GUIDANCE

Guidance on the Application of the CLP Criteria

Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures

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List of abbreviations [This will be transferred to the list of abbreviations in the whole CLP guidance.]

ADME	Absorption, Distribution, Metabolism, Excretion
AMA	Amphibian Metamorphosis Assay
AOP	Adverse Outcome Pathway
BP	Biocidal Products
BPR	Biocidal Products Regulation (Regulation EU 528/2012)
CERAPP	Collaborative Estrogen Receptor Activity Prediction Project
CLP	Regulation on classification, labelling and packaging of substances and mixtures (Regulation EC 1272/2008)
CMR	Carcinogenic, Mutagenic, Reprotoxic
ComPARA	Collaborative Modelling Project for Androgen Receptor Activity
CTA	Comparative thyroid assay
EAMA	Extended Amphibian metamorphosis Assay
EC10	Effect Concentration that causes a measurable adverse effect to 10% of the test organisms comparing to the control group
ED	Endocrine disruptor or endocrine disrupting
ED HH	Endocrine disruptor for human health
ED ENV	Endocrine disruptor for the environment
EffD	Effective Dose
ELS	Early life stages
ER	Estrogen receptor
EATS	Estrogen, Androgen, Thyroid and Steroidogenic
FDA	Food and Drug Administration of United States
FE	first estrus
FFLCTT	Fish full lifecycle toxicity test
FSTRA	Fish short term reproduction assay
GCL	Generic Concentration Limit
GLP	Good Laboratory Practice
GSI	Gonadosomatic index
HPT axis	Hypothalamic–Pituitary–Thyroid axis
IRs & CSA	ECHA Guidance on information requirements and safety assessment
KE	Key Event
KER	Key event relationship
LBD	Ligand binding domain
LDL	Low-Density Lipoprotein (cholesterol)
LOQ	Level of Quantification
MIE	Molecular initiating event
MoA	Mode of Action
MTC	Maximum tolerated concentration
MTD	Maximum tolerated dose
NAMs	New Approach Methodologies
NOEC	No Observed Effect Concentration
NR	Nipple retention
OECD	Organisation for Economic Co-operation and Development
OECD CF	OECD Conceptual Framework
(Q)SAR	(Quantitative) structure-activity relationship
OECD GD 150	Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption
PBTK	Physiologically Based Toxico Kinetic models
PPP	Plant Protection Product
PPPR	Plant Protection Products Regulation (Regulation EC No. 1107/2009)
SAR	Structure-activity relationship
SSC	Secondary Sex Characteristics
SCL	Specific Concentration Limit

SVHC	Substances of Very High Concern
T3	Triiodothyronine
T4	Thyroxine
TBG	Thyroxine binding globulin
TH	Thyroid hormone
TRH	Thyrotropin-releasing hormone
TSH	Thyroid-stimulating hormone
US EPA	United States Environmental Protection Agency
VO	Vaginal opening
VCBA	Virtual cell-based assay
VTG	Vitellogenin
WAT	White adipose tissue
WoE	Weight of Evidence

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3. HH

3.11. Endocrine disruption for human health

Relationship with the ECHA/EFSA ED Guidance on assessing endocrine disrupting properties for biocidal products and plant protection products

The ECHA/EFSA ED Guidance on assessing endocrine disrupting (ED) properties (ECHA/EFSA, 2018), which builds on the '*Revised guidance document 150 on standardised test guidelines for evaluating chemicals for endocrine disruption*' (OECD GD 150; OECD, 2018a), was developed to assist applicants and assessors of the competent regulatory authorities in complying with their obligations to conclude on ED properties for biocidal products (BPs) and plant protection products (PPPs). More specifically, the ECHA/EFSA ED Guidance describes how to gather, evaluate and consider all relevant information for the assessment, apply a weight of evidence (WoE) approach and conduct a mode of action (MoA) analysis, in order to help in establishing whether the substance meets the criteria for approval under the BP[1] and PPP[2] Regulations. Therefore, the ECHA/EFSA ED Guidance remains the key piece of guidance for scientific assessment of ED properties of BPs and PPPs.

In 2023, endocrine disruption was introduced into CLP as a hazard class with sub-categorisation. CLP covers classification of hazardous substances and mixtures across regulations and applies (among others) to industrial substances (subject to the REACH Regulation[3]), BPs and PPPs. Notably, CLP does not require the generation of any new data for the purpose of CLP classification and, therefore, ED classification needs to be based on available data. Consequently, the format of the CLP guidance and that of the ECHA/EFSA ED Guidance are different owing to the regulatory framework. For hazard classification purposes this guidance on the application of the CLP criteria should be followed for all substances and mixtures.

Despite differences in the framework, it is important to note that the current ED criteria for BPs and PPPs are derived from the same basis as the ED hazards in Category 1 for human health (*ED HH 1*) or the environment (*ED ENV 1*) under the CLP criteria. While the format of this guidance on CLP and the ECHA/EFSA ED Guidance may differ due to the differences in scope of the applicable legislation, the guidance to arrive at a conclusion for ED hazards in Category 1 is largely equivalent and based on a similar scientific assessment in both documents.

Accordingly, active substances already concluded to meet the ED criteria under the BP[4]

[1] Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products Text with EEA relevance. OJ L 167, 27.6.2012, p. 1–123. Available online: http://data.europa.eu/eli/reg/2012/528/oj

[2] Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. OJ L 309, 24.11.2009, p. 1–50. Available online: http://data.europa.eu/eli/reg/2009/1107/oj

[3] Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. OJ L 396, 30.12.2006, p. 1–849. http://data.europa.eu/eli/reg/2006/1907/oj

[4] Commission Delegated Regulation (EU) 2017/2100 of 4 September 2017 setting out scientific

and PPP[5] procedures before the criteria in CLP became applicable, should under CLP be assigned to *ED HH 1* or *ED ENV 1*. Similarly, substances identified as Substances of Very High Concern (SVHC) under REACH due to ED properties should also be assigned to *ED HH 1* or *ED ENV 1* under CLP.

3.11.1. Definitions and general considerations for endocrine disruption

The classification for endocrine disruption for human health differs from the other hazard classes in that it refers to a specific (endocrine) MoA which leads to an adverse effect(s). The classification criteria require evidence on three elements, *i.e.* adverse effect(s), endocrine activity, and a biological plausible link between the endocrine activity and the adverse effect(s) consistent with existing knowledge.

> **CLP, Annex I*, Section 3.11.1.1.*** *For the purposes of Section 3.11, the following definitions shall apply:*
>
> (a) *'endocrine disruptor' means a substance or a mixture that alters one or more functions of the endocrine system and consequently causes adverse effects in an intact organism, its progeny, populations or subpopulations;*
>
> (b) *'endocrine disruption' means the alteration of one or more functions of the endocrine system caused by an endocrine disruptor;*
>
> (c) *'endocrine activity' means an interaction with the endocrine system that may result in a response of that system, of target organs or target tissues, and that confers on a substance or the mixture the potential to alter one or more functions of the endocrine system;*
>
> (d) *'adverse effect' means a change in morphology, physiology, growth, development, reproduction or lifespan of an organism, system, population or subpopulation that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences;*
>
> (e) *'biologically plausible link' means the correlation between an endocrine activity and an adverse effect, based on biological processes, where the correlation is consistent with existing scientific knowledge.*

The definitions in CLP, Annex I, Section 3.11.1.1. are further explained below:

(a) The definition of '*endocrine disruptor*' (ED) is based on the WHO/IPCS definition (WHO/IPCS, 2002). It has been modified for the purposes of classification under CLP.

The definition uses the term *'intact organism'*, which is understood to mean that the effect would occur *in vivo*, either observable in a test animal system, epidemiologically or clinically. However, it does not necessarily mean that an adverse effect has to be demonstrated in an intact test animal.

The '*endocrine system*' in this context consists of hormone-producing tissues and their associated hormones that regulate the functioning of the organism.

(b) An '*endocrine disruptor*' may alter one or more functions of the endocrine

criteria for the determination of endocrine-disrupting properties pursuant to Regulation (EU) No 528/2012 of the European Parliament and Council. OJ L 301, 17.11.2017, p. 1–5. Available online: http://data.europa.eu/eli/reg_del/2017/2100/oj

[5] Commission Regulation (EU) 2018/605 of 19 April 2018 setting out scientific criteria for the determination of endocrine-disrupting and amending Annex II to Regulation (EC) 1107/2009. OJ L 101, 20.4.2018, p. 33–36. Available online: http://data.europa.eu/eli/reg/2018/605/oj

system, *e.g.*, hormonal synthesis, transport, signalling, regulation or metabolism.

(c) A substance that has an '*endocrine activity*' has the potential to interact with and alter the function(s) of the endocrine system, target organs and tissues. This interaction may occur at any level in a biologically plausible sequence of events leading to an adverse effect.

(d) The definition of '*adverse effect*' is based on the WHO definition (WHO/IPCS, 2009). The definition of adversity is generic and not specific to the assessment of ED properties. Current practices from other hazard classes for assessing adversity are applicable for deciding whether the observed effects are relevant for human health, treatment-related and should be considered adverse.

(e) The '*biologically plausible link*' relies on an understanding of the fundamental biological processes involved and whether they are consistent with the sequence of the events proposed. The term '*correlation*' used in the definition means that endocrine activity and adverse effect(s) can be plausibly linked (connected) using existing knowledge as the most likely explanation for the observed effects; a causal relationship does not need to be proven.

In a MoA analysis, biological plausibility is considered to be the level of support for the links (connections) between the adjacent key events in the postulated MoA, *i.e.* the key event relationships (KERs); see Section 3.11.2.3.3.

In addition, data with '*equivalent predictive capacity*' are defined as data obtained using alternative methods which can be used with a similar level of confidence as internationally recognised *in vivo* methods or human data, to predict adversity or endocrine activity. Alternative methods do not need to be one-to-one replacements of an internationally recognised *in vivo* method, but can be *e.g.* a set of *in vitro* or *in silico* methods which together meet the requirement of equivalent predictive capacity, see Sections 3.11.2.1.2 and 3.11.2.3.1.

CLP, Annex I, Section *3.11.1.2.1*. *Substances and mixtures fulfilling the criteria of endocrine disruptors for human health based on evidence referred to in Table 3.11.1 shall be considered to be known, presumed or suspected endocrine disruptors for human health unless there is evidence conclusively demonstrating that the adverse effects are not relevant to humans.*

More explicitly, substances or mixtures are classified as '*known or presumed*' or as '*suspected*' endocrine disruptors for human health if they induce adverse effects in humans or animals by altering the function of the endocrine system, *i.e.*, the substance has an endocrine MoA, in accordance with the criteria given in CLP, Annex I, Section 3.11.2.1.

Conclusively demonstrating that the adverse effect is not relevant for humans means that convincing evidence is provided which demonstrates that human relevance can be excluded. This means that persuasive data need to be available clearly indicating that only a well established mechanism not relevant to humans can be attributed to the observed effects and that other, human relevant, mechanisms can be excluded.

CLP, Annex I, Section *3.11.1.2.2*. *Evidence that is to be considered for classification of substances in accordance with other Sections of this Annex may also be used for classification of substances as an endocrine disruptor for human health where the criteria provided in this Section are met.*

In other words, all relevant information for the determination of endocrine disruption for human health is to be considered together. This also includes information that is already

used for classifying the substance or a mixture for carcinogenicity, reproductive toxicity, specific target organ toxicity single or repeated exposure and endocrine disruption for the environment.

The classification of a substance as endocrine disruptor for human health Category 1 or 2 is independent of the classification of the substance for *Carc.*, *Repr.*, or *STOT*. A substance can be classified as endocrine disruptor for human health based on the same set of evidence as used for other hazard classes irrespective of whether the substance is also classified for other hazard classes. For example, based on the same set of evidence, a substance may be classified as endocrine disruptor for human health for adverse effects on the thyroid gland, even though the adverse effect(s) are observed above the suggested guidance values (dose/concentration limits) for classification under *STOT RE*.

The data used for the assessment of ED properties could be overlapping with the data used for other hazard classes. In many cases, the data on adversity may be the same as other hazard classes. However, the ED classification also considers endocrine activity and its link to adversity.

If an effect is considered adverse for *Repr.*, *Carc.* or *STOT* classification. Then this effect is to be considered relevant also for the ED classification. The *ED HH* classification outcome will depend on the overall strength of evidence for a link between adverse effect and endocrine activity.

Some effects may be adverse but not sufficient for *Repr.* classification, *e.g.*, effects on nipple retention and anogenital distance. However, when endocrine activity and its link to the observed adversity is considered together, classification for *ED HH* may be warranted.

Based on the reasons explained above, a substance can be classified as *ED HH 1 or ED HH 2*, even if the substance is already classified in a similar or different Category for reproductive toxicity based on the same adverse effect. This is because evidence for endocrine activity and the biologically plausible link between the endocrine activity and the adverse effect as well as other supportive information, which may not be relevant for *Repr.*, are taken into consideration for classification as ED.

In addition, the allocation of a substance as endocrine disruptor for human health Category 1 or 2 is independent of the allocation of the substance as an endocrine disruptor for the environment, *e.g.*, a substance can be classified as *ED ENV 1, ED ENV 2* or not classified, even if the substance is classified as *ED HH 1 or ED HH 2* and *vice versa*.

Generally, the developing foetus, pups and peripubertal animals are considered to be more sensitive than adults to endocrine perturbations that may potentially lead to adverse effects. The nature of, and sensitivity to, such effects depends on the life-stage investigated. Classification as endocrine disruptor for human health is intended to indicate that a substance may cause an endocrine related adverse effect at any life-stage. Some substances or mixtures may exhibit delayed ED effects, *e.g.* exposure of foetus during gestation or of pups before puberty, which may lead to endocrine disfunction later in life. Some effects may be reversible in adults, but may cause irreversible effects in the developing organism. However, the CLP ED criteria do not mention reversibility as a factor to be considered in the WoE; therefore, an adverse effect, reversible or irreversible, may warrant ED classification.

The concept of ED '*potency*' is considered only in the context of setting specific concentration limits, see Section 0. The CLP criteria for endocrine disruption for human health do not specify any dose above which the production of an adverse effect is outside the criteria which lead to classification. In other words, the criteria apply to all dose levels. Even endocrine-related effects observed at high doses (showing low potency) may still warrant classification.

The ED effect may be a threshold or a non-threshold effect, see the JRC report on `*Thresholds for Endocrine Disrupters and Related Uncertainties*´ (Munn and Goumenou, 2013). When the ED adversity is observed already at very low dose levels (high potency) or alternatively only at very high dose levels (low potency), this guidance considers that potency can be regulated by setting a specific concentration limit, which can be either lower, or in exceptional cases higher than the generic concentration limit. For setting an SCL, a careful assessment on doses or concentrations causing adversity is recommended for all substances.

ED modalities covered by the CLP criteria

While the CLP criteria do not differentiate between different modalities, thus covering all endocrine-disrupting MoAs, it is acknowledged that this guidance mainly addresses the effects caused by estrogen, androgen, thyroid, and steroidogenic (EATS) modalities. This is because the EATS modalities are the pathways for which there is currently the most knowledge available, i.e., there is relatively good mechanistic understanding on how substance-induced perturbations may lead to adverse effects via an endocrine-disrupting MoA.

In addition, only for the EATS modalities there are at present standardised test guidelines for *in vivo* (EATS) and *in vitro* (EAS) testing available where there is broader scientific agreement on the interpretation of the effects observed on the investigated parameters. Further information on EATS modalities can be found in Section 3.11.2.3.1.

However, the general principles outlined in this guidance for evaluation of the data on the different criteria, WoE and decision on classification, are applicable to all endocrine modalities.

The CLP criteria apply to all endocrine modalities. There are at least 50 hormones produced by the classical endocrine glands (i.e. adrenal, hypothalamus, pituitary, (para)thyroid, pineal gland, pancreas, ovary and testes). In addition, there are about 100 hormones produced by other tissues. All hormones that are not covered by EATS-modalities are by definition non-EATS. Examples of non-EATS modalities include, but are not limited to, hormones interfering with the neuroendocrine system, vitamin A and D, peroxisome proliferator-activated receptor-gamma (PPARγ), and the retinoid system (for a detailed review, see OECD GD 150 and OECD, 2021). Other non-EATS modalities include hormones interfering with glucose homeostasis for instance insulin, glucagon and glucagon-like peptide. It should be noted that ligands to some of these receptors (*e.g.*, vitamin D binding to the vitamin D receptor, retinoids binding to the retinoic acid receptor, fatty acids binding to PPARγ) may not fit in the conventional view of a hormone.

Nonetheless, these ligands do fit in the broad definition of a hormone as a substance, originating in one tissue and conveyed by the bloodstream to another tissue to exert a physiological activity (OECD GD 150).

The existing knowledge for non-EATS modalities is not as advanced as that for the EATS modalities. However, in some cases, it may be possible to reach a conclusion on the need to classify the substance based on a non-EATS MoA. For example, in the case of histopathological findings in the islet cells of the pancreas, which are pivotal for glucose homeostasis, scientific knowledge provides mechanistic information that can be linked to adverse effects measured in standard tests, *e.g.* when seeing effects on pancreas histopathology and related changes in glucose levels.

3.11.2. Classification of substances for endocrine disruption for human health

3.11.2.1. Identification of hazard information

CLP does not set information requirements or require further testing of substances and mixtures for classification purposes (CLP, Articles 5, 6 and 9) except for physical hazards (CLP Article 8.2). The assessment is based on the respective criteria and consideration of all available relevant information. All relevant information that addresses endocrine-related adverse effects and activities shall be considered in a WoE approach; this includes guideline and research studies as well as alternative methods such as read-across and *in silico* predictions.

The main ways to gather all available information is collecting studies and data from the registration dossiers, *e.g.* under REACH, BPR, PPPR, and by conducting a literature search or preferably a systematic literature review designed to avoid bias and capture as much as possible of the relevant scientific literature data. Further guidance is available in ECHA/EFSA ED Guidance, Section 3.2 and Appendix F. Additionally, previous regulatory assessments may serve as a starting point for the literature search. Furthermore, information considered for other hazard classes may also provide information relevant for endocrine disruption classification for human health; see Sections 3.6.2.1; 3.7.2.1; 3.9.2.1 and 4.2.2.1.

Upon reviewing the literature, the information is deemed relevant when it investigates or brings information for the assessment of at least one of the three elements; *i.e.* '*endocrine activity', 'adverse effects'* or '*biologically plausible link'*:

- Information on endocrine-related *'adverse effects'* relevant for humans is normally obtained from animal studies with repeated exposures. However, when available non-animal methods or strategies (if providing an equal predictive capacity as animal and/or human data) may bring sufficient information on adversity for decision making on classification, particularly when supported by toxicokinetic data. Information on adversity may also be obtained using read-across or analogy, for example, if the substances by analogy share a common MoA (*e.g.* aromatase inhibitors), or using read-across between substances with a common active metabolite or a different ratio of the same isomers.

- Information on *'endocrine activity'* generally comes from *in vivo* or *in vitro* mechanistic studies. Information may also come from read-across, *in silico* models or *omics*-approaches, if available. In addition, endocrine activity may also be inferred from observed adverse effects known to be mediated by endocrine activity, see '*EATS-mediated*' parameters in Section 3.11.2.3.1.

- *A 'Biologically plausible link'* does not need to be demonstrated with substance specific data. Existing scientific knowledge can be used, *e.g.*, textbooks and peer reviewed scientific literature. AOPs can be helpful to establish biological plausibility, but they are not a prerequisite. Several adverse outcome pathways related to endocrine disruption have been established and endorsed, *e.g.*, OECD Series on AOPs[6]; or EFSA PPPR Panel, 2023. There is continuous development of additional AOPs in various stages in the AOPwiki[7]. It should be noted that the presence of an AOP in the AOPwiki does not necessarily indicate its relevance or reliability. Depending on the stage of development of the AOP in AOPwiki, the amount of data

[6] https://www.oecd-ilibrary.org/environment/oecd-series-on-adverse-outcome-pathways_2415170x
[7] aopwiki.org

needed to support biological plausibility may vary considerably. The validity of an AOP should be considered using expert judgement.

3.11.2.1.1. Identification of human data

Information that is relevant for classification for endocrine disruption may be available for example from case reports, epidemiological studies, medical surveillance and reporting schemes, and national poison centres.

Further information sources are given, for example, in the ECHA Guidance on information requirements and substance safety assessment (Guidance on IRs & CSA), Sections 7.5.3.2., 7.6.3.2. and 7.7.10.2 (ECHA, 2017).

3.11.2.1.2. Identification of non-human data

The OECD '*Revised guidance document on standardised test guidelines for evaluating substances for endocrine disruption*', OECD GD 150 provides widely accepted guidance on the interpretation of effects measured in relevant OECD test guidelines and other standardised test methods, which may arise as a consequence of perturbations of the EATS modalities. It explains how these effects might be evaluated to support identification of endocrine disruptors.

The OECD GD 150 includes the '*OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Substances*' (OECD CF; OECD, 2012) which lists the OECD test guidelines and standardised test methods available that can be used to evaluate substances for endocrine disruption. It is not an exhaustive list and assays other than those described in the list (*i.e.* other published or internationally recognised methods) may also be valuable for assessing substances for endocrine disruption and can also be used for classification if they are relevant and considered predictive for humans. Research studies are an important source of information which must be considered in a WoE approach. New tests are continually being developed, which may provide useful information for classification. In particular endpoints for non-EATS modalities are currently not well covered in the OECD test guidelines.

New approach methodologies (NAMs)

New approach methodologies (NAMs, *e.g. in vitro-, in silico- and omics*-methods; testing strategies; defined approaches etc.) can be used to provide information about adverse effects or endocrine activity if they provide equivalent predictive capacity as animal data from internationally recognised *in vivo* methods or human data. OECD-validated NAMs or internationally recognised methods, if available, may be more relevant than non-validated methods. When the NAMs provide sufficient information on adverse effect(s) or endocrine activity, they can be used for classification purposes.

3.11.2.2. Classification criteria

CLP, Annex I,* Section *3.11.2.1. *Hazard categories*

For the purpose of classification for endocrine disruption for human health, substances shall be allocated to one of two categories.

Table 3.11.1.

Hazard categories for endocrine disruptors for human health

Categories	*Criteria*
CATEGORY 1	*Known or presumed endocrine disruptors for human health*

	The classification in Category 1 shall be largely based on evidence from at least one of the following: *a) human data;* *b) animal data;* *c) non-animal data providing an equivalent predictive capacity as data in points a or b.* *Such data shall provide evidence that the substance meets all the following criteria:* *(a) endocrine activity;* *(b) an adverse effect in an intact organism or its offspring or future generations;* *(c) a biologically plausible link between the endocrine activity and the adverse effect.* *However, where there is information that raises serious doubt about the relevance of the adverse effects to humans, classification in Category 2 may be more appropriate.*
CATEGORY 2	*Suspected endocrine disruptors for human health* *A substance shall be classified in Category 2 where all the following criteria are fulfilled:* *(a) there is evidence of:* *i. an endocrine activity; and* *ii. an adverse effect in an intact organism or its offspring or future generations;* *(b) the evidence referred to in point (a) is not sufficiently convincing to classify the substance in Category 1;* *(c) there is evidence of a biologically plausible link between the endocrine activity and the adverse effect.*

The classification in Category 2 shall also be largely based on evidence from human, animal and non-animal data as described for Category 1. Where there is evidence conclusively demonstrating that the adverse effects are not relevant to humans, the substance should not be considered an ED for human health.

3.11.2.2.1. Classification in the presence of other toxicity

CLP, Annex I: *3.11.2.2.2.* *Adverse effects that are solely non-specific consequences of other toxic effects shall not be considered for the identification of a substance as endocrine disruptor for human health.*

'Other toxicity' refers to adverse effect(s) other than the endocrine-related adverse effect(s). If a substance causes endocrine-related adverse effect(s) which occur as a consequence of other toxicity, classification for endocrine disruption for human health should be applied unless the effect is demonstrated to be *'solely non-specific consequences of the other toxic effects'*. A *'non-specific consequences of the other toxic effects* is understood as:

- an endocrine-related adverse effect that is conclusively demonstrated to occur secondary to excessive toxicity, *i.e.* the co-occurring toxicity is so severe that the animals are prostrate or dying.

In all other cases, *e.g.* when the endocrine activity-related adverse effect is a specific consequence of other toxicity, classification as ED should be applied; see also Section 3.11.2.4 for other situations leading to no classification.

Other toxicity in adult animals

A dose- and temporal concordance between ED-related adverse effects and the other severe toxicity are important to assess if the endocrine system is out of balance solely due to a non-specific consequence of other toxicity. However, the presence of other toxicity shall not be used to dismiss classification, unless it can be justified that the endocrine-related adverse effect(s) are solely non-specific consequences of other toxicity.

If endocrine activity and ED-related adverse effects are observed, it may be difficult to demonstrate that the ED-related adverse effects are solely due to a non-specific consequence of other toxicity. In practice, this can be done only when the other co-occurring toxicity is so severe that the animals are prostrate or dying. It should be noted that according to the international test guidelines, the top dose should not induce excessive toxicity, and studies which cause excessive toxicity should not be conducted.

To consider a potential ED-related adverse effect solely as a non-endocrine MoA-related effect, there must be evidence for a biologically plausible sequence of events demonstrating that it is solely other toxicity that causes the adverse effect and which also excludes the endocrine MoA as a likely cause for the observed adverse effect(s).

Therefore, in such a case data is needed to demonstrate the non-ED MoA induced by other toxic effects and the assessment is best done by a comparative MoA assessment. For further guidance on how to conduct a comparative MoA analysis, see *e.g.* Meek *et al.* 2014a and 2014b. When assessing the potential influence of co-occurring other toxicity to the concurrent endocrine-related adverse effect(s) in adult animals, it may be helpful to also evaluate the co-occurrence at individual animal level considering also the temporal concordance between the potential mechanisms and the different types of effects observed.

The ED-related adverse effects may be dismissed when confounding effects of excessive toxicity at very high dose levels, *e.g.*, prostration, severe inappetence, mortality, are demonstrated using individual animal data. The excessive toxicity should occur at lower or same doses as endocrine–related effect(s). Similarly, excessive toxicity should precede the endocrine-related effect(s). Both dose and temporal concordance are necessary to support a claim that endocrine-related effect(s) are a consequence of the other toxicity; this is best illustrated by a comparative assessment.

An appropriate dose spacing may also help to assess if the effects are solely non-specific consequences of other toxicity. Mortality or other type of adverse findings which occur at similar incidences as in controls at the end of the study in lifetime studies, such as carcinogenicity studies, should not be considered as indication of excessive toxicity and not as treatment-related if these are normal findings in aging animals; see also Sections 3.6, 3.7 and 3.9 on excessive toxicity and non-specific effects.

Other (maternal) toxicity in context of assessing ED-related effects in foetuses and pups

The presence of maternal toxicity shall be considered particularly when evaluating effects in pups or foetuses in reproductive toxicity studies. Other toxicity shall not be used to negate findings of endocrine-related adverse effect(s) in offspring, unless it can be concluded that the endocrine-related effects are solely non-specific consequences of maternal toxicity. Studies generally show that severe weight loss or decrease in body weight gain in dams induced only minor changes in pup weight (Nitzsche *et al.*, 2017).

If maternal toxicity is so severe that it causes over 10% mortality in maternal animals (see CLP, Annex I, 3.7.2.4.4) or severe inanition, or the dams are prostrate and incapable of nursing the pups (see CLP, Annex I, 3.7.2.4.3), the co-occurring adverse effects on the offspring at that dose may be dismissed, because they may be considered to be a result of excessive maternal toxicity. It should be noted that according to the international test guidelines, the top dose should not induce excessive toxicity, and studies which cause excessive toxicity must not be conducted.

Consideration of the adjusted (corrected) maternal body weight change and/or maternal body weight shall be included in the evaluation of maternal toxicity whenever such data are available. When assessing the potential influence of maternal toxicity on the co-occurring endocrine-related effects in offspring, it may be appropriate to evaluate the potential causality at individual animal level. For example, if some of the maternal animals with the endocrine-related effects in foetuses or pups did not have any signs of excessive toxicity, these endocrine-related effects in foetuses or pups should not be dismissed from classification only because other adult animals in the group showed signs of excessive toxicity.

It should be noted that certain types of data should be assessed on a litter basis, not on an individual animal basis; see ECHA, 2023. Even in the presence of excessive toxicity, it is important that the data is reported in a transparent manner so that the data can be assessed on an individual basis, both qualitatively and quantitatively. Therefore, rather than focusing on the numerical results, it is important to understand if the observed endocrine-related effects are a consequence of the excessive toxicity.

In this context, a certain potential endocrine effect can be considered to be a non-specific consequence of one adverse effect, whereas another potential endocrine effect may not be a non-specific consequence of this same toxic effect. For example, a level of maternal toxicity that can be assumed to cause a decrease in pup weight or spontaneous abortions may not be sufficient to explain the presence of some other ED-related effects in pups. To conclude that a certain adverse effect is a non-specific consequence of other toxicity, a careful analysis is needed; see also Section 3.7.2.2.1.2.

Other toxicity when assessing ED related effects in pups and peripubertal animals

Onset of puberty (*i.e.*, age at balanopreputial separation (BPS) in male and vaginal opening (VO) and first estrus (FE) in female rats and mice) is an EAS-mediated parameter. Numerous neuroendocrine factors are involved in sexual maturation, but food restriction studies have shown that body weight may also play a role (Carney *et al.*, 2004; Chernoff *et al.,* 2009; Goldman *et al.*, 2000; Stoker *et al.*, 2000). Some studies suggest that marked changes in body weight may influence mean age of puberty depending on sex, degree and timing of body weight effects. However, due to limitations and inconsistencies in the data available to assess the association between effects on body weight and effects on sexual maturation, no clear thresholds can be given on what level of body weight change can impact to what degree of the onset of sexual maturation.

BPS and VO/FE are sensitive EAS endpoints that may be affected by substance exposure. For example, early VO/FE has been shown in the female rat exposed to estrogenic substances during the perinatal or prepubertal period, unrelated to changes in body weight (Boberg *et al.,* 2023; Goldman *et al.*, 2000, Rogers *et al*., 2023). In female rats, although VO and FE are tightly linked to the first ovulation, they can be uncoupled under certain conditions of housing, growth retardation or treatment (Engelbregt *et al.,* 2002, Posobiec *et al.*, 2015, Firlit and Schwartz, 1977). In contrast to rats, in female mice, these two pubertal markers are not coupled since the FE occurs in general around 10 days after VO. The FE is thus closer to pubertal initiation than VO in mice. Therefore, both parameters should be assessed in female rats and mice for a reliable determination of the age of pubertal onset. Lack of other EAS-related effects does not demonstrate lack of EAS activity

per se, as delayed or advanced sexual maturation may be one of the most sensitive endpoints. A consistent pattern of effects for a certain MoA strengthens the overall WoE based on which the decision on classification is made. The decision for a classification of Category 1, 2 or no classification needs to be based on the overall WoE. In the example above, the delay in sexual maturation can only be dismissed from classification if it can be demonstrated to be solely a non-specific consequence of reduced body weight.

Changes in body weight may also affect other endpoints, such as anogenital distance (AGD) and therefore, should be taken into account when evaluating the data. Since reduced body/fetal/birth weight could be a result of general toxicity and AGD is a measure of length (and size of the animal) its body weight needs to be considered when analysing AGD; *i.e.,* consider the correction index; see further details in '*Guidance document supporting OECD test guideline 443 on the extended one-generation reproductive toxicity test*' (OECD GD 151; OECD, 2013).

Nipple retention (NR) in rats is not dependent on body weight. However, some chemical substances can delay fetal and early postnatal development and also decrease body weight so that nipples would be visible at a later timepoint. Therefore, NR should be assessed in males when nipples (or areolas) are visible in their female littermates (Schwartz *et al.,* 2021, see also further details in OECD GD 151, and the '*Final report of the EOGRTS review project*' (ECHA, 2023).

3.11.2.2.2. Relevant doses for classification

In international test guidelines, the top dose should not induce excessive toxicity. If data are available from studies carried out with doses causing excessive toxicity, this data must be evaluated together with other relevant data and should be interpreted with caution. Because classification is based on all available relevant data, the dose-levels in available studies are as given. All dose-levels, even those tested above the limit dose of a test guideline or above Maximum Tolerated Dose (MTD) may be relevant for classification, *e.g.* if they do not result in such an excessive toxicity that the ED-related effects could be dismissed; see also Section 3.11.2.2.1.

Neither limit dose, top dose nor MTD should be confused with a demarcation above which the results are not relevant for hazard assessment or with a justification that the effect is *solely a non-specific consequence of other toxic effects*. Although a dose of 1000 mg/kg body weight/day is indicated as the limit dose in certain OECD test guidelines via oral route, ED effects at higher doses can be relevant for classification if such data is available. If the top-dose is well below the limit dose of 1000 mg/kg body weight/day and if only minimal or even no toxicity is observed, or in general, the doses are not sufficiently high with regard to tested parameters for endocrine disruption (*i.e.,* not in line with '*Advice on dose-level selection for the conduct of reproductive toxicity studies (OECD TGs 414, 421/422 and 443) under REACH*' (ECHA, 2022) or other considerations given on dose level setting (Hellsten *et al.,* 2023) or in line with standard regulatory testing guidelines and considering human exposure), the studies have limited or no value for hazard identification and the data may be considered inconclusive for classification. Also, improper dose-spacing, may lead to inconclusive data. Furthermore, in case of offspring exposure, lactational transfer and direct dosing need to be considered to ensure a continuous dosing period.

3.11.2.3. Evaluation of hazard information

Appropriate classification will always depend on an integrated assessment of all relevant available data using a WoE approach. This includes positive and negative data from all relevant sources of information, see Section 3.11.2.1. Datasets should be analysed using WoE and expert judgment and the combined, weighted outcome compared with the CLP criteria.

3.11.2.3.1. Evaluation of data on adverse effect(s)

Data on adverse effects are considered similarly to the respective sections of this guidance on carcinogenicity, reproductive toxicity and specific target organ toxicity –single and repeated exposure; see Sections 3.6.2.3, 3.7.2.3, 3.8.2.3 and 3.9.2.3. However, the dose thresholds (*i.e.* guidance values) provided in the STOT hazard classes do not apply to define adverse effect(s) in the context of the ED hazard class. Information on other toxicity shall also be considered in the assessment of adverse effect(s).

The OECD GD 150 provides guidance on how to interpret parameters normally investigated in toxicity studies; ECHA/EFSA ED Guidance. The OECD GD 150 differentiates between:

- '*EATS-mediated*' parameters measured *in vivo* that contribute to the evaluation of adversity, while at the same time (due to the nature of the effect and the existing knowledge, as described in OECD GD 150) they are also considered indicative of an EATS MoA and therefore (in the absence of other explanations) also infer an underlying *in vivo* mechanism. This group includes the parameters mainly labelled in OECD GD 150 as 'endpoints for estrogen-mediated activity', 'endpoints for androgen-mediated activity', 'endpoints for thyroid-related activity' and/or 'endpoints for steroidogenesis-related activity'. Examples of these parameters for human health are effects on uterine weight, disturbed estrous cyclicity, or increases in thyroid gland weight, or changes in histopathology of the follicular cells of the thyroid gland.

- '*Sensitive to, but not diagnostic of, EATS'* parameters measured *in vivo* contribute to the evaluation of adverse effect(s). Due to the nature of the effect and the existing knowledge, these effects cannot be considered diagnostic on their own of any of the EATS modalities. Nevertheless, in the absence of more diagnostic parameters, these effects can indicate an endocrine MoA and be relevant for classification, if they are accompanied with evidence of endocrine activity and the biologically plausible link between the endocrine activity and the observed adverse effect. Examples of these parameters are litter size and gestation length, or changes in spatial associative learning and memory, which alone cannot be considered to be endocrine mediated (*e.g.*, without supportive mechanistic evidence on endocrine activity and evidence of a biologically plausible link between the endocrine activity and the observed adverse effect(s)) .

All the parameters reported in OECD GD 150 are considered to be relevant to support ED-related adverse effects. They are mainly derived from guideline studies, *i.e.* standardised test methods validated for regulatory decision making (*e.g.*, EU test methods/OECD test guidelines or United States Environmental Protection Agency (US EPA)/Food and Drug Administration (FDA) test guidelines).

In addition to results from guideline studies, results from well-performed and reported studies other than those listed in OECD GD 150 may also include '*EATS-mediated*', '*Sensitive to, but not diagnostic of, EATS*' or '*non-EATS*' parameters which may provide relevant information. Therefore, the data used to classify a substance can be drawn from standard studies or other scientific data, *e.g.*, peer reviewed literature studies, Q(SAR) data, internationally recognised databases etc. When evaluating human data, confounding factors should be carefully considered. All relevant data needs to be evaluated carefully in a WoE approach (3.11.2.4.3).

In studies involving routes of administration such as intravenous or intraperitoneal injection, there will be no first pass metabolism of the substance, and also absorption and distribution may be different. Due to possible differences in toxicokinetic, in particular rate of metabolisation and occurrence of metabolites with or without endocrine activity, this may result in different effects observed in such studies compared to studies using oral

exposure. This must be considered when such studies are evaluated and interpreted. Some ED related effects may occur following exposure via breast milk. Also these effects are relevant for ED classification. In addition, if the substance (or its metabolites) are present in breast milk in amounts sufficient to cause concern for the breastfed child, they should be classified for effects on or via lactation.

In case NAMs provide data with equivalent predictive capacity as animal or human data, they can be used to provide sufficient data for adverse effect(s) for classification.

Furthermore, read-across or analogy can also be used to provide information about adversity, *e.g.* if the substances share a common MoA or induce similar adverse effects. When using data from another substance, potential differences in toxicokinetics and toxicodynamics should be considered.

For further details see ECHA/EFSA ED Guidance, tables 13 and 14 which show the assignment of '*EATS-mediated*' parameters; and '*sensitive to, but not diagnostic of, EATS*' parameters from the most common test guidelines, see also Table B.1 in OECD GD 150.

3.11.2.3.2. Evaluation of data on endocrine activity

In terms of endocrine activity, the OECD GD 150 differentiates between:

- *In vitro* mechanistic – parameters measured *in vitro* that provide information on the mechanism through which a substance could be considered endocrine active, *e.g.* by binding to and activating a receptor or interfering with specific enzymes in endocrine pathways.

- *In vivo* mechanistic – parameters measured *in vivo* that provide information on endocrine activity that are usually not considered adverse *per se, e.g.* changes in sex hormone levels are generally considered *in vivo* mechanistic. However, there can be cases where changes in hormone levels may be used as indicators of adversity, e.g. in a case of thyroid hormones.

  As described in Section 3.11.2.3.1 above, '*EATS-mediated*' parameters, are also considered indicative of an EATS MoA and thus (in the absence of other explanations) also infer an underlying *in vivo* mechanism.

- *In silico* approaches as described in Section 3.11.2.3.2.2, also inform on endocrine activity. The applicability domain of the models should be considered.

3.11.2.3.2.1. *In vitro* data

For EAS modalities, there are currently OECD validated *in vitro* tests such as estrogen and androgen receptor transactivation assays and steroidogenesis assays available. These studies provide information on, for example, interaction with a receptor or enzyme. The OECD GD 150 (see CF level 2 studies) describes more in detail the purpose of these *in vitro* assays and their limitations. Also studies from open literature may provide useful information on endocrine activity.

In vitro tests, when used in isolation, lack the complexity of an intact organism. Single assays often identify if a substance is capable of binding to a receptor or interfering with a pathway. Particular attention should be applied to *in vitro* data and the consideration of absorption, distribution, metabolism, excretion (ADME) properties which may not be covered by current *in vitro* test guidelines *e.g.*, those measuring protein binding or disruption of endocrine pathways.

Therefore, when interpreting the results of *in vitro* tests, the possible lack of a metabolising capacity or competence of the system, as well as the possible lack of consideration of other

ADME properties, should be considered. To partly overcome this limitation, metabolism may be addressed when (part of the) metabolising systems are added to the test system, or test data on metabolites of the substance could be directly used. Results from a battery of tests for substances that are not metabolised may in some cases be conclusive on endocrine activity, *e.g.* ToxCast ER model (see below).

Similarly, data may be conclusive if both the parent substance and the metabolites are covered. Therefore, all mechanistic information should be considered together to reach a conclusion on endocrine activity.

Most of the current available *in vitro* assays focus on specific interactions of substances with cellular components, such as nuclear hormone receptors or enzymes in specific pathways such as aromatase. However, not all endocrine-related adverse effects are mediated through a direct action on these molecules. Additionally, compounds might be able to act via more than one mechanism, and some of the pathways, which might be potentially causing an ED adverse effect *in vivo*, might not be covered by the currently available *in vitro* assays. Overall, no single test can be expected to detect all types of endocrine activity. To partly overcome this limitation, several *in vitro* tests investigating different points of perturbation or endocrine pathways can be assessed together. However, the eventual ED effect *in vivo* might be a consequence of disturbance of several pathways simultaneously, some of which might not be covered by available *in vitro* tests.

The capacity of organisms to compensate for a certain level of changes in hormonal regulation may not yet be possible to assess in an *in vitro* system. Further, the applicability domain, as well as overall validity and reliability of *in vitro* tests shall be considered. A negative single *in vitro* result alone cannot be used to exclude endocrine activity.

Because of the inherent limitations of *in vitro* systems such as those highlighted above, conclusions on the endocrine activity of the substance can only be drawn in the context of what the respective *in vitro* assays were developed to evaluate; e.g., receptor binding, enzyme inhibition. Due to limitations of *in vitro* systems, interpretation of results must be carefully considered (in a similar manner as limitations from *in vivo* systems are considered).

Special consideration of the ToxCast ER Bioactivity Model

The output data from the ToxCast ER Bioactivity Model, which builds on a number of in vitro assays, has equivalent predictive capacity as the '*Uterotrophic bioassay in rodents*' (OECD TG 440; OECD, 2007) for substances with no or low metabolising potential; *i.e.*, both methods can detect substances that are estrogen agonists and antagonists *in vivo*.

