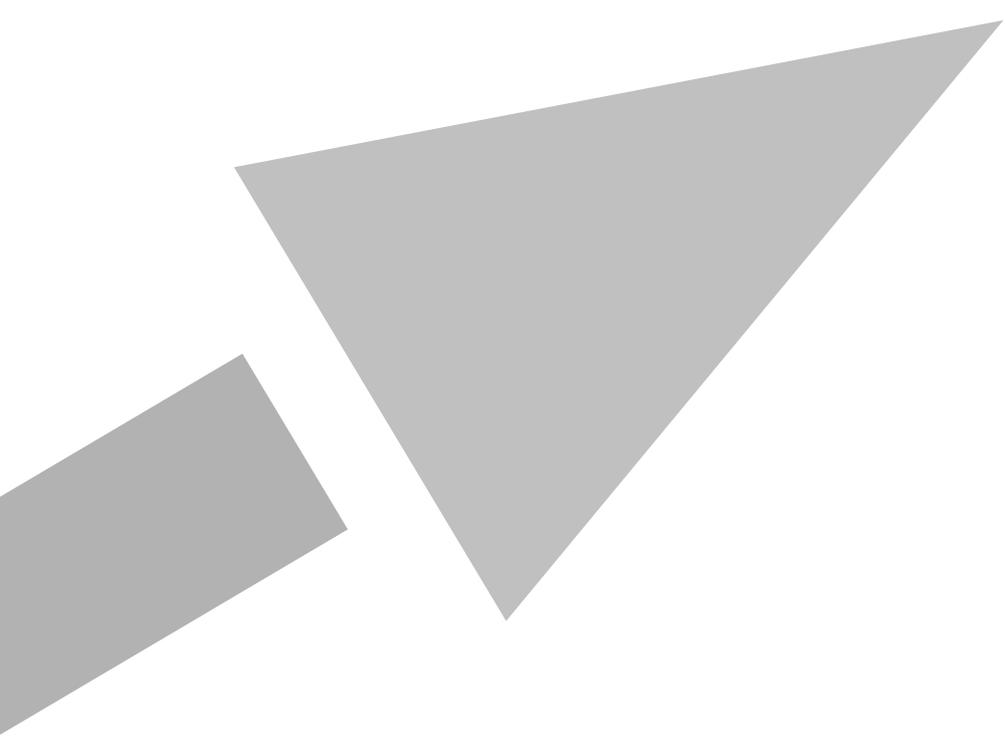




***Guidance on Identifying
Endocrine Disrupting Effects***

Technical Report No. 106



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Guidance on Identifying Endocrine Disrupting Effects

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SUMMARY

Recent revision of the European directive on plant protection products (Directive 91/414/EEC) and new regulations concerning chemicals (Regulation (EC) No. 1907/2006 'REACH') only support the marketing and use of chemical products on the basis that they do not induce endocrine disruption in humans and/or non-target species. However, there is currently no agreed guidance on how to identify and evaluate endocrine activity and disruption. Consequently, this ECETOC task force was formed to provide scientific based criteria that may be used within the context of the plant protection products directive and REACH.

This report reviews and summarises existing definitions for an endocrine disrupter as well as the test methods currently available to identify endocrine activity and/or toxicity. Specific scientific criteria for the determination of endocrine disrupting properties that integrate information from both regulatory (eco)toxicity studies and mechanistic/screening studies are proposed. These scientific criteria rely on the nature of the adverse effects detected in regulatory (eco)toxicity study(ies) that give concern for endocrine toxicity and the description/understanding of the mode of action of toxicity which scientifically support and explain the adverse effects. The criteria developed are presented in the form of flow charts for assessing relevant effects for both human and environmental species. These charts are illustrated using example substances. In addition since all chemicals having endocrine disrupting properties may not represent the same hazard, an element or assessment of potency is also proposed to discriminate chemicals of high concern from those of lower concern.

1. INTRODUCTION

1.1 Background

The chemical industries have been heavily involved in initiatives investigating endocrine disruption for several decades. The first European industry task force to address endocrine disruption was formed by ECETOC in 1995. The objectives of that task force, entitled ‘Environmental Oestrogens’, were to review the available methods for the detection of endocrine disruptors (EDs) and to serve in an advisory capacity to the European chemical industry on how best to detect and assess endocrine disruption. Since then a huge global effort has been invested in validating *in vitro* and *in vivo* targeted test methods under the framework of the Organisation for Economic Co-operation and Development (OECD) or the US Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC). Here also, the chemical industry has contributed heavily to this effort through funding research and validating test methods. Meanwhile research and development in the endocrine systems of humans and wildlife species has benefited from the general momentum created on this topic.

Recent political pressure culminated in a legislative document (EU, 2009) passed by the European parliament that calls for a non-authorisation of plant protection products that have endocrine disrupting properties for human and non-target organisms. However, the fundamental scientific criteria that are necessary to define ‘endocrine disrupting properties’ were not elucidated in the revised directive, despite advances in test method development and in basic endocrine research. Equally, the basis for why an endocrine system toxicant should be considered as more hazardous than other classes of toxicants (e.g. central nervous system, liver or kidney toxicants) has not been provided.

In addition, under REACH (EC, 2006), there are currently no testing strategies and guidance to identify endocrine disrupting effects. Therefore, there is a potential for inappropriate interpretation of ‘endocrine effects’, which may lead to regulatory issues. Such substances might also fall into the category of Substance of Very High Concern (SVHC) and on the ‘Candidate List’ for which restriction, substitution and risk management measures will be applied.

In response to these politically driven regulatory developments, ECETOC has formed a task force to provide guidance on how to identify chemicals with endocrine disrupting properties. The approach is based on the scientific knowledge that has accumulated during the past decades of intensive work (research and test method validation).

It is recognised that endocrine disruption is not a hazard per se, but a mode of action (MoA) of toxicity that could potentially result in a hazard. Since all EDs do not represent the same hazard, an element or assessment of potency is also required to discriminate EDs of high concern from

those of lower concern. This report summarises the main concepts that have emerged from the present task force entitled “Guidance on identifying endocrine disrupting effects”.

This document, similar to the approach of the US EPA’s Endocrine Disrupter Screening Program (EDSP), has limited itself to the assessment of effects on the Hypothalamic-Pituitary-Gonadal (HPG) and Hypothalamic-Pituitary-Thyroid (HPT) axes.

1.2 Terms of Reference

The task force used the following terms of reference in compiling this report:

1. Critically review all available definitions of endocrine disruption, which apply to both human health and other organisms in the environment.
2. Identify key and common themes from all definitions, as well as the relevance of these to chemical classification and risk assessment.
3. Provide guidance on the nature and quality of data required to conclude the induction of endocrine disruption and causation of any adverse effects. This should include the ability to evaluate the potency of any endocrine disruption observed.

2. DEFINING ENDOCRINE ACTIVITY AND ENDOCRINE DISRUPTION

Endocrine activity in the context of this report is defined as the modulation of endocrine processes that may or may not give rise to endocrine toxicity i.e. induction of adverse endocrine effects (see Chapter 3). This chapter provides a summary of the definition of endocrine active substances versus endocrine disrupting substances; and also examples of naturally occurring endocrine processes and substances that perturb them and finally xenobiotic factors that may affect endocrine systems.

2.1 Existing definitions

Endocrine active substances are not the same as endocrine disrupting chemicals. It was not within the scope of this task force to completely re-define the term ‘endocrine disrupter’. Although there are no universally accepted definitions of the term, there are some broadly accepted definitions, which are summarised below:

- Weybridge (1996): *“An endocrine disrupter is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary (consequent) to changes in endocrine function. A potential endocrine disrupter is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism”*.
- European Commission: *“Endocrine disrupters are exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations”*¹.
- International Programme for Chemical Safety (IPCS): *“Endocrine disrupters have been defined as exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations”*.
- US EPA programme on endocrine disrupters: *“An exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body which are responsible for the maintenance or homeostasis, reproduction, development and or behavior”* (Kavlock *et al.*, 1996).
- Japan (Ministry of Environment): *“Injury and/or hazardous effects on organisms caused by exogenous substances through influence on the endocrine system”*.

The definitions above share common elements (i.e. exogenous substances; adverse effects resulting from interference with the endocrine system; and intact organisms). After review, the Weybridge definition was considered to be the most appropriate one. This definition considers the biological plausibility between the whole organism and the MoA which results in the adverse

¹ http://ec.europa.eu/environment/endocrine/definitions/endodis_en.htm

effect. Within the context of ecological risk assessment and in terms of this report the Weybridge definition can be expanded to include adverse population relevant effects that are mediated through the endocrine system of individual organisms. This reflects the differences in protection goals between human health assessments (individual people) and environmental assessments (populations of species).

2.2 *Natural endocrine modulators*

Factors that can modulate endocrine systems may be man-made; however many factors are naturally occurring. In particular a number of components that are present naturally in the diet as well as environmental conditions have endocrine disrupting properties. Three examples are identified below. These are considered to be exogenous, i.e. not produced by the organism itself.

2.2.1 **Phyto-oestrogens**

Some naturally occurring compounds in plants, termed phyto-oestrogens are known to have oestrogenic properties. A phyto-oestrogen is biologically defined as any natural plant compound that is structurally and/or functionally similar to the ovarian and placental oestrogens or their active metabolites. The majority of phyto-oestrogens belong to a large group of substituted phenolic compounds termed flavonoids. Three classes of flavonoids, the coumestans, the prenylated flavonoids and the isoflavones are phyto-oestrogens that possess the most potent oestrogenic activity (COT, 2003).

By definition, phyto-oestrogens are biologically active, and *in vitro* and *in vivo* animal studies have identified the following potential mechanisms through which these effects are mediated:

- Interaction with oestrogen receptors (ERs) to modulate the expression of oestrogen-responsive genes.
- Inhibition of enzymes involved in oestrogen biosynthesis and metabolism.
- Modulation of thyroid hormone biosynthesis.
- Inhibition of protein kinases and interaction with components of the cell cycle as well as proliferation, differentiation and apoptosis pathways.
- Inhibition of topoisomerase.
- Antioxidant reactions.

A classic example of a phyto-oestrogen is genistein and there are many studies describing the biological effects of genistein in mammalian systems, including effects on reproduction and development. The most recent and comprehensive review of these data was completed by NTP-CERHR in 2006 (Rozman *et al*, 2006). Environmental exposure to genistein (and other phyto-

oestrogens) has been reported for the aquatic, but less so for the terrestrial environment (Spengler *et al*, 2001; Kiparissis *et al*, 2001, Kawanashi *et al*, 2004). Most published studies concern possible effects of genistein background levels or effluents containing genistein, specifically pulp and paper mill effluents on fish populations and whether dietary genistein intake can affect farmed fish or fish used experimentally with (weak) oestrogenic compounds (Bennetau-Pelissero *et al*, 2001 ; Denny *et al*, 2005; Kiparissis *et al*, 2003; Panter *et al*, 2002 ; Pelissero and Sumpter, 1992; Scholz *et al*, 2005). This will be further examined in a case study in Chapter 5.

2.2.2 Mycotoxin

Compounds that occur in the diet as fungal contaminants such as the mycotoxin zearalenone are also known to have endocrine disrupting properties. This mycotoxin occurs in food as a result of fungal infection of cereals particularly maize but also oats, wheat, rice, soy-beans. A large number of studies, both *in vitro* and *in vivo*, have shown that zearalenone binds to the ER, inducing oestrogenic effects and interfering with the reproductive process. For example, the adverse effects associated with the ingestion of mouldy feed by pigs include foetal death, infertility, reduced litter size and abortion (Nelson *et al*, 1966; Radnai, 1974). This will be further examined in a case study in Chapter 5.

2.2.3 Physiological stress

Modulation of endocrine systems can also be induced by other natural stressors. Physiological stresses such as parasitism (Allner *et al*, 2009; Jobling and Tyler, 2003; Schabuss *et al*, 2005), temperature (Kime, 1999), hypoxia (Thomas *et al*, 2007; Wu *et al*, 2003), calorific intake (Odum *et al*, 2004) and food restriction (Rehm *et al*, 2008) are known to induce adverse endocrine effects.

Sexual differentiation in many species may be influenced by environmental conditions. Phenotypic sex is determined by external factors and no consistent genetic differences are found between sexes. For example, sex determination in many fish species can be influenced by environmental variables such as temperature, salinity, and/or pH (Baroiller *et al*, 1999).

Temperature-dependent sex determination (TSD) is the prevalent form of environmental sex determination in vertebrates and has been found in reptiles and several fishes. For that reason TSD is one of the most studied sex determination mechanisms in many animals. TSD is very common in reptiles, where the ambient temperature during sensitive periods of early development irreversibly determines whether an individual will be male or female and can produce extreme changes from 100 % females to 100% males and *vice versa* in the offspring (Crews *et al*, 1994).

Some of the effects that may be attributable to exogenous substances operating as EDs might actually occur naturally in response to physiological stress. Therefore, one must always consider that the exogenous agents are operating in a background of constantly changing endocrine processes that are responding to many factors. This is an important consideration when evaluating potential endocrine effects.

2.3 *Xenobiotics*

Xenobiotics are man-made substances. Many such substances are effectively ‘designed’ to be active on the endocrine system for specific applications. Examples of such substances, which have endocrine activity and indeed operate as EDs, have been well documented and concern mainly pharmaceuticals designed to modulate the human endocrine system (e.g. birth control, hormone replacement therapy and prostate cancer treatments).

However, inadvertent exposure to such substances has been shown to induce ‘endocrine disruption’ in non-target species, such as the well documented feminisation of male fish downstream (Purdom *et al*, 1994) from sewage effluent discharges containing breakdown products of the female birth control pill.

Furthermore, a number of other classes of substances not specifically designed to interfere with endocrine system have been demonstrated to have endocrine activity. It is for the assessment of ED properties within these types of substances (typically general chemicals and plant protection products), that this document aims to provide guidance within the confines of the appropriate regulatory frameworks (i.e. REACH and 91/414).

The report will focus upon the inadvertent hazard that endocrine modulators (xenobiotics and naturally occurring chemical substances) may pose to endocrine systems of non-target species.

2.4 *Conclusion*

This chapter has highlighted the common themes for endocrine disruption from several definitions. This has been placed in the context of examples of naturally occurring endocrine processes and substances, and xenobiotics that are also known to impact on endocrine systems.

3. STRATEGIES FOR IDENTIFYING ENDOCRINE EFFECTS

3.1 Introduction

Identifying a compound which has endocrine disrupting properties requires the analysis of regulatory (eco)toxicity data coupled with an understanding of the mode of action underlying the toxicity findings. The analysis also includes identifying the qualitative and/or quantitative differences between species and life stages. Research studies may also be useful but it is important to consider the relevance, reliability and quality of such studies (see Section 3.2.3). Since endocrine disrupting compounds are no different from compounds exerting other modes of toxic action, species differences can be expected as illustrated for thyroid toxicity, which is mediated through liver enzyme induction and is specific to rodents and, therefore non-relevant to humans. Thus, a thorough investigation of the mode of action for toxicity can help in the assessment of qualitative or quantitative differences between species. The task force also acknowledges that a number of endocrine mediated effects are dependent on exposure during critical or sensitive life stages; however the current life-cycle tests (e.g. mammalian two-generation reproduction study, fish full life-cycle) cover, by default all life stages and can therefore be considered maximally sensitive to any mode of action.

3.2 Testing approaches

The current primary toxicology test methods for detecting endocrine mediated toxicity in mammals remain the standard regulatory tests based on the rodent two-generation reproduction study (OECD, 2001), the rodent life-time studies (OECD, 1981a,b,c) and the recently amended short-term toxicity study (OECD, 2006a). These studies are able to interrogate the form and function of multiple biological processes including those endpoints that are vulnerable to endocrine modulation.

In terms of ecotoxicological test methods, the current higher tier tests (e.g. bird reproduction and fish full life-cycle) are useful in addressing the potential population relevant impacts of endocrine disrupters (e.g. reduced fecundity or skewed sex ratio). In contrast to the mammalian studies these do not offer the power to investigate the form and function of the impact (provide less mechanistic insight). However, such methods are currently being validated or are in the process of being developed.

Test methods for invertebrates are not discussed here. Our understanding of invertebrate endocrinology is limited (DeFur *et al*, 1999) therefore there is a reliance on life-cycle methodologies. These measure apical endpoints that are population relevant, therefore, any endocrine specific toxicity should be accounted for in the life-cycle response (e.g. a risk assessment based on these data would be protective of any adverse effect resulting from an endocrine MoA). This is acceptable since generation life-cycle tests with aquatic invertebrates are technically feasible

(generally short generation times) and ethically acceptable. However, it is important to note that any effects observed cannot be considered diagnostic for endocrine disruption since they typically measure growth and reproductive effects only. Since the amount of energy that an individual can invest in maintenance, growth and reproduction is limited (Sibly and Calow, 1986) reductions in energy acquisition and/or increasing energy demand, in response to a toxicant, will result in decreased growth and reproduction (Barata *et al*, 2004). These effects should not be mistaken for an endocrine disrupting MoA (Barata *et al*, 2004). Methods for invertebrate life-cycle assays have been validated and form the basis of current chronic testing strategies for example the *Daphnia* reproduction test (OECD, 2008) and mysid chronic toxicity test (OPPTS 850.1350, 1996). Further invertebrate life-cycle methodologies are currently in development (Gourmelon and Ahtiainen, 2007) including the mysid two-generation toxicity test (US EPA), copepod life-cycle toxicity tests (OECD) and chironomid life-cycle test (Taenzler *et al*, 2007).

The US EPA approach is summarised in Table 1.

Table 1: Summary of the US EPA Endocrine Disrupter Screening Program test methods

TIER 1	TIER 2
<i>In vitro</i>	
ER binding	
ER α transcriptional activation	
AR binding	
Steroidogenesis, H295R	
Aromatase, recombinant	
<i>In vivo</i> (toxicology)	<i>In vivo</i> (toxicology)
Uterotrophic assay	2-generation rat reproduction study
Hershberger assay	(ACSA extended 1-generation study ¹)
Pubertal male assay	
Pubertal female assay	
<i>In vivo</i> (ecotoxicology)	<i>In vivo</i> (ecotoxicology)
Fish short-term screening assay	Fish life-cycle study
Amphibian metamorphosis assay	Amphibian life-cycle study (partial)
	Avian life-cycle (2-generation)
	Invertebrate (mysid)

¹ When validated this will be an acceptable alternative to the rat 2-generation reproduction study.

The OECD proposed approach is more complex in terms of the number of levels employed. However, in the OECD scheme it is possible to enter and exit at any test level depending on the outcome of evaluations. The OECD conceptual framework can be summarised as follows:

Table 2: The OECD conceptual framework was for the testing and assessment of potential endocrine disrupters²

Note: document prepared by the Secretariat of the Test Guidelines Programme based on the agreement reached at the 6th Meeting of the EDTA Task Force.

OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals

<p align="center">Level 1</p> <p>Sorting and prioritization based upon existing information</p>	<ul style="list-style-type: none"> - Physical and chemical properties, e.g. MW, reactivity, volatility, biodegradability - Human and environmental exposure, e.g. production volume, release, use patterns - Hazard, e.g. available toxicological data 	
<p align="center">Level 2</p> <p><i>In vitro</i> assays providing mechanistic data</p>	<ul style="list-style-type: none"> - ER, AR, TR, receptor binding affinity - Transcriptional activation - Aromatase and steroidogenesis <i>in vitro</i> - Aryl hydrocarbon receptor recognition/binding - QSARs 	<ul style="list-style-type: none"> - High throughput prescreens - Thyroid function - Fish hepatocyte VTG assay - Others (as appropriate)
<p align="center">Level 3</p> <p><i>In vivo</i> assays providing data about single endocrine mechanisms and effects</p>	<ul style="list-style-type: none"> - Uterotrophic assay (oestrogenic related) - Hershberger assay (androgenic related) - Non-receptor mediated hormone function - Others (e.g. thyroid) 	<ul style="list-style-type: none"> - Fish VTG (vitellogenin) assay (oestrogenic related)
<p align="center">Level 4</p> <p><i>In vivo</i> assays providing data about multiple endocrine mechanisms and effects</p>	<ul style="list-style-type: none"> - Enhanced OECD 407 (endpoints based on endocrine mechanisms) - Male and female pubertal assays - Adult intact male assay 	<ul style="list-style-type: none"> - Fish gonadal histopathology assay - Frog metamorphosis assay
<p align="center">Level 5</p> <p><i>In vivo</i> assays providing data on effects from endocrine and other mechanisms</p>	<ul style="list-style-type: none"> - 1-generation assay (TG415 enhanced)¹ - 2-generation assay (TG416 enhanced)¹ - Reproductive screening test (TG421 enhanced)¹ - Combined 28 day/reproduction screening test (TG422 enhanced)¹ <p>¹ Potential enhancements will be considered by VMG mamm</p>	<ul style="list-style-type: none"> - Partial and full life cycle assays in fish, birds, amphibians and invertebrates (developmental and reproduction)

Notes to the Framework

Note 1: Entering at all levels and exiting at all levels is possible and depends upon the nature of existing information needs for hazard and risk assessment purposes.

Note 2: In level 5, ecotoxicology should include endpoints that indicate mechanisms of adverse effects, and potential population damage.

Note 3: When a multimodal model covers several of the single endpoint assays, that model would replace the use of those single endpoint assays.

Note 4: The assessment of each chemical should be based on a case by case basis, taking into account all available information, bearing in mind the function of the framework levels.

Note 5: The framework should not be considered as all inclusive at the present time. At levels 3, 4 and 5 it includes assays that are either available or for which validation is under way. With respect to the latter, these are provisionally included. Once developed and validated, they will be formally added to the framework.

Note 6: Level 5 should not be considered as including definitive tests only. Tests included at that level are considered to contribute to general hazard and risk assessment.

² http://www.oecd.org/document/58/0,3343,en_2649_34377_2348794_1_1_1_1,00.html

3.2.1 Purpose, design and endpoints covered by the tests

The individual test methods comprising the two schemes are largely common. However, the OECD scheme has a number of additional test methodologies currently not represented in the US EPA approach (e.g. the fish sexual development test). The testing methods cover a wide range of endpoints that are potential targets for disruption of the normal function of the endocrine system. The following tables provide a summary of the purpose, brief study design and the endpoints addressed by each test method. Table 3 covers the *in vitro* approaches, Table 4 *in vivo* toxicology study types and Table 5 *in vivo* ecotoxicology study types.

Table 3: Summary of targeted *in vitro* assays

Purpose	Design	Summary of endpoints	Proposed use US EPA	Proposed use OECD
<i>Oestrogen receptor binding assay</i>				
To identify chemicals that bind to ER	ER from rat uterus or recombinant protein is incubated with oestradiol and chemical.	Binding curves and IC ₅₀ (molar concentration of chemical which inhibits 50% of binding by oestradiol)	Tier 1	Level 2
<i>ER α transcriptional activation assay</i>				
To identify chemicals that bind to ER and alter gene transcription	HeLa cells (stably transfected with hERα expression construct) are incubated with chemical	Measurement of bioluminescence reflecting changes in gene transcription	Tier 1	Level 2
<i>Androgen receptor binding assay</i>				
To identify chemicals that bind to AR	AR from rat prostate is incubated with R1881 (a strong ligand) and chemical	Binding curves and IC ₅₀ (molar concentration of chemical which inhibits 50% of binding by R1881)	Tier 1	Level 2
<i>Steroidogenesis H295R assay</i>				
To detect chemicals that affect the synthesis of sex steroid hormones	H295R cells contain steroid hormone synthesis pathways. Cells are incubated with chemicals	Oestradiol and testosterone production from H295R cells	Tier 1	Level 2
<i>Aromatase recombinant assay</i>				
To identify chemicals that inhibit aromatase activity	Human recombinant aromatase is incubated with androstenedione and chemical	Formation of ³ H ₂ O, one of the co-reaction products along with oestrone	Tier 1	Level 2

Table 4: Summary of targeted in vivo assays

Purpose	Design	Summary of endpoints	Proposed use US EPA	Proposed use OECD
<i>Uterotrophic assay</i>				
To detect oestrogenic chemicals	Immature or ovariectomised adult female rats are administered chemical for 3 days	Uterine weight is measured and compared with controls	Tier 1	Level 3
<i>Hershberger assay</i>				
To detect chemicals that are androgenic, anti-androgenic or inhibit 5 α -reductase	Immature or castrated rats are administered chemical for 10 days. In the anti-androgen assay testosterone is co-administered	Weights of androgen dependent tissues: ventral prostate, seminal vesicles, levator ani-bulbocavernosus muscle, Cowper's glands and the glans penis	Tier 1	Level 3
<i>Pubertal male assay</i>				
To detect chemicals with anti-thyroid, androgenic, or anti-androgenic activity or alter pubertal development via changes in gonadotropins, prolactin, or hypothalamic function	Male rats are administered chemical from post-natal day (PND) 23 to PND 53 (31 days)	Growth (body weight). Age and weight at preputial separation. Organ weights and histology including sex accessory tissues and thyroid. Serum hormones	Tier 1	Level 4
<i>Pubertal female assay</i>				
To detect chemicals with anti-thyroid, oestrogenic/anti-oestrogenic activity, or alter pubertal development via changes in gonadotropins, prolactin, or hypothalamic function	Female rats are administered chemical from PND 22 to PND 42 (21 days)	Growth (body weight). Age and weight at vaginal opening. Organ weights and histology including uterus, ovaries, thyroid. Serum hormones. Age at first oestrus and oestrus cyclicity	Tier 1	Level 4

Table 4: Summary of targeted in vivo assays (cont'd)

Purpose	Design	Summary of endpoints	Proposed use US EPA	Proposed use OECD
<i>Mammalian 1-generation (OECD TG 415)</i>				
General information on integrity and performance of the male and female reproductive systems and on the growth and development of the offspring	Continuous administration of compound (orally) prior to and during mating, gestation, lactation to termination after weaning of the F ₁ generation	Reproduction, parturition, AGD, lactation, postnatal development. Sperm parameters, oestrus cycles parameters, organ weights, and histopathology		Level 5
<i>Extended single generation (ACSA)</i>				
General information on integrity and performance of the male and female reproductive systems and on the growth and development of the offspring	Continuous administration of compound (orally) prior to and during mating, gestation, lactation to termination. Option to extend study to a second generation	Reproduction, parturition, AGD, lactation, postnatal development. Puberty onset, sexual development, sperm parameters, oestrus cycles parameters, organ weights, histopathology		Level 5

Table 5: Summary of targeted in vivo ecotoxicology assays

Purpose	Design	Summary of endpoints	Proposed use US EPA	Proposed use OECD
<i>OECD fish screening assay / Fish short-term reproduction assay</i>				
<i>In vivo</i> confirmation of endocrine activity on the HPG axis	Groups of reproductively active fish are exposed under flow through conditions for 21 days. A pre-exposure period may be required	In the OECD assay vitellogenin and secondary sexual characteristics are core endpoints. It is currently under debate whether gonad histopathology and fecundity (qualitative / quantitative) should be included. The fish short-term reproduction test includes vitellogenin, fecundity, secondary sexual characteristics, gonad histopathology and other biochemical measures	Tier 1	Level 3

Table 5: Summary of targeted in vivo ecotoxicology assays (cont'd)

Purpose	Design	Summary of endpoints	Proposed use US EPA	Proposed use OECD
<i>Amphibian metamorphosis assay</i>				
<i>In vivo</i> confirmation of effects on the HPT axis	Exposure of <i>Xenopus laevis</i> (NF 51) over the period of metamorphosis	Whole body length, snout-vent length, hind limb length, development stage and thyroid histopathology	Tier 1	Level 3
<i>OECD fish sexual development test</i>				
Information on sexual development, growth and mortality	Groups of newly fertilised embryos are exposed under flow through conditions until sexual maturity	Hatch success, development, sex ratio, vitellogenin and gonadal histopathology (optional)		Level 4
<i>Fish life-cycle tests</i>				
Effects on at least one generation (including reproduction) and development of the second generation	Several designs are under consideration: 1-generation newly fertilised embryos (F ₀) grown on to reproduce and development of the F ₁ . 2-generation adult fish reproducing (F ₀), their progeny continued for assessment of reproduction. Multi-generation freshly fertilised embryos (F ₀) till reproduction of the F ₁ and hatch success of the F ₂	Hatch success, development, growth and reproduction. Additional endpoints include sex ratio, vitellogenin and gonadal histopathology	Tier 2	Level 5
<i>Amphibian life-cycle tests</i>				
Life cycle exposure	Currently unclear	Currently unclear	Tier 2	/
<i>Avian life-cycle tests</i>				
Avian 2-generation study under development by US	Currently unclear	Currently unclear	Tier 2	/
Avian reproduction test OECD 206		Growth, food consumption, gross pathological examination, egg production, eggs set, viability and egg shell thickness		Level 5

Table 6: Summary of supporting and apical in vivo toxicology assays

Purpose	Design	Summary of endpoints	Proposed use US EPA	Proposed use OECD
Updated OECD 407				
To provide data on additional endpoints of sex organ and accessory tissue weights and histology	28-day study in the rat or mouse. Oral administration	Growth. General systemic toxicity. Sex organ and accessory tissue weights and histology. Thyroid hormone measurement an option		Level 4
Chronic / oncongenicity studies (OECD TG 451, 452, 453)				
To provide data on endpoints of sex organ and accessory tissue weights and histology after long-term treatment	Continuous administration of compound (orally) for the majority of the lifespan of the organism	Growth. General systemic toxicity. Sex organ and accessory tissue weights and histology. Tumour formation		
Mammalian 2-generation (OECD TG 416, 2001)				
General information on integrity and performance of the male and female reproductive systems and on the growth and development of the offspring	Continuous administration of compound (orally) prior to and during mating, gestation, lactation to termination after weaning of the F ₂ generation	Reproduction, parturition, AGD, lactation, postnatal development. Puberty onset, sexual development, sperm parameters, oestrus cycles parameters, organ weights, histopathology	Tier 2	Level 5

3.2.2 Modes of Action / Endpoints covered in the testing approaches

The various testing methods cover a range of endpoints from the single and simple target which are usually assessed by means of *in vitro* tests to the full complexity of the integrated organism as reflected by reproduction studies in rodents or fish *in vivo*.

Tables 7 and 8 give a summary of the MoA that the various *in vitro* and *in vivo* tests are able to evaluate.

Table 7: Summary of the modes of action covered by the targeted in vitro test methods

Assays	Validation status	Modes of action covered by assay							
		E	Anti-E	A	Anti-A	Steroidogenesis		HPG	HPT
						T	E		
<i>In vitro</i>									
ER Binding	Complete	x	x						
ER α transcriptional activation	Complete	x							
AR binding	Complete			x	x				
Steroidogenesis H295R	Ongoing					x	x		
Aromatase recombinant	Ongoing						x		

Table 8: Summary of the modes of action covered by the in vivo test methods

Assays	Validation status	Modes of action covered by assay							
		E	Anti-E	A	Anti-A	Steroidogenesis		HPG	HPT
						T	E		
<i>Toxicology</i>									
Uterotrophic	Complete	x	x						
Hershberger	Complete			x	x				
Pubertal Male	Complete			x	x	x		x	x
Pubertal Female	Complete	x	x				x	x	x
OECD 407 - 28 Days	Regulatory	x	x	x	x	x	x	x	x
OECD 451, 452, 453	Regulatory	x	x	x	x	x	x	x	x
OECD 415 - Rodent single generation study	Regulatory	x	x	x	x	x	x	x	x
OECD 416 - Rodent 2-generation study	Regulatory	x	x	x	x	x	x	x	x
Extended single generation	In development	x	x	x	x	x	x	x	x

Table 8: Summary of the modes of action covered by the *in vivo* test methods (cont'd)

Assays	Validation status	Modes of action covered by assay							
		E	Anti-E	A	Anti-A	Steroidogenesis		HPG	HPT
						T	E		
Ecotoxicology									
OECD fish screening assay / Fish short-term reproduction assay	OECD, expected April 2009; US EPA complete	x	x	x	^a	x	x		
Amphibian metamorphosis assay	In development								x
Fish sexual development test	In development	x	x	x	x	x	x	x	
Fish life-cycle study ^b	In development	x	x	x	x	x	x	x	x
Amphibian life-cycle study	In development	x	x	x	x	x	x	x	x
Avian life-cycle tests	In development	x	x	x	x	x	x	x	x

^a Further clarification required on the power of the fish sexual development test to detect this MoA since validation studies were not consistent for the pharmaceutical flutamide. However, a similar assay in the three-spined stickleback can address anti-androgenic activity by measuring the male specific protein spiggin (under androgenic control). This assay is not currently being supported by the OECD.

^b Endocrine specific methodologies are in development. Existing regulatory guidelines exist (US EPA. Fish life-cycle toxicity tests 540/9-86-137) but have not been specifically validated.

E = Oestrogen; A = Androgen; T = Testosterone; HPG = Hypothalamus-pituitary-gonad system; HPT = Hypothalamus-pituitary-thyroid system.

3.2.3 Interpretation and criteria

It is clear that with many separate assays available a holistic evaluation of all data will be needed to assess whether a substance should be regarded as an ED according to the Weybridge definition (Weybridge, 1996). This is commonly referred to as a 'Weight of Evidence Evaluation'. This evaluation often does not only have to contend with varying levels of significance and relevance as found in the studies described above (e.g. the different qualities of *in vitro*, *in vivo* screening and *in vivo* apical data), but, the evaluation also has to incorporate information from studies found in the peer reviewed literature, which will be of varying significance, relevance and quality. It is therefore important that an objective weight of evidence evaluation is conducted.

Several weight of evidence frameworks have been published (Brown *et al*, 2001; LRI-EMSG, 2000) incorporating 'quality' descriptors such as the Klimisch codes (Klimisch *et al*, 1997) and provide an excellent basis for weight of evidence evaluations. In brief the following considerations are important.

Significance and relevance:

- Lowest level of concern – *in vitro* only with no *in vivo* correlating activity;
- low level of concern – an effect on endpoints in *in vivo* studies, but at doses or concentrations that also cause general systemic (and target organ) toxicity;
- low level of concern – *in vitro* and short-term *in vivo* activity but with no activity in apical studies (e.g. reproductive function and reproductive development tests);
- highest level of concern – adverse effects on endocrine endpoints seen in apical studies which are not secondary to general toxicity and which are assumed relevant to humans or directly population relevant in wildlife species.

Quality (including repeatability and reliability):

- Weight given to studies following internationally validated test guidelines, preferably conducted according to the principles of Good Laboratory Practice (GLP);
- well documented studies conducted to scientific rigour;
- poorly documented studies for which the reliability and repeatability cannot be assessed should be given the least priority.

3.3 Conclusion

The use of a weight of evidence assessment technique is considered in this report to be the best scientific approach to evaluate the range of toxicity information available. This is also regarded as an appropriate method for determining endocrine mediated toxicity, as well as other mechanisms of toxicity, as it considers a wide spectrum of data (evidence) that are evaluated for their relevance (weighting). Schematics have been developed, presented as flow charts, to illustrate an approach for the scientific criteria for use in evaluating and identifying endocrine active substances using data from key mammalian and environmentally relevant species. Guidance on how to use these charts is detailed in Chapter 4 and a number of case studies to demonstrate the applicability of this approach are given in Chapter 5.

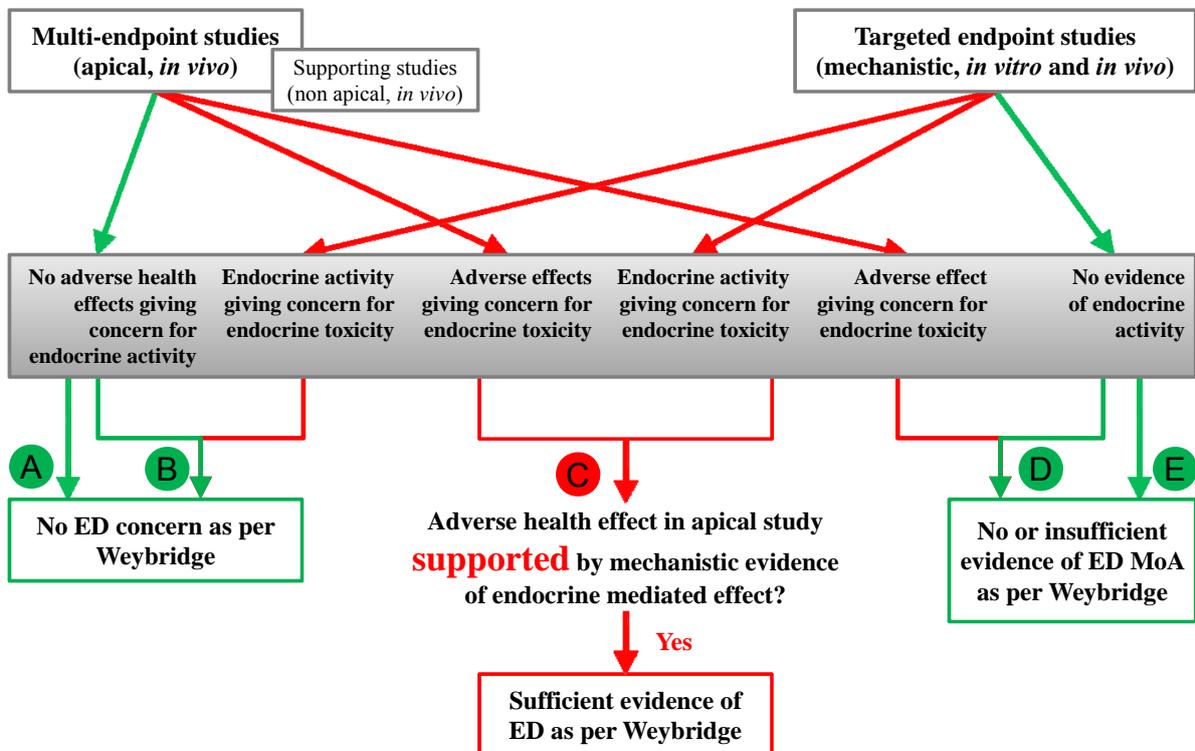
4. GUIDANCE FLOW CHARTS

In order to illustrate the concepts, studies and data discussed in this report, the task force has developed a number of flowcharts for use as guidance to identify endocrine disrupting effects within a dataset. Charts for both human (mammalian) and environmental (fish / amphibians and birds / wild mammals) assessments have been compiled following a similar approach. Each approach guides the evaluation of the available data (or triggers the need to generate specific data) followed by an assessment of endocrine specificity, relevance and potency for identified EDs.

