ECHA
EUROPEAN CHEMICALS AGENCY

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	Adsorption, Distribution, Metabolism, Excretion
AMA	Amphibian Metamorphosis Assay
AOP	Adverse Outcome Pathway
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	Cancerogenic, 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
EffD	Effective Dose
ELS	Early life stages
ER	Estrogen receptor
EATS	Estrogen, Androgen, Thyroid and Steroidogenic
FDA	Food and Drug Administration
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 chemical safety assessment
KE	Key vent
KER	Key event relationship
LBD	Ligand binding domain
LDL	Cholesterol
LOQ	Level of Quantification
MIE	Molecular initiating event
MoA	Mode of Action
MTC	Maximum tolerated concentration
MTD	Maximum tolerated dose
NAM	New Approach Methodologies
NOEC	No Observed Effect Concentration
OECD	Organisation for Economic Co-operation and Development
(Q)SAR	(Quantitative) structure-activity relationship
PBK	Physiologically Based Kinetic models
PPP	Plant Protection Products Regulation (Regulation EC 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
THs	thyroid hormone
TRH	thyrotropin-releasing hormone
TSH	thyroid-stimulating hormone
US EPA	United States Environmental Protection Agency
VCBA	Virtual cell-based assay

VTG	Vitellogenin
WOE	Weight of Evidence

111
112

Disclaimer: This section of the CLP guidance refers to the ECHA/EFSA Guidance (ECHA/EFSA, 2018) in several sub-sections, and further information on can be found in that guidance to assist in concluding on ED properties. However, it is important to make a distinction between that guidance and this one as they serve different purposes.
The ECHA/EFSA 2018 Guidance, which builds on the OECD GD 150, was written assist users to comply with their obligations to conclude on ED properties in accordance with the ED criteria for biocidal products (BP) and plant protection products (PPP), respectively. The ECHA/EFSA 2018 Guidance describes how to gather, evaluate and consider all relevant information for the assessment, conduct a mode of action (MoA) analysis, and apply a weight of evidence (WoE) approach, in order to establish whether the BP or PPP ED criteria are fulfilled. Therefore, the ECHA/EFSA 2018 ED guidance still has a function because it outlines how to conclude on ED properties.

However, in 2023 endocrine disruption was introduced into CLP as a hazard class with subcategorisation. Consequently, for classification purposes this guidance on the application of the CLP criteria is the applicable one which should be followed for all substances subject to CLP, including industrial chemicals and active substances under the BP and PPP Regulations.

[ECHA would also like to note the commenters that all active substances under the BP[1] and PPP[1] Regulations must be classified according to the CLP ED criteria. In this context, it is important to note that the current ED criteria for BP and PPP are essentially the same as ED HH 1 or ED ENV 1 under the CLP criteria. Therefore, in line with the one substance one assessment principles, it is expected that active substances already concluded to meet the ED criteria under the BP and PPP procedures before the criteria in CLP Regulation came applicable, will under CLP Annex VI be assigned to ED HH 1 or ED ENV 1. Similarly, active substances which have been concluded not to meet the ED criteria under the BP and PPP procedures are expected to be assigned to ED HH 2 or ED ENV 2 or no classification unless substantial new information has become available which warrants classification as ED HH 1 or ED ENV 1. Similarly, substances identified as Substances of Very High Concern (SVHC) under REACH due to ED properties are expected under CLP Annex VI be assigned to ED HH 1 or ED ENV 1. This issues above will not be part of the CLP guidance text, but rather considered under respective regulations and guidance's.

The sections for HH and ENV may not be fully aligned, and a better alignment will be considered during the PEG process.
Further, this draft CLP guidance is not necessarily in line with the CLH template ED section and in this case, the guidance should applicable, the template is easy to modify to better reflect the guidance.
In particular, ECHA wishes to receive input and concrete text proposals on the following topics:

- Developing general flow charts and more detailed guidance for
  - Cat 1 Cat 2 (with special attention to thyroid modality) and 'no classification'
  - ED mediated, sensitive to, and non-EATS parameters
- Relation of (developmental) neurotoxicity (and immunotoxicity) to ED classification
- A more detailed paragraph on EAS modalities (similar to specific paragraph on thyroid modality)
- More details on different situations for additivity and non-additivity
- Additional examples on:
  - missing modalities,
  - using in vitro and human data only,
  - read across/grouping,
  - tumours e.g. uterine adenocarcinoma,
  - cross-species considerations and use of AOPs to demonstrate the biologically plausible link,
  - serious doubts about population relevance.]

3. HH

3.11. Endocrine disruption for human health

3.11.1. Definitions and general considerations for endocrine disruption

Annex I*: 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 classification for endocrine disruption for human health differs from the other hazard classes in that it refers to a specific mode of action (endocrine) which will lead to an adverse effect(s), and the classification criteria requires evidence on three empirical definitions, i.e. adverse effect(s), endocrine activity, and a biological plausible link between the endocrine activity and the adverse effect(s); i.e. a correlation[1] between endocrine activity and adverse effect(s) consistent with existing knowledge.

Annex I: *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 mode of action (MoA), in accordance with the criteria given in CLP, Annex I, Section 3.11.2.1. Conclusively demonstrating the adverse effect being not relevant for humans means that robust evidence is provided which unambiguously demonstrates that human relevance can be excluded.

Annex I: *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*

[1] Correlation in this context means that endocrine activity and adverse effect(s) can be linked using existing knowledge as the most likely explanation to the observed effects, for details see Section 3.11.2.3.3.

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 disruption for human health Category 1 or 2 is independent of the classification of the substance as reproductive toxic, carcinogenic or specific target organ toxicant single or repeated exposure. A substance can be classified for endocrine disruption for human health based on the same set of evidence as used for other hazard classes irrespectively of whether the substance is also classified for other hazard classes.

For example, a substance may be classified for endocrine disruption for human health for adverse effects in the thyroid even though the adverse effect(s) are observed above the guidance values for STOT-RE. Another example, a substance can be classified as *ED HH 1*, even if the substance is classified as *Repr.2* for the same adverse effect because also evidence for endocrine activity and the biologically plausible link between the endocrine activity and the adverse effect 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 endocrine disruption for the environment, e.g., a substance can be classified as *ED ENV 1, 2* or not classified, even if the substance is classified as *ED HH 1* and vice versa.

Classification as endocrine disruptor for human health is intended to indicate when a substance may cause harm due to the fact that its effects are mediated by an endocrine MoA in any life stages. The nature and sensitivity to such effects depends on the life-stage investigated. Generally, the developing foetus, pups and peripubertal animals are to be considered more sensitive to endocrine modulation than adults. Some effects may be reversible in adults but may cause irreversible effects in the developing organism. The ED criteria do not mention reversibility as a factor to be considered in the weight of evidence;. therefore, an adverse effect, reversible or not, may warrant ED classification.

The concept of endocrine disrupting "potency" is considered only in the context of setting specific concentration limits (see Section 3.11.2.6). 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. When there is sufficient information that already very low doses or alternatively only very high doses are causing the ED effects, this guidance considers that as a difference in potency which can be regulated by setting a specific concentration limit.

EATS- and non-EATS modalities

Endocrine disrupting modes of action are caused either by estrogen, androgen, thyroid and steroidogenic (EATS) modalities or by so-called non-EATS modalities. Further information on EATS modalities can be found in section 3.11.2.3.1.

Endocrine disrupting modes of action are caused either by estrogen, androgen, thyroid and steroidogenic (EATS) modalities or by so-called Non-EATS modalities. While the CLP criteria do not differentiate among modalities, thus covering all endocrine-disrupting MoAs, i.e., adverse effects which may be caused by any endocrine modality, it is acknowledged that this guidance mainly addresses the effects caused by EATS modalities.

This is because the EATS modalities are the pathways for which there is currently the most knowledge available, i.e., there is a 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* and *in vitro* testing available where there is a broad scientific agreement on the interpretation of the effects observed on the investigated parameters.

However, the general principles outlined in this guidance for evaluation of the data on the different criteria, weight of evidence and decision on classification, are also applicable to other endocrine (non-EATS) modalities. Although the existing knowledge for those modalities is not as advanced as for the EATS modalities, it may, in some cases, be already possible to reach a conclusion on the need to classify the substance on a non-EATS endocrine modality, e.g. where literature data provide mechanistic information, which can be linked to adverse effects measured in standard tests. One example is related to effects interfering with the action of *calciferol*, peroxisome proliferator-activated receptor-gamma (PPARγ), or the retinoid system.

3.11.2. Classification of substances for endocrine disruption for human health

3.11.2.1. Identification of hazard information

The CLP Regulation does not set information requirements or require testing of substances and mixtures for classification purposes (CLP Art. 5, 6 and 9). The assessment is based on the respective criteria and consideration of all available relevant information. Under CLP, no further studies can be requested.

The main ways to gather all available information is by conducting a literature search or a systematic literature review. Additionally, previous regulatory assessments may serve as a starting point for the literature search.

The information is relevant when it investigates at least one of three criteria (endocrine activity, adverse effects and biologically plausible link):

- Information on endocrine related *adverse effects* relevant for humans is normally obtained from animal studies with repeated exposures. Non-animal methods or testing strategies in future, even those which do not necessarily involve an intact organism, may provide sufficient information for decision making on classification. Information may also be obtained using read-across or analogy, e.g., if the substance share a common mode of action.
- Information on *endocrine activity* generally comes from *in vivo* or *in vitro* mechanistic studies. Non-animal methods which provide equivalent predictive capacity of the currently used in vivo mechanistic studies may be used; e.g. the ToxCast ER model. 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.
- For *biological plausibility*, existing scientific knowledge can be used, e.g. textbooks and scientific literature. Several adverse outcome pathways have already been established (see OECD Series on AOPs), and there is continuous development of additional AOPs in the AOPwiki.

3.11.2.1.1. Identification of human data

Information that are relevant for classification as endocrine disruption may be available among others from case reports, epidemiological studies, medical surveillance and reporting schemes, and national poison centres.
Further information is given for example in the ECHA Guidance on information requirements and chemical safety assessment (IRs & CSA), section 7.5.3.2. and 7.6.3.2. (ECHA, 2017).

3.11.2.1.2. Identification of non-human data

All relevant information that addresses endocrine-related adverse effects and activities shall be considered in a weight of evidence approach; this includes guideline and research studies as well as alternative methods such as read across and *in silico* predictions. The OECD 'Guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption', OECD GD 150 (OECD, 2018) 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 estrogen, androgen, thyroid and steroidogenic (EATS) modalities, and 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 Chemicals (OECD CF) which lists the OECD test guidelines and standardised test methods available that can be used to evaluate chemicals for endocrine disruption. The OECD CF is intended to provide a guide to the tests available which can provide information on assessment of endocrine disruption. It is not an exhaustive list and assays other than those described in the list may also be valuable for assessing chemicals for endocrine disruption. New tests are continually being developed, aiming to bring useful information for classification. In particular for any non-EATS modalities, for example adrenal or pancreatic effects, research studies are an important source of information which must be considered in a weight of evidence approach.

Non-animal methods can be used to demonstrate adverse effect(s) if they provide equal predictive capacity as the human or animal data. Validated New Approach Methodologies (NAMs), if available, may be more relevant than non-validated, but also other published /internationally recognised methods can be used for classification to avoid unnecessary animal testing if they are relevant. When the NAMs / *in vitro* / *in silico / omics* models and methodologies / Q(SAR)s / testing strategies etc. provide data with equivalent predictive capacity as the human or animal data, they can be used to provide sufficient data on activity and adverse effect(s) for classification in Category 1 or 2. In general, for endocrine activity, there are more alternative methods available.

Moreover, 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 of this guidance.

3.11.2.2. Classification criteria

Annex I*: 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.*

306 **3.11.2.2.1. Classification in the presence of other toxicity**

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.*

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308 *Other toxicity in adult animals*
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310 "Other toxicity" refers to (adverse) effect(s) other than the endocrine-related adverse
311 effect(s). If a substance causes endocrine-related related adverse effect(s) which occur
312 together with other toxicity, classification for endocrine disruption for human health should
313 be applied unless the effect is demonstrated to be solely a non-specific secondary (indirect)
314 consequence of the other toxicity.
315 As an example, a metal ion has the capacity to replace iron in haemoglobin. This
316 replacement reduces haemoglobin's affinity to oxygen causing hypoxia. As a physiological
317 response to hypoxia, the kidneys release the hormone erythropoietin, which stimulates
318 the production of red blood cells, after sub-chronic exposure erythrocytosis (too many red
319 blood cells in the blood causing sluggish blood flow in organs and tissues) and testicular
320 atrophy is observed. In this example, there are two endocrine-related adverse effects,
321 erythrocytosis and testicular atrophy. Despite this, classification for endocrine disruption
322 for human health is not warranted because it has been demonstrated that the endocrine
323 related effects (erythrocytosis and testicular atrophy) are not caused via direct hormonal
324 activity but they are solely non-specific secondary/indirect effects to other toxicity (in this
325 case due to hypoxia or sluggish blood flow which also cause other severe toxic effects
326 simultaneously). However, classification for reproductive toxicity and STOT-RE may be
327 warranted.
328 To consider an ED-related adverse effect as solely a non-specific consequence of other
329 toxic effects, there must be evidence for a biologically plausible sequence of events which
330 excludes an endocrine mode of action as the most likely explanation to the observed

adverse effect(s). This is best done by a comparative mode of action assessment. When assessing the potential influence of other toxicity to the co-occurring endocrine-related adverse effect(s) in adult animals, it may be helpful to evaluate the cooccurrence at individual animal level. In this context, the other toxic should precede the endocrine-related effect(s), either temporarily or in terms of dose levels, to support that the endocrine-related effect(s) are a consequence of the other toxicity. Mortality at the end of the study in lifetime studies, such as carcinogenicity studies, should not be considered as indication of severe toxicity. See also sections 3.6.2.3.2. on excessive toxicity and 3.9.1 on secondary effects of this guidance.

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

The presence of other toxicity shall be considered particularly when evaluating effects in pups or foetuses in reproductive toxicity studies which can be influenced by maternal toxicity. Other toxicity shall not be used to negate findings of endocrine-related adverse effect(s) in foetuses or pups, unless it can be concluded that the endocrine-related effects are solely non-specific secondary consequences of other toxicity.
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 results, 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 may be dismissed, because they may be considered to be a result of excessive maternal toxicity. When assessing the potential influence of other toxicity to the co-occurring endocrine-related effects, it is may be appropriate to evaluate the potential causality at individual animal level. For example, if 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 another adult animal in the group showed signs of excessive toxicity. Even in the presence of excessive toxicity, it is important that the data is assessed qualitatively rather than quantitatively, that the data is consequently reported in a transparent manner and that the data can be assessed on an individual basis.
In this context, it should be noted that a certain toxic effect can be considered to be a secondary, non-specific cause of one adverse effect, but not of another. For example, a level of maternal toxicity that can be assumed to cause decrease in pup weight or spontaneous abortions may not be sufficient to explain the presence of malformations. To conclude that a certain adverse effect is a secondary, non-specific consequence of other toxicity, a careful analysis is needed. See also section 3.7.2.2.1.2 of this guidance.

3.11.2.2.2. Relevant doses for classification

Because no new tests can be requested under CLP, the dose-setting in available studies are assessed as given. All dose-levels, even those tested above the limit dose of a test guideline or above Maximum Tolerated Dose (MTD) are relevant for classification if they do not result such an excessive toxicity that the ED related effects could be dismissed, see further details in section 3.11.2.2.1 above.

[NOTE for consideration: The text below further explains the difference between MTD and limit dose and how doses are set in toxicological studies. However, for the purpose of CLP no new studies are to be conducted and therefore at this stage of evaluation it is too late to consider what is an appropriate dose setting. Is this text below needed for this Guidance or should this be in a different document?]

Dose selection is considered critical for hazard identification. This guidance is not concerned with the performance of testing, please refer to the relevant test guidelines, OECD GD 116 or regulations such as REACH, BPR and PPPR. Here the evaluation of existing data is discussed. Two different concepts should not be confused, the top dose / MTD to be used in animal studies, and interpretation of data at certain levels of toxicity or at certain doses. Some guidelines for test methods specify a limit dose, others qualify the

limit dose with a statement that higher doses may be necessary if anticipated human exposure is sufficiently high that an adequate margin of exposure is not achieved. Also, due to species differences in toxicokinetics, establishing a specific limit dose may not be adequate for situations where humans are more sensitive than the animal model. Thus, according to many test guidelines the highest oral test dose shall be at least 1000 mg/kg body weight/day or if limited by excessive other toxicity (prostration, severe inappetence, excessive mortality), the highest dose should be chosen with the aim to induce some specific and/or general toxicity (clinical signs or a decrease in body weight) but not death or severe suffering (sometimes referred to as maximal tolerated dose, MTD).

Neither limit dose nor MTD should be confused with a demarcation above which the results are not relevant for hazard assessment. Although 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 bw/d 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 ECHA guidance given on dose level setting 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. 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 weight of evidence (WoE) approach. This includes positive and negative relevant data from all relevant sources of information, as described in Section 3.11.2.1. Datasets should be analysed using weight of evidence 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 mainly similarly to the respective sections of this guidance on carcinogenicity, reproductive toxicity and specific target organ toxicity – repeated exposure (see Sections 3.6.2.3., 3.7.2.3., and 3.9.2.3.). However, the dose thresholds provided in the STOT RE or SE 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 (OECD, 2018) provides guidance on how to interpret parameters normally investigated in toxicity studies (see also the ECHA/EFSA Guidance (ECHA/EFSA, 2018). The OECD GD 150 differentiates between:

- 'EATS-mediated parameters', considered as "diagnostic" parameters, measured *in vivo* that may contribute to the evaluation of adverse effect(s), while at the same time also implying an underlying *in vivo* mechanistic information, thereby providing information on endocrine activity. 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 a uterine adenocarcinoma or an absence of estrous cyclicity

- 'Sensitive to, but not diagnostic of, EATS parameters' measured *in vivo* that may contribute to the evaluation of adverse effect(s), however, 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 might provide indications of an endocrine MoA. Examples of these parameters for human health are litter size and gestation length or changes in brain weight which cannot be alone (e.g., without supportive mechanistic evidence) considered as ED mediated.

All the parameters reported in OECD GD 150 are considered to be relevant to support ED-related adverse effect. 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).

However, studies, other than those listed in OECD GD 150, may also include endpoints that can be affected by an endocrine MoA, and therefore may provide relevant information. In addition to results from guideline studies, results from well-performed and reported studies from the open literature may provide as valuable and useful knowledge as results from the guideline studies. Therefore, the data used to classify a substance can be drawn from standard studies or other scientific data, e.g., robust peer-reviewed publications, literature studies, Q(SAR) data, internationally recognised databases etc.

The current *in silico* and *in vitro* methods cannot fully replace *in vivo* data on adverse effect(s) for endocrine disruption, however, when developed further, they may provide sufficient information for endocrine related adverse effect(s).

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 for the most common test guidelines (ECHA/EFSA, 2018)

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 hormone levels are generally considered *in vivo* mechanistic.

In silico approaches (see Section 3.11.2.3.2.2), such as QSAR models (e.g., ComPARA and CERAPP), physiologically based kinetic (PBK) models and other mathematical models, (e.g., the virtual cell based assay, VCBA), could also be used to support the battery of in vitro assays (Mansouri et. al. 2020; Mansouri et.al 2016; Zaldívar et.al. 2010).

Further information can be found in the ECHA/EFSA Guidance (ECHA/EFSA, 2018).