The ToxCast ER bioassay lacks metabolic capacity; therefore, if the prediction is in conflict with higher tier *in vivo* data, then this *in vivo* data has higher weight, especially data from Level 4 and 5 OECD CF studies. However, several adaptations to consider Phase I metabolism capability are under development and have been applied to over 700 ToxCast substances (Hopperstad *et al.*, 2022). The applicability domain should be considered; see further information on the ToxCast ER Bioactivity model in Browne *et al.*, 2015 and 2017.

3.11.2.3.2.2. In silico data

In silico predictions may be used as supporting information for endocrine modalities within a WoE approach. The different types of *in silico* prediction methods can be grouped as: molecular modelling of receptor interactions, (Q)SAR modelling and other events, profilers based on structural alerts and decision trees; for further details see ECHA/EFSA ED Guidance, Section 4. QSAR predictions may also support read-across.

The evidence from *in silico* predictions is strengthened if the same result is obtained with

independent *in silico* models. Whenever *in silico* methods are used, the general provisions outlined in ECHA Guidance on IRs & CSA, Chapter R.6: QSARs and grouping of chemicals (ECHA, 2008) and *'(Q)SAR Assessment Framework'* (OECD, 2023) should be followed. Attention should be paid to the interpretation of results, for understanding the prediction for each endocrine pathway and for taking into account the performance and the applicability domain of each *in silico* predictive model when drawing conclusions.

3.11.2.3.2.3. *In vivo* data

In vivo studies also provide information on endocrine activity. The '*EATS-mediated*' adverse effects infer an underlying *in vivo* mechanism that should be used for the identification of the endocrine activity; see Section 3.11.2.3.1. The OECD GD 150 also lists assays providing *in vivo* mechanistic information, such as the Uterotrophic (OECD TG 440; OECD, 2007) and Hershberger assays (OECD TG 441; OECD, 2009). Also the *in vivo* mechanistic data have some limitations, and the applicability domain should be carefully assessed. For further details, see ECHA/EFSA ED Guidance.

3.11.2.3.3. Mode of action analysis and evaluation of biologically plausible link

> **CLP, Annex I, Section *3.11.1.1. (e)*** "biologically plausible link" means the correlation between an endocrine activity and an adverse effect, based on biological processes, where the correlation is consistent with existing scientific knowledge.

Guidance on how to postulate and conclude on MoA(s), assess the biological plausibility of a link between endocrine activity and adverse effects as well as to identify which further information could help to clarify the postulated MoA(s) is provided in Section 3.5 of the ECHA/EFSA ED Guidance.

When potential endocrine-related adverse effect(s) and endocrine activity are identified, the link between the two, according to the CLP ED criteria, shall be established and justified based on biological plausibility. To conclude on the biological plausibility of the link, it may not be necessary to have demonstrated the whole sequence of events leading to the adverse effect. Existing knowledge from, *e.g.*, endocrinology and/or toxicology, may be sufficient to conclude on the biological plausibility of the link between adverse effects and the endocrine activity.

Biological plausibility may be demonstrated by conducting a MoA analysis, which shall be determined in the light of current scientific knowledge using all available relevant information in a WoE approach. For classification purposes, knowledge and demonstration of the full MoA is not a requirement. The MoA analysis should aim at establishing biological plausibility based on the consistency and coherence of the responses obtained on measured parameters with a postulated MoA.

The level of information required for a MoA analysis varies depending on which parameters are adversely affected.

For example, '*EATS-mediated*' adversity is considered indicative of an EATS MoA and, thus, also infers an underlying *in vivo* mechanism (in the absence of other explanations). In such cases, the analysis of the biological plausibility may draw conclusions from the broader scientific knowledge. Therefore, less information would be required for a MoA analysis and without recourse to a detailed MoA analysis compared to adversity based on other parameters, *i.e.,* the MoA analysis can be very simple. This is because there is a biologically plausible link between the adverse effect and endocrine activity in an EATS modality which is the most likely explanation of the effects observed. Therefore, in the absence of other explanations, *i.e.* an alternative MoA considered as a more likely explanation, an ED MoA can be considered plausible.

This is in contrast to adversity based on '*sensitive to but not diagnostic of EATS*' and '*non-EATS mediated*' parameters were more evidence is needed to support the KEs in the postulated MoA. In this case, the conclusion will depend on the degree of support provided by the empirical evidence for the KEs in the postulated MoA.

As in all assessments, a consistent pattern of effects strengthens the empirical support for KEs of the postulated MoA. The final WoE conclusion shall consider all available data.

Mode of action analysis

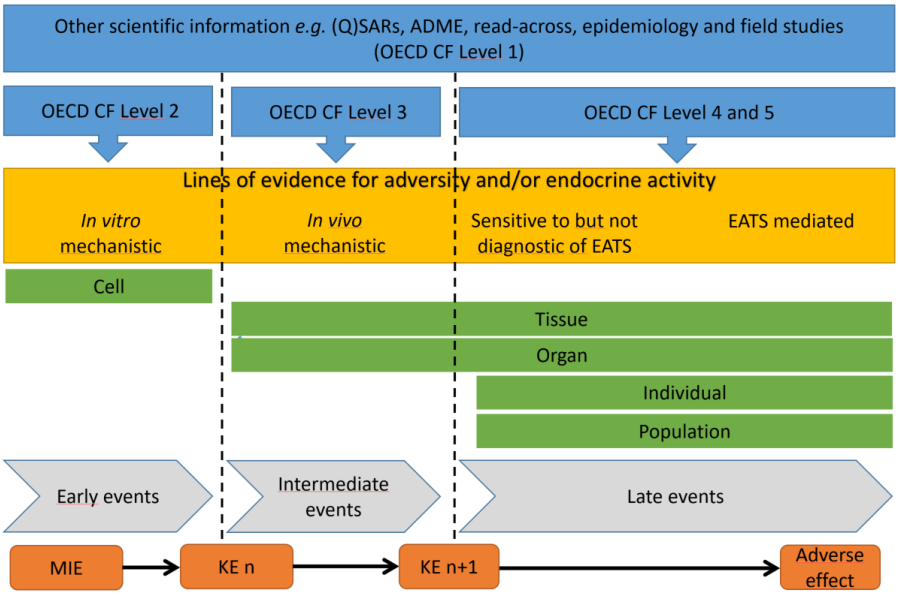
A MoA can be described as a series of biological events, *i.e.*, key events (KEs) that lead to a specific adverse effect. The first KE in the series is referred to as the molecular initiating event (MIE), see Figure 3-11.1.

This guidance uses AOP terminology for the MoA analysis. However, this does not imply that the AOP approach must be used for the MoA analysis.

An endocrine MoA means that the adverse effect is mediated through an alteration of one or more functions of the endocrine system, *e.g.*, hormonal synthesis, transport, signalling, regulation or metabolism, *i.e.*, it is not only mediated via hormone-receptor interactions. Normally, an endocrine MoA contains some earlier KEs (which provide mechanistic information at the molecular or cellular level concerning endocrine activity) and some later KEs (which provide information at the organ or system level, including the adverse effect).

This sequence at least includes one endocrine-mediated KE which may or may not also be adverse (see ECHA/EFSA ED Guidance); *i.e.* the MIE does not need to be known or endocrine related. KEs are those events that are considered essential to the induction of the toxicological response as outlined in the postulated MoA. KEs are empirically observable and measurable steps and can be placed at different levels of biological organisation (at cell, tissue, organ, and individual or population level); see Figure 3.11-1. To support the plausibility of a KE, there needs to be experimental data in which the event is characterised and consistently measured or existing knowledge on which basis the event is understood. KEs are connected to one another, and this linkage is termed a key event relationship (KER).

Figure 3.11-1 Scheme illustrating how the evidence can be organised to support the postulated mode of action. The arrows linking KEs represent the KE relationships. It should be noted that the borders between the different OECD CF levels are not absolute in terms of parameters measured and in their contribution to the weight of evidence.



773

774 KE: key event; MIE: molecular initiating event.

CLP, Annex I, Section *3.11.2.3.3.* Using a weight of evidence determination, the link between the endocrine activity and the adverse effects shall be established based on biological plausibility, which shall be determined in light of available scientific knowledge. The biologically plausible link does not need to be demonstrated with substance specific data.

775 *Evaluation of the biological plausible link between an endocrine activity and an adverse*
776 *effect*

777 The first step in assessing biological plausibility is to gather information from scientific
778 literature / existing knowledge on possible endocrine-related MoAs that are related to the
779 types of adverse effects and endocrine activity observed for the substance or related
780 substances subject to classification; see Section 3.11.2.1. The evidence available for the
781 substance subject to classification shall be assessed against the hypothesis for MoA with
782 its KEs to be able to conclude on a biological plausible link between the observed endocrine
783 activity and adverse effect(s).

784 The conclusion on biological plausibility is based on whether or not the KER is consistent
785 with the general knowledge of biology and what is known about the substance. The
786 analysis of the biological plausibility for the KER refers only to the broader knowledge of
787 the biology, physiology, endocrinology and toxicology involved. In a postulated MoA, the
788 KERs need to be consistent with the current understanding of biology, physiology,
789 endocrinology and toxicology.

790 Existing adverse outcome pathways (AOPs) and modes-of-action can be used as a starting
791 point for the postulated MoA against which the evidence can be systematically organised.
792 Evidence on adverse effect(s) and endocrine activity, assessed for dose and temporal
793 concordance, can provide empirical support to the KEs.

Several adverse outcome pathways related to endocrine disruption have been established and endorsed, *e.g.*, OECD Series on AOPs[6]. There are also numerous AOPs under development in the AOPwiki[7], or published in the literature. The amount of empirical support needed to establish the KERs varies depending on how well developed the AOP in question is. In cases where the MoA is based on a robust[8] or an OECD endorsed AOP, the biological plausibility of the KERs does not need to be demonstrated with experimental data. However, existing data on adversity and endocrine activity should be used to provide the empirical support needed to establish that the postulated MoA is plausible. Lack of a robust or OECD endorsed AOP should not be considered negatively in cases where there is convincing evidence for a biologically plausible link between observed endocrine activity and adversity.

The assessment should, when possible, include consideration of the modified Bradford Hill criteria, *i.e.*, essentiality, dose/incidence and temporal concordance, specificity, consistency, analogy; see further definition in Table 3.11-1. In particular, dose/incidence and temporal concordance are valuable to support or disprove the plausibility of the KERs and should always be assessed. For example, a MIE should occur below or at doses/concentrations where a downstream KE or an adverse outcome is observed. Similarly, early KEs should occur before or at the same time as the adverse outcome. However, since substance specific information on all the Bradford Hill criteria is only very rarely available, the absence of evidence to demonstrate these individual factors should not be used to exclude classification as an ED if the overall picture supports a plausible link to an ED MoA.

It is recognised that there may be cases where the biological relationship between two KEs may be very well established:

- When adverse effects are '*EATS-mediated*'. These parameters provide evidence for adversity, while at the same time (due to the nature of the effect and existing knowledge as described in the OECD GD 150) they are also considered indicative of an EATS MoA and thus (in the absence of other explanations) also infer an underlying *in vivo* mechanism. Where both data on adversity and endocrine activity are provided by the same study, it may be possible to reach a conclusion on the biological plausibility of the link without recourse to a detailed MoA analysis.

- When the MoA analysis is based on a robust or OECD endorsed AOP. In this situation, the biological plausibility is provided by the documentation for the KERs in the AOP used, *e.g.*, OECD Series on AOP No. 13 links inhibition of thyroperoxidase to adverse neurodevelopmental outcomes in mammals (Crofton *et al.*, 2019).

However, for adverse effect(s) based on '*sensitive to, but not diagnostic of, EATS*', the evidence that the adverse effects are caused by an endocrine MoA is not as strong as for adversity based on '*EATS-mediated*' parameters. Therefore, the postulated MoA and its biological plausibility would need to be supported by a more detailed MoA analysis. For example, a decrease in the female fertility index could be considered caused by an endocrine MoA if it were supported by data consistent with AOP: 345[9] in the AOPwiki, *e.g.* evidence of the substance affecting one or more of the MIEs/KEs involved, including inhibition of AR (MIE), decreased AR activation (KE) or reduced granulosa cell proliferation (KE), which would ultimately lead to a decrease in the female fertility index (AO).

Similarly, for adverse effect(s) based on *'non-EATS modalities'* (*i.e.*, adversity resulting

[8] Robust in this context means AOPs that have a broad acceptance in scientific literature.
[9] AOP: 345 - Androgen receptor (AR) antagonism leading to decreased fertility in females; https://aopwiki.org/aops/345

from impairment of endocrine modalities other than E, A, T or S), the evidence that the adverse effect(s) are caused by an endocrine MoA needs to be substantiated with a more extensive MoA analysis than for '*EATS-mediated*' adverse effects; unless the biological plausible link is based on existing scientific knowledge, *e.g.* a robust or OECD endorsed AOP.

A substance may have one or more MoAs, which can be endocrine or non-endocrine. The potential of a substance to elicit more than one MoA can obviously lead to difficulties in concluding on the biological plausibility. If there are indications that a substance may act via multiple MoAs, then the evaluation should first focus on the MoA for which the most convincing evidence is available. The number of potential MoAs to be considered will vary on a case-by-case basis.

Furthermore, there may be more than one MoA which could cause similar effects; hence, it may be necessary to undertake an analysis for more than one postulated MoA for a particular adverse effect. There may be also situations where a pattern, which includes '*EATS-mediated*' adverse effects, has been identified. However, due to the complexity and cross-talk within the endocrine system it may not be possible to identify the specific modality.

In such cases, a biological plausible link should be considered as established for an '*EATS-mediated*' MoA and classification as Category 1 or 2 may be warranted depending on the strength of evidence.

Comparative MoA analysis

To consider an ED-related adverse effect as a specific consequence of another non-endocrine MoA*,* there must be evidence for a biologically plausible sequence of events which excludes an endocrine MoA as a likely explanation for the observed adverse effect(s). To demonstrate this, MoA data is needed on the alternative MoA and the assessment is best done by a comparative MoA assessment. It should be noted that it may be difficult to demonstrate that the effects are solely non-endocrine related because standard studies generally do not provide mechanistic information and thus, further mechanistic studies may be needed. An additional complication is that, substances may have more than one MoA, including an ED MoA. In this situation, the ED MoA should be considered for classification. For further guidance on how to conduct a comparative MoA analysis, see ECHA/EFSA ED Guidance.

Table 3.11-1. Explanations of the terms: analogy, essentiality, consistency, dose and incidence concordance, mode of action, specificity and temporal concordance.

Term	Explanation
Analogy	A consistent observation across (related) substances having a well-defined MoA.
Essentiality	Essentiality is one of the elements that should be considered (when data are available) when performing the WoE analysis using the Bradford Hill considerations. In the context of the MoA/AOP frameworks, essentiality refers to key events. For determining essentiality it should be demonstrated whether or not downstream KEs and/or the adverse effect is prevented/decreased if an upstream event is experimentally blocked. It is generally assessed on the basis of direct experimental evidence of the absence/reduction of downstream KEs when an upstream KE is blocked or diminished (*e.g.*, in null animal models or reversibility studies).
Consistency	Consistency is the pattern of effects across species/organs/test systems that are expected based on the postulated MoA/AOP. In developing a MoA,

	consistency also refers to the repeatability of the KEs in the postulated MoA in different studies. Consistent observation of the same KE(s) in a number of studies with different study designs increases the support.
Dose and incidence concordance	Dose and incidence concordance are elements valuable for the evaluation of the empirical support. In a MoA/AOP context, dose and incidence concordance are verified when the key events are observed at doses or incidences below or similar to those associated with the adverse effect (or key events downstream).
Mode of Action	A biologically plausible sequence of key events at different levels of biological organisation, starting with the exposure to a substance and leading to an observed (adverse) effect.
Specificity	Specificity should be understood as the extent to which the MoA for the adverse effect is likely to be endocrine-related, *i.e.* whether an adverse effect is a consequence of the hypothesised endocrine MoA, and not a result of other non-endocrine MoA, including a result of excessive other toxicity.
Temporal concordance	Temporal concordance increases the empirical support of the biologically plausible link. This is done by evaluating whether key events within the MoA are observed in the hypothesised order.

Assessment of human relevance

CLP, Annex I, Section *3.11.1.2.1.* *Substances and mixtures fulfilling the criteria of endocrine disruptors for human health based on evidence referred to in Table 3.11.1 shall be considered to be known, presumed or suspected endocrine disruptors for human health unless there is evidence conclusively demonstrating that the adverse effects are not relevant to humans.*

It is by default assumed that effects observed in mammalian studies are relevant to humans. The guidance provided by the WHO/IPCS MoA and human relevancy frameworks (WHO/IPCS, 2007) may help in assessing the potential non-relevance to humans. Where it is known that the adverse effects or the MoA are not relevant for humans or is of doubtful relevance to humans, this should be clearly justified. For example, non-tumour thyroid effects observed towards the end of a lifetime study, but not in sub-chronic study, may need to be considered with caution due to possible differences between ageing humans and animals.

Only if a MoA of an endocrine effect is conclusively determined not to be operative in humans may the evidence for that effect be discounted. This requires that it is conclusively demonstrated that only a human non-relevant mechanism can be attributed to the observed effects; and that other human-relevant mechanisms can be excluded. This usually requires additional experimental studies. All available data must be considered to conclude if the endocrine effects are solely induced by a mechanism which has non-relevance for humans. Consequently, the burden of proof is high to substantiate non-relevance to humans. However, where there is information that raises serious doubt about the relevance of the adverse effects to humans, classification in Category 2 may be more appropriate.

3.11.2.3.4. Weight of evidence and expert judgement

According to the ED criteria, WoE and expert judgement must be applied when concluding on the ED criteria (Article 9(3) in conjunction with CLP, Annex I, Sections 1.1.1. and 3.11.2.3.); see guidance on WoE in Section 1.4 of this guidance.

Commented [A1]: Links to other parts of the CLP Guidance to be added

CLP, Annex I, Section *3.11.2.3.1.* *Classification as an endocrine disruptor for human health is*

made on the basis of an assessment of the total weight of evidence using expert judgment (see Section 1.1.1). This means that all available information that bears on the determination of endocrine disruption for human health is considered together, such as:

(a) in vivo studies or other studies (e.g., in vitro, in silico studies) predictive of adverse effects, endocrine activity or biologically plausible link in humans or animals;
(b) data from analogue substances using structure-activity relationships (SAR);
(c) evaluation of substances chemically related to the substance under study may also be included (grouping, read-across), particularly when information on the substance is scarce;
(d) any additional relevant and acceptable scientific data.

A WoE determination means that all available relevant information bearing on the determination of hazard is considered together, including:

(a) human data such as occupational data and data from accident databases, epidemiological and clinical studies and well-documented case reports and observations; relevant animal data such as repeat dose toxicity studies, carcinogenicity studies and reproductive toxicity studies; the results of suitable *in vitro* tests; and relevant *in silico* predictions; these include also relevant peer-reviewed published studies;

(b) (Q)SARs;

(c) information from the application of the Category approach (grouping, read-across); and

(d) any additional acceptable data, for example, information used for the evaluation of the substance as an ED for the environment, including studies in fish, amphibians and birds; physico-chemical or toxicokinetic parameters and information on known metabolites should be considered where relevant.

Formation of a metabolite in mammals with endocrine activity or adversity indicates that exposure to the substance might result in endocrine-related adverse effects. Therefore, endocrine activity or adversity observed with the metabolite shall be considered in the classification of the parent substance. If data are available, quantity and stability of the metabolite(s) formed should be taken into account (*e.g.* if the metabolite is stable for a period long enough to exhibit toxicological properties or if it is an intermediate which is rapidly changed to other metabolites). Even if a substance has been tested as negative for ED it may in certain instances be classified in Category 1 or 2 based on the formation of metabolites with ED properties. If a metabolite is formed in any mammalian species, it should be assumed by default that this metabolite is also formed in humans unless demonstrated otherwise.

CLP, Annex I, Section *3.11.2.3.2.* *In applying the weight of evidence determination and expert judgment, the assessment of the scientific evidence referred to in Section 3.11.2.3.1 shall, in particular, consider all of the following factors:*

(a) *both positive and negative results;*

(b) *the relevance of the study designs for the assessment of adverse effects and of the endocrine activity;*

(c) *the quality and consistency of the data, considering the pattern and coherence of the results within and between studies of a similar design and across different species;*

(d) *the route of exposure, toxicokinetic and metabolism studies;*

(e) *the concept of the limit dose (concentration), and international guidelines on maximum recommended doses (concentrations) and for assessing confounding effects of excessive toxicity.*

The WoE approach for identifying EDs should involve transparently evaluating and

considering together all available data based on factors such as relevance, quality, and consistency; see CLP, Annex I, 1.1.1.3.

Substances can potentially induce endocrine disruption by any route of exposure, but endocrine disruption potential may depend on the conditions of exposure; *e.g.*, route, level, pattern, and duration of exposure; age at the time of exposure. The quality and consistency of the data should be given appropriate weight. Both positive and negative results should be assembled in a single WoE determination, separated for endocrine activity and adversity; see CLP, Annex I, 1.1.1.3 and Section 1.4 of this Guidance. However, negative human data should not normally overrule positive results from animal or *in vitro* studies unless there is *e.g.* a clear mechanistic reason why human data is negative due to species differences.

Commented [A2]: Links to other parts of the CLP Guidance to be added

Although the quality / reliability, validity and applicability domain of a study per se affects the weight given to the study, there are also several other, "external" factors that may influence the WoE assessment, as mentioned above in the green boxes. Information on, *e.g.* toxicokinetics (*e.g.*, saturation, sex differences, accumulation in tissues, information on major metabolites), the route of exposure, physicochemical properties (*e.g.*, vapour pressure, solubility and unspecific binding in *in vitro* test systems), read-across/analogy and availability of substance specific data may have influence on how much weight each piece of information can be given. In general, substance specific information is given more weight than other data, unless there are reasons not to do so. For example, read-across or analogy can sometimes provide stronger evidence for classification than the substance specific data.

The assessment must weigh all the evidence, and be performed on a case-by-case basis using expert judgement. A single positive study can however be sufficient for classification.

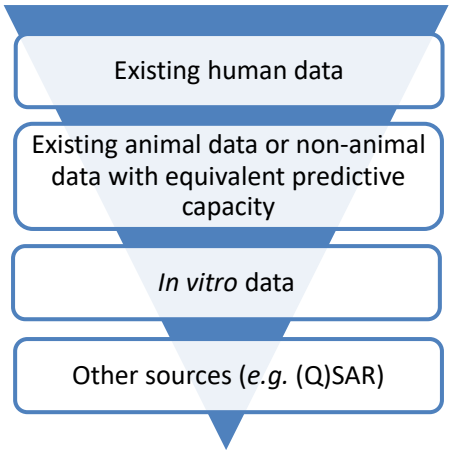
> **CLP, Annex I, Section *1.1.1.4.*** *"Generally, adequate, reliable and representative data on humans […] shall have precedence over other data. However, even well-designed and conducted epidemiological studies may lack a sufficient number of subjects to detect relatively rare but still significant effects, to assess potentially confounding factors. Therefore, positive results from well-conducted animal studies are not necessarily negated by the lack of positive human experience but require an assessment of the robustness, quality and statistical power of both the human and animal data.*

> **CLP, Annex I, Section 3.11.2.2.1.** *Classification shall be made on the basis of the criteria outlined above, and a weight of evidence determination of each of the criteria (see Section 3.11.2.3) and an overall weight of evidence determination (see Section 1.1.1).*

WoE for endocrine disruption must be conducted independently for adverse effect(s) and for endocrine activity. Thereafter, the overall WoE for all these elements together must be conducted in the MoA analysis, also including the conclusion on the biologically plausible link.

Figure 3.11-2 provides an illustration of the relative weight of different types of data. In the case of conflicting results, a decision on the weight to be assigned to the different types of data has to be made. It needs to be noted that the relative weights indicated in Figure 3.11-2 assume comparable quality of the data. WoE considerations need to take into account, on a case-by-case basis, the quality, consistency, nature, severity, relevance and applicability domain of the different types of data available. The figure illustrates a decreasing weight of the information from top to bottom.

Figure 3.11-2 Simplified illustration of the relative weight of the available information with similar or comparable quality



When contradicting data of comparable quality and predictive capacities assessing similar endpoints belongs to different "hierarchical levels", the following considerations should be made:

- When there are relevant positive data which belong to a higher level in the hierarchy than the available negative data, more weight should normally be given to the positive data.

- When the negative data belong to a higher level in the hierarchy than the positive data, more weight should normally be given to the negative data, and a careful evaluation of the reasoning should be conducted considering differences in dose/concentration levels used, species differences, differences in the quality and reliability of data etc. However, as specified earlier, existing good quality positive animal data would normally overrule negative human data. Furthermore, there may be cases where the mechanism investigated at the lower level of the hierarchy (*e.g.*, *in vitro*) is not covered by the investigations at the higher level of the hierarchy (*e.g.*, *in vivo*), or *e.g.* there may be lack of sensitivity in a well conducted *in vivo* study. In such cases negative data at the higher level should not be given higher weight than the positive data at the lower level of the hierarchy.

- Taking inter-species differences into account, results from both human data and *in vitro* data could overrule animal data, assuming that a scientifically justified explanation can be provided and also assuming the same level of quality.

- In all of the above cases, it is important to assess the full data set and a scientifically justified explanation should be provided. In general, positive results that are relevant for classification should not be overruled by negative findings without a scientifically sound and transparent explanation based on the analysis of biological plausibility. All existing evidence should be systematically organised against existing adverse outcome pathways or known modes of action.

3.11.2.3.5. Use of ecotoxicity data when assessing classification as endocrine disruptor for human health

CLP; Annex I, Section *3.11.2.3.4.* *Using a weight of evidence determination, evidence considered for the classification of a substance as an endocrine disruptor for the environment referred to in Section 4.2 shall be considered when assessing the classification of the substance as an endocrine disruptor for human health under Section 3.11.*

Because of the high level of conservation of the endocrine system across taxonomic groups, the non-mammalian data may also be relevant for mammalian toxicity (OECD GD 150), and can be used to support the conclusion on classification for human health. Negative environmental data cannot be used alone as an argument for non-classification on human health. The OECD GD 150 states that: "*Cross-species extrapolations should be considered during data assessment. Endocrine systems with respect to hormone structure, receptors, synthesis pathways, hormonal axes and degradation pathways are well conserved across vertebrate taxa especially in the case of estrogen, androgen and thyroid hormones and steroidogenesis.*"

Furthermore, the EFSA/ECHA ED Guidance specifies that the same database can be used to conclude on the ED properties for human health and the environment: "*The information needed to assess ED properties for humans and non-target organisms may overlap. Mammalian data are always relevant for ED assessment on non-target organisms. Furthermore, there may be information on non-target organisms that could be relevant also for the ED assessment for humans.*" and *"[...] it is recommended to strive for a conclusion on the ED properties with regard to humans and in parallel, using the same database, to strive for a conclusion on mammals as non-target organisms."*

Current advances within development of AOP networks demonstrate that some molecular initiating events and key events are linked to a broad range of adverse outcomes in different species across toxicology and ecotoxicology (for EDs typically rodents, fish and amphibians). By use of well developed AOP networks, cross-species information could be utilised in the evaluation of human health related endocrine disruption to a much higher degree than previously done; *e.g.*, Haigis *et al*, 2023 and Figure 3.11-3 on the AOP network for thyroid effects.

An example of cross-species consideration is a fish study, where a reduction of vitellogenin in females and a decrease of fecundity were recorded. *In vitro* data has demonstrated that the substance is an androgen receptor agonist. The OECD AOP No. 9 (Villeneuve, 2018) outlines a MoA which starts with agonism with androgen receptor, that leads to a reduction of gonadotropins and a decrease of testosterone synthesis, followed by a reduction of 17beta-estradiol synthesis and VTG synthesis, which will impact fish fecundity and spawning. It is known that an androgen receptor agonist can also affect mammals, and thus this can be used as supportive evidence for HH classification, together with some mammalian data.

Any evidence on endocrine MoA in non-mammalian species can be supportive in HH classification. However, the OECD GD 150 also specifies that *"Caution should be exercised, however, when extrapolating in this way, as species differences in exposure pathways, ADME, organ physiology, effects of hormones at different life stages across taxa/classes and other differences should be considered. The consequences of the action of a hormone may be different in different species, even if the molecular initiating event is the same."*

3.11.2.4. Decision on classification

Substances are classified as EDs for human health in Category 1 or 2 when there is sufficient evidence that the three elements (a) endocrine activity, (b) adverse effect and

(c) biological plausible link indicated in CLP, Annex I, Table 3.11.1 (for details see Section 3.11.2.2 of this guidance) are met. If one of the three elements is not met, classification of the substance is not warranted.

The allocation of the substance to Category 1 or 2 or no classification depends on the strength and consistency of the available evidence, *i.e.*, on how convincing the evidence for criteria (a) and (b) is, and whether a plausible link between the two can be established. Allocation to Category 1 is warranted when the evidence for adverse effect(s) and endocrine activity is sufficiently convincing considering all available relevant data in the WoE on the substance. Sufficiently convincing evidence for Category 1 may be based on appropriate and robust read-across, grouping or analogy, when the read-across is sufficiently justified for that particular substance. Also, evidence on a certain pattern of adverse effect(s) observed, which is generally known to be linked to a certain type of endocrine activity, *i.e.,* '*EATS-mediated*', can lead to Category 1 classification.

If there are no human data, then the classification is based on other data. However, negative human data do not normally overrule positive good quality non-human data. Human data are often flawed by too low number of individuals investigated, inadequate exposure assessment, co-exposures and more. Care must be exercised in evaluating the data, in particular the exposure levels in the study, in case it renders the outcome of the human data inconclusive. If human and non-human data both indicate no classification then classification is not required.

When the evidence for either adverse effect(s) or endocrine activity or both is not sufficiently convincing to place the substance in Category 1, then Category 2 or no classification may be warranted. This may be caused by issues related to reliability, dosing/concentration settings, parameters covered, life-stage investigated or exposure duration, incidence of the effects, divergences between results in different studies if not explainable by differences in study designs (*i.e.* lack of consistency), inconsistent pattern of effect etc., or when chance, bias or confounding factors cannot be ruled out with reasonable confidence.

For example, if there are serious concerns regarding the study design or conduct, or the interpretation of existing information, or if there is insufficient information available to make a conclusion on Category 1, or if the adverse effect is considered to be not sufficiently convincing for Category 1 (*e.g.* if a broad range of relevant ED related endpoints are investigated in well-conducted reliable studies, and an ED related effect is observed with a low incidence), classification for Category 2 or no classification may be more appropriate. Evidence on essentiality, consistency, analogy, specificity as well as empirical support for dose-temporal concordance and/or information on lack of human relevance of the postulated MoA may affect the strength of evidence. In cases where two different MoAs, one endocrine and one non-endocrine could explain the same adverse effect, the WoE of both MoAs should be assessed in a comparative analysis, see Section 3.5 of the ECHA/EFSA ED Guidance. However, when the endocrine MoA is the most likely, even in presence of an alternative non-endocrine MoA, the ED MoA should be used for classification. See also examples 6, 7 and 8 in Section 3.11.5 below where data is not sufficiently convincing for Category 1 but the Category 2 criteria are met.

Regarding the reliability of studies, it should be noted that some parameters may be reliably investigated although the study may not be considered fully reliable as regards all parameters due to specific deficiencies which do not affect all the investigated /observed effects. Therefore, reliability should always be assessed with care, and the overall study reliability scores do not necessarily indicate how much weight can be given for a subset of investigations and results in the study in an overall WoE assessment. This applies for the assessment of all types of studies but particularly non-guideline and non-GLP studies.

Sufficient evidence for criterion (c) (a biological plausible link between endocrine activity

and the adverse effect) to classify a substance in Category 1 or Category 2 can be based on *e.g.*:

- understanding of the key event relationships (KER) based on broad acceptance, *e.g.* in scientific literature or in an endorsed Adverse Outcome Pathway (AOP); see OECD Series on AOPs[10], *i.e.* the postulated endocrine MoA and the KEs need to be consistent with the current understanding of physiology, endocrinology and toxicology by addressing structural and/or functional relationships between KEs.

- if the KER is plausible based on analogy with accepted biological relationships even when scientific understanding is not completely established.

- When there are dose and time concordance between early KEs and later KEs.

- existing knowledge on endocrinology / toxicology may be sufficient to assess the biological plausibility (*e.g.*, if the MoA is mainly established and empirically supported on the basis of EATS or other less explored endocrine function mediated parameters).

- when adverse effects are '*EATS-mediated'.* These parameters provide evidence for adversity, while at the same time (due to the nature of the effect and existing knowledge as described in the OECD GD 150) they are also considered indicative of an EATS MoA and thus (in the absence of other explanations) also infer an underlying *in vivo* mechanism. Because both data on adversity and endocrine activity are provided by the same study, it may be possible to reach a conclusion on the biological plausibility of the link without recourse to a detailed MoA analysis.

In general, EATS mediated adverse effects can directly trigger *ED HH 1*, whereas for adverse effects '*sensitive to, but not diagnostic of, EATS*' effects and '*non-EATS mediated*' adverse effects, an ED MoA must be demonstrated in more detail for a classification in *ED HH 1*. Such effects could also potentially lead to an *ED HH 2* (see parameters in Table 14 of ECHA/EFSA ED Guidance. The parameters described in Table 14 may not be considered sufficient in isolation for covering the element of adversity. In such cases, the conclusion on classification relies on a combination of parameters and the observation of a pattern of effects. The following scenarios can be identified:

If **adverse effect(s) are based on '*EATS-mediated parameter(s)*'**, the data provide evidence for adverse effect(s) in an intact organism or its offspring or future generations, endocrine activity and the biologically plausible link between the two, classification for *ED HH 1*; EUH380 is warranted even without specific mechanistic information or identification of the specific MoA. This is the case unless it is conclusively demonstrated that the adverse effect is non-relevant to human; or unless the adverse effect is a non-specific consequence of other toxicity or due to a non-ED MoA. Consideration should be given to the existence of a pattern of effect and a WoE assessment should always be conducted to put any adverse effects into context.

If **adverse effect(s) are based on '*Sensitive to, but not diagnostic of, EATS parameters'* or '*non-EATS mediated*' parameters,** there are several different scenarios that could lead to different classification outcomes for endocrine disruption.

These scenarios depend on:

[10] https://www.oecd-ilibrary.org/environment/oecd-series-on-adverse-outcome-pathways_2415170x

i. the strength of the evidence for the three elements in CLP Annex I: 3.11.2.1;

ii. whether '*EATS-mediated*' parameters (see more details in Section 3.11.2.3.1) have been extensively or partially investigated and found positive or negative and;

iii. the available information on whether other types of endocrine activity not already inferred from the '*EATS-mediated*' parameters is available; and

iv. the WoE.

Classification may also be warranted in cases when there is evidence that criteria indicated in CLP, Annex I, 3.11.2.1., *i.e.,* (a) endocrine activity, (b) adverse effect(s), (c) plausible link are met, even if there is not enough information to postulate which of the endocrine mode(s) of action mediate the adverse outcome due to the lack of thorough mechanistic information. This is for example the case when a pattern of adverse effects has been identified which, based on current knowledge, is concluded to be related to endocrine disruption (effects which are considered EATS mediated or '*sensitive to but not diagnostic of, EATS*' or '*non-EATS mediated*'), but due to the complexity and crosstalk of the endocrine system, it is difficult to identify the specific modality. In this situation, classification as *ED HH 1* or *ED HH 2* may be justified based on the strength of the evidence.

The substance should not be classified for example when:

- no adverse effect(s) are observed. This includes adaptive responses that are demonstrated not to be toxicologically relevant, i.e. not adverse *per se* or not leading to adverse effects; or
- no endocrine activity is observed or cannot be inferred from the adversity; or
- no biological plausible link can be established, *i.e.* adverse effects are observed which cannot be linked to the observed endocrine activity using existing knowledge; or
- adverse effect(s) are solely a non-specific consequence of other toxic effects (see Section 3.11.2.2.1.); *i.e.*, the adverse outcomes are consequences of excessive other toxicity; or
- when a non-endocrine MoA as a result of a comparative MoA analysis has been demonstrated to be the most likely explanation of observed adverse effect(s); or
- adverse effects with a biologically plausible link to endocrine activity are conclusively demonstrated not to be relevant for humans. It should be noted that such results obtained in rodent studies could still be relevant for classification as *ED ENV.*

A distinction may need to be made between whether the data are sufficient to conclude on classification for ED or whether some important data are lacking and therefore the outcome of "no classification" is due to lack of data for the modalities assessed.

To summarise, for Category 2, the situation may be also that Category 1 classification cannot be concluded due to lack of data but the currently available data better supports Category 2 classification.

Ultimately, a WoE approach and expert judgement is needed to decide on the appropriate Category.

3.11.2.4.1. Specific considerations regarding thyroid modality

3.11.2.4.1.1. Background information on thyroid disruption

The thyroid hormones (THs) act on almost all cell types in the body. THs are essential for proper development and differentiation of the cells, and for maintaining metabolic balance and body temperature. THs and their regulation through the hypothalamic–pituitary–thyroid axis (HPT axis) is conserved across evolution in vertebrates. The primary function of the thyroid is production of the iodine-containing hormones triiodothyronine (T3) and thyroxine (T4). The production of THs is primarily regulated by thyroid-stimulating hormone (TSH) released from the anterior pituitary gland. TSH release is in turn stimulated by the thyrotropin-releasing hormone (TRH) from the hypothalamus. The THs provide negative feedback to TSH and TRH: when the THs are high, TSH production is suppressed. Feedback mechanisms are also in place for the regulation of TRH production (Chiamolera and Wondisford, 2009; Hershman and Beck-Peccoz, 2023).

The regulation of serum TH levels and of TH action in various tissues involves a complex interplay of physiological processes which are targets of multiple MIEs which all can lead to the same adverse effects, see Figure 3.11-3. The thyroid function depends on iodine uptake, TH synthesis and storage in the thyroid gland, stimulated release of hormone into and transport through the circulation, hypothalamic and pituitary control of TH synthesis, cellular TH transport, tissue-specific TH de-iodination and degradation of THs by catabolic hepatic enzymes. Substances may interfere in any of these processes which in turn can adversely affect the thyroid function. Figure 3.11-3 is a high-level integration of AOPs into a network. It should be noted that all the thyroid modes of action depicted in the network share a common key event, *i.e.* altered tissue concentration (which is tissue-specific) of THs, which is not normally measured in toxicity studies. Some OECD TGs measure serum TH concentrations which does not directly translate to tissue levels. However, serum TH concentrations may be used as a proxy for tissue concentrations because ultimately changes in serum concentration will be reflected in the tissues since the thyroid target tissues lack the ability to synthesise THs. Proper tissue concentration of THs is crucial for proper tissue function, during all phases of life, but the consequences of improper tissue concentration differ depending on the life-stage exposed. It should be noted that even small changes in fetal thyroid hormone levels (*e.g.* due to decrease of maternal TH levels) may have an influence on adverse outcomes, particularly those related to developmental neurotoxicity. THs are essential for normal human brain development, both prenatally and postnatally, modulating genes critical for normal neuroanatomical development, with subsequent effects on neurophysiology, and finally neurological function (Bernal, 2007; Brosco *et al.*, 2006; Talhada *et al.*, 2019). In early pregnancy the foetus is fully dependent on maternal thyroid hormones; this makes the foetus in this life-stage particularly vulnerable to maternal thyroid disruption (Alemu *et al.*, 2016; Ramprasad *et al.*, 2012; Ghassabian *et al.*, 2011). Therefore, substances that interfere with TH synthesis, and thereby alter circulating TH levels, have the potential to cause TH insufficiency that may result in adverse neurodevelopmental effects in the developing foetus.

Disruption of thyroid function in the mother during pregnancy and in the first years of the child's life can later lead to neurodevelopmental impairments including low IQ scores in children (Päkkilä *et al.*, 2015; Korevaar *et al.*, 2018), cognitive and neurobehavioral defects (Hendrichs *et al.*, 2010), and hearing loss (Crofton, 2004). In adults, THs modulate physiological functions *e.g.*, for maintenance of cellular metabolism and cardiovascular functions (Yamakawa *et al.*, 2021; Mullur *et al.*, 2014).

3.11.2.4.2. Specific considerations regarding thyroid modality with respect to decision on classification

This Section provides additional considerations for the thyroid modality with respect to decision making on classification; all other Sections under 3.11. are still applicable for assessing ED classification based on thyroid modality.

Because of the conserved nature of TH physiology, substances affecting thyroid function or TH signalling in one species may well similarly affect other species, including humans (Haigis *et al.*, 2023; Tan and Zoeller, 2008). Even though there are some inter-species differences with regard to the TH physiology (Haigis *et al.*, 2023; Noyes *et al.*, 2019; Hoshi *et al.*, 2013; Thambirajah *et al.*, 2022) (see below), all thyroid toxicity related mechanisms in *e.g.*, rodents are considered relevant for humans, unless conclusively demonstrated not to be human relevant. More specifically, the HPT axis and the basic physiological processes regulating TH synthesis and release are qualitatively similar across species. However, there are some quantitative differences between species.

Discussions about the rat being an irrelevant model for humans as regards effects on thyroid hormone levels and associated adverse effects are largely based on interspecies differences in the half-lives of adult serum T4 as well as differences in thyroid carrier proteins, which may make rats particularly sensitive to thyroid disturbances. Autonomous regulation of thyroid hormone action at the tissue level, without involvement of the HPT axis, can play an important role, and can affect the organism even without corresponding changes in serum thyroid hormones.

In any case, lower (predicted) sensitivity of humans as compared to the animal model to any effect (*e.g.* due to quantitative differences in the dynamics of the system) does not equal to non-relevance of the effect in the animal model to humans.