4.1 Toxicology charts

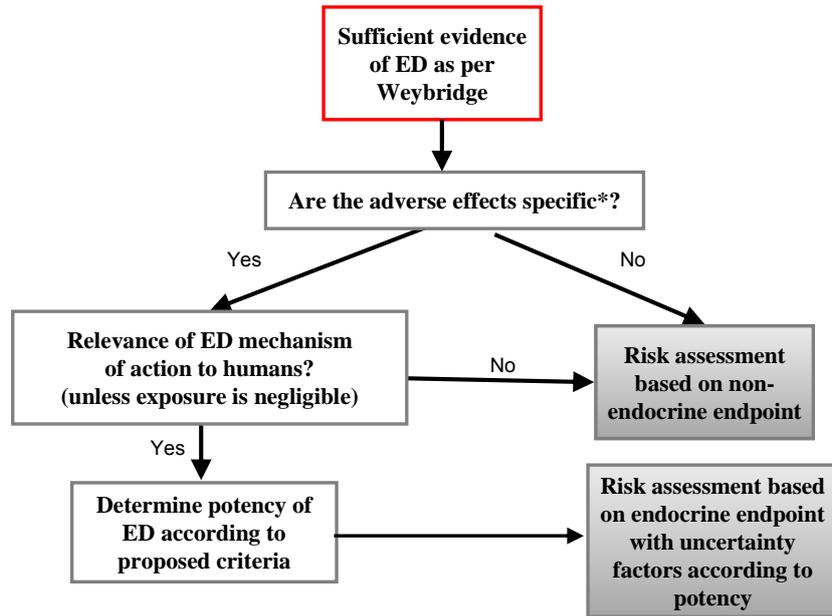
The first half of the flow chart illustrates a 5-step approach to identify an ED from a mammalian dataset. In the following pages, this complex figure is broken down to show the 5 scenarios separately. The second half of the flow chart provides guidance on how to discriminate chemicals of high concern from those of lower concern.

Figure 1a: Flowchart for toxicology - outlines the decision tree to decide if a substance should be considered an ED according to the Weybridge definition (Weybridge, 1996). Scenarios A to E are elucidated further below



Continued overleaf. See Figure 1b.

Figure 1b: Specificity, relevance and potency assessment for substances identified as EDs (See scenario C from previous chart)



*: Endocrine specific adverse effects: Adverse effects on the endocrine system occurring at dose levels lower than any other forms of adverse effects (e.g. neuro-, hepato-, cardio- toxicity).

Several criteria for potency are proposed to assess the level of hazard resulting from endocrine toxicity. These criteria should be considered collectively, using a weight of evidence approach, to determine the potency of the compound as an ED.

1- DOSE LEVEL

The most obvious and simplest criterion to define the potency of a chemical is in terms of the dose level at which adverse effects on endocrine endpoints occur. For example in the "enhanced TG 407" OECD validation exercise with anti-androgens, the NOAEL for endocrine effects on the male reproductive tract for flutamide was 1 mg/kg/day whilst for DDE the NOAEL for the same endpoints was greater than 100 mg/kg/day. In this case therefore, flutamide can be considered as being intrinsically more potent than DDE.

2- EXPOSURE DURATION

Another important criterion of potency is the duration of exposure that is required for an adverse effect to be induced. This aspect of potency was clearly demonstrated during the OECD validation exercise of the "enhanced TG 407" where adverse effects from the potent endocrine disruptors (e.g. flutamide) were readily detected whilst adverse effects from the weak ones (e.g. DDE) were not detected. When endocrine effects are detected in short-term repeat dose general toxicity studies such as TG 407 the endocrine adverse effects are also detected in the subsequent longer term toxicity studies (subchronic, reproductive and life time toxicity studies). For weak endocrine disruptors, adverse effects may sometimes only be detected following life time exposure.

3- TYPE AND SEVERITY OF ENDOCRINE EFFECTS

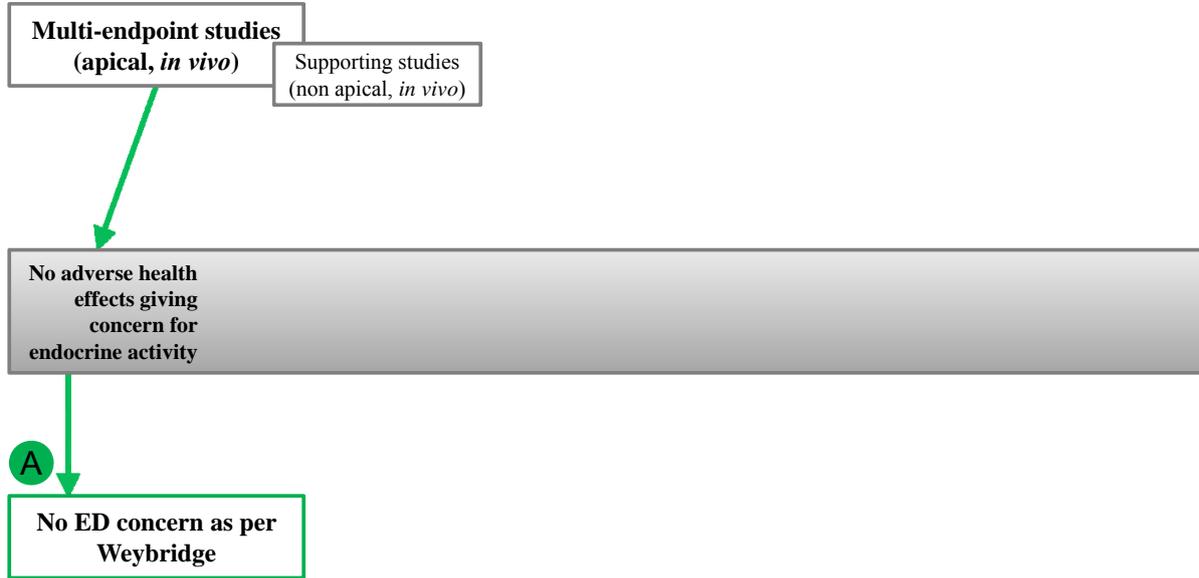
Another aspect to consider is the nature and severity of the adverse effects. For example a slight modification of developmental landmarks (e.g. delay in vaginal opening or preputial separation) only is not as severe as effects on the reproductive function with impairment of fertility.

4- NUMBER OF SPECIES AFFECTED

The last criterion that may also be considered for global evaluation of hazard from endocrine toxicity is the number of animal species from regulatory (eco)toxicity studies showing adverse endocrine effects. Adverse effects only detected in a single species (and one sex) may represent a lower hazard for humans and the environment than those chemicals affecting rat, mouse, rabbit, dog, fish, amphibian and bird.

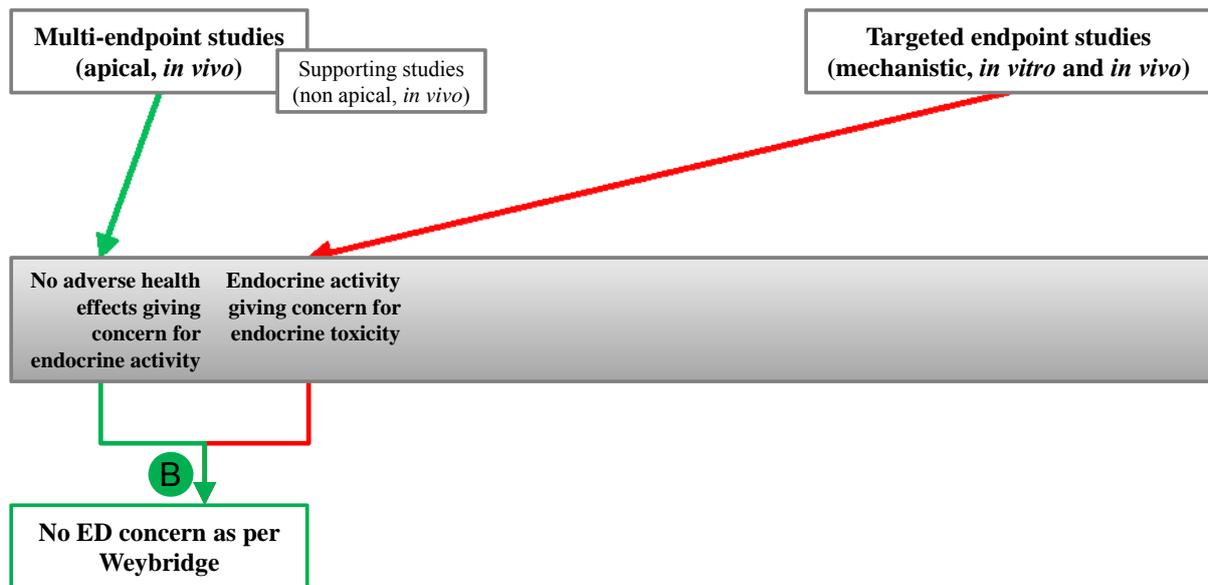
4.1.1 Breakdown of toxicology chart

Scenario A



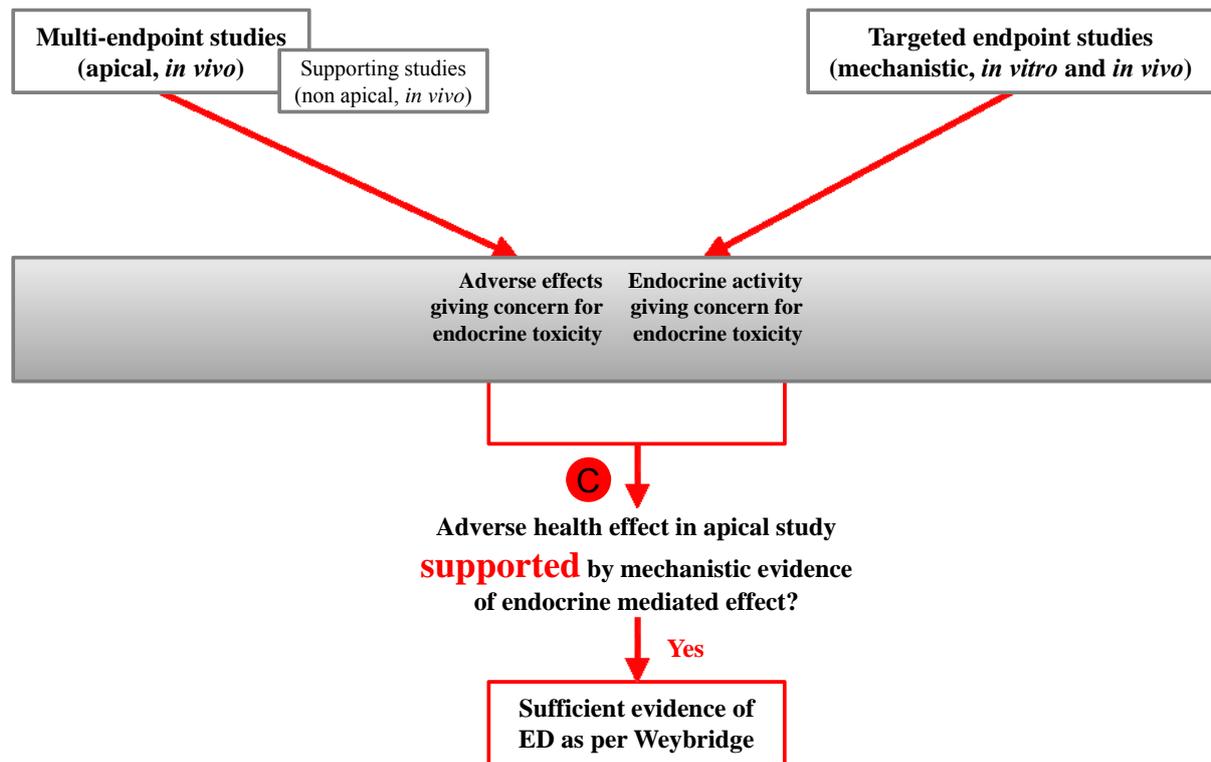
Apical studies are the most relevant highest tier studies to identify and characterise adverse effects that give concern for endocrine toxicity (e.g. two-generation reproductive toxicity study, OECD, 2001). An absence of such adverse effects in the apical studies can be taken as definitive evidence of no endocrine disrupting properties.

Scenario B



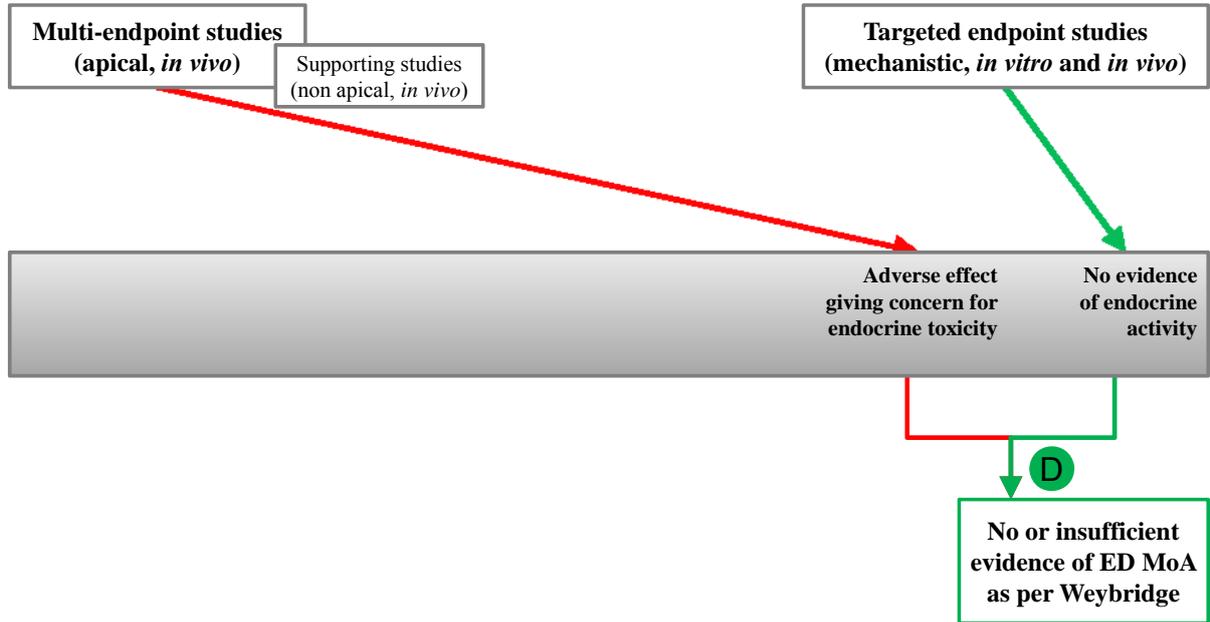
An absence of adverse effect giving concern to endocrine activity in the apical tests can be taken as definitive evidence of no endocrine disrupting properties even if there are positive outcomes from non-apical *in vitro* and/or *in vivo* endocrine targeted endpoint studies.

Scenario C

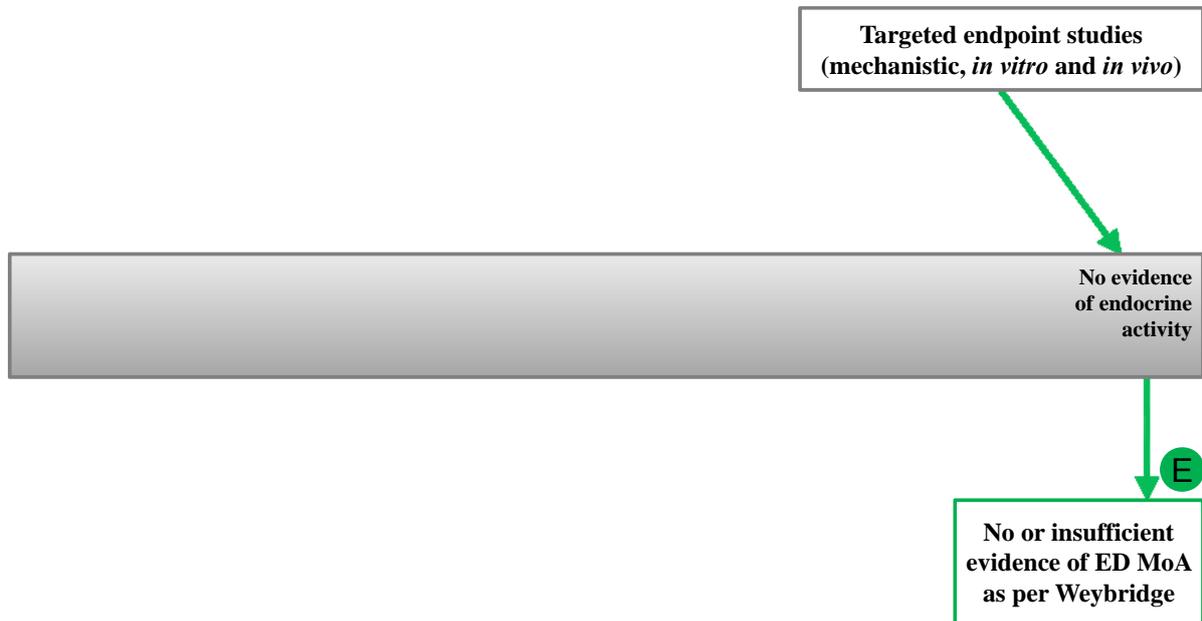


When a positive outcome in one or more endocrine sensitive endpoints in an apical (and/or relevant non-apical *in vivo* studies) study is supported by MoA data (*in vitro* and *in vivo* studies) i.e. the sequence of the biochemical and cellular events that underlies the adverse effect is described and understood, then conclusive proof of endocrine disruption can be considered as established. The next step is to consider the specificity, relevance, and potency of this effect (see Figure 1b for further details).

Scenario D



A positive outcome in one or more endocrine sensitive endpoints in an apical study that raises concern for endocrine activity, cannot by itself, be taken as sufficient evidence of endocrine disruption unless supported by MoA data. If, after exhaustive testing using a battery of *in vitro* and *in vivo* targeted endpoint studies, the sequence of biochemical and cellular events to support an ED mediated mechanism cannot be defined / determined then the adverse effects in the apical studies should not be considered as evidence of endocrine disruption.

Scenario E

In the absence of all other data, negative outcomes in an exhaustive combination of *in vitro/in vivo* targeted endpoint studies can be taken as evidence of the absence of endocrine disrupting properties.

4.2 *Ecotoxicology charts*

For environmental species two sets of charts are necessary; one set for fishes and amphibians and the second for birds and mammals. Assessment for birds and mammals is relevant to the regulation of plant protection products under the revisions to EU directive 91/414 whilst it is likely that the fish and amphibian chart will be useful under a range of EU regulatory directives (e.g. general chemicals REACH, biocidal products 98/8, the Water Framework Directive and plant protection products 91/414). An additional chart has been developed to address relative potency for the assessment of aquatic organisms, birds and mammals. On the subsequent pages the flow charts are broken down to discuss the issues associated with each decision.

