of sensitivity may lead to effects that might be irreversible (depending on the species, the type of effect and the timing of exposure; see section 2.4), whereas exposure to the same concentration of a compound during adulthood might be compensated for (IPCS 2002, Nichols et al. 2011). However, effects may only become apparent when the organisms are mature and reproduction occurs (OECD 2006a, Matthiessen & Johnson 2007, Nichols et al. 2011). This had led to the concern that effects on populations might only be detected considerable time after the exposure has happened. In view of their potential for serious consequences such delayed irreversible effects have caused greatest concern.

One example for a delayed effect that is induced during a sensitive time window of exposure is the occurrence of ovotestes, i.e. testes that contain single or multiple oocytes (Jobling et al. 2006, Wolf 2011). Ovotestes are known to result from exposure to sewage effluents containing estrogenic substances. Their incidence has been studied especially in roach (*Rutilus rutilus*) in the U.K. (e.g. Jobling et al. 1998, 2006). Ovotestes are often accompanied by the presence of a feminised gonadal duct, which forms a female-like ovarian cavity. Fish that exhibit a feminised gonadal duct and / or ovotestes are called intersex fish, i.e. fish that have been partly converted from one gonadal phenotype to the other (Nolan et al. 2001, Wolf 2011). In roach, an increasing degree of intersex was shown to correlate with reductions in sperm mobility, sperm density and fertilisation success (Jobling et al. 2002).

Ovotestes are induced by exposure of male fish to (xeno-) estrogens during the period of gonadal differentiation. However, in laboratory and field studies ovotestes were not detected in younger roach (i.e. in juveniles or newly mature fish) (Sumpter & Johnson 2005, Jobling et al. 2006): in two studies, in which roach were exposed to sewage effluent from 50 to 200 dph (Rodgers-Gray et al. 2001) or from fertilisation to 300 dph (Liney et al. 2005), exposure to sewage effluent led to a feminisation of the gonadal duct, but no ovotestes were observed at the end of the experiments. Only with increasing age of the fish ovotestes become apparent and more intense (Sumpter & Johnson 2005, Jobling et al. 2006). There was concern that such a delayed effect would only be detected using a full life-cycle test including histopathological evaluation of the gonads. However, as mentioned above a feminisation of the gonadal duct could already be discerned at 50 dph (Rodgers-Gray et al. 2001). In addition, vitellogenin was induced in both studies (Rodgers-Gray et al. 2001, Liney et al. 2005).

For invertebrates, there are also indications of a high sensitivity of the early life stages. For instance, larval stages of crustaceans have been shown to be highly sensitive to juvenile

hormone agonists such as methoprene (McKenney 2005). However, for most invertebrates the available information on endocrinology and endocrine disruption is still too sparse for identifying critical developmental periods with sufficient certainty.

2.4 Irreversibility of effects

There has been concern that exposure to endocrine disruptors may cause irreversible effects (IPCS 2002, Nichols et al. 2011). In a number of studies on endocrine disruption in fish, the reversibility of effects was studied after a post-exposure period in control water.

Recovery depends on the test species, the type of effect, the timing and duration of exposure and the exposure concentration (Schäfers et al. 2007, Nichols et al. 2011). In some cases, the effects caused by previous exposure to EDCs are reversible. After short-term exposure to EDCs, recovery of effects on individual endpoints may occur as was e.g. observed for effects of prochloraz on vitellogenin and estradiol levels in female *P. promelas* (Ankley et al. 2009). Recovery was also observed for effects on secondary sexual characteristics in medaka and for a delay in sexual differentiation in zebrafish (see e.g. review of Scholz & Klüver 2009). While mating behaviour often recovers rapidly, effects on fecundity and fertilisation rate need more time to recover (Nichols et al. 2011).

Exposure to endocrine disruptive substances during the critical window of sensitivity may result in permanent effects on organs or organ systems (Knacker et al. 2010, Nichols et al. 2011; see also section 2.3). Examples for effects that persist during extended recovery periods include intersex and disturbed gonadal development (Scholz & Klüver 2009).

In addition, there is evidence of an only incomplete recovery of effects on the reproductive capacities in cases where exposure started during early life stages (Scholz & Klüver 2009). During a recovery period of 8 months in control water male zebrafish, which had been exposed for 120 days (from the embryonic to the adult life stage) to 5 ng/L of EE_2, changed from female to male phenotype (for further details see section 2.5.1). However, when paired with control females, fertilisation rate was considerably below control values (Larsen et al. 2009). Likewise, a very limited recovery of reproduction was observed in zebrafish, which had been exposed for 177 d starting with fertilisation to a mean measured concentration of 9.3 ng/L of EE_2 (during exposure no spawning was observed) and subsequently kept for three months in control water. Fecundity of these fish was below control levels and the fertilisation rate was extremely low (3% compared to 95% in the control). In addition, pathological alterations in the ovaries had not recovered (Schäfers et al. 2007).

2.5 Behavioural effects

Behavioural changes, which are often among the earliest signs of toxicity, might be difficult to detect and, especially, to quantify. Yet, they might lead for example to a reduced capacity to rear offspring or to cope with other stressors, and to a reduced survival in the field (Lyons 2003, 2006, Scott & Sloman 2004). Effects on foraging behaviour, predator avoidance as well as reproductive and social behaviour appear to be particularly relevant (Scott & Sloman 2004).

The assessment of behaviour may be useful for identifying the endocrine mode of action. It is mentioned in several test guidelines for the assessment of endocrine disruption in fish, namely in the short-term reproduction assay (TG 229), the short-term screening assay (TG 230) and the fish full life-cycle test (see Table A11). However, this does not include a quantitative assessment

of behavioural effects. Yet, methods are available that allow to quantify important aspects of fish behaviour (Scott & Sloman 2004). These methods include e.g. direct observation, video analysis systems and computer systems for behavioural analysis. Several commercial systems are available (see e.g. Kane et al. 2004a, b).

Within this project, a systematic review of the effects of endocrine disrupting substances on fish behaviour was not feasible. Instead we have identified a number of examples for effects on fish reproductive behaviour that are mainly based on the data compiled for the model substances and on reviews (e.g. Scott & Sloman 2004, Kortenkamp et al. 2012).

2.5.1 Effects on fish reproductive behaviour

Successful reproduction depends on the appropriate performance of reproductive behaviour (Scott & Sloman 2004, Kortenkamp et al. 2012). Many fish species exhibit a complex reproductive behaviour (Fiedler 1991). This may include selection of a spawning site, defence of this site or a territory, nest building, courtship behaviour, spawning and nest-caring behaviour (Scott & Sloman 2004).

In fish and other vertebrates, reproductive behaviour is controlled by estrogens and androgens (Eckert & Randall 1986). Thus, effects on the levels of these steroid hormones are likely to lead to changes in sexual behaviour. A range of studies has addressed the effects of endocrine disruptive substances (in most cases natural or synthetic hormones) on elements of the reproductive behaviour of fish. In the following, some examples for studies in which effects of EDCs on reproductive behaviour of fish have been addressed will be described. In order to obtain information on (1) the relevance of changes in reproductive behaviour with regard to the reproductive capacity and (2) the relative sensitivity of effects on the reproductive behaviour as compared with other endpoints, we focused on studies in which additional endpoints – preferably including reproductive endpoints (e.g. fertilisation rate) – were addressed. As the setup of these studies is often complex, the studies are described in a relatively detailed form.

Martinović et al. (2007) investigated the effects of estrone, methyltestosterone and sewage effluent on levels of vitellogenin and 11-keto-testosterone, and on reproductive behaviour of fathead minnow (*P. promelas*). In this species, the acquisition of a spawning territory (including a spawning substrate) is a prerequisite for successful reproduction. Dominant male fathead minnows defend this territory against other males; subordinate males that do not acquire a territory often do not reproduce (Danylchuk & Tonn 2001). In their study, Martinović et al. (2007) exposed male fathead minnows for 21 d to sewage effluent with an estrogenic activity of 44 ng/L (measured as estrogen equivalents using a rainbow trout estrogen receptor binding assay), or to nominal concentrations of 50 ng/L of 17β-estradiol[13] (chosen to mimic the estrogenicity of the effluent) or methyltestosterone. Then, exposed males were individually

[13] Mean measured concentration of 17β-estradiol was 31 ng/L.

placed in aquaria (a) with a nest and two unexposed females (non-competitive scenario) or (b) with a nest, two unexposed females and an unexposed male (competitive scenario) for a period of 5 d. In the absence of competition, males that had previously been exposed to sewage effluent needed twice as long as unexposed control fish to acquire a nest, but their reproductive success (measured as mean number of hatched larvae) did not significantly differ from that of the control males. In the presence of a competing unexposed male only 1 out of 10 sewage effluent-exposed males acquired a nest and reproduced. In this scenario, effluent-exposed males exhibited much lower levels of agonistic (e.g. pushing / biting and chasing) and nest-caring behavioural activities than unexposed males. In the non-competitive scenario, previously 17β-estradiol-exposed males initially acquired nests at a similar rate than control males, but on day 5 the number of nest-holding males was significantly lower than in for control males. Despite this fact, their reproductive success did not differ from that of control males. When unexposed competitors were present, only 20% of the estradiol-exposed males acquired nests and even less of them reproduced successfully. Agonistic and nest-caring activities of the estradiol-exposed males were significantly reduced compared to the unexposed males. As can be expected, males that had been exposed to methyltestosterone were more aggressive than control males and acquired more nests. They had a much higher reproductive success than the controls. Both exposure to sewage effluent and 17β-estradiol led to a significant induction of vitellogenin and a significant reduction of 11-ketotestosterone levels. Martinović et al. (2007) concluded that short-term exposure to estrogens could compromise competitive reproductive fitness of male fish.

A similar study was performed by Salierno & Kane (2009). Male fathead minnows were exposed for 21 d to 10, 20 and 40 ng/L of 17α-ethinylestradiol (EE_2). Reproductive behaviour was then assessed using the same competitive scenario. In the presence of an unexposed male and an unexposed female, head-butting activity (pushing with the head towards the rival male) was significantly reduced in males previously exposed to 40 ng/L of EE_2. Males previously exposed to 20 and 40 ng/L of EE_2 exhibited a significantly lower chasing activity than control males and cleaned the spawning substrate significantly less frequently[14]. A range of significant effects on biomarker[15] and morphological endpoints was observed (see Table 9). At all three studied EE_2 concentrations, vitellogenin was induced, while plasma levels of 11-keto-testosterone, estradiol and testosterone were reduced. In addition, gonadosomatic index and male secondary characteristics (nuptial tubercles) were reduced.

Nash et al. (2004) and Larsen et al. (2009) studied the effects of EE_2 on zebrafish (*D. rerio*) reproduction. Zebrafish are group spawners. Sexual behaviour involves males chasing the

[14] It should be noted that the tested EE_2 concentrations were very high (an LC_{50} of 100 ng/L was derived in a 28 d toxicity test with zebrafish; Wenzel et al. 2001a).

[15] The term biomarker is used for a molecular, cellular or physiological response that can be related to exposure to a toxicant or to toxicity (Hutchinson et al. 2006).

females and leading them to an appropriate spawning substrate. Females do not spawn in the absence of males that trigger spawning (Spence et al. 2008).

Nash et al. (2004) performed a two-generation study starting with adult fish (F_0). At a nominal concentration of 5 ng/L EE_2 (measured: 4.8 ng/L), reproduction of the F_0 was not affected. Yet, complete reproductive failure was observed in the offspring (F_1). In the F_1, no phenotypic males were present (based on the absence of yellow/bronze colouration and bright anal fin markings). In addition, the F_1 fish had either female gonads or gonads that had not yet fully differentiated and contained immature ovary-like tissues (as described in section 2.2.3, *D. rerio* is a juvenile hermaphrodite). None of the fish had normal testes. Despite this fact, fish exhibited normal reproductive behaviour and spawning occurred. However, due to the absence of functional testes the eggs were unfertilised.

Larsen et al. (2009) exposed zebrafish for 120 d, from the embryonic stage to adulthood, to a nominal concentration of 5 ng/L of 17α-ethinylestradiol (measured: 5.6 ng/L). At the end of exposure, 95% of the fish were phenotypic females (i.e. had an indistinct anal fin coloration, a large visible urogenital papilla and a round body shape). Twenty-five of the phenotypic females were kept for eight months in clean water. After this post-exposure period, 8 of these fish had changed into a male phenotype (i.e. had for example large anal fins and slim bodies). In breeding trials with unexposed females, these previously ethinylestradiol-exposed fish performed male courtship behaviour. Yet, they only exhibited the first elements of the courtship behaviour. Larsen et al. (2009) assumed that due to the exposure to ethinylestradiol during the early life stages the complete behavioural sequence could not develop. Despite their incomplete courtship behaviour, the previously ethinylestradiol-exposed fish induced spawning of unexposed females. However, fertilisation rate (23%) was significantly below control values (ca. 90%). Histological analysis revealed that 6 out of the 8 fish had poorly developed testes and that the remaining 2 fish had ovaries.

Balch et al. (2004) exposed Japanese medaka (*O. latipes*) from 2–4 d post-hatch to sexual maturity (at the age of 4–6 months) to nominal concentrations of 0.2, 2 and 10 ng/L of EE_2. Following exposure, reproductive trials were performed by pairing exposed males with two unexposed females, and exposed females with an unexposed male. Following previous exposure to 10 ng/L of EE_2, only about 15% of the male fish and no female fish participated in copulatory activity. This effect was associated with a nearly identical reduction of the percentage of breeding pairs that produced fertilised eggs. Both effects were significant. In females that had been exposed to 2 ng/L of EE_2, a slight but non-significant effect on copulatory activity and fertilised eggs was observed. Exposure to EE_2 was neither correlated with the frequency of the individual copulation events within each reproductive trial nor with the duration of each event (these two parameters were highly variable). Exposure to 2 and 10 ng/L of EE_2 led to a significant induction of ovotestis.

Using a similar approach, Gray et al. (1999a) evaluated copulatory behaviour and reproductive success in Japanese medaka. Following exposure from 1 d to 6 months post-hatch to nominal 4-tert-octylphenol concentrations of 10, 25 and 50 µg/L, reproductive trials were carried out with one previously exposed male and three unexposed females[16]. Significant effects on copulatory behaviour were observed at 25 and 50 µg/L of 4-tert-octylphenol. The number of approaches of the males towards the females and the number of copulations were reduced at 50 µg/L, the number of circles (a courtship behaviour performed by the males) was reduced at 25 and 50 µg/L. These effects were associated with a significantly reduced percentage of males that produced fertilised eggs at 25 and 50 µg/L.

The main results of the abovementioned studies are summarised in Table 9. This table includes effects on reproductive behaviour as well as effects on biomarker endpoints, sexual differentiation, secondary sexual characteristics and, most importantly, reproductive endpoints. In the evaluated studies, effects on reproductive success were as sensitive as behavioural effects. This is the case for all studies, in which both behavioural endpoints and reproductive success were assessed (i.e. Gray et al. 1999a, Balch et al. 2004, Nash et al. 2004, Martinović et al. 2007 and Larsen et al. 2009). In the study of Salierno & Kane (2009), effects on reproduction were not evaluated. However, several biomarker endpoints (e.g. vitellogenin) and secondary sexual characteristics (nuptial tubercles) were more sensitive than behavioural effects. In all other evaluated studies, which include biomarker endpoints and / or an evaluation of sexual differentiation and / or secondary sexual characteristics, these endpoints were as sensitive as behavioural effects (Martinović et al. 2007, Nash et al. 2004, Larsen et al. 2009) or even exhibited a higher sensitivity as was the case for intersex in the study of Balch et al. (2004).

In summary, based on the abovementioned studies reproductive behaviour does not appear to be more sensitive than the other evaluated endpoints. This is in agreement with Kortenkamp et al. (2012), who also concluded that behavioural endpoints were not particularly sensitive.

Most of the other endpoints, which were evaluated in the abovementioned experiments and were at least equally sensitive as behavioural endpoints, are included in fish tests for endocrine disruption. Vitellogenin is evaluated in the fish short-term reproduction assay (TG 229), the short-term screening assay (TG 230) and the fish sexual development test (TG 234), secondary sexual characteristics in TG 229 and 230, sexual differentiation / intersex in TG 234, and the gonadosomatic index as well as levels of estradiol and (keto-) testosterone are evaluated in the fish full life-cycle test (see Table 11).

Two issues, which are related to effects on reproductive behaviour, are briefly outlined in the following:

[16] Medaka were also exposed to 100 µg/L of 4-tert-octylphenol. However, due to their reduced growth, reproductive trials with these fish were performed later, following a recovery period. Therefore, reproductive parameters of fish previously exposed to 100 µg/L were less affected than those of fish exposed to 50 µg/L.

There has been concern that the participation of previously EDC-exposed phenotypic males in spawning might result in a population decline. As mentioned above (see description of the study of Nash et al. 2004), these males are able to induce spawning, but fertilisation success is very low (Nash et al. 2004, Larsen et al. 2009). If the endocrine disruptive potential of a compound is evaluated using a fish sexual development test or a full life-cycle test, such an effect would be detected. As mentioned above, the reduced fertility is caused by a feminisation or delayed development of the male gonads (Nash et al. 2004, Larsen et al. 2009). Such effects on gonadal development would be detected in a fish sexual development test. Reduced fertilisation success would be detected in a full life-cycle test, since fertilisation success is an important endpoint of fish life-cycle tests (see Table 11).

Moreover, it was suspected that a disruption of male reproductive behaviour, such as the abovementioned reproductive failure of male fathead minnows following exposure to sewage effluent or 17β-estradiol, could affect the gene pool of the population, as the number of male fish that participate in reproduction is reduced (Martinović et al. 2007). Effects on the gene pool are discussed in section 5.2.

Table 10: Comparison of effects on reproductive behaviour with effects on biomarker endpoints, secondary sexual characteristics and reproduction for the studies described in section 2.5.1 (↑: increased, ↓: reduced). In cases where more than one concentration was tested, LOEC values are indicated. See text for details on the study design.

Species (sex of exposed fish)	Test substance / effluent (concentration)	Effect on behavioural endpoints (reproductive behaviour) (Type of effect and LOEC)		Effect on biomarker endpoint, sexual development and secondary sexual characteristics (Type of effect and LOEC)		Effect on reproductive endpoints (Type of effect and LOEC)	Reference
Pimephales promelas (♂)	Sewage effluent	Significantly reduced agonistic behaviour (pushing / biting, chasing) Reduced nest-caring behaviour		Vitellogenin ↑ 11-Ketotestosterone ↓ Secondary sexual characteristics ↓		Significantly reduced reproductive success in the presence of competitor	Martinović et al. 2007
	17β-Estradiol (50 ng/L)			Vitellogenin ↑ 11-Ketotestosterone ↓		Significantly reduced reproductive success in the presence of competitor	
	Methyltestosterone (50 ng/L)	Increased agonistic behaviour		Trend towards increased secondary characteristics in ♂		Significantly increased reproductive success in the presence of competitor	
P. promelas (♂)	17α-Ethinylestradiol (10, 20, 40 ng/L)	Reduced head-butting	40 ng/L	Induction of vitellogenin	≤10 ng/L [3]	–	Salierno & Kane 2009
		Reduced chasing activity and cleaning of spawning substrate	20 ng/L	Plasma estradiol ↓			
				11-Ketotestosterone ↓			
				Gonadosomatic index ↓			
				Number of nuptial tubercles ↓			
				Induction of ovipositor	40 ng/L		
Danio rerio[1] (♂ and ♀)	17α-Ethinylestradiol (5 ng/L)	Normal reproductive behaviour in F_1		Feminisation or delay in differentiation of ♂ gonads in F_1		Spawning of F_1 occurred, but the eggs were unfertilised	Nash et al. 2004
D. rerio[2] (♂)	17α-Ethinylestradiol (5 ng/L)	Fish that had changed back to ♂ phenotype (see left) only performed first elements of courtship behaviour		Approx. 1/3 of fish that were phenotypic ♀ at the end of exposure had changed into ♂ phenotype after post-exposure		Spawning induced despite incomplete courtship behaviour	Larsen et al. 2009
				Histology: poorly developed testes or ovaries		Fertilisation rate significantly ↓	

Species (sex of exposed fish)	Test substance / effluent (concentration)	Effect on behavioural endpoints (reproductive behaviour) (Type of effect and LOEC)		Effect on biomarker endpoint, sexual development and secondary sexual characteristics (Type of effect and LOEC)		Effect on reproductive endpoints (Type of effect and LOEC)		Reference
Oryzias latipes (♂)	17α-Ethinylestradiol (0.2, 2, 10 ng/L)	Reduced copulatory behaviour	10 ng/L	Induction of intersex	2 ng/L [3]	Percentage of breeding pairs producing fertilised eggs ↓	10 ng/L	Balch et al. 2004
O. latipes (♀)		Suppression of copulatory behaviour	10 ng/L	–		Percentage of breeding pairs producing fertilised eggs ↓	10 ng/L	
O. latipes (♂)	4-tert-Octylphenol (10, 25, 50 ug/L)	Number of approaches ↓	50 ug/L	–		Percentage of ♂ producing fertilised eggs ↓	25 ug/L	Gray et al. 1999a
		Number of circles ↓	25 ug/L					

(1) Two-generation study starting with adult fish (F_0). (2) Exposure for 120 d, followed by 8 months post-exposure. (3) The same effect concentration was obtained for several endpoints (see left).

2.5.2 Effects on other behavioural responses in fish

Endocrine disruptors may also affect other types of behavioural responses, e.g. predator avoidance (Scott & Sloman 2004). Within the present project, a detailed evaluation of such effects was not possible. However, two examples are given in the following. Threespine sticklebacks (*Gasterosteus aculeatus*) exposed to 3 and 9 µg/L of bis(tributyltin)oxide stayed in less protected areas of the water column and showed a delayed and shorter predator avoidance behaviour (Wibe et al. 2001). Exposure of goldfish (*Carassius auratus*) to prochloraz increased agonistic (i.e. aggressive) behaviour towards other fish (Saglio et al. 2001).

2.6 Effects with uncertain population relevance

Endocrine active substances may affect endpoints, for which population relevance is uncertain, e.g. hormone levels, gonad histology and secondary sexual characteristics. In such cases, regulatory decisions cannot be based on the effect concentrations derived for the respective endpoint (ECHA 2008a). There has been intensive discussion on the relevance of some effects at the population level (see e.g. Lyons 2003). Within the present report, effects with uncertain population relevance are evaluated using the example of secondary sexual characteristics in fish.

2.6.1 Secondary sexual characteristics in fish

The development of secondary sexual characteristics in fish is controlled by hormones (OECD 2004a). Therefore, it is not surprising that endocrine disrupters have been shown to affect development of secondary sexual characteristics. Effects on secondary sex characteristics can be evaluated qualitatively or quantitatively (OECD 2004a). In some studies, scoring or rating systems have been used (e.g. Parrott & Blunt 2005, OECD 2009c, d). As mentioned in REACH guidance document R.7b, effects on secondary sexual characteristics are an indication that a chemical has an endocrine mode of action. Yet, they are not considered as evidence for long-term adverse effects (ECHA 2008a).

Examples for effects on secondary sexual characteristics are male-specific gonopodia in female mosquitofish (*Heterandria formosa*) observed in streams dominated by pulp mill effluents (Bortone et al. 1989), premature appearance of male sexual characteristics (e.g. nuptial tubercles) in *P. promelas* exposed to methyltestosterone (Parrott & Wood 2002), a reduced size of nuptial tubercles and fatpads in male fathead minnow (*P. promelas*) exposed to 17β-estradiol (Miles-Richardson et al. 1999, 2000), premature ovipositor development and an increased ovipositor size in female *P. promelas* exposed to 17α-ethinylestradiol (Parrott & Wood 2002, Parrott & Blunt 2005) and feminized male urogenital papillae in sand goby (*Pomatoschistus* sp.) exposed to 17β-estradiol (Kirby et al. 2003).

Based on the literature reviewed within the present project, effects on secondary sexual characteristics appear to be in many cases less sensitive than biomarker responses, such as vitellogenin, or effects on population relevant endpoints, such as fecundity (reviewed by Dang et al. 2011; see also Parrott & Blunt 2005). Yet, there are also cases, in which secondary sexual characteristics proved to be very sensitive. For example, significant reductions of sword length in the green swordtail fish (*Xiphoporus helleri*) were recorded at nominal concentrations of 2 and 20 µg/L of bisphenol A (Kwak et al. 2001). Unfortunately, effects on reproductive success were not investigated in this study. In the European Union risk assessment report (EC 2008a), it

is mentioned that sword length has an influence on mating success of the individual males given that females prefer males with longer swords. However, it is stated that it is not clear what degree of change in sword length would affect mating success. Due to this fact and the lack of supplementary chemical analysis for verification of nominal substance concentrations, it was concluded that the LOEC of 2 µg/L is not suitable for use in the environmental risk assessment (EC 2008a). It should also be noted that Kwak et al. (2001) exposed swordtail fish under static conditions and apparently without replication.

2.7 Low-dose effects, non-monotonic dose-response relationships

There has been considerable debate on the issue of dose-response relationships and low-dose effects, particularly in humans, for example with regard to potential effects of low doses of bisphenol A (IPCS 2002, Crain et al. 2007). It has been argued that the assumption that no significant effect is likely to be seen below a certain threshold dose or concentration may not hold true for endocrine disruptors (Sheehan 2000, Lyons 2003). This issue has caused concern, since in case of low-dose effects combined with non-monotonic dose-response relationships the assumption underlying present risk assessment procedures that is possible to extrapolate from effects seen at higher doses to effects at lower doses does not apply (Matthiessen 2003, Vandenberg et al. 2012). In this context low-dose effects have been considered to occur when (1) significant effects are observed at doses that are lower than the no observed effect levels obtained with the standard toxicological tests and (2) the dose-response relationship is non-monotonous (Melnick et al. 2002). Much of the low-dose discussion has focused on humans, i.e. on individual effects (IPCS 2002, Vandenberg et al. 2012). It should be noted that low-dose effects were, in some cases, not reproducible and that their toxicological relevance is often not known (IPCS 2002, Melnick et al. 2002, Matthiessen & Johnson 2007).