According to the CLP, Annex I, Section 1.1.1.5 (that applies to all human health hazard classes) "*When there is scientific evidence that the mechanism or MoA is not relevant to humans, the substance or mixture should not be classified*". It is thus not sufficient to exclude the human relevance solely by (predicted) differences in sensitivities to effects unless these are so marked that it is certain that the hazardous property cannot be expressed in humans. Thus, to exclude human relevance, human irrelevance of adverse effects observed in animal studies should be demonstrated with substance-specific information.

OECD AOP No. 13 (Crofton *et al.*, 2019) and 14 (Rolaki *et al.*, 2019) may be used to establish a biologically plausible link between the evidence on endocrine associated DNT (impaired learning and memory) and thyroid system-associated endocrine activity. However, it should be noted that validated test methods for detecting the MIEs relating to the thyroid AOPs are currently lacking; for current validation status (Bernasconi et al., 2023). ToxCast 21 / EDSP and scientific literature contain studies which investigate some of the MIEs. Information on the MIE may provide, if available, information on endocrine activity. Given the number of potential MIEs, negative evidence for one or a few MIEs should not negate classification in case there is other evidence fulfilling the CLP criteria for ED for human health.

The evaluation of potential thyroid disruption may be hampered by the limited parameters tested in the available toxicity studies. For example, repeated dose toxicity studies may not investigate the potential MIEs or adverse outcomes manifested as *e.g.* developmental neurotoxicity or cardiovascular toxicity. However, studies such as OECD TG 408 (OECD, 2018b), OECD TG 421 (OECD, 2016), OECD TG 422 (OECD, 2015) and OECD TG 443 (OECD, 2018c) commonly provide information on thyroid weight and histopathology, serum THs and serum total cholesterol and LDL/HDL ratios.

Increased thyroid weight and thyroid follicular cell hypertrophy/hyperplasia are commonly observed in rodent toxicity studies. This may be considered as an indication of reduced serum THs. Reduced serum THs will, in turn, result in reduced tissue concentration of THs which may, depending on the magnitude and timing of the change, ultimately be manifested in adverse outcomes. Furthermore, reduced THs due to hepatic liver enzyme induction resulting in increased liver clearance is a relevant endocrine MoA because the liver metabolism of THs is part of the TH regulation and relevant for ED classification if affected by a substance.

Similarly, changes in the thyroid follicular cells in terms of hypertrophy, hyperplasia and/or a continuum through to thyroid neoplasm, may be interpreted as an indication of persistent TSH stimulation due to low levels of circulating THs (Crofton, 2004) unless there is evidence for another more likely explanation. Altered level of THs provides information about endocrine activity and contribute to the overall assessment pattern of adversity. Due to the complexity of the TH system, it is possible that only TH (T3/T4) level or TSH is altered, not both, and it can still lead to an adverse effect. Therefore, changes in TH levels may provide evidence for classification. However, lack of such effects cannot be used to negate clear evidence of adverse effects on the thyroid gland, *e.g.* adverse microscopic changes.

The production, clearance and transformation of cholesterol is regulated by THs, therefore elevated serum levels of total cholesterol, HDL/LDL ratio and triglycerides may be regarded as an indication of low serum THs together with other thyroid related endpoints (OECD GD 150; Liu and Peng, 2022; Shin and Osborne, 2003). Research shows that also increases in TSH affects lipid metabolism independently of THs (Liu and Peng, 2022). Consequently, hypothyroidism-related dyslipidemia is associated with a decrease of THs and an increase of TSH levels. Therefore, total cholesterol, LDL-cholesterol and triglycerides provide additional evidence that may support decreased THs at the tissue level which is independent and parallel to the effects on the thyroid gland.

Indications of thyroid disruption in adults should be considered as an indication that the same disruption is expected to occur also in earlier life-stages if exposed. For pragmatic reasons the following approach is proposed for classification.

(1) Classification as *ED HH 1*; EUH380 may be warranted *e.g.* when:

There is evidence that the observed pattern of thyroid-related effects lead to the overall conclusion that they constitute an adverse effect. Due to the most often investigated parameters, evidence on thyroid-related effects will normally consist of data on thyroid weight and histopathology since these are the most frequently investigated parameters. When adverse effects are observed on the thyroid gland, additional mechanistic information is not necessarily required to meet the CLP ED criteria. This is because effects on thyroid weight and histopathology, which are '*T-mediated*' parameters, provide intrinsic evidence of adverse effect(s) via endocrine activity.

Nevertheless, the evidence for endocrine activity may be further supported by alteration of specific parameters like reduced serum T4 or T3, increased TSH, increased total cholesterol or altered LDL/HDL ratio, and data on MIEs. In cases where the observed adverse effects could be also mediated via non-endocrine activity (such as DNT effects), information about endocrine activity is needed in addition to the evidence on adverse outcomes.

Ultimately, the differentiation between Category 1 and 2 depends on the strength of evidence. However, when there is information that raises serious doubt about the relevance of the adverse effects to humans, classification in Category 2 or no classification may be more appropriate. Additional mechanistic information, *e.g.*,

positive indications of an endocrine activity-associated MIE, may provide additional support to the classification. However, knowledge of the MIE is not needed for classification in cases where the effects defining adverse effect(s) for the thyroid are '*EATS mediated*' and thus infer inherent endocrine activity which is enough to demonstrate biological plausibility.

The Comparative Thyroid Assay (CTA) is a test for TH disruption in peripheral blood of dams and offspring. Altered TH levels in blood is an indication of endocrine activity. However, disruption of thyroid homeostasis is the initial, critical effect that may lead to adverse effects on the developing nervous system. Therefore, the CTA may provide information for classification on thyroid mediated adversity instead of a rat DNT study (OECD TG 426; OECD, 2007). The study generates mechanistic data on the thyroid which can be used to derive a reference dose that would be protective against the ability of a substance to disrupt thyroid function in pregnant females and in the fetus and in the newborn.

If a CTA is available which provides evidence of alteration of the HPT axis in the foetus or offspring, then classification as *HH ED 1*; EUH380 may be warranted irrespective of the effects in adult animals. This is because there is a well established link between thyroid disruption and developmental neurotoxicity (DNT). For example, OECD AOP No. 13 (Crofton *et al.*, 2019) and 14 (Rolaki *et al.*, 2019) may be used to establish a biologically plausible link between the evidence on endocrine-associated DNT (impaired learning and memory) and thyroid system-associated endocrine activity. Besides the CTA, OECD TGs 421, 422, 443 also investigate THs in offspring.

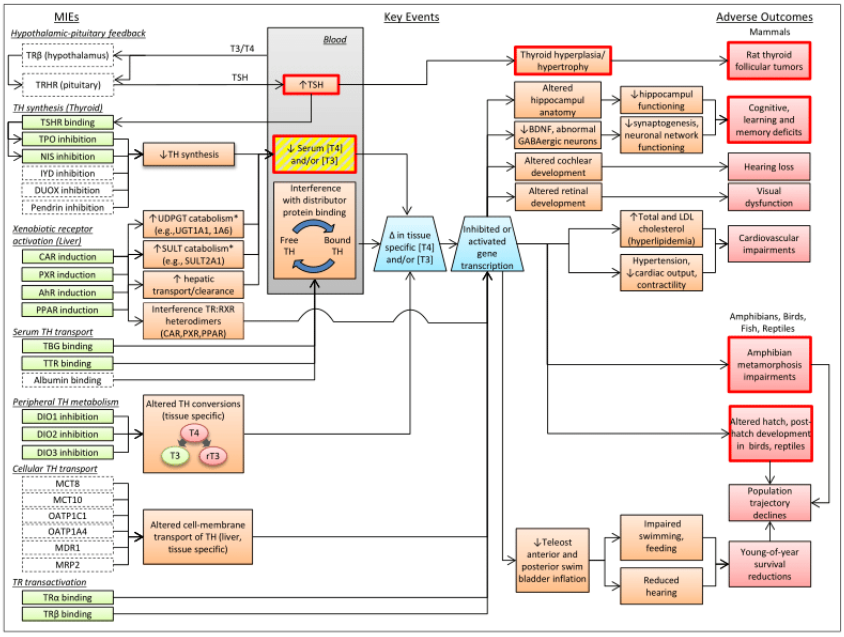
If a CTA is available which provides evidence that the HPT axis is not altered in the foetus or offspring then this result should be considered in the overall WoE for adversity.

(2) Classification as *HH ED 2*; EUH381 may be warranted *e.g.* when:

Evidence of adverse effects on the thyroid gland may be demonstrated for example by changes in organ weight or histopathological findings (follicular cell hypertrophy or hyperplasia) in any vertebrate, but the strength of evidence is not sufficient to classify as Category 1.

Figure 3.11-3 Adverse outcome pathway (AOP) network for induced thyroid activity showing the integration of multiple individual AOPs under development and proposed; from Noyes *et al*., 2019 with permission from the authors. Biological linkages described may be informed by in vitro, in vivo, or computational data and may be causal, inferential, or putative, depending on the strength of the evidence. Boxes with thick, red borders represent in vivo end points that are targeted by U.S. EPA and OECD test guidelines (see the reflection of cochlear-associated hearing loss under Section 3.11.2.4.2. In the left-hand column, MIE boxes with solid borders (shaded green) represent current MIEs with in vitro high-throughput screening (HTS) assays that have demonstrated reliability and are available for use in thyroid activity screens, whereas those with dashed borders represent putative MIEs in the thyroid axis currently without in vitro HTS capabilities. In the key events (KEs) column, the box with the striped background (shaded yellow) depicts changes in serum TH as a KE node that represents a biomarker of thyroid disruption, whereas the trapezoids (shaded blue) represent additional potential KE nodes with limited data. Uppercase nomenclature denoting human protein is shown although present in differing species. Asterisks represent KEs being treated as MIEs. AhR, aryl hydrocarbon receptor; BDNF, brain-derived neurotrophic factor; CAR, constitutive androstane receptor; DIO, iodothyronine deiodinase; DIO1, type 1 deiodinase; DIO2, type 2 deiodinase; DIO3, type 3 deiodinase; DUOX, dual oxidase; IYD, iodotyrosine deiodinase; LDL, low-density lipoprotein; MDR, multidrug resistance protein; MCT, monocarboxylate transporter; NIS, sodium–iodide symporter; OATP, organic anion transporter polypeptide; OECD, Organisation for Economic Co-operation and Development; PPAR, peroxisome

proliferator-activated receptor; PXR, pregnane X receptor; rT3, reverse T3 (3,3',5'-triiodothyronine); RXR, retinoid X receptor; SULT, sulfotransferase; T3, 3,3',5-triiodothyronine; T4, thyroxine; TBG, thyroid binding globulin; TH, thyroid hormone; TPO, thyroperoxidase; TR, thyroid hormone receptor; TRHR, thyrotropin releasing hormone receptor; TSHR, thyroid stimulating hormone receptor; TTR, transthyretin; UDPGT, uridine diphosphate glucuronosyltransferase. Some of the KEs in figure should may be considered as adverse outcomes, such as histopathological changes.



3.11.2.4.3. Specific considerations regarding adverse effects on (developmental) neurotoxicity and immunotoxicity with respect to decision on classification for endocrine disruption

Adverse effects on the (developing) nervous system can be elicited by various mechanisms. These mechanisms may be related to, among others, different types of endocrine activity (not only the hypothalamic-pituitary-thyroid (HPT) system, but also other (neuro)endocrine systems including steroidogenesis modality (see *e.g.* example 4 for *ED HH*)). The endocrine system works also closely with the immune system to influence development from gestation through early life and thus endocrine disruption also may induce developmental immunotoxicity. The immune system is influenced or modulated also throughout all life stages by hormonal activity, and endocrine disruption can cause adverse effects across the life span. The endocrine system is critically important for immune and nervous system formation and functions and vice versa. Endocrine disruption cannot only alter signalling of the immune-neuroendocrine network but can subsequently be detrimental for the entire organism, resulting in an increased risk of both communicable (*i.e.*, increased incidence of infections) and non-communicable diseases (*i.e.*, allergy, autoimmunity, cancer, obesity, neurodegenerative disorders) (Galbiati *et al.*,2021).

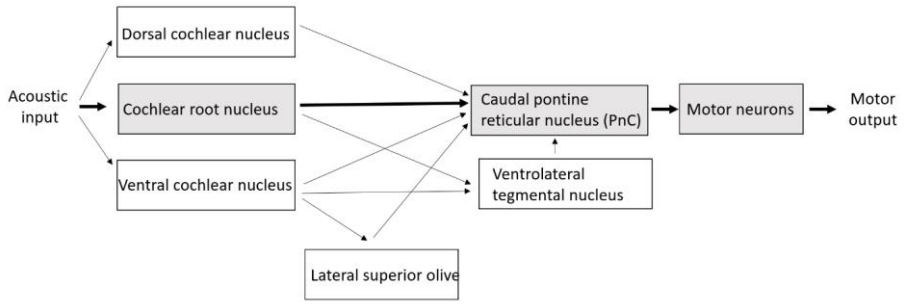
Endocrine disruption may increase the susceptibility to infections and tumors by

immunosuppression or may lead to inflammatory chronic diseases such as allergy, asthma, and autoimmune disorders by immunoenhancement. Steroid hormones (androgens, estrogens, glucocorticoids, and progesterone) are known to act on the immune system by shifting the immune response towards either cell-mediated (*e.g.,* androgens) or humoral immunity and inflammation (*e.g.,* estrogens, progesterone) or anti-inflammation (*e.g.,* glucocorticoids) (Galbiati *et al.,* 2021; Popescu *et al.* 2021). Hormonal influences on the immune system are not limited to steroid hormones, but also hormones such as growth hormone, prolactin, and thyroid stimulating hormone directly or indirectly influence the immune function. Disruption of the hypothalamic-pituitary-gonadal (HPG) axis, the hypothalamic-pituitary-adrenal (HPA) axis, the hypothalamic-pituitary-thyroid (HPT) axis, hypothalamic-growth hormone axis, as well as steroidogenic pathways that intersect with lipid and cholesterol metabolism all play important roles in growth, immunity, and health through direct effects on immunity or indirectly though modulatory effects on another axis (Manley *et al.,* 2018; Christofides *et al.,* 2021; Bansal *et al.,* 2018; Eskandari *et al.,* 2003).

(Developmental) neurotoxic and immunotoxic effects shall be considered as adverse effects relevant for classification as EDs, when there is evidence that they are mediated by endocrine activity and there is evidence of a biologically plausible link between the endocrine activity and the adverse (D)NT or (D)IT effect. Also in the absence of evidence for endocrine activity, DNT and DIT are still relevant for the assessment of developmental toxicity (under reproductive toxicity), and other neurotoxicity and immunotoxicity are relevant for the assessment of STOT SE or RE, depending on whether the adverse effects are caused by a single or repeated exposure, respectively. Figure 3.11-3 shows that one type of adverse outcome associated with altered thyroid hormones are cognitive learning and memory deficits. Such AOPs have already been endorsed in OECD Series on Adverse Outcome Pathways No. 13 (Crofton *et al.*, 2019) and 14 (Rolaki *et al.*, 2019). Altered cochlear development and hearing loss is currently under development in AOP 8 of the AOPwiki[11]. The paper by Noyes *et al.* cited in Figure 3.11-3, indicates that altered cochlear development and hearing loss are not investigated in U.S. EPA and OECD test guidelines. It is to be noted however, that cochlear damage and associated hearing loss could potentially be detected in an acoustic startle response (ASR) test that is a standard test in cohort 2A animals in OECD TG 443. This is because the baseline ASR and its short-term habituation reflect the function of a simple sensory motor pathway consisting of only a few neurons including the auditory nerve (the cochlear nerve) (Figure 3.11-4 below). Lesions in the primary pathway may dramatically decrease the startle amplitude, whereas excitation of this neural pathway elicits a startle response (Bradley and Sabatinelli, 2011).

Figure 3.11-4: The primary ASR pathway (modified from Koch *et al*., 1999). The bold arrows and the lightly shaded boxes symbolize the proposed fastest route of transmission of acoustic input into the motor output.

[11] AOP 8: Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammal AOP-Wiki (aopwiki.org)



The science is continuously developing in this area and therefore, the assessment needs to be done on a case-by-case basis based on the current available scientific knowledge. It is also noted that peripheral circulating TH levels do not necessarily reflect tissue levels and therefore do not predict tissue responses. Thus, if there is evidence on treatment-induced tissue specific molecular initiating events, these may provide information on endocrine activity even in the absence of effects on serum hormone levels.

3.11.2.5. Classification of substances and mixtures containing ED constituents

In analogy to CMRs, from a compositional and a regulatory point of view the situation for substances containing ED constituents, additives or impurities is the same as for mixtures containing components classified for these hazard classes. For this reason the classification procedure for ED endpoints that is foreseen by CLP for mixtures containing ED components, is considered applicable also to substances containing ED constituents, additives or impurities; see Section 3.11.3.1.

Commented [A3]: Links to other parts of the CLP Guidance to be added

As discussed in Section 3.11.3.1 below, mixtures containing components classified as EDs shall be normally classified using only the relevant available information for the individual substances in the mixture. Further, in cases where the available test data on the mixture itself demonstrate positive ED effects which have not been identified from the information on the individual substances, those data shall also be taken into account.

For ED endpoints the lowest incidence possible to detect in the tests should be considered in classification. When testing a diluted substance tests may not be able to detect these low incidences and thus in tests there is a need to use as high a dose as possible to be able to detect a sufficiently high incidence for classification, to compensate for small group sizes in the tests. Thus, the highest test dose shall be the limit dose or the highest possible dose as described in the relevant OECD TG, see further details on dosing in Section 3.11.2.2.2. "Relevant doses for classification". Dilution, as would be the case if mixtures or substances containing ED constituents were tested, would increase the risk that ED hazards would not be detected, *i.e.* dilution might compromise the threshold of detection for CMR and ED hazards. Therefore, negative test data on mixtures containing constituents with these hazards shall not be accepted.

According to Article 10(1), generic and specific concentration limits (GCLs and SCLs) are similarly assigned to substances in other substances and substances in mixtures. A GCL will apply to EDs unless the data justifies setting an SCL.

3.11.2.6. Setting of specific concentration limits

CLP, Article 10(1) *Specific concentration limits and generic concentration limits are limits assigned to a substance indicating a threshold at or above which the presence of that substance in another substance or in a mixture as an identified impurity, additive or individual constituent leads to the classification of the substance or mixture as hazardous.*

Specific concentration limits shall be set by the manufacturer, importer or downstream user where adequate and reliable scientific information shows that the hazard of a substance is evident when the substance is present at a level below the concentrations set for any hazard class in Part 2 of Annex I or below the generic concentration limits set for any hazard class in Parts 3, 4 and 5 of Annex I.

In exceptional circumstances specific concentration limits may be set by the manufacturer, importer or downstream user where he has adequate, reliable and conclusive scientific information that a hazard of a substance classified as hazardous is not evident at a level above the concentrations set for the relevant hazard class in Part 2 of Annex I or above the generic concentration limits set for the relevant hazard class in Parts 3, 4 and 5 of that Annex.

3.11.2.6.1. Procedure

SCLs for ED properties are set based on the potency of the adverse effect, which is a pragmatic approach used in EU laws to further inform the downstream user or supplier on the presence of an hazardous substance in a mixture. However, it should be noted that for some endocrine disruption endpoints, potency may vary. When data allows to set an SCL, the SCLs for ED shall be set following the procedures outlined in this guidance *i.e.*, under Sections 3.6.2.6, 3.7.2.6 or 3.9.2.6, with the following amendments: When the effect subject to ED classification is related to reproductive toxicity, the Section 3.7.2.6 applies, but the potency shall be adjusted to 1, 0.1, 0.01, and 0.001 instead of 3, 0.3, 0.03, and 0.003, and so on, due to the ED GCL value of 0.1 instead of 0.3.

Commented [A4]: Links to other parts of the CLP Guidance to be added

It shall be noted that, for example, for STOT RE, there are guidance values applicable and the GCL is 100 times higher than that for ED. Still, the same formula can be used, with 100-fold lower limits for ED classification. In practise this means that, for example, when the ED Category 1 classification is based on target organ toxicity, such as thyroid toxicity, with an ED MoA, the generic concentration limit for *ED HH 1* classification (0.1%) shall be applied, unless the data suggests a lower or in exceptional cases, a higher SCL, based on the following formula (same formula applies to Cat 2):

$$SCLCat.1 = \frac{EffD}{GV1\ x\ 100} \times 100\%$$

EffD (effective dose) is the dose inducing specific target organ toxicity (single or repeated exposure) and GV1 is the guidance value for Category 1 according to CLP, Annex I, Table 3.9.2 corrected for the exposure duration. The resulting SCL is rounded down to the nearest preferred value[12] (1, 2 or 5).

In exceptional cases a higher SCL than the GCL can also be set for EDs. A higher SCL should only be set where there is adequate, reliable and conclusive scientific information that a hazard of a substance classified as hazardous is clearly above the level of the GCL.

When there are several types of effects and ways to calculate SCLs, the lowest SCL should be selected for the classification. Only one SCL can be set for *ED HH*.

When the calculated SCL or GCL is not considered protective enough (*e.g.* due to a non-

[12] This is the "preferred value approach" as used in the EU and are values to be established preferentially as the numerical values 1, 2 or 5 or multiples by powers of ten.

threshold MoA), the SCL corresponding to the extreme potency group may be set by default, unless an even lower SCL is justified. Due to these above mentioned characteristics for some EDs, the assessment of dose-response related information together with setting SCLs should be conducted with caution.

3.11.2.7. Decision logic for classification of substances

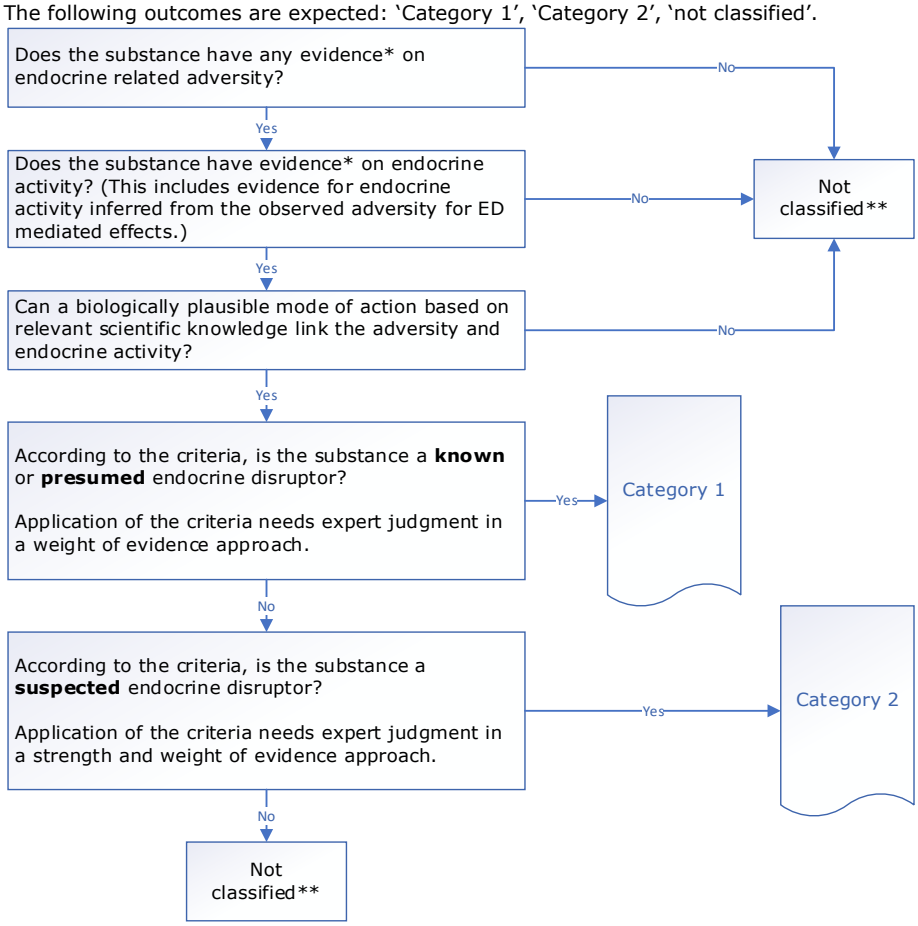
Commented [A5]: Question to CARACAL:
ECHA suggest to delete this whole paragraph and the flowchart since it does not bring any added value.

We would like to hear to opinion of CARACAL on this?

The decision logic which follows, in Figure 3.11-5, is provided here as additional guidance and at a very high level. Therefore, it is strongly recommended that the person responsible for classification study the criteria before and during use of the decision logic.

Figure 3.11-5 Decision logic for endocrine disruption for human health

The following outcomes are expected: 'Category 1', 'Category 2', 'not classified'.



*Evidence in this context does not necessarily need to be substance specific, but can be obtained *e.g.* using read-across or analogy when this is justified.
**In should be noted that when the outcome is 'not classified' it can be for the following reasons not meeting the CLP ED criteria, or 'classification not possible'; i.e. due to lack of or inconclusive data.

3.11.3. Classification of mixtures for endocrine disruption for human health

3.11.3.1. Classification criteria for mixtures

Endocrine disruption classification of mixtures is based on the presence of an ingredient classified for endocrine disruption; see CLP, Article 6(3) and CLP, Annex I, Section, 3.11.3. Only in case there is data available for the mixture itself which demonstrate effects not apparent from the ingredients, might this data be used for classification. In other words, data on tested mixtures shall be used only when it demonstrates classification for endocrine disruption for human health, in line with CLP, Annex I, Section 3.11.3.2.1; *i.e.,* not for "no classification". If such data is not available for the mixture itself, data on a similar mixture can be used in accordance with the bridging principle; see CLP, Annex I, Section 1.1.3. Furthermore, it should be noted that various test guidelines have not been validated for mixtures and therefore, it is questionable if these tests may provide adequate results.

From a compositional and a toxicological point of view, the situation for substances containing ED constituents, additives or impurities is the same as for mixtures containing components classified for these endpoints. For this reason, the classification procedure for ED endpoints that is foreseen by CLP for mixtures containing ED components is considered applicable also to substances containing ED constituents, additives or impurities; see Sections 1.1.6.1, and 3.11.3.1.1 to 3.11.3.2.

Commented [A6]: Links to other parts of the CLP Guidance to be added

CLP, Annex I, Section *3.11.3.1.1.* *A mixture shall be classified as an endocrine disruptor for human health where at least one component has been classified as a Category 1 or Category 2 endocrine disruptor for human health and is present at or above the appropriate generic concentration limit as shown in Table 3.11.2 for Category 1 and Category 2, respectively*.

As such, each component in a mixture classified as an ED is compared separately to their respective generic or specific concentration limit to conclude on the classification of the mixture, unless the additivity principle applies.

The additivity concept may have to be applied for EDs; see Section 1.6.3.3.3. For a given effect, the SCL, if available, needs to be taken into consideration when applying the additivity concept – this will include potency considerations. Exposure to EDs with both similar and dissimilar modes of action can lead to combination effects if they impact the same physiological process(es), or have the same target organs for toxicity. If one single classified substance is present in the mixture above the generic or specific concentration limit, the mixture must be classified for that hazard. If the mixture contains two or more substances each below the generic or specific concentration limits, the mixture will not be classified, unless the additivity concept applies. For endocrine disruption, it is reasonable to assume additivity for substances with a similar or related mechanism or MoA or adverse outcome (*e.g.*, exposure to a combination of anti-androgenic, estrogenic and steroidogenic or even thyroid disrupting substances can lead to additivity), unless there are specific reasons not to do so.

Commented [A7]: Links to other parts of the CLP Guidance to be added

The mechanism does not need to be the same. Similar to most of the other HH hazard classes, the same adverse outcome between substances can already suggest additivity.

It is important in the assessment of potential additivity to consider if constituents with the same biological targets have different effects or mechanism behind the effects (*e.g.,* they may have agonistic or antagonistic activity or even partial activity at the same receptor). In this case a careful assessment is needed since dissimilar modes of action can cause the same adverse outcomes in an additive manner.

CLP, Annex I*, Table 3.11.2.*

Generic concentration limits of components of a mixture classified as endocrine disruptor for human health that trigger classification of the mixture

Component classified as:	*Generic concentration limits triggering classification of a mixture as:*	
Category	*Category 1 endocrine disruptor for human health*	*Category 2 endocrine disruptor for human health*
Category 1 endocrine disruptor for human health	*≥ 0,1 %*	
Category 2 endocrine disruptor for human health		*≥ 1 %* *[Note 1]*

Note: The concentration limits in this Table shall apply to solids and liquids (w/w units) as well as gases (v/v units).

Note 1: If a Category 2 endocrine disruptor for human health is present in the mixture as an ingredient at a concentration ≥ 0,1 % a SDS shall be available for the mixture upon request.

3.11.3.1.1. When data are available for the individual ingredients

CLP, Annex I*, Section 3.11.3.1.1.* *A mixture shall be classified as an endocrine disruptor for human health where at least one component has been classified as a Category 1 or Category 2 endocrine disruptor for human health and is present at or above the appropriate generic concentration limit as shown in Table 3.11.2 for Category 1 and Category 2, respectively.*

Additivity shall be considered on a case-by-case basis, particularly when the data suggests the same/related endocrine MoA or modality or adverse outcome for different ingredients of the mixture.

3.11.3.1.2. When data are available for the complete mixture

CLP, Annex I, Section *3.11.3.2.1.* *Classification of mixtures shall be based on the available test data for the individual components of the mixture using concentration limits for the components classified as endocrine disruptor for human health. On a case-by-case basis, test data on the mixture as a whole may be used for classification when demonstrating endocrine disruption for human health that has not been established from the evaluation based on the individual components. In such cases, the test results for the mixture as a whole must be shown to be conclusive taking into account dose (concentration) and other factors such as duration, observations, sensitivity and statistical analysis of the test systems. Adequate documentation supporting the classification shall be retained and made available for review upon request.*

3.11.3.1.3. When data are not available for the complete mixture: bridging principles

CLP, Annex I, Section *3.11.3.3.1.* *Where the mixture itself has not been tested to determine its endocrine disruption for human health, but there are sufficient data on the individual components and similar tested mixtures (subject to paragraph 3.11.3.2.1) to adequately characterise the hazards of the mixture, those data shall be used in accordance with the applicable bridging principles set out in Section 1.1.3.*

Bridging Principles will only be used on a case-by-case basis; see Section 1.6.3.2. Data on similar tested mixtures shall be used only when it demonstrates classification for endocrine disruption for human health, in line with CLP, Annex I, Section 3.11.3.2.1, *i.e.* not for "no classification". Note that the following bridging principles are not applicable to this hazard class, in line with their non-applicability for CMRs:

- concentration of highly hazardous mixtures
- interpolation within one hazard Category

(see CLP, Annex I, Sections 1.1.3.3 and 1.1.3.4)

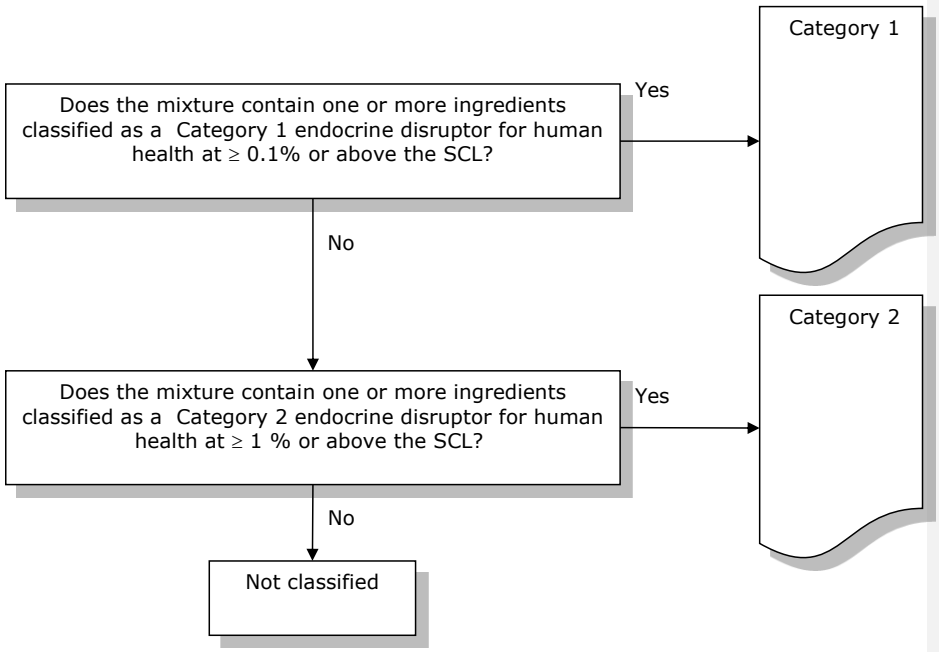
3.11.3.2. Decision logic for classification of mixtures

The decision logic which follows in Figure 3.11-6 and Figure 3.11-7 is provided here as additional guidance. The person responsible for classification should study the criteria before and during use of the decision logic presented below.

Classification of mixtures for endocrine disruption for human health

Classification based on individual ingredients of the mixture

Figure 3.11-6 Decision logic for classification of mixtures based on individual ingredients of the mixture

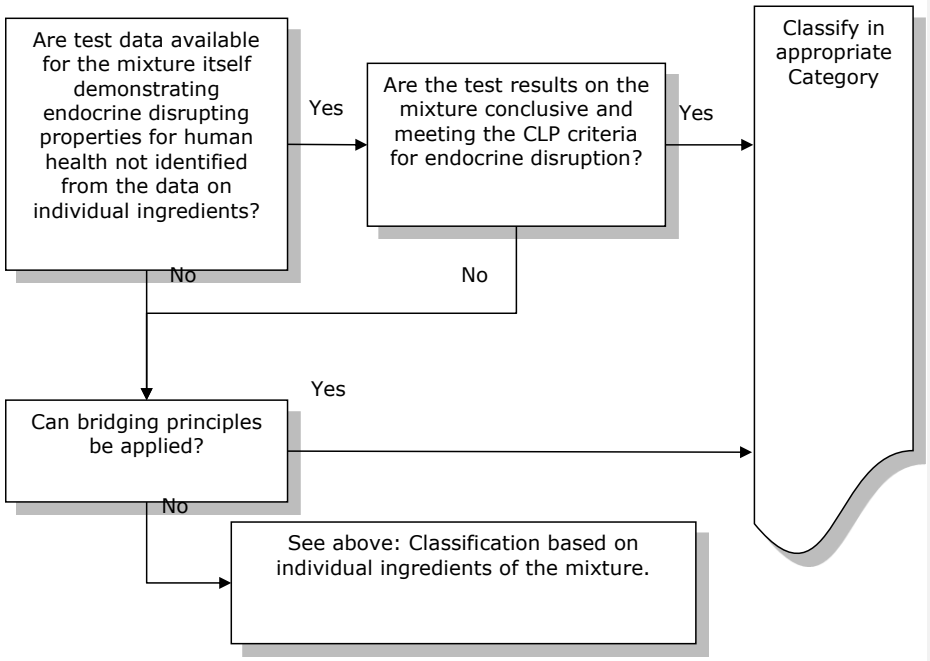


Modified classification when the test data on the mixture itself supports more stringent classification than evaluation based on individual ingredients

Test data on mixtures may be used for classification when demonstrating effects that have not been established from the evaluation based on the individual ingredients; CLP, Article

6(3) and CLP, Annex I, Section 3.11.3.2.1.

Figure 3.11-7 Decision logic for classification of mixtures when the test data on the mixture itself supports more stringent classification then evaluation based on individual ingredients



3.11.4. Hazard communication in the form of labelling for endocrine disruption for human health

3.11.4.1. Pictograms, signal words, hazard statements and precautionary statements

Classification	*Category 1*	*Category 2*
GHS Pictograms	*	*
Signal Word	*Danger*	*Warning*
Hazard Statement	*EUH380: May cause endocrine disruption in humans*	*EUH381: Suspected of causing endocrine disruption in humans*
Precautionary Statement Prevention	*P201* *P202* *P263*	*P201* *P202* *P263*

	P280	*P280*
Precautionary Statement Response	*P308 + P313*	*P308 + P313*
Precautionary Statement Storage	*P405*	*P405*
Precautionary Statement Disposal	*P501*	*P501*

* Pictogram currently unavailable. When included in GHS, but not yet implemented in CLP, it is strongly recommended to be applied.

The wording of the Precautionary Statements is found in CLP, Annex IV, Part 2.

3.11.4.2. Additional labelling provisions

There are no additional labelling provisions for substances and mixtures classified as EDs in CLP. However, there may be provisions laid down in other regulations such as REACH which need to be considered, when relevant.

3.11.5. Examples

Commented [A8]: Question to CARACAL
There are diverging views within the PEG on the usefulness of the examples, and on whether the boundaries between the ED HH 1, ED HH 2 and no classification are correctly illustrated by the examples.

We would like to hear the CARACAL opinion on this.

The substances in the examples are fictitious. They do not represent real cases and are not to pre-empt the classification assessment in concrete cases. These examples are rather only to illustrate what type of data may lead to classification in different categories for ED and to show how an assessment according to this guidance could potentially be approached. Only ED-related data leading to classification or supporting classification or "no classification" is included in the examples but not the whole data set or a detailed description of the effects, nor a full WoE analysis (*e.g.* general toxicity is not included in all examples). The decision on classification is influenced by the strength of overall evidence and should be decided on a case-by-case basis.

List of examples:

Examples *ED HH 1* (see Section 3.11.5.1)

Example 1: Classification as *ED HH 1* based on EAS (estrogenic effect)

Example 2: Classification as *ED HH 1* based on EAS (anti-androgenic effect)

Example 3: Classification as *ED HH 1* based on thyroid effect

Example 4: Classification as *ED HH 1* based on non-EATS (α_2-adrenergic agonist)

Example 5: Classification as *ED HH 1* based on read across

Examples *ED HH* 2 (see Section 3.11.5.2)

Example 6: Classification as *ED HH 2* based on EAS (anti-androgenic effect)

Example 7: Classification as *ED HH 2* based on thyroid effect

Example 8: Classification as *ED HH 2* based on non-EATS (Increased resistance to insulin)

Examples *ED HH* No classification (see Section 3.11.5.3)

Example 9: No classification based on EAS activity

Example 10: No classification based on EAS activity

Example 11: No classification based on thyroid effect

Example 12: No classification based on non-EATS (reduction in cholesterol)

3.11.5.1. Examples *ED HH 1*

3.11.5.1.1. Example 1 *ED HH 1* based on EAS (estrogenic effect)

Available information:

Human data*:*

No information available.

Animal data on adversity:

Species	Type of study	Dose (mg/kg bw/d)	Effect	Indications of excessive general toxicity
Rat	2-generation study OECD TG 416	0, 1.5, 15, 75 (diet)	• P Females: prolonged oestrous cycle, increased number of corpora lutea • not considered indications of excessive toxicity • F1 generation: reduced litter size • F2 generation: reduced litter size	No
Rat	28-day study OECD TG 407	0, 150, 450, 1000 (gavage)	• Increased uterus and ovarian weight at doses ≥ 150 mg/kg body weight/day	No
Rat	Female pre-pubertal assay OPPTS 890.1450	0, 20, 60, 300 (gavage)	• Earlier first oestrus, increased uterus weight and prolonged oestrous cycle at doses ≥ 60 mg/kg body weight/day	No

Data on endocrine activity:

Type of mechanistic data	Type of study		Effect	Comment
In vivo mechanistic study	Uterotrophic assay OECD TG 440	0, 25, 100, 400 Sub-cutaneous injection	• Dose-dependent increase of uterine weight in ovariectomised	Indicative of estrogenicity
In vivo mechanistic study	Hershberger assay OECD TG 441	0, 10, 30, 100 Sub-cutaneous injection	• No androgenic or anti-androgenic activity observed	No indications of androgenicity
In vivo mechanistic,	Female pre-	0, 20, 60,	• Earlier first oestrus, increased uterus weight	Indicative of estrogenicity or

inferred by adverse effect[13]	pubertal assay OPPTS 890.1450	300 (gavage)	and prolonged oestrous cycle at doses ≥ 60 mg/kg body weight/day	altered sterogenisis
In vivo mechanistic, inferred by adverse effect[13]	OECD TG 407	0, 150, 450, 1000 (gavage)	Increased uterus and ovarian weight at doses ≥ 150 mg/kg body weight/day	Indicative of estrogenicity
In vitro mechanistic study	Estrogen Receptor Binding OPPTS 890:1250		• Moderate competitive binding to estrogen receptor 1 (ER1); IC50 1.1 µM compared to 1.2 nM for the positive control oestradiol and 3.5 µM for the weak positive control 19-norethindrone IC50 = 3.46 µM	Indicative of estrogen receptor agonism
In silico prediction	QSAR Toolbox		• the substance is a strong ER binder due to "*cyclic molecular structure with a single non-impaired hydroxyl group*"	Indicative of estrogen receptor agonism

Assessment:

Adverse effect(s):

The adverse effects based on a pattern of effects on uterus and ovarian weight; prolonged oestrous cycle; and age at first oestrus which are '*EAS mediated*' parameters. These provide clear evidence of an endocrine MoA.

Diverging findings on uterus and ovarian weight, oestrous cycle, age at first oestrus, corpora lutea and litter size primarily provide *in vivo* information on adversity but in addition also mechanistic information.

Endocrine activity:

The positive results of the uterotrophic assays indicate an estrogenic activity which is further supported by the QSAR Toolbox and the ER binding assay. The negative Hershberger assay point to lack of (anti-) androgenicity.

Biological plausible link:

There is evidence of a biological plausible link because the parameters measured *in vivo* that contributed to the evaluation of adverse effect(s) also at the same time provide in vivo mechanistic evidence. Due to the nature of the effect and the existing knowledge on mammalian reproductive endocrinology, these '*EAS mediated*' adverse effects are considered diagnostic of an EAS MoA and thus (in the absence of other explanations) also infer an underlying *in vivo* mechanism.

In addition, the ER binding assay provides evidence a MIE which fits with the pattern of effects observed.