3.11.2.3.2.1. *In vitro* data

In general, the *in vitro* tests, when used in isolation, lack the complexity of an intact organism and can identify if a chemical is capable of binding a receptor or interfering with a pathway. The in vitro assays provide little information on whether the effect is operant *in vivo*. Particular attention should be applied to *in vitro* data and the consideration of absorption, distribution, metabolism, excretion (ADME) properties which are not covered by current *in vitro* test guidelines. Therefore, when interpreting the results of *in vitro* tests, the lack of a metabolising capacity of the system, as well as the lack of consideration of other ADME properties, should be considered. To partly overcome this limitation, several

in vitro tests can be run investigating different points of perturbation or endocrine pathways, and metabolism may be addressed by adding (part of the) metabolising systems, potentially metabolising the parent compound into a more active, less active or inactive substance/metabolite, or metabolites of the substance could be directly tested. Results from a battery of tests for substances with low metabolising potential may in some cases be conclusive, 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.

In vitro assays focus on specific interactions of compounds with the molecular machinery of cells, 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 receptors and as compounds might be able to act via more than one mechanism, no single *in vitro* test can be expected to detect all types of endocrine activity. 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 cannot be assessed in an *in vitro* system. Further, the applicability domain 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 highlighted above, conclusions on the endocrine activity of the substance can only be drawn in the context of what the *in vitro* assays can evaluate. Future developments of New Approach Methodologies (NAMs) and the future advancement of, in particular, *in vitro* methods may allow a conclusive assessment on endocrine disruption without *in vivo* data.

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 GD 71); i.e., both methods can detect substances that are estrogen agonists and antagonists *in vivo*. ToxCast data can be used similarly to uterotrophic assay data on endocrine activity. The ToxCast ER bioassay lacks metabolic capacity; therefore, if the prediction is in conflict with higher tier in vivo data then other in vivo data has higher weight.

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. In particular, by providing information on the molecular initiating event (MIE), *in silico* predictions can be used to support the identification of endocrine modes of action. The different types of *in silico* prediction methods can be grouped as: Molecular modelling of receptor interactions, (Q)SAR modelling of receptor-based activity, Profilers based on structural alerts and decision trees; for further details see ECHA/EFSA ED Guidance, section 4 and Table 11 (ECHA/EFSA, 2018).

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, section 6 (ECHA, 2008) should be followed. Attention should be paid in 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.

New *in silico* tools are constantly developed or refined such as but not limited to ComPARA, CERAPP, Leadscope, and Opera, which may provide useful information on endocrine activity.

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

Annex I: ***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 (ECHA/EFSA, 2018).

When potential endocrine-related adverse effect(s) and endocrine activity are identified, the link between the two, according to the 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 or toxicology, may be sufficient to establish the link and conclude on the biological plausibility. The level of information required for a MoA analysis vary depending which parameters are adversely affected, i.e., EATS-mediated, sensitive to but not diagnostic of EATS, or non-EATS.

Biological plausibility may be demonstrated by conducting a mode of action analysis using all available relevant information. For classification purposes, knowledge and demonstration of the full MoA is not a requirement. The MoA analysis should aim at establishing the consistency and coherence of the responses obtained on measured parameters with a postulated MoA.

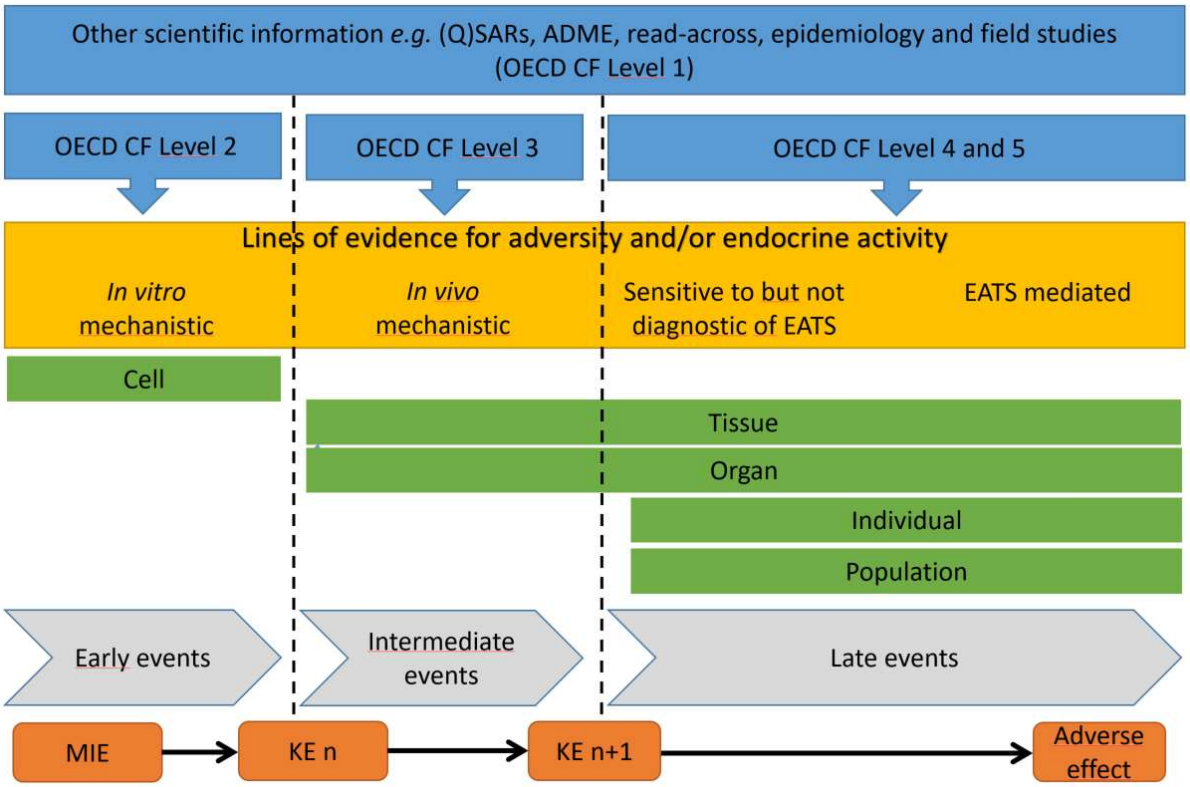
Mode of action analysis

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.

An endocrine mode of action means that the adverse effect is mediated through an alteration of the hormonal synthesis, regulation or metabolism, i.e., is not only about hormone-receptor interactions. Therefore, an endocrine MoA will normally contain some earlier KEs (which provide mechanistic information at the molecular or cellular level) and some later KEs (which provide information at the organ or system level, including the adverse effect).

In the case of endocrine disruption, this sequence at least includes one endocrine-mediated KE which may or may not also be adverse. 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 an event as key, there needs to be experimental data in which the event is characterised and consistently measured. 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



KE: key event; MIE: molecular initiating event.

The first step in assessing biological plausibility and conducting the MoA analysis 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. The evidence available for the substance subject to classification shall be assessed against the hypothesis for mode of action with its key events to be able to conclude on a biological plausible link between the observed endocrine activity and adverse effect(s).
Existing adverse outcome pathways (AOPs) and mode-of-actions can be used as a starting point for the postulated mode of action against which the evidence can be systematically organised. The evidence on adverse effect(s) and endocrine activity provides empirical support to the KEs.

Evaluation of biological plausibility

Annex I: ***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.

The conclusion on biological plausibility may be based on whether or not the KER, as far as it is known, is consistent with what is known in general and specifically for the substance. The analysis of the biological plausibility for the KER refers only to the broader knowledge of the biology involved. In a postulated MoA, the KERs need to be consistent with the current understanding of physiology, endocrinology and toxicology by addressing structural and/or functional relationships between KEs.

The biologically plausible link does not need to be demonstrated with substance specific data but can be explained by existing knowledge. For example, there are numerous AOPs under development in the AOPwiki, these may be used as a starting point for evaluation

biological plausibility. The amount of empirical support needed to establish the KERs vary depending on how well developed the AOP in question is.

The assessment should include, when possible, issues such as essentiality, temporal concordance, specificity, consistency, analogy (see further definition in the table 3.11.1). In particular, dose and temporal concordance, when data are available, 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 the adverse outcome. However, inability to demonstrate these individual factors should not be used as such to exclude classification as an ED if the overall picture supports a plausible link.

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'. For these parameters, the underlying knowledge of the likely endocrine nature the such effects allows for a conclusion on the biological plausibility of the link without recourse to a detailed MoA analysis.

- When the mode of action analysis is based on a well-established AOP, e.g., OECD Series on Adverse Outcome Pathways[2]. In this situation, the biological plausibility is provided by the documentation for the KERs in the AOP used, e.g. OECD AOP 13 links thyroperoxidase to adverse neurodevelopmental outcomes in mammals.

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 mode of action is not as strong as for EATS mediated parameters. Therefore, the conclusion on biological plausibility would need to be supported by mechanistic data. [Example needed]

Similarly, for adverse effect(s) based on non-EATS the evidence that the adverse effects are caused by an endocrine mode of action needs to be substantiated with a more extensive MoA analysis than for EATS-mediated adverse effects.

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 the 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. 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 of 'EATS mediated' adverse effects has been identified which, based on current knowledge, is assumed to be E, A or S but due to the complexity and cross-talk of the endocrine system it is not possible to identify the specific modality. In such cases, a biological plausible link should be considered as established for an endocrine mode of action and classification may be warranted.
When the potentially endocrine-related adverse effects are considered caused by a non-endocrine MoA, a comparative MoA analysis between an ED and non-endocrine mode of action needs to be applied to substantiate a non-ED MoA. The level of empirical support and biological plausibility would need to be very strong to demonstrate that the alternative MoA is the more likely explanation of the adverse effects observed.

Table 3.11.1. Explanations of the terms: analogy, essentiality, consistency, specificity, temporal concordance.

[2] OECD Series on Adverse Outcome Pathways | OECD iLibrary (oecd-ilibrary.org)

Term	Explanation
Analogy	A consistent observation across (related) substances having a well-defined MoA.
Essentiality	Essentiality is one of the elements to be considered when performing the weight of evidence analysis using the evolved 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/other adverse effect is prevented/decreased if an upstream event is experimentally blocked. It is generally assessed, on the basis of direct experimental evidence other absence/reduction of downstream KEs when an upstream KE is blocked or diminished (e.g., in null animal models or reversibility studies).
Consistency	In 3.11. and 4.2 of this guidance, consistency is the pattern of effects across species/strains/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.
Specificity	In 3.11. and 4.2 of this guidance 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 toxicity, including excessive systemic toxicity.
Temporal concordance	Temporal concordance is one of the elements necessary for the evaluation of the empirical observations. Are key events, within the MoA, observed in the hypothesised order.

3.11.2.3.4. Weight of evidence and expert judgement

According to the ED criteria weight of evidence and expert judgement must be applied when concluding on the ED criteria (Article 9 in conjunction with Annex I, Sections 1.1.1. and 3.11.2.1.); see guidance on weight of evidence in Sections 1.4 and 3.9.2.3.4 of this guidance.

Annex I: ***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, such as:

(a) human experience 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 and

reproductive toxicity studies; the results of suitable *in vitro* tests; and relevant *in silico* predictions; these include also peer-reviewed published studies;

(b) (Q)SARs using data from another substance;

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

(d) any additional acceptable data for example physico-chemical or toxicokinetic parameters and information on metabolites should be considered where relevant.

Available information on known metabolites/degradation products should be considered in the WoE.

Formation of an metabolite with endocrine activity indicates an endocrine mechanisms of the parent substance. If a metabolite is formed in one mammalian species, it should be assumed by default that this metabolite is also formed in all mammalian species unless demonstrated otherwise. Therefore, the ED assessment should take into consideration the formation of metabolites with known endocrine activity.

Annex I: ***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.

Chemicals can potentially induce endocrine disruption by any route of exposure (e.g. when inhaled, ingested, applied to the skin or injected), but endocrine disruption potential and potency 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 weight of evidence determination (see CLP, Annex I, 1.1.1.3 and section 1.4 in this guidance). However, negative human data is not normally given much weight in CLP unless there is e.g. a clear mechanistic reason why human data is negative due to species differences.

Although the quality / reliability of a study per se affects the weight given to the study, there are also several other, "external" factors that may influence on weight of evidence assessment, as mentioned above in the green boxes. Information on, e.g. toxicokinetics, physicochemical properties, read-across and availability of substance specific data etc. 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.

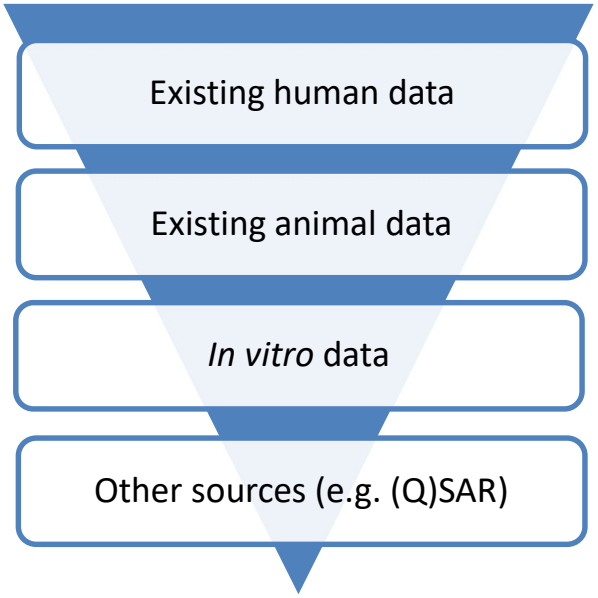
Evaluation must be performed on a case-by-case basis and with expert judgement. However, positive results that are relevant for classification should not be overruled by negative findings.

Annex I, ***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.

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. Weight of evidence for endocrine disruption must be conducted first independently for adverse effect(s), endocrine activity and for biological plausibility. Thereafter, the overall weight of evidence for all these three elements together must be conducted. 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 3.11-1 Simplified illustration of the relative weight of the available information



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

- When there are 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 level which is higher than the positive data, the full available dataset should be assessed in a WoE approach (e.g., existing good quality positive animal data could overrule negative human data and negative good quality *in vitro* data could overrule positive QSAR data).

- 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.

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

Annex I: ***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, 2018), and can be used to support on the classification as ED for human health. The Revised Guidance Document 150 (OECD, 2018) 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, also the EFSA/ECHA ED Guidance (ECHA/EFSA, 2018) specifies that the same database can be used to conclude on the endocrine disrupting 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."*

To support the classification as ED for human health with non-mammalian data, *in silico* tools may be used. As an example, SeqAPASS25 is an *in silico* tool used to assess amino acid sequence conservation across a wide range of species. The level of conservation can be used to predict the likelihood of similar susceptibility of toxicity between species.

However, the OECD 150 (OECD, 2018) 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 endocrine disruptors for human health in Category 1 or 2 when there is sufficient evidence that the three criteria (a) endocrine activity, (b) adverse effect and (c) biological plausible link indicated in CLP, Annex I: Table 3.11.1 (see Section 3.11.2.2) are met. If one of the three criteria 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 are considered relevant for humans. It is by default assumed that effects observed in mammalian studies are relevant to humans. The non-relevance of these effects to humans could be convincingly demonstrated by, for example, applying the guidance provided by the IPCS mode of action and human relevancy frameworks (IPCS 2007). Where it is known that the adverse effects are not relevant for humans or is of doubtful relevance to humans, this should be clearly justified. Where the link is established, the available integrated lines of evidence on adverse effect(s) and endocrine activity must be compared to the classification criteria.

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 clear endocrine (pattern of) changes are identified. Allocation to Category 1 is warranted when the evidence for adverse effect(s) and endocrine activity is conclusive considering all available relevant data in the weight of evidence on the substance or a substance for which a read across or a grouping approach can be performed. Sufficiently convincing evidence for Category 1 may be even based on appropriate and robust read across or analogy, when the read across is sufficiently justified for that particular substance. Also, evidence on certain pattern of adverse effect(s) observed, which is generally known to be linked to a certain type of endocrine activity, can lead to Category 1 classification.

If there are no human data, then the classification is based on the non-human data. If there is human data indicating no classification but there is also non-human data indicating classification then the classification is based on the non-human data unless it is shown that the human data cover the exposure range of the non-human data and that the non-human data are not relevant for humans. If the 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, Category 2 is warranted. This may be caused by issues related to reliability, dosing/concentration settings, parameters covered, life-stage investigated or exposure duration, magnitude of the effects,

divergencies between results in different studies, etc., or when chance, bias or confounding factors cannot be ruled out with reasonable confidence. For example, if there are serious concerns regarding the design, conduct and interpretation of existing information, or if there are insufficient information available to make a determination, or if the magnitude or nature of the adverse effect is considered to be weak, classification for Category 2 or even no classification may be more appropriate. Evidence on essentiality, consistency, analogy, specificity temporal concordance and/or information on 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 weight of evidence of both MoAs should be assessed in a comparative analysis, see section 3.5 of the ECHA EFSA ED Guidance (ECHA/EFSA, 2018). However, when the endocrine MoA is the most likely, even in presence of an alternative non-endocrine MoA, the ED MoA should not be disregarded. See also examples 4 and 5 in Section 3.11.5 below where data is not sufficiently convincing for Category 1 but the Category 2 criteria are met.

The evidence of a plausible biological link between the endocrine activity and the adverse effect (criterion (c)) sufficient for classification is considered as met when there is enough evidence for endocrine mode of action and when the link between adverse effect and endocrine activity is considered biologically plausible based on e.g.:

- understanding of the key event relationship (KER) based on previous documentation e.g. in scientific literature and broad acceptance e.g. in an established Adverse Outcome Pathway (AOP) (see OECD Series on AOPs),

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

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

Category 2 may also be warranted when the biological plausible link between adverse effect(s) and endocrine activity is weak but not contradicting with the existing knowledge. In general, ED mediated adverse effects can directly trigger *ED HH* 1, whereas sensitive to, but not diagnostic effects could more potentially lead to an *ED HH* 2 (see parameters in table 14 of ECHA/EFSA ED Guidance (ECHA/EFSA, 2018)). The parameters described in Table 14 may be sufficient for covering criteria for the adverse effect per se or need further data or a pattern of effects to support classification.

The following scenarios can be identified:

If **adverse effect(s) are based on '*EATS-mediated parameter(s)*'**, the adverse effect(s) observed provide clear evidence for adverse effect(s), endocrine activity and the biological plausible link. Therefore, classification for *ED HH 1*; EUH380 is warranted even without specific mechanistic information or identification of the specific MoA, unless demonstrated not to be ED in a MoA analysis supported by sufficient data.

If **adverse effect(s) are based on '*Sensitive to, but not diagnostic of, EATS parameters*' or non-EATS parameters,** there are several different scenarios that could lead to different classification outcomes for endocrine disruption. These scenarios depend i. on the strength of the evidence for the three criteria; ii. on whether EATS-mediated parameters (see more details in sections 3.11.2.1.2 and 3.11.2.2) have been fully or partially investigated and found positive or negative and; iii. on the available information on whether other types of endocrine activity not already inferred from the EATS-mediated parameters is available and on the weight of evidence. The following scenarios assume that a non-endocrine MoA is not conclusively demonstrated:

(1) 'Adverse effect(s) are based on 'Sensitive to, but not diagnostic of, EATS-parameters' **AND** most of the 'EATS-mediated-parameters' are fully investigated and (borderline) positive **AND** an ED MoA can be postulated => Category 1 or 2 depending on the overall strength of evidence.

(2) Adverse effect(s) are based on 'Sensitive to, but not diagnostic of, EATS parameters' **OR** based on non-EATS parameters (**AND** most of the 'EATS-mediated parameters' have been fully investigated and are negative) **AND** non-EATS endocrine activity positive **AND** an non-EATS ED MoA can be postulated ⇒ Category 1 or 2 depending on the strength of evidence.

(3) Adverse effect(s) are based on 'Sensitive to, but not diagnostic of, EATS parameters' **AND** most '*EATS-mediated parameters*' have not been investigated **AND** (non-)EATS endocrine activity is positive **AND** an (non-)EATS ED MoA can be postulated ⇒ Category 1 or 2 modality depending on the overall strength of evidence. Under this scenario, it may be possible to postulate both an EATS and a non-EATS endocrine MoA.