Non-monotonic (e.g. U-shaped or inverted U-shaped) concentration-response relationships in ecotoxicological tests have been observed for example in molluscs (Matthiessen 2008). Such concentration-response relationships are often caused by the fact that at higher concentrations, endocrine effects are counteracted by systemic toxicity (Matthiessen & Johnson 2007). For example, following exposure of *Chironomus riparius* to 17α-ethinylestradiol or bisphenol A (test concentrations ranged from 10 ng/L to 1 mg/L for both compounds), moulting and growth were affected at the highest substance concentration of 1 mg/L. The highest incidence of deformities of the mouthparts (mentum and mandibles) was observed at intermediate concentrations, while less or no deformities were recorded at higher concentrations (10 µg/L to 1 mg/L for deformities of the mentum, 1 mg/L for deformities of the mandibles; Watts et al. 2003)[17].

[17] The mouthpart deformities are most likely caused by physiological disturbances during the moulting process (OECD 2006a, Soin & Smagghe 2007).

Based on the evaluated literature we have not identified any example for a substance that elicits population relevant effects at low concentrations but no such population relevant effects at higher concentrations.

2.8 Transgenerational / epigenetic effects

Schwaiger et al. (2002) exposed adult male and female rainbow trout (*O. mykiss*) inter-mittently to technical nonylphenol (NP; consisting of 88% 4-nonylphenol, 10% 2-nonylphenol and 2% dinonylphenol). Exposure started four months prior to the spawning period. Trout were exposed for 10 days in each of the four months to nominal concentrations of 1 and 10 µg/L of NP. At the end of this four-month period, effects on plasma vitellogenin levels and gonad histology were evaluated and eggs and sperm of the exposed fish were obtained. Following artificial fertilisation, the offspring was reared in control water (i.e. not exposed) until hatching (offspring of fish exposed to 1 and 10 µg/L of NP) or until sexual maturity at the age of 3 years (offspring of fish exposed to 10 µg/L of NP). The gonads of 6- to 18-month-old offspring were evaluated histologically for potential effects on sex ratio and gonad differentiation. Vitellogenin and sex steroid levels of mature trout were determined in 3-year-old fish at spawning time.

Exposure to both NP concentrations had no effect on gonad histology, but led to a significant increase in vitellogenin levels in male fish at the end of the four months exposure period. In addition, a significantly increased mortality during early embryonic development and a significantly reduced hatching rate were observed at 1 and 10 µg/L of NP. Sex ratio of the offspring of rainbow trout exposed to both nonylphenol concentrations was not significantly affected. However, within the offspring of fish exposed to 10 µg/L of NP a number of fish were noted, which appeared to be males based on their gross morphology but proved to be females when investigated histologically. Moreover, the ovaries of six females in this group contained spermatocysts (i.e. were classified as intersex gonads). Since a similar observation was made for one control fish, it is not clear whether the low percentage of intersex in offspring of fish exposed to 10 µg/L of NP can be considered as transgenerational effect. After being raised for 3 years in control water, plasma vitellogenin and testosterone levels in male offspring of trout exposed to 10 µg/L of NP were at control levels, but plasma estradiol levels were significantly increased. In female offspring of trout exposed to 10 µg/L of NP, plasma estradiol levels were not affected, but vitellogenin and testosterone levels were significantly increased. The mechanism leading to the observed transgenerational effects and the reasons for an increase of vitellogenin levels in female but not in male offspring could not be clarified by Schwaiger et al. (2002).

Transgenerational effects are caused by maternal transfer of the toxicant to the next generation (e.g. Nyholm et al. 2008), by chromosomal alterations or by epigenetic effects that are transferred between generations (Anway et al. 2005). The term epigenetics refers to the study of mitotically and / or meiotically heritable changes in gene function, which are not mediated by alterations in the DNA sequence, but by other molecular mechanisms such as DNA methylation and histone modification (OECD 2011f, Vandegehuchte & Janssen 2011, Head et al. 2012).

Mechanisms as DNA methylation and histone modification are essential to control gene expression (and thereby cell differentiation), in eukaryotes, i.e. to determine which genes are expressed in which cell type (Head et al. 2012). Effects on the epigenetic state of a cell are

passed on from cell to cell during mitotic or, sometimes, meiotic cell divisions, although they are potentially reversible (OECD 2011f, Vandegehuchte & Janssen 2011, Head et al. 2012). Most epigenetic information is not transferred from one generation to the next (Youngson & Whitelaw 2008), but there is some evidence for transgenerational epigenetic effects (Anway et al. 2005, Vandegehuchte & Janssen 2011, Head et al. 2012). Due to the fact that epigenetic effects can be caused by transient exposures and persist in the absence of the stressor until later life stages and, in some cases, successive generations, epigenetics have received considerable attention in the last few years (Head et al. 2012).

A couple of studies have demonstrated effects of substances with endocrine activity (e.g. 17β-estradiol, EE_2, tributyltin, triphenyltin) on the global DNA methylation state in the liver and gonads of different fish species (Anagiu et al. 2008, Wang et al. 2009, see also review of Vandegehuchte & Janssen 2011). Yet, with the exception of a study by Stromqvist et al. (2010), who found a significantly increased DNA methylation in the DNA regions flanking the gene for vitellogenin 1 in zebrafish exposed to 100 ng/L of EE_2, no link between the methylation pattern and gene expression was made[18]. The fungicide vinclozolin, an androgen receptor agonist, affected methylation patterns in *Daphnia magna*, but these effects were not transferred to the next generation (Vandegehuchte et al. 2010).

2.9 'Atypical' effects: immunotoxicity

In addition to their typical effects (e.g. effects on reproduction), endocrine-active substances may also cause 'atypical' effects. A detailed evaluation of such effects was not possible within the present project. Yet one example is briefly mentioned in the following.

The endocrine system is involved in the development and regulation of the immune system in fish. Accordingly, effects on immune parameters in fish were reported for EDCs with an estrogenic, anti-estrogenic, androgenic and anti-androgenic mechanism of action (see e.g. review of Milla et al. 2011). It has therefore been suspected that endocrine disruption might lead to an increased susceptibility to infections (see e.g. Lyons 2003, 2006).

2.10 Mixture effects

In most situations, different endocrine active compounds occur simultaneously in the environment. Therefore, aquatic organisms are more likely to be exposed to mixtures of EDCs than to a single EDC. Thus, if several compounds with the same mode of action are present in the environment, the risk is higher than estimated based on the PEC/PNEC ratio for each single compound (Matthiessen 2003, Santillo & Johnston 2006, Matthiessen & Johnson 2007).

For instance, it was assumed that a high incidence of intersex in juvenile fish (barbel, *Barbus* sp.) in river Po, Italy, might be related to an upstream point source of bisphenol A. The

[18] The tested EE_2 concentration of 100 ng/L corresponds to the LC_{50} derived in a 28 d toxicity test with zebrafish (Wenzel et al. 2001a).

concentrations of bisphenol A in the river water (0.3 µg/L) was most likely not sufficient to cause intersex (see effect concentrations in Table 5), but may have contributed to the overall effect (Vigano et al. 2001, 2006, Crain et al. 2007). Similarly, high mortality in American lobster (*Homarus americanus*) in 1999 in the Western Long Island Sound on the U.S. east coast were assumed to be at least partly caused by methoprene, a juvenile hormone agonist widely used for mosquito control. Environmental concentrations were probably below concentrations leading to mortality in lobster larvae. However, mortality may have been caused by a mixture of substances (Biggers & Laufer 2004, Walker et al. 2005, Kortenkamp et al. 2012). A prominent example for such an exposure to a mixture of EDCs is the well documented case of estrogenic compounds released from sewage treatment plants (e.g. Matthiesen & Sumpter 1998, Körner et al. 2001, Campbell et al. 2006).

For estrogenic substances it was emphasized that environmental risk assessments that do not consider the possible joint effects of these substances are likely to lead to a considerable underestimation of risk (Silva et al. 2002). It was pointed out that mixture effects are complex and that it is therefore difficult or impossible to evaluate whether the currently used assessment factors are sufficiently protective (Santillo & Johnston 2006). In this context, the fact that a mixture of substances present at concentrations below their individual NOEC can induce a significant effect deserves special attention (Rajapakse et al. 2002, Silva et al. 2002, Santillo & Johnston 2006). Such effects are observed when the individual substances produce a small effect that is not statistically significant, and several statistically non-significant effects add up to a statistically significant effect of the mixture (Matthiessen & Johnson 2007, Kortenkamp 2007, Kortenkamp et al. 2009).

2.11 Exposure assessment

Although the present project is focusing on effects assessment, an uncertainty related to exposure assessment shall be mentioned. For substances that mainly enter water bodies through sewage treatment plant effluents, measured concentrations may exhibit considerable seasonal and temporal variations. This is caused, for example, by generally lower biodegradation in winter and by lower dilution in seasons with little rainfall (Sumpter & Johnson 2005). Particular attention should be paid to the fact that worst case exposure situations may coincide with sensitive periods in the development of seasonally reproducing organisms.

3 Regulatory relevance of factors that may increase the uncertainty of the ERA for substances with endocrine activity

In the present chapter, the regulatory relevance of the identified factors that may contribute to an increased uncertainty of the environmental risk assessment is briefly characterised (for a discussion of the resulting uncertainties of the ERA of EDCs see section 5). A summary is given at the end of this section. It should be noted that the different factors are often interrelated. For example, gaps in the knowledge on endocrinology and endocrine disruption in invertebrates are highly relevant for test availability and, thus, implementation of tests, as well as for cross-species extrapolation.

Availability and implementation of tests for assessing endocrine effects

While certain endocrine modes of action have been extensively studied, others have received much less attention. This has implications on the availability and implementation of appropriate tests and, thus, on the ability to detect effects, i.e. on the uncertainties of the ERA. As mentioned in ECHA (2008a), at present no test strategies / test methods are available that specifically detect all effects linked to endocrine disruptive mechanisms. In accordance with the OECD conceptual framework and the related guidance document (OECD 2011a) Appendix 7.8-5 of REACH guidance document R.7b (ECHA 2008a) only covers a limited part of endocrine modes of action, namely estrogen and androgen receptor agonistic and antagonistic effects, effects on steroidogenesis and thyroid effects (see section 2.1). Other endocrine modes of action (e.g. effects on the corticosteroid system and on endocrine control of neural development, see 2.1.2) and, especially, endocrine effects on invertebrates are at present insufficiently covered in the tiered testing strategy in Appendix 7.8-5 of R.7b. Especially in view of the fact that invertebrates are the vast majority of all animal species on earth, this is a crucial shortcoming of the environmental risk assessment for EDCs.

Extrapolation between species / feasibility to select representative test species

Given that it is not feasible to investigate the potential endocrine effects of a chemical on all relevant wildlife species, the selection of representative, sufficiently sensitive and ecologically relevant test species is crucial (OECD 2006a). The difficulty to assess whether the results of toxicity tests with few standard test species are protective for the approximately 9 million wildlife species (Mora et al. 2011) is one of the key factors contributing to an increased uncertainty of the environmental risk assessment of endocrine disrupting compounds.

It has to be emphasized that so far endocrine disruption has only been studied in a relatively limited number of species. Therefore, gaps in the current knowledge on interspecies differences in sensitivity to EDCs appear to be a major factor contributing to this uncertainty.

In teleosts, the largest fish subgroup on which most studies on endocrinology and endocrine disruption have focused, the reproductive endocrine system is relatively conserved. For this reason, mechanisms of action of sexual endocrine disrupting substances are assumed to be the same in all teleost species, a fact that facilitates cross-species extrapolation. However, because of differences in sexual development and compensation potential, effects may manifest in a different way in different species (cf. sections 2.2.2 and 2.2.3). Data analysed in section 2.2.3 and summarised in section 2.2.4 indicate that effect concentrations in different fish species

with similar metabolic capacities are often in the same order of magnitude, while larger differences are observed between species that differ in their metabolic capacities.

Due to the much higher diversity and heterogeneity of invertebrates cross-species extrapolation is far more complex for invertebrates than for fish, especially since endocrine disrupting effects have only been studied in relatively few invertebrate species and the knowledge on underlying endocrine processes is often fragmentary (Oehlmann & Schulte Oehlmann 2003, OECD 2006a; see sections 2.2.5 – 2.2.8). In this context, it should also be noted that invertebrates, which possess vertebrate-type hormones, may exhibit a higher sensitivity to substances interacting with vertebrate-type endocrine processes than vertebrates (see examples in section 2.2.7).

Sensitive time windows for exposure, delayed effects

Endocrine active substances may affect developmental / organisational processes, e.g. sexual differentiation. These effects may manifest only much later in the organism's life cycle, e.g. during reproduction. It is crucial that such effects, which are specific to endocrine active substances, are considered in the environmental risk assessment of EDCs. This means that the test duration has to include both, the critical window of sensitivity and the period in which effects are manifested (i.e. the test duration should generally cover the whole life cycle). Alternatively, screening tests in a tiered testing strategy have to be sufficiently sensitive to predict effects on the apical endpoints of full life-cycle tests.

For fish, such a tiered testing strategy is included in the OECD conceptual framework for estrogen and androgen receptor mediated effects and interference with steroidogenesis (see section 2.1.4). The available screening tests and the fish sexual development test appear to be sufficiently sensitive to predict the occurrence of effects on reproduction (Bosker et al. 2010, Knacker et al. 2010, OECD 2011a).

For invertebrates, the available information on endocrinology and endocrine disruption for most invertebrates is too sparse for identifying critical developmental periods with sufficient certainty (see section 2.3). Therefore, full life-cycle testing is required. However, such tests are not yet implemented in the testing strategy for endocrine disruptive effects in Appendix R.7.8-5 of R.7b, which is a major uncertainty in the ERA of EDCs as outlined above.

Irreversibility of effects

In human health risk assessment, the protection of the individual is crucial and any kind of toxic effect is not accepted. This is different in the environmental risk assessment that focuses on the protection of populations, communities and ecosystems. Adverse effects on the individual are accepted as long as the population is not adversely affected (i.e. endangered species represent an exception). Endpoints that are used in the environmental risk assessment must be indicative of adverse effects that are likely to have consequences on the population level, such as mortality and reproduction (Traas & van Leeuwen 2007, Nichols et al. 2011). Thus, many adverse effects that are considered in traditional ecotoxicological testing are irreversible (e.g. impaired hatching, impaired emergence, mortality). Irreversible effects on individuals, which often occur following exposure to endocrine disrupting substances during critical developmental windows, do not appear to be of higher concern for the population than, for instance, mortality.

It should be noted that at the population level a principally reversible effect might have the same adverse consequence as an irreversible effect. This is the case, if exposure is continuous, i.e. recovery cannot occur. In cases where short-term exposure is assumed to occur, the severity of the effect will depend on its reversibility. However, if exposure is expected to be more or less continuous, the most relevant question is whether the observed effect will have adverse consequences at the population level or not.

Behavioural effects

With regard to EDCs, effects on reproductive behaviour have so far received most attention. Given that intact reproductive behaviour is a prerequisite for successful reproduction (Balch et al. 2004, Scott & Sloman 2004, Kortenkamp et al. 2012), the endpoint reproductive success is usually as sensitive as reproductive behaviour. In the studies of fish reproductive behaviour evaluated in section 2.5.1, effects on reproductive success (which is an endpoint in the short-term reproduction test and the full life-cycle test) were as sensitive as behavioural effects. In addition, a number of biomarker endpoints and secondary sexual characteristics, which are indicative endpoints in fish screening tests, were at least as sensitive as behavioural effects. Based on the evaluated studies (see Table 9) it can therefore be concluded that it is unlikely that reproductive behaviour in fish is significantly affected at concentrations of EDCs, which do not affect indicative and / or apical endpoints in fish screening tests and / or the fish full life-cycle test.

Effects of ECDs on behavioural responses other than reproductive behaviour have been observed (see 2.5.2) but could not be evaluated in detail within the present project.

Effects with uncertain population relevance

In some cases, the most sensitive effect in an environmental risk assessment is not considered as adverse effect. This may for instance be the case for secondary sexual characteristics (ECHA 2008a), such as sword length in swordtail fish (see section 2.6.1). As the environmental risk assessment focuses on the protection of populations, communities and ecosystems (see above), population relevance of effects on secondary sexual characteristics has to be examined on a case by case basis. In some cases, effects on secondary sexual characteristics appear to have direct population relevance, e.g. for gonopodial development in Eastern mosquitofish *Gambusia holbrooki*. In this poeciliid fish, fertilisation is internal. Male *G. holbrooki* use their gonopodium, a modified anal fin, to transfer sperm to the female. A fully developed gonopodium with gonopodial hooks (used to hold the female during copulation) is required for successful fertilisation (Bisazza et al. 1996). Reductions in gonopodium length and in the development of gonopodial hooks as observed by Doyle & Lim (2002) following exposure to nominal concentrations of 100 and 500 ng/L of 17β-estradiol (mean measured concentrations: 102 and 429 ng/L, respectively), can thus be assumed to affect reproductive success.

For sword length in *X. helleri*, such a direct effect on reproductive success is not evident. The sword, which is mainly formed by extension of the ventral caudal fin rays, develops in males reaching sexual maturity. Female swordtail fish prefer larger males (Basolo 1998) and, as mentioned above, males with longer swords (Basolo 1990). Hence, a reduced sword length would result in a lower mating success of affected male swordtail fish. This, in turn, might affect the gene pool of the population (see section 5.2).

In the specific case of estrogenic effects on sword length in *X. helleri*, studies that also include the assessment of effects on reproduction would be useful. In addition, it should be noted that swordtail fish are poeciliids with internal fertilisation. Male *X. helleri* possess a gonopodium (see e.g. Zauner et al. 2003), so that gonopodial development could be used as additional endpoint in studies with potential endocrine disrupting substances.

In case of effects on secondary sexual characteristics, it is indicated in REACH guidance document R.7b (ECHA 2008a) that the observed effects may be the basis for requesting further studies of potential long-term adverse effects. This option is also available for biomarker responses (e.g. effects on hormone levels) and histopathological changes (e.g. effects on spermatogenesis). If further studies are indeed required, the uncertainty that may result from an observed effect with uncertain population relevance appears to be relatively low.

Low-dose effects, non-monotonic dose-response relationships

When effects occur at low doses / concentrations of the toxicant and the dose / concentrations response relationship is non-monotonic, there is a considerable risk that these effects are missed in the risk assessment (see section 2.7). So far, the low-dose discussion has mainly focused on human health risk assessment. As outlined above the protection of the individual is crucial in human health risk assessment, i.e. toxic effects on the individual are not accepted. By contrast, environmental risk assessment aims at the protection of populations, communities and ecosystems. Adverse effects on the individual are accepted, if they are not population relevant.

Based on the literature evaluated within the present project, non-monotonic concentration response relationships in ecotoxicity tests with endocrine active substances are in most cases caused by the fact that at higher concentrations endocrine mediated effects on the respective endpoint are counteracted by systemic toxicity (see section 2.7). In such cases where one type of effect is observed at lower concentrations and a second type of effect at higher concentrations, these effects are detected in tests with (1) sufficient test duration and (2) appropriate apical endpoints given that the population is the protection goal (see above). As mentioned in section 2.7, we have not identified any example for a substance that has population relevant effects only at low but not at high concentrations.

Transgenerational / epigenetic effects

To date, most studies on epigenetics have been performed in the field of biomedical research (e.g. cancer research) and only very few studies in the field of ecotoxicology (Vandegehuchte & Janssen 2011, Head et al. 2012). For this reason, chapter 8 ('Endocrine disrupters and the epigenome') of the OECD draft detailed review paper on the 'State of the science on novel in vitro and in vivo screening and testing methods and endpoints for evaluating endocrine disruptors' (OECD 2011f) focuses on toxicology and does not address specific ecotoxicological aspects such as the high variability of epigenetic processes between invertebrates (Head et al. 2012). The available knowledge on the mechanisms leading to epigenetic changes in different wildlife species, the persistence of these changes, their transfer to the next generation and their consequences at the phenotypic and, especially, population level is still very scarce. Hence it appears that epigenetics effects may increase the uncertainty of the ERA for substances with endocrine effects. Yet further studies are required prior to being able to assess the regulatory relevance of such effects.

'Atypical' effects: immunotoxicity

Further research is required to evaluate the regulatory relevance of 'atypical' effects, such as immunotoxic effects (Milla et al. 2011).

Mixture effects

Since aquatic organisms are very likely to be exposed to complex mixtures of substances with endocrine activity, potential additive effects of EDCs are a relevant factor increasing the uncertainty of the environmental risk assessment.

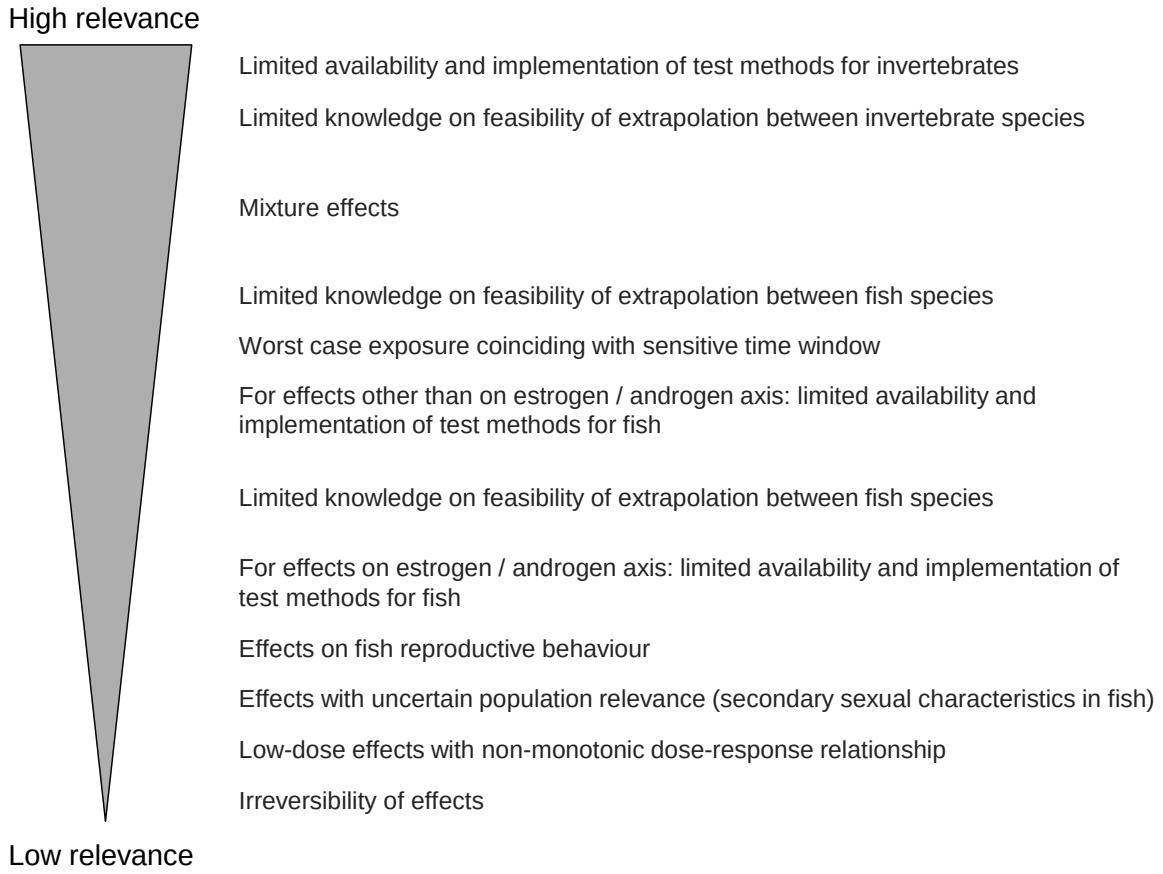
Exposure assessment

Worst case exposure situations (resulting e.g. from low dilution in periods of prolonged drought) that coincide with sensitive periods in the development of seasonally reproducing organisms may lead to an increased uncertainty in the ERA for potential endocrine disrupters.

Summary: most relevant factors increasing the uncertainty of the ERA of EDCs

Based on the evaluation above, two factors appear to be most relevant for the overall uncertainty of the ERA of EDCs: (1) the limited availability and the lack of implementation of test methods for assessing endocrine effects on invertebrates and (2) the limited knowledge on the feasibility of extrapolating between invertebrate species and, thus, of selecting representative test species. Fig. 9 provides a first overview of the relevance of the identified factors. A more detailed overview including information on the specificity of these uncertainties to substances with endocrine activity and the feasibility to reduce the uncertainties is given in Table 10 at the end of section 5.