Type	Brief decription of the key events (KE)	Supporting evidence

[13] Studies which infer endocrine activity based on adversity are reported in both tables.

MIE	Activation of Estrogen receptor	(OPPTS 890:1250; OECD 440)
KE1	Uterine hypertophy	Increased uterine weight (OECD TG 407, OPPTS 890.1450, OECD TG 440)
AO1	Increased ovary weight with increased number of corpora lutea	Increased ovary weight and ovary histopathology OECD TG 407, 416
AO2	Alteration estrus cycle	Prolonged estrus cycle (OECD TG 416, OPPTS 890.1450)

Conclusion:

There is clear evidence for adverse effect on the female reproductive system; there is clear evidence indicating that the substance has estrogenic activity. In addition, knowledge on mammalian reproductive endocrinology supports this conclusion.

Based on the above, Substance X meets the criteria for *ED HH* 1: EUH380.

SCL calculation:

The SCL calculation is based on the potency groups for reproductive toxicity, Section 3.7.2.

(1) There are also ED related adverse effects in the 2 generation reproductive toxicity study. For these effects, the SCL calculation method from Section 3.7.2 was used.

The reproductive is of 60 mg/kg body weight/day effect. The estimated ED_{10} value, based on the top dose of 60 mg/kg body weight/day is suggesting a medium potency group (4 mg/kg body weight/day < ED_{10} value < 400 mg/kg body weight/day), no need for SCL based on effects related to reproductive toxicity, *i.e.* a GCL is warranted.

Conclusion on SCL: The ED GCL of 0.1% applies.

3.11.5.1.2. Example 2 *ED HH 1* based on EAS (anti-androgenic effect)

Available information:

Human data:

No relevant information available

Animal data on adversity:

Species	Type of study	Dose (mg/kg bw/d)	Effect	Indications of excessive general toxicity
Rat	Extended one-generation study	0, 100, 300, 1000	• ↓ AGD in males • Delayed sexual maturation in males	No

	OECD TG 443	(diet)	(preputial separation) without clear relationship to body weight decreases	
Dog	90-day study OECD TG 409	0, 150, 450, 1000 (gavage)	• ↓ prostate weights with histopathological changes • ↑ testes weights	No
Mice	6 week study Non-guideline	0, 100, 300, 1000 (diet)	• No effects on male or female reproduction organs	NO
Mice	Carcinogencity study OECD TG 452	0, 100, 300, 1000 (diet)	• No effects on male or female reproduction organs	No

Data on endocrine activity:

Type of mechanistic data	**Type of study**		**Effect**	**Comment**
In vivo mechanistic, inferred by adverse effect[13]	Extended one-generation study OECD TG 443	0, 100, 300, 1000 (diet)	• ↓ AGD in males • Delayed sexual maturation in males (preputial separation) without clear relationship to body weight decreases	Indicative of estrogenicity, androgenicity or altered sterogenisis
In vivo mechanistic, inferred by adverse effect[13]	90-day study OECD TG 409	0, 150, 450, 1000 (gavage)	• ↓ prostate weights with histopathological changes • ↑ testes weights	Indicative of estrogenicity, androgenicity or altered sterogenisis
In vitro mechanistic study	Androgen receptor transactivation assay OECD TG 458		• AR transactivation assays positive for antagonism	Indicative of androgen receptor antagonism
In silico prediction	ToxCast AR model		• Result from AR Model for antagonist (ToxCast): 0.237	Indicative of androgen receptor antagonism

Assessment:

Adverse effects:

Pattern of adverse effects on male reproduction organs and male puberty entry in rats and dogs, which cannot be attributed to general toxicity. No effects in mice. Human relevance cannot be excluded.

Endocrine activity:

In vitro evidence for anti-androgenicity, no *in vivo* mechanistic study available. However, pattern of effects observed infer endocrine activity which could be explained by anti-androgenicity.

Biological plausibility:

An antagonistic action at the androgen receptor can plausibly lead to anti-androgenic effects *in vivo*.

Type	Brief decription of the key events (KE)	Supporting evidence
MIE	Antagonism of the androgen receptor (AR)	(OECD TG 458, Toxcast AR model)
KE1	Decreased AR activation	Infered by shorter anogenital distance (OECD TG 443) Infered by reduced prostate weight (OECD TG 409)
AO1	Increased ovary weight with increased number of corpora lutea	Reduced prostate weight (OECD TG 409)
AO2	Altered Leydig cell function	Increased testis weight (OECD TG 409)
AO3	Feminisation of male offspring	Shorter anogenital distance (OECD TG 443)

Conclusion:

Delayed puberty, clear pattern of adverse antiandrogenic effects, evidence for anti-androgenic *in vitro*. Clear adversity pattern and endocrine activity, which is biologically plausibly linked. Human relevance cannot be excluded.

Based on the above, the Substance meets the criteria for *ED HH* 1:EUH380.

SCL calculation:

The SCL calculation is based on the potency groups for reproductive toxicity, Section 3.7.2.

(1) There are also ED related adverse effects in the extended one-generation reproductive toxicity study. For these effects, the SCL calculation method from Section 3.7.2 was used.

The reproductive is of 100 mg/kg body weight/day effect. The estimated ED_{10} value, based on the top dose of 100 mg/kg bw/day is suggesting a medium potency group (4 mg/kg body weight/day < ED_{10} value < 400 mg/kg body weight/day), no need for SCL based on effects related to reproductive toxicity, *i.e.* a GCL is warranted.

Conclusion on SCL: The ED GCL of 0.1% applies.

3.11.5.1.3. Example 3 *ED HH* 1 based on thyroid effect

Available information:

Human data:

Type of data	Type of study		Effect	Comment
Analogy			• Substances which inhibit thyroid peroxidase (TPO) are used clinically to manage hyperthyroidism	

Animal data on adversity:

Species	Type of study	Dose (mg/kg bw/d)	Effect	Indications of excessive general toxicity
Rat	90-day study OECD TG 408	0, 10, 50, 250 (diet)	• ↑ Thyroid weight (absolute and relative), statistically significant at top dose only in males. • ↑ Thyroid hyperplasia, statistically significant at top dose only in both males and females. • ↑ TSH, statistically significant at top dose only in both males and females. • ↓ T4, statistically significant in males and females, clear dose-response observed.	No

Data on endocrine activity:

Type of mechanistic data	Type of study		Effect	Comment
In vitro mechanistic study	Human thyroid peroxidase activit (TPO) non-guideline		• thyroid peroxidase inhibition; IC_{50} 0.5 µM compared to 0.1 µM for the positive control methimazole	Indicates of TPO inhibition as the MIE
In vivo mechanistic *In vivo* mechanistic, inferred by adverse effect[13]	90-day study OECD TG 408	0, 10, 50, 250 (diet)	• ↓ T4, statistically significant in males and females, clear dose-response observed. • ↑ TSH, statistically significant at top dose only in both males and females. • ↑ Thyroid weight (absolute and relative), statistically significant at top dose only in males. • ↑ Thyroid hyperplasia, statistically significant at top dose only in both males and females.	Indicative of thyroid disruption Changes in thyroid weight or histopathology can be used as a surrogate for increased TSH, thus indicative of thyroid disruption

Assessment:

Adverse effect(s):

Adverse effects on the thyroid have been observed.

Endocrine activity:

Thyroid effects were accompanied with reduced T4 and increased TSH.

The TPO inhibition assay demonstrate that the substance is a TPO inhibitor with a similar potency as the drug methimazole.

Biological plausibility:

The pattern of effects observed is consistent with current knowledge and the fact that both adverse effect(s) and endocrine activity were observed in the same study at similar doses demonstrates that the effects are biologically plausible. The reduction in T3/T4 is accompanied by a measured and inferred increase in TSH. Observed increase in TSH is consistent with the effects observed on the thyroid gland. The relative potency *in vitro* is in the same order of magnitude as the known TPO inhibitor methimazole TPO inhibition seems to be the most likely MoA. The other possible thyroid MoAs have not been investigated.

Type	Brief description of the key events (KE)	Supporting evidence
MIE	Inhibition of thyroid peroxidase	In vitro mechanistic study
KE1	Decreased TH synthesis	The KE is supported by analogy to other TPO used clinically to reduce TH synthesis in the management of hyperthyroidism
KE2	Decreased serum T4 or T3	Decreased serum T4 OECD TG in 408
KE3	Increase of TSH	Increased TSH supported by thyroid weight and histopathology in OECD TG 408
AO	Increased thyroid hypertrophy and hyperplasia as a result of continuous TSH stimulation	Increased thyroid weight and increased incidence of thyroid hyperplasia in OECD TG 408

Conclusion:

There is clear evidence on thyroid related adverse effect(s) (thyroid follicular cell hyperplasia, increased thyroid weight, and changes in T3/T4 and TSH) from rats and dogs which can be biologically plausibly linked to a MoA based on TPO inhibition.

Human relevance cannot be excluded.

Based on the above, the Substance meets the criteria for *ED HH* 1:EUH380.

In the example above, the MIE is known and used to support the classification as *ED HH* 1. However, knowing the MIE is not a prerequisite for the classification decision it is enough that the substance can be linked to an endocrine MoA. If there are adverse effects on thyroid weight and histopathology, then endocrine activity, and an ED MoA can be inferred from the adverse effects.

SCL calculation:

The adverse effects were observed between 50 and 250 mg/kg body weight/day, *i.e.* there are no evidence that the substance is potent enough for a SCL.

Conclusion on SCL: The ED GCL of 0.1% applies.

3.11.5.1.4. Example 4 *ED HH 1* based on non-EATS (α_2-adrenergic agonist)

The endocrine system extends beyond the EATS modalities. An example of a non-EATS modality is the sympathoadrenal system.

The different subtypes of adrenoreceptors (a_1, a_2, b_1, b_2, b_3) vary in their tissue distribution and their affinity to catecholamines such as adrenalin and noradrenalin. Catecholamines can act both as neurotransmitters and hormones. The regulation and physiological function of adrenoreceptors are known. The general function of catecholamines is to prepare the body for action.

Substances targeting these receptors are extensively used clinically. Indications include asthma, high blood pressure, attention deficit hyperactivity disorder and use in anaesthesia.

The example outlined below is based on the extensive knowledge from human clinical experience on the hazards associated the use of an a_2-adrenergic agonist as a pharmaceutical.

Available information:

Human data: Extensive database in humans with a dose starting at 10 µg/kg bw/day including numerous cases of toxicity observed following overdosing. This data also demonstrates that the substance is a selective a_2-adrenergic agonist.

Animal data: There is animal data available with a LOAEL of 10 µg/kg bw/day, which supports the findings in humans.

Toxicokinetic information: Data available demonstrating that the substance passes the blood-brain-barrier.

Assessment:

Adverse effect(s):

Adverse effects in humans are bradycardia (reduced heart rate), reduced blood pressure, hyperglycaemia, cognitive disorders, and at high doses sedation. These effects are also supported by animal data including pre-clinical toxicity studies.

The MoA outlined below focuses on bradycardia, reduced blood pressure and hyperglycaemia.

Endocrine activity:

The catecholamine noradrenaline functions both as a hormone released by adrenal and a neurotransmitter produced by central nervous system as well as sympathetic nervous system.

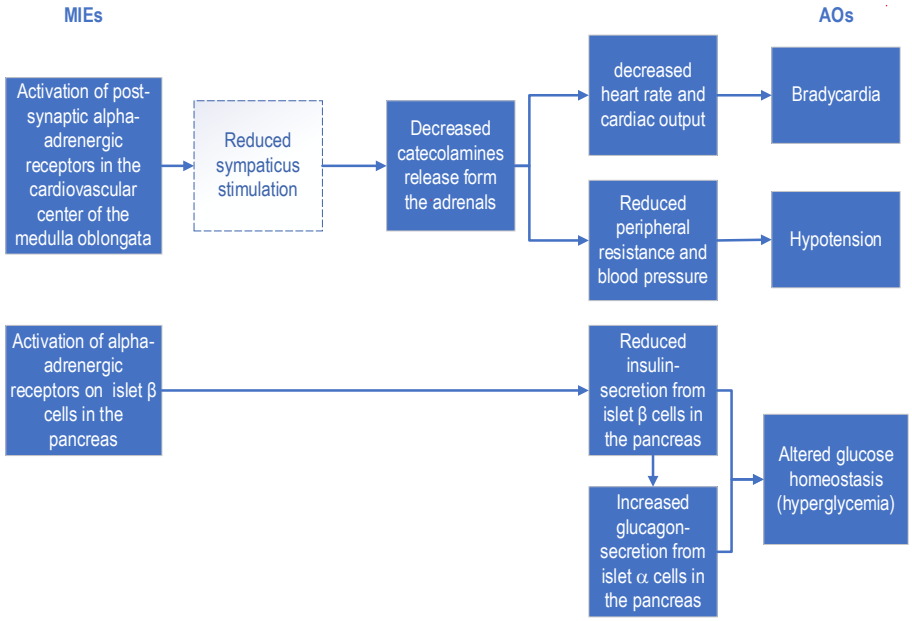
An α2-adrenergic agonist opposes the action of the sympathetic nervous system by exerting negative feedback by inhibiting noradrenalin release from presynaptic neurons. This results in reduced release of catecholamines into the bloodstream form the adrenals, which ultimately results in bradycardia, hypotension and hyperglycaemia. The activation of α2-adrenergic receptors also inhibits the insulin secretion of the pancreatic β-cells, which is the activity initiating the hyperglycaemia adverse effect.

Based on the above, there is clear evidence that the substance has endocrine activity, *i.e.* has the capacity to alter the function of an endocrine system.

Biological plausible link:

The biology of α_2-adrenergic agonists is fully understood. There is clear evidence that the substance is an α_2-adrenergic agonist which reaches the brain. Therefore, the MoA may be inferred by the extensive knowledge available for clinical experience and scientific literature.

Mode of action



Conclusion:

The substance has been specifically designed to bind and activate the a_2-adrenergic receptor which is an integral part of the sympathoadrenal system.

There is clear evidence that the substance supresses the sympathetic nervous system by reducing the neuroendocrine release of noradrenaline in the adrenals, ultimately resulting in bradycardia, hypotension and hyperglycaemia.

There is clear evidence for adverse effects on the cardiovascular system and glucose homeostasis; and there is a clear link because the MoA is fully understood.

Based on the above, the substance meets the CLP criteria for *ED HH 1; EUH380*.

SCL calculation:

The adverse effect is based on an oral EffD of 10 µg/kg body weight from human data an in line with the animal data. Method similar to 3.9.2 for 0.01 mg/kg body weight/day effect. SCL Cat1 = 0.01/(10x100)x100% = 0.001%

Conclusion on SCL: The method similar to 3.9.2 resulted in a high potency group corresponding to an SCL of 0.001%.

3.11.5.1.5. Example 5 ED HH 1 based on read-across

Read across justification

Substance Y is already classified as an ED for human health (*ED HH 1*).

A read across approach is applied from the source Substance Y to the target Substance X. The read-across is supported by the structural similarity of the two substances, the only difference being that Substance X has an isopropyl group at the end of an alkyl chain while Substance Y has a butyl group. The two substances share similarity in physico-chemical properties (molecular weight, lipophilicity, melting point, boiling point) and upon uptake the substances are hydrolysed to the same common metabolite.

Available information :

Substance X

Adverse effects:

No studies available (read-across from Substance Y).

Endocrine activity:

In vivo information: From one male rat ADME single dose study described in the open literature, it appears that Substance X is extensively absorbed orally, slowly metabolised and excreted within 36 hours. Substance X is notably found in several organs including the gonads showing moderate levels of ^{14}C-containing residues.

In vitro information: Substance X binds to the ER (Assessed in hERα and hERb competitive binding assay) and increases ER transactivation (two ER transactivation assays) and estrogen dependent signaling in target cells (estrogen dependent gene and protein expression, downstream effects *e.g.,* cell proliferation in estrogen dependent cell lines) at concentrations below the solubility limit and in absence of cytotoxicity.

In silico information: The Danish QSAR database indicates that Substance X is a strong ER binder and positive for ER activation. The concentration of the test chemical in the target tissue after application of a test dose not expected to cause systemic toxicity is calculated with a PBTK model and compared to the EC_{50} of the *in vitro* effect dose of the parent chemical. It is found that a not systemically toxic test concentration will generate biologically active dose of the parent chemical in the target tissue.

Substance Y

In silico predictions and several *in vitro* and *in vivo* mechanistic studies revealed sufficient evidence for estrogenicity of Substance Y. Several non-guideline reproductive developmental toxicity studies in rats described in the open literature, sc or oral gavage dosing from GD7-PND21. All reliability 2. All the other of these rodent studies using oral gavage or s.c. exposure show moderate-strong evidence for adverse effects of Substance Y on sperm count and quality, whilst one recent developmental study showed no effect on endocrine related endpoints.

Assessment:

Estrogenic activity and adverse effects:

There is strong evidence that Substance X affects ER binding and transactivation and estrogen dependent signaling in target cells *in vitro*.

Commented [A9]: Question for CARACAL:
A CLP specific guide on read-across is foreseen.

Because the use of read-across for classification is a horizontal issue across all hazard classes, ECHA suggests to delete the example in order not to preemt the read-across guidance.

We would like to hear the CARACAL opinion this?

In vivo, there is evidence that Substance X is systemically absorbed and reaches the reproductive organs in significant quantities.

The MoA considered for Substance Y is ER activation leading to decreased sperm count and quality after perinatal exposure shown in several non-guideline reproductive developmental toxicity studies. The lack of effect on endocrine related endpoints seen in a recent developmental study, may reflect differences in bioavailability when using different study designs such as exposure routes and periods. Thus, the adverse findings observed in other studies should not be neglected.

There are no studies for Substance X investigating adverse effects on sperm quality in perinatally exposed rats. Therefore, a read across approach is applied from the source Substance Y to the target Substance X. After consideration of all available *in vivo* results, there is moderate-strong evidence that developmental exposure to Substance Y, and consequently to Substance X, can cause adverse effects on sperm count and quality.

Biological plausibility: The MoA analysis leads to the conclusion that Substance X acts via an estrogenic MoA. Since no information was available on adverse effects of Substance X, information on Substance Y was included in the MoA analysis (perinatal exposure).

The MIE is activation of the ER(s). In developing males, increased ER signaling results in altered testicular development and subsequently altered testicular function in adulthood. In turn, reduced sperm count and quality are observed.

It is biologically plausible that ER activation during development leads to the observed adverse effects on the male reproductive system following perinatal exposure to Substance X. No alternative non-endocrine MoA was demonstrated.

Conclusion:

Several types of *in vitro* assays investigating estrogenicity have been conducted for the two substances: ER binding, ER mediated proliferation, ER mediated gene expression as well as ER transactivational assays. Overall, the response and potency of Substance X is similar to that of Substance Y.

One *in vivo* ADME study was identified from the open literature showing that Substance X is distributed to the gonads following oral administration.

There is strong evidence that Substance X acts via an estrogenic MoA, which in turn leads to adverse effects on the male reproductive system. The observation on Substance X reaching the gonads further supports a classification as ED; however is not a prerequisite for the classification decision.

Considering that there are no studies investigating endocrine-mediated adversity of Substance X, read across from Substance Y to Substance X is supported by similarities of the chemical structures, physico-chemical properties and by comparable estrogenic activity and potency observed *in vitro* of the two substances. Results from QSAR predictions is subject to uncertainties related to their sensitivity and reliability. There are also other factors that may entail differences in metabolism and bioavailability of the two substances *i.e.*, route of exposure, alkyl chain length, isomeric form, and plasma protein binding. However, acceptance of the read across is not influenced by these uncertainties.

Since Substance Y is classified as an ED for human health (*ED HH 1*), Cat. 1 is also justified for Substance X based on the read across approach and effects observed. Therefore, Substance X meets the criteria for classification as *ED HH 1*; EUH380.

SCL calculation:

Overall, the response and potency of Substance X is similar to that of Substance Y. Substance Y has a GCL.

Therefore, the GCL applies and there is no need to derive an SCL for Substance X.

3.11.5.2. Examples *ED HH 2*

3.11.5.2.1. Example 6 *ED HH 2* based on EAS (anti-androgenic effect)

Available information:

Human data:

No relevant information available.

Animal data:

Species	Type of study	Dose (mg/kg bw/d)	Effect	Maternal toxicity
Rat	2-gen old version of the OECD TG 416	0, 25, 100, 400	↓ abs. and rel. to brain epididymis weight (8%) in F2 gen at 400 mg/kg bw d stat. sign.; ↓ rel. to brain prostate weight (6%) in F2 gen at 400 mg/kg bw d stat. sign. ↓ testis weight (4%) F2 gen at 400 mg/kg bw d stat. sign. No changes were seen on epididymis, prostate and testes weights/histopathology in males from the F0 and F1 generation. No changes on sperm quality and offspring numbers.	not excessive
Rat	90-day study	0, 25, 100, 400	↑ testis weight (5%) F2 gen at 400 mg/kg bw d stat. sign. ↓ abs. and rel. to brain seminal vesicles weight (8%) in F2 gen at 400 mg/kg bw d, not stat. sign.;	Not relevant.
Rat	OECD TG 441 (Hershberger Assay)	0, 25, 100, 400	positive for anti-androgenic activity (stat. sign. ↓ organ weights: levator ani plus bulbocavernosus muscles (LABC) 5%, seminal vesicles/coagulating glands 7%)	Not reported.

In vitro data:

ToxCast Pathway estrogen receptor (ER) and androgen receptor (AR) models =>negative

MoA as herbicide: inhibition of the enzyme acetyl-coenzyme-A carboxylase.

Assessment:

Commented [A10]: Question to CARACAL

There are diverging views on the amount of data needed for classification. Examples on ED HH 2 classification will be highly useful. However, at this point in time, in contrast to ED HH 1, we do not have practical experience of type of evidence would result in ED HH 2. Similarly, we do not have practical experience on when 'no classification' is warranted. Based on the above, it may be pre-mature to give guidance on *ED HH 2*.

We would like to hear the CARACAL opinion this?

Adverse effect(s):

Adverse effect(s) on the EAS have been observed in male rats on prostate, epididymis and testes weights in the F2 population in the 2-generation toxicity study. Overall, the pattern of effects observed provide evidence for anti-androgen related adverse effect(s), however, only organ weight changes. No effects observed in females. Some slight support for the effect was seen in a 90 day study, but also contradicting results on testes weight.

Endocrine activity:

Positive Hershberger Assay for anti-androgenic activity effects however ToxCast pathway AR model negative.

Biological plausibility:

The pattern of effects observed is consistent with current knowledge. Both adverse effect(s) and endocrine activity support anti-androgenic MoA.

Conclusion:

It was concluded that the ED criteria for the A-modality are met and that a pattern of A mediated adversity exists, and it is substantiated by evidence of changes observed in prostate, epididymis and testes weights in the F2 population in the 2-generation toxicity study.

For Category 1 classification, effects on male reproductive organ weights should have been observed with higher magnitude (now only slight changes) and more consistently between generations and studies. EOGRTS would have brought added value for Cat 1 classification *e.g.* if nipple retention would have been observed. The adversity is based on the pattern of effects (reduced weights of epididymis, prostate and testes) seen in two studies in male reproductive organs that are consistent with the pattern for anti-androgen effects.

In contrary, no classification is not relevant here, since the pattern is clearly typical for anti-androgenicity, and higher doses, or more sensitive species could demonstrate progression of these effects to more severe and higher incidence.

For the EAS-modalities, positive outcome for the endocrine activity was reported in the Hershberger assay supporting the anti-androgenic MoA. The WoE is indicating some uncertainties for the A-modality because the ToxCast Pathway AR model is negative. However, due to the metabolising capacity of this substance, the Hershberger assay was positive, and this study has more weight that the AR ToxCast pathway model.

Therefore, the substance is suspected of causing endocrine disruption in humans, and the substance meets the criteria for classification as *ED HH 2*; EUH381. It should be noted that Category 1 classification cannot be excluded due to lack of data.

SCL calculation:

Method similar to 3.7.2 for the reproductive LOAEL of 30 mg/kg body weight/day effect. The estimated ED_{10} value, based on the top dose of 30 mg/kg body weight/day is suggesting a medium potency group (4 mg/kg body weight/day < ED_{10} value < 400 mg/kg body weight/day), no need for SCL based on effects related to reproductive toxicity.

(1) There are also ED related adverse effects in reproductive toxicity study. For these effects, the SCL calculation method from Section 3.7.2 was used.

The reproductive effects observed at 30 mg/kg body weight/day effect. The estimated ED_{10} value, based on the top dose of 100 mg/kg body weight/day is suggesting a medium potency group (4 mg/kg body weight/day < ED_{10} value < 400 mg/kg body weight/day), no need for SCL based on effects related to reproductive toxicity.

Conclusion on SCL: The ED GCL applies

3.11.5.2.2. Example 7 *ED HH 2* based on thyroid effect

Available information:

Human data: No relevant information available

Animal data:

Sub-acute toxicity study, 28 day, OECD TG 407, rat, dietary exposure, GLP, reliability 1;

Doses: 0, 100, 300, 1000 mg/kg body weight/day.

- ↑ Thyroid weight (10%, 15%, 17% (absolute), statistically significant at all doses and dose related. Relative weight change was 7%, 8%, 10% at 100, 300, 1000 mg/kg body weight/day, respectively.
- changes in colloid staining (dose-related increase in incidence) and hyperptrophia at top dose in 3 males. No other histopathological changes in thyroid.
- Increase in HDL/LDL ratio (10%) at top dose in same 3 males, not statistically significant.

Extended one-generation reproductive toxicity study (including DNT cohort, OECD TG 443), rat, dietary exposure, GLP, reliability 1; Doses: 0, 30, 100, 300 mg/kg body weight/day. At top dose MTD was reached without excessive toxicity.

- No effect on thyroid weight, histopathology, or other findings related to endocrine disruption.
- No thyroid hormone or TSH measurements were performed in any generation or cohort.

In vitro data:

DIO2 assay negative

Assessment:

Adverse effect(s):

Adverse effect(s) on the thyroid have been observed in rats.

Overall, the pattern of effects observed provide evidence for endocrine-related adverse effect(s).

Endocrine activity:

THs or TSH were not measured in the study which decreases the reliability of the OECD 443 study, however, classification based on T modality for even Cat 1 without data on thyroid hormones according to this guidance is possible. However, the observed thyroid

Commented [A11]: Question to CARACAL:
There are diverging views on whether this is an example example of *ED HH 1, ED HH 2 or 'no classification'*

Are the thyroid effects "weak enough" to justify *ED HH 2* but still strong enough to justify classification?

We would like to hear the CARACAL opinion this?

hypertrophy in a 28-day study infers increased TSH. The increased TSH is likely a compensatory mechanism caused by reduced serum THs. The increased total cholesterol provides supporting evidence for this assumption because this is a key event downstream of reduced serum THs.

The mechanistic information is limited to a DIO2 inhibition assay. The results of this assay suggest that reduced THs due to altered TH metabolism is likely not the cause of the observed effect.

The other possible thyroid MoA have not been investigated and can therefore not be excluded.

Overall, the pattern of effects observed provide evidence for thyroid-related endocrine activity.

Biological plausibility:

The pattern of effects observed is consistent with current knowledge and the fact that both adverse effect(s) and endocrine activity were observed in the same study at similar doses demonstrates that the effects are biologically plausible.

Conclusion:

There is evidence on adverse effect(s) (thyroid follicular cell hypertrophia, increased thyroid weight, and increased total cholesterol indicating reduced THs) indicative of T mediated adversity fromone study, however, results from a highly reliable OECD 443 study in the same dose range show no adverse effects on thyroid weight, histopathology, or other findings related to endocrine disruption. Due to these discrepancies and low magnitude of effects, classification for Cat 1 is not met. Therefore, the substance meets the criteria for classification as *ED HH 2*; EUH381.

SCL calculation:

Method similar to 3.9.2 for 30 mg/kg body weight/day effect. SCL Cat2 = 100/(100x100)x100% = 1.0 %

Conclusion on SCL: GCL of 0.1% applies.

3.11.5.2.3. Example 8 ED HH 2 based on non-EATS (Increased resistance to insulin)

Available information:

Human data:

No relevant information available.

Animal data:

Subacute study, 14 days by oral route (gavage) in Sprague-Dawley Rat, 400-1,500 mg/kg body weight/day:

- No effect on adrenal weights (absolute and relative),
- ↗ statistically significant of relative testes weight from 700 mg/kg body weight/day,
- ↗ statistically significant of cholesterol levels in most females from 700 mg/kg body weight/day and in male rats at the high dose,

Commented [A12]: Question to CARACAL

There are diverging views on the usefulness of this non-EATS example, deletion has been suggested.

ECHA is of the opinion that there may be situations where ED HH 2 classification may be warranted for a non-EATS modality. However, at the given time we are not able to illustrate this in an example (without making it very case-specific). ECHA is in favour of deleting

We would like to hear the CARACAL opinion this?

Subchronic study, 90 days by oral route (gavage) in male Sprague-Dawley Rat, 150 - 1,100 mg/kg body weight/day:

- ↗ adrenal relative weight in males without a clear pattern,
- Significant ↗ of cholesterol levels in most male rats without a clear dose-response relationship,
- No effect on the (absolute and relative) weights of testes, ovaries and adrenals,

Subchronic study, 90 days by oral route (gavage) in male Sprague-Dawley Rat, 150 - 1,100 mg/kg body weight/day:

- No effect on testes weights (absolute and relative) without apparent pathological changes,
- No effect on liver, kidneys, testes, and lungs weights nor histological changes,
- Cholesterol not measured.

Sub-chronic study, 90 days by oral route (drinking water) in male Sprague-Dawley rats, 0; 50, 300 or 1000 µg/L corresponding approximately to 5, 30 and 100 µg/kg body weight/day with an additional group receiving 1000 µg/L of the tested substance + 7.5 mg/L zinc acetate.

- ↗ statistically significant of triglycerides, cholesterol, HDL and LDL plasma levels from the mid or high dose and ↗ statistically significant of fasting glycaemia at the highest tested dose. These effects were counteracted by Zn supplementation,
- ↗ Cu^{2+}/Zn^{2+} plasmatic ratio, C-reactive protein dose-dependently,
- ↗ calcium levels at the highest tested dose and ↘ HDL were not counteracted by Zn supplementation.
- ↘ Ins1 and ins2 (coding for Insulin) gene expression in pancreas (except at the lowest dose), not counteracted by Zn supplementation.

Sub-acute study, 15 or 28 days by oral route (gavage) in Male Sprague-Dawley rats (28–30 day old), 0, 300, 750 and 1700 mg/kg body weight/day:

- ↗ cholesterol at 1700 mg/kg body weight/day after 2 weeks of treatment,
- ↗ cholesterol at the lowest tested dose level (300 mg/kg body weight/day) after 4 weeks of treatment.

<u>Level 3 additional studies</u>

Subacute study, one month by oral route (gavage) in ApoE knockout male mice, 0.1 or 1 mg/kg bw/day, in males fed a high fat diet:

- Signif. ↗ oil red O staining,
- No effect on lipidemia or inflammation.

Sub-chronic study, 14 weeks by oral route (gavage) in male and female mice (8 week-old) receiving standard or high fat diet, 0.15, 0.9 or 90 mg/kg body weight/day:

- **In males fed a high-fat diet:**
  - ↗ visceral white adipose tissue (WAT) weight resulting from adipocyte hypertrophy with induced adipocyte differentiation and reduced insulin sensitivity.
- **In males fed a standard diet**:
  - no metabolic effects.

- **In females fed a standard diet**:
  - ↘ plasma TG , no effect on insulin level, or on the glucose tolerance and insulin metabolic tests.
- **In females fed a high-fat diet:**
  - signif. hypertrophy of the visceral adipocytes at 90 mg/kg body weight/day together with slightly increased sensitivity to insulin (metabolic test).

Additional studies conducted *in-vitro* model:

In vitro studies on 3T3-L1 cell:

- not affect hyperplasia but adipogenesis at concentrations of 0.1 and 1 mM
- ↗ in the red O oil staining: increase of intracellular lipids,
- ↗ Fatty acid binding protein 4 (FABP4) and PPARG mRNA levels.
- ↘ glucose uptake indicating ↘ insulin sensitivity (↘ index of insulin sensitivity).

Assessment:

Adversity:

The following adverse effects were observed *in vivo*:

- increased adrenal weight (not consistent in studies)
- increased cholesterol
- increased WAT
- increased intracellular lipids
- visceral adipocyte hypertrophy
- decreased insuline sensitivity
- increased foam cell formation in the atherosclerotic plaques.

Clinical chemistry effects such as changes in cholesterol and insulin levels, can be adverse when of biological relevance.

Endocrine activity:

Positive indications for endocrine activity stem from the effect on PPARG and FABP4 (previously called aP2) expression in an appropriate cell line. Furthermore, changes in expression of Ins1 and Ins2 were observed *in vivo*.

Biological plausibility:

The literature available on FABP and in particular FABP4, its negative feedback loop exerted to control PPARγ receptor signaling may explain the loss of insulin sensitivity, as PPARγ induces insulin sensitivity. Furthermore Fabp4$^{-/-}$ mice are protected from obesity-induced insulin resistance, whereas exogenous FABP4 administration impairs insulin sensitivity. In human beings, a polymorphism in the promoter of FABP4 that results in reduced expression of this gene was associated with reduced risk of developing type 2 diabetes mellitus and reduced risk of coronary heart disease among patients with obesity. In addition, adipocyte dysfunction may have also consequences to sensitivity to insulin. Thus, the biological plausibility that the identified MoA (*via* FABP4 and PPARγ) induces insulin resistance is considered sufficient.

Even if decreased insulin sensitivity is not yet described as an ED-mediated adverse effect, existing knowledge describes the involvement of these nuclear receptors in decreased insulin sensitivity and diabetis mellitus.

Overall, based on current understanding of endocrinology and physiology, the close interaction between FABP4 and PPARG and the contribution of FABP4 to the pathogenesis

of diabetes mellitus, the criteria for biological plausibilty are fulfilled

Conclusion:

It should be noted that effects on glucose homeostasis cannot clearly be demonstrated in guideline studies and are normally studied using specific functional tests such as the glucose tolerance test (GTT) and the insulin tolerance test (ITT) (Kozlova *et al.*, 2023). Note that a high fat diet is not necessarily required to study effects on glucose homeostasis. There is evidence on adversity with a decreased insulin sensitivity and increased visceral fat mass especially if mice were fed a high-fat diet. Adipocyte dysfunction was also observed which may have consequences to sensitivity to insulin. Lastly, a rather consistent increase of cholesterol levels was observed in repeated toxicity studies as well as a promoted foam cell formation in the atherosclerotic plaques.

There is evidence of endocrine activity based on the interaction of this substance with FABP4 and PPARG. However, the endocrine activity is reported in a single study which increases uncertainty supported also by absence of pattern of effects and endocrine activity, and the evidence is therefore not sufficiently convincing to classify as *ED HH 1* but. Therefore, the criteria for classification as *ED HH 2*; EUH381 are fulfilled.

SCL calculation:

150 mg/kg body weight/day was used in SCL calculation since it was lowest dose causing adversity subject to classification (an increase of visceral WAT weight resulting from adipocyte hypertrophy with induced adipocyte differentiation and reduced insulin sensitivity). Method similar to 3.9.2 for 150 mg/kg body weight/day effect. SCL Cat2 = 150/(100x100)x100% = 0.15 %

Conclusion on SCL: The method similar to 3.9.2 resulted in a high potency group corresponding to a SCL of 0.15 %.

3.11.5.3. Examples no classification

If the overall strength of evidence is not convincing enough to place a substance in *ED HH 2* then no classification is warranted.

Commented [A13]: Question to CARACAL:
ECHA suggests to delete the no classification examples as they can all be summarised with one sentence: "*If the overall strength of evidence is not convincing enough to place a substance in ED HH 2 then no classification is warranted.*"

Based on the CLP ED criteria there must be evidence on all three elements: adversity, endocrine activity and a biologically plausible link. If one of them is missing the ED criteria are not met.

We would like to hear the CARACAL opinion this?

3.11.5.3.1. Example 9 ED HH No classification (EAS activity)

Available information:

Human data:

No relevant information available.

Animal data:

Combined Repeated Dose Toxicity Study with the Reproductive/ Developmental Toxicity Screening Test (OECD TG 422), GLP, reliability 1, Doses: 0, 100, 300, 1000 mg/kg body weight/day

- ↑ Post-implantation loss at highest dose (non-significant)
- ↑ Epididymis weight (relative),
- No effect on estrous cycle, sperm count and other sperm parameters, sexual organ histopathology or fertility

Extended one-generation reproductive study (OECD TG 443) with F2, GLP, reliability 1, Doses: 0, 100, 300 and 1000 mg/kg body weight/day

- F1 pups (highest dose): significantly reduced body weight, ↓ and slight AGD but no significant effect on AGDi. and Non-significantly higher nipple retention in F1 pups (but significantly lowerno effect in F2). but no variation of AGDi.
- No effect on estrous cycle, ovaries, uterus, testis/epididymis weight, sperm count and other sperm parameters or fertility

A Hershberger assay of low reliability showed slight anti-androgenic activity.

Other information:

CLH conclusion on reproductive toxicity: no classification for sexual function and fertility or development.

In vitro data:

Several in vitro studies showed a weak estrogenic activity (ER binding and activation). Contradictory results on anti-androgenic activity.

No other literature data available.

Assessment:

Adverse effect(s):

There are no adverse effects in well conducted guideline studies. The well performed and reliable EOGRTS did not show obvious adverse effect, except in F1 pups of the highest dose group, for which AGD was slightly reduced. However, the body weight was reduced in F1 pups leading to an absence of effect on the AGDi. The effects on nipple retention were apparently contradictory (significantly lower in F2 and higher in F1 pups).

Overall, these effects are too weak and give only some indications of adverse effects.

Endocrine activity:

The substance shows weak estrogenic and anti-androgenic activity.

Biological plausibility:

Does not apply here since there are no clear adverse effects, the biological plausible link cannot be demonstrated.

Conclusion:

Slight inconsistent EAS-mediated effects on AGD and nipple retention have been observed in the higher tier study of good quality, but they are considered weak and inconclusive. Although supported by other mechanistic alerts raised in *in vitro* studies and *in vivo* bad quality studies, they are not considered sufficient altogether to demonstrate an endocrine adverse effect and classification.

Therefore, the substance cannot be identified as ED HH.

3.11.5.3.2. Example 10 ED HH No classification (EAS activity)

Available information:

Human data:

No relevant information available.

Animal data:

Extended one-generation reproductive toxicity study, EOGRTS (OECD TG 443) in rat, 100, 300 and 1000 mg/kg body weight/day, all parameters measured F1, F2, Cohorts 2A, 2B and 3.

- Extended transient oestrus cycles at weeks 1 and 2 after VO, no effects on cycle parameters thereafter
- No other effects on any EAS-related parameters (AGD, NR, semen quality, reproductive organ weight or histopathology, timing of sexual maturation, or ovarian follicle count) were seen in the TG443 in any cohorts or generation.

Uterotrophic study in ovariectomised rats (OECD TG 440) 100, 300 and 1000 mg/kg body weight/day

- No change in uterus weight (uterotrophic assay negative) , hormonal measurements all negative in females.

Hershberger assay (OECD TG 441) in castrated male rats (broadly in line with OECD 441) also measured serum LH and FSH 100, 300 and 1000 mg/kg body weight/day. Measurements of testosterone, oestradiol, FSH and LH in males for 14 days were all negative.

- No change in androgen-sensitive organ weights (Hershberger negative)
- levels of 17a-hydroxyprogesterone was the only positive effect

In vitro data:

- ↓ aromatase activity

- Evidence for agonism and/or antagonism of AER inconsistently observed across multiple non-guideline studies

- Evidence for antagonism (but not agonism) of AR across multiple nonguideline studies androgen receptor assays

- ↓ progesterone, estradiol, estrone, and testosterone synthesis across multiple non-guideline studies

Assessment:

Adverse effects:

No consistent pattern of adverse effects.

Endocrine activity:

Some evidence for endocrine activity without a clear direction in level 2 studies, but no effects in level 3 studies.

Biological plausibility:

No clear link between endocrine activity and adverse effects.

Conclusion:

In vitro evidence of steroidogenesis and AER interaction without *in vivo* correlate, since there was no adverse effect observed *in vivo* except slight transient effect on estrous cycle on first two weeks after VO which was not considered adverse in this case since it was slight, transient and single isolated finding which is not considered sufficient for classification. Evidence of *in vitro* activity that does not manifest as effects *in vivo*.

3.11.5.3.3. Example 11 ED HH No classification (thyroid)

Available information:

Human data:

No relevant information available.

Animal data:

Short-term repeated dose toxicity study (OECD TG 407), GLP, reliability 1, 0, 100, 300, 1000 mg/kg body weight/day.

- ↑ Absolute and relative weight of thyroid, only at 1000 mg/kg body weight/day in both male and females, not statistically significant.
- Thyroid follicular cell hypertrophy (severity: mild) observed in 2/5 males and 1/5 females at top dose, not statistically significant. 3/10 animals with mild thyroid hypertrophy did not have individually more than +5% increased weight in thyroid.
- THs were not investigated

In vitro data:

No relevant information available

Assessment:

Adverse effect(s):

Overall, the pattern of effects observed provide weak evidence for thyroid-related adverse effect(s) which are not sufficient for classification in the absence of further supporting evidence on adverse effect(s).

Endocrine activity:

Endocrine activity is inferred by the thyroid-related adverse effect(s).

Overall, there are evidence for thyroid-related endocrine activity.

Biological plausibility:

The pattern of effects observed is consistent with current knowledge. Adverse effect(s) are thyroid-mediated and imply endocrine activity.

and the fact that both adverse effect(s) and endocrine activity were observed in the same study at similar doses supports that the effects are biologically plausible.