(4) Adverse effect(s) are based on 'Sensitive to, but not diagnostic of, EATS parameters' **AND** most of the 'EATS-mediated parameters' have been fully investigated[3] and are negative ⇒ no classification based on EATS modalities.

(5) Adverse effect(s) are based on 'non-EATS parameters' **AND** non-EATS endocrine activity positive **AND** an non-EATS ED MoA can be postulated ⇒ Category 1 or 2 depending on the strength of evidence.

However, 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, however there is not enough information to postulate a detailed mode of action 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 assumed to be EATS mediated, but due to the complexity and crosstalk of the endocrine system, it is difficult to identify the specific modality. In these cases, classification as *ED HH 1*; EUH380 or *ED HH 2*; EUH381 may be justified based on the strength of the evidence.

The substance should normally not be classified for example when:

- adverse effect(s) are not demonstrated, or
- endocrine activity is not observed ("observed" covers also situations when only ED mediated adverse effect(s) are observed i.e. endocrine activity is inferred by the adverse effect(s) observed, see examples under 3.11.2.3.1 under "ED mediated parameters"); or
- adverse effects are observed which cannot be linked to the observed endocrine activity using existing knowledge, therefore, a biological plausible link cannot be established; or
- adverse effect(s) are solely a non-specific consequence of other toxic effects (see CLP, Annex I, Section **Error! Reference source not found.**); i.e., a non-

[3] As defined in the ECHA/EFSA ED Guidance (2018), i.e., an EOGRTS with extension of cohort 1B to produce the F2 generation to investigate EAS and OECD 407, 408, 409, 414, 416/443 and 451-3 to investigate T.

endocrine MoA has been demonstrated to be the most likely explanation of observed adverse effect(s); or

- when a non-endocrine MoA has been demonstrated to be the most likely explanation of observed adverse effect(s)

- adverse effects are conclusively demonstrated to be due to an endocrine mode of action that is not relevant to humans

It is important to clarify if endocrine disruption is sufficiently investigated for classification purposes. If sufficient data were not provided to allow conclusion, it should be noted that no classification is warranted due to the lack of data. Ultimately, a weight of evidence approaches and expert judgement is needed to decide on the appropriate category.

3.11.2.4.1. Specific considerations regarding thyroid modality with respect to decision making 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.

The thyroid hormones (THs) act on almost all cell types in the body. THs are essential for proper development and differentiation of all cells of the body, and for maintaining metabolic balance and body temperature. TH and their regulation through the hypothalamic–pituitary–thyroid axis (HPT axis) is highly conserved across evolution in vertebrates. Because of the highly conserved nature of TH physiology, substances affecting thyroid function or TH signalling in one species may well similarly affect others, including humans [REF]. All thyroid toxicity related mechanisms in e.g. rodents are considered relevant for humans, unless conclusively demonstrated not to be human relevant.

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 [REF].

The regulation of serum TH levels and of TH action in various tissues involves a complex interplay of physiological processes which includes multiple MIEs which all can lead to the same adverse effect, see Figure 3.11-3 (Noyes et al., 2019). 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 all these processes which in turn adversely affect the thyroid function.

Figure 3.11-3 is a high-level integration of currently available 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. 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. In theory, all the molecular initiating events (MIEs) mentioned in the figure could lead to the same adverse outcomes. However, what the figure does not show is the magnitude, timing or length of initiating or key events needed to trigger the adverse outcomes.

THs are essential for normal human brain development, both prenatally and postnatally, modulating genes critical for a normal neuroanatomical development, with subsequent effects on neurophysiology, and finally neurological function [REF]. In early pregnancy the foetus is fully dependent on maternal thyroid hormones; this makes the foetus in this life-stage particularly vulnerable to thyroid disruption [REF]. Therefore, chemicals that interfere with TH synthesis have the potential to cause TH insufficiency that may result in adverse neurodevelopmental effects in developing foetus.

In children, thyroid disruption during pregnancy and early years of life can lead to neurodevelopmental impairments including low IQ scores [REF], cognitive and neurobehavioral defects [REF], and hearing loss (Crofton, 2004). In adults, THs are responsible e.g. for maintenance of cellular metabolism and cardiovascular functions [REF].

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 nor adverse outcomes manifested e.g. as developmental neurotoxicity. However, studies commonly provide information on thyroid weight and histopathology, serum THs and serum total and LDL cholesterol.

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 an adverse outcomes. Furthermore, reduced THs due to increased liver clearance is recognized as a relevant endocrine mode-of-action in OECD Guidance Document 150 (OECD, 2018). Similarly, changes in the thyroid follicular cells in terms of hypertrophy, hyperplasia and/or continuum through 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.

TH measurements may support classification by providing evidence for endocrine activity. Generally, the specificity and sensitivity of TH measurements are not high; thus, there may uncertainties in results. In addition, due to complexity of the TH system, it is possible that only hormone (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 supporting evidence for classification. However, lack of such effects cannot be used to negate adverse effects on the thyroid gland.

The production, clearance and transformation of cholesterol is regulated by THs, therefore elevated serum levels of total cholesterol, LDL-cholesterol and triglycerides may be regarded as an indication of low serum THs (Liu & Peng, 2022; Shin & Osborne, 2003). Research shows that also TSH affect in lipid metabolism independently of TH [REF]. Consequently, hypothyroidism-related dyslipidemia is associated with the decrease of TH and the increase of TSH levels. Therefore, total cholesterol, LDL-cholesterol and triglycerides provide additional evidence that may support KEs that support decreased THs at the tissue level which is independent and parallel to the to the effects on the thyroid gland.

The validated methods for detecting the MIEs relating to the thyroid AOPs are currently lacking. The scientific literature contains studies which investigate some of the MIE. Information on the MIE may provide, if available, supporting evidence for the classification. Given the number of potential MIEs, negative evidence for one or a few MIE should not negate classification in case there is other evidence fulfilling the CLP criteria for ED for human health.

Given the highly conserved nature of TH physiology, indications of interference with

thyroid function or TH signalling in one species may well indicate similar effects in others, including humans. Therefore, indications of thyroid disruption in one species should be considered a concern also for other species, including humans, unless there is data to disprove this. Similarly, indications of thyroid disruption in adults should be considered indicative of 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 *HH ED 1*; EUH380 may be warranted when:

If there is evidence that the observed pattern of thyroid-related effects lead to the overall conclusion that they constitute an adverse toxicologically significant effect.

Evidence on thyroid-related adverse effects will normally consist of data on thyroid weight and histopathology. Thyroid effects observed in more than one study has more weight than effects observed in one study, however adverse effects in a single study may warrant classification. Similarly, thyroid effects observed in more than one mammalian species further strengthen the evidence.

When adverse effects are observed on the thyroid gland, additional mechanistic information is not necessarily required to meet the ED criteria. This is because effects on thyroid weight and histopathology, which are 'EATS-mediated parameters', provide by themselves evidence of adverse effect(s) via endocrine activity. However, the evidence for endocrine activity may be further supported by toxicologically significant alteration of specific parameters like reduced serum T4 and/or T3, increased TSH, increased total cholesterol or LDL-cholesterol, and data on MIEs. However, when there is information that raises serious doubt about the relevance of the adverse effects to humans, classification in Category 2 may be more appropriate. Ultimately, the differentiation between Category 1 and 2 depends on the strength of evidence.

Additional mechanistic information, e.g. positive indications of a endocrine activity-associated MIE, may provide additional support to the classification. However, knowledge of the MIE is not needed for classification as the effects defining adverse effect(s) for the thyroid are 'EATS mediated' and thus contain inherent endocrine activity which is enough to demonstrate biological plausibility.

If a Comparative thyroid assay (CTA)[4] is available which provides evidence of alteration of the HPT axis and/or histopathology 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 the foetus and new-born animals are representing the target population for the adverse outcome of concern, e.g., brain development.

There is a link between thyroid disruption and developmental neurotoxicity (DNT). E.g. OECD AOP 13 and 14 may be used to establish a biologically plausible link between the evidence on ED-associated DNT (impaired learning and memory the adverse outcome in AOP 13 and 14) and thyroid system-associated endocrine activity. Evidence of effects on thyroid hormone levels, thyroid weight and/or histopathology (potentially supported by altered cholesterol levels) may indicate endocrine activity. In addition, it should be highlighted that if the function of a brain-specific deioninase of transporter is impaired, then adverse effects on neurodevelopment may occur with (see OECD AOP 14) or without [REF] altering

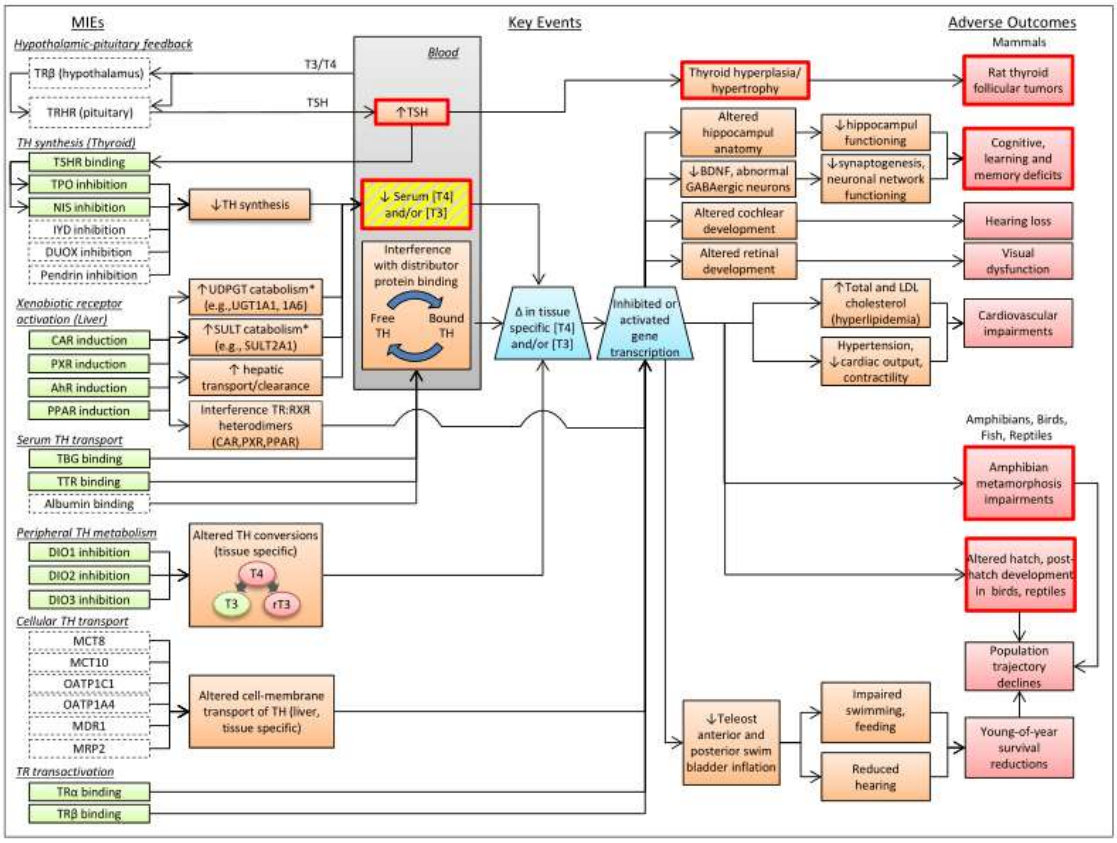
[2]US EPA 2005 https://www.epa.gov/sites/default/files/2015-06/documents/thyroid_guidance_assay.pdf

serum TH levels.

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

Evidence of adverse effects on the thyroid gland may be demonstrated by changes in organ weight or histopathological findings (follicular cell hypertrophy or hyperplasia) in any vertebrate provided that these changes result in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences, but the strength of evidence is not sufficient to classify as Category 1.

Figure 3.11-3 Adverse outcome pathway (AOP) network for chemically induced thyroid activity showing the integration of multiple individual AOPs under development and proposed. 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. 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. Figure from Noyes et al. (2019) Reproduced from Environmental Health Perspectives with permission from the authors.



3.11.2.4.2. Specific considerations regarding adverse effects on (developmental) neurotoxicity and immunotoxicity with respect to decision making 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 hormone systems (see e.g. example 3 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. (Developmental) neurotoxic and immunotoxic effects shall be considered as adverse effects relevant for classification as endocrine disruptors, similar to the other ED-mediated adverse effects 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. Please note that 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 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.

Currently, there are several indications of ED-related mechanisms causing (developmental) neuro- or immunotoxicity in scientific literature. The science is continuously developing on this area and therefore, the assessment needs to be done on a case by case basis based on the current available scientific knowledge.

3.11.2.5. Classification of substances containing CMR or ED constituents

From a compositional and a regulatory point of view the situation for substances containing CMR or 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 CMR and ED endpoints that is foreseen by CLP for mixtures containing CMR or ED components, is considered applicable also to substances containing CMR or ED constituents, additives or impurities (see sections **Error! Reference source not found.** and 3.11.3.1.1 to 3.11.3.2 of this guidance). As discussed in section **Error! Reference source not found.** below, mixtures containing components classified as endocrine disruptors 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 CMR or ED effects which have not been identified from the information on the individual substances, those data shall also be taken into account. For CMR or ED endpoints the lowest incidence possible to detect in the tests is by far unacceptable in humans. Thus, the highest test dose shall be the limit dose as described in the relevant OECD TG, see further details on dosing in section 3.11.2.3.1.1. "Relevant doses for classification". Dilution, as would be the case if mixtures or substances containing CMR or ED constituents were tested, would increase the risk that CMR or ED hazards would not be detected. Therefore, negative test data on mixtures containing constituents with these hazards shall not be accepted.
According to Article 10 (1), substances in other substances and substances in mixtures are treated in the same way regarding the use of generic and specific concentration limits (GCLs and SCLs). A GCL will apply to EDs unless the data justifies setting an SCL.

3.11.2.6. Setting of specific concentration limits

> ***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. SCLs for ED shall be set following the procedures outlined in this guidance paragraphs 3.6.2, 3.7.2. and/or 3.9.2, with the following amendments: When the effect subject to ED classification is related to reproductive toxicity, the paragraph 3.7.2 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 0.1 instead of 0.3. For carcinogenicity related ED effects such as testicular or ovarian tumours, 3.6.2 applies and for other target organ ED effects, 3.9.2 applies. It shall be noted that for STOT RE and SE, 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[5] (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 not evident at a level above the concentrations set for the relevant hazard class.

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

When there is sufficient and conclusive data available that the ED effect is a non-threshold effect or, with a non-monotonic dose response curve, the SCL corresponding to the extreme potency group may be set by default, unless an even lower SCL is justified. Due to these typical characteristics for many EDs, the assessment of dose-response related information together with setting SCLs should be conducted with caution.

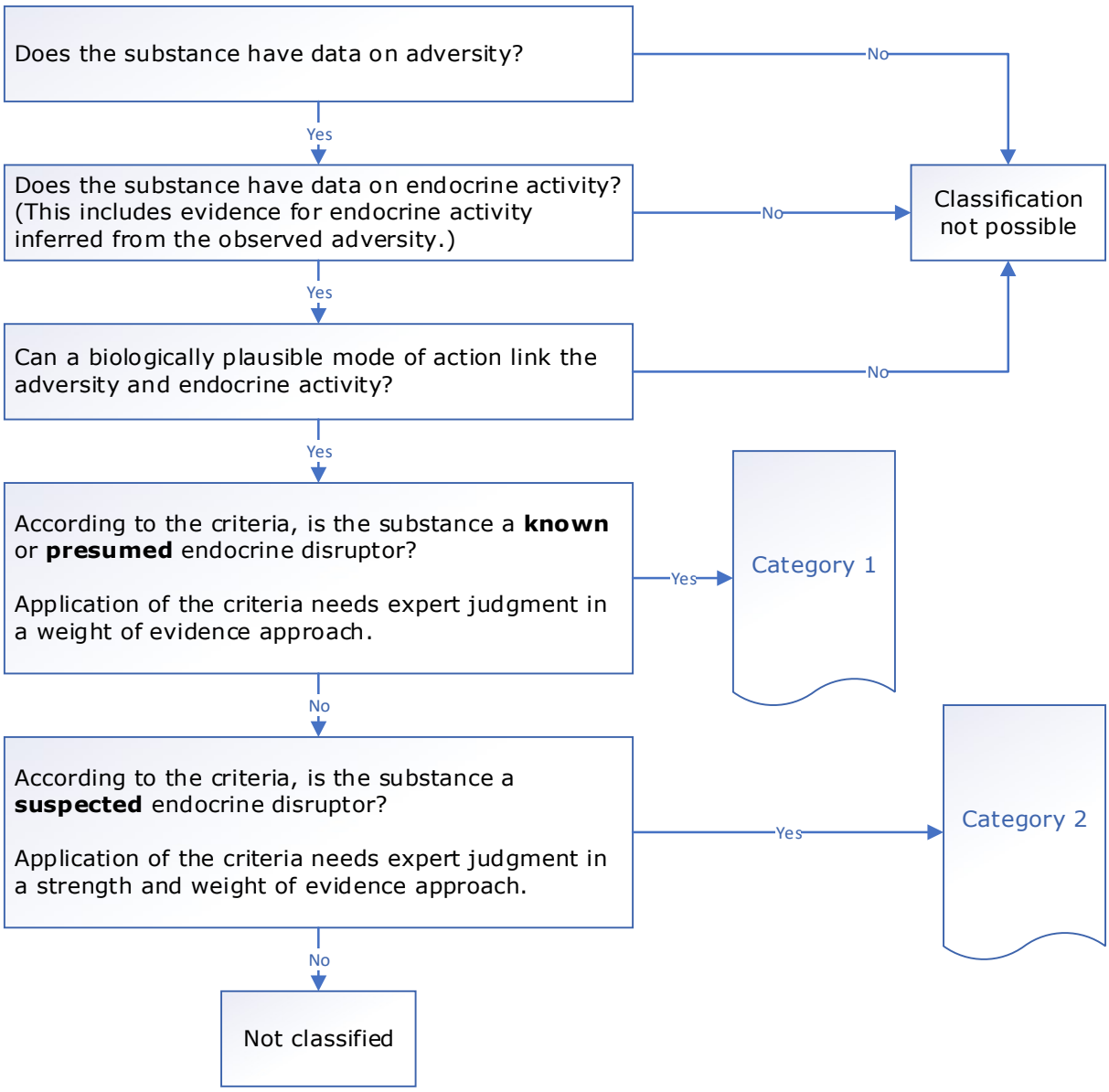
3.11.2.7. Decision logic for classification of substances

The decision logic which follows, in Figure 3.11-4, is provided here as additional guidance. It is strongly recommended that the person responsible for classification study the criteria before and during use of the decision logic.

Figure 3.11-4 Decision logic for endocrine disruption for human health

Decision logic for endocrine disruption for human health. The following outcomes are expected: 'Category 1', 'Category 2', 'not classified'; i.e., not meeting the ED criteria, or 'classification not possible'; i.e. due to lack of or inconclusive data.

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



[A placeholder for a more detailed flow-chart where more detailed information on possible scenarios which are leading to different categories or no classification. Examples of scenarios where Cat 2 would be more appropriate despite criteria a, b and c are met: Increased uncertainty due to:

- inconsistent results withing study or among studies (e.g. positive and negative / pointing towards different directions)
- low quality of study/studies (e.g. low reliability of study/studies, issues with study design such a dose level setting)
- lack of enough data to increase certainty]

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, 3.11.2). Only

in case there is data available for the mixture itself which demonstrate effects not apparent from the ingredients, this data might be used for classification. Data from tests with the mixtures might be considered in the event that an ED concern for human health then becomes apparent (and does not occur in the measurements of the individual constituents). 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, 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).