Fig. 9: **Overview of the relevance of the identified factors increasing the uncertainty of the environmental risk assessment for substances with endocrine activity. Please note that some of the identified factors (behavioural effects other than fish reproductive behaviour, transgenerational / epigenetic effects, 'atypical' effects: immunotoxicity, effects on the gene pool) are not included in the figure, since further studies are required to evaluate their relevance.**



4 Hazard-based assessment of PBT, vPvB and CMR substances

4.1 The precautionary principle

As mentioned in section 1.1.1, the criteria for identifying substances of very high concern (SVHC) shall ensure a high level of protection, both for humans and for the environment (EC 2007). The underlying rationale for the identification of substances of very high concern is the precautionary principle (EC 2007). The terms 'precautionary principle' or 'precautionary approach' were defined in 1987 by the second international conference on the North Sea to describe the obligation to control the most dangerous substances before a definitive causal link is evident between the chemical and health / environmental effects (Krimsky 1998, Commission of the European Communities 2000, Maeder 2004). In other words, the essential element of the precautionary principle is that preventive actions shall be taken, if there is a threat of serious or irreversible damage, even if full scientific certainty is lacking (Commission of the European Communities 2000, van Leeuwen 2007). Scientific uncertainty may be caused by the lack of relevant data, qualitative or quantitative elements of the risk analysis or controversies regarding available data. The precautionary principle is applied as part of the risk management process (Commission of the European Communities 2000).

4.2 Hazard-based assessment of PBT and vPvB substances

The identification of hazardous substances which are persistent, likely to bioaccumulate and toxic (PBT) or very persistent and very likely to bioaccumulate (vPvB) is part of various national and international programmes: the UNEP Stockholm Convention on persistent organic pollutants (POPs; UNEP 2009), the OSPAR Hazardous Substances Strategy (OSPAR 1992), the REACH Regulation (EC 2007) and the former US EPA Persistent Bioaccumulative and Toxic (PBT) Chemical Program (see also van Wijk et al. 2009). In all of these programmes, the identification of PBT and vPvB substances is based on their intrinsic properties: lack of degradability, bioaccumulation potential and toxicity. The critical values or cut-off values for each property are hazard-based (ECETOC 2006). They deviate between programmes (see review of Moermond et al. 2012).

4.2.1 Rationale for PBT and vPvB assessment

Due to their persistence combined with the potential for accumulation in the environment and biota it is not possible to derive a 'safe' concentration for PBT and vPvB substances in the environment with sufficient reliability using traditional quantitative risk assessment methodologies (EC 2007, ECHA 2008d, ESIS 2011, Führ et al. 2011). The major concerns have been summarised in the 'Technical guidance document on risk assessment' (EC 2003), by van Wijk et al. (2009), in ESIS (2011) and by Moermond et al. (2012):

- PBT and vPvB substances are likely to accumulate to high levels in the environment. Even when emissions are reduced, such accumulation would be difficult or impossible to reverse. It might lead to long-term effects that are not predictable.
- As PBT substances often require extended time periods to reach steady state accumulation in organisms, their long-term toxicity is difficult to predict and can be underestimated in standard chronic studies.

- Especially for vPvB substances, unpredictably high accumulation may be reached in humans or wildlife (especially top predators) over extended time periods. In such cases, long-term effects cannot be excluded even when no toxicity has been observed in laboratory tests.
- Due to their persistence, PBT and vPvB substances can be transported over long ranges in the environment. Hence, they will also reach areas far away from the site where they were produced or used. Remote areas such as marine environments, where the risks are more difficult to estimate than in local or regional assessment, and pristine areas should be protected from these substances.
- In view of the fact that their accumulation in the environment is difficult or impossible to reverse (see above), an underestimation of a possible risk to the environment is more problematic for persistent substances than for substances that degrade rapidly.

Thus, the aspect of irreversibility is an important element of the rationale for PBT and vPvB assessment. The unacceptably high uncertainty in predicting reliable environmental concentrations (PECs) via established exposure models and/or in establishing the predicted no effect levels (PNECs) based on standard laboratory tests was also emphasized by EMA (2008, 2010).

4.2.2 Intrinsic properties of PBT and vPvB substances

The intrinsic properties of PBT substances are persistence and bioaccumulation potential for PBT and vPvB substances and, additionally, toxicity for PBT substances. The criteria for identification of PBT and vPvB substances are described in Annex XIII of the REACH Regulation (EC 2007) as amended by EC (2011b)[19].

Briefly, the 'persistent' and 'very persistent' criteria are fulfilled, if a certain degradation half-time is exceeded (40 d in freshwater, 60 d in marine water, 120 d in freshwater sediment and soil, and 180 d in marine sediment for 'persistent'; 60 d in freshwater and marine water, and > 180 d in freshwater and marine sediments and soil for 'very persistent'). Classification as 'bioaccumulative' is justified by a bioconcentration factor (BCF) of > 2000, classification as 'very bioaccumulative' by a BCF of > 5000. The toxicity criterion is fulfilled if a long-term NOEC for aquatic organisms is < 10 µg/L, if a substance is classified as carcinogenic, mutagenic or toxic for reproduction, or if there is evidence of chronic toxicity as identified by the classification as T, R48 or Xn, R48. In the context of the present project, it should be noted that long-term adverse effects like endocrine disruption can be regarded as equivalent level of concern for toxicity.

[19] Further screening level criteria can be found in ECHA (2008d).

4.3 Hazard-based assessment of CMR substances

Following Articles 57 (a) – (c) of the REACH Regulation (EC 2007), substances may be included in Annex XIV, if they are carcinogenic (C), mutagenic (M) or toxic for reproduction (R). CMR substances represent three categories of substances of very high concern, with each category of toxicity (i.e. C, M or R) standing for a toxic endpoint, the fulfilment of which is sufficient for inclusion in Annex XIV.

4.3.1 Rationale for CMR assessment

Assessment of carcinogenicity, mutagenicity and toxicity for reproduction exclusively focuses on humans. This implies that effects on the individual are considered and not effects on the population as in the environmental risk assessment.

As for PBT and vPvB substances (see section 4.2.1), the underlying rationale for CMR assessment is the precautionary principle (EC 2007). In the REACH regulation (EC 2007), it is stated that for "mutagenicity and carcinogenicity, the available information may not enable a threshold, and therefore a DNEL, to be established". Theoretically, a single molecule may cause DNA damage, e.g. the formation of a DNA adduct, which may induce a mutation. Therefore, the prudent assumption was adopted that there is no threshold for effects of mutagens, i.e. no DNELs can be derived (Parry 2000, Marzin 2007, COM 2011). This assumption of a lack of a threshold concentration also applies to genotoxic carcinogens (ECHA 2007, Marzin 2007, Speit 2009).

However, it has been suggested that for mutagens, which do not induce mutations at low concentrations, threshold doses for effects (and thus safe levels) can be derived. This applies to non-DNA-reactive genotoxins, for example substances inducing aneuploidy (i.e. an abnormal number of chromosomes) due to interference with the spindle apparatus during cell division, to topoisomerase inhibitors and DNA polymerase inhibitors (Parry 2000, Bolt et al. 2004, Marzin 2007, Speit 2009, COM 2011). Therefore, it was proposed that a threshold concentration should be derived, if there is evidence of a mechanism of action with a demonstrated threshold. In all other cases, the precautionary assumption that there is no threshold for mutagenicity should be applied (COM 2011).

For carcinogens, the mode of action also has to be taken into account when reflecting on possible threshold doses. According to Foth et al. (2004), exposure levels at which no relevant human cancer risks are anticipated can be defined for non-genotoxic carcinogens. Such levels can also be defined for genotoxic substances that are not DNA-reactive, but have a mutagenic mechanism of action that allows deriving a threshold (see previous paragraph).

Reproductive toxicity is generally considered to have an underlying threshold mechanism. For substances that are toxic to reproduction, a threshold dose (DNEL) for effects on fertility or developmental toxicity can thus be derived (EC 2008b).

4.3.2 Intrinsic properties of CMR substances

The intrinsic properties of C, M, and R substances are carcinogenicity, mutagenicity, and toxicity to reproduction, respectively. Briefly, classification of substances as 'mutagenic' is predominantly based on mutagenic effects found in human germ cells. Results from in vivo and in vitro mutagenicity and genotoxicity tests with mammalian germ cells and somatic cells are also considered. As emphasized in Annex I of Regulation (EC) No 1272/2008 (EC 2008b), this scheme is used to classify substances according to their hazard and not for quantitative risk

assessment. Since no threshold concentrations can be derived for mutagenicity (see section 4.3.1), there are no specific concentration limits for mutagenicity (EC 2007, ECHA 2011).

A substance is classified as 'carcinogenic' when it is known to induce cancer or to increase the incidence of cancer in humans. Substances which have been shown to induce tumours in animal studies are also considered as human carcinogens unless it is shown that the mechanism of tumour formation is not relevant for humans. According to Article 10.1 of the Regulation (EC) No 1272/2008 (EC 2008b), specific concentration limits are used to describe the carcinogenic potency of a substance. The EU has adopted the T25 concept (Dybing et al. 1997, EC 1999).

Toxicity for reproduction (R) includes adverse effects on sexual function and fertility in adults and developmental toxicity in the offspring. Adverse effects on sexual function and fertility include all adverse effects that have the potential to interfere with sexual function and fertility in all relevant life-stages, e.g. effects on the reproductive system, the onset of puberty, gamete production, the reproductive cycle, sexual behaviour, fertility, parturition, pregnancy outcomes, and premature reproductive senescence. Developmental toxicity focuses on effects induced during pregnancy or as a result of parental exposure and include death of the developing organism, structural abnormality, altered growth, and functional deficiency (EC 2008b).

5 Discussion

The objective of the present project was to evaluate whether a 'safe' concentration (i.e. a PNEC) can be derived for substances with endocrine activity with an acceptable level of uncertainty. To this aim, the most relevant factors increasing the uncertainty of the environmental risk assessment (or, more specifically, the assessment of environmental effects) of EDCs are discussed (section 5.1). In section 5.2, the specificity of the identified uncertainties for substances with endocrine activity is evaluated. Section 5.3 deals with the feasibility to reduce the uncertainty in the ERA for EDCs.

5.1 Uncertainties in the ERA of EDCs

Availability and implementation of tests for assessing endocrine effects

With regard to potential uncertainties in the environmental risk assessment of endocrine disrupting chemicals, the amount of available data on potential endocrine activity is a crucial issue. The amount of data is directly related to the availability of appropriate tests for identifying endocrine effects and to the implementation of such tests within REACH (see section 2.1).

Test development efforts in the last decade have mainly focused on vertebrates. With regard to fish, estrogen and androgen receptor agonistic / antagonistic effects and effects on steroidogenesis are covered reasonably well by the tiered testing strategy in Appendix 7.8-5 of REACH guidance document R.7b (see section 2.1.4). Thyroid effects are detected in the amphibian metamorphosis assay (OECD 2009b). Yet, a number of endocrine modes of action (e.g. effects on the corticosteroid system and on the endocrine control of neural development, see also section 2.1.2) will most likely not be detected with the available fish screening tests. If aquatic vertebrates shall be protected from all adverse endocrine effects, this is a shortcoming of the current testing framework.

Invertebrates are at present not sufficiently covered in the testing strategy in Appendix 7.8-5 of R.7b (ECHA 2008a). Consequently, endocrine disruptive effects in invertebrates may be missed because of the very limited availability of appropriate tests (Oehlmann & Schulte-Oehlmann 2003, Kortenkamp et al. 2012). Substantial progress in the development of test methods for invertebrates has been made in the last few years (see section 2.1.5). Given that there is limited knowledge on the sensitivity of different developmental stages of most invertebrates to EDCs, there is general agreement that invertebrate tests should include all life stages and all population relevant endpoints (Ingersoll et al. 1999, OECD 2006a, Hutchinson 2007; cf. also section 2.3). Due to the relatively short life cycles of many invertebrates, such an approach is feasible (OECD 2006a, 2010a). Protocols are available for several invertebrate species. Moreover, a number of protocols are currently being developed and have already been included in the OECD conceptual framework (see 2.1.5).

It should be noted that the apical endpoints of such life-cycle tests allow the identification of adverse effects of EDCs, but do not provide causal evidence of an endocrine mode of action (OECD 2006a, 2010a, LeBlanc 2007). In order to unequivocally identify endocrine disruption as the underlying mode of action, specific diagnostic endpoints are required. However, considerable gaps in the knowledge on endocrine system of most invertebrate taxa with the exception of insects, crustaceans (DeFur et al. 1999b, IPCS 2002) and, partly, gastropods hamper

the identification and development of appropriate diagnostic endpoints (see sections 2.1.5 and 2.2.6). As emphasised for example by Soin & Smagghe (2007), more research is needed in most cases to allow a mechanistic understanding of the relationship between the mechanism of action of the substance and the adverse effect in invertebrates. Given that the (endocrine) mode of action of a chemical is often not completely known (in some cases not known at all) and that a single substance may have different (endocrine and / or non-endocrine) modes of action as exemplified by tributyltin (OECD 2010a), the development of a comprehensive set of tests that allows to identify ED as underlying mode of action is an extremely difficult task (see e.g. Barata et al. 2004, Oehlmann et al. 2007). This applies especially to invertebrates, because the current knowledge on invertebrate endocrinology is still too limited and there are considerable differences between and even within invertebrate phyla. For the detection of endocrine disruption in invertebrates – including causal evidence for an endocrine mode of action – additional knowledge of the endocrinology of this very diverse group of animals and further test development are required (LeBlanc et al. 1999, Hutchinson 2007).

In summary, the fact that invertebrates are not adequately covered in the testing strategy according to Appendix 7.8-5 of R.7b is a crucial shortcoming of the ERA for EDCs. The situation is currently improved by the invertebrate tests, which have been developed recently or are in development and which have already been included in the OECD testing framework. The apical endpoints of these tests allow the identification of adverse effects, i.e. these tests are suitable for deriving a PNEC for aquatic invertebrates, even if they would in most cases not provide evidence on endocrine disruption as underlying mode of action.

Extrapolation between species / feasibility to select representative test species

Generally, extrapolation between species is most feasible within related taxonomic groups where structure and function of hormones have been conserved. As the reproductive endocrine system is relatively conserved within teleost fish, the primary effects of substances with sexual endocrine activity on different teleost species are comparable. Yet, it should be noted that different endpoints vary in their sensitivity between fish species.

If a number of endpoints is studied (as is done in the fish screening tests for endocrine effects, the fish sexual development test and the fish full life-cycle test) effect concentrations in different fish species are often in the same order of magnitude for species with similar metabolic capacity: Based on the evaluation of a relatively limited dataset – the results of laboratory tests with the estrogen agonists 17α-ethinylestradiol, bisphenol A and 4-tert-octylphenol and the aromatase inhibitor prochloraz on different fish species – effects on apical endpoints and, in most cases, also on indicative endpoints were observed at similar concentrations. Variation between results of several tests with the same species was often in the same order of magnitude as variation between fish species. Yet two issues deserver further attention:

First, an assessment based on results of tests with fish species with a higher metabolic capacity, such as medaka and guppy, might underestimate the risk for fish species with slower metabolism (e.g. salmonids).

Second, effects on spermatogenesis and oogenesis, i.e. non-standard endpoints, exhibited a particularly high sensitivity to bisphenol A, especially for trout. The LOEC derived for effects of bisphenol A on sperm density and sperm motility in brown trout at the beginning and in the

middle of the spawning season (Lahnsteiner et al. 2005) was by a factor of 9 lower than the LOEC for spermatogenesis in fathead minnow (Sohoni et al. 2001). Lahnsteiner et al. (2005) concluded that this effect is associated with a delay in gamete maturation. Based on their study, the delayed gamete maturation would be by a factor of approx. 90 more sensitive in the seasonal spawner brown trout than in the continuous spawner zebrafish (see Table 5). For seasonal spawners, timing of reproduction is crucial to ensure survival of the offspring (Crain et al. 2007). Therefore, risks for seasonal spawners might be underestimated when the ERA is based on a LOEC obtained with zebrafish.

It should also be noted that no data are available on endocrine disruption in minor taxonomic groups of fish (e.g. lampreys, hagfish and cartilaginous fish). Thus, it is not possible to assess whether an assessment based on teleosts is protective for these taxonomic groups.

While it can generally be assumed that the mechanisms of action of EDCs are the same in all teleost species, this is not the case for invertebrates. For example, structurally similar hormones may have different functions in different invertebrate taxa (Lafont 2000, OECD 2006a). In addition, knowledge on the mechanisms of action of many endocrine disrupters in invertebrates is still limited. Consequently, there is considerable uncertainty in extrapolating from a single or few invertebrate test species to wildlife invertebrates.

The specificities in invertebrate endocrinology (e.g. the importance of ecdysteroids and terpenoids) are likely to result in specific susceptibilities to endocrine disrupting chemicals (IPCS 2002). In combination with the fact that invertebrates are not adequately represented in Appendix 7.8-5 of REACH guidance document R.7b (see above), there is a high risk to miss effects of an EDC on certain invertebrate species / taxa (Lafont 2000, Oehlmann & Schulte-Oehlmann 2003, OECD 2006a, Hutchinson 2007), i.e. a considerable uncertainty with regard to the protection of wildlife invertebrates. This is exemplified by the high sensitivity of molluscs to tributyltin and triphenyltin and the associated population declines in many prosobranch species. In addition, molluscs, copepods and echinoderms proved to be particularly sensitive to bisphenol A and 4-tert-octylphenol, i.e. invertebrates possessing vertebrate-type hormones may exhibit a higher sensitivity to substances interacting with vertebrate-type endocrine processes than vertebrates.

A comprehensive review of available data on the effects of EDCs on aquatic invertebrates and further studies of the sensitivities of different species / taxa to EDCs with different endocrine mechanisms of action would be required to systematically evaluate interspecies differences in sensitivity (cf. sections 2.2.3 and 2.2.7). As outlined by Ingersoll et al. (1999), the outcome of such a comparative review could be the identification of suitable test species and endpoints, which can be used with appropriate assessment factors in the ERA procedures. Yet, it is also possible that sensitivity differences across species are so large that test species and endpoints, which are suitable to predict effects on a wide range of wildlife species with an acceptable level of uncertainty, cannot be identified.

An environmental risk assessment procedure for potential endocrine disrupters should be based on tests with representatives from the most relevant taxonomic groups, including cnidarians, annelids, crustaceans, insects, molluscs and echinoderms (Hutchinson 2002, Matthiessen 2003, Oehlmann & Schulte-Oehlmann 2003, OECD 2006a). Ideally, representatives from all major taxa are needed (Oehlmann & Schulte-Oehlmann 2003) as long as the

comparative sensitivity of different species / taxa to ECDs with different endocrine mechanisms of action has not been systematically evaluated as outlined above.

One of the main questions is whether an acceptable set of representative test organisms / regulatory tests for EDCs is likely to be available in the near future. While doubts have been expressed by some scientists (e.g. Sumpter & Johnson 2005), others are confident that an acceptable set of tests will be available within 1 to 5 years (e.g. Matthiessen 2010). Although such an environmental risk assessment procedure relying only on a few representative, sensitive and ecologically relevant test species may not protect all wildlife species, it has been assumed that this approach would provide "some degree of protection to critical parts of the ecosystem" (OECD 2006a).

As mentioned above, the situation is considerably improved by the invertebrate tests that have been developed recently or are being developed, i.e. a partial and a full life-cycle test with molluscs, a *Daphnia* multi-generation test, a copepod reproduction and development test, a mysid life-cycle test and a sediment-water chironomid life-cycle test (cf. Table 1). Implementation of these tests in the testing strategy in Appendix 7.8-5 of R.7b (ECHA 2008a) would be a major advancement of the ERA procedure for chemicals with potential endocrine activity. However, as discussed above it still remains unclear if these test species are sufficiently representative for all invertebrate species.

Sensitive time windows for exposure, delayed effects

As detailed in sections 2.3 and 3 endocrine active substances may affect developmental / organisational processes and the resulting effects may manifest much later in the organisms' life cycle. Such effects are only detected in (1) screening tests that have been proven to be sufficiently sensitive and (2) life-cycle tests that include the critical window of sensitivity and the period in which effects are manifested, i.e. cover the whole life cycle and start with the most sensitive life stage.

With regard to fish, a tiered testing strategy with screening tests and a full life cycle test is available for detecting estrogen and androgen receptor mediated effects and interference with steroidogenesis. If this test strategy is used, delayed effects of EDCs are likely to be detected, i.e. the uncertainty of the ERA can be considered as low.

For most invertebrates, information on critical developmental periods is too sparse and sufficiently sensitive screening tests are not available. Therefore, whole life cycle testing should be performed. In view of the relatively short life cycles of invertebrates, this approach is feasible (OECD 2006a, 2010a). However, as outlined above invertebrates are currently not adequately covered in the testing strategy in Appendix 7.8-5 of R.7b (ECHA 2008a). Thus, uncertainty in the ERA is caused by the lack of tests as detailed above. If life-cycle tests with invertebrates will be implemented, delayed effects of EDCs will be detected.

Irreversibility of effects

The potential to cause irreversible effects has been mentioned as a concern for endocrine active substances that might parallel the concern caused by PBT and vPvB substances (Santillo et al. 1999). For PBT and vPvB substances this concern is related to the accumulation in the environment, which – due to the persistence of these substances – is difficult or impossible to reverse. Therefore, possible long-term effects in the environment might persist (see section

4.2.1). For endocrine disrupters, the aspect of irreversibility relates to effects on the individual organism (e.g. effects on gonadal differentiation, which may lead to a shift in sex ratio) that are often caused by exposure during sensitive time windows (cf. sections 2.3 and 2.4).

In human risk assessment, which aims at protecting the individual, reversibility of an effect on the individual organism is an important criterion. However, given that environmental risk assessment focuses on the protection of populations, communities and ecosystems, the crucial question is whether an effect is relevant at the population level (cf. section 3): upon continuous exposure, which is assumed in the ERA procedure for chemicals, no recovery will occur even though an effect on individual organisms is principally reversible. Hence, such principally reversible effects are likely to have the same adverse consequences at the population level as irreversible effects. In this context, it should also be noted that many adverse effects on the individual that are evaluated in the ERA are irreversible (e.g. impaired emergence and mortality; see section 3).

In consequence, potentially irreversible effects of endocrine active substances do not appear to justify a concern that is comparable to the concern caused by PBT and vPvB substances, as long as the basic concept of environmental risk assessment remains valid that effects on the individual are tolerated if the population is not affected.

Behavioural effects

It has been suggested that due to their potentially serious consequences behavioural effects of endocrine disrupting substances deserve more attention and that even subtle behavioural alterations should be considered as adverse effects (Lyons 2006).

So far, effects of EDCs on reproductive behaviour have received most attention. Therefore, the present project mainly focused on the effects of EDCs on reproductive behaviour of fish. Based on reviews and the evaluation of a number of studies within the present project it is concluded that effects on indicative and / or apical endpoints of fish screening tests and the fish full life-cycle test are generally at least as sensitive as effects on fish reproductive behaviour that are evaluated in current standard test (see sections 2.5.1 and 3). Thus, there is a low risk that significant effects of sexual endocrine disruptors on fish are missed, if the assessment of endocrine effects is based on the tiered testing strategy as included in Appendix 7.8-5 of R.7b (ECHA 2008a).

Effects on behavioural responses other than reproductive behaviour have also been observed for EDCs. Such effects could not be evaluated in detail within the present project (see 2.5.2). However, it should be noted that they might lead to a reduced capacity to avoid predation or to cope with other stressors and, consequently, to a reduced survival in the field (Scott & Sloman 2004). The fact that such behavioural responses are at present not covered in the ERA would increase the uncertainty of the assessment, if the observed effects are more sensitive than effects on the standard test endpoints (e.g. growth).

Effects with uncertain population relevance

There has been intensive debate on the relevance of some effects at the population level (see e.g. Lyons 2003, ECHA 2008a). Examples are effects on secondary sexual characteristics (e.g. sword length in *X. helleri*; cf. section 2.6.1) and histopathological effects. If population relevance cannot be demonstrated, regulatory decisions cannot be based on the effect

concentration that was derived for the respective endpoint (ECHA 2008a). However, as mentioned in section 3, such effects can be used as basis for requesting further studies (ECHA 2008a). For example in the case of effects of sword length in *X. helleri*, a partial of full life-cycle test with this fish species could be requested (also in view of the fact that the study of Kwak et al. 2001 has a number of shortcomings as mentioned in section 2.6.1). Although no standardised test protocol is available, such a test is feasible (see e.g. Schäfers 1991).

If further studies are requested in all cases where an effect with uncertain population relevance is the most sensitive effect, the uncertainty that may result from an effect with uncertain population relevance appears to be relatively low.

Low-dose effects, non-monotonic dose-response relationships

The low-dose discussion has so far mainly focused on effects on the individual as evaluated in human health risk assessment (see sections 2.7 and 3). While the protection of the individual is crucial in human health risk assessment, the ERA aims at protecting populations (see above). Within the present project, we have not identified any example for a substance that causes population relevant effects at low concentrations, while no population relevant effects are observed at higher concentrations. Yet, we have identified examples for endocrine effects that are only observed at low concentrations, but counteracted by systemic toxicity at higher concentrations (see 2.7). If tests with sufficiently long duration and appropriate (apical) endpoints are used, such effects should be detected.

In summary, low-dose effects as discussed with regard to human health do not seem to be relevant in environmental risk assessment as long as the basic concept of the ERA remains valid that effects on the individual are tolerated when the population is not affected.

Transgenerational / epigenetic effects

As mentioned in section 3 very few studies on epigenetics have been carried out in the field of ecotoxicology. Fundamental research is required prior to (1) evaluating the uncertainty in the ERA that is caused by epigenetic effects and (2) including epigenetic effects in environmental risk assessment procedures (OECD 2011f, Vandegehuchte & Janssen 2011, Head et al. 2012).

'Atypical' effects: immunotoxicity

It is of note that besides their typical effects, EDCs may also cause 'atypical' effects such as effects on immune parameters (see 2.9). Further studies are required prior to evaluating the uncertainty caused by such effects (see section 3).