Conclusion:

There is very little evidence for T-mediated adversity and activity both inferred from a slight increase in thyroid follicular hypertrophy. There is a low severity (5% thyroid weight increase ats 1000 mg/kg body weight/day) and low incidence of histological effects (the

latterboth not even statistically significant). No further mechanistic information (such as TH and TSH measurements, or studies on the MIE) is available. Since hypertrophy was mild, not statistically significant, and observed in a very few animals at very high dose and the thyroid weight increase was also very mild without statistical significance and no other thyroid related effects were observed, there is no clear evidence that the effects would be treatment related or sufficient concern for being adverse to be able to classify for Cat. 2.

Therefore, no classification is warranted due to overall insufficient evidence/information and lack of data. A conclusive classification decision cannot be made due to missing CF level 4 and 5 studies.

3.11.5.3.4. Example 12 ED HH No classification (non-EATS, reduction in cholesterol)

Available information:

Human data:

No relevant information available

Animal data:

Repeated dose toxicity study with reproduction/developmental toxicity screening Subchronic study (OECD TG 422), 42 (m)/63 (f) days by oral gavage in Sprague-Dawley rats, 0, 50, 300, 1000 mg/kg body weight/day:

- No treatment-related mortalities occurred.
- ↓bw in males at 1000 mg/kg body weight/day.
- ↑ liver enzymes (ALT and AST) at 1000 mg/kg body weight/day in both sexes.
- ↓ cholesterol at 300 mg/kg body weight/day and above in both sexes, without clear dose-response. No statistical significant effect on other clinical chemistry parameters, including triglycerides.

Sub-chronic study (OECD TG 408), 90 days by oral gavage in Sprague-Dawley rats, 170, 750, 3000 mg/kg body weight/day:

- No treatment-related mortalities occurred.
- ↑hepatocellular hypertrophy and slight increase in absolute and relative liver weight at 750 and 3000 mg/kg body weight/day in both sexes.
- ↑ liver enzymes (ALT and AST) at 3000 mg/kg body weight/day in both sexes.
- ↑ cholesterol at 3000 mg/kg body weight/day in females. Triglycerides not measured.

Sub-acute study (OECD TG 414), GD6-19 by oral gavage in pregnant Sprague-Dawley rats, 50-1000 mg/kg body weight/day

- No treatment-related mortalities occurred.
- No organ weight or histopathology performed on dams.
- Cholesterol not measured.

I*n vitro* data:

ToxCast Attagene TRANS-FACTORIAL HepG2 Human Peroxisome Proliferator-activated Receptor Gamma (PPARg) Activation Assay (ATG_PPARg_TRANS_up), concentrations of 0, 4, 10, 40, 125, and 500 µM in triplo.

- At 500 µM, a 1.4-fold induction was seen compared to the control, which was above the assay's cut-off of 1.15.

- At 500 µM, cytotoxicity was seen as well, with 80% cell viability compared to the control.

Assessment:

Adversity:

A change decrease in serum cholesterol was found in one study in both sexes (OECD TG 422) and increase in another study (OECD TG 408) in one sex only (females). Mild liver toxicity was also observed in these studies. However, effects on cholesterol seen in the two studies, were not consistent in the direction of the effect, and decrease in cholesterol alone is not considered adverse with biological relevance. No statistically significant effect on triglycerides was measured in the OECD TG 422, but no effect was observed up to the highest dose tested. In the OECD TG 408, triglyceride levels were not evaluated.

Endocrine activity:

Using the US EPA Chemistry dashboard, one *in vitro* study was identified, noting a mild induction of PPARgamma at a cytotoxic concentration. No other mechanistic data is available for this substance.

Biological plausibility:

Activation of PPARgamma is associated with changes in lipid and glucose homeostasis. Biological plausibility between the change in cholesterol and the activation of PPARgamma activation is generally associated with an increase in plasma high-density lipoprotein cholesterol, a decrease in plasma triglycerides. It is however difficult to establish a clear biological plausible link because of the uncertainty both related to the evidence on adversity and endocrine activity.

Conclusion:

There is not sufficient information for either the criteria on adversity or endocrine activity to be met. Because 1) endocrine activity was observed *in vitro*, but only at a concentration that also induced cytotoxicity 2) a inconsistent change in cholesterol was noted in two studies *in vivo* where decrease in cholesterol was more clear than an increase. Decrease in cholesterol alone is not considered adverse with biological relevance and inconsistency 3) a biologically plausible link could not clearly be established because of a lack of endocrine activity.

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4. ENV

4.2. Endocrine disruption for environment

Relationship with the ECHA/EFSA ED Guidance on assessing endocrine disrupting properties for biocidal products and plant protection products

The ECHA/EFSA ED Guidance on assessing endocrine disrupting (ED) properties (ECHA/EFSA, 2018), which builds on the '*Revised guidance document 150 on standardised test guidelines for evaluating chemicals for endocrine disruption*' (OECD GD 150; OECD, 2018a), was developed to assist applicants and assessors of the competent regulatory authorities in complying with their obligations to conclude on ED properties for biocidal products (BPs) and plant protection products (PPPs). More specifically, the ECHA/EFSA ED Guidance describes how to gather, evaluate and consider all relevant information for the assessment, apply a weight of evidence (WoE) approach and conduct a mode of action (MoA) analysis, in order to help in establishing whether the substance meets the criteria for approval under the BP[14] and PPP[15] Regulations. Therefore, the ECHA/EFSA ED Guidance remains the key piece of guidance for scientific assessment of ED properties of BPs and PPPs.

In 2023, endocrine disruption was introduced into CLP as a hazard class with sub-categorisation. CLP covers classification of hazardous substances and mixtures across regulations and applies (among others) to industrial substances (subject to the REACH Regulation[16]), BPs and PPPs. Notably, CLP does not require the generation of any new data for the purpose of CLP classification and, therefore, ED classification needs to be based on available data. Consequently, the format of the CLP guidance and that of the ECHA/EFSA ED Guidance are different owing to the regulatory framework. For hazard classification purposes this guidance on the application of the CLP criteria should be followed for all substances and mixtures.

Despite differences in the framework, it is important to note that the current ED criteria for BPs and PPPs are derived from the same basis as the ED hazards in Category 1 for human health (*ED HH 1*) or the environment (*ED ENV 1*) under the CLP criteria. While the format of this guidance on CLP and the ECHA/EFSA ED Guidance may differ due to the differences in scope of the applicable legislation, the guidance to arrive at a conclusion for ED hazards in Category 1 is largely equivalent and based on a similar scientific assessment in both documents.

[14] Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products Text with EEA relevance. OJ L 167, 27.6.2012, p. 1–123. Available online: http://data.europa.eu/eli/reg/2012/528/oj

[15] Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. OJ L 309, 24.11.2009, p. 1–50. Available online: http://data.europa.eu/eli/reg/2009/1107/oj

[16] Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. OJ L 396, 30.12.2006, p. 1–849. http://data.europa.eu/eli/reg/2006/1907/oj

Accordingly, active substances already concluded to meet the ED criteria under the BP[17] and PPP[18] procedures before the criteria in CLP became applicable, should under CLP be assigned to *ED HH 1* or *ED ENV 1*. Similarly, substances identified as Substances of Very High Concern (SVHC) under REACH due to ED properties should also be assigned to *ED HH 1* or *ED ENV 1* under CLP.

4.2.1. Definitions and general considerations for endocrine disruption

The classification for endocrine disruption for the environment, similar to classification for ED for human health, refers to a specific (endocrine) MoA which leads to an adverse effect(s). The classification criteria require evidence on three elements, *i.e.*, adverse effect(s), endocrine activity, and a biological plausible link between the endocrine activity and the adverse effect(s) consistent with existing knowledge.

> **CLP, Annex I, Section *4.2.1.1.*** *For the purposes of Section 4.2., the following definitions shall apply:*
>
> *(a) 'endocrine disruptor' means a substance or a mixture that alters one or more functions of the endocrine system and consequently causes adverse effects in an intact organism, its progeny, populations or subpopulations;*
>
> *(b) 'endocrine disruption' means the alteration of one or more functions of the endocrine system caused by an endocrine disruptor;*
>
> *(c) 'endocrine activity' means an interaction with the endocrine system that may result in a response of that system, of target organs or target tissues and that confers on a substance or mixture the potential to alter one or more functions of the endocrine system;*
>
> *(d) 'adverse effect' means a change in morphology, physiology, growth, development, reproduction or lifespan of an organism, system, population or subpopulation that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences;*
>
> *(e) 'biologically plausible link' means the correlation between an endocrine activity and an adverse effect, based on biological processes, where the correlation is consistent with existing scientific knowledge.*

The definitions in CLP Annex I, Section 4.2.1.1. are further explained below:

(a) The definition of '*endocrine disruptor*' (ED) in this guidance is based on the WHO/IPCS definition (WHO/IPCS,2002). It has been modified for the purposes of classification under CLP.

The definition uses the term "*intact organism"* which is understood to mean that the effect would occur *in vivo*, observable in a test animal system. However, it does not necessarily mean that an adverse effect has to be demonstrated in an intact

[17] Commission Delegated Regulation (EU) 2017/2100 of 4 September 2017 setting out scientific criteria for the determination of endocrine-disrupting properties pursuant to Regulation (EU) No 528/2012 of the European Parliament and Council. OJ L 301, 17.11.2017, p. 1–5. Available online: http://data.europa.eu/eli/reg_del/2017/2100/oj

[18] Commission Regulation (EU) 2018/605 of 19 April 2018 setting out scientific criteria for the determination of endocrine-disrupting and amending Annex II to Regulation (EC) 1107/2009. OJ L 101, 20.4.2018, p. 33–36. Available online: http://data.europa.eu/eli/reg/2018/605/oj

test animal.

The 'endocrine system' in this context consists of hormone-producing tissues and their associated hormones that regulate the functioning of the organism.

(b) An ED may alter one or more functions of the endocrine system, *e.g.*, hormonal synthesis, transport, signalling, regulation or metabolism.

(c) A substance that has an '*endocrine activity*' has the potential to interact with and alter the function(s) of the endocrine system, target organs and tissues. This interaction may occur at any level in a biologically plausible sequence of events leading to an adverse effect.

(d) The definition of '*adverse effect*' is based on the WHO definition (WHO/IPCS, 2009). The definition of adversity is generic and not specific to the assessment of ED properties. Current practices from other hazard classes for assessing adversity are applicable for deciding whether the observed effects are treatment-related and should be considered adverse.

(e) The '*biologically plausible link*' relies on an understanding of the fundamental biological processes involved and whether they are consistent with the sequence of the events proposed. The term '*correlation*' used in the definition means that endocrine activity and adverse effect(s) can be plausibly linked (connected) using existing knowledge as the most likely explanation for the observed effects; a causal relationship does not need to be proven.

In a MoA analysis, biological plausibility is considered to be the level of support for the links (connections) between the adjacent key events in the postulated MoA, *i.e.* the key event relationships (KERs); see Section 4.2.2.3.4.

In addition, data with '*equivalent predictive capacity*' are defined as data obtained using alternative methods which can be used with a similar level of confidence as internationally recognised *in vivo* methods or human data, to predict adversity or endocrine activity. Alternative methods do not need to be one-to-one replacements of an internationally recognised *in vivo* method, but can be *e.g.* a set of *in vitro* or *in silico* methods which together meet the requirement of equivalent predictive capacity, see Sections 4.2.2.1.2 and 4.2.2.3.1.

CLP, Annex I, *Section 4.2.1.2.1.* *Substances and mixtures fulfilling the criteria of endocrine disruptors for the environment based on evidence referred to in Table 4.2.1 shall be considered to be known, presumed or suspected endocrine disruptors for the environment unless there is evidence conclusively demonstrating that the adverse effects identified are not relevant at the population or subpopulation level.*

More explicitly, substances or mixtures are classified as '*known or presumed'* or as '*suspected*' EDs for the environment if they induce adverse effects in wildlife which have a consequence on the maintenance of the population or subpopulation[19], by altering the function of the endocrine system, *i.e.*, the substance has an endocrine MoA, in accordance

[19] The term "subpopulation" is of predominant relevance with respect to humans where it indicates a subset of the population with distinguishing characteristics (for example children are the most sensitive subpopulation). However, it may also be used in the environmental context to refer to a subset of the population within an ecosystem in a particular region or habitat with corresponding genetic clustering. The term population will be used in the subsequent Sections of this guidance to mean population or subpopulation.

with the criteria given in CLP, Annex I, Section 4.2.2.1.

> ***CLP, Annex I, Section 4.2.1.2.2.*** *Evidence that is to be considered for classification of substances in accordance with other Sections of this Annex may also be used for classification of substances as an endocrine disruptor for the environment where the criteria provided in this Section are met.*

In other words, all relevant information for the determination of endocrine disruption for the environment is to be considered together. This includes information also considered in relation to the criteria for aquatic toxicity, information from other aquatic or non-aquatic species (*e.g.*, birds, invertebrates) and information related to endocrine disruption for human health (see Section 4.2.2.3.5 of this guidance).

Classification as ED for the environment is intended to indicate that a substance may cause an endocrine related adverse effect. The sensitivity to such effects may depend on the life-stage investigated. Depending on the type of effect some life stages may be more sensitive than others.

In order to classify a substance as ED for the environment, the adverse effects need to be relevant at the population level. See Section 4.2.2.3.2 on population relevance.

It is sufficient that the substance meets the CLP ED criteria in one taxonomic group in order to conclude that a substance meets the CLP ED criteria for the environment.

The classification for endocrine disruption for the environment is independent of the classification of other hazard classes, including classification as *ED HH*. A substance may or may not be classified for endocrine disruption for environment using the same evidence irrespectively of whether the substance is also classified for other hazard classes.

In addition, the classification of a substance as an ED for the environment Category 1 or 2 (or no classification) is independent of the classification of the substance for human health *ED HH 1* or *2* or no classification. Therefore, a classification for *ED ENV* does not automatically translate into a classification for *ED HH* and vice versa. For example, a substance can be classified as *ED ENV 2* or not classified, even if it is classified as *ED HH 1*.

The concept of ED "potency" is considered only in the context of setting specific concentration limits, see Section 4.2.2.6. The CLP criteria for endocrine disruption for the environment do not specify any dose/concentration above which the production of an adverse effect is considered to be outside the criteria which lead to classification. In other words, the criteria apply to all dose/concentration levels. Even endocrine-related effects observed at high doses/concentrations (showing low potency) may still warrant classification.

The ED effect may be a threshold or a non-threshold effect, see the JRC report on `Thresholds for Endocrine Disrupters and Related Uncertainties´ (Munn and Goumenou, 2013). When the ED adversity is observed already at very low doses/concentrations (high potency) or alternatively only at very high doses/concentrations (low potency), this guidance considers that potency can be regulated by setting a specific concentration limit, which can be either lower, or in exceptional cases higher, than the generic concentration limit. For setting an SCL, a careful assessment on doses or concentrations causing adversity is recommended for all substances.

ED modalities covered under CLP

The CLP criteria apply to all endocrine modalities. While the CLP criteria do not

differentiate between different modalities, thus covering all endocrine-disrupting MoAs, it is acknowledged that this guidance mainly addresses the effects caused by estrogen, androgen, thyroid, and steroidogenic (EATS) modalities.

This is because the EATS modalities are the pathways for which there is currently the most knowledge available, *i.e.*, there is relatively good mechanistic understanding on how substance-induced perturbations may lead to adverse effects via an endocrine-disrupting MoA. In addition, only for the EATS modalities there are at present standardised test guidelines for *in vivo* (EATS) and *in vitro* (EAS) testing available where there is broad scientific agreement on the interpretation of the effects observed on the investigated parameters. Further information on EATS modalities can be found in Section 4.2.2.3.1.

However, the general principles outlined in this guidance for evaluation of the data on the different criteria, WoE and decision on classification, are also applicable to non-EATS modalities. The existing knowledge for those modalities is not as advanced as that for the EATS modalities and future research is needed for a better understanding of non-EATS modalities. However, in some cases it may be possible to reach a conclusion on the need to classify the substance based on a non-EATS MoA. For example, where scientific knowledge provides mechanistic information, that can be linked to adverse effects measured in standard tests. One example is related to effects on fecundity that could potentially occur also due to inhibition of retinoic acid. Other examples of non-EATS modalities can involve *e.g.*, juvenile hormones, ecdysone or peroxisome proliferator-activated receptor-gamma (PPARγ) related endocrine disruption. It should be noted that ligands to some of these receptors (*e.g.*, retinoids binding to the retinoic acid receptor, fatty acids binding to PPARγ) may not fit the conventional view of a hormone. Nonetheless, these ligands do fit the broad definition of a hormone as a substance, originating in one tissue and conveyed by the bloodstream to another tissue to exert physiological activity (OECD, 2012).

4.2.1.1. Taxa covered

Based on the current knowledge and understanding of the endocrine system as well as on the available testing methods, the current guidance, in line with the ECHA/EFSA ED Guidance, focuses on vertebrate organisms, mainly fish and amphibians. For other vertebrate taxa (besides mammals), like birds and reptiles, there are, currently, no standard methods which investigate endocrine specific endpoints. Similarly, due to the scarce knowledge on the endocrinology for invertebrates, this guidance does not specifically cover those organisms.

Nevertheless, the general principles outlined in this guidance for evaluation of the data on the different criteria, WoE and decision on classification, are also applicable to those organisms. Therefore, if available, information on invertebrates, birds and reptiles should be assessed and can be used to conclude on the need to classify the substance as *ED ENV*.

Data and effects on plants are not under the scope of this hazard class.

4.2.2. Classification of substances for endocrine disruption for the environment

4.2.2.1. Identification of hazard information

CLP does not set information requirements or require further testing of substances and mixtures for classification purposes (CLP, Articles 5, 6 and 9) except for physical hazards (CLP, Article 8.2). The assessment is based on the respective criteria and consideration of

all available relevant information. All relevant information that addresses endocrine-related adverse effects and activities shall be considered in a WoE approach; this includes guideline and research studies as well as alternative methods such as read-across and *in silico* predictions.

The main ways to gather all available information is collecting studies and data from the registration dossiers, *e.g.* under REACH, BPR, PPPR, and by conducting a literature search or preferably a systematic literature review designed to avoid bias and capture as much as possible relevant scientific literature data. Further guidance is available in ECHA/EFSA ED Guidance, Section 3.2 and Appendix F. Additionally, previous regulatory assessments may serve as a starting point for the literature search. Furthermore, information considered for other hazard classes may also provide information relevant for endocrine disruption classification for environment; see 3.6.2.1; 3.7.2.1; 3.9.2.1; 4.2.2.1.

Commented [A14]: Links to other parts of the CLP Guidance to be added (relevant sections indicated in yellow)

Upon reviewing the literature, the information is deemed relevant when it investigates or brings information for the assessment of at least one of the three elements: *i.e.* endocrine activity, adverse effects or biologically plausible link:

- Information on endocrine-related *'adverse effects'* for the environment is normally obtained from animal chronic studies. However, when available, non-animal methods or strategies (if providing an equal predictive capacity as animal data) may bring sufficient information on adversity for decision making on classification, particularly when supported by toxicokinetic data. Information on adversity may also be obtained using read-across or analogy, *e.g.* if the substances by analogy share a common MoA, or using read-across between substances with a common active metabolite, or compounds with a different ratio of the same isomers, etc.

- Information on *'endocrine activity'* generally comes from *in vivo* or *in vitro* mechanistic studies. Information may also come from read-across, *in silico* models or omics approaches, if available. In addition, endocrine activity may also be inferred from observed adverse effects known to be mediated by endocrine activity, see '*EATS-mediated*' parameters in Section 4.2.2.3.1.

- A *'Biological plausible link'* does not need to be demonstrated with substance specific data, but existing scientific knowledge can be used, *e.g.*, textbooks and peer-reviewed scientific literature. AOPs can be helpful to establish biological plausibility, but they are not a prerequisite. Several AOPs related to endocrine disruption have been endorsed, see *e.g.*, OECD Series on AOPs[20]. There is continuous development of additional AOPs in various stages in the AOPwiki[21]. It should be noted that the presence of an AOP in the AOPwiki does not necessarily indicate its relevance or reliability. Depending on the stage of development of the AOP in AOPwiki, the amount of data needed to support biological plausibility may vary considerably. The validity of an AOP should be considered using expert judgement.

4.2.2.1.1. Identification of animal data

Information considered for other hazard classes *e.g.*, hazardous to aquatic environment, (Section 4.1 of this guidance) as well as information relevant for endocrine disruption for human health (Section 3.11 of this guidance) and information on birds, reptiles, or invertebrates may also provide information relevant for endocrine disruption for the

[20] https://www.oecd-ilibrary.org/environment/oecd-series-on-adverse-outcome-pathways_2415170x
[21] aopwiki.org

environment.

All relevant information that addresses endocrine-related adverse effects and activities shall be considered in a WoE approach; this includes guideline and research studies as well as alternative methods such as read-across and *in silico* predictions.

Animal studies to be considered for classification of substances as EDs for the environment are outlined in the OECD GD 150 'Revised guidance document on standardised test guidelines for evaluating substances for endocrine disruption'. This document provides widely accepted guidance on the interpretation of effects measured in relevant OECD test guidelines, and other standardised test methods, which may arise as a consequence of perturbations of the EATS modalities. It explains how these effects may be evaluated to support identification of EDs.

The OECD GD 150 includes the OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Substances (OECD CF; OECD, 2012) which lists the OECD test guidelines and standardised test methods available in 2018 that can be used to evaluate substances for endocrine disruption. It is not an exhaustive list and tests and assays other than those described in the list (*i.e.* other published or internationally recognised methods) may also be valuable for assessing substances for endocrine disruption and can also be used for classification if they are relevant and considered predictive for wildlife. Research studies are an important source of information which must be considered in a WoE approach. New tests are continually being developed, which may provide useful information for classification. In particular, endpoints for non-EATS modalities are currently not well covered in the OECD test guidelines.

New approach methodologies (NAMs)

New approach methodologies (NAMs, *e.g. in vitro-, in silico- and omics*-methods; testing strategies; defined approaches, etc.) can be used to provide information about adverse effects or endocrine activity if they provide equivalent predictive capacity as animal data from internationally recognised *in vivo* methods or human data. OECD-validated NAMs or internationally recognised methods, if available, may be more relevant than non-validated methods. When the NAMs provide sufficient information on adverse effect(s) or endocrine activity, they can be used for classification purposes.

4.2.2.2. Classification criteria

CLP, Annex I, *4.2.2.1. Hazard categories*

For the purpose of classification for endocrine disruption for the environment, substances shall be allocated to one of two categories.

Table 4.2.1

Hazard categories for endocrine disruptors for the environment

Categories	*Criteria*
CATEGORY 1	*Known or presumed endocrine disruptors for the environment* *The classification in Category 1 shall be largely based on evidence from at least one of the following:* *a) animal data;* *b) non-animal data providing an equivalent predictive capacity as data in point a.* *Such data shall provide evidence that the substance meets all the following criteria:*

	(a) endocrine activity; *(b) an adverse effect in an intact organism or its offspring or future generations;* *(c) a biologically plausible link between the endocrine activity and the adverse effect.* *However, where there is information that raises serious doubt about the relevance of the adverse effects identified at population or subpopulation level, classification in Category 2 may be more appropriate.*
CATEGORY 2	*Suspected endocrine disruptors for the environment* *A substance shall be classified in Category 2 where all the following criteria are met:* *(a) there is evidence of:* *i. an endocrine activity; and* *ii. an adverse effect in an intact organism or its offspring or future generations;* *(b) the evidence referred to in point (a) is not sufficiently convincing to classify the substance in Category 1;* *(c) there is evidence of a biologically plausible link between the endocrine activity and the adverse effect.*

The classification in Category 2 shall also be largely based on evidence from animal and non-animal data as described for Category 1. Where there is evidence conclusively demonstrating that the adverse effects are not relevant at the population level, the substance should not be considered an ED for the environment (see Section 4.2.2.3.2).

4.2.2.2.1. Classification in the presence of other toxicity

CLP, Annex I, Section *4.2.2.2.2.* *Adverse effects that are solely non-specific consequences of other toxic effects shall not be considered for the identification of a substance as endocrine disruptor for the environment.*

"Other toxicity" refers to (adverse) effect(s) other than the endocrine-related adverse effect(s). If a substance causes endocrine-related adverse effect(s) which occur as a consequence of other toxicity, classification for endocrine disruption for the environment should be applied unless the effect is demonstrated to be '*solely non-specific consequences of the other toxic effects'*. A '*non-specific consequences of the other toxic effects'* is understood as:

- an endocrine-related adverse effect that is conclusively demonstrated to occur secondary to excessive toxicity *i.e.* the co-occurring toxicity is so severe that the animals are suffering[22] or dying.

In principle, ED related adverse effect(s) seen only at very high dose/concentration levels in animal studies (for example doses/concentrations that induce severe suffering, excessive mortality) without being the consequence of an endocrine activity would not normally lead to classification. In all other cases, it needs to be demonstrated on a case-by-case assessment that ED related adverse effect(s) are solely a non-specific

[22] Examples of suffering include lethargy, observations that the animals stay on the bottom of the tank, or lie motionless.

consequence of other toxic effects to be able to justify no classification.

A concentration/dose and temporal concordance between ED and other severe toxicity are important to assess if the endocrine system is out of balance solely due to a non-specific consequence of other toxicity. However, the presence of other toxicity or stress shall not be used to dismiss classification unless it can be unequivocally demonstrated that the potentially endocrine related adverse effects are solely non-specific consequences of other toxicity. In reality, this may be difficult to demonstrate and therefore, dismissing the substance from ED classification (when there is evidence of the endocrine activity, and adverse effect and the biologically plausible link between the two) may in many cases be done only when the other co-occurring toxicity is so severe that the animals are suffering or dying. In this respect, it is important to evaluate carefully the life stages affected by mortality or suffering. If for example, post-hatch mortality is observed in F1 generation of a multi-generation study, this should not question potential ED related effects in the parent F0 generation, in absence of mortality or suffering in adults of the F0 generation.

To consider an ED-related adverse effect solely as a non-specific consequence of other toxic effects, there must be evidence for a biologically plausible sequence of events demonstrating that it is solely other toxicity that causes the adverse effect, and which also excludes the endocrine MoA as the most likely cause for the observed adverse effect(s). Therefore, in such a case, data is needed to demonstrate the non-ED MoA induced by other toxic effects and the assessment is best done by a comparative MoA assessment. For further guidance on how to conduct a comparative MoA analysis, see *e.g.* Meek *et al.* 2014a and 2014b.

When assessing the potential influence of co-occurring other toxicity to the concurrent endocrine-related adverse effect(s) in animals, the empirical support needs to be evaluated carefully. In particular, it may be helpful to assess the different types of effects observed and evaluate the temporal and dose/concentration-concordance between the potential mechanisms and the different types of effects observed. It must be noted that in the case of non-mammalian data, the empirical support will be mainly based on the evaluation of the dose/concentration-response relationship due to the available data set not often allowing for the evaluation of the temporal concordance and consistency among species (often only studies on a single species are available).

To consider that the potentially endocrine-related effect(s) are a consequence of the other toxicity, the other (potentially excessive) toxic effect should precede the endocrine-related effect(s) in time (other toxicity should precede or co-occur with the endocrine-related effects) or should occur at lower or same dose/concentration levels as endocrine–related effects. An evaluation of the appropriateness of the dose/concentration spacing may also help to assess if the effects are solely the non-specific consequence of other toxicity.

However, these cases should be evaluated on a case-by-case basis taking into consideration aspects such as the dose/concentration-response in the endocrine-related adverse effects, the severity of the other toxicity observed and whether the potential endocrine-related effects are only observed at the same doses/concentrations where other toxicity is observed. Aspects such as analogy with other substances, the overall (eco) toxicological data package suggesting a specific non-endocrine MoA etc, may be considered to substantiate that the potential endocrine-related adverse effects are most likely non-specific consequence of other toxicity.

Considering the complexity of the endocrine system, the conclusion that a certain adverse effect is a non-specific consequence of other toxicity needs to be assessed carefully and on a case-by-case basis considering the full picture and pattern of effects.

4.2.2.2.2. Relevant concentrations for classification

The interpretation of adverse effects observed at certain concentrations or at certain levels of toxicity should not be confused with the top dose/concentration to be used in animal studies. The former pertains to the evaluation of existing data, while the latter refers to the selection of the doses/concentrations when performing a study.

Test guidelines specify the approach to determine the highest test dose/concentration to be tested. The top dose/concentration selected for the ecotoxicological studies should provide information on substance toxicity at an exposure of the tested agent that should be compatible with animal survival and permits data interpretation in the context of the use of the study. In ecotoxicology, this can be assessed by using the concept of the maximum tolerated concentration (MTC). For tests on aquatic organisms, the maximum concentration defined through a range finding test or from other toxicity data, or the limit concentration as defined in the relevant OECD guidelines, should be considered to establish this value.

The MTC should not be confused with a demarcation above which the results are not relevant for classification purposes. Although a MTC is aimed at when performing an ecotoxicological study to investigate the endocrine-related adverse effect of a substance, endocrine-related adverse effects are not relevant for classification only when they are the non-specific consequence of other toxicity (see Section 4.2.2.2.1).

There are no generic dose/concentration or toxicity levels that can be used as universal demarcation limits for such effects.

4.2.2.3. Evaluation of hazard information

Appropriate classification will always depend on an integrated assessment of all relevant available data using a WoE approach. This includes positive and negative data from all relevant sources of information, see Section 4.2.2.1. Datasets should be analysed using WoE and expert judgment and the combined, weighted outcome compared with the CLP criteria.

4.2.2.3.1. Evaluation of data on adverse effect(s)

Data on adverse effect(s) are considered similarly to the respective Sections of this guidance on the hazard to the aquatic compartment. All parameters related to effects on reproduction (*e.g.* fertility, fecundity, etc.) in the case of EAS modalities, on development/growth (hindlimb length, developmental stage, time to metamorphosis) for the T modality, and behavioural effects that are considered to be population relevant, shall be considered in the assessment of adversity (see Tables 15 and 16 of ECHA/EFSA ED Guidance. It should be highlighted that some individual parameters may not be considered adverse in isolation. In any case, the conclusion on adversity relies on a combination of parameters and the observation of a pattern of effects across studies. Information on other toxicity shall also be considered in the assessment of adverse effect(s).

The OECD GD 150 provides guidance on how to interpret parameters normally investigated in (eco)toxicity studies; see ECHA/EFSA ED Guidance. The OECD GD 150 differentiates between:

- '*EATS-mediated*' parameters, which are parameters measured *in vivo* that contribute to the evaluation of adversity, while at the same time (due to the nature of the effect and the existing knowledge as described in OECD GD 150, section B2) they are also considered indicative of an EATS MoA and therefore (in the absence of other explanations) also infer an underlying *in vivo* mechanism. This group includes the parameters mainly labelled in OECD GD 150 as 'endpoints for estrogen-mediated

activity', 'endpoints for androgen-mediated activity', 'endpoints for thyroid-related activity' and/or 'endpoints for steroidogenesis-related activity'. Examples of these parameters for environment are sex ratio and some changes in gonad histology[23].

- '*Sensitive to, but not diagnostic of, EATS*' parameters measured *in vivo* that contribute to the evaluation of adverse effect(s). Due to the nature of the effect and the existing knowledge, these effects cannot be considered diagnostic on their own of any of the EATS modalities. Nevertheless, in the absence of more diagnostic parameters, these effects can indicate an endocrine MoA and be relevant for classification, if they are accompanied with evidence of endocrine activity and the biologically plausible link between the endocrine activity and the observed adverse effect. Examples of these parameters for the environment are fecundity, growth, hatching success, behaviour (*e.g.*, stickleback nesting, courtship, mating, aggressiveness).

All the parameters reported in OECD GD 150 are considered to be relevant to support ED-related adverse effects. They are mainly derived from guideline studies, *i.e.* standardised test methods validated for regulatory decision making (*e.g.*, EU test methods/OECD test guidelines or United States Environmental Protection Agency (US EPA)/Food and Drug Administration (FDA) test guidelines).

In addition to results from guideline studies, results from well-performed and reported studies other than those listed in OECD GD 150 may also include '*EATS-mediated*', '*Sensitive to, but not diagnostic of, EATS*' or '*non-EATS*' parameters which may provide relevant information. Therefore, the data used to classify a substance can be drawn from standard studies or other scientific data, *e.g.*, peer reviewed literature studies, Q(SAR) data, internationally recognised databases etc. All relevant data needs to be evaluated carefully in a WoE approach (4.2.2.3.5).

In case NAMs provide data with equivalent predictive capacity as animal data, they can be used to provide sufficient data for adverse effect(s) for classification.

Furthermore, read-across or analogy can also be used to provide information about adversity, *e.g.* if the substances share a common MoA or induce similar adverse effects. When using data from another substance, potential differences in toxicokinetics and toxicodynamics should be considered.

For further details see ECHA/EFSA ED Guidance - Tables 15 and 16 are useful as they show the assignment of '*EATS-mediated*' parameters, and '*sensitive to, but not diagnostic of, EATS*' parameters from the most common test guidelines, see also Table B.1 in OECD GD 150.

4.2.2.3.2. Population relevance

CLP, Annex 1, Section: ***4.2.1.2.1.*** *Substances and mixtures fulfilling the criteria of endocrine disruptors for the environment based on evidence referred to in Table 4.2.1 shall be considered to be known, presumed or suspected endocrine disruptors for the environment unless there is evidence conclusively demonstrating that the adverse effects identified are not relevant at the population or subpopulation level.*

CLP, Annex 1, Section: ***4.2.2.1****. Where there is evidence conclusively demonstrating that the adverse effects identified are not relevant at the population or subpopulation*

[23] More detailed guidance on specific gonad histopathology examination in fish is given in OECD (2010a).

level, the substance shall not be considered an endocrine disruptor for the environment.

The criteria stipulate that substances and mixtures fulfilling the criteria shall be considered as EDs for the environment unless there is evidence conclusively demonstrating that the adverse effects identified are not relevant at the population level. The criteria also stipulate that only when there is evidence conclusively demonstrating that the adverse effects are not relevant at the population level, the substance shall not be considered an ED for the environment.

In applying the WoE approach, the assessment of the scientific evidence shall consider if the adverse effects identified may impact the maintenance of wildlife populations in terms of abundance/biomass and in terms of structure. This consideration is in line with the general level of protection in ecotoxicology where the entity to be protected is the population of wildlife. If data from multiple species are available, the population relevance of the observed adverse effect should be assessed taxon by taxon.

When assessing the effects observed in the available (eco)toxicological studies, relevant parameters for the effects on wildlife are those parameters expected to show adverse effects on the population in the environment. Effects on growth (body weight and length), development and reproduction (such as fecundity, fertility, sex ratio, hatching success and offspring survival) in single species in laboratory studies are generally regarded relevant for the maintenance of the wild population (European Commission, 2011; Marty *et al.*, 2017). Effects observed in toxicity studies conducted in the laboratory, in some circumstances, may be even more severe in the field where animals need also to cope with additional stressors, *e.g.*, predation, food availability, etc. Therefore, when effects are observed in those parameters the relevance at the level of population is inferred unless the contrary is proven.

Behavioural changes and impaired ability to cope with additional stress are factors implicitly covered by the definition of adverse effect(s), since they could affect development and reproductive performance, hence impact the population stability. Therefore, they should be considered to be relevant at the population level (Agerstrand *et al.*, 2020). However, it is acknowledged that current standard tests are not specifically designed to capture all behavioural effects (European Commission, 2011) and the ability to cope with stressors.

Effects in reproductive organs (*e.g.* gonads) are considered as population relevant since they are expected to have a direct impact on reproduction. On the other hand, effects in non-reproductive organs, are considered as relevant at the level of population when accompanied by a pattern of effects including other apical parameters. If effects in non-reproductive organs are the only effects available in the data package for the substance, and apical effects were not investigated, the population relevance of those effects cannot be excluded.

When evaluating mammalian data to reach a conclusion on the classification for the environment, further consideration is needed to evaluate whether some ED-related adverse effects observed in mammals can be considered adverse for mammals as wildlife species at the level of population. With regard to adverse effects in mammalian species, it has to be noted that the entity to be protected in mammalian toxicology is the individual organism, while for wild mammals the entity to be protected is the population. This means that, although to conclude on wild mammals the same dataset is used as the one used to conclude on human health, each effect and parameter must be considered from a different perspective, *i.e.* relevance of the effect observed for wild mammal populations. Therefore, in the evaluation of the ED potential in mammals, the assessment for human health may consider as adverse changes at organ level which may or may not impact the maintenance

of the population. To this respect, it is recommended that effects at organ level should be considered in a WoE approach together with any other effects observed which could be related to the same pattern of effects.

It should be noted that effects observed in rodents are of high concern for wildlife species with a natural low reproductive output, including top predators and other mammals (including endangered species), as negative effects on reproduction have an even higher potential for causing long term negative effects at the population level for such taxa.

Effects on growth, development and reproduction should generally be regarded relevant for the maintenance of the wild population if they are statistically significant compared to the controls. However, the lack of statistical significance should not be the only reason for concluding a lack of treatment-related effect. Statistical significance and biological relevance[24] should be considered together when assessing the presence/absence of treatment related effects. Considering the two aspects together (i.e. biological relevance and statistical significance) is of particular importance in those situations where a trend of effect is observed without being statistically significant. This, for example, could happen in the case of screening studies which are known to have a limited statistical power. Besides the two aspects mentioned above (statistical significance and biological relevance), the overall dataset should be carefully considered to understand whether a pattern of effects is observed. If a pattern of effects is observed, changes observed below a certain magnitude, which in isolation would not be considered relevant, could still contribute to the assessment of adversity, if considered to be part of the pattern identified.

When evaluating mammalian data to reach a conclusion on the classification on wild mammals, although the same dataset is used as the one used to conclude on human health, as indicated above, each effect and parameter must be considered from a different perspective. Therefore, in the evaluation of the ED potential in mammals, the assessment for human health may consider as adverse changes observed with very low incidence, but considered severe enough to establish the adverse effect(s) (*e.g.* tumours). Those effects, however, may not be relevant for the population of wild mammals, as they are not expected to occur at a high enough prevalence in the population to impact population survival/maintenance.

Future developments in the field of effect and population models may be considered as valuable tools in better understanding the population relevance of the observed adverse effects. The advancement of novel techniques such as AOPs and population modelling may facilitate the comprehension of the connection between disruptions in endocrine systems at the lower levels of biological organization and their repercussions at the population level.

Specific considerations related to the thyroid modality - mammals

As explained above, when evaluating mammalian data to reach a conclusion on the classification for the environment, further consideration is needed to evaluate whether some ED-related adverse effects observed in mammals can be considered adverse for mammals as wildlife species at the level of population. In the case of the thyroid modality, as for the other modalities, the thyroid endpoints are not looked at in isolation, but the whole data package is evaluated holistically. In particular, organ level endpoints should be assessed using a WoE approach considering apical endpoints in the dataset. The level of effect (as to population relevance) and the type/directionality of the effect (as to whether

[24] Guidance on biological relevance can be found in the Scientific opinion of the EFSA Scientific Committee on "Statistical Significance and Biological Relevance", EFSA Journal 2011: 9(9): 2372 and in the Guidance on the assessment of the biological relevance of data in scientific assessments, EFSA Journal 2017. Doi: 10.2903/j.efsa.2017.4970

this matches the proposed MoA) should be specifically addressed. Therefore, in order to reach a conclusion on the need to classify the substance, it may be necessary to reconsider the mammalian data package to further understand whether there are other effects which may be due to the same ED MoA and can further support the conclusion in population relevance. For example, thyroid histopathological findings observed in rats are relevant at population level if observed together with impairment of growth/development and/or reproduction or with support of other data in a WoE approach.

However, if the data package does not contain information on other effects potentially related to the same MoA because those were not investigated, it cannot be excluded that thyroid histopathological findings observed in the rats are of population relevance.

Specific considerations related to the thyroid modality - non-mammalian organisms

In the case of amphibians, normally apical endpoints are investigated together with thyroid histopathology. Therefore, in such situation changes in thyroid histopathology are considered adverse at the population level when observed together with effects on development (*e.g.*, accelerated or asynchronous). Population relevance can be excluded only if thyroid histopathology is observed and development was investigated, but no concomitant effects were observed, provided that the power of the test is sufficient to elicit an effect. This is because thyroid histopathology often exhibits compensation to thyroid insufficiency (Marty *et al*., 2017). In rare cases the thyroid histology may be the only endpoint assessed, and therefore case-by-case consideration should be made to come to a conclusion on the relevance of that effect at the population level. Nevertheless, changes in development in amphibians, even if observed in the absence of investigation of thyroid histopathology, are considered population relevant effects.

Several potential endpoints for disruption of the HPT axis in fish have been described in the scientific literature, such as thyroid histopathology, thyroid hormone levels, gene expression, swim bladder development and inflation, neurodevelopment, eye development, behaviour. There are also a number of AOPs under development where histopathology of the thyroid is linked to adverse and population relevant effects also in fish, *e.g.* eye development (AOPwiki 363) and swim bladder inflation (AOPwiki 156, 158, 159). Although it is acknowledged that those endpoints are currently not included in standard fish tests, future developments in this field may provide a better understanding of the population relevance of the observed adverse effects.

> **CLP, Annex 1, Section *4.2.2.1***. *(Table 4.2.1) However, where there is information that raises serious doubt about the relevance of the adverse effects identified at population or subpopulation level, classification in Category 2 may be more appropriate.*

According to the criteria, classification as Category 2 may be more appropriate when effects are observed, either in mammalian data or in non-mammalian species, but there are serious doubts that those effects would be relevant at the population level, *i.e.* that the observed effects would impede the maintenance of the population. This conclusion needs to be taken with caution using a WoE approach.

4.2.2.3.3. Evaluation of endocrine activity

In terms of endocrine activity, the OECD GD 150 differentiates between:

- *In vitro* mechanistic – parameters measured *in vitro*, that provide information on the mechanism through which a substance could be considered endocrine active (*e.g.*, by binding to and activating a receptor or interfering with specific enzymes in endocrine pathways).