The additivity concept can be applied for endocrine disruptors (see also Section 1.6.3.4.3. of this guidance). Exposure to endocrine disruptors with both similar and dissimilar modes of action can lead to combination effects. 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 similar mechanism or mode of action or adverse outcome (e.g. exposure to a combination of anti-androgenic, estrogenic and steroidogenic disrupting substances can lead to additivity), unless there are specific reasons not to do so. Modality or the MIE does not need to be the same, similar to most of the other HH hazard classes where the same adverse outcome between substances can already suggest additivity.

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

Annex I*: 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 endocrine MoA or modality for different ingredients of the mixture.

3.11.3.1.2. When data are available for the complete mixture

Annex I: ***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

Annex I: ***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 of this guidance). Data on similar tested mixtures shall be used only when it demonstrates classification for endocrine disruption for human health, in line with 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

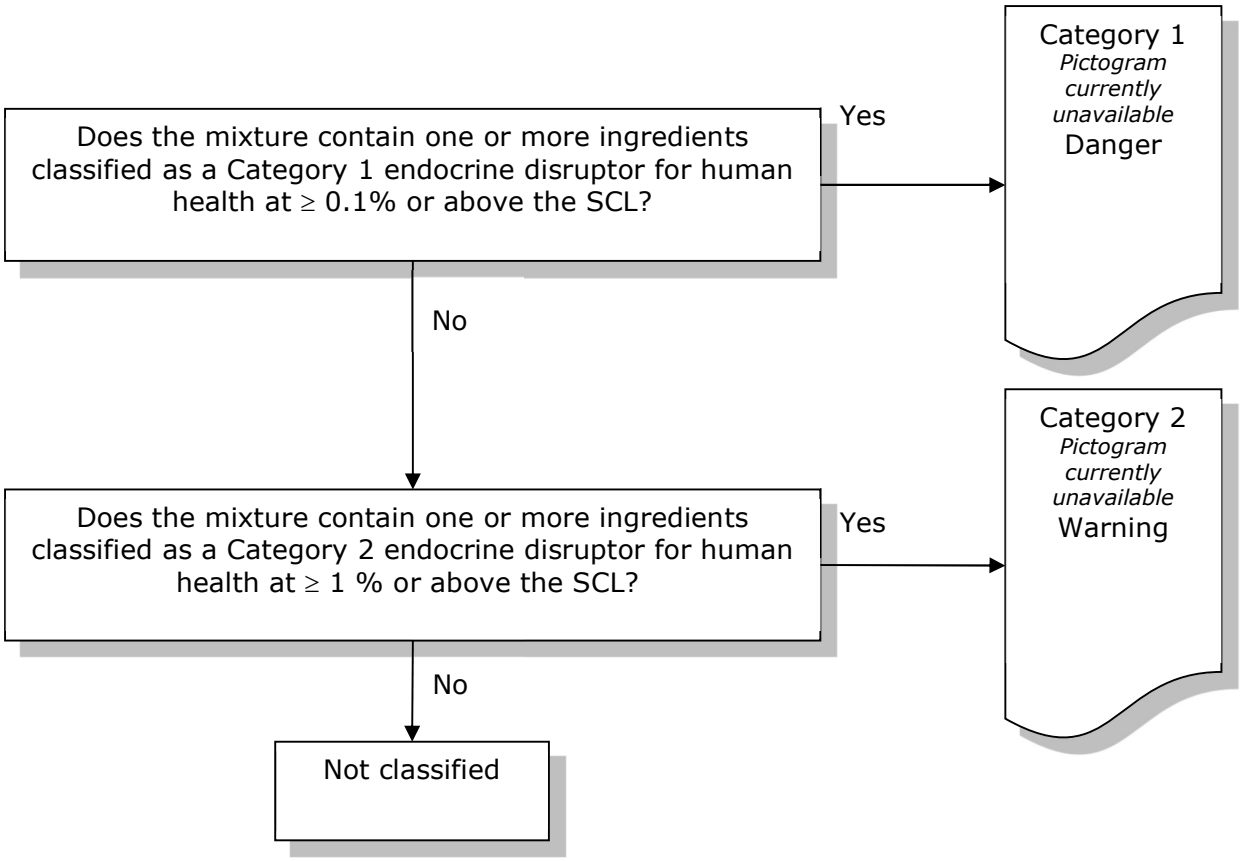
(see CLP, Annex I, Sections 1.1.3.3 and 1.1.3.4)

3.11.3.2. Decision logic for classification of mixtures

The decision logic which follows 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

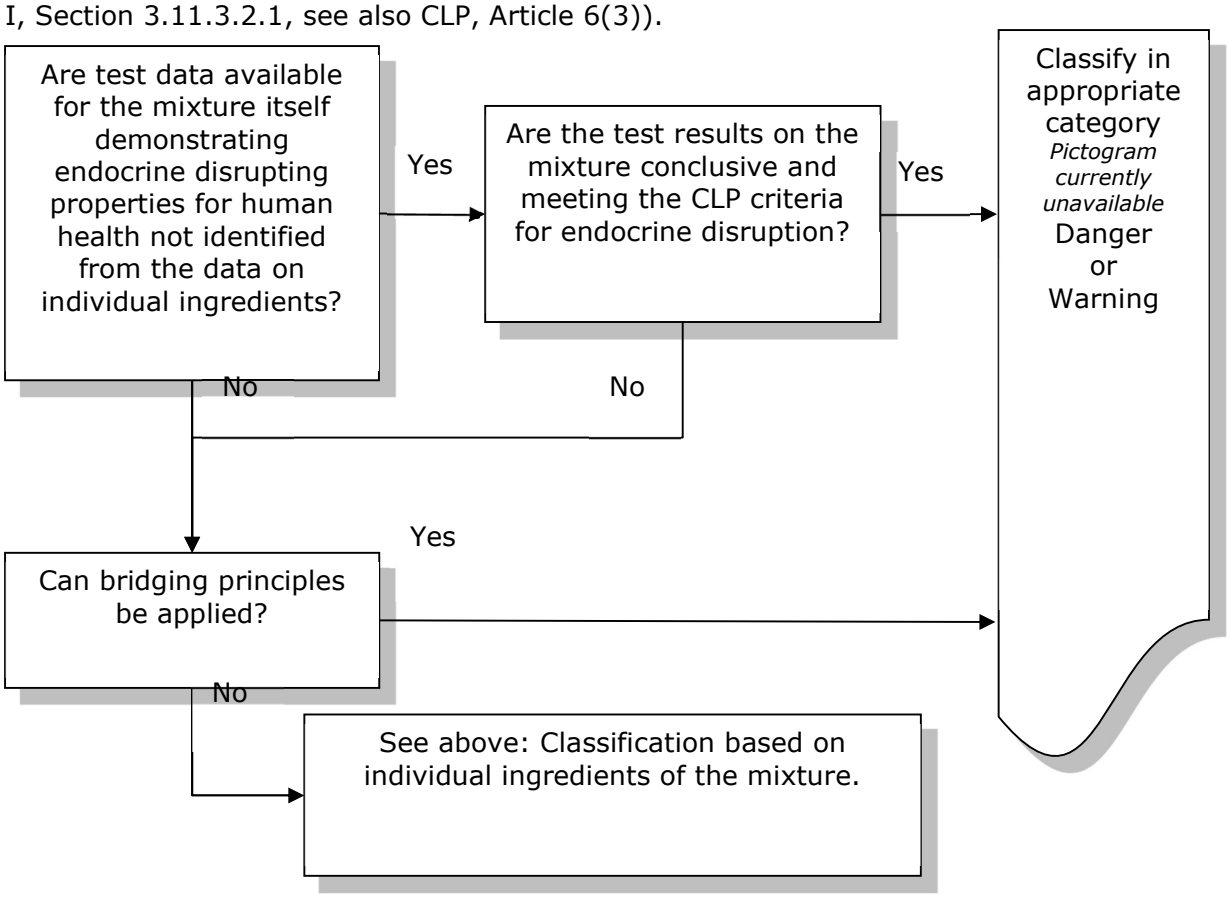
Classification based on individual ingredients of the mixture


Does the mixture contain one or more ingredients classified as a Category 1 endocrine disruptor for human health at ≥ 0.1% or above the SCL?
Yes
Category 1
Pictogram currently unavailable
Danger
No
Does the mixture contain one or more ingredients classified as a Category 2 endocrine disruptor for human health at ≥ 1 % or above the SCL?
Yes
Category 2
Pictogram currently unavailable
Warning
No
Not classified

1191
1192

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, Annex I, Section 3.11.3.2.1, see also CLP, Article 6(3)).



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* *P280*	*P201* *P202* *P263* *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 endocrine disruptors in CLP, however there may be provisions laid out in other regulations such as REACH which need to be considered, when relevant.

3.11.5. Examples

These examples are only to illustrate what type of data may lead to classification in different categories for endocrine disruption. Only ED related data leading to classification/ supporting classification or "no classification" is included but not the whole data set or a detailed description of the effects, nor a full weight of evidence analysis. The template for conducting full assessment based on lines of evidence can be found on the ECHA website in the ECHA CLH template and ECHA/EFSA Guidance on endocrine disruptors (2018). It also should be noted that 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 thyroid effect

Example 3: Classification as *ED HH* 1 based on non-EATS (neurotoxicity,α_2-adrenergic agonist)

Examples *ED HH* 2 (see Section 3.11.5.2)

Example 4: Classification as *ED HH* 2 based on EAS (anti-androgenic effect)

Example 5: Classification as *ED HH* 2 based thyroid effect

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

Example 6: no classification based thyroid effect

3.11.5.1. Examples *ED HH* 1

3.11.5.1.1. Example 1

Adverse effect(s):
The following effects are observed at top dose in a two-generation reproductive toxicity study (OECD TG 416 with very recent protocol) in rats; GLP, reliability 1; 0, 1.5, 15, and 75 mg/kg body weight/day in the diet:

- P Females: prolonged oestrous cycle, reduced number of corpora lutea
- F1 generation: reduced litter size
- F2 generation: reduced litter size

Decreased uterus and ovarian weight ≥ 150 mg/kg body weight/day observed in sub-acute and sub-chronic toxicity studies (OECD TG 407, reliability 1; 0, 150, 450, 1000 mg/kg body weight/day; and OECD TG 408, reliability 1; 0, 50, 150, 300 mg/kg body weight/day).
Earlier first oestrus, decreased uterus weight and prolonged oestrous cycle observed in a female pre-pubertal assay at doses ≥ 60 mg/kg body weight/day (OPPTS 890.1450; reliability 1; 0, 20, 60, 300 mg/kg body weight/day).
Based on the above, Substance X meets the criteria for Repro. 1B:H360F.
Endocrine activity:
In silico information*:*

- The QSAR Toolbox indicates the substance is a strong ER binder due to "*cyclic molecular structure with a single non-impaired hydroxyl group*".

In vitro information:

Moderate competitive binding to estrogen receptor 1 (ER1); IC_{50} 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 (OPPTS 890:1250, reliability 1).

In vivo information:

- Dose-dependent increase of uterine weight in ovariectomised rats (OECD TG 440; reliability 1; 0, 75, 125, 250 and 500 mg/kg body weight/day in the diet).

- No androgenic or anti-androgenic activity observed in a Hershberger assay (OECD TG 441; reliability 1; 0, 10, 30 and 100 mg/kg body weight/day subcutaneous injection).
- Adverse effects on uterus and ovarian weight, oestrous cycle, sperm count, age at first oestrus, corpora lutea and litter size provide *in vivo* mechanistic information.

The adverse effects on uterus and ovarian weight, oestrous cycle and sperm count are '*EAS mediated*' parameters and age at first oestrus is an '*EA mediated*' parameter. These provide clear evidence of an endocrine MoA. This is further supported by the observations in parameters which are '*sensitive to but not diagnostic of EAS*' indicating a wider pattern of effects likely to be EAS mediated.
The results of the uterotrophic assays indicate an estrogenic activity which is further supported by the QSAR Toolbox and the ER binding assay. The Hershberger assay excludes androgenicity.
Therefore, it is considered that estrogenicity is the most likely MoA. It should be noted that in this case the endocrine activity data gives additional support for the classification but is not necessary to have due to the type of adverse effect(s) observed.

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 evidence for specific EAS modes of action. Due to the nature of the effect and the existing knowledge on mammalian reproductive endocrinology and human contraception, these EATS mediated adverse effects are considered diagnostic of an EAS mode of action and thus (in the absence of other explanations) also imply underlying *in vivo* mechanistic information.

Conclusion *ED HH*:
There is clear evidence for an adverse effect on the female reproductive system; there is clear evidence indicating that the substance has estrogenic activity; and there is a clear link because both adverse effect(s) and endocrine activity have been observed in the same study in a dose and temporal concordant manner. In addition, knowledge on mammalian reproductive endocrinology and human contraception supports this conclusion.
Based on the above, Substance X meets the criteria for *ED HH* 1:H380.

SCL calculation:
Two methods were used, since there were ED effects in parental animals where the SCL calculation method modified from 3.9.2 shall be used as well as ED effects where the SCL calculation method similar to 3.7.2 shall be used. The most conservative SCL will then be selected.
Method similar to 3.7.2 for the reproductive LOAEL of 75 mg/kg bw/day effect. The estimated ED10 value, based on the top dose of 75 mg/kg bw/day is suggesting a medium potency group (4 mg/kg bw/day < ED10 value < 400 mg/kg bw/day), no need for SCL based on effects related to reproductive toxicity. See further information on ED10 and potency groups in 3.7.2 of this guidance.
Method similar to 3.9.2 for parental LOAEL of 60 mg/kg bw effect from female pre-pubertal assay. SCL Cat1 = (60/(10x100)x100% = 6%, rounded to 5%.
Conclusion on SCL: The method similar to 3.7.2 resulted in a medium potency group which corresponds to a GCL of 0.1% whereas the method similar to 3.9.2 resulted in a low potency group corresponding to an SCL of 5%. The lower should be selected, which is the GCL 0.1%. Thus, no SCL will be set.

3.11.5.1.2. Example 2

Available information:
Human data: Other substances with the same MoA, i.e. thyroid peroxidase (TPO) inhibition, are used clinically to treat hyperthyroidism.

Animal data: Sub-chronic toxicity study (90 day, OECD TG 408), rat, dietary exposure, GLP, reliability 1; Doses: 0, 10, 50, 250 mg/kg body weight/day.

- ↑ 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.

Sub-chronic toxicity study (90 day, OECD TG 409), dog, diet, GLP, reliability 1; Doses: 0, 2, 10, 50 mg/kg body weight/day.

- ↑ Thyroid weight (absolute and relative), statistically significant at all doses in both males and females, dose-response observed.
- ↑ Thyroid hyperplasia, statistically significant at all doses in both males and females, severity increase with dose.
- ↓ T4 and T3, statistically significant in males and females measured at top dose only.

Assessment:

Adverse effect(s): Adverse effects on the thyroid have been observed in two species. Thyroid effects were accompanied with reduced T4/T3 and increased TSH. Dogs are more sensitive than rats.
Overall, the pattern of effects observed provide clear evidence for endocrine-related adverse effect(s).

Endocrine activity: 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.
The mechanistic information is limited to measurements of thyroid hormones in the available *in vivo* studies and a TPO inhibition assay. T3/T4 is significantly reduced in both rats and dogs. The reduction in T3/T4 is accompanied by an expected increase in TSH
Given that the relative potency is in the same order of magnitude as the known TPO inhibitor methimazole, TPO inhibition seems to be the most likely mode of action.
The other possible thyroid MoAs have not been investigated. However, increased hepatic clearance of THs due to enzyme induction can be excluded because this is a rodent specific effect; in this case there are adverse effects also in dogs. (It should be noted that, while this information is good to have, the classification of this substance could be concluded without consideration of this issue).
There is an existing AOP supporting the MoA analysis.
Overall, the pattern of effects observed provide evidence for human relevant thyroid MoA, i.e. TPO inhibition.

Biological plausibility: AOP 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 fact that TPO inhibitors are used clinically to treat hyperthyroidism provides additional support for the human relevance of the MoA.

Based on current understanding of endocrinology and physiology, considering the pattern of effects observed, there is clear evidence for TPO inhibition as the MoA.

Conclusion:

There is clear evidence on thyroid related adverse effect(s) (thyroid follicular cell hyperplasia, increased thyroid weight, decreased colloid, and changes in thyroid hormones) (changes in T3/T4 and TSH) and positive indications of TPO in liver microsomes from rats and dogs. Even if the effects were seen in one species, Cat 1 would be warranted based on the effects observed. Therefore, the substance meets the criteria for classification as *ED HH 1*; EUH381.

SCL calculation:

Method similar to 3.9.2 for 2 mg/kg bw effect. SCL Cat1 = 2/(10x100)x100% = 0.2%

Conclusion on SCL: The method similar to 3.9.2 resulted in a medium potency group very close to a GCL of 0.1%. Thus, no SCL will be set.

3.11.5.1.3. Example 3

Adverse effect(s):

Substance Y is a veterinary drug used as a surgical anaesthetic and analgesic. It is also used as an enhancer in a biocidal product. Substance Y induces transient general narcosis (lethargy and ataxia) at oral doses between 10-100 µg/kg body weight depending on the species. The substance also reduces blood pressure.

Substance Y meets the CLP criteria for classification as STOT-SE 3 (H336) for narcosis.

There is clear evidence that the substance induces adverse effects.

Endocrine activity:

The catecholamine noradrenaline functions both as a hormone and a neurotransmitter. The general function of noradrenaline is to prepare the body for action. In the brain, noradrenaline increases among others arousal, alertness and focuses attention. In the rest of the body noradrenaline increases heart rate, glycolysis and increases blood pressure.

Substance Y is an α_2-adrenergic agonist which opposes the effects of the sympathetic nervous system by reducing signal transmission in noradrenaline neurons. The MoA of the substance is well documented in scientific literature.

Based on the above, there is clear evidence that the substance has endocrine activity.

Biological plausible link:

The biology of catecholamines is fully understood. There is clear evidence of a (neuro)endocrine MoA based on an α_2-adrenergic agonist

Conclusion *ED HH*:

There is clear evidence for an adverse effect on the central nervous system; there is clear evidence that Substance Y interferes with noradrenaline signalling; and there is a clear link because the MoA is fully understood.

Based on the above, Substance Y meets the criteria for *ED HH* 1:H380.

SCL calculation:

The adverse effect was observed at oral LOAEL of 10 µg/kg body weight. Method similar to 3.9.2 for 0.01 mg/kg bw 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%.

3.11.5.2. Examples *ED HH* 2

3.11.5.2.1. Example 4

Available information:

Human data: No relevant information available

Animal data: Combined Repeated Dose Toxicity Study with the Reproduction/ Developmental Toxicity Screening Test (OECD TG 422), GLP, reliability 1, 0, 25, 100, 400 mg/kg/day + 14-day recovery group for control and high dose.

P0 animals:

- ↑ Absolute and relative weight of testes, +8% ($p<0.05$) at 400 mg/kg/day
- ↓ Absolute weight of prostate, -11% ($p<0.05$) at 400 mg/kg/day
- ↓ Absolute and relative weight of seminal vesicles, -10% ($p<0.05$) at 400 mg/kg/day
- Changes in organ weights were partially recovered in the recovery group.

F1 animals:

- Nipple retention in males:
  - In controls, 0.25 retained nipples per male pup (16/43)
  - At 25 mg/kg/day, 0.12 retained nipples per male pup (5/40)
  - At 100 mg/kg/day, 0.42 retained nipples per male pup (18/43)
  - At 400 mg/kg/day, 1.54 ($p<0.05$) retained nipples per male pup (60/39)
- Anogenital distance in males:
  - No effects

Assessment:

Adverse effect(s): Adverse effect(s) are observed both in the P0 and F1 generation. However, the study provides screening level information on adverse effect(s) with low statistical power. In addition, no histopathological effects were observed.
Overall, the pattern of effects observed provide some evidence for endocrine-related adverse effect(s).