Figure 2: Flowchart for fish and amphibians - outlines the decision tree to decide if a substance should be considered an ED according to the Weybridge definition (Weybridge, 1996), incorporating population relevance

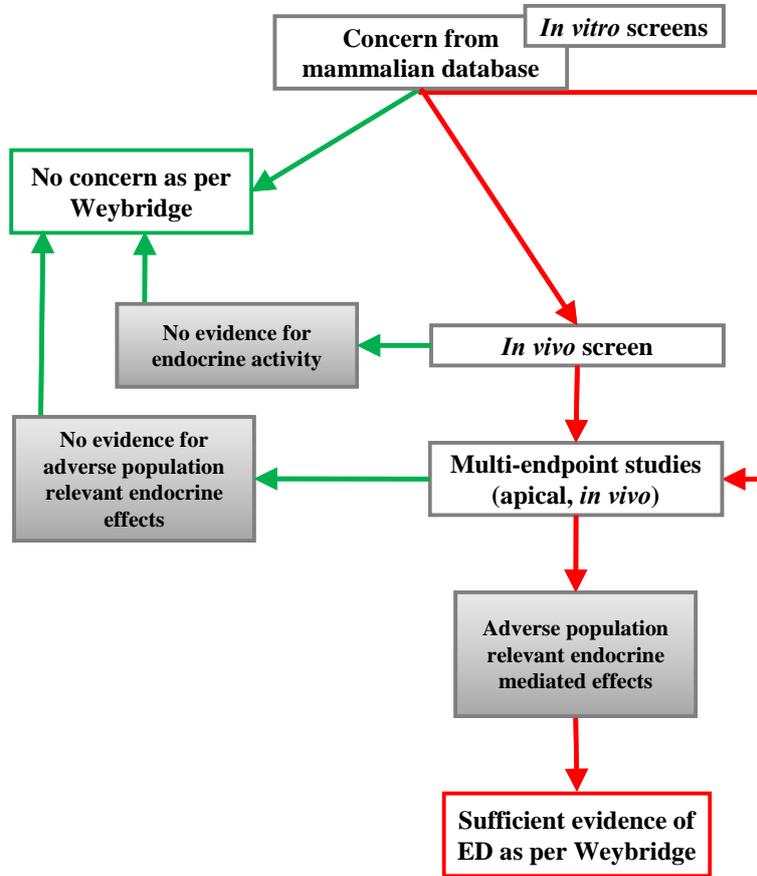


Figure 3: Flowchart for birds and mammals - outlines the decision tree to decide if a substance should be considered an ED according to the Weybridge definition (Weybridge, 1996) incorporating population relevance

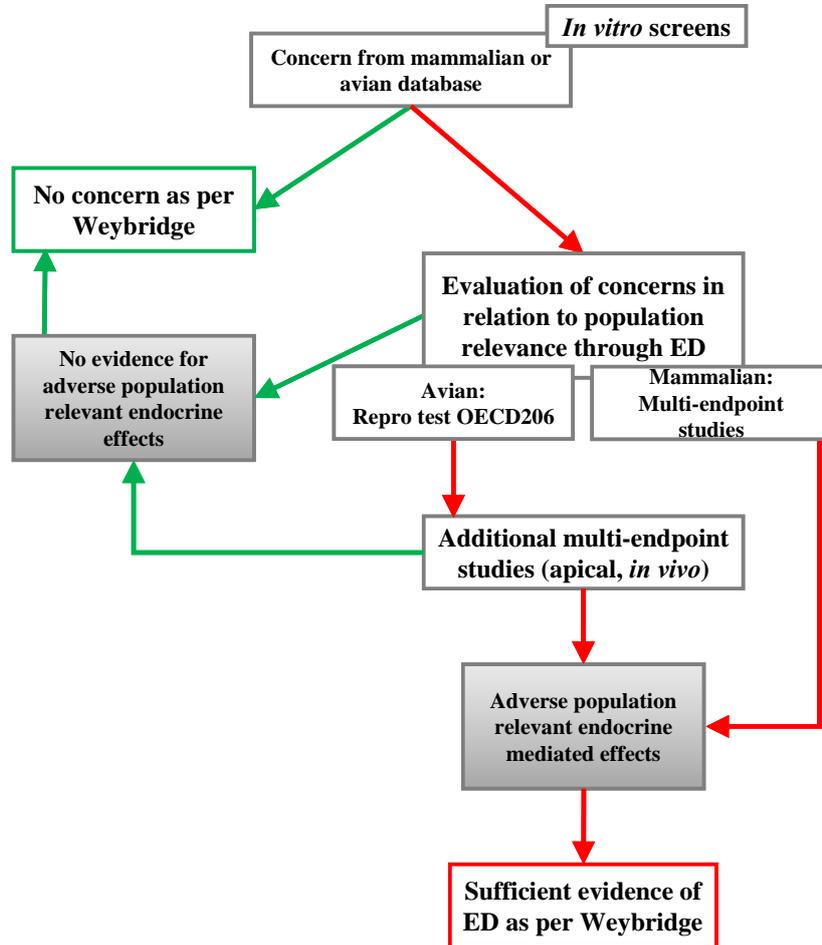
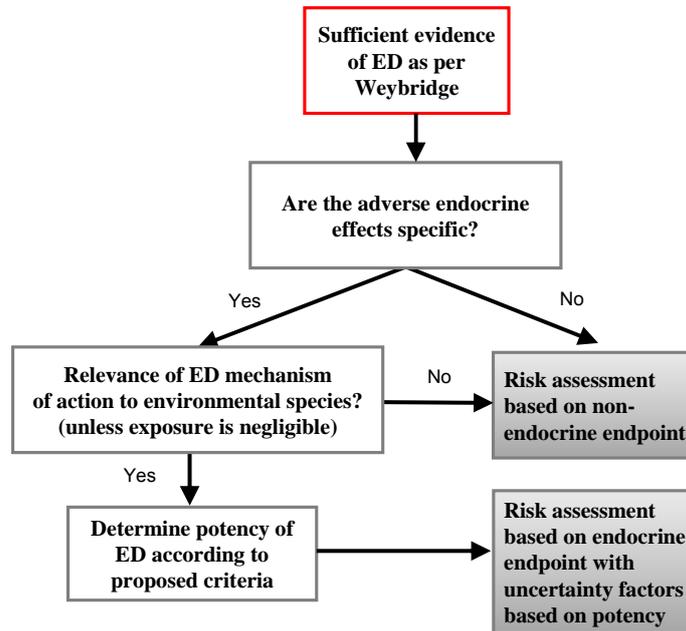


Figure 4: Assessment of relevance and potency of substances identified as EDs for environmental species



* Endocrine specific adverse effects: adverse effects on the endocrine system occurring at dose/concentrations lower than any other forms of adverse effects (systemic toxicity).

Several criteria for potency are proposed to assess the level of hazard resulting from endocrine toxicity. These criteria should be considered collectively, using a weight of evidence approach, to determine the potency of the compound as an ED.

1- DOSE/CONCENTRATION

The most obvious and simplest criterion to define the potency of a chemical is in terms of the dose/concentration at which adverse effects on endocrine endpoints occur. For example in fish full life-cycle studies the NOEC for sex ratio effects for ethinylestradiol was 1 ng/L (Lange *et al*, 2001), whilst for 4-nonylphenol the NOEC for the same end point was 17700 ng/L (Yokota *et al*, 2001).

2- EXPOSURE DURATION

Another important criterion of potency is the duration of exposure that is required for an adverse effect to be induced. For example, in a given study adverse effects from potent endocrine disrupters may be readily detected, whereas adverse effects from weak ones may not.

3- SPECIFICITY OF ENDOCRINE EFFECTS IN RELATION TO OTHER TAXONOMIC GROUPS

In the environmental assessment consideration may also be given to the position of the endocrine effect in relation to which endpoints may drive the overall risk assessment. For instance an endocrine effect in fish may be orders of magnitude above (at higher concentrations) than general toxic effects found in other species (e.g. algae).

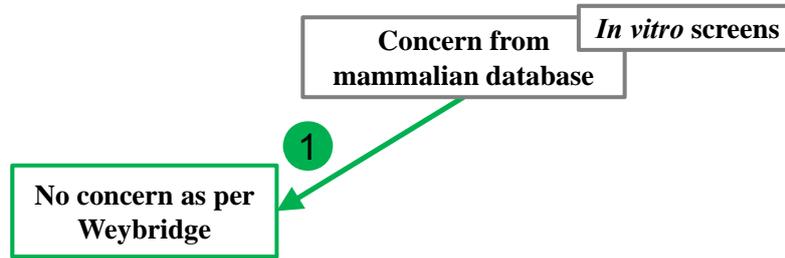
4- TYPE AND SEVERITY OF ENDOCRINE EFFECTS

Another aspect to consider is the nature and severity of the adverse effects. For example slight delays in time to sexual maturity alone are not as severe as effects on the reproductive function with impairment of fertility.

5- NUMBER OF SPECIES AFFECTED

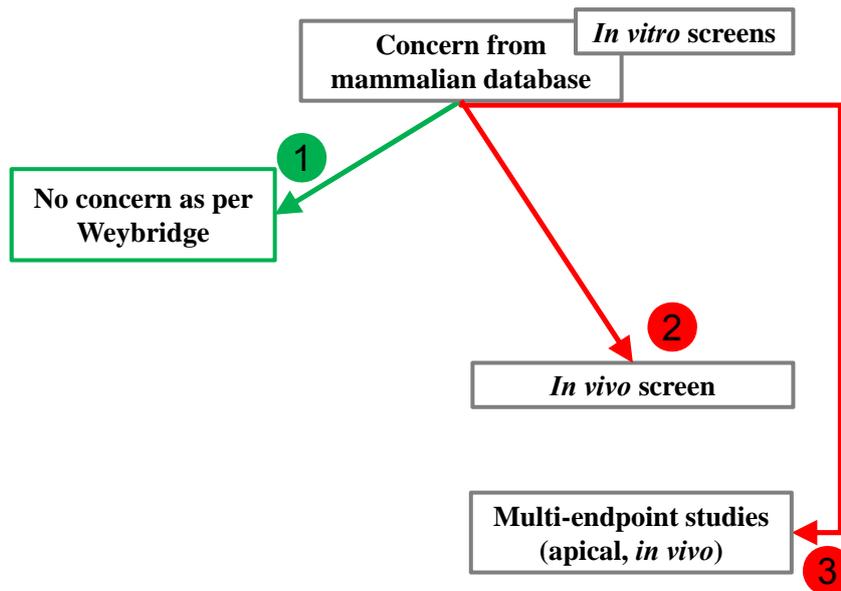
The last criterion, that may also be taken into consideration for global evaluation of hazard from endocrine toxicity, is the number of animal species from regulatory (eco)toxicity studies showing adverse endocrine effects. Adverse effects only detected in a single species (and one sex) may represent a lower hazard for humans and the environment than those chemicals affecting more than one species (e.g. rat, mouse, rabbit, dog, fish, amphibian and bird).

4.2.1 Fish and amphibian flowchart



It is most likely that endocrine specific testing in ecotoxicological species will be triggered by concerns from the mammalian toxicology database in combination with relevant *in vitro* studies (where available). Concerns from mammalian database represent consistent adverse (see toxicology chart) findings from toxicology studies indicating potential impacts on the endocrine system. It is important to note that evaluation of the toxicology database should give precedence to internationally validated test protocols (e.g. OECD and US EPA) preferably conducted to GLP. A weight of evidence evaluation is likely to be necessary where peer reviewed literature are available to incorporate an assessment of study reliability and relevance (see Section 3.2.3). Read across from structurally similar substances may also be considered. *In vitro* screen data refers to methods developed by the US EPA and OECD programmes (tier I and level 2, respectively). Although mammalian in origin these tests address basic molecular mechanisms so can also be considered relevant to environmental species. Where *in vitro* data are concerned the potential for metabolic activation should also be evaluated.

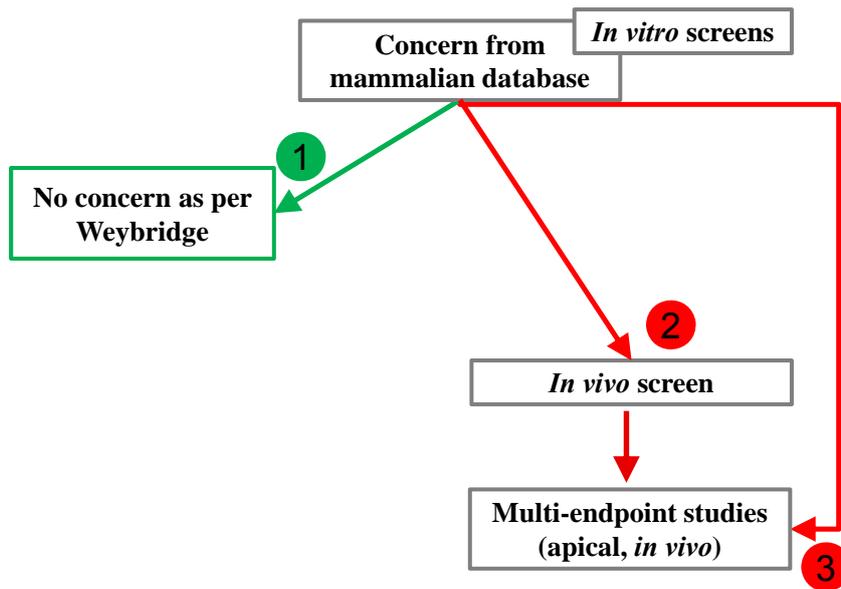
1 If after such an evaluation there is no concern for potential endocrine activity it should be possible to stop the assessment without the need for specific endocrine ecotoxicity testing (screening or definitive testing).



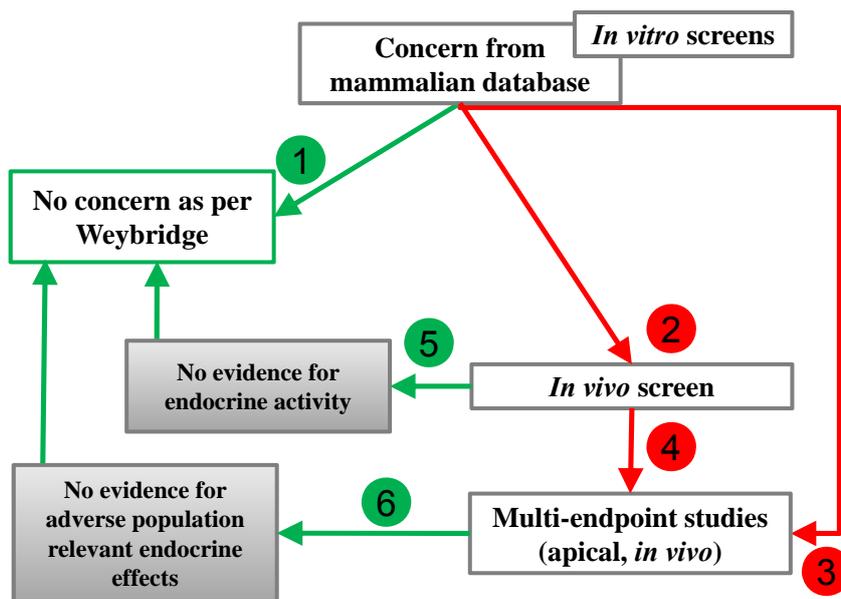
If an evaluation of the mammalian toxicology database and any *in vitro* data identify concerns that are confirmed by a rigorous weight of evidence evaluation of all the data further confirmatory testing will be required at either the screening or apical level. *In vivo* screen refers to methods developed by the US EPA and OECD programmes (tier I and level 3-4, respectively), specifically the OECD 21-day fish endocrine screening assay, fish short-term reproduction assay and amphibian metamorphosis assay. Apical test – refers to methods under development by the US EPA and programmes (tier 2 and level 5, respectively). However, it may be necessary to conduct study variants based on standard established practice (e.g. the US fish full life-cycle test). Specifically the tests currently available or in development are the fish sexual development test, fish life-cycle test and amphibian partial life-cycle test.

2 Concern identified: An option is to conduct an *in vivo* endocrine screening assay. The assay chosen will depend on the nature of the endocrine activity identified from the existing mammalian and *in vitro* data. Knowledge preceding this step should be used to determine which screen is most appropriate. For example suspicion of thyroid activity would require the screen to be the amphibian metamorphosis assay. Effects on the HPG axis would suggest a fish screening assay is more appropriate. The purpose of the *in vivo* screen is to confirm if endocrine activity is expressed in an intact organism.

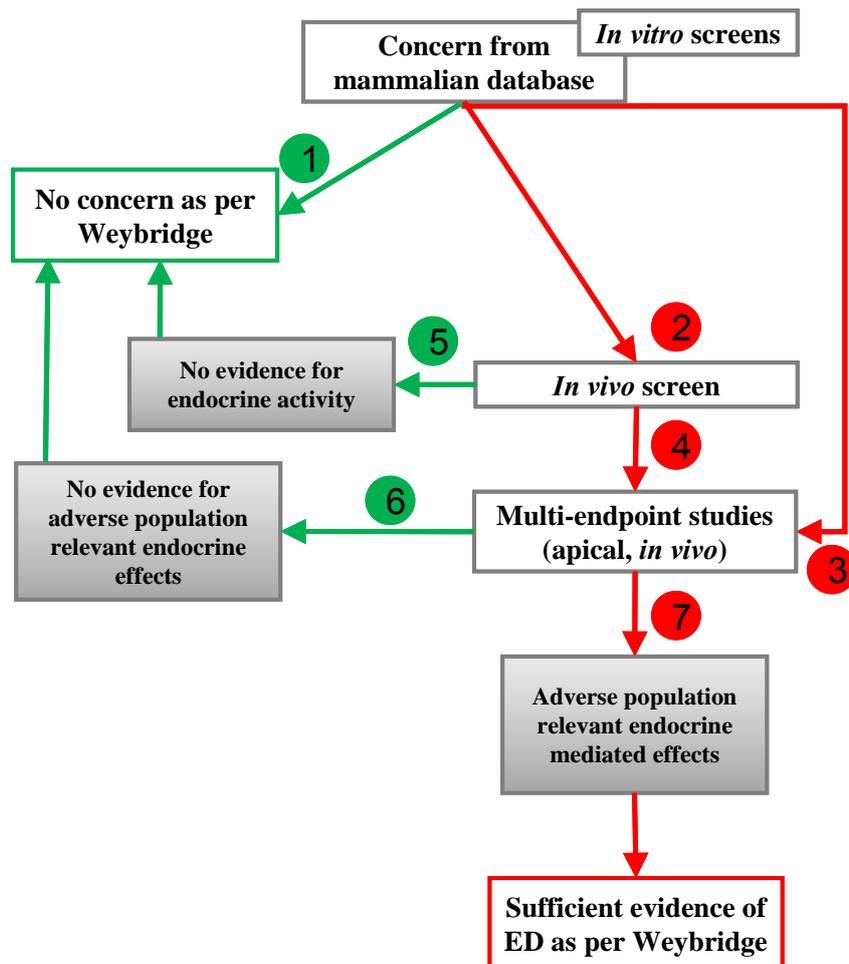
3 Concern identified: Alternatively it is possible to proceed directly to the apical or definitive test without conducting any *in vivo* screening assays. This may be the case where the effect is clearly relevant to environmental species.