- *In vivo* mechanistic – parameters measured in vivo that provide information on endocrine activity that are usually not considered adverse per se. Changes in sex hormone levels are generally considered *in vivo* mechanistic. An example of these parameters for environment is vitellogenin (VTG). As described in Section 4.2.2.3.1. above, '*EATS-mediated*' parameters are also considered indicative of an EATS MoA and thus (in the absence of other explanations) also infer an underlying *in vivo* mechanism.

In silico approaches as described in Section 4.2.2.3.3.2 also inform on endocrine activity. The applicability domain of the models should be considered.

4.2.2.3.3.1. *In vitro* data

Currently, there are standardised *in vitro* assays based on non-mammalian receptors and/or enzymes. However, since the endocrine system is known to be conserved across vertebrates, *in vitro* assays with mammalian cells can be used in a WoE approach to give indications on possible MIEs or interference with a certain pathway also for non-mammalian species, see further information in Section 3.11.2.3.2.1. Moreover, the OECD GD 150 clearly indicates that: "*The in vitro screens in question (although at present based largely on mammalian receptors and/or enzymes) are generally capable of providing information applicable to both humans and vertebrate wildlife (OECD, 2010b). Such extrapolation of in vitro information is generally qualitative (...)*".

The *in vitro* tests, when used in isolation, lack the complexity of an intact organism. Single assays often identify if a substance is capable of binding to a receptor or interfering with a pathway. Particular attention should be paid to *in vitro* data and the considerations of absorption, distribution, metabolism, excretion (ADME) properties which may not be covered by current *in vitro* test guidelines *e.g.*, those measuring protein binding or disruption of endocrine pathways. Therefore, when interpreting the results of *in vitro* tests, the possible lack of a metabolising capacity or competence of the system, as well as the possible lack of consideration of other ADME properties, should be considered. To partly overcome this limitation, metabolism may be addressed when (part of the) metabolising systems are added to the test system, or test data on metabolites of the substance could be directly used. Results from a battery of tests for substances that are not metabolised may in some cases be conclusive on endocrine activity. Similarly, data may be conclusive if both the parent substance and the metabolites are covered. Therefore, all mechanistic information should be considered together to reach a conclusion on endocrine activity.

Most of the current available *in vitro* assays focus on specific interactions of substances with cellular components, such as nuclear hormone receptors or enzymes in specific pathways (*e.g.* aromatase). However not all endocrine related adverse effects are mediated through a direct action on these molecules. Additionally, compounds might be able to act via more than one mechanism and some of the pathways, which might be potentially causing an ED adverse effect *in vivo*, might not be covered by the currently available *in vitro* assays. Overall, no single test can be expected to detect all types of endocrine activity.

To partly overcome this limitation, several *in vitro* tests investigating different points of perturbation or endocrine pathways can be assessed together. However, the eventual ED effect *in vivo* might be a consequence of disturbance of several pathways simultaneously, some of which might not be covered by available *in vitro* tests.

The capacity of organisms to compensate for a certain level of changes in hormonal regulation may not yet be possible to assess in an *in vitro* system. Further, the applicability domain as well as overall validity and reliability of *in vitro* tests shall be considered. A negative single *in vitro* result alone cannot be used to exclude endocrine activity.

Because of the inherent limitations of *in vitro* systems such as those highlighted above, conclusions on the endocrine activity of the substance can only be drawn in the context of what the respective *in vitro* assays were developed to evaluate (*e.g.*, receptor binding, enzyme inhibition).

Due to limitations of *in vitro* systems, interpretation of results must be carefully considered (in a similar manner as limitations from *in vivo* systems are considered).

4.2.2.3.3.2. *In silico* data

In silico predictions may be used as supporting information for endocrine modalities within a WoE approach. The different types of *in silico* prediction methods can be grouped as: molecular modelling of receptor interactions, (Q)SAR modelling and other events, and profilers based on structural alerts and decision trees; for further details see Section 4.1 of the ECHA/EFSA ED Guidance. QSAR predictions may also support read-across.

The evidence from *in silico* predictions is strengthened if the same result is obtained with independent *in silico* models. Whenever *in silico* methods are used, the general provisions outlined in ECHA Guidance on IRs & CSA, Chapter R.6: QSARs and grouping of chemicals (ECHA, 2008) and *'(Q)SAR Assessment Framework'* (OECD, 2023) should be followed. Attention should be paid to the interpretation of results to understand the specific basis and scope of the prediction for each endocrine pathway, taking into account the performance and the applicability domain of each *in silico* predictive model when drawing conclusions.

4.2.2.3.3.3. *In vivo* data

In vivo studies also provide information on endocrine activity. The '*EATS-mediated*' adverse effects infer an underlying *in vivo* mechanism that should be used for the identification of the endocrine activity; see section 4.2.2.3.1. The OECD GD 150 also lists assays providing *in vivo* mechanistic information. Also, the *in vivo* mechanistic data have some limitations, and the applicability domain should be carefully assessed. For further details, see ECHA/EFSA ED Guidance.

4.2.2.3.4. Mode of action analysis and evaluation of biological plausibility

> **CLP, Annex I, Section *4.2.1.1.*** *"biologically plausible link" means the correlation between an endocrine activity and an adverse effect, based on biological processes, where the correlation is consistent with existing scientific knowledge.*

Guidance on how to postulate and conclude on MoA(s), assess the biological plausibility of a link between endocrine activity and adverse effects as well as to identify which further information could help to clarify the postulated MoA(s), is provided in Section 3.5 of the ECHA/EFSA ED Guidance.

When potential endocrine-related adverse effect(s) and endocrine activity are identified, the link between the two, according to the CLP ED criteria, shall be established and justified based on biological plausibility. To conclude on the biological plausibility of the link, it may not be necessary to have demonstrated the whole sequence of events leading to the adverse effect. Existing knowledge from, *e.g.*, endocrinology and/or (eco)toxicology, may

be sufficient to conclude on the biological plausibility of the link between adverse effects and the endocrine activity.

Biological plausibility may be demonstrated by conducting a MoA analysis, which shall be determined in the light of current scientific knowledge using all available relevant information in a WoE approach. For classification purposes, knowledge and demonstration of the full MoA is not a requirement. The MoA analysis should aim at establishing biological plausibility based on the consistency and coherence of the responses obtained on measured parameters with a postulated MoA.

The level of information required for a MoA analysis varies depending on which parameters are adversely affected.

For example, '*EATS-mediated*' adversity is considered indicative of an EATS MoA and, thus, also infers an underlying *in vivo* mechanism (in the absence of other explanations). In such cases, the analysis of the biological plausibility may draw conclusions from the broader scientific knowledge. Therefore, less information would be required for a MoA analysis and without recourse to a detailed MoA analysis compared to adversity based on other parameters, *i.e.,* the MoA analysis can be very simple. This is because there is a biologically plausible link between the adverse effect and endocrine activity in an EATS modality which is the most likely explanation of the effects observed. Therefore, in the absence of other explanations, *i.e.* an alternative MoA considered as a more likely explanation, an ED MoA can be considered plausible.

This is in contrast to adversity based on '*sensitive to but not diagnostic of EATS*' and '*non-EATS mediated*' parameters where more evidence is needed to support the KEs in the postulated MoA. In this case, the conclusion will depend on the degree of support provided by the empirical evidence for the KEs in the postulated MoA.

As in all assessments, a consistent pattern of effects strengthens the empirical support for KEs of the postulated MoA. The final WoE conclusion shall consider all available data.

Mode of action analysis

A MoA can be described as a series of biological events, *i.e.*, key events (KEs) that lead to a specific adverse effect. The first KE in the series is referred to as the molecular initiating event (MIE), see Figure 4-2.1.

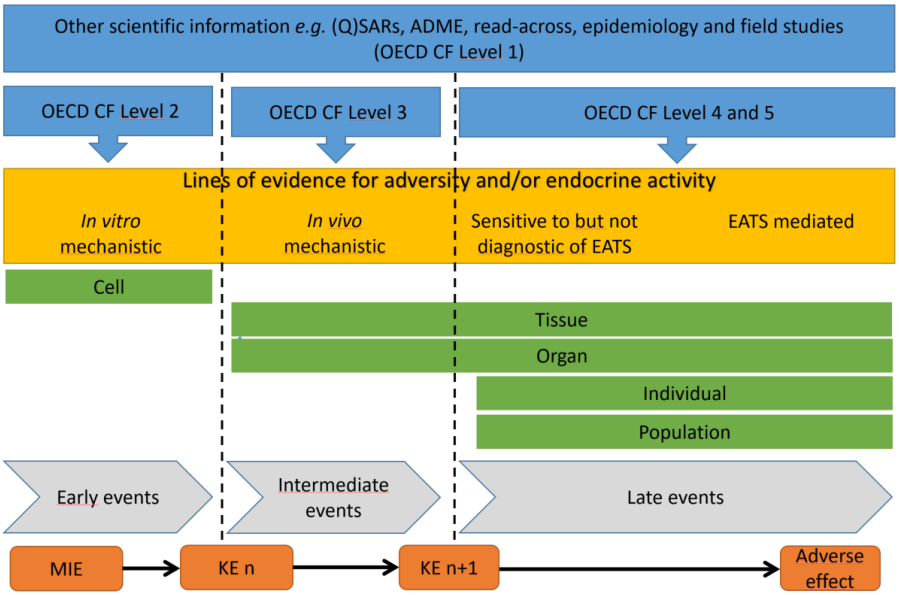
This guidance uses AOP terminology for the MoA analysis. However, this does not imply that the AOP approach must be used for the MoA analysis.

An endocrine MoA means that the adverse effect is mediated through an alteration of one or more functions of the endocrine system, *e.g.*, hormonal synthesis, transport, signalling, regulation or metabolism, *i.e.*, it is not only mediated via hormone-receptor interactions. Normally, an endocrine MoA contains some earlier KEs (which provide mechanistic information at the molecular or cellular level concerning endocrine activity) and some later KEs (which provide information at the organ or system level, including the adverse effect).

This sequence at least includes one endocrine-mediated KE which may or may not also be adverse (see ECHA/EFSA ED Guidance); *i.e.* the MIE does not need to be known or endocrine related. KEs are those events that are considered essential to the induction of the (eco)toxicological response as outlined in the postulated MoA. KEs are empirically observable and measurable steps and can be placed at different levels of biological organisation (at cell, tissue, organ, and individual or population level); see Figure 4.2-1. To support the plausibility of a KE, there needs to be experimental data in which the event is characterised and consistently measured or existing knowledge on which basis the event is understood. KEs are connected to one another, and this linkage is termed a key event

relationship (KER).

Figure 4.2-1 Scheme illustrating how the evidence can be organised to support the postulated mode of action. The arrows linking KEs represent the KE relationships. It should be noted that the borders between the different OECD CF levels are not absolute in terms of parameters measured and in their contribution to the WoE.



KE: key event; MIE: molecular initiating event.

CLP, Annex I, Section *4.2.2.3.3.* *Using a weight of evidence determination, the link between the endocrine activity and the adverse effects shall be established based on biological plausibility, which shall be determined in light of available scientific knowledge. The biologically plausible link does not need to be demonstrated with substance specific data.*

Evaluation of the biological plausible link between an endocrine activity and an adverse effect

The first step in assessing biological plausibility is to gather information from scientific literature / existing knowledge on possible endocrine-related MoAs that are related to the types of adverse effects and endocrine activity observed for the substance or related substances subject to classification; see Section 3.11.2.1. The evidence available for the substance subject to classification shall be assessed against the hypothesis for MoA with its KEs to be able to conclude on a biological plausible link between the observed endocrine activity and adverse effect(s).

The conclusion on biological plausibility is based on whether or not the KER is consistent with the general knowledge of biology and what is known about the substance. The analysis of the biological plausibility for the KER refers only to the broader knowledge of the biology, physiology, endocrinology and toxicology involved. In a postulated MoA, the KERs need to be consistent with the current understanding of biology, physiology, endocrinology and toxicology.

Existing adverse outcome pathways (AOPs) and MoAs can be used as a starting point for the postulated MoA against which the evidence can be systematically organised. Evidence on adverse effect(s) and endocrine activity, assessed for dose and temporal concordance, can provide empirical support to the KEs.

Several adverse outcome pathways related to endocrine disruption have been established and endorsed, *e.g.*, OECD Series on AOPs[6]. There are also numerous AOPs under development in the AOPwiki[7], or published in the literature. The amount of empirical support needed to establish the KERs varies depending on how well developed the AOP in question is. In cases where the MoA is based on a robust[25] or an OECD endorsed AOP, the biological plausibility of the KERs does not need to be demonstrated with experimental data. However, existing data on adversity and endocrine activity should be used to provide the empirical support needed to establish that the postulated MoA is plausible. Lack of a robust or OECD endorsed AOP should not be considered negatively in cases where there is convincing evidence for a biologically plausible link between observed endocrine activity and adversity.

The assessment should, when possible, include consideration of the modified Bradford Hill criteria, *i.e.*, essentiality, dose/incidence and temporal concordance, specificity, consistency, analogy; see further definition in Table 4.2.1. In particular, dose/incidence and temporal concordance are valuable to support or disprove the plausibility of the KERs and should always be assessed. For example, a MIE should occur below or at doses/concentrations where a downstream KE or an adverse outcome is observed. Similarly, early KEs should occur before or at the same time as the adverse outcome. However, since substance specific information on all the Bradford Hill criteria is only very rarely available, the absence of evidence to demonstrate these individual factors should not be used to exclude classification as an ED if the overall picture supports a plausible link to an ED MoA.

It must be also noted that in the case of non-mammalian data, the empirical support will be mainly based on the evaluation of the dose/concentration-response relationship due to the available data set not often allowing for the evaluation of the temporal concordance and consistency among species (often only studies on a single species are available).

It is recognised that there may be cases where the biological relationship between two KEs may be very well established:

- When adverse effects are '*EATS-mediated*'. These parameters provide evidence for adversity, while at the same time (due to the nature of the effect and existing knowledge as described in the OECD GD 150) they are also considered indicative of an EATS MoA and thus (in the absence of other explanations) also infer an underlying *in vivo* mechanism. Where both data on adversity and endocrine activity are provided by the same study, it may be possible to reach a conclusion on the biological plausibility of the link without recourse to a detailed MoA analysis.

[25] Robust in this context means AOPs that have a broad acceptance in scientific literature.

- When the MoA analysis is based on a robust or endorsed AOP, *e.g.*, OECD Series on Adverse Outcome Pathways[26]. In this situation, the biological plausibility is provided by the documentation for the KERs in the AOP used, *e.g.* OECD series on AOP No. 4 links aromatase inhibition to reproductive dysfunction in fish.

However, for adverse effect(s) based on '*Sensitive to, but not diagnostic, of EATS*', the evidence that the adverse effects are (exclusively) caused by an endocrine MoA is not as strong as for adversity based on *'EATS-mediated'* parameters. Therefore, postulated MoA and its biological plausibility would need to be supported by a more detailed MoA analysis. For example a decrease in fecundity in fish together with a reduction in VTG concentration in females could be considered caused by an endocrine MoA, *i.e.* AR agonism or aromatase inhibition, if it were supported by mechanistic data such as those described in the endorsed OECD AOP 4 or 9[27], respectively.

Similarly, for adverse effect(s) based on non-EATS modalities (*i.e.*, adversity resulting from impairment of endocrine modalities other than E, A, T or S), the evidence that the adverse effect(s) are caused by an endocrine MoA needs to be substantiated with a more extensive MoA analysis than for '*EATS-mediated*' adverse effects; unless the biological plausible link is based on existing scientific knowledge, *e.g.* a robust or OECD endorsed AOP.

A substance may have one or more MoAs, which can be endocrine or non-endocrine. The potential of a substance to elicit more than one MoA can obviously lead to difficulties in concluding on the biological plausibility. If there are indications that a substance may act via multiple MoAs, then the evaluation should first focus on the MoA for which the most convincing evidence is available. The number of potential MoAs to be considered will vary on a case-by-case basis.

Furthermore, there may be more than one MoA which could cause similar effects; hence, it may be necessary to undertake an analysis for more than one postulated MoA for a particular adverse effect. There may be also situations where a pattern, which includes 'EATS mediated' adverse effects, has been identified. However due to the complexity and cross-talk within the endocrine system it may not be possible to identify the specific modality.

In such cases, a biological plausible link should be considered as established for an 'EATS-mediated' MoA and classification as Category 1 or 2 may be warranted depending on the strength of evidence.

Comparative MoA analysis

To consider an ED-related adverse effect as a specific consequence of another non-endocrine MoA*,* there must be evidence for a biologically plausible sequence of events which excludes an endocrine MoA as the most likely explanation for the observed adverse effect(s). To demonstrate this, MoA data is needed on the alternative MoA and the assessment is best done by a comparative MoA assessment. It should be noted that it may be difficult to demonstrate that the effects are solely non-endocrine related because standard studies generally do not provide mechanistic information and thus, further mechanistic studies may be needed. An additional complication is that substances may have more than one MoA, including an ED MoA. In this situation, the ED MoA should be considered for classification. For further guidance on how to conduct a comparative MoA analysis, see ECHA/EFSA ED Guidance.

[26] OECD Series on Adverse Outcome Pathways | OECD iLibrary (oecd-ilibrary.org)
[27] AOP 4 (OECD series, endorsed): Aromatase Inhibition leading to Reproductive Dysfunction (in Fish); AOP 9 (OECD Series, endorsed): Androgen receptor agonism leading to reproduction dysfunction (in repeat-spawning fish)

Table 4.2.1. Explanations of the terms: analogy, essentiality, consistency, dose and incidence concordance, MoA, specificity and temporal concordance.

Term	Explanation
Analogy	A consistent observation across (related) substances having a well-defined MoA.
Essentiality	Essentiality is one of the elements that should be considered (when data are available) when performing the WoE analysis using the Bradford Hill considerations. In the context of the MoA/AOP frameworks, essentiality refers to key events. For determining essentiality, it should be demonstrated whether or not downstream KEs and/or the adverse effect is prevented/decreased if an upstream event is experimentally blocked. It is generally assessed on the basis of direct experimental evidence of the absence/reduction of downstream KEs when an upstream KE is blocked or diminished (*e.g.*, in null animal models or reversibility studies).
Consistency	Consistency is the pattern of effects across species/ organs/test systems that are expected based on the postulated MoA/AOP. In developing a MoA, consistency also refers to the repeatability of the KEs in the postulated MoA in different studies. Consistent observation of the same KE(s) in a number of studies with different study designs increases the support.
Dose and incidence concordance	Dose and incidence concordance are elements valuable for the evaluation of the empirical support. In a MoA/AOP context, dose and incidence concordance are verified when the key events are observed at doses or incidences below or similar to those associated with the adverse effect (or key events downstream).
Mode of Action	A biologically plausible sequence of key events at different levels of biological organisation, starting with the exposure to a substance and leading to an observed (adverse) effect.
Specificity	Specificity should be understood as the extent to which the MoA for the adverse effect is likely to be endocrine-related, *i.e.* whether an adverse effect is a consequence of the hypothesised endocrine MoA, and not a result of other non-endocrine mediated MoA, including results of excessive other toxicity.
Temporal concordance	Temporal concordance increases the empirical support of the biologically plausible link. This is done by evaluating whether key events within the MoA are observed in the hypothesised order.

4.2.2.3.5. Weight of evidence and expert judgement

According to the CLP ED criteria, WoE and expert judgement must be applied when concluding on the CLP ED criteria (CLP, Article 9 in conjunction with CLP, Annex I, Sections 1.1.1. and 4.2.2.1.); see guidance on WoE in Sections 1.4 of this guidance.

CLP, Annex I, *Section 4.2.2.3.1.* *Classification as an endocrine disruptor for the environment is made on the basis of an assessment of the total weight of evidence using expert judgment (see Section 1.1.1.). This means that all available information that*

bears on the determination of endocrine disruption for the environment is considered together, such as:

(a) in vivo studies or other studies (e.g., in vitro, in silico studies) predictive of adverse effects, endocrine activity or biologically plausible link in animals;
(b) data from analogue substances using structure-activity relationships (SAR),

(c) evaluation of substances chemically related to the substance under study may also be included (grouping, read-across), particularly when information on the substance is scarce;
(d) any additional relevant and acceptable scientific data.

A WoE determination means that all available, relevant information bearing on the determination of hazard is considered together, including:

(a) relevant animal data; the results of suitable *in vitro* tests; and relevant *in silico* predictions;
(b) information from the application of the Category approach (grouping, read-across); (Q)SARs etc.;
(c) peer-reviewed published studies; and
(d) any additional data, for example information used for the evaluation of the substance as an ED for human health, including physico-chemical parameters and information on known metabolites or degradation products should be considered where relevant.

Formation of a metabolite with endocrine activity or adversity indicates that exposure to the substance might result in endocrine-related adverse effects. Therefore, endocrine activity or adversity observed with the metabolite shall be considered in the classification of the parent substance. If data are available, quantity and stability of the metabolite(s) formed should be taken into account (*e.g.* if the metabolite is stable for a period long enough to exhibit toxicological properties or if it is an intermediate which is rapidly changed to other metabolites). Even if a substance has been tested as negative for ED it may in certain instances be classified in Category 1 or 2 based on the formation of metabolites with ED properties.

Considering the similarity of metabolization across vertebrates (Auer *et al*., 2017), if a metabolite is formed in any vertebrate species, it is assumed by default that this metabolite is also formed in other vertebrates unless demonstrated otherwise.

If a substance degrades (biotically or abiotically) in the environment and the degradation (or transformation or breakdown) product shows endocrine activity and/or adverse effect(s), this should be taken into account in the assessment of classification for the parent substance.

CLP, Annex I, Section *4.2.2.3.2.* *In applying the weight of evidence determination and expert judgement, the assessment of the scientific evidence referred to in Section 4.2.2.3.1 shall, in particular, consider all of the following factors:*

(a) both positive and negative results;
(b) the relevance of the study design for the assessment of adverse effects and its relevance at the population or subpopulation level, and for the assessment of the endocrine activity;
(c) the adverse effects on reproduction, growth/development, and other relevant adverse effects which are likely to impact on populations or subpopulations;
(d) the quality and consistency of the data, considering the pattern and coherence of the results within and between studies of a similar design and across different

species;
(e) the route of exposure, toxicokinetic and metabolism studies;
(f) the concept of the limit dose (concentration), and international guidelines on maximum recommended doses (concentrations) and for assessing confounding effects of excessive toxicity;
(g) where available, adequate, reliable and representative field or monitoring data or results from population models.

The WoE approach for identifying EDs should involve transparent assessment and consideration of all available data based on factors such as relevance, quality, and consistency, see CLP, Annex I, 1.1.1.3.

The quality and consistency of the data should be given appropriate weight. Both positive and negative results should be assembled in a single WoE determination, separated for endocrine activity and adversity; see CLP, Annex I, 1.1.1.3 and Section 1.4 in this guidance).

Although the quality, reliability, validity and applicability domain of a study per se affects the weight given to the study, there are also several other, "external" factors that may influence the WoE assessment, as mentioned above in the green boxes. Information on toxicokinetics, (*e.g.*, sex differences, accumulation in tissues, information on major metabolites), physicochemical properties (*e.g.*, vapour pressure, solubility and unspecific binding in *in vitro* test systems), read-across/analogy and availability of substance specific data may have influence on how much weight each piece of information can be given. In general, substance specific information is given more weight than other data unless there are reasons not to do so. For example, read-across or analogy can sometimes provide stronger evidence for classification than the substance-specific data.

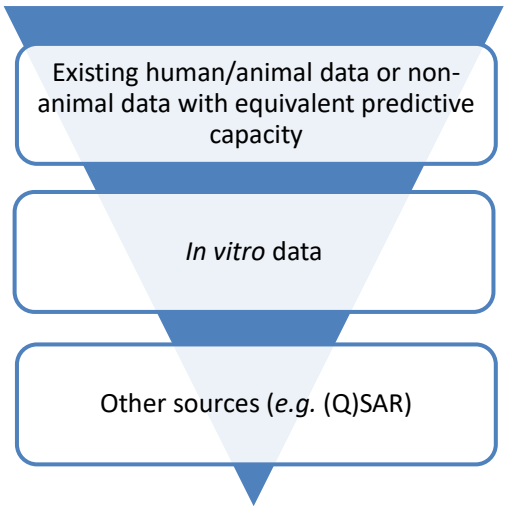
The assessment must weigh all evidence and be performed on a case-by-case basis using expert judgement. A single positive study can however be sufficient for classification.

CLP, Annex I, Section 4.2.2.2.1 *Classification shall be made on the basis of the appropriate criteria outlined above, and a weight of evidence determination of each of the criteria (see Section 4.2.2.3) and an overall weight of evidence determination (see Section 1.1.1).*

WoE for endocrine disruption must be conducted independently for adverse effect(s) and for endocrine activity. Thereafter, the overall WoE for all these elements together must be conducted in the MoA analysis, also including the conclusion on the biologically plausible link.

Figure 4.2-2 provides an illustration of the relative weight of different types of data. In the case of conflicting results, a decision on the weight to be assigned to the different types of data has to be made. It needs to be noted that the relative weights indicated in Figure 4.2-2 assume comparable quality of the data. WoE considerations need to take into account, on a case-by-case basis, the quality, consistency, nature, severity, relevance and applicability domain of the different types of data available. The figure illustrates a decreasing weight of the information from top to bottom.

Figure 4.2-2 Simplified illustration of the relative weight of the available information with similar or comparable quality



When contradicting data of comparable quality and predictive capacities assessing similar endpoits belongs to different "hierarchical levels", the following considerations should be included in the WoE approach:

- When there are relevant positive data which belong to a higher level in the hierarchy than the available negative data, more weight should normally be given to the positive data.

- When the negative data belong to a higher level in the hierarchy than the positive data, more weight should normally be given to the negative data, and a careful evaluation of the reasoning should be conducted considering differences in dose/concentration levels used, species differences, differences in the quality and reliability of data etc. Furthermore, there may be cases where the mechanism investigated at the lower level of the hierarchy (*e.g. in vitro*) is not covered by the investigations at the higher level of the hierarchy (*e.g. in vivo*), or *e.g.* there may be lack of sensitivity in a well conducted *in vivo* study. In such cases negative data at the higher level should not be given higher weight than the positive data at the lower level of the hierarchy.

- In all of the above cases, it is important to assess the full data set and a scientifically justified explanation should be provided. In general, positive results that are relevant for classification should not be overruled by negative findings without a scientifically sound and transparent explanation based on the analysis of biological plausibility. All existing evidence should be systematically organised against existing adverse outcome pathways or known modes of action.

Field or monitoring studies can also contribute to the WoE, for more information see in Section 3.2 of the ECHA/EFSA ED Guidance.

4.2.2.3.6. Use of evidence considered for classification as endocrine disruptor for human health when assessing classification as endocrine disruptor for the environment

CLP, Annex I, Section *4.2.2.3.4*. *Using a weight of evidence determination, evidence considered for the classification of a substance as an endocrine disruptor for human health referred to in Section 3.11. shall be considered when assessing the classification of the substance as an endocrine disruptor for the environment under Section 4.2.*

Because of the high level of conservation of the endocrine system across taxonomic groups, the conclusion on the classification as ED for the environment makes use of all available data in mammalian and non-mammalian species in a holistic approach. The Revised Guidance Document 150 states that: "*Cross-species extrapolations should be considered during data assessment. Endocrine systems with respect to hormone structure, receptors, synthesis pathways, hormonal axes and degradation pathways are well conserved across vertebrate taxa especially in the case of estrogen, androgen and thyroid hormones and steroidogenesis.*" And: "*When interpreting data for endocrine assessment, this conservation should be borne in mind as results from tests using human in vitro or non-human mammalian (in vitro and in vivo) systems may be highly relevant for vertebrate wildlife species and vice versa. In addition, results from non-human mammalian studies are also highly relevant for mammalian wildlife species.*"

Furthermore, the EFSA/ECHA ED Guidance (2018) specifies that the same database can be used to conclude on the ED properties for human health and the environment: "*The information needed to assess ED properties for humans and non-target organisms may overlap. Mammalian data are always relevant for ED assessment on non-target organisms. Furthermore, there may be information on non-target organisms that could be relevant also for the ED assessment for humans.*" and *"[...] it is recommended to strive for a conclusion on the ED properties with regard to humans and in parallel, using the same database, to strive for a conclusion on mammals as non-target organisms."*

Current advances within development of AOP networks demonstrate that some molecular initiating events and key events are linked to a broad range of adverse outcomes in different species across toxicology and ecotoxicology (for EDs typically humans, rodents, fish and amphibians). By use of well-developed AOP networks, cross-species information could be utilised in the evaluation of environmental endocrine disruption to a much higher degree than previously done for environmental ED assessment, see *e.g.*, Haigis *et al.*, 2023 and Figure 3.11-3 on the AOP network for thyroid effects.

Therefore, effects on mammals can also give information on endocrine disruption in non-mammalian vertebrates and data on mammals and other taxa should be considered together in a holistic approach as part of the available evidence for reaching a conclusion on the need to classify the substance. See also population relevance (Section 4.2.2.3.2 of this guidance).

4.2.2.4. Decision on classification

Substances are classified as EDs for the environment in Category 1 or 2 when there is sufficient evidence that the three empirical elements (a) adverse effect(s) (relevant at the population level) (b) endocrine activity and (c) the biological plausible link as indicated in CLP, Annex I: Table 4.2.1 (for details see Section 4.2.2.2 of this guidance) are met. If one of the three elements is not met, classification of the substance is not warranted.

To be able to meet the classification criteria, it is highly important to understand the biologically plausible link between endocrine activity and observed adverse effect(s) that is relevant at the population level (see more information on population relevance under

Section 4.2.2.3.2).

Where there is evidence conclusively demonstrating that the adverse effects are not relevant at the population level, no classification is warranted. If there are serious doubts based on all available information about the relevance of the adverse effects at the population level, this should be taken into account in the classification, and Category 2 classification should be considered.

The allocation of the substance to Category 1 or 2 or no classification depends on the strength and consistency of the available evidence, *i.e.* on how convincing the evidence for criteria (a) and (b) is as well as whether a plausible link between the two can be established. Allocation to Category 1 is warranted when the evidence for adverse effect(s) and endocrine activity is sufficiently convincing considering all available relevant data in the WoE on the substance. Sufficiently convincing evidence for Category 1 may also be based on appropriate and robust read-across or analogy or grouping, when those approaches are sufficiently justified for that particular substance and a biologically plausible link is established. Also evidence on a certain pattern of adverse effect(s) observed, which is generally known to be linked to a certain type of endocrine activity (*i.e.* '*EATS-mediated*'), can lead to Category 1 classification.

When the evidence for either adverse effect(s) or endocrine activity or both is not sufficiently convincing to place the substance in Category 1, then Category 2 or no classification may be warranted. This may be caused by issues related to reliability, dosing/concentration settings, parameters covered, life-stage investigated or exposure duration, serious doubts on the relevance at the level of population, incidence of the effects, divergencies between results in different studies if not explainable by differences in study design (*i.e.* lack of consistency), inconsistent pattern of effects, etc., or when chance, bias or confounding factors cannot be ruled out with reasonable confidence.

For example, if there are serious concerns regarding the study design or conduct or the interpretation of existing information, or if there is insufficient information available to make a conclusion on Category 1, or if the adverse effect is considered to be not sufficiently convincing for Category 1 (*e.g.* if a broad range of relevant ED related endpoints are investigated in well-conducted reliable studies, and the ED related effect(s) is observed with low incidence), classification for Category 2 or no classification may be more appropriate.

Evidence on essentiality, consistency, analogy, specificity as well as empirical support for dose-temporal concordance may affect the strength of evidence. In cases where two different MoAs, one endocrine and one non-endocrine could explain the same adverse effect, the WoE of both MoAs should be assessed in a comparative analysis, see 3.5 of the ECHA/EFSA ED Guidance. However, when the endocrine MoA is the most likely, even in presence of an alternative non-endocrine MoA, the ED MoA should be used for classification. See also examples in Section 4.2.5 below where data is not sufficiently convincing for Category 1, but the Category 2 criteria are met.

Regarding the reliability of studies, it should be noted that some parameters may be reliably investigated although the study may not be considered fully reliable as regards all parameters due to specific deficiencies which do not affect all the investigated /observed effects. Therefore, reliability should always be assessed with care, and the overall study reliability scores do not necessarily indicate how much weight can be given for a subset of investigations and results in the study in an overall WoE assessment. This applies for the assessment of all types of studies but particularly non-guideline and non-GLP studies.

Sufficient evidence for the empirical element (c) (a biological plausible link between endocrine activity and the adverse effect) to classify a substance in Category 1 or Category 2 can be based on *e.g.*:

- understanding of the key event relationships (KER) based on broad acceptance, *e.g.*, in scientific literature or in an endorsed Adverse Outcome Pathway (AOP), see OECD Series on AOPs[28]), *i.e.* the postulated endocrine MoA and the KEs need to be consistent with the current understanding of physiology, endocrinology and (eco)toxicology by addressing structural and/or functional relationships between KEs

- if the KER is plausible based on analogy with accepted biological relationships even when scientific understanding is not completely established

- When there are dose and time concordance between early KEs and later KEs.

- existing knowledge on endocrinology / toxicology may be sufficient to assess the biological plausibility (*e.g.* if MoA is mainly established and empirically supported on the basis of EATS or other less explored endocrine function mediated parameters).

- When adverse effects are '*EATS-mediated*'. These parameters provide evidence for adversity, while at the same time (due to the nature of the effect and existing knowledge as described in the OECD GD 150) they are also considered indicative of an EATS MoA and thus (in the absence of other explanations) also infer an underlying *in vivo* mechanism. Because both data on adversity and endocrine activity are provided by the same study, it may be possible to reach a conclusion on the biological plausibility of the link without recourse to a detailed MoA analysis.

In general, EATS mediated adverse effects can directly trigger *ED ENV 1*, whereas for adverse effects '*sensitive to, but not diagnostic of, EATS*' effects and '*non-EATS mediated*' adverse effects, an ED MoA must be demonstrated in more detail for a classification in *ED ENV 1*. Such effects could also potentially lead to an *ED ENV 2* classification (see parameters in Tables 15 and 16 of ECHA/EFSA ED Guidance). It should be highlighted that some individual parameters described in Tables 15 and 16 may not be considered sufficient in isolation for covering the element of adversity. In such cases, the conclusion on classification relies on a combination of parameters and the observation of a pattern of effects.

The following scenarios can be identified.

If **adverse effect(s) are based on *'EATS-mediated parameter(s)'***, the pattern of adverse effect(s) observed provide evidence for both adverse effect(s), endocrine activity and the biologically plausible link. Therefore, classification *ED ENV 1*; EUH430 or *ED ENV 2* EUH431 is warranted depending on the strength of the available evidence even without specific mechanistic information or identification of the specific MoA, unless demonstrated not to be ED in a MoA analysis (with a fully developed non-ED MoA) supported by sufficient data. Consideration should be given to the existence of a pattern of effect and a WoE assessment should always be conducted to put any adverse effects into context.

If **adverse effect(s) are based on *'Sensitive to, but not diagnostic of, EATS parameters'***, or **'non-EATS mediated parameters'**, there are several different scenarios that could lead to different classification outcomes for endocrine disruption.

These scenarios depend on:

[28] https://www.oecd-ilibrary.org/environment/oecd-series-on-adverse-outcome-pathways_2415170x

i. the strength of the evidence for the three elements in CLP Annex I: 4.2.2.1,

ii. whether '*EATS-mediated*' parameters have been extensively or partially investigated and found positive or negative and,

iii. the available information on whether other types of endocrine activity, including activity not already inferred by '*EATS-mediated*' parameters, is available and

iv. the WoE.

Classification may also be warranted in cases when there is evidence that the elements indicated in CLP, Annex I 4.2.2.2 *i.e.* (a) endocrine activity, (b) adverse effect(s), (c) plausible link are met, however there is not enough information to postulate a detailed MoA due to the lack of thorough mechanistic information. This is for example the case when a pattern of adverse effects has been identified which, based on current knowledge, is concluded to be related to endocrine disruption (adverse effects which are considered EATS mediated or '*sensitive to, but not diagnostic of, EATS*' or '*non-EATS mediated*'), but due to the complexity and crosstalk of the endocrine system, it is difficult to identify the specific modality. In this situation, classification as *ED ENV 1*; EUH430 or *ED ENV 2*; EUH431 may be justified based on the strength of the evidence (see Section 4.2.6.2.6. example 6).

The substance should not be classified, for example, when:

- no adverse effect(s) are observed. This includes adaptive responses that are demonstrated not to be ecotoxicologically relevant, *i.e.* not adverse per se or not leading to adverse effects), or
- adverse effect(s) are not relevant at the population level, or
- no endocrine activity is observed, or
- no biological plausible link can be established, *i.e.* adverse effects are observed which cannot be linked to the observed endocrine activity using existing knowledge, or
- if adverse effect(s) are solely a non-specific consequence of other toxic effects (see CLP, Annex I, Section 4.2.2.2.2.) *i.e.* observed adverse effects are a consequence of excessive other toxicity, or
- when a non-endocrine MoA as a result of a comparative MoA analysis has been demonstrated to be the most likely explanation of the observed adverse effect(s).

A distinction may need to be made between whether the data are sufficient to conclude on classification for ED or whether some important data are lacking and therefore the outcome of "no classification" is due to lack of data for the modalities assessed.

To summarise, for Category 2, the situation may be also that Category 1 classification cannot be concluded due to lack of data but the currently available data better supports Category 2 classification.

Ultimately, a WoE approach and expert judgement is needed to decide on the appropriate Category.

4.2.2.4.1. Specific considerations related to the thyroid modality with respect to decision on classification

As mentioned in Section 3.11.2.3.1 of this guidance, the thyroid system is highly conserved across vertebrates, therefore, indications of interference with thyroid function or thyroid hormone signalling in one species may well lead to similar affects in others, including in wildlife species such as amphibians.

The classification of a substance as *ED ENV* can, in some situations, already be reached considering the available evidence on the thyroid modality from mammals if that evidence allows to classify the substance as *ED HH.*

This is the case when the adverse effect(s) observed in mammals leading to the classification for HH are considered to be population relevant (for example if (neuro)developmental effects in mammals are observed). In such case, classification for *ED ENV* is also warranted (see Section 4.2.2.3.2 on population relevance).

If adverse effect(s) observed in mammals, taking into account the whole data package in a WoE approach, are considered not relevant at the population level, classification for environment is warranted only when there is other information specific for the environment proving the population relevance of the effects. The allocation to Category 1 or 2 will depend on the type of evidence available and on the strength of that evidence.

In case there is no evidence from mammals, or the substance is not classified for *ED HH* for the thyroid modality, classification as Category 1 is only warranted if there is at least one *in vivo* long-term test in a non-mammalian species showing evidence of adverse effects relevant at the population level, or non-animal data providing an equivalent predictive capacity to the *in vivo* data. When the *in vivo* information is available only at the screening level, classification in either Category 1 or Category 2 should be considered on a case-by-case basis, depending on whether it is positive for adverse effect(s) or only for endocrine activity. If only mechanistic information is available and positive, due to the absence of evidence on adverse effect(s), no classification is warranted.

4.2.2.5. Classification of substances and mixtures containing ED constituents/components

In analogy to the approach used for CMR substances, from a compositional and a regulatory point of view the situation for substances containing ED constituents, additives or impurities is the same as for mixtures containing components classified for these hazard classes. For this reason, the classification procedure for ED endpoints that is foreseen by CLP for mixtures containing ED components, is considered applicable also to substances containing ED constituents, additives or impurities (see Sections 4.2.3.1 and 4.2.3.2 of this guidance).

As discussed in Section 4.2.3.2 below, mixtures containing components classified as EDs shall be normally classified using only the relevant available information for the individual substances in the mixture. Further, in cases where the available test data on the mixture itself demonstrate positive ED effects which have not been identified from the information on the individual substances, those data shall also be taken into account.

Dilution, as would be the case if mixtures or substances containing ED components/constituents were tested, would increase the risk that ED hazards would not be detected, *i.e.* dilution might compromise the threshold of detection for ED hazards. Therefore, negative test data on mixtures containing components with these hazards shall not be accepted.

According to Article 10(1), generic and specific concentration limits (GCLs and SCLs) are similarly assigned to substances in other substances and substances in mixtures. A GCL will apply to EDs unless the data justifies setting an SCL.

4.2.2.6. Setting of specific concentration limits

> **CLP, Article 10(1)** *Specific concentration limits and generic concentration limits are limits assigned to a substance indicating a threshold at or above which the presence of that substance in another substance or in a mixture as an identified impurity, additive or individual constituent leads to the classification of the substance or mixture as hazardous.*
>
> *Specific concentration limits shall be set by the manufacturer, importer or downstream user where adequate and reliable scientific information shows that the hazard of a substance is evident when the substance is present at a level below the concentrations set for any hazard class in Part 2 of Annex I or below the generic concentration limits set for any hazard class in Parts 3, 4 and 5 of Annex I.*
>
> *In exceptional circumstances specific concentration limits may be set by the manufacturer, importer or downstream user where he has adequate, reliable and conclusive scientific information that a hazard of a substance classified as hazardous is not evident at a level above the concentrations set for the relevant hazard class in Part 2 of Annex I or above the generic concentration limits set for the relevant hazard class in Parts 3, 4 and 5 of that Annex.*

The concept of applying the SCL is described in Section 1.5 of this guidance. The information on GCL of a mixture classified as ED for the environment is described in Section 4.2.3.1.

To align the protection levels for EDs for human health and the environment the SCLs for ED effects for the most potent substances need to be derived. As explained in Section 4.2.1, the concept of ED "potency" is considered only in the context of setting specific concentration limits.

4.2.2.6.1. Procedure

In general, the SCLs for ED properties are set based on the potency of the adverse effect, which is a pragmatic approach used in EU laws to further inform the downstream user or supplier on the presence of a hazardous substance in a mixture. However, it should be noted that for some endocrine disruption endpoints, potency may vary. When data allows to set an SCL, the SCLs for ED shall be set following the procedure described below instead of using the GCL. The way of setting the SCL for ED for environment will depend on the source of data used to classify a substance for this hazard class.