Endocrine activity: Positive indications for endocrine activity stem from the adverse effects observed. Effects on testes, prostate and seminal vesicle weights are all sensitive to but not diagnostic of EAS activity. However, the pattern of effects is indicative of an anti-androgenic activity which is further supported by the effects on nipple development.
Overall, the pattern of effects observed provide evidence for anti-androgenic 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.
Based on current understanding of endocrinology and physiology, considering the pattern of effects observed, there is clear evidence for anti-androgenic MoA.

Conclusion:

There is some evidence on adverse effect(s) (decreased organ weights and nipple retention) in a screening study. However, the study design does not allow a robust conclusion on adverse effect(s), given the low number of animals used and that no

histopathological effects were observed. There is convincing evidence of endocrine activity based on a pattern of *in vivo* mechanistic effects consistent with existing knowledge for an anti-androgenic MoA. Therefore, the substance meets the criteria for classification as *ED HH 2*; EUH381.

SCL calculation:
Method similar to 3.7.2 for 100 mg/kg bw/day effect. ED10 value is suggesting a medium potency group (4 mg/kg bw/day < ED10 value < 400 mg/kg bw/day), no need for setting SCL.

3.11.5.2.2. Example 5

Human data: No relevant information available
Animal data: Sub-acute toxicity study (28 day, OECD TG 407), rat, diet, GLP, reliability 1; Doses: 0, 30, 100, 300 mg/kg body weight/day.

- ↑ Thyroid weight (17% (absolute), statistically significant at all doses and dose related
- changes in colloid staining (dose-related increase in incidence)

Animal data: Extendend one-gerenration reproductive toxicity study (including DNT, OECD TG 442), rat, diet, GLP, reliability 1; Doses: 0, 30, 100, 300 mg/kg body weight/day.

- Dose-related change in morphometric measurements
- No effect on thyroid weight

Invitro DOI2 assay
Biological plausibility supported by AOP

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. However, the fact that thyroid hyperplasia was observed is suggestive evidence of 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 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.
The mechanistic information is limited to a TPO inhibition assay. The results of this assay suggest that reduced THs due to reduced TH synthesis is likely not the cause of the effect observed.
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 clear evidence on adverse effect(s) (thyroid follicular cell hyperplasia, increased thyroid weight, and increased total cholesterol indicating reduced THs). Since the human relevance of the effects observed cannot be excluded (not all human relevant thyroid-modes of action can be excluded), the substance meets the criteria for classification as *ED HH2*; EUH381.

SCL calculation:

Method similar to 3.9.2 for 30 mg/kg bw effect. SCL Cat1 = 30/(10x100)x100% = 3 %

Conclusion on SCL: The method similar to 3.9.2 resulted in a low potency group corresponding to a SCL of 3 %. Thus, a higher SCL of 3% will be set.

3.11.5.3. Examples no classification

3.11.5.3.1. Example 6

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/day.

- ↑ Absolute and relative weight of thyroid, +5% at 1000 mg/kg/day in both male and females.
- Thyroid follicular cell hypertrophy observed in 2/5 males and 1/5 females.
- THs were not investigated

In vitro data: No relevant information available

Assessment:

Adverse effect(s): The study provides screening level information on adverse effect(s) with low statistical power. In addition, no histopathological continuum observed, i.e. findings are confined to histopathological diagnosis of follicular cell hypertrophy without evidence of evolution of follicular cell hypertrophy to hyperplasia. 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 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 not sufficient evidence for thyroid-related adverse effect(s) because no histopathological continuum was observed. In the absence of an adverse effect the ED criteria are not met.

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4. ENV

4.2. Endocrine disruption for environment

Disclaimer: *This section of the CLP guidance refers to the ECHA/EFSA Guidance (ECHA/EFSA, 2018) in several sub-sections, and further information can be found in that guidance to assist in concluding on ED properties. However, it is important to make a distinction between that guidance and this one as they serve different purposes.*
The ECHA/EFSA 2018 Guidance, which builds on the OECD GD 150, was written to assist users to comply with their obligations to conclude on ED properties in accordance with the ED criteria for biocidal products (BP) and plant protection products (PPP), respectively. The ECHA/EFSA 2018 Guidance describes how to gather, evaluate and consider all relevant information for the assessment, conduct a mode of action (MoA) analysis, and apply a weight of evidence (WoE) approach, in order to establish whether the BP or PPP ED criteria are fulfilled. Therefore, the ECHA/EFSA 2018 ED guidance still has a function because it outlines how to conclude on ED properties.

However, in 2023 endocrine disruption was introduced into CLP as a hazard class with subcategorisation. Consequently, for classification purposes this guidance on the application of the CLP criteria is the applicable one which should be followed for all substances subject to CLP, including industrial chemicals and active substances under the BP and PPP Regulations.

[ECHA would also like to note the commenters that all active substances under the BP[1] and PPP[1] Regulations must be classified according to the CLP ED criteria. In this context, it is important to note that the current ED criteria for BP and PPP are essentially the same as ED HH 1 or ED ENV 1 under the CLP criteria. Therefore, in line with the one substance one assessment principles, it is expected that active substances already concluded to meet the ED criteria under the BP and PPP procedures before the criteria in CLP Regulation came applicable, will under CLP Annex VI be assigned to ED HH 1 or ED ENV 1. Similarly, active substances which have been concluded not to meet the ED criteria under the BP and PPP procedures are expected to be assigned to ED HH 2 or ED ENV 2 or no classification unless substantial new information has become available which warrants classification as ED HH 1 or ED ENV 1. Similarly, substances identified as Substances of Very High Concern (SVHC) under REACH due to ED properties are expected under CLP Annex VI be assigned to ED HH 1 or ED ENV 1. This issues above will not be part of the CLP guidance text, but rather considered under respective regulations and guidance's.

The sections for HH and ENV may not be fully aligned, and a better alignment will be considered during the PEG process.
Further, this draft CLP guidance is not necessarily in line with the CLH template ED section and in this case, the guidance should applicable, the template is easy to modify to better reflect the guidance.
In particular, ECHA wishes to receive input and concrete text proposals on the following topics:

1. Developing general flow charts and more detailed guidance for
    1. Cat 1 Cat 2 (with special attention to thyroid modality) and 'no classification'
    2. ED mediated, sensitive to, and non-EATS parameters
2. Relation of (developmental) neurotoxicity (and immunotoxicity) to ED classification
3. A more detailed paragraph on EAS modalities (similar to specific paragraph on thyroid modality)
4. More details on different situations for additivity and non-additivity
5. Additional examples on:
    1. missing modalities,
    2. using in vitro and human data only,
    3. read across/grouping,
    4. tumours e.g. uterine adenocarcinoma,
    5. cross-species considerations and use of AOPs to demonstrate the biologically plausible link,
    6. serious doubts about population relevance.]

4.2.1. Definitions and general considerations for endocrine disruption

Annex I*: 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 classification for endocrine disruption for the environment, similar to classification for ED for human health, refers to a specific mode of action (endocrine) which will lead to an adverse effect(s) and in that the criteria requires evidence on three different aspects, i.e. adverse effect(s), endocrine activity, and a biological plausible link between the endocrine activity and the adverse effect(s); i.e. a correlation[6] between endocrine activity and adverse effect(s) consistent with existing knowledge.

Annex I: 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*' endocrine disruptors for the environment if they induce adverse effects in wildlife which have a consequence on the maintenance of the population by altering the function of the endocrine system, i.e., the substance has an endocrine mode of action (MoA), in accordance with the criteria given in CLP, Annex I, Section 4.2.2.1.

Annex I: 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).
This classification is intended to indicate when a substance may cause harm due to the fact that its effects are mediated through an endocrine MoA. The sensitivity to such effects depends 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 endocrine disruptor for the environment, the adverse effects need to be relevant at the population or subpopulation level. See section 4.2.2.3.2 on population relevance.

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

[6] Correlation in this context means that endocrine activity and adverse effect(s) can be linked using existing knowledge as the most likely explanation to the observed effects, for details see Section 4.2.2.3.4

In addition, the classification of a substance as endocrine disruptor 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*. (See example 7 in Section 4.2.5.2).

The concept of endocrine disrupting "potency" is considered only in the context of setting specific concentration limits (see Section 4.2.2.5 of this guidance), and 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, i.e., the criteria apply to all dose/concentration levels. In other words, even endocrine related effects observed at high doses/concentrations (showing low potency) are still relevant for classification. When there is sufficient information that already very low doses/concentrations or alternatively only very high doses/concentrations are causing the ED effects, this guidance considers that as a difference in potency which can be regulated by setting a specific concentration limit.

EATS- and non-EATS modalities

Endocrine disrupting modes of action are caused either by estrogen, androgen, thyroid and steroidogenic (EATS) modalities or by so-called Non-EATS modalities. Further information on EATS modalities can be found in section 3.11.2.3.1.

Endocrine disrupting modes of action are caused either by estrogen, androgen, thyroid and steroidogenic (EATS) modalities or by so-called non-EATS modalities. While the CLP criteria do not differentiate among modalities, thus covering all endocrine-disrupting MoAs, i.e., adverse effects which may be caused by any endocrine modality, it is acknowledged that this guidance mainly addresses the effects caused by EATS modalities.

This is because the EATS modalities are the pathways for which there is currently the most knowledge available, i.e., there is a 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* and *in vitro* testing available where there is a broad scientific agreement on the interpretation of the effects observed on the investigated parameters.

However, the general principles outlined in this guidance for evaluation of the data on the different criteria, weight of evidence and decision on classification, are also applicable to other endocrine (non-EATS) modalities. Although the existing knowledge for those modalities is not as advanced as for the EATS modalities, it may, in some cases, be already possible to reach a conclusion on the need to classify the substance on a non-EATS endocrine modality, e.g., where literature data provide mechanistic information, which 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 retinoid acid related endocrine disruption.

4.2.1.1. Species 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, 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, weight of evidence and decision on classification, are also applicable. 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 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

The CLP Regulation does not set information requirements or require testing of substances and mixtures for classification purposes (CLP Art. 5, 6 and 9). The assessment is based on the respective criteria and consideration of all available relevant information. Under CLP, no further studies can be requested.

The main ways to gather all available information is by conducting a literature search or a systematic literature review. Additionally, previous regulatory assessments may serve as a starting point for the literature search.

The information is relevant when it investigates at least one of three criteria (endocrine activity, adverse effects and biologically plausible link):

- Information on endocrine related *adverse effects* for the environment is normally obtained from animal studies. In the future there may be non-animal methods which may provide equivalent predictive capacity to the currently used animal studies; however, currently no such methods are available. Information may also be obtained using read-across or analogy, e.g. if the substance share a common mode of action.
- Information on *endocrine activity* generally comes from *in vivo* or *in vitro* mechanistic studies. Also non-animal methods which provide equivalent predictive capacity of the currently used in vivo mechanistic studies may be used; e.g. the ToxCast ER model. 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.
- For *biological plausibility*, existing scientific knowledge can be used, e.g. textbooks and scientific literature. Several adverse outcome pathways have already been established (see OECD Series on AOPs), and there is continuous development of additional AOPs in the AOPwiki.

4.2.2.1.1. Identification of animal data

Information considered for other hazard classes e.g., hazardous to aquatic environment, may also provide information relevant for endocrine disruption for the 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.

All relevant information that addresses endocrine-related adverse effects and activities shall be considered in a weight of evidence 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 endocrine disruptors for the environment are outlined in the OECD GD 150 'Guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption' (OECD 2018). 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 estrogen, androgen, thyroid and steroidogenesis (EATS) modalities, and how these effects may be evaluated to support identification of endocrine disruptors.

The OECD GD 150 (2018) includes the OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals (OECD CF) which lists the OECD test guidelines and standardised test methods available that can be used to evaluate chemicals

for endocrine disruption. The OECD CF is intended to provide a guide to the tests available which can provide information on assessment of endocrine disruption, but it is not intended to be a testing strategy. It is not an exhaustive list and tests and assays other than those described in the list may also be valuable for assessing chemicals for endocrine disruption provided that they use scientifically acceptable methods. New tests are continually being developed, aiming to bring useful information for classification. In particular, for non-EATS modalities, research studies are an important source of information which must be considered in a weight of evidence approach.
In addition to animal and experimental data outlined in OECD GD 150 (2018), other relevant and validated OECD studies as well as literature studies of good quality may also provide information relevant for endocrine disruption classification for the environment.

4.2.2.1.2. Identification of non-animal data

Data from non-animal approaches can be used instead of animal data for classification purposes provided the data have an equivalent predictive capacity to animal data.
As described in the previous section, a non-animal study or testing strategy may provide equivalent predictive capacity to an *in vivo* study even if it is not done in an intact organism. Currently, for adverse effect(s), there are no such studies or testing strategies available which provide data with equal predictive capacity as the animal data. For endocrine activity, there are more alternative methods available. The developments of new *in vitro*/*in silico* models may in the future provide data with equivalent predictive capacity as animal data.
Information obtained using read-across from similar substances can also be used, where appropriate, e.g. if information on the substance itself is scarce.

Validated New Approach Methodologies (NAMs), if available, and also other published /internationally recognised methods can be used for classification to avoid unnecessary animal testing if they are relevant. When the NAMs (*in vitro, in silico* models, omics approaches and methodologies, QSARs, testing strategies etc.) provide data with equivalent predictive capacity as the animal data they can be used to provide sufficient data on activity and adverse effect(s) for classification in Category 1 or 2.

4.2.2.2. Classification criteria

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.*

4.2.2.2.1. Classification in the presence of other toxicity

Annex I: ***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 an endocrine-related effect occurs together with "other toxicity", classification for ED should normally be applied. Substances shall however, not be classified for endocrine disruption if an ED related adverse effect occurs solely as a non-specific secondary (indirect) consequence of other toxic effects.

The presence of other toxicity shall not be used to negate findings of endocrine-related adverse effect, unless it can be concluded that the endocrine-related adverse effects are solely secondary (indirect) non-specific effects of other toxicity.

In practice, the differentiation between a secondary non-specific effect of other toxicity and a specific endocrine-related adverse effect is done by assessing whether the other toxicity can influence the occurrence of the endocrine-related adverse effect.

If other toxicity, co-occuring with endocrine related adverse effects, is so **severe/excessive** that it causes e.g. ≥10% mortality, at this dose level the endocrine related effects that can be clearly attributed to non-endocrine specific MoA, can be reasonably expected to be solely a secondary non-specific consequence of the other toxicity.

If the severity of co-occurring other toxicity is **less than excessive**, it shall normally not influence the classification. Ideally, to be considered as a consequence of other toxicity the endocrine-related adverse effects must be observed at higher concentrations than the concentrations at which the other toxicity (such as mortality or sublethal clinical effects[7]) is observed. 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 and the severity of the other toxicity observed (e.g. less than 10%

[7] For a list of clinal signs observed that can be used to identify sublethal effects see Table 1 of Annex 4 of the OECD TG 203 for fish, and paragraph 41 in OECD TG 231 for amphibians.

mortality or much less than 10% mortality or only sub-lethal effects). Endocrine-related adverse effects observed below the concentration where other toxicity is observed, can be considered as secondary to other (non-endocrine) toxicities only if there is evidence for a biologically plausible sequence of events which excludes an endocrine mode of action as the most likely explanation to the observed adverse effect(s). This is best done by a comparative mode of action assessment. Considering the complexity of the endocrine system, the effects observed in the presence of excessive toxicity need to be assessed with caution and on a case-by-case basis.

For example, if <10% mortality is observed, but still close to this threshold, and the endocrine related adverse effects are only observed concomitantly to this other toxicity, it is likely that the endocrine-related adverse effects are solely a secondary non-specific consequence of the other toxicity. Aspects such as analogy with other chemicals, the overall (eco) toxicological data package suggesting a specific non-endocrine MoA etc, may be considered to substantiate that the endocrine related adverse effects are likely secondary non-specific consequence of other toxicity. However, if there is <10% mortality and the endocrine related adverse effects are observed in a dose/concentration-response manner and the other toxicity is only observed at the highest tested dose/concentration, those effects should be considered for classification purposes.

If there is <<10% mortality or only sublethal effects and the endocrine related adverse effects are only observed at the highest tested dose/concentration, those effects should not be ignored by default for classification. Aspects such as analogy with other chemicals, the overall (eco) toxicological data package suggesting a specific non-endocrine MoA etc, may be considered to substantiate that the endocrine related adverse effects are likely secondary non-specific consequence of other toxicity. However, if there is <<10% mortality or only sublethal effects and the endocrine related adverse effects are observed in a dose/concentration response manner and the other toxicity is only observed at the highest tested dose/concentrations, those effects should be considered for classification purposes.

4.2.2.2.2. Relevant concentrations for classification

[This paragraph is for clarification purposes only and it is not meant to stay in the final version of the guidance.]

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 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 tolerated without inducing significant chronic physiological dysfunctions, be compatible with animal survival and permits data interpretation in the context of the use of the study. In ecotoxicology, this is assessed by using the concept of the maximum tolerated concentration (MTC) which is defined as the highest test dose/concentration of the chemical which results in less than 10% mortality (Hutchinson et al., 2009; Wheeler et al., 2013; Ankley and Jensen, 2014) or in any other relevant effects (such as mortality or sublethal clinical effects) which might be clearly attributed to general toxicity. For tests on aquatic organisms, the maximum solubility in water, or the limit concentration as defined in the relevant OECD guidelines, should be considered.

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 (including studies to investigate the endocrine-related adverse

effect of a substance), endocrine-related adverse effects at higher doses/concentrations can be relevant for classification if such data is available.

An adverse effect can sometimes be a secondary, non-specific effect of other toxic effects. Such an effect would not be considered an adverse effect in the context of the ED assessment. See Section 4.2.2.2.1. There is no generic 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 weight of evidence (WoE) approach. This includes positive and negative data from all relevant sources of information, as described in section 4.2.2.1. Datasets should be analysed using weight of evidence 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 as to the sections of this guidance on the hazard to the aquatic compartment. All adverse effect(s) related to effects on reproduction (e.g. fertility, fecundity, etc.) in the case of EAS modalities and on developmental/growth (hindlimb length, developmental stage, time to metamorphosis, thyroid histopathology) for the T modality shall be assessed (see Tables 15 and 16 of ECHA/EFSA ED Guidance (ECHA/EFSA, 2018). Information on other toxicity shall also be considered in the assessment of adverse effect(s).

For the EATS modalities, the OECD GD 150 (OECD, 2018) provides guidance on how to interpret parameters normally investigated in (eco)toxicity studies (see also the ECHA/EFSA Guidance (ECHA/EFSA, 2018)). The OECD GD 150 differentiates between:

- 'EATS-mediated parameters', considered as "diagnostic" parameters, measured *in vivo* that may contribute to the evaluation of adverse effect(s), while at the same time also implying an underlying *in vivo* mechanistic information, thereby providing information on endocrine activity. 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[8].

- 'Sensitive to, but not diagnostic of, EATS parameters' measured *in vivo* that may contribute to the evaluation of adverse effect(s), however, due to the nature of the effect and the existing knowledge, and thus 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 might provide indications of an endocrine MoA. Examples of these parameters for environment are fecundity, hatching success, behaviour (e.g. stickleback nesting, mating, predator avoidance).

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).

However, studies, other than those listed in OECD GD 150, may also include endpoints

[8] It should be noted that some specific gonad histopathological findings are EATS-mediated, but some others are not (e.g. oocyte atresia). More detailed guidance on specific gonad histopathology examination in fish is given in OECD (2009).

that can be affected by endocrine MoA, and therefore may provide relevant information. In addition to results from guideline studies, results from well-performed and reported studies from the open literature may provide just as valuable and useful knowledge as results from guideline studies. Therefore, the data used to classify a substance can be drawn from standard studies or other scientific data, e.g. robust peer-reviewed publications, literature studies, Q(SAR) data, internationally recognised databases etc.