Mixture effects

Aquatic organisms are exposed to complex mixtures of substances with endocrine activity, and there is evidence of additive effects of substances with similar mechanisms of action. One of the most salient examples for the shortcomings in the present ERA procedure is the fact that several individual effects, which are not statistically significant (i.e. below the LOEC), may add up to a significant effect (cf. section 2.10). Consequently, the risk resulting from cumulative exposure to endocrine active substance in the environment is very likely to be underestimated when mixture effects are not considered in the assessment (see e.g. review of Kortenkamp 2007).

Exposure assessment

Delayed population relevant effects may be caused by transient exposure to endocrine active substances during sensitive developmental periods (see 2.3). Therefore, the uncertainty of the ERA is increased, if worst case exposure situations (e.g. during prolonged drought) coincide with these sensitive time windows.

5.2 Are the identified uncertainties specific to EDCs?

Some of the main uncertainties addressed in section 5.1 are specific to substances with endocrine effects. This is the case for effects on reproduction that are caused by exposure during sensitive time windows during early development (cf. 2.3).

Yet, most of the relevant uncertainties also apply similarly to substances with non-endocrine modes of action (in most cases specific modes of action). For instance, the limited availability and implementation of tests is also likely to increase the uncertainty for substances with other specific modes of action (e.g. immunotoxicity). As is the case for EDCs the effects of such substances are unlikely to be detected in the standard tests performed according to REACH.

For a number of uncertainties, the specificity to EDCs will be evaluated in more detail below.

Extrapolation between species / feasibility to select representative test species

Uncertainties associated to interspecies variations in sensitivity and the extrapolation from a few test species to other wildlife species are not specific to endocrine disrupting substances, but apply to environmental risk assessment in general (see e.g. Celander et al. 2011). As emphasized by Rubach et al. (2011) it is often not known to what extent test species are representative for the respective taxonomic group. Notably, uncertainties in extrapolation between species are more pronounced for substances with a specific mode of action, for which higher toxicity and higher variation in toxicity between species can be expected as is outlined in the following.

Vaal et al. (1997a, b) analysed acute toxicity data for aquatic species and substances with different modes of action (narcotics, polar narcotics, reactive substances, and substances with a specific mode of action). Interspecies variation was strongly associated to the mode of action. Substances with a narcotic mode of action had the lowest toxicity, and interspecies differences in sensitivities to these substances were low. Substances with a specific mode of action were most toxic. This higher toxicity was associated with a much higher interspecies variation in sensitivity. Highest interspecies variations were observed for acetylcholinesterase inhibiting pesticides (e.g. trichlorfon and dichlorvos). It was assumed that these differences were at least partly due to interspecies differences in target sites, metabolic activation and detoxification of these pesticides in the different test organisms.

Based on the results of their evaluation, Vaal et al. (1997a, b) concluded that for estimating safe environmental concentration with equal accuracy more species have to be tested in the ERA of substances with a specific mode of action than in the ERA of narcotic substances. They also concluded that data sets including a much higher number of species and substances should be used for analysing patterns in interspecies variation in sensitivity and for identifying the most sensitive species for each mode of action. A similar analysis of chronic toxicity data was strongly limited by the lack of available toxicity data, especially for substances with specific modes of action (Van der Wal et al. 1995).

Vaal et al. (1997a, b) analysed interspecies variation in sensitivity across all taxonomic groups (and not within single taxonomic groups). However, other studies also provide evidence for variation within taxonomic groups. In accordance with the results of Vaal et al. (1997a, b) variation seems to be most pronounced within those taxonomic groups in which highest toxicity is observed (Roex et al. 2000, Breitholtz et al. 2001, Forbes & Calow 2002). This is, for example, the case for ivermectin, a parasiticide that affects glutamate-gated chloride channels of invertebrates. With 48 h-EC_{50} values of 1.2 – 10.7 ng/L and a 21 d-NOEC of 0.0003 ng/L for growth, reproduction and sex ratio ivermectin is extremely toxic to *D. magna* (Garric et al. 2007). While sensitivity of the mysid *Neomysis integer* was also relatively high (48 h-LC_{50}: 26 ng/L), other crustaceans such as *Artemia salina* (24 h-LC_{50} > 300 µg/L) and *Carcinus maenas* (96 h-LC_{50}: 957 µg/L) were far less sensitive to the parasiticide (Grant & Briggs 1998). In this case the standard test organism *D. magna* was the most sensitive organisms. Yet, there are also examples where this is not the case as Irgarol 1051. For this antifouling herbicide, which inhibits the electron transfer in the photosystem II, clear interspecies differences in sensitivity of primary producers were observed. Irgarol 1051 is considerably more toxic to the freshwater macroalga *Chara vulgaris* ($EC_{50\ growth}$: 0.012 µg/L; Lambert et al. 2006) than to microalgae such as *Chlorella vulgaris* ($EC_{50\ growth}$: 0.5 µg/L) or *Pseudokirchneriella subcapitata* ($EC_{50\ growth}$: 3.3 µg/L; Bérard et al. 2003) and higher aquatic plants such as *Apium nodiflorum* ($EC_{50\ growth}$: 0.2 µg/L) and *Myriophyllum spicatum* ($EC_{50\ growth}$: 2.0 µg/L, Lambert et al. 2006).

Based on the work of Vaal et al. (1997a, b) it can be concluded that the uncertainties associated to interspecies variations in sensitivity and to the extrapolation from a few test species to other wildlife species are very likely to be higher for endocrine disrupters than for substances with a narcotic mode of action.

The question whether interspecies differences are more relevant for endocrine disrupting substances than for substances with other specific modes of action cannot be systematically addressed within the present project. This would require a comprehensive evaluation of interspecies differences in sensitivity to (1) endocrine disrupting substances with different endocrine mechanisms of actions and (2) substances with other specific mechanisms of action, i.e. a similar but more extensive compilation and review of data as outlined section 2.2.7 with regard to the evaluation of interspecies differences in sensitivity of aquatic invertebrates to EDCs. As mentioned in section 2.2.7, the availability of data on endocrine disruption in invertebrates is limited to relatively few species / taxa. This also applies to ecotoxicity data for substances with other specific modes of action (Ingersoll et al. 1999, De Lange et al. 2009). Therefore, it is very likely that such a compilation of data for evaluating interspecies differences would have to be complemented by further systematic investigations of the sensitivity of different invertebrate species / taxa to substances with different endocrine and non-endocrine mechanisms of action.

In order to improve the prediction of potential adverse effects for a wider range of species, a framework for traits-based assessment was proposed (Baird et al. 2008, Rubach et al. 2011). This framework is based on species vulnerability (see section 2.2). A preliminary list of species traits (i.e. physiological, morphological and ecological characteristics of species / taxonomic groups, which contribute to species vulnerability; Baird et al. 2008), was developed and knowledge gaps were identified. From this list, it is obvious that there are numerous data gaps. For example, while information on target sites and interaction of toxicants with these target sites is available for model species, the availability of such data in other species has been considered as

low. The same applies to data on biotransformation and elimination potential and on compensatory mechanisms (Rubach et al. 2011). The degree of uncertainty in extrapolation across species is likely to be related to evolutionary distance (Hahn 2011). However, for many traits it is still not clear to what extent they are correlated with phylogeny (Rubach et al. 2011).

Due to the higher interspecies variation in toxicity as compared to baseline toxicants (Vaal et al. 1997a, b; see above) the requirement that an environmental risk assessment procedure should be based on tests with representatives from the most relevant taxonomic groups (see section 5.1) also applies to the ERA procedures for substances with other (i.e. non-endocrine) specific modes of actions. As far as possible, the biological traits of the selected species should be representative for the respective taxonomic group (EC 2011a). Yet, as noted above, only a relatively small number of species has ever been evaluated in toxicity tests (Vaal et al. 1997a, b, Ingersoll et al. 1999). In most cases, the selection of test species for the current ERA procedures has not been based on a comprehensive evaluation of the sensitivity of species / taxonomic groups. Likewise physiological and life history traits have in most cases not been considered when selecting the test species. Rather, practical reasons such as availably of test organisms, the ease of laboratory culture and partly also commercial importance (e.g. in the case of *O. mykiss*) have significantly contributed to the selection of currently used test species (Celander et al. 2011). In most cases it is not known if a test species is a sufficiently sensitive representative of the respective taxonomic group (Rubach et al. 2011).

Behavioural effects

Reproductive behaviour is not only affected by substances with an endocrine mode of action, but also by compounds with other specific modes of action. For example, exposure to the pyrethroid esfenvalerate led to a delayed onset of reproductive behaviour. Reduced frequencies or intensities of courtship behaviour were also reported upon exposure to lindane and phenol. Homing of fish to natal streams may be affected by pollutants as shown for cadmium and copper (reviewed by Scott & Sloman 2004).

Likewise, other types of behaviour can be affected by a variety of pollutants (Scott & Sloman 2004). Predator avoidance behaviour was shown to be affected by a number of metals (e.g. cadmium, copper and mercury) and organic pollutants (e.g. atrazine, carbaryl, chlordane, and diazinon). Toxicants (e.g. mercury and carbaryl) may also disrupt schooling behaviour and hence lead to an increased risk of predation (Scott & Sloman 2004).

Given that sensory, neurological and metabolic systems contribute to the performance of the appropriate behaviour, interference with each of these systems may result in behavioural changes. This has been demonstrated for effects on olfaction (caused e.g. by a number of metals and pesticides) and neurotransmission (caused e.g. by acetylcholinesterase inhibitors and substances affecting the levels of the neurotransmitters serotonin and dopamine; Scott & Sloman 2004).

In summary, behavioural endpoints are also affected by a range of substances with other (i.e. non-endocrine) specific modes of action.

Transgenerational / epigenetic effects

Epigenetic effects are not only caused by substances with endocrine activity, but also by other contaminants such as metals (e.g. Ni, Cu, Zn, Cd), halogenated organics and solvents (reviewed by Vandegehuchte & Janssen 2011 and Head et al. 2012).

'Atypical' effects: immunotoxicity

Immunotoxic effects are caused by a wide range of pollutants including metals (e.g. Cd, Cu and Mn) and organics (e.g. lindane, dichlorvos and phenol; see e.g. O'Halloran et al. 1998).

Effects on the gene pool

Effects on the gene pool are not an issue that is specific to endocrine disrupters given that all toxicants exert a selective pressure on populations and thus affect the gene pool (see e.g. Anderson et al. 1994, Evenden & Depledge 1997). Effects on the gene pool occur in all cases where the contribution of individuals to reproduction is affected. Therefore, they are likely to occur before significant population relevant effects such as reduced survival and reduced fecundity are visible. The implications of effects on the gene pool have, for example, been discussed for antibiotics (e.g. Chee-Sanford et al. 2009).

Mixture effects

The issue of mixture toxicity does not only apply to substances with endocrine activity, but also to substances with other modes of action.

5.3 Feasibility to reduce the uncertainties in the ERA of EDCs

It was not the objective of the present project to develop recommendations on how to reduce the uncertainties in the environmental risk assessment of EDCs. However, the feasibility of some options for reducing the identified uncertainties shall be briefly addressed in the following. Table 10 gives an overview of the relevance of the identified factors for environmental risk assessment, the specificity of the respective factor to endocrine active substances and the possibility to address this factor within the environmental risk assessment, i.e. to reduce the associated uncertainty.

Availability and implementation of tests for assessing endocrine effects

As mentioned in section 2.1, the current tiered testing strategy for aquatic vertebrates covers estrogen and androgen receptor agonistic and antagonistic effects, effects on steroidogenesis and thyroid effects. If other endocrine modes of action shall be detected, the development and validation of screening tests for a tiered testing strategy is required. This is feasible, although it is a very complex task, comparable to the development of the tiered testing strategy that is presently available. With regard to the selection of test species, it should be kept in mind that sensitivity to endocrine active substances may differ considerably between species (see sections 2.2 and 5.1).

For invertebrates, extrapolation from tests with mammals is difficult or impossible (depending on the species). In vitro screening tests are largely unavailable. In view of the limited knowledge on endocrinology and endocrine disruption for many invertebrate taxa, and the currently incomplete knowledge on differences in sensitivity between invertebrate species,

fundamental research is required prior to the development of screening tests that cover the critical developmental periods and include appropriate diagnostic endpoints (OECD 2010a).

The uncertainty of the ERA would be reduced significantly, if full life-cycle testing with invertebrates would be included as a general approach (Ingersoll et al. 1999, OECD 2006a, Hutchinson 2007). Life-cycle tests include all critical life stages and all biochemical / physiological processes that might be affected by EDCs (including developmental and reproductive impairment) and, thus, provide the most comprehensive information for environmental risk assessment (OECD 2006a, 2010a). This approach would require further test development and implementation of these tests in the current ERA procedure for chemicals. It would not only cover potential endocrine disrupting effects, but also adverse effects caused by other, less studied modes of action that might be equally relevant. However, as outlined in section 5.1 a comprehensive analysis of interspecies differences in sensitivity is required to account for uncertainties that are related to the extrapolation between invertebrate species.

Worst case exposure coinciding with sensitive developmental periods

Given that short-term exposure during a sensitive time window may cause delayed adverse effects, the suggestion of Crain et al. (2007) to use maximum measured concentrations or, alternatively, maximum predicted environmental concentrations for evaluating potential risks in the environment appears to be very useful.

Effects with uncertain population relevance

For effects with uncertain population relevance, it is crucial that further testing is triggered in all cases where an effect with uncertain population relevance is the most sensitive effect. Alternatively, population relevance should be assumed as a precautionary approach. Further studies evaluating the relevance of the respective effect at population level would be desirable.

Mixture effects

It has been recommended that potential additive effects of EDCs in mixtures should be considered in environmental risk assessment (OECD 2006a). The summation of PEC/PNEC quotients as a worst-case approach is currently under discussion for application in the risk assessment of mixtures and has already been adopted for the evaluation of biocidal products (ECB 2008). Another option under discussion is the inclusion of an additional assessment factor in order to take mixture effects into account (Kortenkamp et al. 2009).

Table 11: Relevance and specificity of the factors that may contribute to an increased the uncertainty of the ERA for substances with an endocrine mode of action.

Factor that may contribute to increased uncertainty		Relevance for environmental risk assessment	Specificity to EDCs	Feasibility to address this factor and to reduce the uncertainty that is causes
Limited availability and implementation of test methods	Fish	Low [1] / Medium [2]	Yes	High (but partly longer-term) [2]: further test development and standardisation / validation, implementation of tests
	Invertebrates	High	Yes	High (but partly longer-term) [4]: further test development, implementation of tests
Limited knowledge on feasibility of extrapolation between species	Fish	Low – medium	No	Medium – high (but longer-term): systematic evaluation, further studies
	Invertebrates	High	No	Medium (longer-term): systematic evaluation, further studies
Sensitive time windows for exposure, delayed effects	Fish	Low [1]	Yes	Not required: tiered testing framework with appropriate tests available [1]
	Invertebrates	Medium	Yes	Life-cycle testing in invertebrates
Irreversibility of effects		Low	No	Not required
Behavioural effects	Fish reproductive behaviour	Low [1]	Yes	Not required
	Other behavioural effects	(?) [5]	No	(Further investigations required)
Low-dose effects with non-monotonic dose-response relationship		Low	Yes	Not required
Effects with uncertain population relevance (secondary sexual characteristics in fish)		Low	Yes	High: triggering of further testing
Transgenerational / epigenetic effects		(?) [5]	No	(Further investigations required)
'Atypical' effects: immunotoxicity		(?) [5]	No	(Further investigations required)
Effects on the gene pool		(?) [5]	No	(Further investigations required)
Mixture effects		Medium – high	No	Medium to high (but partly longer-term)
Exposure assessment		Low – medium	No	High: worst case exposure estimates

(1) For estrogen receptor mediated effects, androgen receptor mediated effects and interference with steroidogenesis. (2) For other endocrine mechanisms of action. (3) Due to the lack of diagnostic endpoints in invertebrates. (4) For life-cycle tests without or with few specific diagnostic endpoints. (5) Further research is required to evaluate the relevance of these factors.

6 Conclusions

The aim of the present project was to contribute to the evaluation if a PNEC can be derived for substances with endocrine modes of action with an acceptable level of uncertainty and to identify factors that increase the uncertainty of the ERA for such substances. When addressing uncertainties in the environmental risk assessment of endocrine active substances it should be kept in mind that with the present ERA procedures risks are generally assessed in a very simplified way and that, consequently, uncertainty is inherent in the risk assessment process (van Leeuwen 2007).

As discussed in section 5.1, the following two key factors contribute most to an increased uncertainty of the environmental risk assessment of endocrine active substances as compared to baseline toxicants: (1) the limited availability of test methods and (2) the limited knowledge on the feasibility of cross-species extrapolation. Both factors have highest relevance for aquatic invertebrates and a lower relevance for fish as outlined in the following.

The availability of appropriate tests is crucial for the uncertainty of the ERA. Due to substantial differences in test availability, our ability to detect endocrine effects greatly differs depending on the mode of action of an endocrine active compound. For effects on the estrogen / androgen and thyroid axis of aquatic vertebrates, the uncertainty is acceptable given that these effects are covered reasonably well by a tiered testing strategy. For other endocrine modes of action (e.g. effects on the corticosteroid system) in aquatic vertebrates, such a testing strategy is not available. Consequently, the resulting uncertainty of the ERA is higher.

For aquatic invertebrates, extrapolation from tests with mammals is difficult or impossible. The uncertainty of the ERA is high when no results of tests with invertebrates or only results of the two tests mentioned in Appendix 7.8-5 of R.7b (a *Daphnia* magna reproduction test and a development and reproduction test with marine copepods) are available. The uncertainty is reduced when results of those tests that have been developed recently or are currently developed are also available (i.e. a partial and a full life-cycle test with molluscs, a *Daphnia* multi-generation test, a mysid life-cycle test and a chironomid life-cycle test). However, further research is needed to systematically evaluate if test results obtained with these species are sufficiently protective for other invertebrate groups. It is of note that invertebrates with vertebrate-type hormones may be more sensitive to substances interacting with vertebrate-type endocrine processes than vertebrates.

The difficulty to assess if the results of toxicity tests with few standard species are protective for the large number of species in the environment is the second key factor contributing to an increased uncertainty of the ERA of endocrine active substances. As previously discussed cross-species extrapolation is more feasible for fish than for aquatic invertebrates. In most cases, effect concentrations for the most commonly tested fish species are comparable, if a number of endpoints is studied. However, PNECs derived using tests with fish species exhibiting a high metabolic capacity may not be protective for species with slower metabolism. In addition, potential risks to seasonally spawning fish species (e.g. brown trout) may be underestimated when the PNEC is derived based on effects on standard test species such as zebrafish. It should also be mentioned that it is currently not possible to assess whether an ERA based on tests with teleosts will protect minor taxonomic fish groups.

For invertebrates, extrapolation between species is far more complex than for fish. This is due to the much higher diversity and heterogeneity of invertebrates and to the often fragmentary knowledge on endocrine effects and the underlying processes in invertebrate species.

The following two factors also increase the uncertainty of the ERA of EDCs. Given that aquatic organisms are very likely to be exposed to complex mixtures of substances with endocrine activity, potential additive effects of EDCs are relevant. Worst case exposure situations coinciding with sensitive periods in the development of seasonally reproducing organisms may also be a relevant factor.

By contrast, the following four factors appear to be of low relevance with regard to the overall uncertainty in the ERA of EDCs. Low dose effects as discussed with regard to human health do not appear to be relevant in environmental risk assessment as long as the basic concept of the ERA remains valid that effects on the individual are tolerated when the population is not affected. Irreversible effects on individuals, which often occur following exposure to endocrine active substance during critical developmental windows, are not of higher concern for the population than other adverse effects evaluated in standard ecotoxicological tests (e.g. mortality). Significant effects on fish reproductive behaviour are unlikely to occur at concentrations of endocrine active substances that do not affect indicative and / or apical endpoints of fish screening tests and the fish full life-cycle test. The uncertainty that might results from effects with uncertain population relevance (e.g. effects on secondary sexual characteristics) appears to be low, if further studies on potential long-term adverse effects are required in all cases where such an effect with uncertain population relevance is the most sensitive effect.

Further studies are required prior to evaluating the relevance of uncertainties caused by effects on behavioural endpoints other than fish reproductive behaviour, transgenerational / epigenetic effects, effects on the gene pool and ‘atypical’ effects such as immunotoxicity (see section 5.1).

As discussed in section 5.2, most of the identified uncertainties are not specific to substances with endocrine activity, but apply to the environmental risk assessment in general. For example, the limited availability of tests is also likely to increase the uncertainty for chemicals with other (i.e. non-endocrine) specific modes of action. Uncertainties associated to cross-species extrapolation are also relevant for chemicals with other specific modes of action and, to a lower extent, for baseline toxicants. These uncertainties have to be addressed in a broader context. For example, although an intact immune system is critical to disease resistance and, thus, to survival (reviewed e.g. by Demas et al. 2011), immunotoxic effects are not considered in the present ERA procedure for chemicals. Similarly, the issue of mixture toxicity has to be addressed – and is already addressed – in a wider context (see section 5.3).

It appears feasible to reduce some of the most relevant uncertainties in the environmental risk assessment of endocrine active compounds. However, this would require considerable effort (see section 5.3 and Table 10). One option to address the overall uncertainty could be to increase the assessment factor. For chemicals with endocrine activity this option has explicitly been mentioned by ECHA (2008a, b). The selection of an appropriate assessment factor should be based on a systematic review of the available data on endocrine disruption and available ERAs for EDCs.

Based on the present project it is concluded that the overall uncertainty in the environmental risk assessment is higher for endocrine disrupters than for baseline toxicants. The most relevant factors contributing to an increased uncertainty of the ERA for EDCs – (1) the limited availability of test methods for invertebrates, and (2) the limited knowledge on the feasibility of cross-species extrapolation for invertebrates – are also relevant for substances with other specific modes of action, but less relevant for baseline toxicants.

A comparison of the overall uncertainty of the ERA for EDCs with the uncertainty of the ERA for substances with other specific mechanisms of action is an extremely broad issue that could not be addressed within the present project. In view of the fact that endocrine disruption has been more intensively studied than many other specific modes of action, it is also very likely that further investigations on specific non-endocrine modes of action are required before this issue can be addressed.

In order to systematically evaluate whether the uncertainties in the environmental risk assessment of endocrine disrupters accumulate in a specific way that might lead to an unacceptably high uncertainty in the derivation of the PNEC, a number of case studies with different substances would be required. These should include substances with different endocrine and non-endocrine mechanisms of action. For each substance, all uncertainties in the ERA should be evaluated, i.e. a complete ERA should be available or should be performed. This should include an evaluation of (1) the uncertainty of each step of the ERA and (2) the overall uncertainty. To our knowledge no such evaluation is available so far. Since the uncertainty of the ERA crucially depends on the amount and quality of available data (see section 5.1), it may vary strongly between different substances with the same endocrine mode of action and has to be addressed on a case-by-case basis.

7 Outlook / further open questions

Within the present project, it was assumed that based on their endocrine disrupting properties the substances to be considered were already classified as substances of very high concern (see section 1.2). Yet, an important shortcoming of the environmental risk assessment procedure for potential EDCs according to REACH should at least be mentioned: Given that information on potential endocrine activity is not part of the standard information requirements according to REACH Annexes VII – X (EC 2007, ECHA 2008a; section 2.1.1), the initial assessment of the potential for ED is only based on an evaluation of available information. When there is concern of possible ED, specific studies may be requested on a case-by-case basis (ECHA 2008a). This means that the likelihood of an initial concern for potential ED depends on the availability of (1) information on potential endocrine activity of the substance that has been generated e.g. within public screening programmes or research projects and (2) toxicological information on potential ED for the respective substance. For data-poor substances, there is thus a high risk that endocrine activity of a chemical is not identified.

In this context, it is also of note that toxicological information is relevant for wildlife vertebrates, since the endocrine system is relatively conserved within the vertebrates (Vos et al. 2000, OECD 2011a). Yet, due to the large differences in endocrinology between vertebrates and invertebrates, this information is only of limited use for invertebrates (IPCS 2002, Matthiessen & Johnson 2007). For chemicals, which exclusively affect specific endocrine processes of invertebrates, the likelihood to miss endocrine effects is therefore high as long as life-cycle testing with invertebrates is not included as a general approach (see section 5.3).

In the present report, differences in sensitivity between fish species were mainly evaluated on the basis of laboratory tests. Yet, factors other than the intrinsic sensitivity, which are responsible for interspecies differences observed in laboratory studies, are relevant for the vulnerability of a species in the field, namely the extent of exposure to the toxicant and the population's potential to recover from a toxic effect (cf. section 2.2). This is for example illustrated by the results of the whole lake study with 17α-ethinylestradiol (section 2.2.3) and the effects of tributyltin on different prosobranch species (section 2.2.7). This issue – the extrapolation from laboratory data to effects in the field – is too broad to be addressed in the present project. However, it should be noted that this issue is not specific to endocrine active substances, but applies to environmental risk assessment in general (Rubach et al. 2010).

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9 Annex

This annex contains the following tables:

- Table 11: Relevant endpoints for endocrine disruption in fish tests included in the 'Guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption' (OECD 2011a)
- Table 12: Required long-term aquatic toxicity tests according to ECHA (2008)
- Table 13. : Relevant endpoints of partial and full life-cycle tests with invertebrates, which are currently being developed or have been developed recently and are included in the OECD Conceptual Framework
- Table 14: Effect concentrations of bisphenol A in aquatic invertebrates and fish
- Table 15: Effect concentrations of 4-tert-octylphenol in aquatic invertebrates and fish
- Table 16: : Effect concentrations of 17α-ethinylestradiol in fish
- Table 17: Effect concentrations of prochloraz in fish
- Table 18: Effect concentrations of tributyltin in aquatic invertebrates and fish
- Table 19: Effect concentrations of triphenyltin in aquatic invertebrates and fish

All abbreviations used in the tables are included in the list of abbreviations (p. VI ff.). The cited references are included in section 8.