ED effect level (*e.g.*, EC10, NOEC, LOEC or DNEL from any relevant studies[29] where adverse effect(s) are observed with sufficient confidence) for adverse endpoints can be considered for setting the SCLs (see Section 4.2.2.3.1. of this guidance), but the CLP criteria for ED for the environment do not specify any concentrations above which the production of an adverse effect is considered to be outside the criteria which lead to classification, provided that the used concentrations are still within the recommendations for test concentrations set by the corresponding OECD test guidelines.

When the *ED ENV* classification is based on the mammalian data used for the *ED HH* classification and there is no relevant non-mammalian information, derivation of the SCLs should be calculated according to the same principles as described in Section 3.11.2.6

[29] SCL may also be calculated based on effect levels derived from the screening studies. It has to be noted however that such provisional NOECs, EC_{10}, etc can be higher than the effect values derived in the definitive studies.

above.

However, when the *ED ENV* classification is based on information on non-mammalian organisms the following scenarios for the derivation of concentration limits are possible.

a. When the adverse effect used for the *ED ENV* classification comes from the non-mammalian toxicity study from which the EC_{10} or NOEC value[30] for the specific ED parameters indicating adverse effects can be derived and is below 0.1 mg/L, the SCL should be calculated as presented in Table 1 below:

   i. For substances with EC_{10} or NOEC ≤ 0.00001 mg/L, the SCL that is 100-fold lower than GCL should be considered on a case-by-case basis. This is introduced to cover extremely potent ED substances.

   ii. For substances with 0.00001 mg/L < EC_{10} or NOEC ≤ 0.001 mg/L, the SCL should be 10-fold lower than a default GCL.

   iii. For substances with 0.001 mg/L < EC_{10} or NOEC ≤0.1 mg/L, the GCL as presented in the CLP, Annex I, table 4.2.2 should be applied.

b. When the adverse effect used for *ED ENV* classification comes from the non-mammalian toxicity study from which the EC_{10} or NOEC value is above 0.1 mg/L, the GCL as indicated in the CLP, Annex 1, Table 4.2.2. should be used.

Table 4.2.2. SCL derivation based on non-mammalian data

Potency	Effect leading to adverse effect(s) (Non-mammalian study) [mg/L][a, b]	SCL (Cat1)	SCL (Cat2)
Very high potency (see bullet point a.i. above)	EC10 or NOEC≤0.00001	GCL/100 = 0.001%	GCL/10 = 0.01%
High potency (see bullet point a.ii. above)	0.00001<EC10 or NOEC≤ 0.001	GCL/10 = 0.01%	GCL/10 = 0.1%
Medium potency (see bullet point a.iii. above)	0.001<EC10 or /NOEC≤0.1	no SCL derived, GCL =0.1%	no SCL derived, GCL =1%
Low potency (see bullet point b. above)	EC10 or /NOEC>0.1 mg/L	no SCL derived, GCL =0.1%	no SCL derived, GCL =1%

[a] *When the adverse effect used for ED ENV classification would come from the non-aquatic non-mammalian toxicity study where the results are expressed in mg/kg (e.g., bird reproduction studies), the SCLs should be calculated based on the same principles as described in Section 3.11.2.6, particularly following a method similar to 3.7.2 above.*

[b] *If a NOEC value is not available, the LOEC may be used to calculate the SCL, however, when calculating the SCL it should be taken into account that the NOEC value would be lower than the LOEC.*

[30] If available, EC10 is preferred over NOEC, see Section 4.1.3.1.1

In exceptional cases a higher SCL than the GCL can also be set for EDs. A higher SCL should only be set where there are adequate, reliable and conclusive scientific information that a hazard of a substance classified as hazardous is clearly above the level of GCL.

When there are several types of effects and ways to calculate SCLs, the lowest SCL should be selected for the classification. Only one SCL can be set for *ED ENV*.

When the calculated SCL or GCL is not considered protective enough, the SCL corresponding to very high potency group may be set by default, unless an even lower SCL is justified. Due to these above-mentioned characteristics for some EDs, the assessment of dose-response related information together with setting SCLs should be conducted with caution.

4.2.2.7. Decision logic for classification of substances

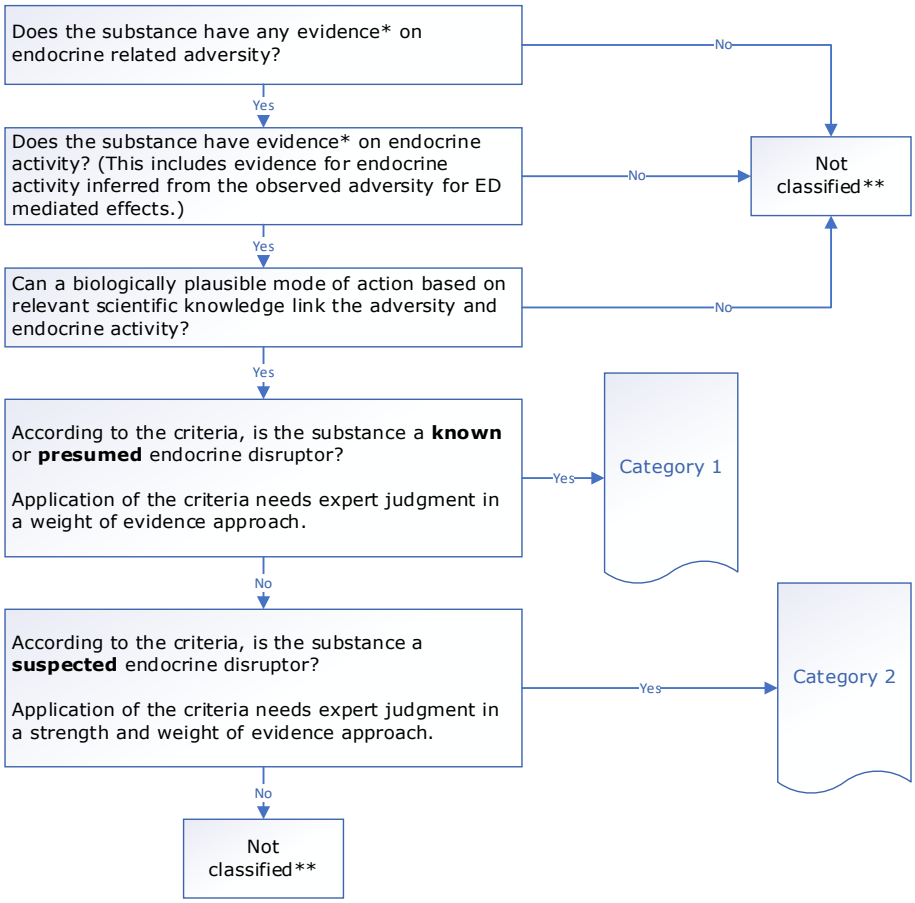
Commented [A15]: Question to CARACAL:
ECHA suggest to delete this whole paragraph and the flowchart since it does not bring any added value.

We would like to hear to opinion of CARACAL on this.

The decision logic which follows in Figure 4.2-3, is provided here as additional guidance and at a very high level. Therefore, it is strongly recommended that the person responsible for classification study the criteria before and during use of the decision logic.

Figure 4.2-3 Decision logic for endocrine disruption for the environment

The following outcomes are expected: 'Category 1', 'Category 2', 'not classified'.



*Evidence in this context does not necessarily need to be substance specific, but can be obtained *e.g.* using read-across when this is justified.

**In should be noted that when the outcome is 'not classified' it can be for the following reasons not meeting the CLP ED criteria, or 'classification not possible'; *i.e.* due to lack of or inconclusive data.

4.2.3. Classification of mixtures for endocrine disruption for environment

4.2.3.1. Classification criteria for mixtures

Endocrine disruption classification of mixtures is based on the presence of an ingredient classified for endocrine disruption (see CLP, Article 6(3) and CLP, Annex I, Section, 4.2.3). Only in case there is data available for the mixture itself which demonstrate effects not apparent from the ingredients, might this data be used for classification. In other words, data on tested mixtures shall be used only when it demonstrates classification for endocrine disruption for the environment, in line with CLP, Annex I, Section 4.2.3.2.1. *i.e.*, not for "no classification". If such data is not available for the mixture itself, data on a

similar mixture can be used in accordance with the bridging principle; see CLP, Annex I, Section 1.1.3. Furthermore, it should be noted that various test guidelines have not been validated for mixtures and therefore, it is questionable if these tests may provide adequate results.

From a compositional and an (eco)toxicological point of view, the situation for substances containing ED constituents, additives or impurities is the same as for mixtures containing components classified for these endpoints. For this reason, the classification procedure for ED endpoints that is foreseen by CLP for mixtures containing ED components is considered applicable also to substances containing ED constituents, additives, or impurities (see Sections 1.1.6.1, and 3.11.3.1.1 to 3.11.3.2 of this guidance).

CLP, Annex I, Section: ***4.2.3.1.1.*** *A mixture shall be classified as an endocrine disruptor for the environment where at least one component has been classified as a Category 1 or Category 2 endocrine disruptor for the environment and is present at or above the appropriate generic concentration limit as shown in Table 4.2.2 for Category 1 and Category 2, respectively.*

As such, each component in a mixture classified as an ED is compared separately to their respective generic or specific concentration limit to conclude on the classification of the mixture, unless the additivity principle applies.

The additivity concept may have to be applied for EDs; see also Section 1.6.3.4.3. For a given effect, the SCL, if available, needs to be taken into consideration when applying the additivity concept - this will include potency considerations. Exposure to EDs with both similar and dissimilar modes of action can lead to combination effects if they impact the same physiologic process(es), or have the same target organs for toxicity. If one single classified substance is present in the mixture above the generic or specific concentration limit, the mixture must be classified for that hazard. If the mixture contains two or more substances each below the generic or specific concentration limits, the mixture will not be classified, unless the additivity concept applies. For endocrine disruption, it is reasonable to assume additivity for substances with a similar or related mechanism or MoA or adverse outcome (*e.g.*, exposure to a combination of anti-androgenic, estrogenic and steroidogenic or even thyroid disrupting substances can lead to additivity), unless there are specific reasons not to do so.

The mechanism does not need to be the same. Similarly, to most of the HH hazard classes, the same adverse outcome between substances can already suggest additivity.

It is important in the assessment of potential additivity to consider if constituents with the same biological targets have different effects or mechanism behind the effects (*e.g.* they may have agonistic or antagonistic activity or even partial activity at the same receptor). In this case a careful assessment is needed since dissimilar modes of actions can cause the same adverse outcomes in an additive manner.

CLP, Annex I*: Table 4.2.2*

Generic concentration limits of components of a mixture classified as endocrine disruptor for the environment that trigger classification of the mixture

Component classified as:	*Generic concentration limits triggering classification of a mixture as:*	
	Category 1 endocrine disruptor for the	*Category 2 endocrine disruptor for the*

	environment	*environment*
Category 1 endocrine disruptor for the environment	*≥ 0,1 %*	
Category 2 endocrine disruptor for the environment		*≥ 1 %* *[Note 1]*
Note: The concentration limits in this Table shall apply to solids and liquids (w/w units) as well as gases (v/v units). *Note 1: If a Category 2 endocrine disruptor for the environment is present in the mixture as an ingredient at a concentration ≥ 0,1 % a SDS shall be available for the mixture upon request.*		

3888 **4.2.3.1.1. When data are available for the individual ingredients**

CLP, Annex I, Section *4.2.3.1.1.* *A mixture shall be classified as an endocrine disruptor for the environment where at least one component has been classified as a Category 1 or Category 2 endocrine disruptor for the environment and is present at or above the appropriate generic concentration limit as shown in Table 4.2.2 for Category 1 and Category 2, respectively.*

3889 Additivity shall be considered on a case-by-case basis, particularly when the data suggests
3890 the same/related endocrine MoA or modality or adverse outcome for different ingredients
3891 of the mixture.

3892 **4.2.3.1.2. When data are available for the complete mixture**

CLP, Annex I, Section *4.2.3.2.1.* *Classification of mixtures shall be based on the available test data for the individual components of the mixture using concentration limits for the components classified as endocrine disruptor for the environment. On a case-by-case basis, test data on the mixture as a whole may be used for classification when demonstrating endocrine disruption for the environment that has not been established from the evaluation based on the individual components. In such cases, the test results for the mixture as a whole must be shown to be conclusive taking into account dose (concentration) and other factors such as duration, observations, sensitivity and statistical analysis of the test systems. Adequate documentation supporting the classification shall be retained and made available for review upon request.*

3893 **4.2.3.1.3. When data are not available for the complete mixture: bridging**
3894 **principles**

CLP, Annex I, Section *4.2.3.3.1.* *Where the mixture itself has not been tested to determine its endocrine disruption for the environment, but there are sufficient data on the individual components and similar tested mixtures (subject to paragraph 4.2.3.2.1.) to adequately characterise the hazards of the mixture, those data shall be used in accordance with the applicable bridging principles set out in Section 1.1.3.*

3895 Bridging Principles will only be used on a case-by-case basis (see Section 1.6.3 of this
3896 guidance). Data on similar tested mixtures shall be used only when it demonstrates

classification for endocrine disruption for environment, in line with CLP, Annex 1, Section 4.2.3.2.1. *i.e.* not for "no classification". Note that the following bridging principles are not applicable to this hazard class:

- concentration of highly hazardous mixtures
- interpolation within one hazard Category

(see CLP, Annex I, Sections 1.1.3.3 and 1.1.3.4)

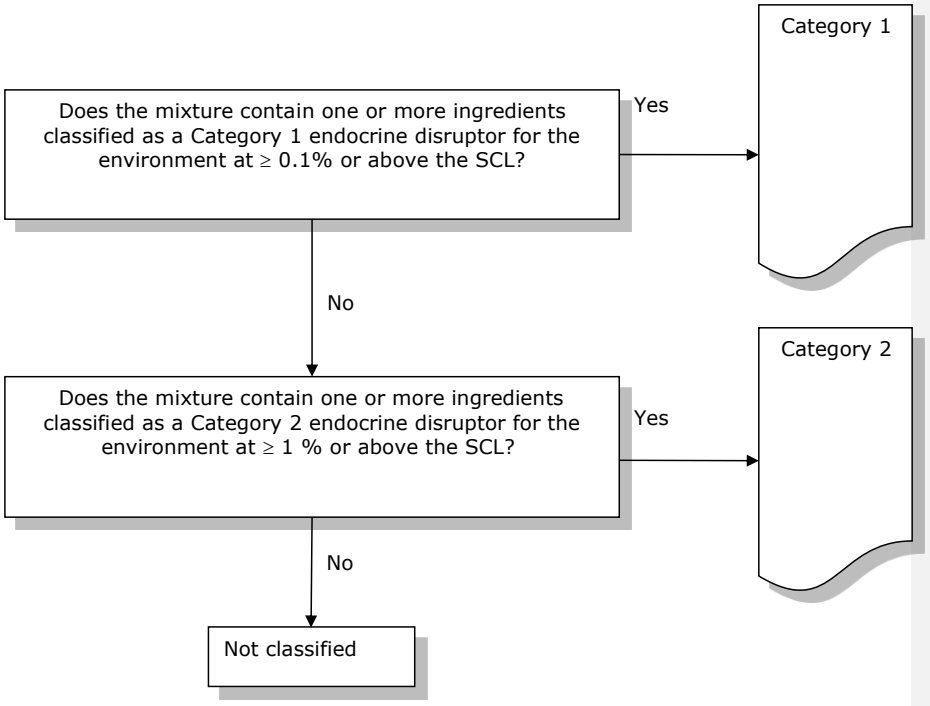
4.2.3.2. Decision logic for classification of mixtures

The decision logic for classification of mixtures in Figure 4.2-4 and Figure 4.2-5 is provided here as additional guidance. The person responsible for classification should study the criteria before and during use of the decision logic presented below.

Classification of mixtures for endocrine disruption for environment

Classification based on individual ingredients of the mixture

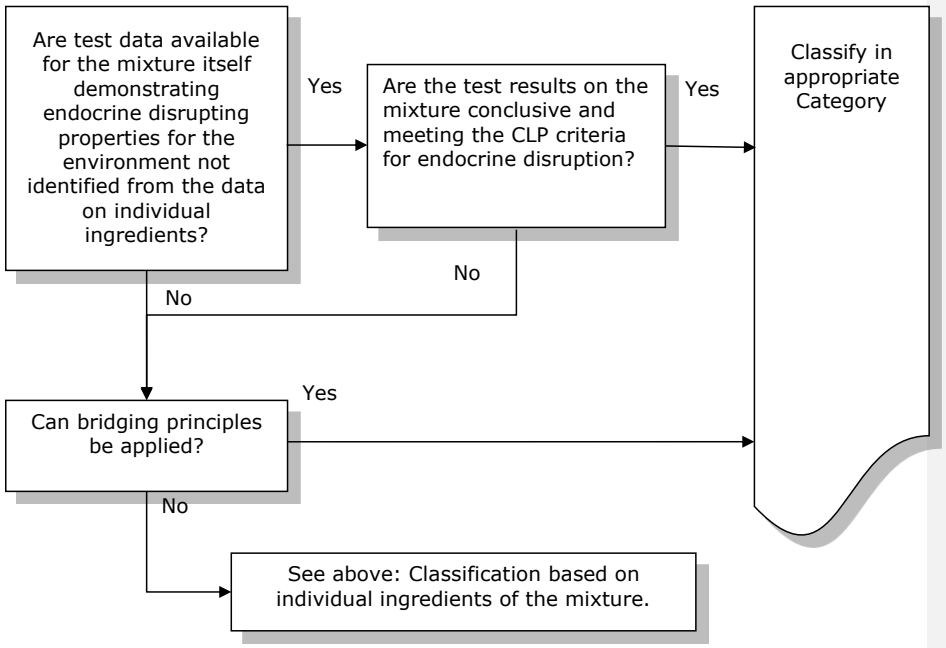
Figure 4.2-4 Decision logic for classification of mixtures based on individual ingredients of the mixture



Modified classification when the test data on the mixture itself supports more stringent classification than evaluation based on individual ingredients

Test data on mixtures may be used for classification when demonstrating effects that have not been established from the evaluation based on the individual ingredients; CLP, Article 6(3) and CLP, Annex I, Section 4.2.3.2.1.

Figure 4.2-5 Decision logic for classification of mixtures when the test data on the mixture itself supports more stringent classification then evaluation based on individual ingredients



4.2.4. Hazard communication in the form of labelling for endocrine disruption for environment

4.2.4.1. Pictograms, signal words, hazard statements and precautionary statements

Classification	*Category 1*	*Category 2*
GHS Pictograms	*	*
Signal Word	*Danger*	*Warning*
Hazard Statement	*EUH430: May cause endocrine disruption in the environment*	*EUH431: Suspected of causing endocrine disruption in the*

		environment
Precautionary Statement Prevention	*P201* *P202* *P273*	*P201* *P202* *P273*
Precautionary Statement Response	*P391*	*P391*
Precautionary Statement Storage	*P405*	*P405*
Precautionary Statement Disposal	*P501*	*P501*

*Pictogram currently unavailable. When included in GHS but not yet implemented in CLP, it is strongly recommended to be used.

The wording of the Precautionary Statements is found in CLP, Annex IV, Part 2.

4.2.4.2. Additional labelling provisions

There are no additional labelling provisions for substances and mixtures classified as EDs in CLP. However, there may be provisions laid out in other regulations such as REACH which need to be considered, when relevant.

4.2.5. Examples

Commented [A16]: Question to CARACAL
There are diverging views within the PEG on the usefulness of the examples, and on whether the boundaries between the ED ENV 1, ED ENV 2 and no classification are correctly illustrated by the examples.

We would like to hear the CARACAL opinion on this.

The examples are presented using a format starting with listing all the information available for a substance (*in vivo*, *in vitro*, *in silico*), followed by an assessment for each of the three criteria, adverse effect(s), endocrine activity and biological plausible link between adverse effect(s) and endocrine activity, and a section with the reasoning behind the conclusion on the classification.

The substances in the examples are fictitious. They do not represent real cases and are not to pre-empt the classification assessment in concrete cases. These examples are rather only to illustrate what type of data may lead to classification in different Categories for ED and to show how an assessment according to this guidance could potentially be approached. Only the ED-related information leading to classification, supporting classification or resulting in "no classification" is included in the examples, but not the whole data set or a detailed description of the effects, or a full WoE analysis. All the endocrine-related effects reported for the different examples leading to classification are considered adverse *i.e.* statistically significant compared to the control and biologically relevant. The reliability reported for the studies is according to Klimisch score. The concentration settings in the examples are considered acceptable unless stated otherwise. The decision on classification is influenced by the strength of the overall evidence and should be decided on a case-by-case basis.

List of examples:

Examples *ED ENV* 1 (see Section 4.2.5.1)

Example 1: Classification as *ED ENV 1* of a substance already classified as *Repr. 1B* and *ED HH 1*. There are no data available in fish or other wildlife organisms, therefore classification is solely based on data on mammals showing adverse effect(s) at population

level. The example is focused on EAS modalities (SCL is the same as calculated for *ED HH* classification).

Example 2: Classification as *ED ENV 1* based on fish data. The example is focused on EAS modalities (SCL calculation: GCL to be applied as no SCL is derived) for a data-rich substance.

Example 3: Classification as *ED ENV 1* based on fish data. The example is focused on EAS modalities (SCL calculation: GCL to be applied as no SCL derived) for a data-poor substance.

Examples *ED ENV* 2 (see Section 4.2.5.2)

Example 4: Classification as *ED ENV 2* based on fish data. The example is focused on EAS modalities. Adverse effect(s) observed are not convincing enough to place the substance in Category 1 (GCL to be applied).

Example 5: Classification as *ED ENV 2* based on fish data. The example is focused on EAS modalities. Adverse effect(s) observed are based on '*Sensitive to, but not diagnostic of, EATS*' parameters (SCL calculation: GCL to be applied as no SCL derived).

Example 6: Classification as *ED ENV 2* based on fish data. The example is focused on EAS modalities (GCL to be applied).

Example 7: Classification as *ED ENV 2* for the thyroid modality (GCL to be applied).

Example 8: Classification as *ED ENV 2* for non-EATS modalities (GCL to be applied).

Example 9: Classification of a metal compound as *ED ENV 2* based on fish data. The example is focused on EAS modalities. (GCL to be applied).

Examples *ED ENV* No classification (see Section 4.2.5.3)

Example 10: no classification as no adverse effect(s) (the only effects are observed in the presence of other toxicity) and no endocrine activity identified. The example focuses on EAS modalities.

Example 11: no classification as no adverse effect(s) and no endocrine activity identified. The example focuses on EATS modalities.

4.2.5.1. Examples *ED ENV 1*

4.2.5.1.1. Example 1 - *ED ENV 1* (EAS modalities)

Available information in mammals and conclusion for classification as *ED HH 1*.
See information in example 1 in Section 3.11.5.1.1.

Available information for environment:
There is no aquatic *in vivo* long-term data for fish and other aquatic vertebrates.

The assessment for the environment is based on the mammalian data used for the human health assessment.

There is no additional mechanistic information available which was not considered with regard to human health.

Assessment:

Adverse effect(s):
The adverse effects on uterus and ovarian weight, and oestrous cycle are considered '*EAS mediated*'. The effect on age at first oestrus is an '*EAS mediated*' parameter and provides clear evidence of an endocrine MoA. This is further supported by the observed effects on corpora lutea and litter size that are considered '*sensitive to, but not diagnostic of, EAS*' parameters, indicating a wider pattern of effects likely to be EAS mediated. All effects are observed in the absence of other toxicity. The pattern of effects identified is considered relevant at the level of population for wild mammals.

Endocrine activity:
In the absence of additional information specific to the environment, the assessment with regard to human health is fully applicable for environment. As explained in Section 3.11.5.1.1., there is a positive uterothrophic assay indicating estrogenic activity, further supported by QSAR predictions and ER binding capacity.

Biological plausible link:
There is evidence of a biological plausible link because the parameters measured *in vivo* that contribute to the evaluation of adverse effect(s) at population level at the same time provide evidence for specific EAS modes of action. Due to the nature of the effect and the existing knowledge on mammalian reproductive endocrinology, these adverse effects are considered diagnostic of an EAS MoA and thus (in the absence of other explanations) also infer an underlying *in vivo* mechanism.

Conclusion:
The substance caused significant effects on fertility (such as reduction in number of corpora lutea, reduced number of implantation sites, reduced litter size) in reproductive toxicity studies leading to a reduced number of offspring.
As effects on growth, development and reproduction in single species are generally regarded relevant for the maintenance of wild populations, the observed effects on reproduction and pubertal development in rats are relevant for mammalian populations in the environment (wild mammals).

Therefore, it is concluded that the substance meets the CLP criteria for *ED ENV 1*.

SCL calculation:
The ED classification is derived based on the mammalian data, therefore the SCL as calculated for the *ED HH* classification should be used. For details on calculation of SCL see HH example 1, Section 3.11.5.1.1 of this guidance. According to mammalian data no SCL needs to be set for this substance.

4.2.5.1.2. Example 2 - *ED ENV 1* (EAS modalities)

Available information:
The substance was concluded not to meet the CLP criteria as *ED HH* due to the absence of a pattern of '*EATS-mediated*' adversity.

In vivo information:

- Fish full lifecycle test conducted with sheepshead minnow (FFLCT, OPPTS 850.1500, reliability 1, 100 days exposure, measured test concentrations: 0, 0.016, 0.038, 0.068, 0.15, 0.29, 0.55 mg/L):
    - No effects on hatching success or survival of F0.

- Effects on hatching success in F1 generation observed, but at concentrations where reproduction was severely decreased and thus this information in the F1 is likely to reflect the quality of eggs produced.
- No effects on weight and length of larvae of F0.
- Reproduction (fecundity) significantly reduced at 0.15, 0.29 and 0.55 mg/L (NOEC = 0.068 mg/l (mean measured)).
- F1 hatching success significantly reduced at 0.29 and 0.55 mg/L.
- F1 28-day post-hatch survival significantly reduced at 0.55 mg/L.
- Gonad histopathology not assessed.

- Fish full lifecycle test conducted with fathead minnow (FFLCT, OPPTS 850.1500, reliability 1, 256 days exposure, test concentrations: 0, 0.0078, 0.022, 0.063, 0.188 and 0.558 mg/L) with inclusion of all the parameters foreseen to be investigated in the OECD TG 240:
  - No effects on hatching success or fertility of F1 or F2 generations.
  - No statistically significant effects on weight and length of F1 generation.
  - No statistically significant effects on sex ratio in the F1 generation.
  - Reproduction significantly reduced at 0.558 mg/L in both the F0 and F1 reproductive groups (NOEC = 0.188 mg/L).
  - Delayed maturation/time to first spawn in F1 generation at 0.558 mg/L.
  - Increased gonado-somatic-index (GSI) in F1 males at 0.558 mg/L.
  - Increased GSI in F1 females at 0.063, 0.188 and 0.558 mg/L.
  - Increased tubercle score in F1 males at 0.022, 0.063, 0.188 and 0.558 mg/L.
  - Statistically significant decrease in F1 Female VTG plasma concentration starting from 0.188 mg/L.
  - No effects on F1 male VTG plasma concentration.
  - Gonadal histopathology results:
    - Decreased yolk formation, decreased post-ovulatory follicles, and decreased mean ovarian stage scores in the ovaries of females at 0.558 mg/L;
    - Increased interstitial cell hyperplasia (number)/hypertrophy (volume) at 0.063, 0.188 and 0.558 mg/L, and increased spermatozoa at 0.558 mg/L in male testis
  - Liver histopathology results:
    - Increased nuclear pleomorphism, multi-nucleation, cystic degeneration, necrosis, pigmented macrophages, aggregates and anisocytosis in hepatocytes of males and females at 0.558 mg/L.
    - Instances of nuclear pleomorphism in males at 0.188 mg/L.
    - Decreased basophilia (vitellogenesis) in female hepatocytes at 0.558 mg/L.
    - No effects on basophilia in male livers.

- Fish short term reproduction assay with fathead minnow (FSTRA, OECD TG 229, reliability 1, 21-day exposure, test concentrations: 0, 0.01, 0.12 and 1.0 mg/L):
  - Decreased fecundity and fertilisation success at 1.0 mg/L (note increased fecundity observed at 0.12 mg/L but this was not deemed biologically significant).
  - Increased male and female GSI at 1.0 mg/L.
  - Decreased vitellogenin in females at 1.0 mg/L.

- Study with elements of OPPTS Guideline 890.1350 and OECD 229 with fathead minnow (21-day exposure, test concentrations: 0, 0.005, 0.05, 0.5 and 1 mg/L, reliability 1):
  - No effects on nuptial tubercles.
  - Increased male and female GSI at 0.5 and 1 mg/L.

- Decrease in cumulative number of eggs per female at 0.5 and 1 mg/L (a decrease was also noted at 0.05 mg/L but without concentration-response).
- Decreased 17β-estradiol in females at 0.5 and 1 mg/L.
- Decreased vitellogenin in females at 0.05, 0.500 and 1 mg/L.
- Gonad histopathological results:
  - increased prevalence of spermatozoa,
  - distended seminiferous tubules at 1 mg/L.
- Some limited increase and decrease in ovarian expression of several genes related to steroidogenesis (increase in: fshr, star, cyp11a, cyp17, and cyp19a1a; decrease in: hmgr and cyp51). These were generally inconsistent and very small changes in most instances ≤1 fold difference and were considered not biologically significant. The up-regulation observed in genes coding for cyp19a1a was around 2-3 fold at 0.5 and 1 mg/L. This was statistically significant and could be considered biologically significant.
- Some limited increases and decreases in hepatic expression of several genes coding for proteins related to metabolism (increases in: cy3a; decreases in: hmgr, fasn, fdps and cyp51). These changes were generally small and inconsistent and the Limit of quantification (LOQ) of the methodology could not be established. Statistically significant up-regulation in the gene coding for cyp1a1 (xenobiotic metabolising enzyme) at all concentrations appeared dose responsive and was up-regulated in the region 4-fold in the highest concentration.

- Non-guideline study with newly fertilised fathead minnow *Pimephales promelas* embryos exposed to concentrations of 0.069, 0.12, 0.21, 0.43 and 0.97 mg/L for 4 days and after hatching were exposed for a further 31 days (study reliability 2):
  - No effects on hatching success.
  - Larval growth (length and weight) significantly reduced at concentrations of 0.97 mg/L.
  - Larval survival significantly reduced at concentrations of 0.97 mg/L.
  - Any growth/development effects only observed at concentrations equivalent to those at which effects on survival were observed.

- Rapid Androgen Disruption Activity Reporter assay with spg1-gfp transgenic medaka eleutheroembryos (RADAR assay, OECD TG 251, reliability 1, 3 days exposure, test concentrations: 0, 0.003, 0.009, 0.027, 0.081, 0.243 mg/L):
  - No mortality at any test concentration. No observation of malformations or behavioral effects.
  - Unspiked condition: no statistically significant change in fluorescence.
  - Spiked condition: statistically significant concentration-dependent decrease in fluorescence indicating inhibition of 17α-methyltestosterone-induced spiggin production in transgenic medaka eleutheroembryos.

In vitro information:
All assays reported below have a reliability 1-2 and no cytotoxicity was reported.

- Inhibition of CYP19 activity (IC50=6.5 uM) in human placental microsomes.
- Competitive inhibition of CYP19 activity in H295R cell line.
- Positive in recombinant human microsome aromatase activity inhibition assay
- Inconclusive results on aromatase activity inhibition in a JEG-3 cell line.
- Negative for agonism and positive for antagonism modulation of testosterone-in MCF-7 cell line proliferation assay.

- 185-fold selectivity for inhibition of yeast (Candida albicans) CYP51 compared to human CYP51 in Yeast and human CYP51 expressed in bacteria.
- Binding to zebrafish CYP51 with a much lower affinity than yeast.
- Negative for both agonism and antagonism ER activation in human ERα or ERβ transfected into CHO cell line.
- Weak positive for agonism ER activation in Yeast estrogen screen.
- Negative for agonism, positive for antagonism ER activation in MCF-7 Cell line proliferation assay.
- Negative for binding in rat uterine ER.
- Weak positive for agonism, negative for antagonism ER activation in MVLN cell line.
- Positive for AR binding in immuno-immobilised human AR.
- Negative for agonism, positive for antagonism AR activation in human AR transfected into CHO, CHO-K1, and MDA-kb2 cell lines.
- Inhibition of estrone biosynthesis in human ovarian granulosa tumour cells.
- Decreased oestradiol and testosterone biosynthesis in H295R cell line.
- Decreased estrogen biosynthesis at ≥1000 µg/L (≥3 µM).
- No effect on testosterone biosynthesis in ovary explants from fathead minnow.
- Positive toxcast in NCGC_ERalpha_Antagonist and NVS_NR_hAR.

Assessment

Adverse effect(s):
A pattern of potentially endocrine-related adverse effects relevant at the population level was observed across studies and species: decrease in fecundity was observed, accompanied by changes in gonad histopathology in both males and females.

The endocrine-related adverse effects were observed in the absence of other toxicity. Although some effects in liver were observed in one of the available studies, currently there is no proven correlation between hepatotoxicity and effects due to endocrine disruption.

Endocrine activity:
Several *in vitro* assays are available showing positive evidence for androgen antagonism and aromatase inhibition (inhibition of CYP19).

In addition, a FSTRA and a 21-d assay were available. In one of the 2 available FFLCTTs *in vivo* mechanistic parameters were also measured.

Estradiol and testosterone were only measured in the 21-d assay. Decrease in the level of estradiol was observed in a dose response manner (0.5 and 1 mg/L) both *ex vivo* and in plasma. A decrease in testosterone was only observed *ex vivo* at the highest tested concentration.

VTG was measured in 3 studies and a decrease was observed in females in all of them. The decrease observed is empirically supported by the dose response. Difference between studies can be explained by the study design and dose spacing.

The endocrine activity gives indication of activity through A and S modalities.

Biological plausible link:
Considering the observed endocrine activity and adverse effect(s), two MoAs can be postulated: aromatase inhibition leading to reproductive failure and androgen antagonism leading to reproductive failure.
For the first MoA:

	Brief description of key event	**Supporting evidence**

MIE	Inhibition of aromatase	Several *in vitro* assays showing positive evidence
KE1	Decreased level of estradiol ex vivo in ovaries	Decrease observed in one 21-day assay with fish
KE2	Decreased level of estradiol in plasma	Decrease observed in one 21-day assay with fish
KE3	Decreased VTG level in plasma	Decrease observed in 2 level 3 studies and one FFLCTT
KE4	Change in female gonad histopathology	Change in gonad histopathology observed in 1 level 3 study and one FFLCTT
Adverse effect	Decrease in fecundity	Decrease observed in 2 FFLCTTs and 2 level 3 studies

An additional MoA for androgen antagonism was postulated. However, this is not completely supported by the available data. No decrease in testosterone was observed *in vivo*. No changes in male secondary sex characteristics were recorded or on fertility. Therefore, the substance is not likely to be acting as an androgen antagonist. The most plausible MoA is the aromatase inhibition leading to reproductive failure.

Conclusion:
Overall, in all the available studies and in two species, a decrease in fecundity was observed in a dose response manner. When assessed, this was accompanied by changes in female gonad histopathology.
Endocrine activity, *i.e.*, inhibition of aromatase was also observed *in vitro* and *in vivo*.
Considering all the available information on *in vitro* and *in vivo* mechanistic parameters and EAS-mediated parameters it can be concluded that the substance meets the CLP ED criteria Category 1 for the EAS-modalities for the environment.

SCL calculation:
No observed effect concentration ($NOEC_{reproduction}$ = 0.05 mg/L), thus according to Table 1, Section 4.2.2.5.1 of this guidance, substances with 0.001 mg/L<NOEC≤0.1 mg/L result in a medium potency group corresponding to a GCL (0.1%). Therefore, no SCL will be set.

4.2.5.1.3. Example 3 - *ED ENV 1* (EAS modalities)

Available information:
The substance was concluded not to meet the CLP criteria as *ED HH* due to the absence of a pattern of '*EATS-mediated*' adversity.
In vivo information

- Fish sexual developmental test with *Pimephales promelas* (study similar to OECD 234, exposure over 128 days, test concentrations of 0, 9.6, 27, 83, 255 µg/L, reliability 1):
  - Secondary sexual characteristics (proportion of male fish with a pigmented spot on dorsal fin, with pigmentation on the nose/lip, with a fatpad present, fatpad score of male fish, proportion of male fish with one or more tubercles present) in male fish significantly decreased at 27, 83, 255 µg/L (NOEC = 9.6 µg/L).
  - No effect on sex ratio.
  - One fish with testis-ova observed at 255 µg/L. This fish also had feminized gonadal ducts.
  - Retained peritoneal attachments/gonadal duct feminization of the testis: at 255 µg/L almost all male fish (42 out of 45) exhibited feminization of gonadal ducts.
  - Stage testis development affected with the highest proportion of fish in all treatments in entirely immature phase or even juvenile phase (54 to 69 %) compared to control fish with 33 %.

- Length and weight slightly reduced at 27 µg/L and higher concentrations in males and females; (NOEC=9.6 µg/L).
- Time to hatch significantly increased at 255 µg/L.
- Significant decrease (90%) in larvae/juvenile survival from post-hatch to thinning on day 33 at 255 µg/L.
- Statistically significant VTG induction observed only in females at 83, 255 µg/L.

- Modified juvenile growth test with *Sander lucioperca* (fish were exposed from 60 dph to 88 dph and further reared without exposure until 144 dph, test concentrations 0, 10, 100, 200 µg/L, reliability 2 as well conducted study but no raw data available):
  - Statistically significant and concentration dependant sex ratio shift towards more females and less males at 10 µg/L and above (from 58% females at 10 µg/L to 98% females at 200 µg/L).
  - No males were observed at the highest test concentrations (100 and 200 µg/L).
  - Results at day 144 show that the effects on sex ratio persist even after exposure has ceased.
  - Statistically significant and concentration dependant VTG induction observed both in males and females in all treatments.

- Modified reproduction assay with *Oryzias latipes* (14 days, tested concentrations 0, 151, 453, 1510 µg/L, reliability 3):
  - Significant decrease in number of hatchings and unfertilized eggs at the lowest concentration of 151 µg/L.
  - Reduced average number of hatchings at higher concentrations (453 and 1510 µg/L), but not significant due to high replicate variances.

In vitro information:
All assays reported below have a reliability 1-2 and no cytotoxicity was reported.

- All available competitive binding assays using fish receptors showed that the substance binds to the ER receptor. The relative binding affinity (RBA) was 1.4 – 7.7E-5.
- Binding to sex steroid binding proteins (in plasma of rainbow trout).
- Dose-dependent increase in vitellogenin expression in primary fish hepatocytes.
- Weak ER agonist in a reporter gene assay based on recombinant yeast cells.
- Induction of human breast cancer cell (MCF-7) proliferation in four studies and thus acts as ER agonist in these cells.
- No interference with growth or survival of the immature rat ovarian follicles (from 14- day-old rat) but decreased estradiol and testosterone secretion in a dose-dependent manner.

Assessment

Adverse effect(s):
A pattern of potentially endocrine-related adverse effects relevant at the population level was observed across studies and species: change in sex ratio and decreased secondary sex characteristics in males accompanied by changes in male gonad histopathology. Decreased fertility was observed in one study not considered reliable. The effects were observed below the concentration at which excessive toxicity was observed.

Endocrine activity:
In vitro data unambiguously show that the substance acts as a ligand of the estrogen receptor in fish and mammalian cells. Modulation of ER-mediated gene expression was observed on transcriptional, protein and cell physiological levels showing that the

substance activates fish and mammal estrogen receptors. Moreover, based on the available mechanistic information (*e.g.* VTG) it can be concluded that the substance has the potential to exert estrogen-like effects and disrupt endocrine homeostasis.

Biological plausible link:
Information on endocrine activity on the substance points to an estrogenic mechanism of action. Endpoints indicative for an estrogenic MoA were assessed in three fish species (*P. promelas*, *S. lucioperca* and *O. latipes*) and a pattern of adverse effects was observed. A change in the sex ratio towards females was observed in at least one species (*S. lucioperca).* This change is both indicative for an endocrine MoA and adverse. This substantiates that the substance alters the function of the endocrine system in fish via an estrogenic MoA. Such an effect was observed in at least one species (*S. lucioperca).*

Conclusion:
There is convincing evidence for endocrine-related adverse effects in different fish species such as reduction of secondary sexual characteristics in males, accompanied by changes in gonad histopathology in one species and sex ratio shift towards more females and less males in another species; there is convincing evidence indicating that the substance has estrogenic activity; there is a plausible link with both adverse effect(s) and endocrine activity observed in the same study.

Based on the above, the substance meets the CLP criteria for *ED ENV 1*.

SCL calculation:
The No observed effect concentration is $NOEC_{growth}$=9.6 µg/L = 0.0096mg/L,, thus according to Table 1, Section 4.2.2.5.1 of this guidance, substances with 0.001<NOEC≤0.01 result in a medium potency group corresponding to a GCL. Therefore, no SCL will be set.

It should be noted that a LOEC in a juvenile growth test (10 µg/L) is similar to NOEC from fish sexual development study used for SCL calculation. Thus the actual NOEC value could be significantly lower.

4.2.5.2. Examples *ED ENV 2*

4.2.5.2.1. Example 4 - *ED ENV 2* (EAS modalities)

Available information:
The substance was concluded not to meet the CLP criteria as *ED HH* due to the absence of a pattern of '*EATS-mediated*' adversity.

In vivo information:
- Fecundity test on zebrafish (similar to a partial life cycle test, reliability 2, adult fish were exposed over 21 days, eggs were collected at 1h post fertilisation and incubated until 6 dpf test concentrations of 0, 0.001, 0.01, 0.1 and 1 mg/L):
  - No mortality observed in the parental generation.
  - Decrease in egg production of parental fish only at 0.01 mg/L.
  - No significant changes on secondary sex characteristics were observed.
  - Decrease in hatching and survival rates of their offspring at 1 mg/L.
  - Increase of hepato-somatic index at 1 mg/L in males and females, and decrease of gonado-somatic index (GSI) at 1 mg/L in males and females in absence of effects on body weight.
  - Alteration of the testis tubules and a decrease in the amount of mature spermatids at 1 mg/L, however the way the histopathological data were reported was not fully appropriate and did not allow to exclude artefacts.
  - No effect on female gonad histopathology.