The current *in silico* and *in vitro* methods cannot fully replace *in vivo* data on adverse effect(s) for endocrine disruption, when developed further, they may provide sufficient information for endocrine related adverse effect(s).

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 for the most common test guidelines (ECHA/EFSA, 2018).

4.2.2.3.2. Population relevance

> **Annex 1**: ***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.*

> **Annex 1**: ***4.2.2.1****. Where there is evidence conclusively demonstrating that the adverse effects identified are not relevant at the population or subpopulation 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 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. The criteria also stipulate that only when there is evidence conclusively demonstrating that the adverse effects are not relevant at the population or subpopulation level, the substance shall not be considered an endocrine disruptor 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. 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.

To understand whether a change in a given parameter may be relevant at the level of population, two aspects should be considered: the relevance of the affected parameters and the effect level.

Relevance of the affected parameters

When assessing the effects observed in the available (eco)toxicological studies, relevant parameters for the effects on wildlife are those parameters that show an expectation of adverse effects on the population in the environment. This means that when extrapolating an effect from what is observed in the laboratory to a field situation, there are some parameters which are considered relevant at the level of population, e.g. effects on reproduction, growth/development. This is concluded because 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. Effects on growth (body weight and length), development, reproduction (such as fecundity, sex ratio, hatching success and offspring survival) in single species are generally regarded relevant for the maintenance of the wild population (European Commission, 2011; Marty, 2017). Such changes in fish, amphibians and mammals when caused by an ED MoA are considered to pose unacceptable effects to the environment. 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 would affect development and the reproductive performance. Therefore, if behavioural changes are observed they should be considered in the definition of adverse effect(s) and relevant at the population level.

On the other hand, other parameters, e.g. effects in non-reproductive organs, are not generally considered as relevant at the level of population unless accompanied by a pattern of effects on other more apical parameters.

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. This means that 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). Those effects, however, may not be relevant for the population of wild mammals, as the level will not likely result in high enough prevalence in the population to impact population survival/maintenance.

It should be noted that effects observed in rats 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.

Effect level

Regarding the effect level that should be observed to consider a change as an adverse effect at population level, a statistically significant difference compared to the control and the biological relevance of the observed change should be considered. 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, even changes with low prevalence may be considered as adverse.

Future developments in the field of effect models may be considered as valuable tools in better understanding the population relevance of the observed adverse effects.

Specific considerations related to the thyroid modality

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. For example, thyroid histopathological findings observed in the rat are likely not relevant at population level if observed in isolation without impairment of growth/development and/or reproduction or without support of other data in a WoE approach.

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 more apical (see definition for apical in ECHA/EFSA Guidance 2018) effects which may be due to the same ED MoA. Similarly, in the case of amphibians, changes in thyroid histopathology should be considered adverse at the population level only when observed together with effects on development (i.e., delay or acceleration). However, if the effects on development were not investigated, they can be inferred based on the changes in thyroid histopathology. This is because thyroid histopathology often exhibits compensation to thyroid insufficiency (Marty et al., 2017). Nevertheless, changes in development in amphibians even if observed in the absence of investigation of thyroid histopathology are considered population relevant effects.

Annex 1: ***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 wildlife 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. For example, if adverse effects on fertility or fecundity are observed in fish, but they are not statistically significant and of low biological relevance, this might raise serious doubts that these effects would impact the maintenance of the population.

Another example could be if in an amphibian study a statistically significant delay in metamorphosis is observed, but the delay is very short with no dose/concentration response and no clear change in thyroid histology. Such short delay may raise serious doubts that it would have an effect at population level and therefore Category 2 may be more appropriate.

One more example is the case where adverse effects such as uterine adenocarcinoma are observed only in old animals that are unlikely to reproduce, it is excluded that tumours or pre-stages of tumour occurred earlier in life and there were no effects on reproduction in the available reproductive mammalian studies. In this case, it would be unlikely that those effects would impede the maintenance of the population. Therefore, also in this case, classification in Category 2 might be more appropriate.

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 hormone levels are generally considered *in vivo* mechanistic. An example of these parameters for environment is vitellogenin (VTG).

In silico approaches (see Section 3.11.2.2.3.2), such as QSAR models (e.g. ComPARA and CERAPP), physiologically based kinetic (PBK) models and other mathematical models, (e.g. the virtual cell based assay, VCBA), could also be used to support the battery of *in vitro* assays (Mansouri et.el. 2020; Mansouri et.al 2016; Zaldívar et.al. 2010).

EATS-mediated, sometimes referred to as "diagnostic" of ED or EATS, parameters that contribute to the evaluation of adverse effect(s) (see section 4.2.2.3.1. of this guidance), at the same time (due to the nature of the effect and the existing knowledge as described in OECD GD 150) are also considered indicative of an EATS MoA and thus (in the absence of other explanations) also imply underlying *in vivo* mechanistic information. Further

information can be found in the ECHA/EFSA Guidance (ECHA/EFSA, 2018).

4.2.2.3.3.1. In vitro data

Currently, there are no *in vitro* assays with non-mammalian cells. However, since the endocrine system is known to be conserved across vertebrates, *in vitro* assays with mammalian cells can be used in a weight of evidence approach to give indications on possible MIEs also for non-mammalian species. 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, 2010). Such extrapolation of in vitro information is generally qualitative (...)*".
In general, the *in vitro* tests, when used in isolation, lack the complexity of an intact organism and can identify if a chemical is capable of binding a receptor or interfering with a pathway. However, the in vitro assays provide little information on whether the effect is operant *in vivo*. Particular attention should be applied to *in vitro* data and the considerations of ADME properties which are not covered by current *in vitro* test guidelines. Therefore, when interpreting the results of *in vitro* tests, the lack of a metabolising capacity of the system, as well as the lack of consideration of other ADME properties, should be considered. To partly overcome this limitation, several *in vitro* tests can be run investigating different points of perturbation or endocrine pathways, and metabolism may be addressed by adding (part of the) metabolising systems, potentially metabolising the parent compound into a more active, less active or inactive substance/metabolite, or metabolites of the substance could be directly tested. Therefore, all mechanistic information should be considered together to reach a conclusion.

In vitro assays focus on specific interactions of compounds with the molecular machinery of cells, 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 receptors and as compounds might be able to act via more than one mechanism, no single *in vitro* test can be expected to detect all types of endocrine activity.

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.

Because of the inherent limitations of *in vitro* systems highlighted above, conclusions on the endocrine activity of the substance can only be drawn in the context of what the *in vitro* assays can evaluate.

Results from a battery of tests for substances with low metabolising potential may in some cases be conclusive, e.g., ToxCast ER model. Similarly, data may be conclusive if both the parent substance and the metabolites are covered. The capacity of the organisms to compensate for a certain level of changes in hormonal regulation cannot be assessed in *in vitro* system. Further, the applicability domain of *in vitro* tests shall be considered.

Future developments of NAMs and the possible rapid advancement of, in particular, *in vitro* methods may allow a conclusive assessment of endocrine disruption without *in vivo* data.

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. In particular, by providing information on the molecular initiating event (MIE), *in silico* predictions can be used to support the identification of endocrine modes of action. The different types of *in silico* prediction methods can be grouped as: Molecular modelling of receptor interactions, (Q)SAR modelling of receptor-based activity, and Profilers based on structural alerts and decision trees. For further details see section 4.1

of the ED Guidance (ECHA/EFSA, 2018).

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 R6 should be followed (ECHA, 2008). Attention should be paid in 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.

New *in silico* tools are constantly being developed, and new tools not specified in the ECHA/EFSA Guidance or other available guidance documents, such as, but not limited to, ComPARA, CERAPP, Leadscope, and Opera can also provide useful information for the assessment.

4.2.2.3.4. Mode of action analysis and evaluation of biological plausibility

Annex I: ***4.2.1.1. (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 (ECHA/EFSA, 2018).

When potential endocrine-related adverse effect(s) and endocrine activity are identified, the link between the two, according to the 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 or toxicology, may be sufficient to establish the link and conclude on the biological plausibility. The level of information required for a MoA analysis vary depending on which parameters are adversely affected, i.e., EATS-mediated, sensitive to but not diagnostic of EATS, or non-EATS.

Biological plausibility may be demonstrated by conducting a mode of action analysis using all available relevant information. For classification purposes, knowledge and demonstration of the full MoA is not a requirement. The MoA analysis should aim at establishing the consistency and coherence of the responses obtained on measured parameters with a postulated MoA.

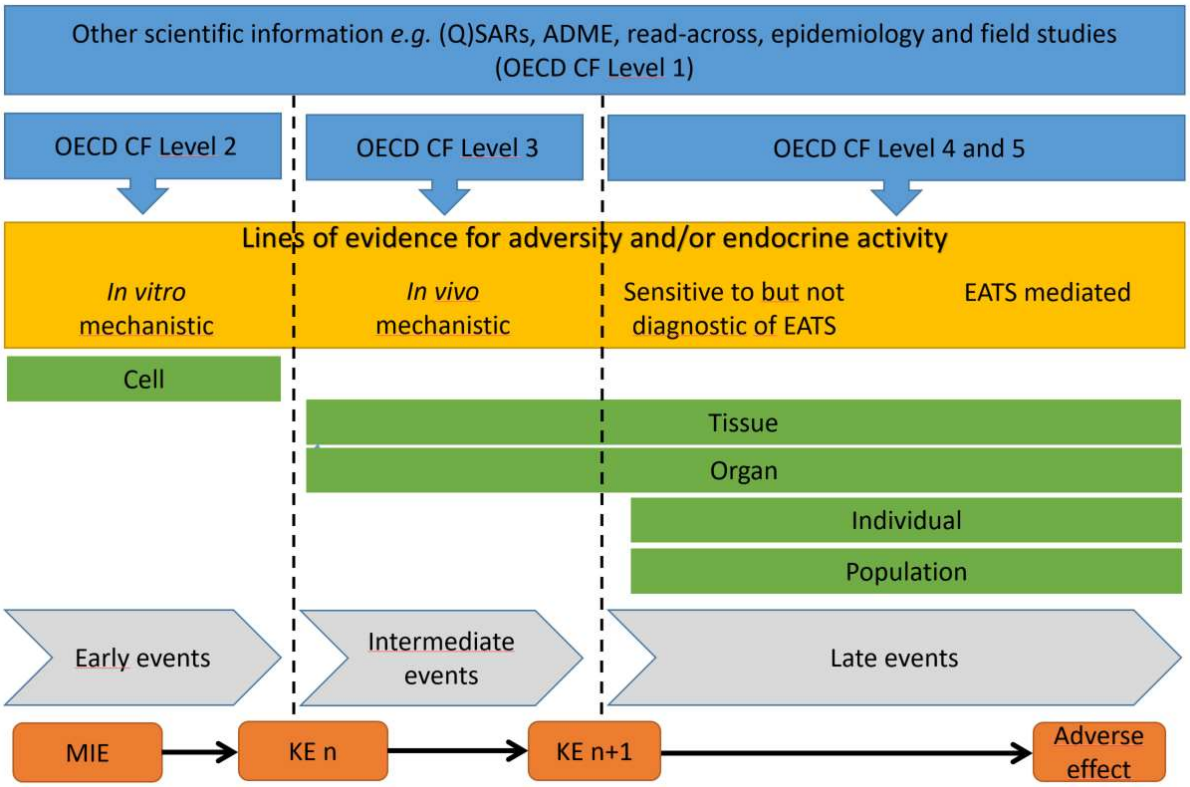
Mode of action analysis

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.

An endocrine mode of action means that the adverse effect is mediated through an alteration of the hormonal synthesis, regulation or metabolism, i.e., is not only about hormone-receptor interactions. Therefore, an endocrine MoA will normally contain some earlier KEs (which provide mechanistic information at the molecular or cellular level) and some later KEs (which provide information at the organ or system level, including the adverse effect).

In the case of endocrine disruption, this sequence at least includes one endocrine-mediated KE which may or may not also be adverse. 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 an event as key, there needs to be experimental data in which the event is characterised and consistently measured. 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. (ECHA/EFSA, 2018)



KE: key event; MIE: molecular initiating event.

The first step in assessing biological plausibility and conducting the MoA analysis 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. The evidence available for the substance subject to classification shall be assessed against the hypothesis for mode of action with its key events to be able to conclude on a biological plausible link between the observed endocrine activity and adverse effect(s).
Existing, adverse outcome pathway (AOPs) and mode-of-actions can be used as a starting point for the postulated mode of action against which the evidence can be systematically organised. The evidence on adverse effect(s) and endocrine activity provides empirical support to the KEs.

Evaluation of biological plausibility

Annex I: ***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.*

The conclusion on biological plausibility may be based on whether the KER, as far as it is known, is consistent with what is known in general and also what is known for the substance, specifically. The analysis of the biological plausibility for the KER refers only to the broader knowledge of the biology involved. In a postulated MoA, the KERs need to be consistent with the current understanding of physiology, endocrinology and toxicology by addressing structural and/or functional relationships between KEs.

The biologically plausible link does not need to be demonstrated with substance specific data but can be explained by existing knowledge. For example, there are numerous AOPs under development in the AOPwiki, these may be used as a starting point for evaluation of the biological plausibility. However, the amount of empirical support needed to establish

the KERs vary depending on how well developed the AOP in question is.

The assessment should include, when possible, issues such as essentiality, temporal concordance, specificity, consistency, analogy (see further definition in the table 4.2.1). In particular, dose and temporal concordance, when data are available, 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 the adverse outcome. However, inability to demonstrate these individual factors should not be used as such to exclude classification as an ED if the overall picture supports a plausible link.

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).

Table 4.2.1 Explanations of the terms analogy, essentiality, consistency, specificity, temporal concordance.

Term	**Explanation**
Analogy	A consistent observation across (related) substances having a well-defined MoA.
Essentiality	Essentiality is one of the elements to be considered when performing the weight of evidence analysis using the evolved Bradford Hill considerations. In the context of the MoA/AOP frameworks, essentiality refers to key events. For determining essentiality, it should be demonstrated whether 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	The pattern of effects across species/strains/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.
Specificity	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 toxicity, including excessive systemic toxicity.
Temporal concordance	Temporal concordance is one of the elements necessary for the evaluation of the empirical observations. Are key events, within the MoA, observed in the hypothesised order?

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'. For these parameters, the underlying knowledge of the likely endocrine nature of such effects allows for a conclusion on the biological plausibility of the link without recourse to a detailed MoA analysis.

- When the mode of action analysis is based on a well-established AOP, e.g., OECD Series on Adverse Outcome Pathways[9]. In this situation, the biological plausibility is provided by the documentation for the KERs in the AOP used, e.g. OECD AOP 25 links inhibition of gonadal activity in female fish and reproductive dysfunction.

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 mode of action is not as strong as for EATS mediated parameters. Therefore, the conclusion on biological plausibility would need to be supported by additional mechanistic data.

Similarly, for adverse effect(s) based on non-EATS the evidence that the adverse effects are caused by an endocrine mode of action needs to be substantiated with a more extensive MoA analysis.

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 the 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. 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 of 'EATS mediated' adverse effects has been identified which, based on current knowledge, is assumed to be E, A or S but due to the complexity and cross-talk of the endocrine system it is not possible to identify the specific modality. In such cases, a biological plausible link should be considered as established for an endocrine mode of action and classification may be warranted.
When the potentially endocrine-related adverse effects are considered secondary to other non-endocrine related toxic effects, a comparative MoA analysis between an ED and non-endocrine mode of action needs to be applied to substantiate a non-ED MoA. The level of empirical support and biological plausibility would need to be very strong to demonstrate that the alternative MoA is the more likely explanation of the adverse effects observed.

4.2.2.3.5. Weight of evidence and expert judgement

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

> **Annex I:** ***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.*

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

[9] OECD Series on Adverse Outcome Pathways | OECD iLibrary (oecd-ilibrary.org)

(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 including physico-chemical parameters and information on metabolites or degradation products should be considered where relevant.

Available information on known metabolites/degradation products should be considered in the WoE.

Formation of an endogenous metabolite with endocrine activity indicates an endocrine mechanism of the parent substance. If a metabolite is formed in one mammalian species, it should be assumed by default that this metabolite is also formed in all mammalian species and other vertebrates unless demonstrated otherwise. Therefore, the ED assessment should take into consideration the formation of known endogenous metabolites.

If a substance degrades 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.

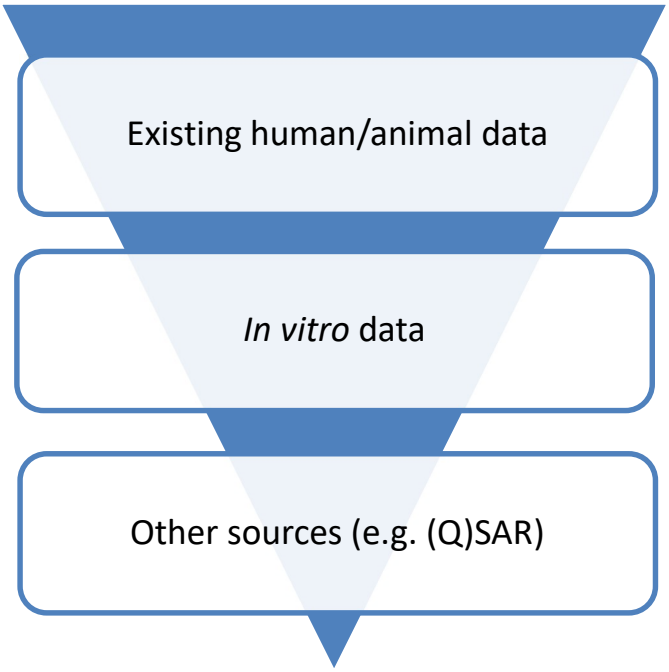
Annex I: ***4.2.2.3.2.*** *In applying the weight of evidence determination and expert judgement, the assessment of the scientific evidence referred to in section 4.2.2.3.1 shall, in particular, consider all of the following factors:*

(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 quality and consistency of the data should be given appropriate weight. Both positive and negative results should be assembled in a single WoE determination (see CLP, Annex I, 1.1.1.3 and Section **Error! Reference source not found.** in this guidance).
Although the quality / reliability of a study per se affects the weight given to the study, there are also several other, "external" factors that may influence on WoE assessment, as mentioned above in the green boxes. Information on toxicokinetics, physicochemical properties, read-across and availability of substance specific data etc. 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.
Evaluation must be performed on a case-by-case basis and with expert judgement. However, positive results that are relevant for classification should not be overruled by negative findings.

The following 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. WoE for endocrine disruption must be conducted first independently for adverse effect(s), endocrine activity and for biological plausibility. Thereafter, the overall weight of evidence for all these three elements together must be conducted. It needs to be noted that the relative weights indicated in the 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



When contradicting data of comparable quality belongs to different "hierarchical levels", the following considerations should be included in the WoE approach:

- When there are 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 level which is higher than the positive data, the full available dataset should be assessed (e.g., negative good quality *in vitro* data could overrule positive QSAR data).

Field or monitoring studies can also contribute to the WoE, for more information see in section 3.2 of the ECHA/EFSA ED GD (2018).

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

Annex I: *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 mammalian data may also be relevant for other vertebrates (OECD, 2018), and can be used to support or to conclude on the classification as ED for the environment. The Revised Guidance Document 150 (OECD, 2018) 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, also the EFSA/ECHA ED Guidance (2018) specifies that the same database can be used to conclude on the endocrine disrupting 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."* 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 endocrine disruptors for the environment in Category 1 or 2 when there is sufficient evidence that the three criteria (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 (see Section 4.2.2.2 of this guidance) are met. If one of the three criteria 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 are relevant at the population level.

Where it is proven that the adverse effects are not relevant at the population level, no classification is warranted. If there are serious doubts 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.