Table 12: Relevant endpoints for endocrine disruption in fish tests included in the 'Guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption' (OECD 2011a). Grey shading indicates apical endpoints. A draft guideline for a fish multi-generation assay is included, for which only provisional guidance is provided in OECD (2011a).

Level in OECD CF	Test (guideline)	Endpoint	Type of effect	Indicated effect / endocrine disruption (ED) modality [1]	Remark
3	Fish short-term reproduction assay (OECD TG 229)	Vitellogenin in males	Induction	ER agonism	
		Vitellogenin in females	Depression	ER antagonism / steroidogenesis related activity	In the absence of systemic toxicity
		Secondary sexual characteristics in males (fathead minnow, medaka)	Reduction	ER agonism / AR antagonism	
			Induction	AR agonism	
		Specific gonad histopathological changes		ER agonism / antagonism, AR agonism / antagonism or steroidogenesis related activity	As detailed in OECD (2010d)
		Fecundity [2]	Reduction	Not diagnostic of ED modality	
		Behaviour			
		Growth (length, weight)			
3	21-Day fish assay (OECD TG 230)	Vitellogenin in males	Induction	ER agonism	
		Vitellogenin in females	Depression	ER antagonism / steroidogenesis related activity	In the absence of systemic toxicity
		Secondary sexual characteristics in males (fathead minnow, medaka)	Reduction	ER agonism / AR antagonism	
			Induction	AR agonism	
		Behaviour		Not diagnostic of ED modality	
3	Androgenised female stickleback screen (OECD GD 140)	Spiggin	Induction	AR agonism	
			Depression	AR antagonism	
4	Fish sexual development test (OECD TG 234)	Phenotypic sex ratio [3]	Female-biased	ER agonism / AR antagonism	
			Male-biased	ER antagonism / AR agonism or steroidogenesis related activity	
		Percentage of sexually undifferentiated fish	Increase	ER antagonism	
		Intersex	Induction	AR antagonism	
		Vitellogenin in males and females	Induction	ER agonism	
			Depression	AR agonism / steroidogenesis related activity	
		Vitellogenin in females	Depression	ER antagonism	
			Induction	AR antagonism	

Level in OECD CF	Test (guideline)	Endpoint	Type of effect	Indicated effect / endocrine disruption (ED) modality [1]	Remark
4	Fish sexual development test (continued)	Specific gonad histopathological changes		ER agonism / antagonism or AR agonism / antagonism	As detailed in OECD (2010d)
		Morphological abnormalities		Not diagnostic of ED modality	
		Hatching			
		Survival			
		Growth (length, weight)			
5	Fish life cycle toxicity test (US EPA OPPTS 850.1500) [4]	Phenotypic sex ratio [3]	Female-biased	ER agonism	
			Male-biased	AR agonism	
		Vitellogenin in males	Induction	ER agonism	
		Vitellogenin in females	Depression	Steroidogenesis related activity	
		Levels of estradiol / (keto-)testosterone		Effect on ER / AR	
		Levels of thyroid hormones		Thyroid-related activity	
		Hatching success		Not diagnostic of ED modality	
		Growth (length, weight)			
		Behaviour			
		Gross morphology			
		Gonado-somatic index			
		Multiple organ histopathology			
		Time to maturity (time to first spawn)			
		Fecundity			
		Fertilisation success			
5	Fish (medaka) multi-generation test (draft OECD TG)	Phenotypic sex ratio	Female-biased	ER agonism	Assay not yet fully validated
		Vitellogenin in males	Induction	ER agonism	
		Vitellogenin in females	Depression	Steroidogenesis related activity	
		Altered levels of estradiol and / or (keto-) testosterone		Effect on ER / AR	
		Altered levels of thyroid hormones		Thyroid-related activity	
		Hatching success		Not diagnostic of ED modality	
		Growth (length, weight)			

Level in OECD CF	Test (guideline)	Endpoint	Type of effect	Indicated effect / endocrine disruption (ED) modality[1]	Remark
5	Fish (medaka) multi-generation test (continued)	Behaviour			Assay not yet fully validated (see above)
		Gross morphology			
		Gonado-somatic index			
		Multiple organ histopathology			
		Time to maturity (time to first spawn)			
		Fecundity			
		Fertilisation success			

(1) For many tests, individual endpoints alone may not indicate an endocrine disruption modality, but a combination of endpoints or assays in a weight of evidence assessment is required to identify the ED modality. (2) Effects on fecundity observed in OECD TG 229 could be used in the ERA. Yet, due to the high variability of fecundity, the relative short test duration and the fact that only three concentrations are tested in this assay no reliable NOEC or EC_x for fecundity can be derived. Therefore, a positive test result would usually trigger a fish life-cycle or medaka multi-generation test. (3) Determination of genotypic sex ratio (in medaka, zebrafish or stickleback) allows a more powerful detection of effects on phenotypic sex ratio. However, sufficient power can be achieved with phenotypic sexing alone when using an appropriate number of animals. (4) With optional endocrine-sensitive additions. At present, no endpoints for estrogen and androgen receptor agonistic and antagonistic effects, effects on steroidogenesis and thyroid effects are included in the guideline. Such endpoints could be added.

Table 13: Required long-term aquatic toxicity tests according to ECHA (2008). Only the recommended OECD test methods are mentioned, alternative test methods based on national test guidelines are not included.

Test	Test guideline	Endpoint	Relevant endpoint for	Required for tonnage	Remark / Reference
Long-term toxicity testing on invertebrates (preferably *Daphnia*)	*Daphnia magna* reproduction test (OECD TG 211)	Survival		≥ 100 t/a	
		Reproduction (number of living offspring per animal and day)			
		Growth (length)			
		Time to production of first brood (and subsequent broods)			Optional endpoint
		Number and size of broods per animal			Optional endpoint
		Number of aborted broods			Optional endpoint
		Presence of male neonates			Optional endpoint
		Presence of ephippia			Optional endpoint
		Intrinsic rate of population increase			Optional endpoint
Long-term toxicity testing on fish	Fish early-life stage (FELS) toxicity test (OECD TG 211)	Hatching rate		≥ 100 t/a	
		Survival			
		Appearance (observations)			
		Behaviour (observations)			
		Growth (length, weight)	Aromatase inhibitors		Teigeler et al. 2007
	Fish, juvenile growth test (OECD TG 215)	Growth (weight)	Aromatase inhibitors	≥ 100 t/a	Teigeler et al. 2007
Long-term toxicity to sediment organisms	Sediment-water chironomid toxicity test using spiked sediment (OECD TG 218)	Development time (time to emergence) for ♂ / ♀		≥ 1,000 t/a	
		Number of emerged ♀ and ♂ midges			
		Larval survival			
		Larval growth (length, weight)			
	Sediment-water chironomid toxicity test using spiked water (OECD TG 219)	Development time (time to emergence)		≥ 1,000 t/a	
		Number of emerged ♀ and ♂ midges			
		Larval survival			
		Larval growth			
	Sediment-water *Lumbriculus* toxicity test using spiked sediment (OECD TG 225)	Survival / reproduction (total number of worms)		≥ 1,000 t/a	
		Biomass			

Table 14: Relevant endpoints of partial and full life-cycle tests with invertebrates, which are currently being developed or have been developed recently and are included in the OECD Conceptual Framework (CF).

Level in OECD CF	Test	Endpoint	Reference
4	Mollusc partial life-cycle test with *Potamopyrgus antipodarum*	Reproductive success: total number of shelled and unshelled embryos [1]	OECD 2010a
		Mortality	
5	Harpacticoid copepod development and reproduction test with *Amphiascus tenuiremis*	Mortality	OECD 2011c
		Moulting	
		Developmental rate	
		Sex ratio	
		Body length	
		Reproductive success (fecundity)	
		Ecdysone levels	
5	Mollusc full life-cycle test with *Lymnea stagnalis*	Reproductive success of F_0 and F_1: number of egg clutches per day; number of viable eggs per clutch	OECD 2010a
		Hatching success	
		Time to 50% hatch	
		Time to first reproduction of F_1	
		Mortality	
5	Mysid two-generation reproductive and developmental toxicity test with *Americamysis bahia*	Mortality	OECD 2006a, Verslycke et al. 2007
		Developmental rate	
		Growth	
		Time to sexual maturation	
		Time to first brood release	
		Total number of offspring	
		Sex ratio	
		Percentage of females that are reproductively active	
		Steroid metabolims (optional)	
		Vitellogenin levels (optional)	
5	Water-sediment-water chironomid life-cycle test (OECD TG 233)	Emergence	OECD 2010c
		Time to emergence	
		Sex ratio	
		Reproduction: number of egg ropes / female, number of fertile egg ropes / female [2]	

(1) Developmentally more advanced embryos already possessing shells are distinguished from developmentally less advanced embryos not yet possessing shells (OECD 2010a). (2) An egg rope is considered fertile, if larvae hatch out of at least 1/3 of the eggs (OECD 2010c).

Table 15: Effect concentrations of bisphenol A (BPA) in aquatic invertebrates and fish (classification of validity based on Klimisch et al. 1997, EC 2003 and 2011a).

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity [1]	Reference
Porifera											
Heteromyenia sp.	Growth test starting with gemmules (9 d)	BPA (n.i.)	n.i.	0.16, 1.6, 16, 80, 160 mg/L	n.i.	Growth rate on d 6	LOEC	16 mg/L	No germination at 80 and 160 mg/L	2-3	Hill et al. 2002
Hydrozoa											
Hydra vulgaris	Regeneration test with injured animals (72 h)	BPA (n.i.)	Ethanol (100-500 ul/L)	n.i.	0.002, 0.02, 0.04, 7.8, 42, 460, 1000, 2200 and 4600 ug/L	Regeneration of injured region (isolated digestive region)	Inhibition of regeneration at > 1000 ug/L		Clear effect, no statistical analysis	2-3	Pascoe et al. 2002
Hydra oligactis	Chronic test, semi-static, 10°C, separate exposure of ♂ (35 d), and ♀ (50 d) with (1) starved and (2) fed polyps. Test water only exchanged on d 12, 24 and 36	BPA (n.i.)	DMSO (50 ul/L)	0.5, 1, 2, 3, 4 mg/L	n.i. (variation in measured concentrations: 6%, no information on time of sampling and number of analysed samples; probably only stock solutions analysed)	Reduced percentage of polyps with testes in starved ♂	LOEC	1 mg/L		3	Fukuhori et al. 2005
						Reduced percentage of polyps with testes in fed ♂	Significant effect only at 0.5, 1 and 4 mg/L		No clear concentration-effect relationship		
						Reduced percentage of polyps with eggs in starved ♀	LOEC	1 mg/L			
	Asexual reproduction test with ♂ polyps, semi-static, at 10 and 20°C (35 d). Test water exchanged 3 times /week	BPA (n.i.)	DMSO (50 ul/L)	0.5, 1, 2, 3, 4 mg/L		Nº of buds/polyp at 10°C	Stimulation at 0.5 and 1 mg/L, reduction at 2, 3 and 4 mg/L			2-3	
						Nº of buds/polyp at 20°C	Stimulation at 1 mg/L, reduction at 2, 3 and 4 mg/L				

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Vali-dity	Reference
Nematoda											
Caenorhabditis elegans	6-d Test with age-synchronous worms on agar plates	BPA (n.i.)	Ethanol (3 ml/L)	0.023, 0.23, 2.3, 23, 228, 2283 ug/L agar	None	Increase in percentage of germ cells	LOEC	≤ 0.23 ug/L	High solvent conc. Test on agar plates	2–3	Hoshi et al. 2003
Mollusca											
Marisa cornuarietis	Reproduction test starting with adults (F_0), semi-static, 22°C (5 mo)	BPA (n.i.)	Ethanol (12.5 ug/L)	1, 5, 25 and 100 ug/L	None	Increase in spawning mass production	LOEC	≤ 1 ug/L		2	Oehlmann et al. 2000, Schulte-Oehlmann et al. 2001
						Increase in egg production					
						Induction of superfemales					
						Mortality					
	Life-cycle test: egg clutches (F_1) from previous test exposed for further 12 mo, semi-static, 22°C	BPA (n.i.)	Ethanol (12.5 ug/L)	1 and 100 ug/L	None	Hatching success	Not affected				
						Increase in spawning mass production	LOEC	≤ 1 ug/L			
						Increase in egg production					
						Mortality					
						Induction of superfemales	At both concentrations				
						Imposex	LOEC	100 ug/L			
M. cornuarietis	Reproduction test starting with adults, semi-static, 22°C (6 mo)	BPA (n.i.)	Ethanol (12.5 ug/L)	50, 100, 250, 500, 1000 ng/L	7.9, 48, 104, 205 and 404 ng/L	Induction of superfemales	At 7.9, 104, 205 and 404, but not at 48 ng/L		High variation between measured conc. All effect concentrations based on measured conc.	2	Schulte-Oehlmann et al. 2001, Oehlmann et al. 2006a
						Clutch production (d 0 to d 60)	LOEC	48 ng/L			
							EC_{10}	15 ng/L			
							EC_{50}	60 ng/L			
						Egg production (d 0 to d 60)	LOEC	48 ng/L			
							EC_{10}	14 ng/L			
							EC_{50}	63 ng/L			
						Imposex	Not observed				

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
M. cornuarietis	Reproduction test starting with adults, semi-static (5 mo) (1) at 20°C	BPA (n.i.)	Ethanol (12.5 ul/L)	250, 500, 1000, 5000 ng/L	106, 224, 465, 2170 ng/L	Egg production	LOEC	≤ 106 ng/L	High variation between measured conc. All effect conc. based on meas. conc. (some EC_{10} values extra-polated beyond range of tested conc.)	2	Oehlmann et al. 2006a
							EC_{10}	15 ng/L			
						Clutch production	LOEC	≤ 106 ng/L			
							EC_{10}	18 ng/L			
	(2) at 27°C				97.5, 205, 436, 1990 ng/L	Egg production	LOEC	436 ng/L			
							EC_{10}	998 ng/L			
						Clutch production	LOEC	> 1990 ng/L			
							EC_{10}	2090 ng/L			
M. cornuarietis	Reproduction test starting with adults, flow-through, 25°C (12 weeks)	BPA (n.i.)	None	0.1, 1, 16, 160, 640 ug/L	0.1, 1.01, 13.7, 155, 607 ug/L	Mortality	No effect		GLP study	1	Forbes et al. 2007
						Egg production (egg/♀/month)	LOEC	> 607 ug/L			
	Hatchability /juvenile growth test starting with egg masses from the reproduction test (continued until 60 dph)				0.14, 1.22, 12.0, 157, 682 ug/L	Hatching success	LOEC	> 682 ug/L			
						Time to first hatch					
						Juvenile growth					
Nucella lapillus	Chronic test starting with adults collected from field, semi-static, 14°C (3 mo)	BPA (n.i.)	Glacial acetic acid	1, 25, 100 ug/L	None	Mortality	No effect		Note that *N. lapillus* does not produce egg capsules when transferred from field to laboratory	2	Oehlmann et al. 2000, Schulte-Oehlmann et al. 2001
						Reduced penis length	LOEC	≤ 1 ug/L			
						Reduced prostate length					
						Reduced amount of stored sperm					
						Oocyte production					
						Increased weight of pallial gland					
						Capsule gland length					

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
Nassarius reticulatus	Chronic sediment test (spiked sediment) starting with adults collected from field (3 mo)	BPA (n.i.)	Glacial acetic acid (5 mg/kg sediment dw)	10, 50 and 1000 ug/kg sediment dw	None	Increased weight of pallial gland	LOEC	≤ 10 ug/kg dw		2	Schulte-Oehlmann et al. 2001
Potamopyrgus antipodarum	Reproduction test, semi-static (28 d) (1) at 7°C	BPA (≥97%)	None	5, 10, 20 and 40 ug/L	4.8, 9.3, 19.1 and 39.4 ug/L	Total number of embryos / ♀	LOEC	9.3 ug/L		2	Sieratowicz et al. 2011
	(2) at 16°C				4.6, 8.9, 19.4 and 38.7 ug/L	Total number of embryos / ♀	LOEC	38.7 ug/L			
	(3) at 25°C				1.4, 1.7, 7.2, 21.6 ug/L	Total number of embryos / ♀	LOEC	1.7 ug/L			
P. antipodarum	Reproduction test, static with spiked sediment, 15°C (8 weeks)	BPA (>97%)	Ethanol	1, 10, 30, 100, 300 ug/kg sediment dw	None	Number of unshelled embryos (8 weeks) ↑	LOEC	≥ 1 ug/kg dw		2	Duft et al. 2003b
						Total number of embryos (8 weeks) ↑					
P. antipodarum	Reproduction test, semi-static, 14°C, (9 weeks)	BPA (n.i.)	Ethanol (12.5 ug/L)	1, 5, 25 and 100 ug/L	None	Embryo production	Significant effect at 5 and 25 ug/L, but not at 1 and 100 ug/L (inverse U-shaped curve)			2	Schulte-Oehlmann et al. 2001
P. antipodarum	Reproduction test, semi-static (9 weeks)	BPA (n.i.)	n.i.	1, 5, 25 and 100 ug/L	None	Mortality	No effect			2	Jobling et al. 2004
						Increased embryo production (d 21 and d 42)	Significant effect at 1, 5 and 25, but not at 100 ug/L				
						Increased embryo production (d 63)	Significant effect at 5 and 25, but not at 1 and 100 ug/L				

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Vali-dity	Reference
Rotifera											
Brachionus calyciflorus	Reproduction test, static (48 h)	BPA (n.i.)	n.i.	n.i.	n.i.	Reproduction	NOEC	1800 ug/L	NOEC based on measured concentrations. GLP study	1	Springborn Smithers Laborato-ries 2006a (cited in EC 2008a)
Crustacea											
Acartia tonsa	Partial life cycle test starting with eggs, semi-static (11 d). Test substances were added with food (algae) to facilitate sorption to food	BPA (>99%)	None	0.2, 2, 20 ug/L	None	Egg production, d 10	LOEC	20 ug/L	No effect on d 9 and d 11	2	Andersen et al. 1999
Daphnia magna	9-d Test starting with adult females. Number of male offspring in third brood determined	BPA (n.i.)	Ethanol (100 ul/L)	10 mg/L	None	Induction of male offspring	No effect		Only 1 test conc.	2-3	Wang et al. 2005
D. magna	Exposure of ♀ starting with < 1 h old animals, semi-static (n.i.). Evaluation of duration of first moulting	BPA (n.i.)	Ethanol (100 ul/L)	n.i. (approx. 20 concentrations ranging from 1 to 10 mg/L)	None	Intermolt duration	Increased at conc. ≥ 8 mg/L (no statistical evaluation; only 1 individual per conc.)			3	Mu et al. 2005
	Reproduction test (21 d)					Total offspring produced	Clear reduction at 10 mg/L (no statistical evaluation; only 1–2 individuals per conc.)				

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Vali-dity	Reference
Ceriodaphnia dubia	Survival and reproduction test (7 d)	BPA (n.i.)	Methanol	0.94, 1.88, 3.75, 7.5, 15, 30 mg/L	None	Reproduction inhibition	LOEC	1.88 mg/L		2	Tatarazako et al. 2002
							EC_{25}	3.92 mg/L			
Tigriopus japonicus	2-Generation test starting with < 24 h old nauplii, semi-static. F_0 exposed for 21 d, F_1 (first brood) exposed for further 21 d	BPA (98–99%)	DMSO (max. 10 ul/L)	0.01, 0.1, 1 and 10 ug/L	None	F_0: Delayed naupliar development	LOEC	0.1 ug/L		2	Marcial et al. 2003
						F_0: Delayed maturation	LOEC	1.0 ug/L			
						F_0: Fecundity	LOEC	> 10 ug/L			
						F_0: Sex ratio					
						F_0: Survival					
						F_1: Delayed naupliar development	LOEC	≤ 0.01 ug/L			
						F_1: Delayed maturation					
						F_1: Fecundity	LOEC	> 10 ug/L			
						F_1: Sex ratio					
						F_1: Survival					
Gammarus pulex	Short-term exposure (24 h) followed by behavioural assay	BPA	Ethanol, c_{max} (max. 5 ml/L), i.e. very high max. solvent conc.	0.01, 0.1, 1.0, 10, 100, 1000, 10000, 20000 ug/L	0.0146, 0.0538, 0.36, 5.1, 56, 830, 8400, 19400 ug/L	Delay in median time to re-pairing after pairs were separated	LOEC	8400 ug/L	LOEC close to 24-h LC_{50} (12.8 mg/L) and above 48-h LC_{50} (5.6 mg/L)	2	Watts et al. 2001
G. pulex	Subchronic test started with pre-copula pairs , semi-static (14 d)	BPA (n.i.)	Ethanol (100 ul/L)	1, 10, 100, 1000 ug/L	None	Survival	Affected at 1000 ug/L			2	Johnson et al. 2005
						Juvenile production	LOEC	>1000 ug/L			
						Moulting	LOEC	>1000 ug/L			

Hyalella azteca	Reproduction test (42 d)	BPA (n.i.)	n.i.	n.i.	0.12, 0.22, 0.49, 1.0, 2.2 mg/L	Cumulative number of offspring per female	LOEC	1000 ug/L	GLP study	1	Springborn Smithers Laboratories 2006b (cited in EC 2008a)
Test species	**Test method (duration)**	**Substance (purity)**	**Solvent (max. conc.)**	**Nominal conc.**	**Measured conc.**	**Endpoint**	**Effect conc.**		**Remark**	**Validity**	**Reference**
Echinodermata											
Strongylocentrotus purpuratus	Developmental toxicity test starting with freshly fertilised eggs (96 h)	BPA (n.i.)	DMSO (n.i.)	n.i.	None	Developmental toxicity (teratogenicity) at the pluteus stage	EC_{50}	226.5 ug/L	No information provided on tested conc.	2-3	Roepke et al. 2005
Pisces											
Danio rerio	Short term screening test with adults, flow-through (96 h)	BPA (>99%)	None	0.01, 0.1, 1.0, 10, 100 ug/L	0.013, 0.14, 0.97, 7.5, 81 ug/L	Vitellogenin in ♂	LOEC	7.5 ug/L		2	Villeneuve et al. 2012
						Vitellogenin in ♀	LOEC	> 81 ug/L			
D. rerio	Short-term screening test with adult ♂, flow-through (10 d)	BPA (>99%)	None	20, 63, 200, 632 ug/L	8, 17, 72 and 165 ug/L	Vitellogenin in ♂	Clear increase in highest test conc., but no significance due to large variation between fish			2	Duis & Knacker 2003
D. rerio	Fish life cycle test starting with freshly fertilized eggs, semi-static with three exchanges of test solutions per week (205 d)	BPA (98%)	n.i.	94, 187, 375, 750, 1500 ug/L	Geometric means: 12, 24, 40, 86, 157 ug/L (see remark)	F_0: Vitellogenin in ♂, d 205	LOEC	40 ug/L	Effect conc. based on gemetric means of conc. measured in freshly prepared and old test solutions (before renewal; C. Schäfers, pers. comm.	2	Schäfers & Wenzel 2000, Segner et al. 2003a, b, Wenzel et al. 2001b, Teigeler et al. 2007
						F_0: Histologic alterations in gonads					
						F_0: Growth, 75 dpf	LOEC	157 ug/L			
						F_0: Time to first spawn					
						F_0: Mating behaviour	Altered at 157 ug/L				
						F_0: N° of eggs/♀	LOEC	157 ug/L			
						F_0: Fertilization success					

						F_1: Hatching and survival until 35 dpf	LOEC	> 157 ug/L			
Pimephales promelas	Short term screening test with adults, flow-through (96 h)	BPA (>99%)	None	0.01, 0.1, 1.0, 10, 100 ug/L	0.013, 0.14, 0.97, 7.5, 81 ug/L	Vitellogenin in ♂	LOEC	81 ug/L		2	Villeneuve et al. 2012
						Vitellogenin in ♀	LOEC	7.5 ug/L			
						Plasma estradiol level in ♀					
Test species	**Test method (duration)**	**Substance (purity)**	**Solvent (max. conc.)**	**Nominal conc.**	**Measured conc.**	**Endpoint**	**Effect conc.**		**Remark**	**Validity**	**Reference**
P. promelas	Long-term reproductive test starting with adults fish (F_0), flow-through (164 d), F_1 only evaluated until hatch	BPA (n.i.)	n.i.	1, 16, 160, 640, 1280 ug/L	70 – 96% of nominal conc.	F_0: Length of ♂, d 71 and 164	LOEC	640 ug/L		2	Sohoni et al. 2001
						F_0: Weight of ♂, d 71 and 164					
						F_0: Vitellogenin in ♂, d 43					
						F_0: Vitellogenin in ♂, d 71	LOEC	160 ug/L			
						F_0: Vitellogenin in ♂, d 164					
						F_0: GSI in ♀, d 164	LOEC	640 ug/L			
						F_0: GSI in ♂↑, d 43	LOEC	1 ug/L	Transient effect (see line below)		
						F_0: GSI ↓ in ♂, d 164	LOEC	640 ug/L			
						F_0: Reduced % of spermatozoa in testes	LOEC	16 ug/L			
						F_0: Cumulative fecundity (total nº of egg/♀)	LOEC	1280 ug/L			
						F_1: Hatching	LOEC	640 ug/L			