4 If the screening study indicates an interaction with the endocrine system (typically biomarker or histopathological responses) in the absence of systemic toxicity further testing should be required (in the absence of appropriate pre-existing apical data). It is important to note that an effect in a screening assay does not constitute definitive evidence that a substance is an endocrine disrupter or that the observed effect will necessarily result in an adverse effect (population relevant). If the screening study indicates endocrine activity an appropriate apical test should be conducted. Apical studies must address population relevant endpoints, for example reproductive effects (gonadal development and differentiation).

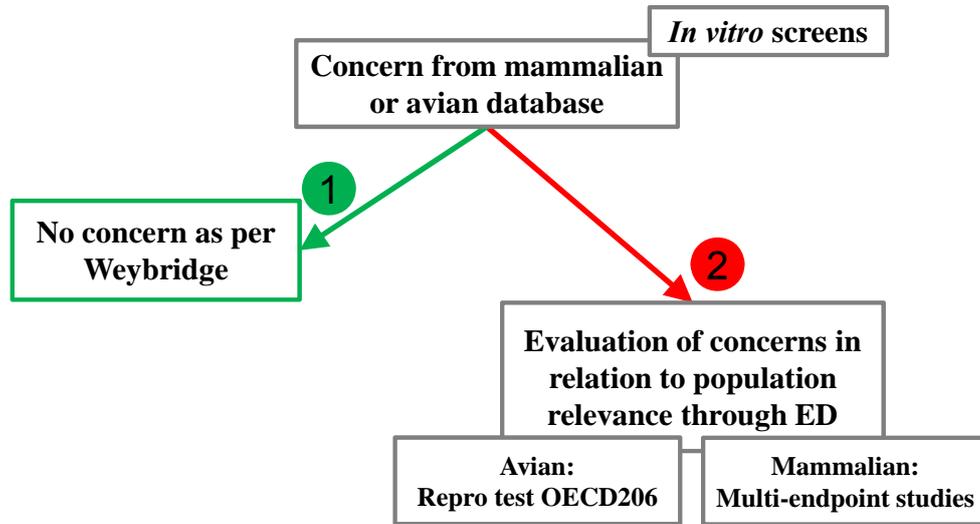


- 5 If the results from the screen indicate that there is no endocrine mediated effect, then no further endocrine specific testing should be required.
- 6 If the apical test demonstrates that there is not an endocrine mediated adverse population relevant effect there is no evidence that the substance is an endocrine disrupter according to the Weybridge definition incorporating population relevance (Weybridge, 1996).



- 7 If the apical study demonstrates an adverse (population relevant) effect mediated through the endocrine system the substance should be classified as an endocrine disrupter according to the Weybridge definition incorporating population relevance (Weybridge, 1996).

4.2.2 Bird and mammal flow chart breakdown

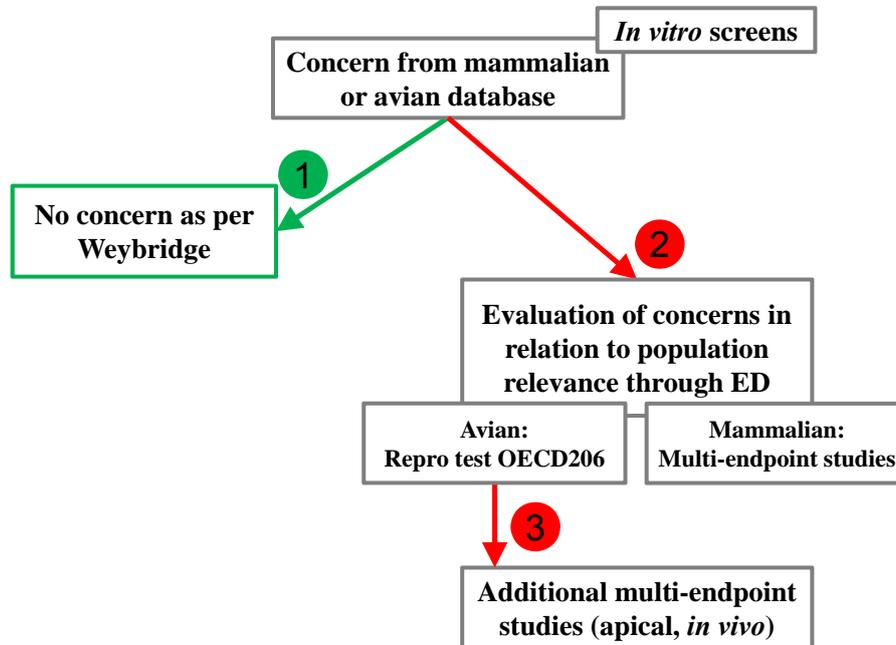


Concerns from the mammalian database represent consistent adverse (see toxicology chart) findings from toxicology studies indicating potential impacts on the endocrine system. The only difference from the toxicology approach being that human relevance is not important for the evaluation for environmental species. Similarly the avian database, where available, should be interrogated for relevant indicators (Appendix C). It is important to note that evaluation of the avian and mammalian database should give precedence to internationally validated test protocols (e.g. OECD and US EPA) preferably conducted to GLP. A weight of evidence evaluation is likely to be necessary where peer reviewed literature are available to incorporate an assessment of study reliability and relevance (see chapter 3). Read across from structurally similar substances may also be considered. *In vitro* screen data refers to methods developed by the US EPA and OECD programmes (tier I and level 2 respectively). Although mammalian in origin these tests address basic molecular mechanisms so can also be considered potentially relevant to environmental species. Where *in vitro* data are concerned the potential for metabolic activation should also be evaluated. Differences between the endocrinology of birds and mammals should be considered.

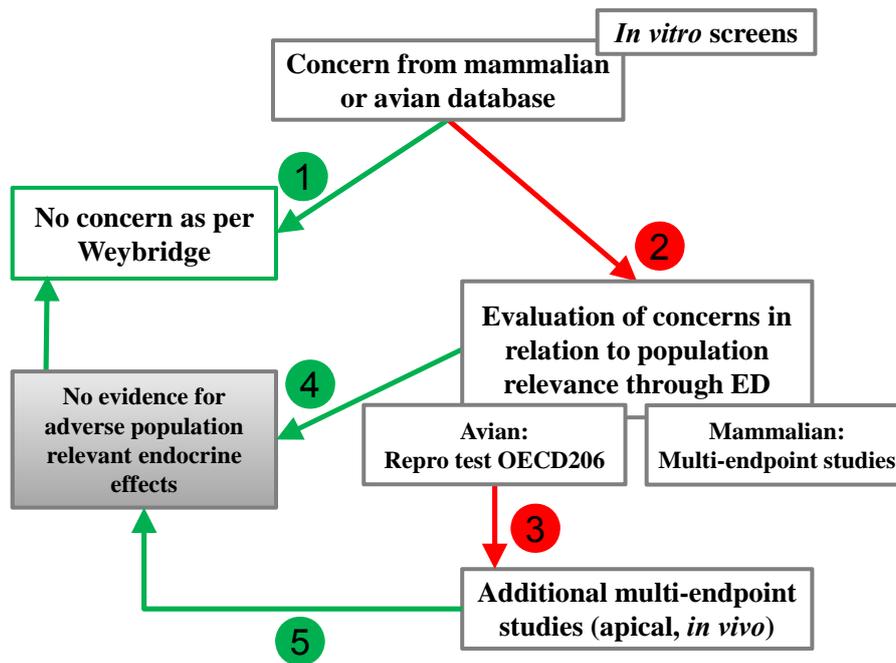
1 If, after such an evaluation, there is no concern for potential endocrine activity it should be possible to stop the assessment without the need for further evaluation or data generation.

2 If concerns from the mammalian and avian database appear to indicate endocrine activity a second evaluation step is necessary. This should consider the population relevance of the observed effects. This is in line with the protection goal of the bird and mammal assessment – the protection of bird and mammal populations (not individuals). In particular there should be a

detailed emphasis on the population relevant studies. For birds the relevant study is OECD 206 Avian Reproduction Test. For mammals reference can be made to all multi-endpoint studies under the toxicology evaluation.

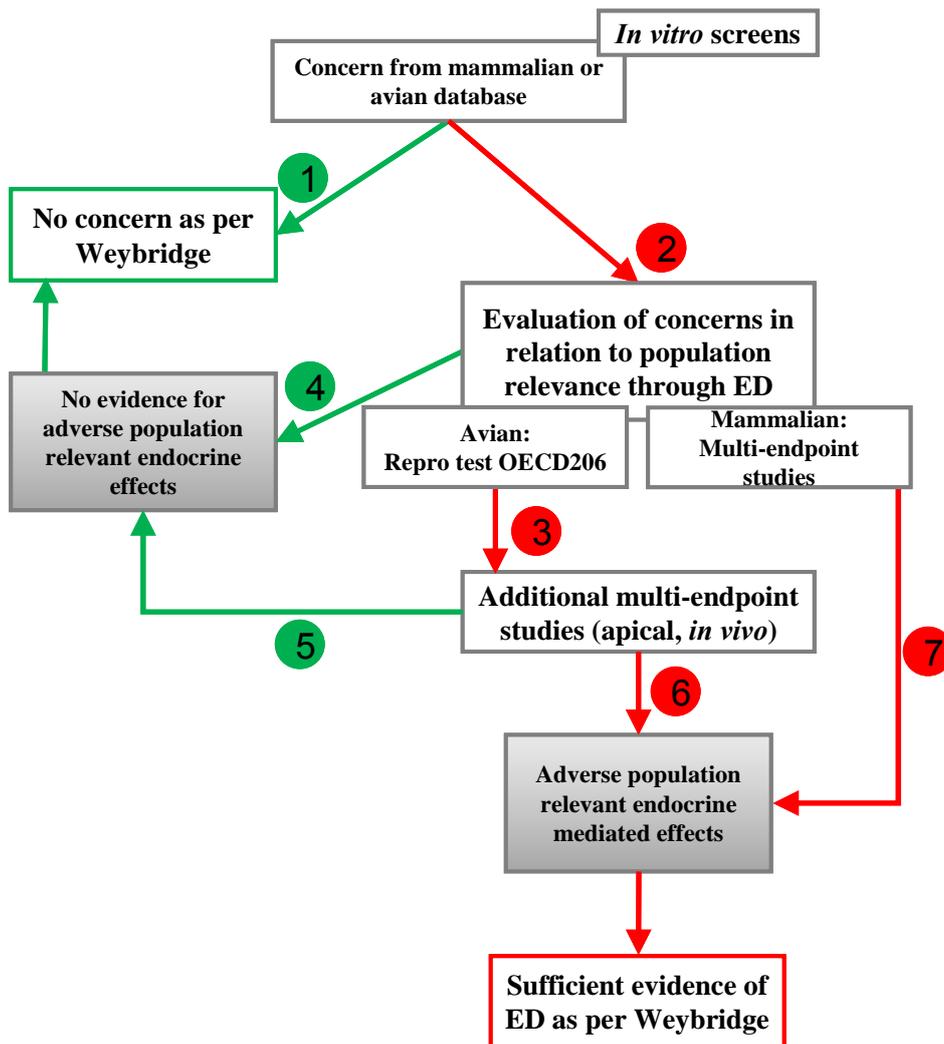


3 Since the one-generation avian reproduction test does not include exposure during all relevant stages of bird development or endocrine responsive endpoints further testing may be required. Additional multi-endpoint avian studies may be designed (e.g. partial or critical life stage tests) addressing endpoints not currently included in OECD 206 (e.g. behaviour) (OECD, 1984). Further, a two-generation avian test with Japanese quail is being considered by the OECD (OECD, 2006b).



4 If the evaluation demonstrates that there is not an endocrine mediated adverse effect leading to population relevant impacts there is no concern according to the Weybridge definition incorporating population relevance.

5 If the additional apical test demonstrates that there is not an endocrine mediated adverse effect leading to population relevant impacts there is no concern according to the Weybridge definition incorporating population relevance.



6 If the apical avian studies demonstrate an adverse (population relevant) effect mediated through the endocrine system the substance should be classified as an endocrine disrupter according to the Weybridge definition incorporating population relevance.

7 If the apical study mammalian studies demonstrate an adverse (population relevant) effect mediated through the endocrine system the substance should be classified as an endocrine disrupter according to the Weybridge definition incorporating population relevance.

5. EXAMPLES / CASE STUDIES

To illustrate the soundness of the proposed schemes they have been applied to a number of reference chemicals that have well documented toxicity profiles.

5.1 Toxicology charts: Case studies

5.1.1a Scenario A - BASF Herbicide: Flow chart for effects in mammals

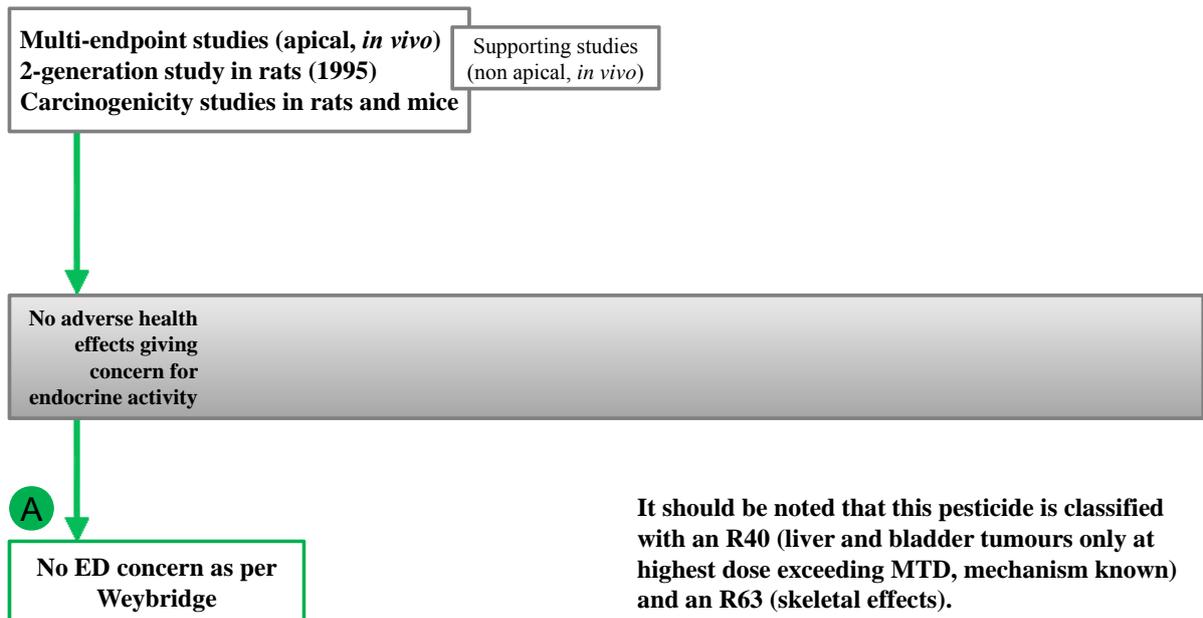


Table 9: BASF Herbicide: Toxicity database focused on mammalian ED effects

Study	Species	Adverse effect or endocrine activity giving concern for endocrine toxicity	NO(A)EL	Other effects (not endocrine activity or toxicity)	NO(A)EL	
Apical / definitive multi-endpoint	24-month carcinogenicity	Rat	-	-	Haematological changes, bile duct hyperplasia, hepatocellular adenomas, urinary bladder neoplasms (transitional cell papillomas and carcinomas)	5 mg/kg
	2-generation study (1995)	Rat	-	-	Pup effects: decreased weights, delays in physiological developmental landmarks	224 mg/kg reproductive function for parental animals 11 mg/kg general systemic toxicity 11 mg/kg developmental toxicity (F ₁ a, F ₁ b) 55 mg/kg developmental toxicity (F ₂)
Supporting studies	90 days	Rat	-	-	Haematological changes, liver hypertrophy	100 ppm
	Prenatal	Rat	-	-	Reduced foetal weights, skeletal malformation, skeletal variations and retardations	180 mg/kg 60 mg/kg maternal toxicity
	Prenatal	Rabbit	-	-	Reduced foetal body weights, skeletal variations, external malformations (one litter only)	50 mg/kg
<i>In vivo</i> (targeted)	-	-	-	-	-	-
<i>In vitro</i> (targeted)	-	-	-	-	-	-

5.1.1b Scenario A - Sucralose: Flow chart for effects in mammals

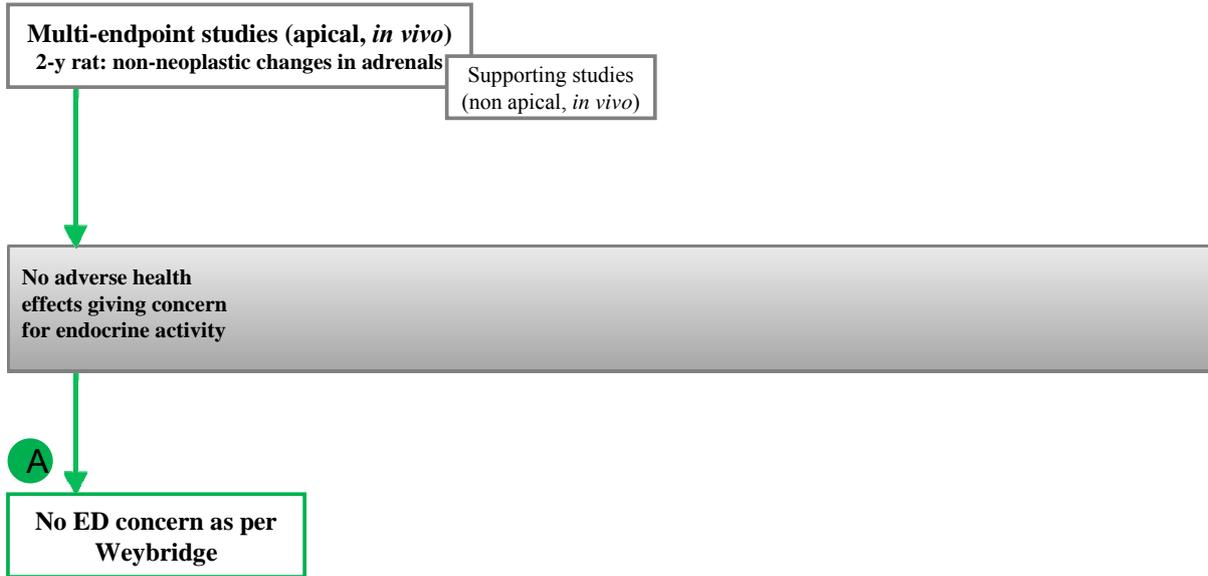


Table 10: Sucralose: Toxicity database focused on mammalian ED effects

	Study	Species	Adverse effect or endocrine activity giving concern for endocrine toxicity	NO(A)EL	General findings	NO(A)EL	Reference
Apical	Carcinogenicity	Rat	Non-neoplastic changes in adrenals	10000 ppm	Non-neoplastic changes in kidney	10000 ppm	Mann <i>et al</i> , 2000
	2-generation study	Rat	None		Kidney and thymus weight changes		Kille <i>et al</i> , 2000
Supporting studies	4 weeks and 8 weeks	Rat	Changes in weights of testes, prostate, ovaries adrenals but no histopathology data to support weight changes		Spleen and thymus changes		Goldsmith, 2000
Targeted endpoints	None found						