- Malformations (*e.g.* abnormal curvature of larvae) in the F1 generation at 1 mg/L.
- VTG induction in males at the highest and lowest concentration but not at intermediate concentrations.
- No changes in VTG in females.
- In males, statistical significant decrease in T and increase in P at 0.1 mg/L, increase in plasmatic E2 content significant from 0.01 mg/L. In females, decreased T concentration at 1 mg/L, and increased E2 concentration at 0.01, 0.1 and 1 mg/L (statistically significant for both).
- Significant and dose-dependent induction of gnrhr1, gnrhr2, fshβ, lhβ, ERα, cyp19b in male brain while only a few genes were significantly repressed at the maximal dose in female brain. In testes, dose-dependent induction of fshr, lhr, cyp11a, 3βhsd and cyp19a gene expression while cyp17 and 17βhsd transcript levels decreased (only at 1 mg/L). Significant induction of hepatic vtg gene expression in male liver at 0.1 mg/L.
- Fertility was not measured.

- In a developmental toxicity study, not similar to any OECD guideline (reliability 4), malformation and death of zebrafish embryos were observed after exposure started on day 1 until 6 dpf and were associated with developmental disturbances.

- No other *in vivo* data available on HH side.

In vitro information:

[All assays reported below have a reliability 1-2 and no cytotoxicity was reported]

- The substance can displace 17β-Estradiol (E2) from its binding site with half the maximal inhibitory concentration (IC50) of 1.08 µM and a relative binding affinity (RBA) to E2 of 0.086%.
- The substance binds to human ER from breast cancer cells to bovine ER from uterus membrane and to recombinant mouse ERα ligand binding domain (LBD) with IC50 ranging from 0.023 µM to 0.43 µM.
- The substance induced an estrogenic response in the transactivation assay based on yeast cells stably transfected with human hERα, with an EC50 evaluated from 1.73 to 5 µM rat ERα or based on medaka ERα with an EC50 of 0.59 µM.
- The substance is able to competitively bind AR from different species (human, rat) with an IC50 in the µM range (2.2 to 37.5 µM).
- No human AR binding was observed in either human cells, mouse NIH3T3 cells, hamster CHO-K1 cells, yeast cells or with human nuclear receptor in a radiolabelled ligand binding assay.
- The two H295R assays performed show that the substance affects steroidogenesis by decreasing androgen levels (androstenedione and testosterone) and increasing estrone levels, combined with a decrease of cortisol.

Assessment:

Adverse effect(s):

A clear pattern of endocrine-related adverse effects was not observed. Effects in fecundity were observed only at one concentration level with weak empirical support and not accompanied by change in female gonad histopathology. Furthermore, there were no changes observed in secondary sex characteristics of the fish. The change observed in male gonad histopathology was not considered fully reliable. No mortality was observed in the parental generation while sublethal effects on early life stages are reported across studies.

Endocrine activity:

The estrogenic activity is well established with a large body of *in vitro* data showing that

ER signalling pathways are activated by the substance. Positive indication of endocrine activity also comes from the modification of hormone levels, upregulation of hepatic vitellogenin gene expression and the altered expression of key genes involved in the HPG axis and steroidogenesis observed in fish.

Biological plausible link:
VTG induction in males and changes in gonadal staging such as increased proportion of early sperm stages in fish, are diagnostic for the estrogen MoA. In addition, reduction of GSI in male fish is regarded as a sensitive parameter in reproductive studies with estrogenic substances, as GSI is a general measure of gonad maturation and spawning readiness. Based on current understanding of endocrinology and physiology, the adverse effects observed in fish exposed to the substance are biologically plausibly linked to its endocrine activity as an estrogen agonist. This is the most plausible MoA of the substance. In addition, effects are seen at concentrations where no systemic toxicity was observed.

Conclusion:
There is evidence of endocrine activity *in vitro* pointing to an estrogenic MoA, however uncertainties remain because no dose response was observed in the VTG induction in males. There is some evidence on adverse effect(s), however uncertainties remain because the change observed in male gonad histopathology was not considered fully reliable and the effect on fecundity was only observed at the third highest test concentration. Therefore, the substance meets the CLP criteria for classification as Category 2.

SCL calculation:
Based on the screening study on fecundity on zebrafish the provisional No Observed Effect Concentration can be derived (NOEC = 0.01 mg/L). According to Table 1, Section 4.2.2.5.1 of this guidance, substances with 0.001 mg/L <NOEC≤ 0.1 mg/L result in a medium potency group corresponding to a GCL. Thus, no SCL will be set.

In addition, it has to be noted that the provisional NOEC derived based on a screening study can be higher than the relevant effect values derived in the definitive studies.

4.2.5.2.2. Example 5 - *ED ENV 2* (EAS modalities)

Available information:
In vivo information (see table below for a comparative summary assessment of the main parameters in the different studies):

- Fish sexual development test with Zebrafish (OECD 234, reliability 1, 73 days exposure, test concentrations: 1.11 – 3.01 – 7.76 – 33.3 – 76.8 µg/L):
    - No signs of other toxicity at all concentration levels.
    - No significant change in sex ratio.
    - Increase in body weight in a conc.-dependent manner with a stat. signif. increase for the highest conc. in males and the two highest conc. in females (NOEC=7.76 µg/L).
    - Conc.-dependent decrease in plasma E2 levels in females (no measurements on males), signif. difference at the highest conc.; strong conc.-dependent increase in 11-KT in males stat. sign.
    - Stat. signif. increase in VTG in males at 33. µg/L with no dose response.
    - Stat. signif. increase in VTG in females at 33. µg/L and 76.8 µg/L.
    - Conc. dependant acceleration of gonad maturation in both sexes.
    - Conc.-dependent increase in all ovarian pathologies (oocyte atresia, egg debris, granulomatous inflammation), but without stat. signif. in any group.
    - Liver histopathological analysis revealed a dose-dependent decrease in hepatocyte lipid inclusions in females. In males, a dose-dependent increase in bile duct proliferation and inflammatory foci.

- Non-guideline study with adult zebrafish *Danio rerio* (21-day exposure using a single test concentration corresponding to less than 10% of the LC50, *i.e.* 80 µg/L (reliability 2):
    - Statistically significant increase in the hepatosomatic index (HSI) by a factor of 1.8 and 2.2 for males and females, respectively.
    - Decrease in the gonadosomatic index (GSI) in males and an increase in females (not quantified).
    - Histopathological changes: increase in the early stages of sex cells in testes and ovaries and, decrease in the more developed stages in both sexes indicating an inhibition of gametogenesis.
    - No effect on plasma hormone levels (T and E2), although E2/T ratio significantly decreased in exposed females.
    - No change in VTG in males, but a decrease is observed in females.
    - Statistically significant decrease in number of eggs laid, without significant consequences on the fertilisation and hatching rate of the remaining eggs.

- Non-guideline study with adult Zebrafish *Danio rerio* (14-day exposure, semi-static exposure, test concentrations: 0.04, 0.2 and 1 mg/L, no analytical measurement, reliability 2):
    - At 1 mg/L estrogen levels stat. signif. decrease in both male and female fish compared to controls. 11-ketotestosterone and testosterone levels were statistically significantly increased in male fish, but no effects on these hormones occurred in females.
    - In both male and female fish, statistically significant upregulation of the gonad gene (CYP17, CYP19A) transcription seen only at 1 mg/L.
    - Statistically significant upregulation of the VTG-1 gene transcription seen at all three test concentrations in male fish, and statistically significant down-regulation at the highest concentration in female fish.
    - effect on the number of spawning events at both 0.2 and 1 mg/L, while effects on hatchability only at 1 mg/L.
    - No statistically significant effect on fertilisation success.

- Study similar to OECD TG 229 Fish Short Term Reproduction Assay, with adult *Danio rerio (*21-day exposure, semi-static exposure, test concentrations: 0, 0.04, 0.2 and 1 mg/L, reliability 2):
    - No mortality occurred.
    - No effects on fish growth.
    - No effects on gonadosomatic index (GSI) nor hepatosomatic index (HSI)
    - statistically significant increase in estrogen levels in female fish at 1 mg/L, with a statistically significant decrease in 11-ketotestosterone and testosterone levels.
    - In male fish, no effects for 11-ketotestosterone and testosterone.
    - Statistically significant increase of estrogen levels in males at the middle concentration (nominal 0.2 mg/L) but not at 1 mg/L.
    - Increase in VTG levels in both male fish (1 mg/L) and female fish (0.2 and 1 mg/L) but not statistically significant.
    - Decrease on fecundity but not statistically significant.

- Non-guideline study with *Danio rerio* covering development from embryos through to adult fish (120-day exposure, test concentrations: 0, 0.005, 0.05, 0.50 mg/L, flow-through, reliability 2):
    - Statistically significant elevation in estrogen levels in female fish at 0.005 and 0.50 mg/L, but not 0.05 mg/L, and only at the lowest concentration in male fish (0.005 mg/L) (LOEC=0.005 mg/L).

- Statistically significantly decrease in 11-ketotestosterone levels in both male (at all concentrations) and female fish (at 0.50 mg/L only). Testosterone was not measured.
- No mortality occurred.
- No effects on female fish growth, but male fish growth affected at 0.05 mg/L and 0.5 mg/L. Female GSI affected at 0.005 and 0.50 mg/L but not 0.05 mg/L. Male GSI unchanged in the test.
- No significant difference in sex ratio amongst the treatment groups compared to the controls.

test	Test item concentration (µg/L)	E2 Plasma Female	E2 plasma Male	11-KT Plasma Female	11-KT Plasma Male	Body Weight Female	Body Weight Male	Liver histopathology Female	Liver histopathology Male	Vitellogenin Female	Vitellogenin Male	Gonad histopathology Female	Gonad histopathology Male	Sex ratio	Secondar Sexual Chracteristics Male	Fecundity	Fertility
FSDT 73 days	1.11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
FSDT 73 days	3.01	-	-	-	-	-	-	-	-	-	-	-	-				
non-GD 120 days	5	↑	↑	-	↓	-	-						-				
FSDT 73 days	7.76	-	-	-	-	-	-	-	-	-	-	-	-				
FSDT 73 days	33.3	-	-	-	-	↑	-	↓	↑	↑	↑	↑	↑	-			
TG 229 (rel 2)	40	-	-	-	-	-	-			-	↓						
non-GD 14 days	40	-	-	-	-					-	↑					-	-
non-GD 120 days	50	-	-	-	↓	-	↓							-			
FSDT 73 days	76.8	↑	-	-	-	↑	↑	↓	↑	↑	-	↑	↑	-			
non GD 21 days	80	-	-							↓	-	-				↓	-
TG 229 (rel 2)	200	-	↑	-	-	-	-			↑	-						
non-GD 14 days	200	-	-	-	-				-	-	↑					-	-
non-GD 120 days	500	↑	-	↓	↓	-	↓							-			
TG 229 (rel 2)	1000	↑	-	↓	-	-	-			↑	↑					↓	
non-GD 14 days	1000	↑	↑	-	↓					↓	↑					-	-

Legend: "-" no significant change detected," ↑" significant increase, "↓" significant decrease.
Blank cell means the parameter was not measured.

In vitro information:

- Toxcast: 8 of the 16 assays indicated ER-mediated activity, although all above the reported cytotoxicity threshold.
- Toxcast: One out of 8 androgen assays showed AR-mediated activity, but this was above the cytotoxicity threshold.
- No binding affinity to the E2 receptor detected in the MVLN cells.

Assessment:

Adverse effect(s):

A clear pattern of endocrine-related adverse effects was not observed. A decrease in fecundity accompanied by an alteration of gametogenesis with a reduction of maturation stage was observed in a limit test, while no effect (only a non-statistically significant decrease at the highest test concentration) on fecundity was observed in a reliable fish short term reproduction assay.

Endocrine activity:
Depending on the development stage and concentrations tested, effects were observed leading to perturbation of circulating sex hormone concentrations. The circulating estradiol and 11-KT concentrations are not consistent across studies. Also, there are conflicting results on VTG levels in females across studies.

Biological plausible link:
The most plausible MoA is associated with estrogen receptor agonism leading to reproductive dysfunction: increase of estradiol concentration and decrease of 11-KT, followed by increase of VTG in males, alteration of gametogenesis with reduction of mature stage fish which consequently leads to reduction of fertility and reproductive success. However, the available data do not strongly support the above postulated MoA: there is no evidence for interaction with the ER receptor, there is no induction of VTG in males, and the effects on reproductive success are not consistent across studies with the same species.
There was not sufficient evidence to postulate other (ED) MoA.

Conclusion:
All available studies show that the substance exerts an effect on the endocrine system of fish. Overall, the substance shows endocrine activity in fish, with adverse effects on fertility and reproduction. However, the available evidence is not very convincing as for both adverse effect(s) and endocrine activity there are conflicting results across studies with the same species. Therefore, the substance meets the criteria for classification as *ED ENV 2*.

SCL calculation:
The lowest no observed effect concentration related to effects subject to ED classification was selected (NOEC = 0.005 mg/L =). Thus, according to Table 1, Section 4.2.2.5.1 of this guidance, substances with 0.001 mg/L <NOEC≤ 0.1 mg/L result in a medium potency group corresponding to a GCL. Therefore, no SCL will be set.

4.2.5.2.3. Example 6 - *ED ENV 2* (EAS modalities)

Available information:
In vivo information:

- Modified OECD 229 with Zebrafish (non GLP, 21 days exposure, hatching rate and hatching success measured at 5 dpf, test concentrations: 0, 5, 50, 500 µg/L, reliability 2):
  - Decreased egg production at 50 and 500 µg/L.
  - Egg diameter was significantly decreased at 50 and 500 µg/L.
  - Decreased hatching success and embryo survival rates in offspring.
  - Decreased number of post-ovulatory follicles in females was the only change observed in the gonad histopathology.
  - Significant decrease in GSI in male at the two highest concentrations.
  - Plasma concentrations of 17β-estradiol (E2) significantly increased in both sexes of fish, and testosterone (T) levels increased in male fish but not significantly.
  - No VTG measured, but in females *vtg1, vtg3* gene transcription was significantly up regulated after exposure at the top concentration, while no significant effect on the transcription of *vtg1, vtg3* observed in male livers.
  - No mortality nor other toxicity observed in adults.

- Non guideline study with embryos of Japanese medaka (14 day exposure, non GLP, test concentrations: 0, 5, 50, 500 µg/L, reliability 2):
  - Decreased hatchability, delayed time to hatch, and increased occurrence of gross abnormalities at the highest concentration.

- Significantly decreased heart rate and body length at the highest two concentrations.
- Transcription levels for several genes used as biomarkers for developmental neurotoxicity (*gap43*, *mbp*, and *gfap)* significantly altered following exposure to the top concentration.
- No examination of steroid hormone levels nor of transcription of genes involved in steroidogenesis, or other markers of EAS-related mechanisms of action.

- No other *in vivo* data available on HH side.

In vitro information:

- Increase in both E2 and T concentrations in H295R cells.
- Reduced expression of genes related to T synthesis in Leydig cells *in vitro.*

Assessment:

Adverse effect(s):
In the available study with zebrafish, a convincing pattern of adverse effects was not observed. A decrease in fecundity in absence of a clear dose response accompanied by a decrease in post-ovulatory follicles[31] was observed. The study with Medaka does not provide evidence for endocrine-related adversity in the absence of additional information to support an ED MoA.

Endocrine activity:
There is indication of endocrine activity, with a good correspondence between the altered transcriptional levels of steroidogenic genes along the HPG axis and the disturbance of the plasma E2 and T levels.

Biological plausible link:
The molecular initiating event was not investigated. The most plausible MoA is associated with perturbation of the E/T ratio. The ratio of T/E2 is a sensitive biomarker of disturbed sex hormones in fish and it has been demonstrated that disequilibrating the balance between T and E2 can influence reproduction, sex development, and sex differentiation. The MoA cannot be postulated in detail due to the absence of information. However, since an alternative non-endocrine MoA is unlikely, an endocrine MoA is the most plausible explanation for the effects observed.

Conclusion:
There is neither a convincing pattern of endocrine-related adverse effects nor strong indication of endocrine activity. The limited information on adverse effect(s) and endocrine activity is consistent with a MoA based on perturbation of the E/T ratio. Even though a detailed endocrine MoA cannot be postulated, classification is still warranted because a non-endocrine explanation is unlikely. Because the available evidence is not convincing enough for the substance to be placed in Category 1, the substance should be classified as Category 2.

SCL calculation:
Based on the screening study (Modified OECD 229 with Zebrafish) the provisional No Observed Effect Concentration can be derived (NOEC = 0,005 mg/L). According to Table 1, Section 4.2.2.5.1 of this guidance, substances with 0.001 mg/L <NOEC≤ 0.1 mg/L result in a medium potency group corresponding to a GCL. Thus, no SCL will be set.

In addition, it has to be noted that the provisional NOEC derived based on a screening

[31] The decrease in post-ovulatory follicles is considered a consequence of the effects in fecundity rather than a clear endocrine mediated effect.

study can be higher than the relevant effect values derived in the definitive studies.

4.2.5.2.4. Example 7 - *ED ENV 2* (T modality)

Available information:
The substance is not classified for HH.
There is a reliable ADME study available in rat showing the formation of the metabolite MetW. However, metabolism studies in poultry and goat did not show the formation of MetW.

In vivo information in non-mammalian species:
There is one Xenopus Eleutheroembryos study (XETA) with the substance W. All other available studies are with the metabolite MetW.

Study with W:

- Xenopus Eleutheroembryonic Thyroid Assay with THb/zip-gfp transgenic *Xenopus laevis* eleutheroembryos (XETA, OECD TG 248, reliability 1, 3 days exposure, test concentrations: 0, 10, 30, 90 mg/L):
  - No mortality at any test concentration.
  - No observation of malformations or behavioral effects.
  - Unspiked condition: statistically significant increase in fluorescence lower than 12% (8.8%) at the highest test concentration.
  - Spiked condition: no statistically significant change in fluorescence.

Studies with MetW:

- Amphibian Metamorphosis Assay study with *Xenopus laevis* (AMA, OECD TG 231; 21days, test concentrations: 0, 5, 10, 22, 50, 100 mg/L, reliability 2):
  - Decrease in developmental stage at 22 mg/l and above in a dose response manner.
  - No effect on mortality, body and tail length.
  - No other parameters measured (*e.g.* thyroid histopathology).
  - Not all performance criteria were within the acceptable limits.

- Study with *Xenopus laevis* similar to AMA with some modifications (OECD ring-test of the method; 28 days, stage 48-50, test concentrations: 0, 5, 10, 25, 50, 100 mg/L, reliability 3):
  - Development completely inhibited at 50 mg/l and above.
  - No effect on mortality.
  - Effects on body length at 50 mg/l and above.
  - Effects on tail length at 25 mg/l and above.
  - No other parameters measured (*e.g.* thyroid histopathology).
  - No analytical measurements provided; results not fully reproducible across different laboratories involved.

- Modified Amphibian Metamorphosis Assay study with *Xenopus laevis* (90 days, initiation stage 51, test concentrations: 1, 2.5, 10, 25 and 50 mg/l, reliability 2):
  - Metamorphic development retarded in a dose response manner.
  - The highest tested concentration caused a complete inhibition of development with animal at premetamorphic stage 53/54.
  - Fore Limb Emergence completely inhibited at 50 mg/l while at 25 mg/l only 83% of tadpoles exhibited fore limb emergence after 90 days.

- Changes in thyroid histopathology observed in a dose response manner, *e.g.*, partial depletion of colloid, distension of follicles, enlargement of thyroid gland, follicular cell hypertrophy and hyperplasia.
- No effects on mortality and body weight.
- Analytical measurements at the beginning and at the end of the study for one of the concentrations, only.

- Non guideline study with *Xenopus laevis*, stage 48-50 (12 days, test concentrations: 0, 50 mg/L, reliability 2):
  - Development completely inhibited.
  - Statistically significant decrease in Hind limb length.
  - Changes in thyroid histopathology observed, *e.g.*, partial depletion of colloid, follicular cell hypertrophy and hyperplasia.
  - No effects on wet body weight.

- Non guideline study with fish eleutheroembryos of zebrafish (3 days, tests concentrations 0, 10, 25 and 50 mg/l, reliability 2):
  - Dose-dependent decrease of T4 in follicles across concentrations,
  - analytical measurements only at the beginning of the test.
  - No information on the method used for measuring T4.

In silico information*:*

No available information.

In vitro information:

Not available for the parent compound. The metabolite MetW was positive in the TPO ToxCast assay (TPO_AUR_dn).

Assessment

Adverse effect(s) for non-mammalian species:

No relevant studies (*i.e.*, studies measuring relevant parameters for an ED assessment) were available with the parent compound W in non-mammalian species.

Regarding the metabolite MetW, although all the studies showed limitations mainly related to the lack of proper analytical measurements, they all showed a consistent pattern of endocrine-related adverse effects: delay in development, completely inhibited at concentrations above 50 mg/l, and changes in thyroid histopathology, when investigated.

Endocrine activity:

The metabolite MetW was positive in the TPO ToxCast assay (TPO_AUR_dn) and decreased T4 in the zebrafish eleutheroembryo assay. No evidence of endocrine activity was available with the parent compound W, except for the XETA. However, even if the eleutheroembryos are metabolically competent and in principle the metabolite (MetW) would be formed in the test, the XETA is not able to detect TPO inhibitors. Therefore, given that the in vitro information indicates that MetW is a TPO inhibitor, the XETA does not bring any relevant information and the negative outcome of XETA cannot be used to dismiss any endocrine activity elicited by the parent and/or by the metabolite.

Biological plausible link:

Based on the available data, one of the plausible MoAs is: MetW inhibits TPO activity, decreases THs levels, leading to changes in thyroid histopathology and delay in metamorphosis development. It is well established that a substance acting as TPO inhibitor will induce delay in metamorphosis in amphibians, since metamorphosis is a process controlled by thyroid. However, uncertainties have been identified in the available data which do not allow to properly substantiate the postulated MoA. In addition, there are no

studies showing that the substance W metabolises into MetW in vertebrates. Therefore, there is uncertainty on the postulated MoA that has the formation of the metabolite MetW as MIE.

Conclusion:
No studies are available with the parent compound W in non-mammalian species.
All the available studies were done with the metabolite MetW. All studies showed a consistent pattern of effects and endocrine activity, *i.e.*, delay in development coupled with changes in thyroid histopathology, when assessed.
MetW is one of the metabolites observed in one metabolism study in rat; however, MetW was not formed in metabolism studies inpoultry and goat.
Overall, it is concluded that Substance W meets the CLP criteria for classification for ED cat. 2 as the level of uncertainties in the available data and MoA is considered too high to place it in Cat 1.

SCL calculation:
The *ED ENV* classification is based on assays for which the NOEC value is not available therefore, as indicated in Section 4.2.2.5.1 above, no SCL will be calculated and the GCL will be applied.

4.2.5.2.5. Example 8 - *ED ENV 2* (non-EATS modalities)

Available information:
The substance is not ED for EATS modalities for either HH or ENV.

In vivo information:

- Sub-chronic toxicity study with Japanese quail (OECD draft for sub-chronic study with birds; 6-week exposure, test doses: 0, 500, 1000, 2000 ppm, reliability 1):
  - Decrease[32] in eggshell thickness in a dose response manner at all tested doses.
  - No effect on egg strength.
  - No other parameters measured.

- Sub-chronic toxicity study with Japanese quail (OECD draft for sub-chronic study with birds, 8-week exposure, test doses: 0, 48, 100, 225, 500 ppm, reliability 1):
  - Decrease in eggshell thickness at 100 ppm and above, but without a clear dose response.
  - No other parameters measured.

- Sub-chronic toxicity study with Mallard duck (Avian reproduction test, OECD TG 206; 20-week exposure, test doses: 0, 500, 2000, 4000 ppm, reliability 1):
  - Decrease in eggshell thickness in a dose response manner at all tested doses.
  - No effects in all the other measured parameters, *i.e.*, mortality, body weight, egg production, cracked eggs, egg viability (% viable embryo of egg set), embryo viability (embryonic day 15), hatchability, number of 14 day-old survivors.
  - Historical control data on eggshell thickness were available but not considered reliable.

- Sub-chronic toxicity study with Northern bobwhite (Avian reproduction test, OECD TG 206; 20-week exposure, test doses 0, 500, 2000, 4000 ppm, reliability 1)

[32] In the OECD TG 206, eggshell thickness normal values are reported to be in the range of 0.19-0.23 for the Japanese quail

- Decrease in eggshell thickness at all tested doses with clear dose response.
- Increase in the percentage of cracked eggs/eggs laid at 2000 ppm and above.
- Decrease in percentage of 14-d old survivor/hatchings, hatchlings/maximum set and 14-d old survivor/maximum[33] set at the highest tested dose.
- No effects in all the other measured parameters, *i.e.*, mortality, body weight, egg production, egg viability (% viable embryo of egg set), embryo viability (embryonic day 15), hatchability.
- Historical control data on eggshell thickness were available but not considered reliable.

In vitro information:
No information relevant for non-EATS modalities.

Assessment

Adverse effect(s):
In all the available studies with birds, a consistent pattern of adverse effects on eggshell thickness–was observed across studies and species. In one of the available studies with quail a pattern of adverse effect(s) was seen as the effects on eggshell thickness were coupled with an increase in the number of cracked eggs and a decrease in hatchling/maximum set and 14-d old survivors/maximum set[33]. The other available studies with quails had a shorter exposure duration which could explain why no effects on the more apical parameters were observed in those studies.
Although in some cases the effects on eggshell thickness were not statistically significant, those were considered biologically relevant. In nature, eggs are normally incubated by bird parents (adult birds sit on the eggs to keep them warm until hatching) while this does not happen in the laboratory. Therefore, compared to what is observed in laboratory studies, effects on eggshell thickness in the field may be more critical and may be more often accompanied by egg breakage.

Endocrine activity:
No evidence of endocrine activity was available with the parent compound. However, one of the metabolites of the parent substance found in rat urine is sulfonamide which is a known inhibitor of cyclooxygenase. It is assumed that, in absence of evidence proving the contrary, the same metabolite is also formed in birds.

Biological plausible link:
It is known that effects on eggshell thickness may be due to a non-EATS MoA. The postulated MoA is described below.

	Brief description of the Key event	**Brief description of the observed effects/positive findings**	**Supporting Evidence**
MIE	Inhibition of the cyclooxygenase activity	Sulfonamide, which is a known inhibitor of cyclooxygenase is a metabolite of the parent substance found	Analogy to rat

[33] The number of hatchlings per female divided by the largest number of eggs set from any one female and the number of 14-day old survivors per pen divided by the largest number of eggs set.

		in rat urine	
KE1	Reduction of the prostaglandin E2 concentration	Predicted based on literature	Well established consequence of cyclooxygenase inhibition
KE2	Reduction of Ca^{2+} and HCO_3 transport to shell gland	Not evaluated	Not evaluated
KE3	Reduction of eggshell thickness	Decrease of eggshell thickness	Effects observed in the two available reproductive toxicity studies with birds. Effects observed in a dose-response manner. As additional supportive evidence, in two studies (6-week and 8-week exposure) the same effects were observed.
AO	Reproductive failure[34]	Increase of the number of cracked eggs and decrease of the number of 14-day survivors	Effect observed in one of the species (Northern bobwhite quail) tested in dose-response manner.

Conclusion:
For the postulated MoA, data on the MIE is based on analogy with rats and for KE1 on textbook knowledge. For the adverse outcome, data on the substance indicates a decrease in eggshell thickness, together with an increase in the number of cracked eggs and decrease in 14-d old survivors. Although information on the endocrine activity is not available, the information about the metabolite sulfonamide and the availability of an AOP under development especially for the earlier KEs support the biological plausibility that the adverse effects observed may be caused by a non-EATS ED MoA via the formation of the sulfonamide metabolite. Therefore, classification as *ED ENV 2* is warranted.

SCL calculation
When the adverse effect used for *ED ENV* classification would come from the non-aquatic non-mammalian toxicity study where the results are expressed in mg/kg (*e.g.*, bidrs reproduction studies), the SCLs should be calculated based on the same principals as described in Section 3.11.2.6, particularly following method similar to 3.7.2 above. In conclusion no SCL need to be set for this substance.

4.2.5.2.6. Example 9 - *ED ENV 2* (EATS modalities)

Available information:
The substance was concluded not to meet the criteria as ED HH.

In vivo information:

- A 21-day study with Persian sturgeon (*Acipenser persicus* – sexually mature males and spawning females) (concentrations 0, 0.4, 0.8 and 1.2 mg/L, reliability 2).

[34] Effects mainly leading to impairment of population maintenance

- No mortality in the controls or treatments.
- Reduced plasma VTG in females at 0.8 and 1.2 mg/L on day 21 but not statistically significant,
- no change in VTG in males measured at any concentration.
- Significantly reduced egg production but not statistically significant in comparison to the control at 0.8 and 1.2 mg/L.
- Significantly reduced hatching rate at 0.8 mg/L and at 1.2 mg/L.
- Secondary sex characteristics not reported.
- No histopathology conducted.

- A 30-day study with zebrafish (*Danio rerio* – sexually mature males and spawning females) (concentrations 0, 0.02, 0.2 and 2 mg/L, reliability 2).
  - No mortality in the controls or treatments.
  - No change in vitellogenin (VTG) gene expression on day 30.
  - Female gonad histopathology: reduced growth and development of the oocyte at 0.02, 0.2, and 2 mg/L (most severe).
  - Significantly reduced egg production at 2 mg/L.
  - Significantly reduced hatching rate at 2 mg/L.

In vitro information:

- There is one in vitro assay available showing significant inhibition of ER DNA-binding activity observed at 100 mg/L only. No inhibition observed at lower test concentrations.

Assessment

Adversity:

There are indications of adverse effects in the available studies. One study reported reduced oocyte development. Significant and consistent reductions in fecundity (hatch rate, egg production) were observed in both available *in vivo* studies.

Endocrine activity:

There is some evidence available from binding assays with fish receptors to indicate the substance can bind to, and inhibit, the ER receptor. However, the inhibition was only observed at very high concentrations, and no inhibition was observed at lower test concentrations. There are inconsistent results on the *in vivo* mechanistic parameter vitellogenin (diagnostic of an EAS MoA), with one study showing no change in vitellogenin gene expression, and significant reductions in plasma vitellogenin levels in the other study (in females only). However, protein level in vivo is a more relevant parameter than gene expression because a gene expression result does not automatically translate into an effect in protein level.

Biological plausible link:

The available information on endocrine activity for the substance points to an estrogenic MoA, involving inhibition of estrogen receptors and reduced vitellogenin. The MoA is postulated following the AOP 30 (estrogen receptor antagonism leading to reproductive dysfunction (aopwiki.org)). In the postulated MoA, the substance directly binds to the estrogen receptor and prevents activation by other molecules, leading to reduced vitellogenin concentrations and impaired oocyte development. This MoA is supported by the complementary evidence for the adverse effects from two studies showing reduced fecundity and hatching success. However, the inconsistent evidence for endocrine activity does reduce the WoE for this MoA.

Level of organisation	Brief description of key event	Supporting evidence
MIE	Binding to estrogen receptor	Indication of inhibition of ER – *limited relevance due to high concentration*
KE1	Reduced VTG mRNA expression	*Non supporting evidence: No change in VTG gene expression in standard test species*
KE2	Reduced VTG protein synthesis	
KE3	Reduced VTG concentrations	Reduced plasma VTG in females (non-standard test species)
KE4	Reduced VTG uptake and impaired development of the oocyte	Reduced oocyte development
AE1	Decreased spawning and cumulative fecundity	Reduced fecundity & hatch success (dose-response)
AE2	Decline in population growth	

Conclusion:
There is a clear pattern of adverse effects on fish reproduction and some evidence for adverse effects on the ovaries. However, there is limited evidence of endocrine activity, and the consistency of the data between two studies cannot be assessed. A MoA fitting the observed effects is postulated (antagonism of the estrogen receptor) following a well-established AOP. However, as the available evidence on endocrine activity is not sufficiently convincing, the substance meets the criteria for classification as *ED ENV 2.*

SCL calculation:
Based on the screening study (30-day study with zebrafish) the provisional Lowest Observed Effect Concentration can be derived (LOEC = 0,02 mg/L). According to Table 1, Section 4.2.2.5.1 of this guidance, substances with 0.001 mg/L <NOEC≤ 0.1 mg/L result in a medium potency group corresponding to a GCL value. Thus, no SCL will be set.

In addition, it has to be noted that as the NOEC value is not available, the SCL is derived based on provisional LOEC derived based on a screening study and when available the relevant NOEC value derived in the definitive studies could be significantly lower.

4.2.5.3. Examples no classification

4.2.5.3.1. Example 10 - *ED ENV* no classification (EAS modalities)

Available information:
The substance was concluded not to meet the CLP criteria as *ED HH*.

In vivo information:

- Fish short term reproduction assay with zebrafish (FSTRA, OECD TG 229, 21-day exposure, test concentrations: 0, 3.2, 10, 32 µg/L, reliability 1):
  - No effects on survival, fecundity, VTG concentrations and wet weight.
  - Histopathology and secondary sex characteristic analysis were not performed.
    Uncertain whether the MTC was reached based on the available evidence from chronic studies.
- Fish full lifecycle test with *Fathead minnow* (FFLCT, OPPTS 850.1500, 136 days exposure, test concentrations: 0, 0.32, 1.0, 3.2, 10 µg/L, reliability 1), the test design of the study was adapted to include such as sex ratio of adults, fecundity and fertility, time to sexual maturity, secondary sex characteristics in males and females, gonad histopathology and VTG concentrations:
  - VTG was measured, but not considered reliable in both generations assessed.
  - No treatment related effects on sex ratio in the F2 generation.
  - In F1 generation slight (but not statistically significant) increase in the percentage of males at the highest test concentration), but, at this concentration, also significant effects on mortality.
  - No findings in histopathology.
  - For body weight, length, fertility, liver histopathology and time to maturity, significant effects observed at the highest test concentration, but also clear effects on survival at that concentration.
  - Effects on fertility observed in the F1, but seen in presence of other toxicity.

- three Early life stage studies available in rainbow trout (tested concentrations 1, 3.2, 10, 32, 100, 320 µg/L), sheepshead minnow (tested concentrations 0.9, 1.9, 3.8, 7.5, 15, 30 µg/L), and fathead minnow (tested concentrations 0.01, 0.03, 0.09, 0.28, 0.8, 2.5 mg/L) (all studies reliability 2). In the last two species, significant effects seen on parameters '*sensitive to, but not diagnostic of, EATS*' at concentrations below those where effects on other toxicity (*i.e.* survival) were observed.

- prolonged toxicity test (28 days) with rainbow trout, significant effects on parameters '*sensitive to, but not diagnostic of, EATS*' were observed at the same concentrations where there were effects on mortality (concentrations tested 0, 0.5, 1.2, 2.5, 5, 10 mg/L).

In silico:

Negative ER model.

In vitro information:

ToxCast negative for aromatase inhibition, no indication for AR (reliability 1).

Assessment

Adverse effect(s):

Some effects on reproduction parameters were noted in the FFLCT. A reduction in fertility was observed in the F1 generation, however this was observed in presence of other toxicity, therefore, there is not sufficient evidence of endocrine-related adverse effect(s) based on this parameter. Other parameters such as sex ratio and VTG were considered not reliable from this test. For some of the parameters '*sensitive to, but not diagnostic of, EATS*' (*e.g.*, body weight, length, fertility, liver histopathology and time to maturity), significant effects were observed at the highest test concentration. However, there were also clear effects on survival at that concentration. Therefore, the effects observed could

be considered as indicative of other toxicity to the test organisms rather than as an endocrine-related adverse effect. In the FSTRA, no effects on fecundity were observed. Overall, no evidence of endocrine-related adverse effect(s) were observed.

Endocrine activity:
The level 3 FSTRA is overall negative. The only *in vivo* mechanistic parameter assessed was VTG which was considered inconclusive. Secondary sex characteristics were not assessed since that parameter cannot be easily assessed and quantified in zebrafish. ToxCast data were considered overall negative.
Overall, there is no evidence of endocrine activity *in vitro* and *in vivo*.

Biological plausible link:
Not applicable.

Conclusion:
There is no evidence of endocrine-related adverse effect(s) because the effects observed are a non-specific consequence of other toxicity, and there is no evidence of endocrine activity. By considering all the available information on *in vivo* mechanistic parameters and EAS-mediated parameters in the available FSTRA (level 3) and FFLCT (level 5), it can be concluded that the substance does not meet the CLP ED criteria for the EAS-modalities for the environment.

4.2.5.3.2. Example 11 - *ED ENV* no classification (EATS modalities)

Available information:
The substance was concluded not to meet the CLP criteria as *ED HH*.

EAS modalities
In vivo information:

- Fish short term reproduction assay with *Fathead minnow* (FSTRA, OECD TG 229, , 21-day exposure, test concentrations: 0, 0.018, 0.18 and 1.2 mg/L, reliability 1):
  - No mortality observed at any concentration.
  - Increase of GSI and VTG in females, but not statistically significant, at 1.2 mg/L.
  - No effect on GSI and VTG in males.
  - No effects on SSC in males.
  - Effects on egg production (no eggs produced) at 1.2 mg/L.
  - No effects on gonad histopathology in both sexes at any concentration.

- Fish full lifecycle test with *Fathead minnow* (FFLCT, OPPTS 850.1500, test concentrations: 0, 25, 50, 100, 200 and 400 µg/L, reliability 1), the test design of the study was adapted to include 'EAS-mediated' parameters foreseen to be investigated in OECD TG 240:
  - No indications of adverse effects on growth, development or survival in any generation.
  - No effects on sex ratio.
  - No effects on secondary sex characteristics (SSCs).
  - In F1 generation, significant decrease in egg production in females at 200 µg/L.
  - No effect on egg production at 400 µg/L.
  - No effects on fertility.
  - Effects on ovary histopathology (slight increase of oocyte atresia not statistically significant) at 400 µg/L, no without change in the ovarian stage scores.
  - Increase in VTG in females only at 100 µg/L.

- One early life stage test in fathead minnow is available which does not cover all possible life stages wherein adverse effect(s) could occur but does not indicate EAS-mediated adverse effect(s). The only effects seen were on post-hatch survival at 1.9 mg/L (EC50 estimated at 1.3 mg/L), and length and weight (growth) at 486 µg/L.

- No evidence of EAS-mediated adverse effect(s) nor activity in mammals (Uterotrophic, Hershberger and two prepubertal assays were also all negative).

In vitro information:
[All assays reported below have a reliability 1-2 and no cytotoxicity was reported]

- Negative *in vitro* estrogen receptor (ER) binding, aromatase and steroidogenesis assays.
- Equivocal results in three runs of androgen receptor (AR) binding assay. In first run reduced binding of the radiolabelled ligand, but results were found to be variable and not dose specific. Negative results in second and third runs.

Assessment

Adverse effect(s):
In the FLCTT, there were no significant effects on sex ratio, fertility or fecundity noted. There is only a slight effect on ovary histopathology which is not statistically significant. Overall, there is no strong evidence of endocrine-related adverse effect(s) in fish in the FLCTT nor in the FSTRA.

Endocrine activity:
The effects on VTG were observed in the FSTRA and FLCTT only at the highest test concentration and were not statistically significant. There were also no indications of sex ratio changes or biologically relevant SSC effects which might be considered indicative of EAS activity.
Overall, the indications of endocrine activity in fish are equivocal.

Biological plausible link:
Not applicable.

Conclusion:
By considering all the available information, it can be concluded that the substance does not meet the CLP ED criteria for the EAS-modalities for the environment as there is no evidence of endocrine-related adverse effect(s).

Available information:

T modality
In vivo information in mammals

- In 90-days studies in rats and dogs increase in thyroid weight
- In rats, the relative thyroid/parathyroid weight significantly increased by 23% and 20% in the mid- and high-dose in males, respectively.
- In dogs, thyroid weight increased >20% in males at 2000 mg/kg bw/day, in females at 400 mg/kg bw/day, but not statistically significant.
- No indication of brain or pituitary toxicity or adverse neurodevelopment in any of the available studies.
- No evidence of thyroid-related adverse effect(s) in the mammalian dataset.
- No effects on thyroid pathway in males and female pubertal assay.

In vivo information in amphibians

- Amphibian metamorphosis assay (AMA, OECD TG 231, 21-day exposure, test concentrations 0, 0.015, 0.15 and 1.5 mg/L, with *Xenopus laevis*, reliability 1):
  - Body weight statistically significantly reduced by 22% at the highest tested concentration on day 21.
  - Snout-vent length statistically significantly reduced by 8% at the highest tested concentration on day 21.
  - No effects on normalized hind limb length.
  - No effects on developmental stage.
  - No effect on thyroid histopathology.
  - No evidence of other toxicity.

In vitro information

- No *in vitro* studies available.

Assessment

Adverse effect(s):
There is no evidence of thyroid-related adverse effect(s) in the mammalian or non-mammalian datasets. There is an effect on thyroid weight in rats and dogs, but thyroid weight changes are not considered adverse if not confirmed by thyroid histopathology.

Endocrine activity:
There is no evidence of thyroid activity in the mammalian dataset. There is also no evidence of thyroid activity in the non-mammalian dataset.

Biological plausible link:
Not applicable.

Conclusion:
In mammals, there are no indications of thyroid activity in the *in vivo* dataset, including two prepubertal assays. In amphibians, an AMA was available which showed no evidence of thyroid activity. By considering all the available information on the substance, it can be concluded that the substance does not meet the CLP ED criteria for the T-modality for the environment as there is no evidence of endocrine activity nor of adverse effect(s).

4.2.6. **Reference list**

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