P. promelas	Short term screening test with adults, flow-through (14 d)	BPA (99%)	Dimethyl-formamide (n.i.)	7.5, 15, 30, 45, 75, 150 ug/L	4.1 / 6.1, 9.6 / 12, 19 / 22, 43 / 32, 79 / 41, 150 / 110 ug/L (2 measure-ments per conc.)	Vitellogenin in ♂	EC_{50}	158 ug/L	Extrapolated slightly beyond range of tested concentrations	2	Brian et al. 2005
Oryzias latipes	Fish sexual development assay starting 1 dph, semi-static (approx. 100 d)	BPA (n.i.)	Acetone	10, 50, 100, 200 ug/L	None	Intersex	LOEC	> 200 ug/L		2	Metcalfe et al. 2001
						Testes histology	Histological changes (e.g. fibrosis, reduced number of spermatozoa) at ≥ 50 ug/L (no statistical evaluation)				
Test species	**Test method (duration)**	**Substance (purity)**	**Solvent (max. conc.)**	**Nominal conc.**	**Measured conc.**	**Endpoint**	**Effect conc.**		**Remark**	**Vali-dity**	**Reference**
O. latipes	Reproduction test with adults, flow-through (21 d)	BPA (>99%)	None	1000, 2000, 4000 ug/L	837, 1720 and 3120 ug/L	Cumulative nº of eggs/pair	LOEC	> 3120 ug/L		2	Kang et al. 2002
						Fertilisation rate					
						GSI in ♂ and ♀					
						Intersex/ovotestes	Intersex induced in 1 of 8 males at 837 ug/L, in 6 of 7 males in 1720 ug/L and in 4 of 8 males at 3120 ug/L (no statistical evaluation)				
						Vitellogenin in ♂	LOEC	3120 ug/L			
						Vitellogenin in ♀	LOEC	> 3120 ug/L			
O. latipes	Fish sexual development test starting with freshly fertilised eggs, semi-static until hatch, then flow-through (approx. 70 d)	BPA (>99%)	None	3.2, 16, 800, 400, 2000 ug/L	2.28, 13.0, 71.2, 355, 1820 ug/L	Length	LOEC	1820 ug/L		2	Yokota et al. 2000
						Weight					
						Sex ratio					
						Intersex	Intersex induced at 1820 ug/L in 6 out of 19 fish (no statistical evaluation)				

Xiphophorus helleri	Short term screening test starting with 30-d-old juveniles, static (60 d)	BPA (n.i.)	Ethanol (100 ul/L)	0.2, 2, 20 ug/L	None	Reduced sword length in ♂	LOEC	2 ug/L	Exposure under static conditions, apparently no replication	3	Kwak et al. 2001
Carassius auratus	Short-term screening assay for endocrine effects with adult ♂, semi-static (28 d)	BPA (n.i.)	Ethanol (100 ul/L)	1, 10, 100, 1000 ug/L	None	Vitellogenin in ♂ (d 7)	LOEC	1000 ug/L	Vitellogenin determined using antibody against *Cyprinus carpio* lipo-vitellin	2	Ishibashi et al. 2001
						Vitellogenin in ♂ (d 28)	LOEC	100 ug/L			
						GSI of ♂		No effect			
Oncorhynchus mykiss	Screening test for endocrine effects with juveniles, flow-through (12 d)	BPA (n.i.)	Ethanol (n.i.)	10, 40, 70, 100, 500 ug/L	9.0, 37.6, 70.2, 106, 556 ug/L	Vitellogenin (d 6)	LOEC	556 ug/L	Clear effects already at ≥70 ug/L, but not significant	2	Lindholst et al. 2000, 2003
							EC_{50}	69 ug/L			
						Vitellogenin (d 12)	LOEC	556 ug/L			
							EC_{50}	95 ug/L			
Test species	**Test method (duration)**	**Substance (purity)**	**Solvent (max. conc.)**	**Nominal conc.**	**Measured conc.**	**Endpoint**	**Effect conc.**		**Remark**	**Validity**	**Reference**
Salmo trutta f. *fario*	Chronic test with late prespawning and spawning adults (2 mo)	BPA	DMSO (max. 510 ug/L)	1.75, 2.4, 5.0 ug/L	None	Sperm density ↓	LOEC	≤ 1.75 ug/L	[2]	2	Lahnsteiner et al. 2005
						Sperm motility ↓			[3]		
						Swimming velocity of sperm ↓			[2]		
						Semen mass in ♂ ↓	LOEC	5.0 ug/L			
						Suppression of ovulation in ♀					

(1) Classification of validity: 1 = valid without restrictions, 2 = valid with restrictions, 3 = not valid, 4 = validity not assingnable. (2) Significant reduction at the beginning and in the middle of the spawning period, but not at the end of the spawning period. (3) Significant reduction at the beginning of the spawning period, in the middle of the spawning period significant effect only at next higher concentration (2.4 ug/L), at the end of the spawning period no significant effect.

Table 16: Effect concentrations of 4-tert-octylphenol (4-tert-OP) in aquatic invertebrates and fish (classification of validity based on Klimisch et al. 1997, EC 2003 and 2011a).

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
Mollusca											
Marisa cornuarietis	Reproduction test starting with adults (F_0), semi-static, 22°C (5 mo)	4-tert-OP [1] (n.i.)	Ethanol (n.i.)	1, 5, 25, 100 ug/L	None	Spawning mass production ↑	LOEC	≤ 1 ug/L	U-shaped conc.-response curves	2	Oehlmann et al. 2000
						Egg production ↑					
						Induction of superfemales					
						Mortality					
	Life-cycle test: egg clutches (F_1) from previous test exposed for further 12 mo, semi-static, 22°C			1 and 100 ug/L		Hatching success	Not affected				
						Spawning mass production ↑	LOEC	≤ 1 ug/L			
						Egg production ↑					
						Mortality					
						Induction of superfemales					
						Imposex	LOEC	> 100 ug/L			
Nucella lapillus	Chronic test starting with adults collected from field, semi-static, 14°C (3 mo)	4-tert-OP [1] (n.i.)	Glacial acetic acid	1, 25, 100 ug/L	None	Mortality	No effect		Note that *N. lapillus* does not produce egg capsules when transferred from field to laboratory	2	Oehlmann et al. 2000
						Penis length ↓	LOEC	≤ 1 ug/L			
						Prostate length ↓					
						Amount of stored sperm ↓					
						Weight of pallial glands ↑					
						Oocyte production ↑					
						Capsule gland length↑					
Potamopyrgus antipodarum	Reproduction test, semi-static, (9 wk)	4-tert-OP (n.i.)	n.i.	1, 5, 25, 100 ug/L	None	Mortality	No effect			2	Jobling et al. 2004
						Increased embryo production (d 63)	Significant effect at 5 and 25 ug/L, but not at 1 and 100 ug/L (inverted U-shaped conc.-response curve)				

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
P. antipodarum	Reproduction test, static, with spiked sediment 15°C (8 wk)	4-tert-OP (>98%)	Ethanol (n.i.)	1, 10, 30, 100, 300 ug/kg sediment dw	None	Number of unshelled embryos (8 wk) ↑	LOEC	1 ug/kg sed. dw	No significant effect at 300 ug/kg dw (inverted U-shaped conc-response curve)	2	Duft et al. 2003b
						Total number of embryos (8 wk)↑	Significant effect only at 1 ug/kg sediment dw				
Crustacaea											
Daphnia magna	Life-cycle toxicity test (21 d)	4-tert-OP (99.3%)	Acetone	30, 60, 120, 250, 500 ug/l	37, 62, 120, 230, 510 ug/L	See remark	LOEC	62 ug/L	GLP study. Secondary source, overall LOEC based on survival, reproduction and mean length of adults.	1	Analytical Bio-Chemistry Laboratories, Inc. 1988, cited in OECD 1995b and IUCLID 2000
D. magna	Subchronic test starting with 12 h-old neonates, semi-static (7 d)	4-Octylphenol (no further specification; purity n.i.)	Acetone (16-315 mg/L)	10, 20 and 40 ug/L	None	Interference with molting (increase in time needed to accomplish four moults)	No effect		Test substance not sufficiently specified. Up to 17% mortatlity in control	3	Zou & Fingerman 1997b
Acartia tonsa	Larval development test starting with eggs (5 d)	4-tert-OP (90%)	Acetone (max. 100 ul/L)	n.i.	Yes, but no data presented	Inhibition of naupliar development	EC_{10}	5.2 ug/L	EC_{10} and EC_{50} based on measured conc.	2	Andersen et al. 2001
							EC_{50}	13 ug/L			
Tigriopus japonicus	2-Generation test starting with < 24 h old nauplii, semi-static. F_0 exposed for 21 d, F_1 (first brood) exposed for further 21 d	4-tert-OP (n.i.)	DMSO (10 ul/L)	0.01, 0.1, 1, 10 ug/L	None	F_0: Delayed naupliar development	Significant effect at 0.1 and 1, but not at 10 ug/L			2	Marcial et al 2003
						F_0: Delayed maturation	LOEC	> 10 ug/L			
						F_0: Fecundity	Significant increase at 1.0 ug/L				
						F_0: Sex ratio	LOEC	> 10 ug/L			
						F_0: Survival					

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
T. japonicus	2-Generation test (continued)	See above	See above	See above	None	F_1: Delayed naupliar development	Significant effect at 0.01, 1.0 and 10, but not at 0.1 ug/L			2	Marcial et al 2003
						F_1: Delayed maturation	LOEC	1.0 ug/L			
						F_1: Fecundity	LOEC	> 10 ug/L			
						F_1: Sex ratio					
						F_1: survival					
Echinodermata											
Arbacia lixula	Embryo-larval toxicity test starting with freshly fertilised eggs (72 h)	Octylphenol (no further specification; purity n.i.)	DMSO (16 ml/L)	5, 10, 20, 40, 80, 160 ug/L	None	Larval malformations	LOEC	20 ug/L	Test substance not sufficiently specified. Extremely high solvent (DMSO) concentration.	3	Arslan & Parlak 2007
						Developmental arrest in blastula / gastrula					
Paracentrotus lividus	Embryo-larval toxicity test starting with freshly fertilised eggs (72 h)	Octylphenol (p.a.) (no further specification)	DMSO (5.3 ml/L)	5, 10, 20, 40, 80, 160 ug/L	None	Delayed larval development	LOEC	> 160 ug/L	Test substance not sufficiently specified. Extremely high solvent (DMSO) concentration	3	Arslan et al. 2007
						Malformations of larvae	LOEC	≤ 5 ug/L			
						Developmental arrest in blastula / gastrula		40 ug/L			
	Evaluation of fertilisation success					Fertilisation success ↓	LOEC	≤ 5 ug/L			
Strongylocentrotus purpuratus	Developmental toxicity test starting with freshly fertilised eggs (96 h)	4-OP (no further specification, purity not indicated)	DMSO (n.i.)	0.01, 0.1, 5.0 ug/L	None	Developmental toxicity (teratogenicity) at the pluteus stage	EC_{50}	0.174 ug/L	Test substance not sufficiently specified. Few test concentrations for EC_{50} determination	2-3	Roepke et al. 2005

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
Pisces											
D. rerio	Short-term exposure of adult ♂ and ♀, semi-static (21 d) followed by 5-d cross-breeding trial (exposed ♂ with unexposed ♀; exposed ♀ with unexposed ♂) in control water	4-tert-OP (97%)	Methanol (1 ml/L)	12.5, 25, 50, 100 ug/L	Fresh solutions (immediately after renewal): measured conc. = 75% of nominals; 24 h later: meas. conc. = 56% of nominals	% Spawning ♀	No effect		Very high solvent concentration. Mortality up to 30%. Evaluation of effects on GSI at end of 5-d post-exposure period. No effect on total plasma protein content in ♂ and ♀, therefore vitellogenin not analysed	3	van den Belt et al. 2001
						GSI of non-spawning ♀	LOEC	25 ug/L			
						GSI of spawning ♀	LOEC	> 100 ug/L			
						% ♂ with fertilisation success >70%					
						GSI of ♂					
D. rerio	Fish sexual development test starting with freshly fertilised eggs, ending 60 dph (OECD draft TG 234)	4-tert-OP	Lab 1: none. Lab 2, 4: solvent used (not further specified)	Lab 1: 32, 100, 320 ug/L Lab 2: 32, 100, 200 ug/L Lab 4: 10, 32, 100, 320 ug/L	Lab 1: 13.8, 40.6, 73.1 ug/L Lab 2: 5.7, 17.6, 42.5 ug/L Lab 4: 9.5, 26.0, 91.5, 298.1 ug/L	Vitellogenin	LOEC	Lab 1: 40.6 ug/L Lab 2: 42.5 ug/L Lab 4: 26.0 ug/L		1	OECD 2011d
						Sex ratio	LOEC	Lab 1: ≤ 13.8 ug/L Lab 2: 17.6 ug/L Lab 4: 26.0 ug/L			
						Hatching	LOEC	Lab 4: 298.1 ug/L			
D. rerio	Fish life cycle test starting with fertilized eggs, flow-through (185 d)	4-tert-OP (99% monomeric isooctyl-phenols, 90% 4-tert-OP)	None	1.2, 3.7, 11.9, 38 ug/L	1.2, 3.2, 12 and 35 ug/L	F_0: Juvenile growth (d 42–78)	LOEC	35 ug/L		1	Wenzel et al. 2001a
						F_0: Time to 1st spawn					
						F_0: Number of eggs / ♀ and d					
						F_0: Fertilisation rate					
						F_0: Sex ratio	LOEC	> 35 ug/L			
						F_1: Survival to d 28					
						F_1: Growth to d 28					

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
Pimephales promelas	Short-term screening test with adults, flow-through (14 d)	4-tert-OP (97%)	Dimethyl-form-amide (n.i.)	2.25, 4.5, 9.0, 13.5, 22.5, 45 ug/L	1.5 / 2.4, 2.5 / 5.1, 4.5 / 8.2, 11 / 12, 20 / 14, 35 / 32 ug/L (2 measure-ments / conc.)	Vitellogenin induction in ♂	EC_{50}	48.2 ug/L	Extrapolated slightly beyond range of tested concentrations	2	Brian et al. 2005
P. promelas	Fish short-term reproduction test, flow-through (14–22 d pre-exposure, 21 d exposure)	4-tert-OP	Triethyle ne glycol (approx. 50 uL/L)	1.0, 50 and 150 ug/L	Lab A: 0.6, 37 and 120 ug/L Lab B: 0.6, 31 and 98 ug/L Lab C: 0.8, 42 and 120 ug/L	Vitellogenin induction in ♂	LOEC	Lab A: 37 ug/L Lab B: 31 ug/L Lab C: 0.8 ug/L	GLP-study. Interlaboratory validation study for U.S. EPA	1	Biever et al. 2007
						Reduced testos-terone level in ♂	LOEC	Lab A 37 ug/L Lab B: 98 ug/L Lab C: 42 ug/L			
						Secondary sexual characteristics in ♂ (tubercle score)	LOEC	Lab A: 37 ug/L Lab B 98 ug/L Lab C: 42 ug/L			
						Fecundity (eggs per ♀ and day)	LOEC	Lab A: 120 ug/L Lab B: 98 ug/L Lab C: > 120 ug/L			
						Fertisation rate	LOEC	Lab A: 120 ug/L Lab B: 98 ug/L Lab C: > 120 ug/L			
						Suvival of ♂ and ♀	LOEC	Lab A 120 ug/L	Lab C and D: no effect on survival		
Oryzias latipes	Fish sexual development test starting with freshly fertilised eggs, ending 60 dph (OECD draft TG 234)	4-tert-OP	Lab 5, 9: none. Lab 4: solvent used (not further specified)	Labs 4 and 5: 10, 32 and 100 ug/L Lab 9: 6.25, 12.5, 25, 50, 100 ug/L	Lab 4: 11.2, 31.7 and 105 ug/L Lab 5: 12.1, 30.6, 89.6 ug/L Lab 9: 6.2, 12.3, 23.6, 50.4, 100.6 ug/L	Vitellogenin	LOEC	Lab 4: 105 ug/L Lab 5: ≤ 12.1 ug/L Lab 9: 12.3 ug/L		1	OECD 2011d
						Sex ratio	LOEC	Lab 4 ≤ 11.2 ug/L Lab 5: 30.6 ug/L Lab 9: 50.4 ug/L			

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.	Remark	Validity	Reference
O. latipes	Exposure of adult ♂, flow-through (21 d) followed by 2 d recovery and 9-d cross-breeding trial of exposed ♂ with unexposed ♀ in control water. Development of offspring monitored for 7 d	4-tert-OP (97%)	Methanol and tri-ethylene glycol (n.i)	20, 50, 100, 300 ug/L	20, 41, 74, 230 ug/L	Vitellogenin in ♂ (d 21)	Vitellogenin levels positively correlated to conc. of 4-tert-OP (no LOEC derived)		2	Gronen et al. 1999
						Number of eggs/day	LOEC ≤ 20 ug/L			
						Fertilisation rate	Significant correlation between increasing 4-tert-OP conc. and decrease in % fertilised eggs (no LOEC derived)			
						Survival of offspring	Significant correlation between increasing 4-tert-OP conc. and decrease in % survival (no LOEC derived)			
						Abnormal development of offspring	LOEC ≤ 20 ug/L			
O. latipes	Screening test for endocrine disruption with adult ♂, semi-static, (36 d)	4-tert-OP (technical grade)	Acetone (n.i.)	200 and 300 ug/L	Estimated: 50% of nominal conc. (see remark)	Induction of intersex (18 d)	No effect	Chemical analysis only in parallel vessels without fish. Measured conc. in these vessels: approx. 50% of nominals	2	Gray et al. 1999b
						Induction of intersex (36 d)	In 16% (1 out of 6 fish), no statistical evaluation			
	Sexual development test starting 1 dph, semi-static, (3 mo)			100 ug/L		Induction of intersex (1 and 2 mo)	No effect			
						Induction of intersex (3 mo)	In 6% (3 out of 50 fish), no statistical evaluation			
	Sexual development test starting 1, 3, 5, 7, 21 and 35 dph, semi-static (100 d)			100 ug/L		Sex ratio	No effect			
						Intersex induction	Significant effect only in exposure starting 3 dph			

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Vali-dity	Reference
O. latipes	Partial life-cycle test starting 1 dph, semi-static (6 mo) followed by reproduction test in control water with (1) previously exposed ♂ and unexposed ♀, (2) previously exposed ♀ and unexposed ♂. Effects on all endpoints evaluated after reproduction tests	4-tert-OP (technical grade; 99%)	Acetone (n.i.)	10, 25, 50, 100 ug/L	Estimated: 50–60% of nominal conc. (see remark)	Sex ratio	No significant effect		Chemical analysis only in a parallel 168 h test without fish and a 72 h test in 5 L of water with 30 medaka. Measured concentrations in these parallel test were approx. 50–70% of nominals	2	Gray et al. 1999a
						Intersex	One fish at 50 and 100 ug/L, respectively (no statistical evaluation)				
						Reproductive behaviour of exposed ♂: nº of approaches	LOEC	50 ug/L			
						Reproductive behaviour of exposed ♂: nº of circles	LOEC	25 ug/L			
						Nº of copulations for previously exposed ♂	LOEC	50 ug/L			
						Percentage of previously exposed ♂ producing fertilised eggs	LOEC	25 ug/L			
						Fertilisation rate (%) for exposed ♂	LOEC	50 ug/L			
						Fertilisation rate (%) for exposed ♀	Significant effect at 10, 25 and 100 ug/L, but not at 50 ug/L				
						Sum of develop-mental problems in offspring of exposed ♂	Significant effect at 10 and 25 ug/L, but not at 50 and 100 ug/L				
						Sum of developmental problems in offspring of exposed ♀	Significant effect only at 10 ug/L				

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
O. latipes	Fish sexual development test starting with eggs < 12 hpf, flow-through (60 d)	4-tert-OP (97.6%)	DMSO (100 ug/L)	6.25, 12.5, 25, 50, 100 ug/L	6.94, 11.4, 23.7, 48.1, 94.0 ug/L	Hatchability	No effect			2	Seki et al. 2003
						Mortality	Significant increase at 23.7 ug/L, no significant effect at all other concentrations (increased growth = consequence of reduced fish density)				
						Total length					
						Body weight					
						Sex ratio based on secondary sex characteristics	LOEC	48.1 ug/L			
						Sex ratio based on gonad histology					
						Intersex/ovotestes	Observed at ≥ 11.4 ug/L (no statistical evaluation)				
						Vitellogenin in ♂ ↑	LOEC	11.4 ug/L			
						Vitellogenin in ♀ ↑	LOEC	48.1 ug/L			
O. latipes	Full life-cycle test (no details available)	4-tert-OP (n.i.)	n.i.	n.i.	n.i.	Vitellogenin in ♂	NOEC	4.3 ug/L	Secondary source	4	Japanese Ministry of the Environment 2006 as cited in OECD 2011a
						Ovotestis	NOEC	9.9 ug/L			
Gasterosteus aculeatus	Fish sexual development test starting with freshly fertilised eggs, ending 60 dph (OECD draft TG 234)	4-tert-OP	Solvent (not specified) used in both labs	Lab 6: 10, 32, 100 ug/L Lab 8: 32, 100, 320 ug/L	Lab 6: 12.2, 22.2 and 66.9 ug/L Lab 8: 41.9, 130.6 and 488.9 ug/L	Vitellogenin	LOEC	Lab 6: 66.9 ug/L Lab 8: > 41.9 ug/L	Lab 8: 100% mortality at > 41.9 ug/L	1	OECD 2011d
						Sex ratio	LOEC	Lab 6: > 66.9 ug/L Lab 8: > 41.9 ug/L			
						Hatching	LOEC	Lab 8: 130.6 ug/L			
						Survival	LOEC	Lab 8: 130.6 ug/L			
Poecilia reticulata	Exposure of adult ♂, flow-through (60 d) followed by cross-breeding trial with unexposed ♀	4-tert-OP (n.i.)	Acetone (72 ul/L)	100, 300, 900 ug/L	Max. 14% deviation from nominal conc. (data not presented)	F_0: Testes histology (d 60)	At 900 ug/L increased nº of spermatozeugmata, reduced nº of spermatogenic cysts (no statistical evaluation)		Exposure to 900 ug/L ended on d 30 due to 60% mortality. Control: 15% mortality	2-3	Toft & Baatrup 2001, Kinnberg & Toft 2003
						Sperm count ↑ (d 30)	LOEC	100 ug/L	No effect at 900 ug/L due to general toxicity		
						Sperm count ↑ (d 60)	LOEC	300 ug/L			

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
P. reticulata	Exposure of adult ♂ followed by cross-breeding trial (continued)	See above	See above	See above	See above	Coloration index ↓ (d 30, d 60)	LOEC	300 ug/L		2-3	Toft & Baatrup 2001, Kinnberg & Toft 2003
						Gonopodial index	No effect				
						N° of offspring / ♀	Reduced for ♂ previously exposed to 900 ug/L				
P. reticulata	Exposure of adult fish for 28 d (♂) and 26-36 d (♀: until birth of offspring), flow-through. Offspring raised in control water (70 d) for evaluation of sexual development	4-tert-OP (n.i.)	Acetone (60 ul/L)	n.i.	26 ± 8 ug/L	F_0: GSI of ♂	No effect		Only one test conc. 20% Mortality in control, 27% mortality in 4-tert-OP exposed fish	2-3	Kinnberg et al. 2003
						F_0: GSI of ♀					
						F_0: Testis histology	Increased n° of spermatozeugmata, reduced n° of spermatogenic cysts				
						F_0: Ovary histology	Reduced amout of yolk				
						F_1: Gonad development	No significant effect on gonad stages, but tendency towards faster development				
						F_1: Sex ratio based on 2nd sexual characteristics	No effect				
						F_1: Sex ratio based on gonad histology					
						F_1: Gonopodium index					
						F_1: Total length					
						F_1: Weight					
P. reticulata	Sexual development test starting with max 6 d old offspring, flow-through (90 d) followed by 24-48 h recovery in control water and behavioural trial	4-tert-OP (n.i.)	Acetone	First experiment: 1, 10, 100 ug/L Second experiment: 100 and 200 ug/L	First experiment: 1.7, 11.7, 149 ug/L (only single measurement) Second experiment: None	Sex ratio	LOEC	> 200 ug/L	Results of both experiments pooled for evaluation	2	Toft & Baatrup 2003
						Body lenght of ♂ ↑	LOEC	200 ug/L			
						Gonopodium length relative to body length	Significant effect at 100 but not at 200 ug/L				

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
P. reticulata	Sexual development test (continued)	See above	See above	See above	See above	Coloration index ↓	LOEC	200 ug/L	See above	2	Toft & Baatrup 2003
						GSI of ♀	Significant effect at 100 but not at 200 ug/L				
						GSI of ♂	LOEC	> 200 ug/L			
						Nº of mature oocytes and embryos	Reduced at all conc. (no statistical evaluation)				
						Sperm count ↑	Significant effect at 100 but not at 200 ug/L				
						Increased time spent in posturing behaviour					
Zoarces viviparus	Exposure of pregnant ♀ (starting with embryos in late yolk-sac phase), flow-through (35 d)	4-tert-OP (n.i.)	Isopropanol (n.i.)	25 and 100 ug/L	14 and 65 ug/L	F_0: Vitellogenin in ♀ ↑	LOEC	14 ug/L	Fish caught from the wild. Seawater	2	Rasmussen et al. 2002
						F_0: GSI in ♀	Significant reduction at 14 but not at 65 ug/L				
						F_1: Survival ↓	LOEC	65 ug/L			
						F_1: Lenght ↓	LOEC	≤ 14 ug/L			
						F_1: Weight ↓	LOEC	≤ 14 ug/L			
						F_1: Gonad development	Reduced % of ♂ and occurrence of ovotestes at 65 ug/L				
Z. viviparus	Sreening test with adult ♂, flow-through (3 wk)	4-tert-OP (n.i.)	Isopropanol (n.i.)	10, 50, 100 ug/L	9, 35, 63 ug/L	Vitellogenin in ♂ ↑	LOEC	35 ug/L	Fish caught from the wild. Seawater	2	Rasmussen et al. 2005
						GSI in ♂ ↑					
						Histological effects on testes (spermatogenesis ↓, degeneration of lobular structure)	At all tested concentrations (no statistical evaluation)				
Oncorhynchus mykiss	Early life stage test starting post hatch, flow-through (60 d)	4-tert-OP (99.2%)	Acetone (n.i.)	6.2, 12, 25, 50, 100 ug/L	6.1, 11, 22, 51, 91 ug/L	Growth	LOEC	11 ug/L	GLP-study. Embryonic stages not included. Secondary source	2	Analytical Bio-Chemistry Laboratories 1986 cited in IUCLID 2000