5.1.2a Scenario B - Coumarin: Flow chart for effects in mammals

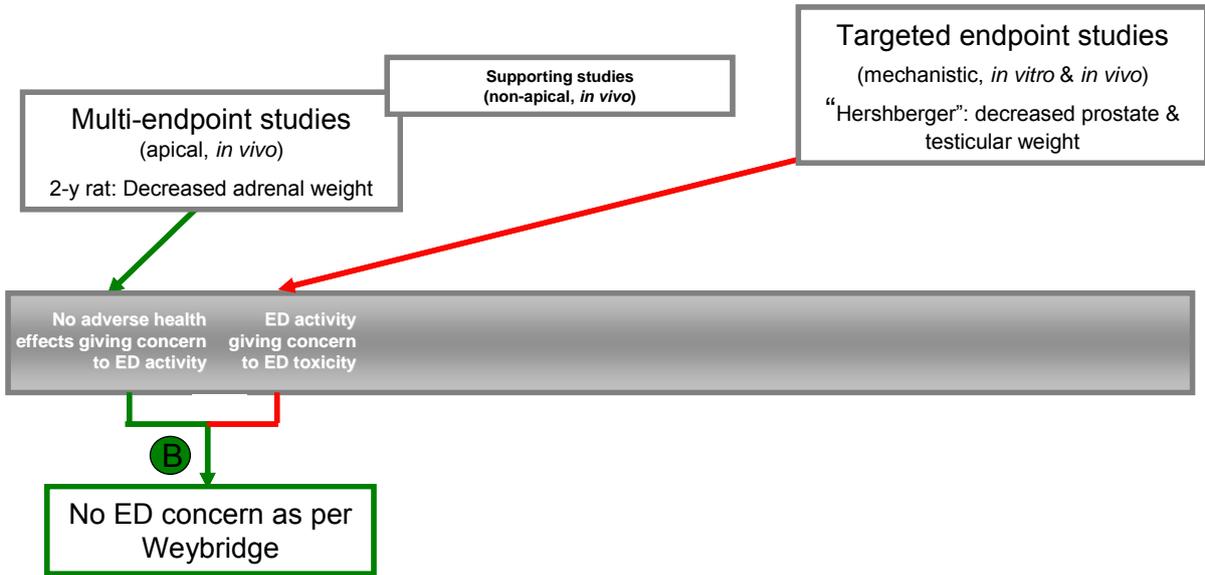


Table 11: Coumarin - Toxicity database focused on mammalian ED effects

	Study	Species	Adverse effect or endocrine activity giving concern for endocrine toxicity	NO(A)EL	General findings	NO(A)EL	Reference
Apical	Carcinogenicity	Rat	Decreased adrenal weight	333 ppm	Liver tumours	87/107 mg/kg/d	Carlton <i>et al</i> , 1996
		Mouse	None		Pulmonary tumours	100 mg/kg/d	NTP, 1993 a, b
	2-generation study	Rat	None		Kidney and thymus weight changes		Kille <i>et al</i> , 2000
Supporting studies	7 days	Rat	None		Liver weight, histology and histochemistry changes	45 mg/kg/d	Grasso <i>et al</i> , 1974
Targeted endpoints	Hershberger	Rat	Decreased prostate and testicular weights	10 mg/kg/d			Omarbasha <i>et al</i> , 1989

5.1.2b Scenario B - Glyphosate: Flow chart for effects in mammals

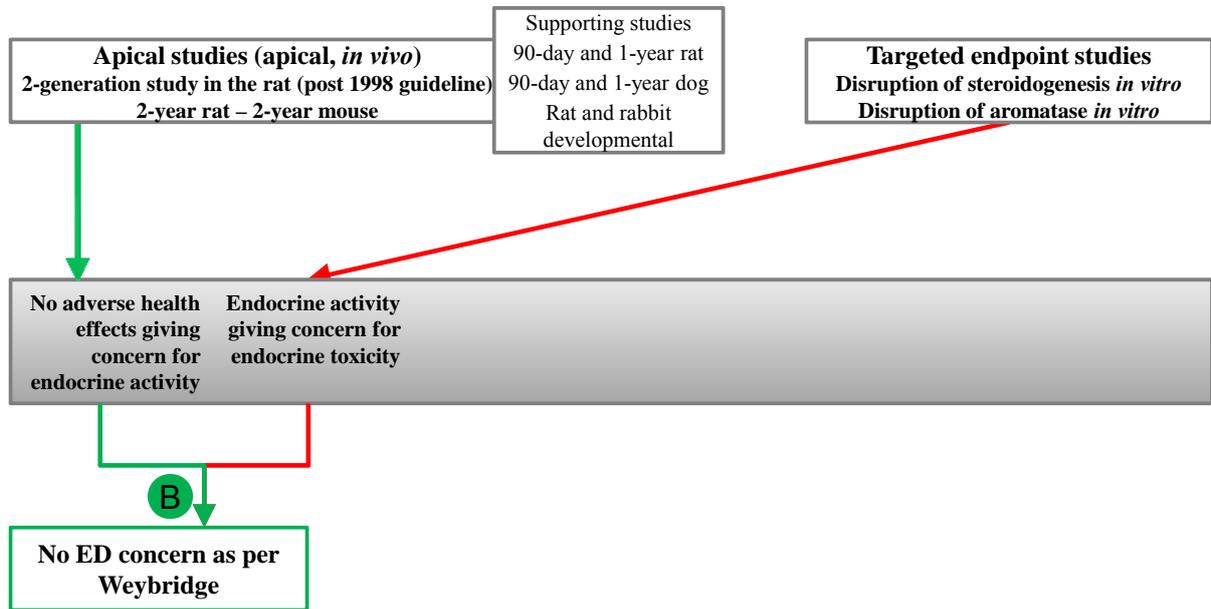


Table 12: Glyphosate: Toxicity database focused on mammalian ED effects Apical and supporting in vivo studies. Sub-acute and chronic toxicity and oncogenicity.

Species, study duration and route of administration	Dose at which no toxicity was observed (NOAEL)		Lowest dose at which toxicity was observed (LOAEL)		Observed toxicity
	ppm	mg/kg/day	ppm	mg/kg/day	
Rat 90-day dietary (male / female)	5000	414/447	20000	1693/1821	Reduced bodyweight and food utilisation efficiency in males, minor clinical chemistry changes
Rat 90-day dietary (male / female)	-	300	-	1000	Changes in clinical chemistry parameters in females, decreased urinary pH in males
Rat 90-day dietary (male / female)	20000 (HDT)	1267/1263	-	-	No treatment-related effects
Mouse 90-day dietary (male / female)	-	>4500 (HDT)	-	-	No treatment-related effects
Mouse 90-day dietary (male / female)	10000	1870/2740	50000	9710/14800	Reduced bodyweight gains
Dog 90-day dietary (male / female)	10000	323/343	50000	1680/1750	Reduced bodyweight females only
Dog 1-year dietary male	30000	906 (HDT)	-	-	No evidence of toxicity at HDT
Dog 1-year dietary female	15000	447	30000	926.2	Minimal reduction in bodyweight
Dog 1-year capsule (male / female)	-	300	-	1000	Changes in fecal consistency

Table 12: Glyphosate: Toxicity database focused on mammalian ED effects (cont'd)
Apical and supporting in vivo studies.
Sub-acute and chronic toxicity and oncogenicity.

Species, study duration and route of administration	Dose at which no toxicity was observed (NOAEL)		Lowest dose at which toxicity was observed (LOAEL)			
Dog 1-year capsule (male / female)	-	500 (HDT)	-	-	-	No treatment-related effects
Rat 1-year dietary (male / female)	2000	141/167	8000	560/671		Reduced bodyweight and food utilisation efficiency
Rat 1-year dietary Glyphosate acid (male / female)	6000	361/437	20000	1214/1498		Reduced bodyweight and pathology changes in liver and kidney
Rat 2-year dietary Glyphosate acid (male / female)	-	300	-	1000		Reduced bodyweight, pale faeces, changes in clinical chemistry parameters, decreased urinary pH
Rat 2-year dietary Glyphosate acid (male / female)	8000	362/457	20000	940/1183		Reduced bodyweight in females, degenerative lens changes in males
Mouse 2-year dietary (male / female)	-	1000 (HDT)	-	-	-	No treatment-related effects
Mouse 2-year dietary (male / female)	5000	814/955	30000	4841/5874		Reduced bodyweight, histological changes in the livers of males only

NOAEL = No observed adverse effect level

LOAEL = Lowest observed adverse effect level

HDT = Highest dose tested

**Table 13: Glyphosate: Toxicity database focused on mammalian ED effects
Reproduction and developmental studies**

Species, study type and route		NOAEL	LOAEL	
		mg/kg/day	mg/kg/day	Observed toxicity
Rat Multi-generation reproduction dietary (males/females)		293/323 (3000 ppm)	1054 (10000 ppm)	Reduced bodyweight in F ₁ a pups
Rat two-generation reproduction dietary (males/females)		197/226 (3000 ppm)	668/752 (10000 ppm)	Slightly decreased bodyweights in F ₁ males at second generation selection
Rat two-generation reproduction dietary (males/females)		777/666 (10000 ppm) (HDT)	-	Reduced bodyweight, soft faeces, equivocal effect on litter size
Rat three-generation reproduction dietary (males/females)		30 (HDT)	-	Reduced bodyweight in F ₁ a pups
Rat developmental	Maternal	1000 (HDT)	-	No evidence of toxicity at HDT
	Developmental	1000 (HDT)	-	No adverse developmental effects seen
Rat developmental	Maternal	300	1000	Decreased bodyweight gain; noisy respiration
	Developmental	300	1000	Delayed ossification (at a dose that produced maternal toxicity)
Rat developmental	Maternal	1000	3500	Mortality, reduced bodyweight gain, diarrhoea
	Developmental	1000	3500	Mortality, no evidence of teratogenicity
Rabbit developmental	Maternal	100	175	Diarrhoea, reduced food consumption, reduced bodyweight
	Developmental	175	300	Slightly reduced foetal weight
Rabbit developmental	Maternal	50	150	Gastrointestinal disturbances, decreased bodyweight gain and food consumption
	Developmental	450 (HDT)	-	No adverse effects
Rabbit developmental	Maternal	175	350	Mortality, diarrhoea
	Developmental	350	-	No adverse effects

HDT = Highest dose tested

**Table 14: Glyphosate: Toxicity database focused on mammalian ED effects
Literature references to *in vitro* publications**

Publication	Publication conclusion	Publication critiques
Walsh <i>et al</i> , 2000	Glyphosate formulation disrupts steroidogenic acute regulatory (StAR) protein expression <i>in vitro</i>	Subsequent publication (Levine <i>et al</i> , 2007) shows non-endocrine disruption mechanism
Levine <i>et al</i> , 2007	Glyphosate and glyphosate formulations have no effect on StAR protein expression. Surfactants in general cause perturbation of mitochondrial membrane, causing down stream effects measured by Walsh <i>et al</i> (2000)	Non-endocrine mechanism of membrane disruption is evident when surfactants are applied at high concentrations to <i>in vitro</i> systems.
Richard <i>et al</i> , 2005	Glyphosate and formulation disrupt aromatase activity <i>in vitro</i>	Methodological flaws. Multiple instances of bias in arguments and data interpretation (Commission d'Etude de la Toxicité: Procès-verbal de la réunion du 14 décembre 2005) ^a .

^a *In vitro* studies have appeared in the scientific literature reporting adverse effects by glyphosate or glyphosate containing formulations through endocrine mediated mechanisms. However, these studies were conducted using non-standard and non-validated methodology. Subsequently in scientific reviews and/or in additional peer-reviewed publications these studies have been shown to have methodological flaws and bias in arguments and errors in data interpretation.

Glyphosate has undergone a comprehensive battery of *in vivo* toxicology and ecotoxicology testing that cover a broad spectrum of endocrine endpoints that are sufficient to detect endocrine disruption. This testing included a tiered battery of acute, sub-chronic and chronic tests. Furthermore, these studies have robust experimental designs, follow internationally accepted protocols, have a high level of replication and a long history of use in hazard identification and risk assessment. The results from these studies show no evidence of endocrine-mediated effects by glyphosate.

5.1.3a Scenario C - Dibutyl phthalate: Flow chart for effects in mammals

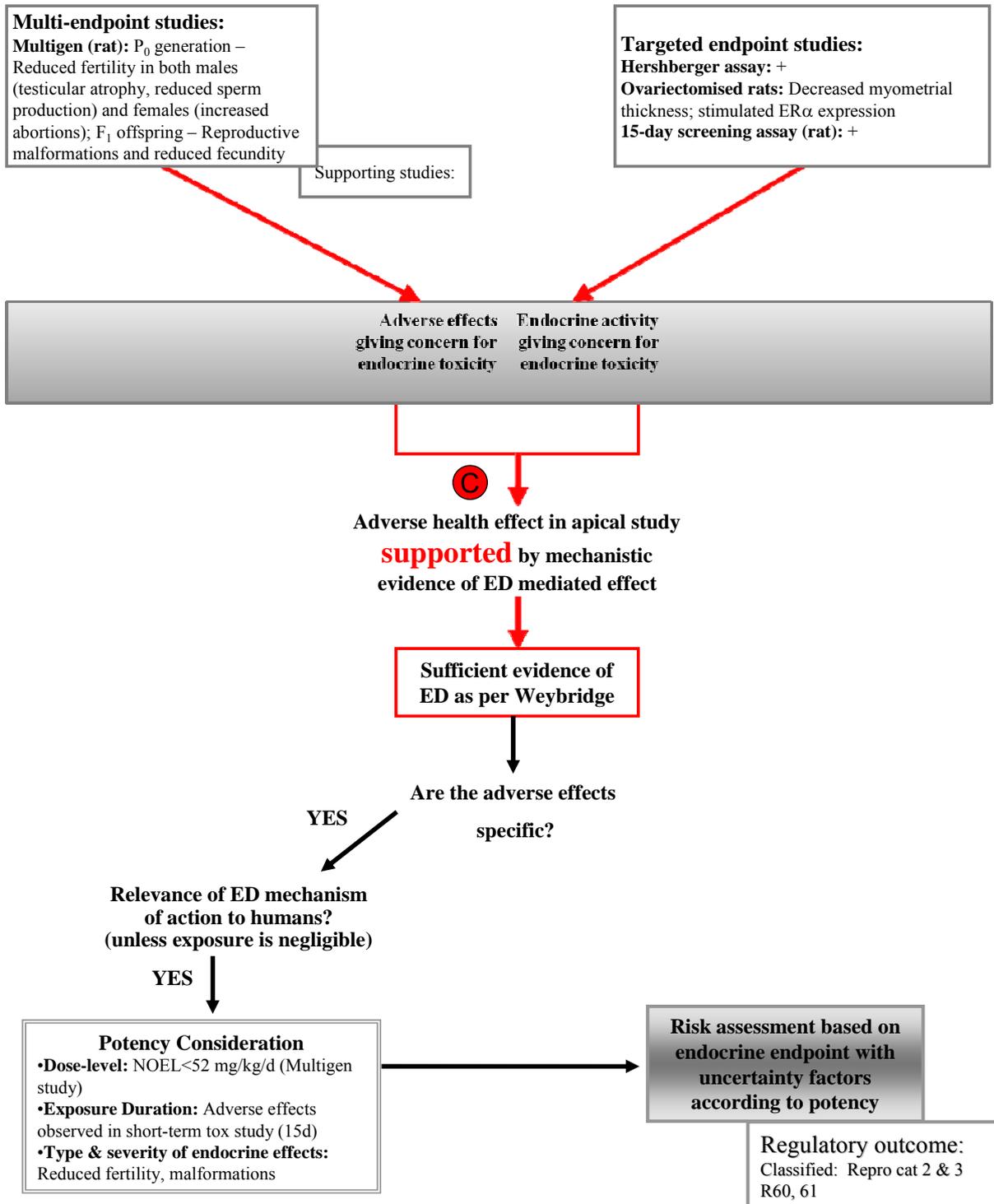


Table 15: Dibutyl phthalate - Toxicity database focused on mammalian ED effects

Method	Results	Reference
Multi-endpoint studies		
Multigeneration study using LE hooded rats treated with 250, 500 and 1000 mg/kg/day	Reduced fertility in P ₀ generation: both males (testicular atrophy, reduced sperm production) and females (increased abortions) at 500 and 1000 mg/kg/day; F ₁ offspring – Reproductive and non-reproductive malformations and reduced fecundity	Gray <i>et al</i> , 1999
Continuous breeding study using Sprague-Dawley rats treated with up to 1% w/w (dose ranges of 52-509 ♂ or 80-794 ♀ mg/kg/day)	Reduced litter survival and pup weight in all groups. F1 showed decreased mating, pregnancy performance and fertility. Effects included: F0 males (decreased sperm counts, testicular degeneration, defective epididymides) and females (decreased pregnancy, low live birth frequency and reduced pup weights). NOAEL not derived as lowest dose was LOAEL = 52 mg/kg bw/day based on embryotoxicity..	Wine <i>et al</i> , 1997
Targeted endpoint studies		
Hershberger assay : administration of 20, 100 or 500 mg/kg/day DBP for 10 days to testosterone proportionate-treated Sprague-Dawley CrI:CD castrated rats by oral gavage	Reduced ventral prostate weights at all doses; no effect on serum testosterone or luteinising hormone (LH) levels	Lee and Koo, 2007
Uterotrophic assay : Administration of 20-2000 mg/kg/day DBP to (im)-mature ovariectomised Sprague-Dawley rats by oral gavage	No effect on uterine weight or vaginal epithelial cell cornification.	Zacharewski, 1998
Treatment of ovariectomised Sprague-Dawley with 92.5 mg/kg/day or 462.5 mg/kg/day DBP	Decreased myometrial thickness (both doses); simulated ER α expression (high dose); inhibition of gene expression of complement protein C3 (both doses); reduced bone mineral density of metaphysis of tibia (low dose); inhibition of cornifin gene expression (low dose)	Seidlova-Wuttke <i>et al</i> , 2004
15-day screening assay : Treatment of male Sprague-Dawley rats with 250, 500, 750 and 1000 mg/kg/day by oral gavage	Antiandrogen-like activity: Bilateral testicular degeneration; decreased dihydrotestosterone, testosterone, oestradiol; increased prolactin, follicle stimulating hormone (FSH) and luteinising hormone (LH)	O'Connor <i>et al</i> , 2002
Other miscellaneous assays : These studies contribute to the database of information about this chemical, but may provide misleading information about the mode of action as further work has since shown that phthalates are not receptor agonists or oestrogenic.		
DNA microarray containing oestrogen responsive genes (EstrArray®) to examine gene expression profiles in MCF-7 cells treated with 10 μ M DBP followed by correlation analysis	DBP: correlation with 17 β -oestradiol; correlation coefficient $r = 0.36$ (10 μ M butylbenzyl phthalate, $r = 0.85$)	Parveen <i>et al</i> , 2008
ZR-75 proliferation assay	Oestrogenic effect	Harris <i>et al</i> , 1997
ZR-75 proliferation assay	Oestrogenic activity: Mitogenic effects on cell growth at 10 ⁻⁵ M	Jobling <i>et al</i> , 1995

Table 15: Dibutyl phthalate - Toxicity database focused on mammalian ED effects (cont'd)