In addition, for both categories there needs to be evidence of a plausible biological link between the endocrine activity and the adverse effect. Criterion (c) is considered as met when there is enough evidence for endocrine MoA and when the link between adverse effect and endocrine activity is considered biologically plausible based on e.g.:

- understanding of the key event relationship (KER) based on previous documentation and broad acceptance (e.g. in an established Adverse Outcome Pathway (AOP)(see OECD Series on AOPs),

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

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

Where the link is established, the available evidence on adverse effect(s) and endocrine activity must be compared with the classification criteria.
Alternative MoAs, essentiality, consistency, analogy, specificity and 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 weight of evidence of both MoAs should be assessed in a comparative analysis, see 3.5 of the ECHA/EFSA ED Guidance (ECHA/EFSA, 2018). 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.

Category 2 may also be warranted when the biological plausible link between adverse effect(s) and endocrine activity cannot unequivocally be established.
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 clear endocrine (pattern of) changes is identified. Allocation to Category 1 is warranted when the evidence for adverse effect(s) and endocrine activity is conclusive considering all available relevant data in the weight of evidence on the substance or a substance for which a read across or a grouping approach can be performed. The sufficiently convincing evidence for Category 1 may be even based on appropriate and robust read-across or analogy, when the read across is sufficiently justified for that particular substance. Also evidence on certain pattern of adverse effect(s) observed, which is generally known to be linked to a certain type of endocrine activity, 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, Category 2 is warranted. This may be caused by issues related to reliability, dosing/concentration settings, parameters covered, life-stage investigated or exposure duration, magnitude of the effects, divergencies between results in different studies, etc., or when chance, bias or confounding factors cannot be ruled out with reasonable confidence. For example, if there are serious concerns regarding the design, conduct and interpretation of existing information, or if there are insufficient information available to make a determination, or if the magnitude or nature of the adverse effect is considered to be weak, classification for Category 2 or even no classification may be more appropriate.

The following scenarios can be identified.

If **adverse effect(s) are based on *'EATS-mediated parameter(s)'***, the adverse effect(s) observed provide clear evidence for both adverse effect(s), endocrine activity and the biological plausible link. Therefore, classification *ED ENV 1*; EUH430 or *ED ENV 2* EUH431 is warranted depending on the strength of the available evidence.

If **adverse effect(s) are based on *'Sensitive to, but not diagnostic of, EATS parameters'***, there are several different scenarios that could lead to different classification outcomes for endocrine disruption. These scenarios depend i. on the strength of the evidence for the three criteria, ii. on whether EATS-mediated parameters have been fully or partially investigated and found positive or negative and, iii. on the available information on endocrine activity, including the one not already inferred by EATS-mediated parameters, in case some of this have been investigated:

> **Scenario a)**: **EATS-mediated parameters have not been investigated.** In this case either no classification or classification as Category 1 or 2 is warranted depending on whether there is endocrine activity information available and whether it is possible to postulate an endocrine MoA linking the adverse effect(s) based on "sensitive to parameters" and the observed endocrine activity and depending on the strength of the available evidence.

Scenario b): all EATS-mediated parameters have been investigated[10] and found negative. In this case classification for EATS modalities is normally not warranted because the most diagnostic parameters (EATS-mediated) have been measured and did not show evidence of ED related adverse effect(s) and endocrine activity. However, there may be exceptions to this, i.e. cases where classification as Category 1 should be considered even if the EATS-mediated parameters were negative. This is the case for example of aromatase inhibitors for which no effects on Secondary sex characteristics and sex ratio are expected, but effects are observed on VTG, fecundity and gonad histopathology.

However, if other type of endocrine activity information is available, not inferred from the EATS-mediated parameters, and it is possible to postulate a non-EATS MoA linking that observed endocrine activity and the adverse effects, also Category 1 or 2 depending on the strength of evidence could be considered.

Scenario c): **not all EATS-mediated parameters have been investigated, and those investigated were found negative.** In this case either no classification or classification as Category 1 or 2 is warranted depending on whether there is additional endocrine activity information available and whether it is possible to postulate an endocrine MoA linking the adverse effect(s) based on "sensitive to parameters" and the observed endocrine activity. Also in this case there may be exceptions as mentioned above under scenario b) e.g. for aromatase inhibitors, as it depends on which EATS-mediated parameters have been measured and found negative.

To note that, if other type of endocrine activity information is available not already inferred from the EATS-mediated parameters, and it is possible to postulate a non-EATS MoA linking that observed endocrine activity and the adverse effects, also category 1 could be considered.

However, classification may also be warranted in cases when there is evidence that criteria 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 mode of action due to the lack of thorough mechanistic information. This is for example the case when a pattern of adverse effect has been identified which, based on current knowledge, is assumed to be EATS mediated, but due to the complexity and cross-talk of the endocrine system, it is difficult to identify the specific modality. In these cases, 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:

- adverse effect(s) are not demonstrated, or
- adverse effect(s) are not relevant at the population level, or
- endocrine activity is not observed, or
- when adverse effects are observed which cannot be linked to the observed endocrine activity using existing knowledge, therefore, a biological plausible link cannot be established, 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. a non-endocrine MoA has been demonstrated to be the most likely explanation of the observed adverse effects.

[10] According to the specifications of the ECHA/EFSA ED guidance, section 3.4.1

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 making 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. However, the classification of a substance as *ED ENV* if it is already classified as *ED HH 1* based on evidence on the thyroid modality from mammals is not automatically warranted. This is because the observed effects in mammals might not always be population relevant for wild mammals.

For example, if the adverse effect(s) observed in mammals leading to the classification for HH includes neurodevelopmental toxicity effects, those effects can be assumed to be population relevant. In such case, classification in category 1 for *ED ENV* is also warranted.

If adverse effect(s) in mammals are only based on histopathology in thyroid, thus not relevant at the population level, classification in Category 1 for environment is warranted only when there is information specific for the environment proving the population relevance of the effects. This is the case if there is at least one *in vivo* test in non-mammalian species (e.g. amphibians) showing evidence of adverse effects relevant at the population level, or non-animal data providing an equivalent predictive capacity to the *in vivo* data. In the case where only screening level *in vivo* information is available, classification in either Category 1 or 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, together with positive mammalian data on thyroid adverse effect(s), classification as Category 2 on a case-by-case basis could be considered based on a weight of evidence approach because, considering the highly conserved nature of thyroid hormone physiology, it is expected that the substance would elicit adverse effects relevant at the population level if tested in an *in vivo* long-term test.

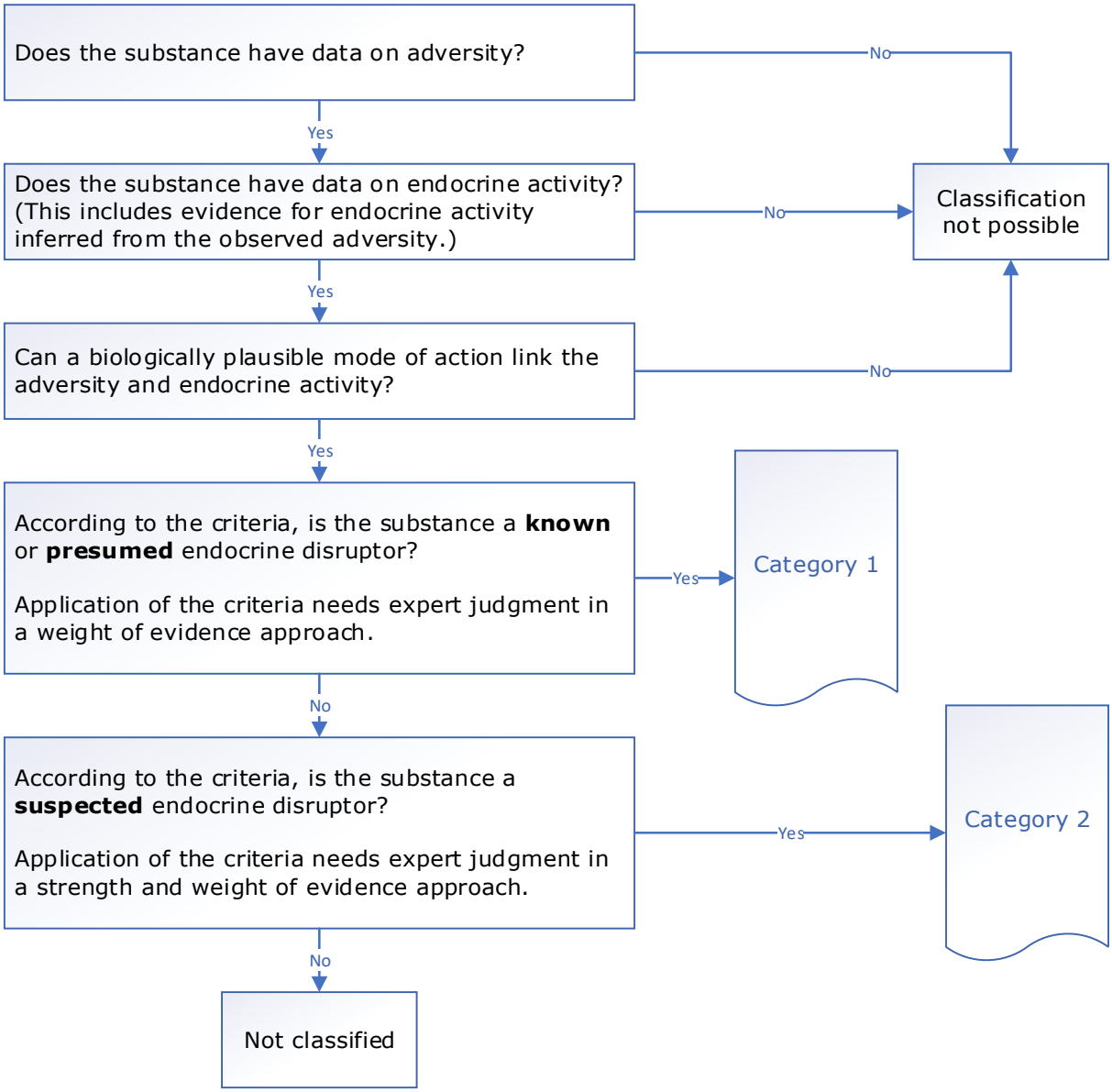
In case there is no evidence from mammals, or the substance is not classified for *ED HH* for the thyroid modality, classification as Category 1 is only warranted if there is at least one *in vivo* long-term test in a non-mammalian species showing evidence of adverse effects relevant at the population level, or non-animal data providing an equivalent predictive capacity to the *in vivo* data. In the case 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. Decision logic for classification of substances

The decision logic which follows in Figure 4.2-3, is provided here as additional guidance. 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'; i.e., not meeting the ED criteria, or 'classification not possible'; i.e. due to lack of or inconclusive data.



[A placeholder for a more detailed flow-chart where more detailed information on possible scenarios which are leading to different categories or no classification. Examples of scenarios where Cat 2 would be more appropriate despite criteria a, b and c are met: Increased uncertainty due to:

- inconsistent results withing study or among studies (e.g. positive and negative / pointing towards different directions)
- low quality of study/studies (e.g. low reliability of study/studies, issues with study design such a dose level setting)
- lack of enough data to increase certainty]

4.2.2.6. Classification of substances containing CMR or ED constituents

From a compositional and a regulatory point of view the situation for substances containing CMR or 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 CMR and ED endpoints that is foreseen by CLP for mixtures containing CMR or ED components, is considered applicable also to substances containing CMR or ED constituents, additives or impurities (see Sections **Error! Reference source not found.** and 4.2.2.1 to 4.2.2.2 of this guidance). As discussed in Section 4.2.3.2 below, mixtures containing components classified as endocrine disruptors 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 CMR or ED effects which have not been identified from the information on the individual substances, those data shall also be taken into account. For ED endpoint the lowest incidence possible to detect in the tests is by far unacceptable for the environment. Thus, the highest test dose/concentration shall be the limit concentration as described in the relevant OECD TG, see further details on dosing/concentrations in Section 4.2.2.2.2. "Relevant concentrations for classification". Dilution, as would be the case if mixtures or substances containing CMR or ED constituents were tested, would increase the risk that CMR or ED hazards would not be detected. Therefore, negative test data on mixtures containing constituents with these hazards shall not be accepted.

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

4.2.2.7. Setting of specific concentration limits

> ***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.*

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

The concept of applying the SCL is described in Chapter 1.5 of this guidance.

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

4.2.2.7.1. Procedure

In general, the SCLs for ED properties are set based on the potency of the adverse effect, however 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.

Endocrine disrupting effect level (e.g. EC10, NOEC, LOEC or DNEL from any relevant studies where adverse effect(s) are observed at 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 endocrine disruptor for the environment do not specify any dose above which the production of an adverse effect is considered to be outside the criteria which lead to classification.

When the ED classification for the environment is based on the mammalian data used for the ED classification for human health 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 above.

However, when the ED classification for the environment 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}/NOEC value 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}/NOEC ≤ 0.00001, 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 < EC_{10}/NOEC ≤ 0.001, the SCL should be 10-fold lower than a default GCL.

   iii. For substances with 0.001 < EC_{10}/NOEC ≤0.1, 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}/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 1. SCL derivation based on non-mammalian data

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

* *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., birds 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 exceptional cases a higher SCL than the GCL can also be set for EDs [does the PEG agree?]. 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 not evident at a level above the concentrations set for the relevant hazard class.

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

When there is sufficient and conclusive data available that the ED effect is a non-threshold effect or with a non-monotonic dose response curve, in this situation the SCL corresponding to extreme potency group may be set by default, unless even lower SCL is justified. Due to these typical characteristics for many EDs, the assessment of dose-response related information together with setting SCLs should be conducted with caution.

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, 4.2.3). Only in case there is data available for the mixture itself which demonstrate effects not retrieved from the ingredients, this data might 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, 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).

The additivity concept can be applied for endocrine disruptors (see also Section 1.6.3.4.3. of this guidance). Exposure to endocrine disruptors with both similar and dissimilar modes of action can lead to combination effects. 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 similar mechanism or mode of action or adverse outcome (e.g., exposure to a combination of anti-androgenic, estrogenic and steroidogenic disrupting substances can lead to additivity), unless there are specific reasons not to do so. Modality or the MIE does not need to be the same, similar to most of the HH hazard classes where the same adverse outcome between substances can already suggest additivity.

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 environment	*Category 2 endocrine disruptor for the environment*
Category 1 endocrine disruptor for the environment	*≥ 0,1 %*	
Category 2 endocrine disruptor for the		*≥ 1 %* *[Note 1]*

environment		
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.*		

4.2.3.1.1. When data are available for the individual ingredients

Annex I: ***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.*

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

4.2.3.1.2. When data are available for the complete mixture

Annex I*: 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.*

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

Annex I: ***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.*

Bridging Principles will only be used on a case-by-case basis (see Section 1.6.3 of this 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, 4.2.3.2.1. i.e. not for "no classification". Note that the following bridging principles are not applicable to this hazard class:

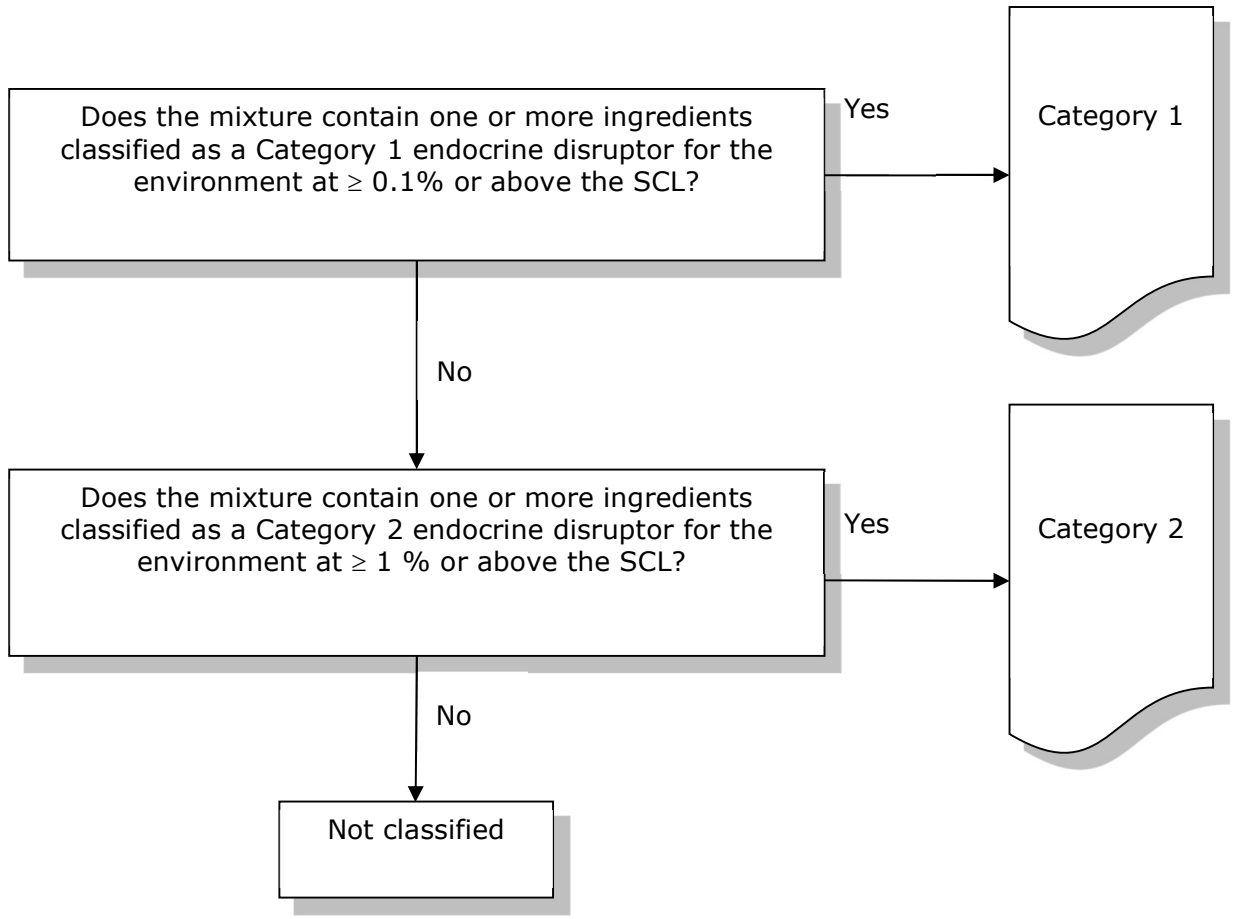
- concentration of highly hazardous mixtures
- interpolation within one hazard category

(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 is provided here as an additional guidance. The person responsible for classification should study the criteria before and during use of the decision logic presented below.