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
O. mykiss	Screening test with adult ♂, flow-through (21 d)	4-tert-OP (96% 4-substituted isomers)	Methanol	First experiment: 30 ug/L	First experiment:39 ug/L	Vitellogenin ↑	Effect at 39 ug/L		First experiment in May (onset of testes growth)	2	Jobling et al. 1996
						GSI ↓					
						Spermatogenesis ↓					
				Second experiment: 0.5, 1.32, 3.5, 9.3, 24.5 and 65 ug/L	Second experiment: 0.3, 0.6, 1.6, 4.8, 14.6 and 43.9 ug/L	Vitellogenin ↑	LOEC	4.8 ug/L	Second experiment in November (testes fully grown)		
						GSI	Significant reduction only at 4.8 ug/L, no effect at lower and higher concentrations				
O. mykiss	Exposure of all-female trout starting with newly hatched fish, flow-through (22 d) followed by 86 d in control water	4-tert-OP (n.i.)	Methanol (max. 5 ul/L)	1, 10, 50 ug/L	None	Body weight ↓ (d 108)	LOEC	≤ 1 ug/L (Effect decreases with increasing concentration)	Evaluation of endpoints after recovery	2	Ashfield et al. 1998
	Exposure of all-female trout starting with newly hatched fish, flow-through (35 d) followed by 431 d in control water			1, 10, 30 ug/L		Growth (length and body weight)	No consistent effect (reduction at 10 ug/L, but growth at 30 ug/L similar to control values)		Evaluation of endpoints on d 24, 55, 84, 108, 144, 220, 300 and 466		
						GSI in ♀ (d 466)	No effect				
O. mykiss	Screening test with adult ♂, flow-through (21 d)	4-tert-OP (>99%)	Methanol (≤50 ul/L)	1, 10, 100 ug/L	1.0 (1.4 / 0.6), 8.7 (11.3 / 6) and 109 (49–149) ug/L	Vitellogenin ↑	LOEC	10 ug/L	Trout and roach exposed in same tank (physically separated)	2	Routledge et al. 1998
Rutilus rutilus	Screening test with adults, flow-through (21 d)					Vitellogenin in ♂ ↑	LOEC	100 ug/L			
						Vitellogenin in ♀ ↑	LOEC	> 100 ug/L			

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
Cyprinodon variegatus	Screening test with adult ♂, flow-through (24 d)	4-tert-OP (n.i.)	Tri-ethylene gycol (n.i.)	20, 40 and 80 ug/L	11.5, 33.6 and 61.1 ug/L	Vitellogenin in ♂ ↑	LOEC	≤ 11.5 ug/L	Seawater (14-16‰)	2	Karels et al. 2003
	Cross-breeding trial (10 d) with ♂ from screening test above and unexposed ♀ in control water, evaluation of offspring until 3 dph					Vitellogenin in ♂ ↑ (10 d post-exposure)	LOEC	≤ 11.5 ug/L			
						Increased % of ♂ fish with testes anomalies	LOEC	33.6 ug/L			
						Reduced % of viable eggs	LOEC	33.6 ug/L			

(1) Substance identity: pers. comm. U. Schulte-Oehlmann (2011).

Table 17: Effect concentrations of 17α-ethinylestradiol (EE_2) in fish (classification of validity based on Klimisch et al. 1997, EC 2003 and 2011a).

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
Danio rerio	Short-term fish screening test for endocrine effects with adult ♂, flow-through (8 d)	EE_2 (99.1%)	Ethanol (n.i.)	1, 2, 4, 8, 12,16, 20, 30 and 100 ng/L	0.72, 2.20, 3.58, 6.58, 10.1, 13.5, 17.2, 26.1, 90.1 ng/L	Vitellogenin in ♂ ↑	LOEC	3.58 ng/L		2	Rose et al. 2002
							EC_{10}	0.92 ng/L			
D. rerio	Fish sreening test for endocrine effects with adult ♂, flow-through (10 d)	EE_2 (≥98%)	Ethanol (50 ul/L)	0.1, 0.3, 1.0. 3.2 ng/L	n.d. / n.d., 0.2 / n.d., 0,8 / n.d., 1.5 / 0.7 ng/L	Vitellogenin in ♂ ↑	LOEC	1.1 ng/L	Chemical analysis: results of 2 measure-ments. LOEC based on mean measured conc.	2	Duis & Knacker 2003
D. rerio	Fish screening test for endocrine effects with ♂ and ♀ adults, semi-static (24 d)	EE_2 (≥98%)	Methanol (1 ml/L)	10, 25 ng/L	Fresh solutions: 9.4, 18 ng/L Old solutions: 8.7 and 11.6 ng/L	Vitellogenin in ♂ (d 3, 6, 12 and 24)	LOEC	≤ 9.1 ng/L	Very high solvent concentration. LOECs based on mean measured conc.	2–3	van den Belt et al. 2002
						GSI in ♂ (d 24)					
						Testes histology (only evaluated for 9.1 ng/L)	Affected on d 24				
						Vitellogenin in ♀	LOEC	≤ 9.1 ng/L			
						GSI in ♀ (d 6, 12 and 24)					
						Ovary histology (only evaluated for 9.1 ng/L)	Increased atresia starting on d 3				

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
D. rerio	Short-term exposure of adult ♂ and ♀, semi-static (21 d) followed by 5 d cross-breeding trial (exposed ♂ with unexposed ♀; exposed ♀ with unexposed ♂) in control water	EE_2 (≥98%)	Methanol (1 ml/L)	5, 10, 25, 50 ng/L	Fresh solutions (immediately after renewal): measured conc. = 99% of nominals; 24 h later: meas. conc. = 76% of nominals	% Spawning ♀	Reduced at ≥ 5 ng/L (no statistical evaluation)		Very high solvent concentration. Mortality up to 60%. Evaluation of effects on GSI at end of 5-d post-exposure period. No effect on total plasma protein content in ♂ and ♀, therefore vitellogenin not analysed	3	van den Belt et al. 2001
						GSI of non-spawning ♀	LOEC	10 ng/L			
						GSI of spawning ♀	LOEC	> 10 ng/L			
						% ♂ with fertilisation success >70%	Reduced to 0% at 5 ng/L				
						GSI of ♂	LOEC	10 ng/L			
						Vitellogenin in ♀					
						Vitellogenin in ♂	Clear effect at ≥ 5 ng/L (no statistical evaluation)				
						GSI males	LOEC	10 ng/L			
D. rerio	Partial life-cycle test starting with embryos, 3 mo exposure, semi-static, followed by 5 mo of recovery, then assessment of reproduction in a cross-breeding trial (exposed ♀ with non-exposed ♂)	EE_2 (>98%)	Methanol (1 ml/L)	0.1, 1, 10, 25 ng/L	None	Total body length (month 3)	LOEC	10 ng/L	Very high solvent concentration. At 25 ng/L: edema in approx. 17% of the fish, malformed spine in 51% of the fish. Mortality up to 40% in cross-breeding trial for ♀ previously exposed to 25 ng/L	2-3	van den Belt et al. 2003
						Body weight (month 3)	LOEC	25 ng/L			
						Gonad morphology	At all EE_2 concentrations increased % of fish without macroscopically visible gonads				
						Vitellogenin ↑	LOEC	10 ng/L			
						% Spawning ♀	Reduced at 10 ng/L, no spawning at 25 ng/L				
						Fecundity (total nº of eggs)	LOEC	10 ng/L			

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
D. rerio	Short-term fish sexual development test starting 20 dph, semi-static (40 d)	EE_2 (n.i.)	Ethanol (max. 100 ul/L)	1, 2, 5, 10, 25 ng/L	< 0.6, 1.5, 6.8, 9.9, 23 ng/L	Vitellogenin (38 dph)	LOEC	1.5 ng/L		2	Örn et al. 2003
						Delayed sexual differentiation: higher % of female-type gonads 60 dph	LOEC	≤ 0.6 ng/L			
D. rerio	2-Generation test starting with fertilised eggs, flow-through (315 d)	EE_2 (98%)	Acetone (n.i.)	0.05, 0.28, 1.7, 10 ng/L	F_0 period: 0.05, 0.3, 1.1, 10 ng/L F_1 period: 0.1, 0.3, 2.0, – (see remark)	F_0: Juvenile survival (d 42-78)	LOEC	10 ng/L	At highest nom. concentration (10 ng/L): no reproduction → no evaluation of F_1	1	Wenzel et al. 2001a, b
						F_0: Juvenile growth (d 42-78)	LOEC	1.1 ng/L			
						F_0: Time to first reproduction					
						F_0: Fecundity (n° of eggs / ♀ and day)					
						F_0: Fertilisation rate					
						F_1: Juvenile growth (d 35-75)	LOEC	0.3 ng/L			
						F_1: Time to first reproduction	LOEC	2.0 ng/L			
						F_1: Fecundity (n° of eggs / ♀ and day)					
						F_1: Fertilisation rate					

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
D. rerio	2-Generation-test starting with adult fish, semi-static. F_0 exposed for 40 d. F_1 embryos from end of F_0 period exposed until adulthood. After exposure of F_1 for 210 dpf, assessment of reproductive success of F_1 during 10 d. F_2 further exposed until 100 hpf for evaluation of embryo survival / integrity	EE_2 (n.i.)	Ethanol (max. 0.05 ul/L)	0.5, 5 and 50 ng/L	0.5, 4.5 ng/L, – (50 ng/L: not analysed)	F_0: Reproductive success (n° of viable embryos 14 hpf) d 6-10 and d 11-15	LOEC	50 ng/L	At 50 ng/L, reproduction ceased after 10 d exposure → treatment terminated, F_1 not evaluated	2	Nash et al. 2004
							No survival of offspring until 100 hpf				
						F_0: Vitellogenin in ♂ ↑, d 40	LOEC	0.5 ng/L			
						F_0: Vitellogenin in ♀ ↑, d 40	LOEC	5 ng/L			
						F_1: Reproductive success (n° of viable embryos 14 hpf), d 240	LOEC	5 ng/L			
						F_1: Vitellogenin in ♂, d 310	No significant effect				
						F_1: Vitellogenin in ♀, d 310	No significant effect				
Pimephales promelas	Short-term reproduction test (gonadal recrudescence test[1]), flow-through (21 d exposure of ♂ and ♀ in separate aquaria). Reproduction evaluated in subsequent 3-wk period in control water	EE_2 (≥98%)	DMSO (max. 10 ul/L)	0.1, 1, 3, 10, 100 ng/L	Only for 1 and 10 ng/L: 0.7 / 0.8 ng/L (for ♂ and ♀ aquaria with nom. 1 ng/L), 8.1 and 7.8 ng/L (for ♂ and ♀ aquaria with nom. 10 ng/L)	Condition factor in ♂ ↓	LOEC	10 ng/L		2	Pawlowski et al. 2004
						Condition factor in ♀ ↓	LOEC	100 ng/L			
						GSI in ♂ ↓	LOEC	10 ng/L			
						GSI in ♀ ↓	LOEC	100 ng/L			
						Vitellogenin in ♂ ↑	LOEC	1 ng/L			
						Vitellogenin in ♀ ↑	LOEC	1 ng/L			
						N° of nuptial tubercles in ♂	LOEC	1 ng/L			

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
P. promelas	Short-term reproduction test (continued)	See above	See above	See above	See above	Fecundity (n° of eggs/ spawning pair)	At 0.1 and 1 ng/L significant increase, at 100 ng/L significant decrease			2	Pawlowski et al. 2004
						Fertilization rate	LOEC	10 ng/L			
						Histologic alterations in testes	No sperm detected at 10 and 100 ng/L				
						Ultrastructure of testes	Necrotic spermatogonia at ≥ 1 ng/L				
P. promelas	Full life-cycle test (US EPA 540/9-86-137) with additional analysis of histology and vitellogenin levels, starting < 24 hpf, flow-through (301 d)	EE_2 (100%) and ^{14}C-EE_2 (99.5%) for analytical monitoring of test concen-trations	None	0.2, 1.0, 4.0, 16, 64 ng/L	RIA: 0.14, 0.58, 2,75, 12.7 and 53.6 ng/L; LSC: 0.16, 0.76, 2.80, 12.1 and 46.8 ng/L (see remark)	F_0: Length, d 28	LOEC	16 ng/L	Monitoring of test conc. using (1) radio-immunoassay (RIA) and (2) liquid scintillation counting (LSC). Reproduction not evaluated at ≥ 4 ng/L due to lack of phenotypic ♂ at these conc.	1	Länge et al. 2001
						F_0: Length, d 56	LOEC	4.0 ng/L			
						F_0: Weight, d 56	LOEC	> 64 ng/L			
						F_0: Ovotestes, d 56	LOEC	4.0 ng/L			
						F_0: Ovotestes, d 172					
						F_0: Vitellogenin, d 172	LOEC	16 ng/L			
						F_0: Egg production	LOEC	> 1.0 ng/L			
						F_1: Survival, d 28					
						F_1: Length, d 28	LOEC	≤ 0.2 ng/L			
						F_1: Weight, d 28	LOEC	1.0 ng/L			
						F_1: Gonad histology, d 28	Slight, but not concentration related increase in % of ♀ at 0.2 and 1.0 ng/L				
P. promelas	Full life-cycle test starting with eggs, flow-through (125 d)	EE_2 (n.i.)	Ethanol (1 ul/L)	0.32, 1.0, 3.2, 10, 32 ng/L	None	Length (60 dph) ↓	LOEC	32 ng/L		2	Parrott & Wood 2002
						Ovipositor size (60 dph) ↑	LOEC	3.2 ng/L			
						Male secondary sexual characteristics	Reduced at 1 ng/L. At 3.2–32 ng/L: complete external feminisation				

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
P. promelas	Life-cycle test, starting 48–60 hpf, flow-through (approx. 155 d). F_1 only evaluated until hatch	EE_2 (98%)	Ethanol	0.32, 0.96, 3.2, 9.6, 32 ng/L	(n.d.), (n.d.), 3.54, 9.55, 22.7 ng/L (see remark)	F_0: Length, 60 dph	LOEC	32 ng/L	EE_2 conc. measured by radioimmuno assay (LOD: 0.74–1.5 ng/L). Measured conc. at two lowest nominal conc. < LOD	2	Parrott & Blunt 2005
						F_0:Weight, 60 dph	LOEC	> 32 ng/L			
						F_0: Ovipositor index, ♀, 60 and 150 dph	LOEC	3.2 ng/L			
						F_0: Fertilisation rate (%)	LOEC	≤ 0.32 ng/L			
						F_0: Sex ratio F_0 (feminisation)					
						F_0: LSI of ♀	LOEC	9.6 ng/L			
						F_0: GSI of ♀	LOEC	3.2 ng/L			
						F_0: Secondary sex characteristics in ♂	LOEC	0.96 ng/L			
Oryzias latipes	Partial life cycle test: 2 mo exposure starting with newly-hatched fish, followed by 6 wk recovery period; all effects evaluated at the end of the recovery period	EE_2 (98%)	Acetone (50 ul/L)	1, 10 and 100 ng/L	None	GSI of ♀	LOEC	10 ng/L		2	Scholz & Gutzeit 2000
						Sex ratio	LOEC	100 ng/L			
						Fecundity (n° of eggs / ♀ and day)	LOEC	10 ng/L			
O. latipes	Short term screening test for endocrine effects with adult ♂, semi-static (4 wk)	EE_2 (97%)	DMSO (50 ul/L)	10, 100 ng/L	None	Vitellogenin in ♂ ↑	LOEC	≤ 10 ng/L	Water exchange only once per wk	2-3	Scholz et al. 2004

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
O. latipes	Short term reproduction test with breeding pairs (F_0), semi-static (14 d) followed by evaluation of hatching success, survival and sex ratio of offspring (F_1) raised in control water	EE_2 (n.i.)	Ethanol (70 ul/L)	0.2, 5, 500, 2000 ng/L	None	F_0: Fecundity (n° of eggs / spawning pair and d)	Significant increase at 0.2 ng/L, significant decrease at 500 ng/L		At 2000 ng/L: mortality during 2nd week of exposure. Endpoints not evaluated	2	Tilton et al. 2005
						F_0: Fertilisation rate ↓	LOEC	500 ng/L			
						F_0: Spawning frequency ↓					
						F_0: Vitellogenin in ♀					
						F_0: Vitellogenin in ♂					
						F_0: pPlasma estradiol in ♀ ↑	LOEC	5 ng/L			
						F_0: Plasma estradiol in ♂ ↑					
						F_0: Plasma testosterone in ♂ and ♀	No significant effect				
						F_0: Ovarian estradiol release	LOEC	0.2 ng/L			
						F_0: Testicular testosterone release	LOEC	5 ng/L			
						F_1: Hatching rate ↓	LOEC	500 ng/L			
						F_1: Sex ratio based on fin morphology	No effect				
O. latipes	Reproduction test, flow-through (21 d)	EE_2 (100%)	Acetone	31.3, 62.5, 125, 250, 500 ng/L	32.6, 63.9, 116, 261, 488 ng/L	Fecundity (n° of eggs/ spawning pair and d)	LOEC	488 ng/L	At 261 ng/L, 1 out of 12 fish died, at 488 ng/L 5 out of 12 fish	2	Seki et al. 2002
						Fertilisation rate	LOEC	> 488 ng/L			
						Vitellogenin in ♂	LOEC	63.9 ng/L			
						Intersex (ovotestis)	Increased at 63.9 ng/L and higher, but no statistical evaluation				

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
Pomatoschistus minutus	Chronic test starting with juveniles and including period of gonad maturation and 8 week-breeding period (7 mo)	EE_2 (n.i.)	Methanol (17 ul/L)	6 ng/L	None	Vitellogenin induction	Significant effect at 6 ng/L		Only one test concentration (EE_2 was used as positive control in this study). High mortality (approx. 50% in solvent control, approx. 49% in EE_2 treatment) due to acclimation stress	3	Robinson et al. 2003
						Fecundity (fertile eggs/♀) ↓					
						Fertilization rate					
						Secondary sex characteristics					
						Reproductive behaviour of ♂					
Oncorhynchus mykiss	Chronic test for endocrine effects with juvenile fish at 11.4 and 17.4°C (28 wk)	EE_2 (n.i.)	None	0.1, 0.3, 1.0 ng/L	None (nominal conc. were below detection limit of analytical method)	Vitellogenin in ♀ at 11.4 and 17.4°C	LOEC	> 1 ng/L		2	Sheahan et al. 1994
						Vitellogenin in ♂ (11.4°C)	LOEC	0.3 ng/L			
						Vitellogenin in ♂ (17.4°C)	LOEC	1.0 ng/L			
						GSI in ♂ and ♀	No effect				
O. mykiss	Screening test with ♂ fish, flow-through (10 d) at 16.5°C	EE_2 (n.i.)	n.i.	0.1, 0.5, 1.0 and 10 ng/L	None	Vitellogenin in ♂ ↑	LOEC	≤ 0.1 ng/L		2	Purdom et al. 1994
Salvelinus namaycush	Screening test for endocrine disruption with juveniles, flow-through (21 d)	EE_2 (n.i.)	Ethanol	4, 40 and 400 ng/L	15, 35 and 373 ng/L	Vitellogenin in ♂↑	LOEC	≤ 15 ng/L	EE_2 measured by radio-immunoassay	2	Werner et al. 2003
						Vitellogenin in ♀↑					
						GSI in ♂					
						GSI in ♀					

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
Cyprinodon variegatus	Screening test for endocrine effects with adult ♂, flow-through (16 d)	EE_2 (n.i.)	Tri-ethylene glycol (50 ul/L)	20, 100, 200, 500, 1000 ng/L	24, 109, 192, 416, 832 ng/L	Vitellogenin in ♂ ↑	Clear effect starting at 109 ng/L, but no statistical evaluation			2	Folmar et al. 2000
C. variegatus	Partial life-cycle test: exposure of F_0 starting with juveniles (43 or 59 d), followed by reproductive trials (for 0.2, 2, 20, 200 ng/L) in control water (ending on d 73); F_1 raised for 7 d in control water	EE_2 (n.i.)	Tri-ethylene glycol (approx. 8 ul/L)	0.2, 2, 20, 200, 400, 800, 1600, 3200 ng/L	Test solutions only analysed for 200, 400 and 800 ng/L: 117 ng/L, 328 ng/L, 723 ng/L (lower EE_2 conc.: only stock solutions analysed)	Testes histology: fibrosis (d 57, 73)	Observed at ≥ 2 ng/L		Seawater (approx. 20‰). At 1600 and 3200 ng/L approx. 79% mortality → all remaining fish sacrificed on d 17. At 400 and 800 ng/L: 50 and 70% mortality until d 42	2	Zillioux et al. 2001
						Ovary histology: atresia (d 57, 73)					
						Ovotestes (d 73)	Observed at ≥ 20 ng/L				
						Fecundity (eggs / ♀ and day)	Reduced at 20 and 200 ng/L (no statistical evaluation)				
						Hatching rate	Reduced at 200 ng/L (no statistical evaluation)				
Gobiocypris rarus	Short-term screening test for endocrine effects with juveniles, semi-static (7 d)	EE_2 (n.i.)	DMSO (100 ul/L)	0.6, 0.8, 1, 2, 4, 8 ng/L	None	Vitellogenin ↑ (indirect ELISA for *C. carpio*)	LOEC	2 ng/L	Comparison of three different ELISA techniques	2	Liao et al. 2006
						Vitellogenin ↑ (competitive ELISA for *C. carpio*)	LOEC	1 ng/L			
						Vitellogenin ↑ (competitive ELISA for *G. rarus*)	LOEC	0.8 ng/L			

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
Gasterosteus aculeatus	4-Wk exposure starting 7 dpf, flow-through, followed by 50 wk recovery in control water, then evaluation of reproduction	EE_2 (n.i.)	Methanol (n.i.)	10, 100 ng/L	1.75 and 27.7 ng/L	Sex ratio	Skewed (more ♀, no ♂), intersex fish observed at 27.7 ng/L (no statistical evaluation)		All endpoints evaluated at the end of the reproduction trial	2	Maunder et al. 2007
						GSI of ♀	LOEC	27.7 ng/L			
						GSI of ♂	LOEC	> 27.7 ng/L			
						Average number of nests per ♂↓	LOEC	≤ 1.75 ng/L			
						Average number of eggs normalised to number of ♂↓	LOEC	27.7 ng/L			
						Increased % of dead eggs					
Rutilus rutilus	Sexual development test starting with freshly fertilised eggs, flow-through (84 d)	EE_2 (n.i.)	n.i.	0.1, 1, 10 ng/L	n.d., 0.3, 4 ng/L	Vitellogenin	LOEC	4 ng/L		2	Katsu et al. 2007
						Morphological sex ratio / feminization	At 0.3 ng/L, no males were discernible, at 4 ng/L 95% of the fish had female-like gonads (no statistical evaluation)				
Cyprinus carpio	Screening test with juvenile fish, flow-through (10 d) at 9.5°C	EE_2 (n.i.)	n.i.	1, 10, 25, 50 ng/L	None	Vitellogenin ↑	LOEC	10 ng/L	Relatively low temperature for carp	2	Purdom et al. 1994
Fundulus heteroclitus	Short-term reproduction test, semi-static (28 d)	EE_2 (98%)	Ethanol (33 ul/L)	0.1, 1, 10, 100 ng/L	Measured conc. at nom. 10 ng/L: 18.1 (0 h), 10.4 ng/L (12 h). Conc. at lower nominals: below detection limit (10 ng/L)	GSI in ♂ (d 21)	LOEC	100 ng/L	Animals caught from field 6 mo before experiments. Seawater (20‰)	2	Peters et al. 2007
						Vitellogenin in ♂ (d 28)					
						Fecundity (total n° of eggs)					
						Fertilisation rate					
						Plasma estradiol levels in ♀ (28 d)	LOEC	10 ng/L			

(1) In gonadal recrudescence assays, mature *P. promelas*, which have been maintained under simulated winter conditions (short day length, low temperatures) and therefore exhibit regressed secondary sex characteristics and gonad maturation, are subjected to increasing photoperiod and temperature regime and exposed to a test substance to determine potential effects on gonadal recrudescence, i.e. maturation of the gonad from its regressed form (Pawlowski et al. 2004).

Table 18: Effect concentrations of prochloraz in fish (classification of validity based on Klimisch et al. 1997, EC 2003 and 2011a).