MCF-7 Cell Line assays		
Luciferase-mediated reporter gene assay with transfected MCF-7 or HeLa using DBP (0.1, 1 and 10 µM)	Induction of luciferase activity by 10 µM DBP (effect was completely inhibited by 100 nM of a pure ER antagonist)	Zacharewski <i>et al</i> , 1998
E-Screen (MCF-7 proliferation assay)	7-fold proliferation upon treatment with 10 ⁻⁴ M (17β-oestradiol: 9-fold upon 10 ⁻⁹ M)	Hong <i>et al</i> , 2005
MCF-7 proliferation assay	Oestrogenic effect	Harris <i>et al</i> , 1997
ER Ligand-Binding assays		
Oestrogen receptor ligand-binding assays with oestrogen receptor ERα and ERβ from cytosolic porcine uterine cells	No binding affinity at ERα; weak binding affinity at ERβ	Seidlova-Wuttke <i>et al</i> , 2004
ER binding assay with cytosolic extract from rainbow trout liver	Oestrogenic activity: DBP reduced binding of 17β-oestradiol to receptor, authors called for <i>in vivo</i> confirmation	Jobling <i>et al</i> , 1995
Oestrogen Receptor Transactivation assays		
Recombinant yeast screen	Oestrogenic response (35% compared to 17β-oestradiol 100%)	Harris <i>et al</i> , 1997
Luciferase-mediated reporter gene assay with transfected MCF-7	Oestrogenic activity: Stimulation of transcription at 10 ⁻⁴ to 10 ⁻⁵ M	Jobling <i>et al</i> , 1995
Thyroid Hormone Screening assays		
Ligand-specific thyroid hormone (TH) inducible luciferase assay in a permanent <i>Xenopus laevis</i> recombinant cell line XL58-TRE-Luc	T3-antagonistic activity: Inhibition of expression of the endogenous primary T3 response TH nuclear receptor β (TRβ) gene (IC ₅₀ : 39 ± 1 µM).	Sugiyama <i>et al</i> , 2005

5.1.3b Scenario C - Phenobarbital: Flow chart for effects in mammals

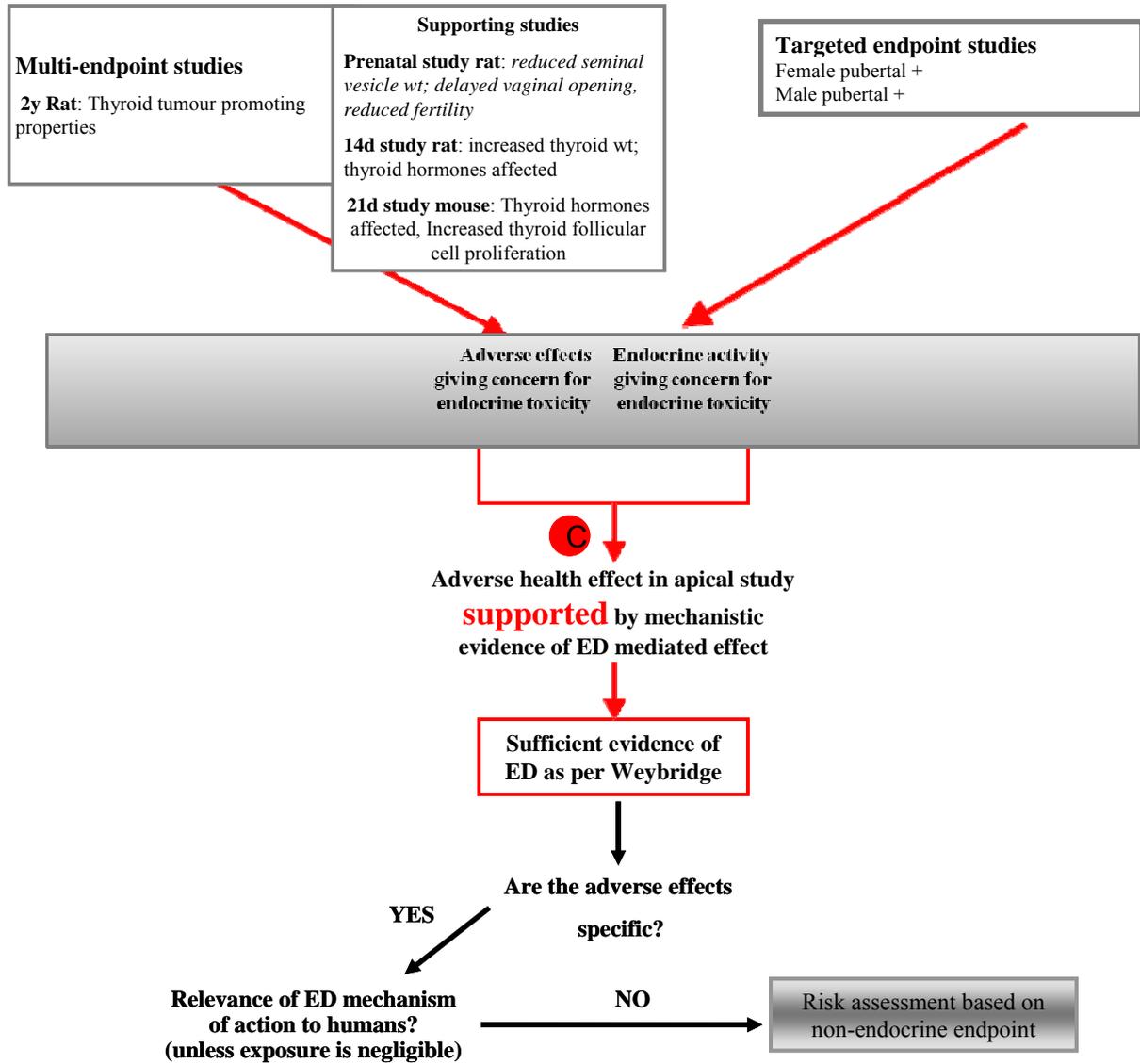


Table 16: Phenobarbital - Toxicity database focused on mammalian ED effects

	Study	Species	Adverse effect or endocrine activity giving concern for endocrine toxicity	NO(A)EL	General findings	NO(A)EL	Reference
Apical	Carcinogenicity	Rat	None		Hepatocellular adenomas	<500 mg/l	Rossi <i>et al</i> , 1977
		Mouse	None		Hepatocellular adenomas and carcinomas	<500 mg/kg/d	Thorpe and Walker, 1973
	1-generation study	Mouse	None		Cleft palate; heart, skeletal and urogenital defects		Finnell <i>et al</i> , 1987
Supporting studies	Prenatal exposure study (GD6-GD15)	Rat	Males: Decreased seminal vesicle weight; reduced fertility; Females: Delayed VO, reduced fertility, altered oestrous cycles	<40 mg/kg/d			Gupta <i>et al</i> , 1980
	14 days	Rat	Increased thyroid weight, reduced T3 and T4, increased TSH		Increased liver weight		McClain <i>et al</i> , 1989
	21 days	Mouse	Reduced T3 and T4, increased TSH, increased thyroid follicular cell proliferation				Hood <i>et al</i> , 2003
	Thyroid tumour promoting studies	Rat	Increased incidence of thyroid tumours in rats pre-treated with DHPN				Kanno <i>et al</i> , 1990 McClain <i>et al</i> , 1988
Targeted endpoints (<i>in vivo</i>)	Prepubertal	Rat female	VO delayed, extended dioestrus, increased thyroid and adrenal weights	LOAEL = 25 mg/kg/d			Goldman <i>et al</i> , 2000
		Rat male	Delayed PS, reduced SAT weight, non-stat significant changes in thyroid weight and TSH				Stoker <i>et al</i> ; 2000
	Hershberger assay	Rat	Slight LABC weight decrease	<125 mg/kg/d			Yamada <i>et al</i> , 2004
Targeted endpoints (<i>in vitro</i>)	Aromatase assay		Inhibition	Lowest positive concentration: 0.29 mM (50% reduction at 1.57 mM)	-	-	Jacobsen <i>et al</i> , 2008
	AR binding assay		Negative up to 1 mM		-	-	US EPA, 2007

Table 16: Phenobarbital - Toxicity database focused on mammalian ED effects (cont'd)

Study	Species	Adverse effect or endocrine activity giving concern for endocrine toxicity	NO(A)EL	General findings	NO(A)EL	Reference
Non-relevance of phenobarbital thyroid effects to man		Plasma T4 half-life in rats shorter than in humans				Döhler <i>et al</i> , 1979
		Rat-specific increased thyroid hormone catabolism through UDP-GT induction				Capen, 2001

5.1.3c Scenario C - Flutamide: Flow chart for effects in mammals

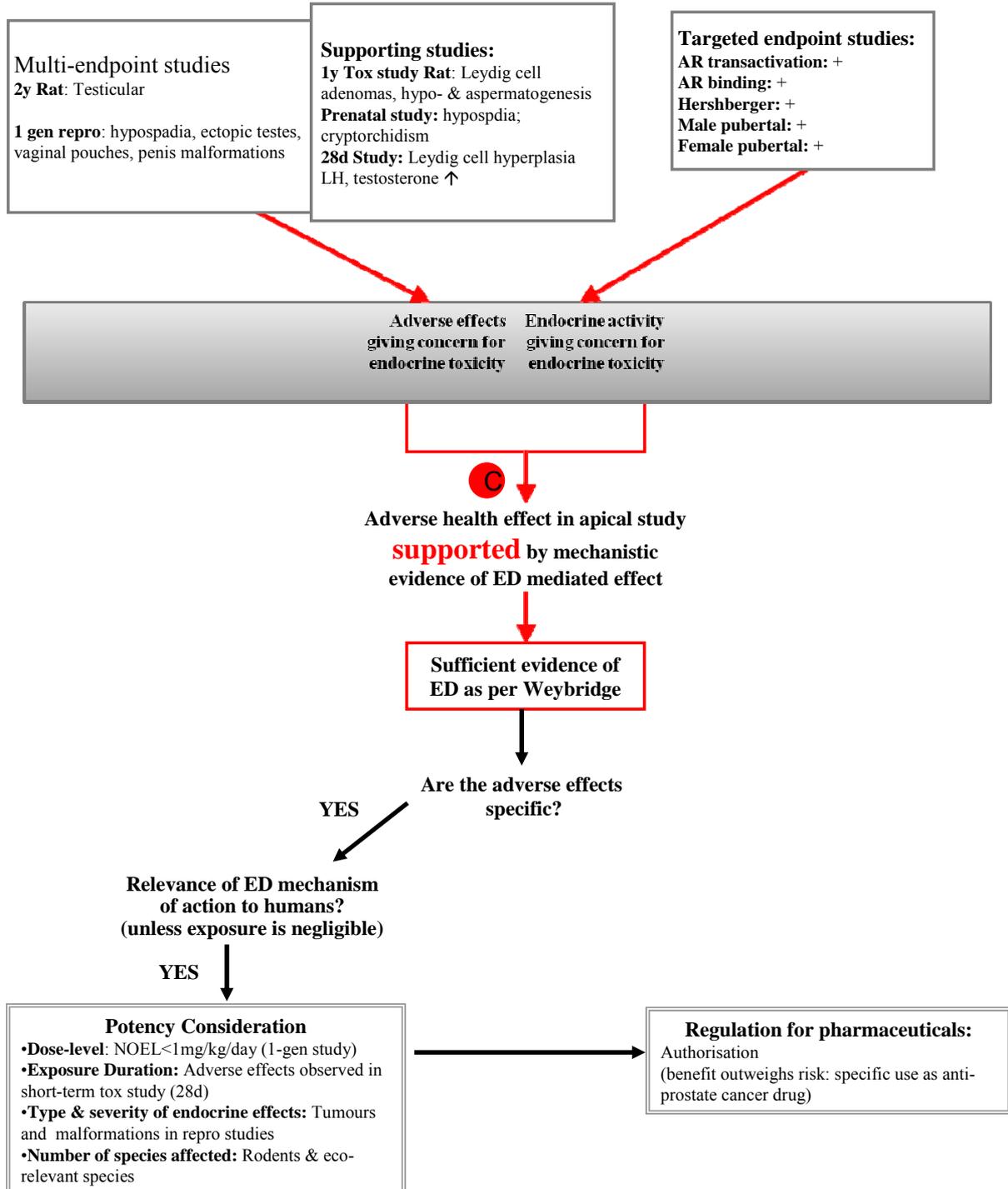


Table 17: Flutamide - Toxicity database focused on mammalian ED effects

	Study	Species	Adverse effect or endocrine activity giving concern for endocrine toxicity	NO(A)EL	General findings	NO(A)EL	Reference
Apical	2-year carcinogenicity	Rat	Leydig cell tumours.	?			Capen, 2001
	1-generation study	Rat	Nipple retention, hypospadias, vaginal pouches, penis malformation, unilateral ectopic testis, decrease of organ weights	0.6 mg/kg/d (NOEL)			Miyata <i>et al</i> , 2002
Supporting studies	1-year toxicity	Rat	Leydig cell adenomas, hypo- and aspermatogenesis, atrophy of seminal vesicles and prostate	<300 mg/kg/d	Increased liver, adrenal and spleen weights	?	Physicians Desk Review, 1994
	Prenatal exposure study (GD12-GD21)	Rat	Hypospadias; cryptorchidism; agenesis of the prostate, epididymis, and <i>vas deferens</i> ; degeneration of the seminiferous epithelium; and interstitial cell hyperplasia of the testis	<100 mg/kg			Mylchreest <i>et al</i> , 1999
	28 days	Rat	SAT weight decreases, testosterone and LH increases, Leydig cell hyperplasia	1 mg/kg/d			Rouquié <i>et al</i> , 2009
Targeted endpoints (<i>in vivo</i>)	Prepubertal	Rat female	VO delayed, extended dioestrus, decreased ovarian weight; TSH, T3 increased	5 mg/kg/d			Kim <i>et al</i> , 2002
		Rat male	SAT weight decreases, testosterone and oestradiol increases	1 mg/kg/d			Shin <i>et al</i> , 2002
	Hershberger assay	Rat	SAT weight decreases	<0.1 mg/kg/d			Owens <i>et al</i> , 2006
Targeted endpoints (<i>in vitro</i>)	AR trans-activation assay and AR binding assay		Significant inhibition of response to R-1881 in transactivation assay; weak affinity for AR in binding assay	10 ⁻⁸ M (NOEC for trans-activation); IC ₅₀ >10µM			Charles <i>et al</i> , 2005

5.1.3d Scenario C - Genistein: Flow chart for effects in mammals

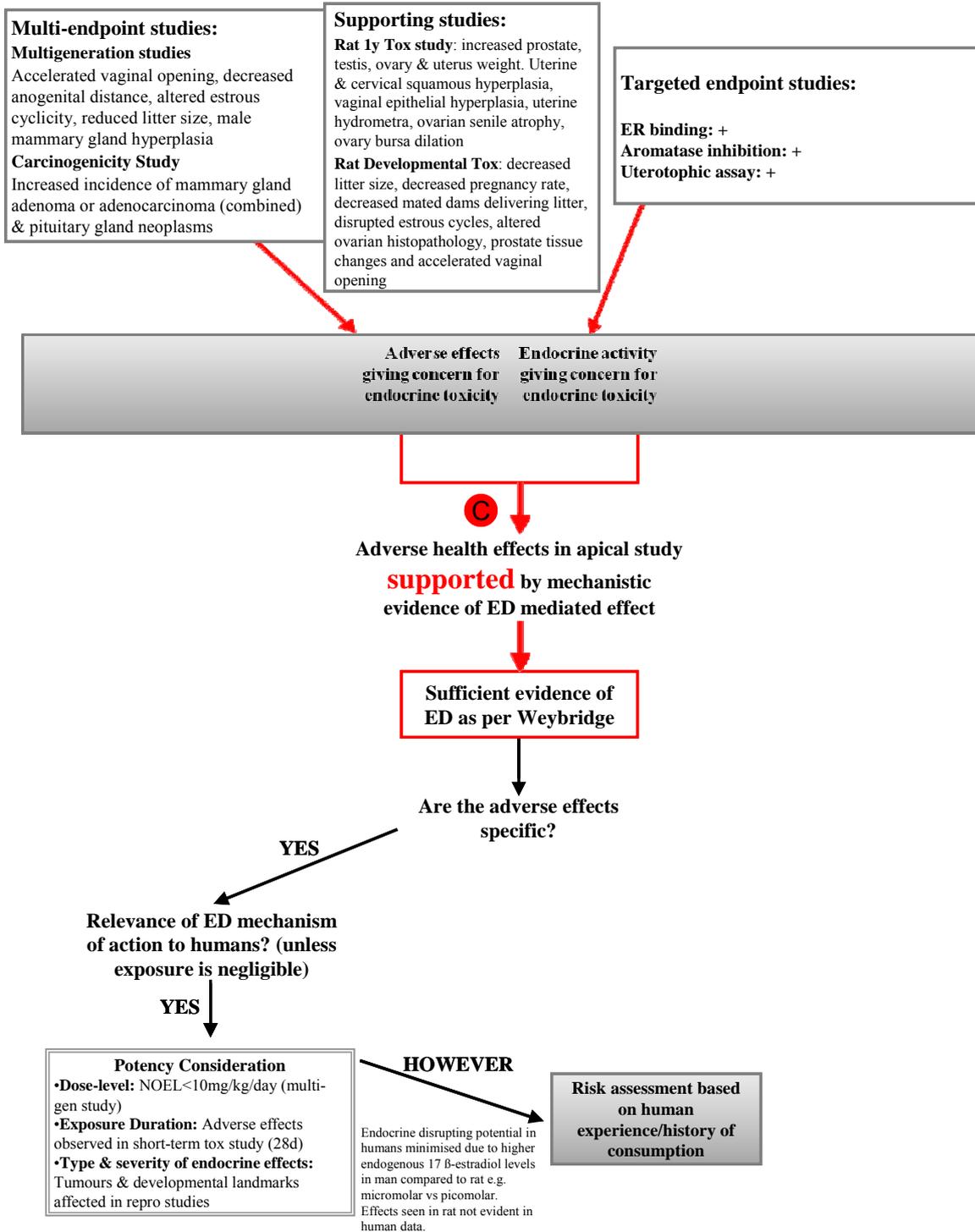


Table 18: Genistein - Toxicity database focused on mammalian ED effects

Study	Species	Adverse effect or endocrine activity giving concern for endocrine toxicity	NO(A)EL	Other effects (not endocrine activity or toxicity)	NO(A)EL	Reference
Apical / definitive multi-endpoint	Multi-generation study	Rat Accelerated vaginal opening, decreased anogenital distance, altered oestrous cyclicity, reduced litter size, male mammary gland hyperplasia	7-10 mg/kg/d (LOEL)	Reduced body weight		NTP, 2008a
	Carcinogenicity	Rat Mammary gland adenoma or adenocarcinoma Positive trend for increased mammary tumour incidence (5-500ppm) in female rats and pituitary gland adenoma / carcinoma in female rats	500 ppm male rats			NTP, 2008b
Supporting studies	Developmental toxicity studies	Rat Decreased litter size, pregnancy rate, mated dams delivering. Disrupted oestrous cycles, altered ovarian histopathology, prostate tissue changes and accelerated vaginal opening	35 mg/kg/d (LOAEL)			Rozman <i>et al</i> , 2006
	1-year toxicity study	Rat Increased prostate, testis, ovary and uterus weight. Uterine and cervical squamous hyperplasia, vaginal epithelial hyperplasia, uterine hydrometra, ovarian senile atrophy and ovary bursa dilation	NOEL not identified	Minimal bile duct proliferation and increased γ -GT	50 mg/kg/d	McClain <i>et al</i> , 2006
<i>In vivo</i> (targeted)	Uterotrophic assay	Rat	Increased uterine weight			Kanno <i>et al</i> , 2003
<i>In vitro</i> (targeted)	ER receptor binding assay		Positive			Rozman <i>et al</i> , 2006
	Aromatase assay		Aromatase inhibition			Aldlercreutz <i>et al</i> , 1993
Human data						Rozman <i>et al</i> , 2006 COT, 2003