2729 Classification of mixtures for endocrine disruption for environment
2730 *Classification based on individual ingredients of the mixture*



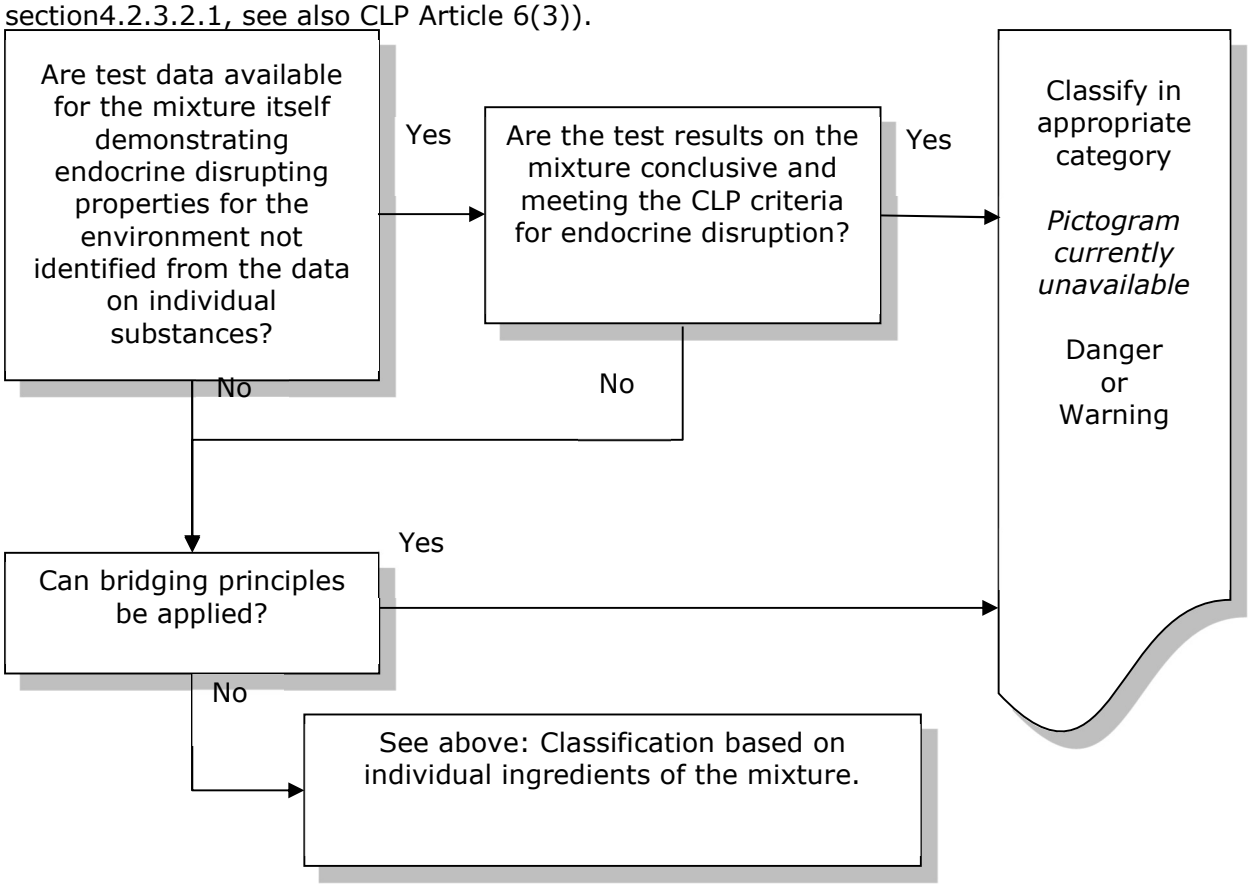
2731

2732

2733

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 more stringent effects than the one established based on the individual ingredients (CLP, Annex I, section4.2.3.2.1, see also CLP Article 6(3)).



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 endocrine disruptors 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

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 and are not real cases. The examples are illustrative of what type of data may lead to classification of different categories for endocrine disruption. The examples do not attempt to present the exhaustive information

package for a substance, but only the ED related information leading to classification, supporting classification or "no classification" is included, but not the whole data set or a detailed description of the effects, or a full weight of evidence analysis. All the endocrine related effects reported for the different examples leading to classification are considered adverse i.e. statistically significant from the control and biologically relevant. It also should be noted that the decision on classification is influenced by the strength of the overall evidence and should be decided on a case-by-case basis.

The template for conducting full assessment based on lines of evidence can be found on the ECHA website in the ECHA CLH template and ECHA/EFSA Guidance on endocrine disruptors (2018).

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 *Repro* 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 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 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* based on fish data. The example is focused on EAS modalities. Adverse effect(s) observed are associated to 'Sensitive to, but not diagnostic of, EATS' parameters (SCL calculation: GCL to be applied as no SCL derived).

Example 6: Classification as *ED ENV* based on fish data. The example is focused on EAS modalities (GCL to be applied).

Example 7: Classification as *ED ENV* 2 of a substance already classified as *ED HH* 1 for the thyroid modality (GCL to be applied).

Example 8: Classification as *ED ENV* 2 for non-EATS modalities.

Examples *ED ENV* No classification (see Section 4.2.5.3)

Example 9: 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 10: 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

Application of criteria for evaluation/classification and decision on classification:
ED ENV 1 – EAS modalities
Available information in mammals and conclusion for classification as *ED HH* 1.
See information in example 3 in section 3.11.5.1.3.
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 '*EA mediated*' parameter and provides clear evidence of an endocrine mode of action. 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.

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 and human contraception, these adverse effects are considered diagnostic of an EAS mode of action and thus (in the absence of other explanations) also imply underlying *in vivo* mechanistic information.

Conclusion:
The substance caused significant effects on fecundity and 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 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 3, section 3.11.5.1.3 of this guidance. According to mammalian data no SCL need to be set for this substance.

4.2.5.1.2. Example 2

Application of criteria for evaluation/classification and decision on classification: *ED ENV* 1 - EAS modalities

Available information:

The substance was concluded not to meet the criteria as *ED HH*.

In vivo information:

- Fish full lifecycle test conducted with *Sheepshead minnow* (FFLCT, OPPTS 850.1500, reliability 1, 100 days exposure, test concentrations: 0, 0.55, 0.29, 0.15, 0.068, 0.038 and 0.016 mg/L) with inclusion of all the parameters foreseen to be investigated in the OECD TG 240:
    - 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 biologically 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
    - Increase GSI in F1 males at 0.558 mg/L
    - Increased GSI in F1 females at 0.063, 0.188 and 0.558 mg/L
    - Increase tubercle score in F1 males at 0.022, 0.063, 0.188 and 0.558 mg/L
    - Decrease in F1 Female VTG plasma concentration at 0.558 mg/L (statistical decrease observed at 188 µg/L, but not considered to be biologically significant)
    - 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 558 µg/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 was not considered biologically significant)
    - Decrease 17β-estradiol in females at 0.5 and 1 mg/L
    - Decrease 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 decreases 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 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 *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.

In vitro information:

- 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 microsomes 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 endocrine related adverse effects was observed across studies and species: changes in gonad histopathology in both males and females accompanied by decrease in fecundity.

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 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 (500 and 1000 µg/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 decrease was observed 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	Decrease in fecundity	Decrease observed in 2 FFLCTTs

effect		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 ED criteria category 1 for the EAS-modalities for the environment.

SCL calculation:

The lowest effect value ($NOEC_{reproduction}$ = 0.068 mg/L), the substance is non rapidly degradable; according to Table 1, section 4.2.2.5.1 of this guidance, substances with 0.001<NOEC≤0.1 result in a medium potency group corresponding to a GCL (0.1%). Thus, no SCL will be set.

4.2.5.1.3. Example 3

Application of criteria for evaluation/classification and decision on classification: *ED ENV* 1 - EAS modalities

Available information :

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.
  - VTG induction observed only in females at 83, 255 µg/L.

- Modified juvenile growth test with *Sander lucioperca* (144 days, test concentrations 0, 10, 100, 200 µg/L, reliability 2):
  - 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
- 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 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 endocrine related adverse effects relevant at the population level was observed across studies and species: change in sex ratio, decreased fertility, decreased secondary sex characteristics in males accompanied by changes in male gonad histopathology.

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 *C. carpio*). In all species all endpoints assessed were positive. This substantiates that the substance alters the function of the endocrine system in fish via an estrogenic MoA. A change in the sex ratio toward females is both indicative for an endocrine mode of action and adverse. Such an effect was observed in 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 criteria for *ED ENV* 1.

SCL calculation:

The lowest effect value ($NOEC_{SSC}$=9.6 µg/L = 0.0096mg/L), the substance is non rapidly degradable; 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 (0.1%). Thus, no SCL will be set.

4.2.5.2. Examples *ED ENV* 2

4.2.5.2.1. Example 4

Application of criteria for evaluation/classification and decision on classification: *ED ENV* 2 – EAS modalities
Available information:

In vivo information:

- Fecundity test on zebrafish (similar to a partial life cycle test, reliability 2, exposure over 21 days, 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 without dose-response
  - 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 effects 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
  - Dose-dependent responses of testosterone (T), estradiol (E2) and progesterone (P) in males, but not statistically significant. In males, significant decrease in T and significant increase in P at 0.1 mg/L, increase in plasmatic E2 content significant from 0.01 mg/L. In females, decrease T concentration at 1 mg/L, and increase E2 concentration at 0.01, 0.1 and 1 mg/L.
  - 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 1-6 dpf and were associated with developmental and reproductive disturbances.

- No other *in vivo* data available on HH side.

In vitro information:

- 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 agonism 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 with a weak empirical support and not accompanied by change in female gonad histopathology. The change observed in male gonad histopathology was not considered 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.
The anti-androgenic activity is demonstrated in vertebrate cells including human cells but has not been confirmed *in vivo*.

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. 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.
The possible activity via androgen antagonism could also be linked to the observed adverse effects. These endocrine disruption pathways are highly inter-related and therefore the substance may act via different MoAs.

Conclusion:

There is evidence of endocrine activity *in vitro* pointing to an estrogenic MoA. There is some evidence on adverse effect(s), however a clear pattern of effects was not identified. Therefore, the substance meets the criteria for classification as Category 2.
SCL calculation:

The *ED ENV* classification is based on a study 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.

4.2.5.2.2. Example 5

Application of criteria for evaluation/classification and decision on classification: ***ED ENV* 2 – EAS modalities**
Available information:

In vivo information:

- 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 increase 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 not 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-dependant 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, resp.;
  - 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 (by about -20%), 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. elevated in both male and female fish compared to controls. 11-ketotestosterone and testosterone levels were statistically significantly decreased 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.
    - With respect to fecundity, an effect on cumulative egg numbers only at 0.2 mg/l; 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 4):
    - 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.
    - Statistically significant increase in VTG levels in both male fish (1 mg/L) and female fish (0.2 and 1 mg/L).
    - No effects on fecundity.

- 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 including the controls.

- OECD TG 229 test with adult Japanese Medaka *Oryzias latipes* using four concentrations (21-day, test concentrations: 2.13, 7.19, 17.1 and 44.9 µg/L, flow-through, reliability 2):
    - No significant mortality occurred.
    - No effects on male fish growth, but female fish growth affected at the highest measured test concentration (0.045 mg/L).
    - HSI affected in male fish at the three highest test concentrations (0.007, 0.017 and 0.045 mg/L, measured) but female fish unaffected.
    - GSI unchanged in both male and female fish.
    - VTG levels unchanged in male fish at all concentrations, but a statistically significant reduction occurred in female fish (at 0.007 mg/L and above).
    - Fecundity in female fish (number of eggs and number of fertile eggs) reduced at the highest test concentration (0.045 mg/L, measured).
    - No effects on secondary sexual characteristics in male fish at all concentrations.

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. In the available studies a decrease in fecundity, in some cases accompanied by an alteration of gametogenesis with a reduction of maturation stage, was observed. However this was not consistent across studies with the same species.

Endocrine activity:
Depending on the development stage, species and concentrations, effects were observed leading to modulations of circulating sex hormone concentrations. The available studies showed an increase in circulating estradiol concentrations in two species (Zebrafish & Medaka) with a decrease in 11-KT (except in the FSDT) and an increase in VTG.
However, there is a contradictory result on VTG level in female Zebrafish with both increase and decrease of VTG in reliable studies conducted with different species. Additionally no increase in VTG levels in males is observed.

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 different species. Therefore, the substance meets the criteria for classification as *ED ENV* 2.

SCL calculation:
The lowest effect value ($NOEC_{body\_weight}$= 7.76µg/L = 0.0077mg/L), the substance is non rapidly degradable. According to Table 1, Section 4.2.2.5.1 of this guidance, substances with $0.001<NOEC\leq0.1$ result in a medium potency group corresponding to a GCL 0.1%. Thus, no SCL will be set.

4.2.5.2.3. Example 6

Application of criteria for evaluation/classification and decision on classification:
***ED ENV* 2 – EAS modalities**
Available information:
In vivo information:
- Modified OECD 229 with Zebrafish (non GLP, 21 days exposure, hatching rate and survival success measured at 5 dpf, test concentrations: 0, 5, 50, 500 µg/L, reliability 2):

- Decreased egg production without dose response
- 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
- GSI in male significant 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
- Analytical measurements performed only at the beginning of the study.

- 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

<u>*In vitro information:*</u>

- 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[11] was observed. No clear endocrine related adverse effects were observed in the study with Medaka.

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 modulation 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 details due to the absence of information. However, since an alternative non-endocrine MoA is unlikely, an endocrine mode of action is the most plausible explanation for the effects observed.

Conclusion:

There is neither a convincing pattern of endocrine related adverse effects nor strong

[11] The decrease in post-ovulatory follicles is considered a consequence of the effects in fecundity rather than a clear endocrine mediated effect.

indication of endocrine activity.The limited information on adverse effect(s) and endocrine activity is consistent with a MoA based on modulation 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:

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 derived and the GCL will be applied.

4.2.5.2.4. Example 7

Application of criteria for evaluation/classification and decision on classification:
ED ENV 2 – T modality
Available information:

The substance is classified as *ED HH* 1 (see example 1 in section 3.11.5.1 of this guidance).

Adverse effect(s) in wild mammals:

The parent compound W showed changes in thyroid histopathology across species and studies. This adverse effect, as explained in section 4.2.2.4.1 of this guidance, is not considered relevant at the level of population.

For MetW, an endogenous metabolite of the substance formed in all vertebrate metabolism studies, effects on thyroid histopathology were observed. In the available DNT study, effects on brain morphometry were also observed which could be linked to TH deficiencies. However, this could not be confirmed/dismissed. Therefore, also for MetW the population relevance of the observed adverse effect(s) could not be confirmed.

In vivo information in non-mammalian species:

No study on amphibians with substance W are available. All available studies are with the metabolite MetW.

- Amphibian Metamorphosis Assay study with *Xenopus laevis* (AMA, OECD TG 231; 28-days, 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, 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

- Extended Amphibian Metamorphosis Assay study with Xenopus laevis (EAMA, 90-day, 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 only at the beginning of the study for one of the concentrations, only.

- Non guideline study study with Xenopus laevis (12-day exposure, test concentrations: 0, 50 mg/L, reliability 1):
  - 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 day exposure, reliability 3):
  - Dose-dependent decrease of T4 in follicles across concentrations
  - No analytical measurements, no information on the concentrations tested
  - No information on the method used for measuring T4

In silico information:

No available information.

In vitro information:

Not available.

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:

No evidence of endocrine activity was available with the parent compound W. The metabolite MetW was positive in the TPO ToxCast assay (TPO_AUR_dn).

Biological plausible link:

Based on the available data, one of the plausible MoAs is: MetW formation leading to TPO inhibition, changes in THs levels, changes in thyroid histopathology and delay in development and metamorphosis. 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, major uncertainties have been identified in the available data which do not allow to properly substantiate the postulated MoA.

Source of Uncertainty	Explanation
Metabolism study in amphibians	Amphibian metabolism studies are not available. Although all the available metabolism studies in vertebrates show a consistent qualitative metabolism, the level of MetW which may be formed in amphibians is uncertain.
Level of metabolite MetW formed in metabolism studies	All the available metabolism studies in vertebrates have shown that MetW is always formed (below 10%) but it is also rapidly metabolised.
Cross species extrapolation	The extrapolation between species and taxa is challenging when considering both metabolism and possible expected endocrine effects. Although, a high level of conservation of the endocrine system and similar metabolic pathways are expected across vertebrates, both are true from a qualitative point of view. However, uncertainty exists on whether, quantitatively, similar patterns can be expected both in terms of metabolism and effects.
Concentrations to be reached for eliciting adverse effects/ endocrine activity	In the available screening and long-term studies, MetW has shown adverse effects and/or endocrine activity in a consistent manner. However, from the available information, effects were observed at concentrations above 25 mg/L. This may raise uncertainty that endocrine adverse effect/endocrine activity through MetW formation would not be observed if the parent substance is tested up to the maximum test concentration (i.e. 100 mg/L).

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 metabolism studies in rat, poultry and goat. In all metabolism studies, a similar pattern was shown; the parent compound is extensively metabolised and converted to MetW, however, this was always formed below the critical threshold of 10%.
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.
For the SCL calculation based on mammalian data see example 1 in section 3.11.5.1 of this guidance, no SCL will be calculated.
In conclusion, no SCL will be set, and the GCL will be applied.

4.2.5.2.5. Example 8

Application of criteria for evaluation/classification and decision on classification:
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 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.

- 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)
  - Decrease in eggshell thickness at all tested doses with no 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[12] 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.

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[12]. 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.

[12] 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.

Endocrine activity:
No evidence of endocrine activity was available with the parent compound. However, one of the metabolites of the parent substance is found in rat urine is sulfonamide which is a known inhibitor of cyclooxygenase.

Biological plausible link:
It is known that effects on eggshell thickness may be due to a non-EATS MoA. The MoA below is postulated following the AOP 28 (cyclooxygenase inhibition leading to reproductive failure).

	Brief description of the Key event	**Brief description of the observed effects/positive findings**	**Supporting Evidence**
MIE	Inhibition of the cyclooxygenase activity	Not evaluated	Not evaluated
KE1	Reduction of the prostaglandin E2 concentration	Not evaluated	Not evaluated
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[13]	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 are only available in relation to later KEs and for the adverse outcome (decrease in eggshell thickness, increase in the number of cracked eggs and decrease in 14-d old survivors). However, although information on the endocrine activity is not available, the information about the metabolite sulfonamide and the availability of an AOP 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

[13] Effects mainly leading to impairment of population maintenance

conclusion no SCL need to be set for this substance.

4.2.5.3. Examples no classification

4.2.5.3.1. Example 9

Application of criteria for evaluation/classification and decision on classification: *ED ENV* no classification (EAS modalities)
Available information:

The substance was concluded not to meet the 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 and acute to chronic ratio.

- 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 adverse 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, sheepshead minnow, and fathead minnow (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 with rainbow trout, significant effects on parameters 'sensitive to, but not diagnostic of EATS' were observed at the same doses where there were effects on mortality.

In silico:

Negative ER model.

In vitro information:

ToxCast negative for aromatase inhibition, no indication for AR.

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:
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 ED criteria for the EAS-modalities for the environment.

4.2.5.3.2. Example 10

Application of criteria for evaluation/classification and decision on classification:
***ED ENV* no classification for EATS modalities**
Available information:
The substance was concluded not to meet the 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):
  - Concentration setting considered acceptable
  - No mortality observed at the highest dose
  - Significant increase of GSI and VTG starting at 180 µg/L
  - Effects on SSC in males (decreased tubercles and fatpad), egg production in females (no eggs produced) and gonad histopathology in both sexes at 1.2 mg/L

- 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 at 200 and 400 µg/L, including slightly increased oocyte atresia, decreased post-ovulatory follicles, increased 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:

- 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):

The effects on ovary histopathology observed in the FLCTT might indicate inhibited spawning. However, as there were no significant effects on fertility or fecundity noted in either concentration, and considering the last spawning of the fish at the two top concentrations influenced the ovary histopathology, it is likely that these findings had to do with the periodic nature of fathead minnow spawning and the timing of the end of the test.

Overall, there is no strong evidence of endocrine related adverse effect(s) in fish in the FLCTT at concentrations where potential endocrine activity was determined in the FSTRA.

Endocrine activity:

The effects on VTG observed in the FSTRA were not replicated in the FLCTT study, despite similar dosing and the same species, as an increase in VTG in females was observed at the 100 µg/L concentration only. It is noted that the developmental stage/exposure is different (as adult fish are exposed in the FSTRA, whereas the F1 generation of the FLCTT is exposed continually throughout growth and development). 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. Effects indicating endocrine activity are inconsistent between the developmental stages/tests, though the same species was tested.

Biological plausible link:

Not applicable.

Conclusion:

By considering all the available information, it can be concluded that the substance does not meet the 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

- No effects on the thyroid were observed in the available animal studies
- 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 amphibians, 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 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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