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
Danio rerio	Fish screening assay with adult fish, flow-through (21 d) (draft OECD TG 230)	Prochloraz (99.5%)	–	20, 100 and 300 ug/L	Lab 6: 7, 54, 217 ug/L Lab 12: 15, 67, 166 ug/L Lab 13: 19, 83, 194 ug/L	Vitellogenin ↓ in ♀	LOEC	Lab 6: > 217 ug/L Lab 12: 67 ug/L Lab 13: 83 ug/L		1	OECD 2006b
						Vitellogenin in ♂	No effect				
D. rerio	Fish sexual development test starting 24 hpf, flow-through (60 d)	Prochloraz (Pestanal®)	–	20, 100 and 300 ug/L	16, 65, 202 ug/L	Sex ratio	LOEC	202 ug/L	No signifikant effects on growth of ♂ and ♀	2	Kinnberg et al. 2007
						Incidence of intersex gonads ↑					
						Vitellogenin ↑ in ♂	Signifikant at 16 and 65 ug/L				
						Vitellogenin ↓ in ♂	LOEC	202 ug/L			
						Vitellogenin ↓ in ♀					
						Gonad histology ♀	Significant effects at 16 and 202 ug/L				
D. rerio	Fish sexual development test, flow-through, starting 24 h post fertilization, ending 60 dph	Prochloraz (99.5%)	–	Lab 2: 32, 100, 320 ug/L Labs 3 and 4: 38, 75, 150, 300, 600 ug/L	Lab 2: 15, 48, 320 ug/L Lab 3: 22, 44, 99, 197, 434 ug/L Lab 4: 60, 135, 183, 233, 1166 ug/L	Vitellogenin ↓ in ♀	LOEC	Lab 2: 48 ug/L Lab3: 99 ug/L Lab 4: 183 ug/L	Lab 3: Effect at 197 ug/L not significant	1	Holbech et al. 2012 (see also OECD 2011e)
						Vitellogenin ↓ in ♂	LOEC	Lab3: 44 ug/L Lab 4: 135 ug/L	Lab 2: No effect		
						Sex ratio	LOEC	Lab 2: 320 ug/L Lab 3: 99 ug/L Lab 4: < 60 ug/L	Lab 3: Effect at 434 ug/L not significant		
D. rerio	Fish sexual development test, flow-through, starting with embryos < 24 hpf, ending 60 dph	Prochloraz (99.1%)	–	32, 100, 320 ug/L	32, 82, 297 ug/L	Total length ♂	LOEC	297 ug/L		1	Thorpe et al. 2011
						Increased % of indifferent gonads	LOEC	82 ug/L			
						Sex ratio					
						Vitellogenin ↓ in ♂	LOEC	297 ug/L			
						Vitellogenin ↓ in ♀	Reduction at 297 ug/L (no statistical evaluation as n = 1)				

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
Pimephales promelas	Short-term screening test with adult ♂ and ♀, flow-through (8 d exposure, 8 d recovery)	Prochloraz (99.4%)	–	30 and 300 ug/L	22 and 284 ug/L	GSI	LOEC	> 284 ug/L		2	Ankley et al. 2009
						Vitellogenin in ♂	No effect				
						Vitellogenin ↓ in ♀ (d 4 and 8 of exposure)	LOEC	284 ug/L	Recovery after 8 d post-exposure		
						Plasma estradiol ↓ in ♀ (d 8 of exposure)					
						Plasma testosterone ↓ in ♂ (d 8 of exposure)	LOEC	< 22 ug/L			
P. promelas	Fish screening assay with adult ♂and ♀, flow-through (21 d) (draft OECD TG 230)	Prochloraz (99.5%)	–	20, 100 and 300 ug/L	Lab 4: 24, 121, 382 ug/L Lab 8; 20, 98, 299 ug/L Lab 11: 15, 69, 275 ug/L	Vitellogenin ↓ in ♀	LOEC	Lab 4: 121 ug/L Lab 8: 299 ug/L Lab 11: 275 ug/L		1	OECD 2006b
						Vitellogenin in ♂	No effect				
						Secondary sex characteristics (nuptial tubercles)	LOEC	Lab 8: 299 ug/L	No effect in Lab 4 and Lab 11		
P. promelas	Short-term reproduction test with adults, flow-through (21 d)	Prochloraz (99.5%)	–	30, 100 and 300 ug/L	32, 116 and 311 ug/L	Vitellogenin ↓ in ♀	LOEC	116 ug/L	Test design very close to OECD 229	1	Ankley et al. 2005
						Vitellogenin in ♂	No effect				
						Cumulative fecundity (total nº of eggs / ♀)	LOEC	116 ug/L			
						Plasma estradiol ↓ in ♀	LOEC	311 ug/L			
						Plasma estradiol in ♂	LOEC	> 311 ug/L			
						Plasma testosterone in ♀	No effect				
						Plasma testosterone ↓ in ♂	LOEC	311 ug/L			
						Plasma 11-ketotesto-sterone↓ in ♂					

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
P. promelas (continued)	Short-term reproduction test (see above)	See above	–	See above	See above	Brain aromatase in ♀	No effect			1	Ankley et al. 2005
						Brain aromatase ↓ in ♂	LOEC	311 ug/L			
						Fertilisation rate	No treatment-related effect				
						Hatching success of F_1	No treatment-related effect				
P. promelas	Short-term reproduction test, flow-through (14–22 d pre-exposure, 21 d exposure)	Prochloraz	–	20, 100 and 300 ug/L	Lab A: 16, 77, 220 ug/L Lab B: 15, 83, 230 ug/L Lab C: 23, 90, 270 ug/L	Vitellogenin in ♂	No effect		GLP-study. Interlaboratory validation study for U.S. EPA	1	Biever et al. 2007
						GSI ↑ in ♂	LOEC	Lab A: No effect Lab B: 230 ug/L Lab C: 90 ug/L			
						Secondary sexual characteristics in ♂ (tubercle score)	LOEC	Lab A: 77 ug/L Lab B: 230 ug/L Lab C: 90 ug/L			
						Vitellogenin ↓ in ♀	LOEC	Lab A: No effect Lab B: 83 ug/L Lab C: ≤ 23 ug/L			
						Fecundity (eggs/♀ and day)	LOEC	Lab A: 220 ug/L Lab B: 230 ug/L Lab C: 270 ug/L			
						Fertisation rate (%)	No effect				
P. promelas	Fish sexual development test, flow-through, starting 24 hpf, ending 60 dph (lab 5) and 120 dph (lab 2)	Prochloraz (99.5%)	–	32, 100 and 320 ug/L	Lab 2: 31, 106, 301 ug/L Lab 5: 29, 96, 284 ug/L	Vitellogenin ↓ in ♀ (60 dph)	LOEC	Lab 5: ≤ 29 ug/L	Validation study for OECD TG 234	1	Holbech et al. 2012 (see also OECD 2011e)
						Vitellogenin ↓ in ♀ (120 dph)	LOEC	Lab 2: 106 ug/L			
						Sex ratio (60 dph)	LOEC	Lab 5: 284 ug/L			
						Sex ratio (120 dph)	LOEC	Lab 2: 301 ug/L			

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
P. promelas	Fish sexual development test, flow-through, starting with embryos < 24 hpf, ending 125 dph	Prochloraz (99.1%)	–	32, 100, 320 ug/L	34, 88, 294 ug/L	Total length of ♀	Reduction at 294 ug/L (no statistical evaluation as n = 1)		Test design very similar to OECD TG 234	1	Thorpe et al. 2011
						Total length of ♂	LOEC	88 ug/L			
						Sex ratio	LOEC	294 ug/L			
						Gonad maturity stage ↓ in ♂					
						Gonad maturity stage ↓ in ♀	LOEC	> 294 ug/L			
						Vitellogenin in ♂	LOEC	88 ug/L			
						Vitellogenin in ♀					
Oryzias latipes	Short-term reproduction test with adult ♂ and ♀, semi-static (7 d)	Prochloraz (n.i.)	DMSO (n.i.)	3, 30, 300 ug/L	No chemical analysis	Fecundity (cumulative nº of eggs / ♀)	LOEC	30 ug/L		2–3	Zhang et al. 2008
						Expression of vitellogenin I in liver of ♀ ↓	LOEC	300 ug/L			
						Expression of vitellogenin II in liver of ♀ ↓	LOEC	≤ 3 ug/L			
						Expression of aromatase (cyp19A) in gonads of ♀ ↑	LOEC	30 ug/L			
O. latipes	Fish screening assay with adult fish, flow-through (21 d) (draft OECD TG 230)	Prochloraz (99.5%)	–	20, 100, 300 ug/L	Lab 1: 18, 93, 279 ug/L Lab 2: 20, 95, 284 ug/L Lab 4: 23, 100, 296 ug/L Lab 6: 7, 54, 217 ug/L	Vitellogenin in ♀ ↓	LOEC	Lab 1: 18 ug/L Lab 2: 95 ug/L Lab 4: 296 ug/L Lab 6: 217 ug/L		1	OECD 2006b
						Vitellogenin in ♂	No consistent conc. response relationship				

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.	Remark	Validity	Reference
Oncorhynchus mykiss	Screening test with juveniles, flow-through (14 d exposure). Gonad maturation evaluated after 3 and 9 wk post-exposure in control water	Prochloraz (95%)	Ethanol (n.i.)	9.98, 99.8 ug/L	8.66, 62.5 ug/L	Vitellogenin (after 14 d exposure)	No effect		2	Le Gac et al. 2001
						GSI (3 wk post-exposure)	No effect			
						Gonadal maturation in ♂ (3 wk post-exposure)	Delay at 8.66 and 62.5 ug/L, no statistical evaluation			
						Gonadal maturation in ♂ (9 wk post-exposure)	No effect			

Table 19: Effect concentrations of tributyltin (TBT) in aquatic invertebrates and fish (classification of validity based on Klimisch et al. 1997, EC 2003 and 2011a).

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
Nematoda											
Caenorhabditis elegans	6-d Test with age-synchronous worms on agar plates	TBT-Cl (n.i.)	Ethanol (3 ml/L)	0.12, 1.2, 12, 120, 1200 ug Sn/L agar	None	Reduction in percentage of germ cells	LOEC	0.12 ug Sn/L agar	High solvent conc. Test on agar plates	2–3	Hoshi et al. 2003
Mollusca											
Potamopyrgus antipodarum	Reproduction test, water/ sediment system (with spiking of sediment), static (8 wk)	TBT-Cl (>97%)	Ethanol	10, 25, 50, 125, 250, 500 ug Sn/kg sed. dw	Chemical analysis at test end only: 14.9, 20.1, 13.8, 70.6, 95.4, 152 and 396 ug Sn/kg sed. dw. Degradation products (mono- and dibutyltin) also detected	Mortality (wk 8)	LC_{50}	431 ug Sn/kg sed. dw	100% Mortality at 500 ug Sn/kg sed. dw	2	Duft et al. 2003a
						N° of embryos without shell (wk 8)	LOEC	25 ug Sn/kg sed. dw			
							EC_{10}	2.98 ug Sn/kg sed. dw			
							EC_{50}	64 ug Sn/kg sed. dw			
						N° total embryos (wk 8)	LOEC	≤ 10 ug Sn/kg sed. dw	No significant effect at 75 ug/kg sed. dw		
							EC_{10}	3.5 ug Sn/kg sed. dw			
							EC_{50}	93.9 ug Sn/kg sed. dw			
P. antipodarum	Reproduction test, static (8 wk)	TBT-Cl (n.i.)	n.i.	30, 60, 125, 250, 500 ng Sn/L	n.i.	N° of embryos	EC_{10}	37.8 ug Sn/L	Few information provided on experimental details. Apparently static exposure	4	Albanis et al. 2006, Duft et al. 2007
							EC_{50}	115 ug Sn/L			
Marisa cornuarietis	Partial life cycle assay with adults, semi-static (6 mo), 25°C	TBT-Cl (n.i.)	Ethanol	50 and 200 ng Sn/L	None	Imposex (VDSI)	Clear effect at 200 ng Sn/L (no statistical evaluation)			2	Schulte-Oehlmann et al. 1995
						Penis sheath length in ♀					
						Testosterone / 17β-estradiol-ratio ↑	LOEC	200 ng Sn/L			

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
M. cornuarietis	Partial life cycle test (150 d)	TBT-Cl (n.i.)	n.i.	30, 60, 125, 250 and 500 ng Sn/L	Yes, but results not indicated	Imposex (VDSI)	LOEC	≤ 16.5 ng Sn/L	Effect conc. based on measured conc. Few information provided on experimental details	4	Albanis et al. 2006, Duft et al. 2007
							EC_{10}	3.42 ng Sn/L			
							LOEC	98.2 ng Sn/L			
						Total nº of embryos	LOEC	≤ 16.5 ng Sn/L			
							EC_{10}	10.4 ng Sn/L			
							EC_{50}	64.9 ng Sn/L			
Nassarius reticulatus	Chronic sediment test (spiked artificial sediment; 30 d), 15°C	TBT-Cl (n.i.)	Glacial acetic acid (n.i.)	10, 25, 50, 75, 125, 250 and 500 ug Sn/kg sed. dw	n.i.	Imposex (VDSI)	LOEC	50 ug Sn/kg sed. dw	Artificial seawater. EC_{50} = value leading to an increase of VDSI to 150% of control value	2	Tillmann 2004, Duft et al. 2007
							EC_{50}	16.9 ug Sn/kg sed. dw			
Nucella lapillus	Chronic test with adult snails, flow-through with simulated tidal conditions (52 wk)	TBT oxide (n.i.)	Ethanol (n.i.)	0.8, 3.3, 13 and 52 ng Sn/L	< 1.1, 3.2, 14 and 51 ng Sn/L	Mortality	No effect		Seawater	2	Davies et al. 1997
						Imposex (VDSI)	LOEC	≤ 1.1 ng Sn/L			
Hexaplex trunculus	Chronic test with adult snails, semi-static (2 mo)	TBT-Cl (95%)	Ethanol (100 ul/L)	2.1, 20.5 ng Sn/L	None	Proportion of imposex snails	LOEC	20.5 ng Sn/L	Individuals collected from the field. Seawater	2	Abidli et al. 2012
						Penis length in ♀					
						VDSI					
						Relative penis length index in ♀					
						Penis length in ♂	LOEC	> 20.5 ng Sn/L			
						Free testosterone in ♀ ↑	LOEC	2.1 ng Sn/L			
						Free estradiol in ♀ ↑					

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
H. trunculus (continued)	Chronic test with adult snails (see above)	See above	See above	See above	See above	Ratio of free testosterone to free estradiol	Not affected in ♂ and ♀		See above	2	Abidli et al. 2012
Bolinus brandaris	Chronic test with adult snails, semi-static (2 mo)	TBT-Cl (95%)	Ethanol (100 ul/L)	2.1 and 20.5 ng Sn/L	None	Proportion of imposex snails	LOEC	> 20.5 ng Sn/L	Individuals collected from the field. Seawater	2	Abidli et al. 2012
						Penis length in ♀					
						VDSI	LOEC	20.5 ng Sn/L			
						Relative penis length index in ♀	LOEC	> 20.5 ng Sn/L			
						Penis length in ♂	LOEC	20.5 ng Sn/L			
						Free testosterone in ♀ ↑					
						Free estradiol in ♀ ↑	LOEC	> 20.5 ng Sn/L			
						Ratio of free testosterone to free estradiol	Not affected in ♀. In ♂: significant effect at 2.1, but not at 20.5 ng Sn/L				
Ilyanassa obsoleta	Chronic test with adult snails, semi-static (6 mo)	TBT-Cl (n.i.)	DMSO (130 ul/L)	0.1, 1.0, 10 ng Sn/L	None	Percentage of imposex ♀ (month 6)	LOEC	1.0 ng Sn/L	Animals collected from field	2	Gooding et al. 2003
						Level of free testosterone in ♀ ↑ (month 3)	LOEC	10 ng Sn/L			
						Fatty acid esterification of testosterone in ↓ (month 3)					

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
Crustacea											
Daphnia magna	Two-generation test starting with < 24 h-old neonates (42 d)	TBT-Cl (n.i.)	Ethanol (100 ul/L)	0.11, 0.23, 0.46, 0.91 ug Sn/L	None	F_0: Survival	LOEC	0.91 ug Sn/L	60% Mortality at 0.91 ug Sn/L	2	Oberdörster et al. 1998
						F_0: Average n° of moults per / ♀	LOEC	> 0.46 ug Sn/L	Due to high mortality at 0.91 ug Sn/L, this conc. was not included in the evaluation		
						F_0: Offspring / ♀					
						F_1: Adverse effects					
						F_0: Ratio of metabolic androgenisation [1]	Increased at 0.11, 0.23 and 0.46 ug Sn/L, but effect not significant				
						F_1: metabolic androgenisation ratio	No effect				
Macrobrachium rosenbergii	Short-term reproduction test (7 d). Offspring (F_1) raised in control water for 10 d for assessment of hatching and development	TBT without further specification (95%)	Ethanol (n.i.)	0.32, 0.64, 1.28 mg Sn/L	None	F_1: Hatching rate	LOEC	≤ 0.32 mg Sn/L	Very high TBT concentrations	2-3	Revathi & Munuswamy 2010
						F_1: Larval deformities	LOEC	0.64 mg Sn/L			
						F_1: Larval growth (d 10)	LOEC	≤ 0.32 mg Sn/L			

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
Uca pugilator	Limb regeneration test, semi-static (approx. 3 wk). 1st Experiment: early July, 2nd experiment: late August	TBTO (n.i.)	Acetone (n.i.)	0.2, 2.1 and 20.5 ug Sn/L	None	Delay in limb regeneration (d 14)	Observed at ≥ 0.2 ug Sn/L (1st experiment: stronger effect on ♂, 2nd experiment: stronger effect on ♀)		Crabs collected from field directly before exposure. Seawater (25‰). Gaps in description of methods and results	2-3	Weis et al 1987
						Delay in moulting (d 24)	Observed at ≥ 0.2 ug Sn/L (1st experiment; 2nd experiment: no data presented)				
						Deformities of regenerated limbs	Observed at ≥ 0.2 ug Sn/L (1st experiment; 2nd experiment: no data presented)				
Insecta											
Chironomus riparius	Developmental test starting with larvae in stages 5-6 (48 h)	TBTO (p.a.)	Ethanol (50 ul/L)	10, 50, 200, 1000 ng Sn/L	None	Development	♂ Larvae: tendency towards faster development ♀ Larvae: tendency towards slower development			2	Hahn & Schulz 2002
	Acute toxicity test starting with larvae in stages 5-6 (48 h)			10, 20, 30, 100 ng Sn/L		Mortality	LC_{50}	25 ug Sn/L			
Echinodermata											
Strongylocentrotus purpuratus	Developmental toxicity test starting with freshly fertilised eggs (96 h)	TBT, not further specified (purity n.i.)	None	0.0004, 0.041, 0.41 ug Sn/L	None	Developmental toxicity (teratogenicity) at the pluteus stage	EC_{50}	0.37 ug Sn /L	Few test conc. for EC_{50} determination	2-3	Roepke et al. 2005
Lytechinus anamesus						Developmental toxicity (teratogenicity) at the pluteus stage	EC_{50}	0.02 ug Sn /L			

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Vali-dity	Reference
Pisces											
Cyprinodon variegatus	Fish life-cycle test starting with embryos < 24 hpf, flow-through (180 d)	TBTO (97.9% TBTO, 1.0% dibutyltin-Cl_2, 1.05% tetra-butyltin)	None	0.25, 0.49, 0.98, 2.0 and 4.0 ug Sn/L	0.17, 0.27, 0.5, 1.3 and 2.2 ug Sn/L	F_0: Embryo survival ↓	LOEC	2.2 ug Sn/L	Seawater (15‰). Complete mortality at 4.0 ug Sn/L on d 7 → reproduction not evaluated	2	Manning et al. 1999
						F_0: Survival ↓(hatch – d 30)	LOEC	1.3 ug Sn/L			
						F_0: Survival ↓ (d 30 – d 163)	LOEC	0.27 ug Sn/L			
						F_0: Reproduction	Reduced at 0.5 and 1.3 ug Sn/L, but difference not significant.				
						F_1: survival to d 30	LOEC	1.3 ug Sn/L			
Danio rerio	Fish sexual development test, starting 1 d before hatch, flow-through (70 d). Following exposure fish were raised to maturity in control water	TBT (n.i.)	Acetone (10 ng/L)	0.004, 0.041, 0.41, 4.0 and 41 ng Sn/L	Only highest nominal conc. analysed, but results not presented (lower conc.: below limit of detection)	Sex ratio (% ♂ at maturity ↑)	LOEC	0.041 ng Sn/L		2	McAllister & Kime 2003
						Sperm motility ↓	LOEC	0.41 ng Sn/L			
						% Abnormal sperm (lacking flagella) ↑	LOEC	0.041 ng Sn/L			
						Milt volume	LOEC	41 ng Sn/L	Not analysed at 0.041 and 4 ug Sn/L Sn/L		

(1) Hydroxylation and conjugation of testosterone results in inactivation / elimination, whereas reduced / dehydrogenated products may serve as androgens / androgen precursors. Hence, the ratio of the rate of production of reduced / dehydrogenated metabolites to the rate of production of hydroxylated / conjugated metabolites was derived as indicator of metabolic androgenisation (Oberdörster et al. 1997).

Table 20: Effect concentrations of triphenyltin (TPT) in molluscs (classification of validity based on Klimisch et al. 1997, EC 2003 and 2011a).

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
Marisa cornuarietis	Reproduction test starting with adults, semi-static, 22°C (4 mo)	TPT-Cl (n.i.)	Ethanol (max. 12.5 ug/L)	75, 150, 250, 500 ng Sn/L	43, 93, 163 and 471 ng Sn/L	VDSI (month 4)	LOEC	≤ 75 ng Sn/L	EC_{10} values extrapolated slightly beyond range of tested conc.	2	Schulte-Oehlmann et al. 2000
							EC_{10}	18 ng Sn/L			
						Penis sheath length in ♀ (month 4)	LOEC	≤ 75 ng Sn/L			
							EC_{10}	23 ng Sn/L			
						Reduced penis length in ♂	LOEC	250 ng Sn/L			
						Fecundity (nº of spawning masses)	LOEC	≤ 75 ng Sn/L			
							EC_{10}	14 ng Sn/L			
						Nº of eggs per spawning mass	LOEC	≤ 75 ng Sn/L			
						Impairment of spermatogenesis	Only evaluated at 500 ug Sn/L: severely disturbed				
Nucella lapillus	Chronic test with adults, semi-static, 14°C, 35‰ (3 mo)	TPT-Cl (n.i.)	Glacial acetic acid (max. 10 ug/L)	5, 50 and 100 ng Sn/L	None	VDSI	No effect		Adults collected from field. Effects on fecundity were not assessed, as upon transfer from field to lab, *N. lapillus* usually does not reproduce		
						Reduced length of prostate gland in ♂	LOEC	100 ng Sn/L			
						Reduced length of penis in ♂	LOEC	100 ng Sn/L			
						Incidence of tissue excrescences ↑ (epithelial hyper-plasia on gills, osphradium or pallial sexual organs)	LOEC	≤ 5 ng Sn/L			

Test species	Test method (duration)	Substance (purity)	Solvent (max. conc.)	Nominal conc.	Measured conc.	Endpoint	Effect conc.		Remark	Validity	Reference
Potamopyrgus antipodarum	Reproduction test, static (8 wk)	TBT-Cl (n.i.)	n.i.	30, 60, 125, 250 and 500 ng Sn/L	n.i.	Fecundity (total nº of embryos per ♀)	LOEC	≤ 30 ng Sn/L	Few information on experimental details. EC_{10} for fecundity extrapolated slightly beyond range of tested conc. Apparently static exposure	4	Albanis et al. 2006, Duft et al. 2007
							EC_{10}	20 ng Sn/L			
						Nº of shelled embryos per ♀	LOEC	60 ng Sn/L			
							EC_{10}	60 ng Sn/L			
						Nº of unshelled embryos per ♀	LOEC	≤ 30 ng Sn/L			
							EC_{10}	30 ng Sn/L			
							EC_{10}	0.03 ng Sn/L			
P. antipodarum	Reproduction test, water / sediment system (with spiking of sediment), static (8 wk)	TPT-Cl (>98%)	Ethanol	10, 25, 50, 75, 125, 250 and 500 ug Sn/kg sed. dw	Chemical analysis at test end only: most TPT had degraded to mono- and diphenyltin. TPT only detected at nom. conc. of 10, 75 and 250 ug Sn/kg sediment dw: 4.33, 28.4 and 75.4 ug Sn/kg sed. dw, respectively	Nº of unshelled embryos (8 wk)	LOEC	≤ 10 ug Sn/kg sed. dw	Artificial sediment (95% quartz sand, 5% beech leaves). EC_{10} and EC_{50} values extrapolated beyond range of tested conc.	2	Duft et al. 2003a
							EC_{10}	0.03 ug Sn/kg sed. dw			
							EC_{50}	0.74 ug Sn/kg sed. dw			
						Total nº of embryos (8 wk)	LOEC	≤ 10 ug Sn/kg sed. dw			
							EC_{10}	0.05 ug Sn/kg sed. dw			
							EC_{50}	23.6 ug Sn/kg sed. dw			
Nassarius reticulatus (formerly *Hinia reticulata*)	Chronic test with adults, water / sediment system, semi-static, 14°C (3 mo)	TPT-Cl (n.i.)	Glacial acetic acid (max. 5 mg/kg dw)	50, 125 and 500 ug Sn/kg sed. dw	None	VDSI	No effect		Adults collected from field. Seawater (35‰), artificial sediment (90% quartz sand, 10% peat)	2	Schulte-Oehlmann et al. 2000
						Reduced length of penis in ♂	No effect				
						Atrophy in ovaries ↑	LOEC	≤ 50 ug Sn/kg sed. dw			
						Atrophy in testes ↑	LOEC	≤ 50 ug Sn/kg sed. dw			
						Incidence of tissue excrescences[1]	No effect				