5.1.3e Scenario C - Zearalenone: Flow chart for effects in mammals

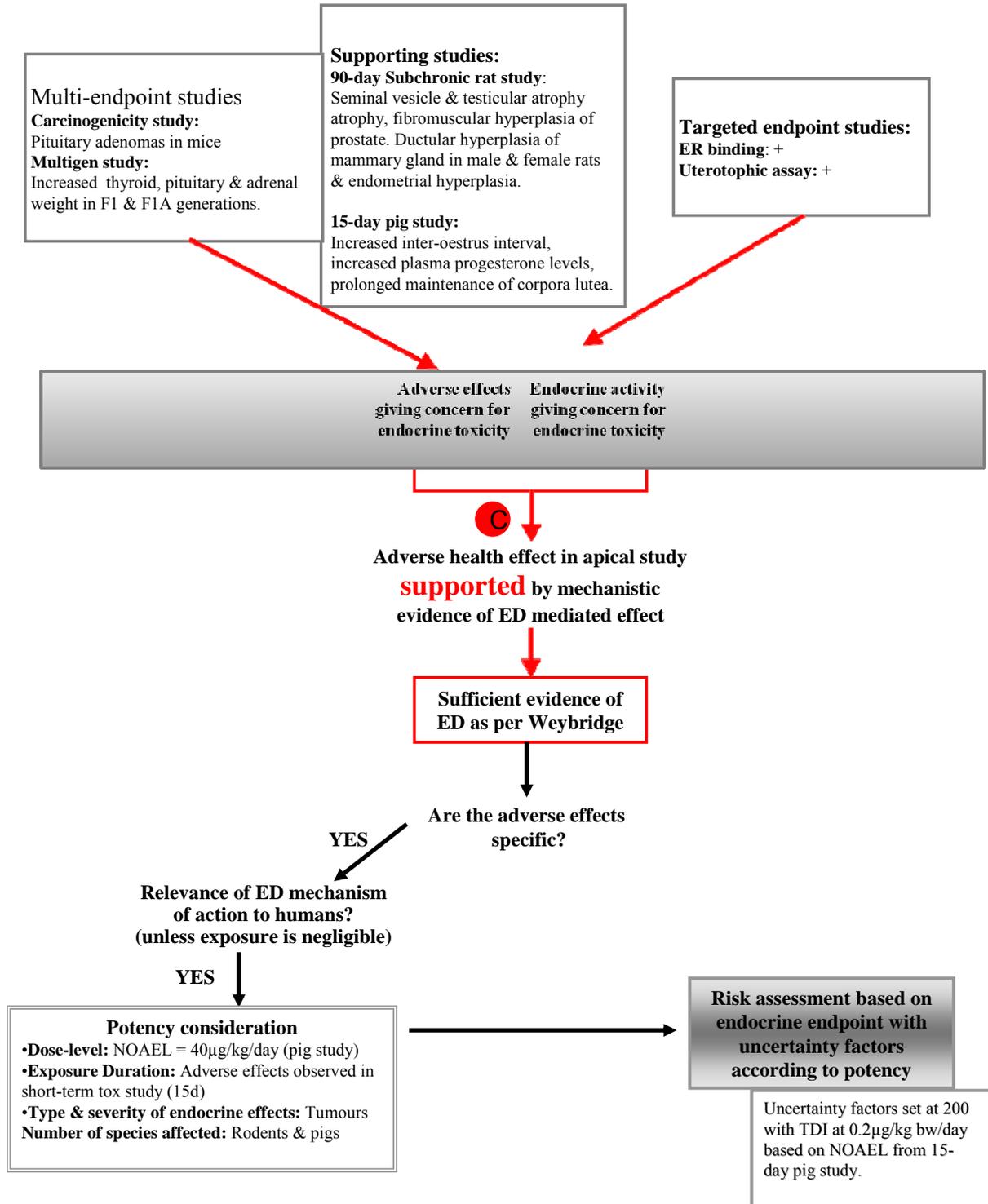


Table 19: Zearalenone - Toxicity database focused on mammalian ED effects

Study	Species	Adverse effect or endocrine activity giving concern for endocrine toxicity	NO(A)EL	Other effects (not endocrine activity or toxicity)	NO(A)EL	Reference
Apical / definitive multi-endpoint	Carcinogenicity	Mouse	Pituitary adenomas	7-10 mg/kg bw/d but positive trend for tumours across all dose levels	Hepatocellular adenomas	NTP, 1982
	Multi-generation study	Rat	Increased thyroid, pituitary and adrenal weights	0.1 mg/kg bw/d	Increased medullary trabeculation of femur	Becci <i>et al</i> , 1982
Supporting studies	90-day study	Rat	Testicular and seminal vesicle atrophy, fibromuscular hyperplasia of prostate. Ductular hyperplasia of mammary gland and endometrial hyperplasia. Osteopetrosis	300 ppm for males. No NOAEL for females		NTP, 1982
	90-day study	Mouse	Testicular and seminal vesicle atrophy, cytoplasmic vacuolisation of adrenal, squamous metaplasia of prostate and endometrial hyperplasia. Osteopetrosis	30 ppm		NTP, 1982
	15-day study	Pig	Increased inter-oestrus interval, increased plasma progesterone levels, prolonged maintenance of <i>corpora lutea</i>	40 µg/kg bw/d		Edwards <i>et al</i> , 1987
<i>In vivo</i> (targeted)	Uterotrophic effect	Rat / Mouse	Increased uterine weight			Christensen <i>et al</i> , 1965 Ueno <i>et al</i> , 1974
<i>In vitro</i> (targeted)	Binding to oestrogen receptor		Affinity for ER in binding assay			Kuiper <i>et al</i> , 1998
Regulatory risk assessment						SCF, 2000

5.1.4 Scenario D - 1,3-dinitrobenzene: Flow chart for effects in mammals

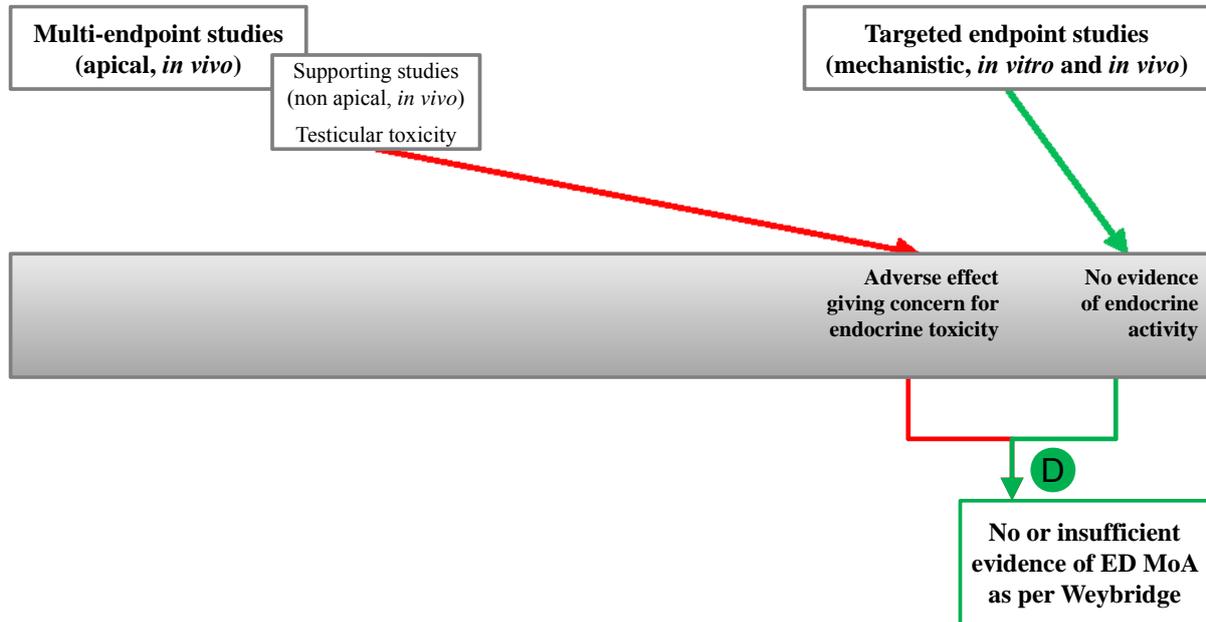


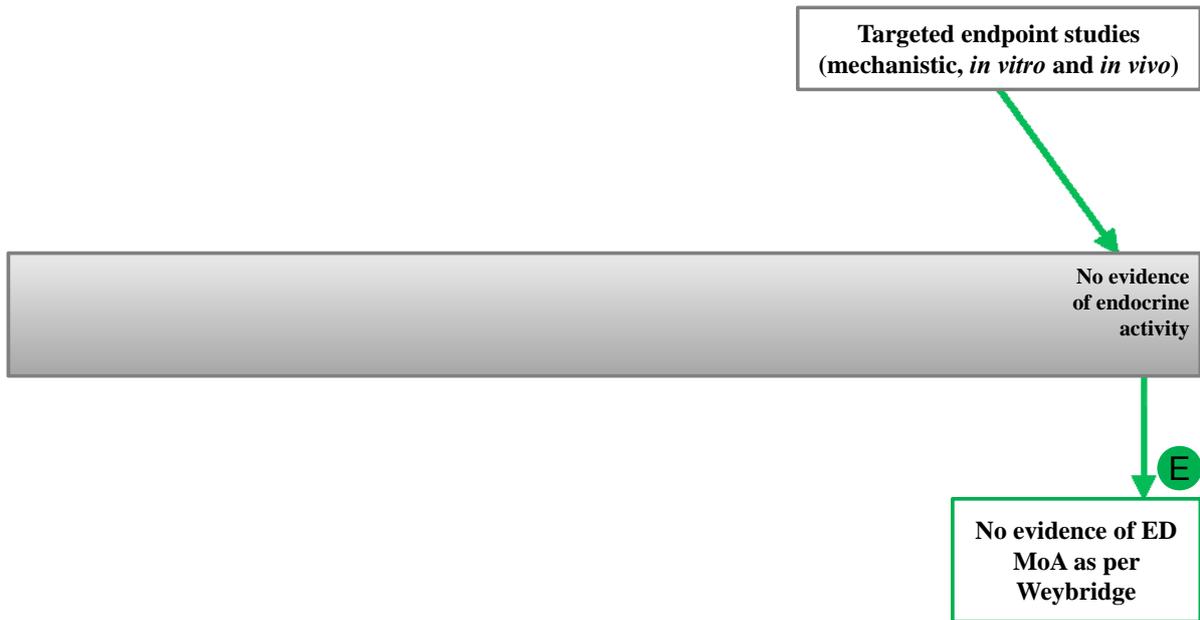
Table 20: 1,3-dinitrobenzene - Toxicity database focused on mammalian ED effects

	Study	Species	Adverse effect or endocrine activity giving concern for endocrine toxicity	NO(A)EL	General findings	NO(A)EL	Reference
Apical	None conducted						
Supporting studies	16 weeks and 8 weeks	Rat	Decreased testicular weight; decreased spermatogenesis				Cody <i>et al</i> , 1981
	4 weeks	Rat	Reduced testicular and epididymal weight; macroscopic atrophy; decreased number of spermatocytes; degeneration/necrosis; giant cell formation; vacuolation; reduced sperm counts				Irimura <i>et al</i> , 2000
	7 days	Rat	Degenerating round spermatids; increased cellular debris; epithelial cell vacuolisation; sperm head malformations; genes associated with ubiquitin-proteasome pathway affected				Tengowski <i>et al</i> , 2007
	4 days	Rat	Sertoli cell vacuolation; germ cell degeneration, giant multinucleated cells. No effects on plasma testosterone concentrations				LRI-EMSG46, 2008

Table 20: 1,3-dinitrobenzene - Toxicity database focused on mammalian ED effects (cont'd)

	Study	Species	Adverse effect or ED activity giving concern for ED toxicity	NO(A)EL	General findings	NO(A)EL	Reference
Targeted endpoints	<i>In vitro</i> steroidogenesis		No effects on testosterone secretion				LRI-EMSG46, 2008
	ER binding		Negative (theoretical)				
	AR binding		Negative (theoretical)				
	Aromatase		Negative (theoretical)				
	Uterotrophic assay		Negative (theoretical)				
	Hershberger assay		Negative (theoretical)				
	Pubertal male		Negative (theoretical)				
	Pubertal female		Negative (theoretical)				

5.1.5 Scenario E - Theoretical example



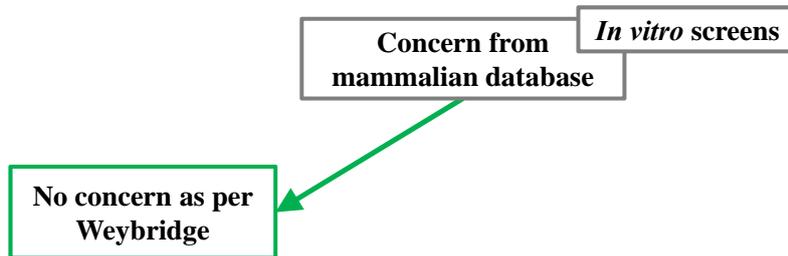
In the absence of all other data, negative outcomes in an exhaustive combination of *in vitro* / *in vivo* targeted endpoint studies can be taken as evidence of the absence of endocrine disrupting properties.

5.2 Ecotoxicology charts: Case studies

The case studies for eco examples do not follow exactly the same structure as for the toxicology examples because each scenario (i.e. A to E) does not necessarily apply to environmental species. Hence examples are presented for the same compounds as the toxicology scenarios below where data are available.

5.2.1 Glyphosate

- Flow chart for effects in fish and amphibians



- Flow chart for effects in birds and mammals

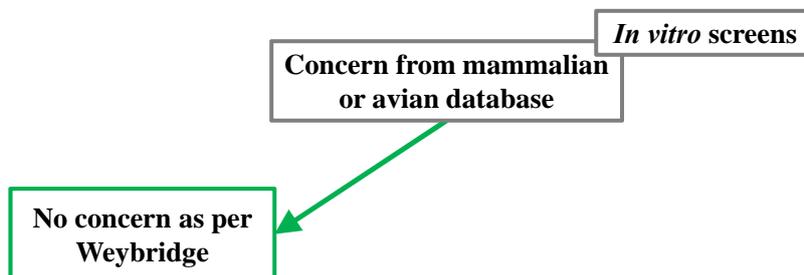


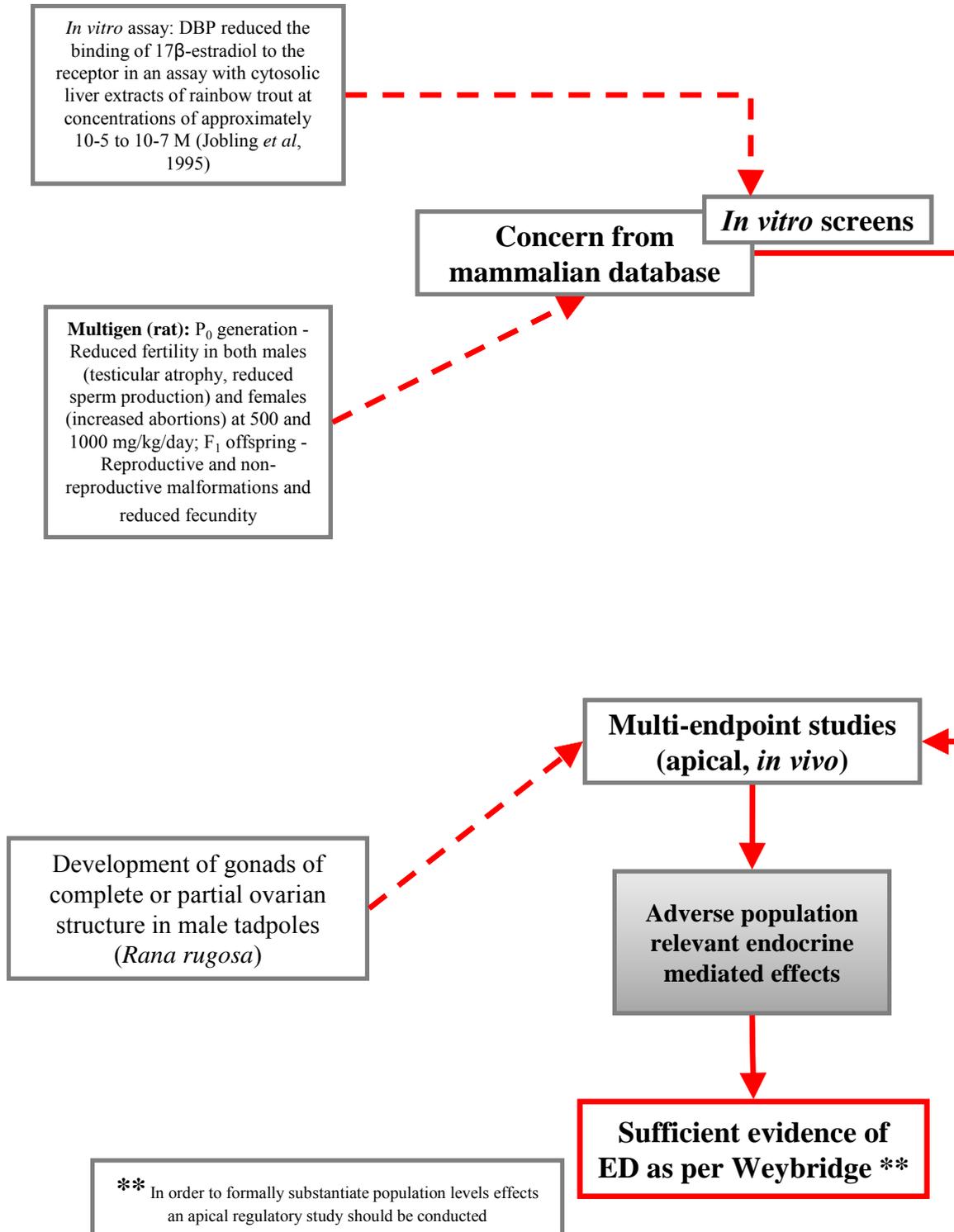
Table 21: Glyphosate : Avian toxicity database

Species, study type and route	NOAEL		LOEL
	mg/kg	mg/kg	Observed toxicity
Mallard duck, Reproduction dietary (EPA FIFRA Sub-division E, Guideline 71-4; OECD 206)	2250 (HDT)	-	No effects observed on eggs laid, eggs damaged/laid, eggs set, viable embryos, live 3 week embryos, hatchlings, 14-day survivors, eggs laid/female, eggs laid/female/day, 14-day survivors/female or egg shell thickness
Northern bobwhite, Reproduction dietary (EPA FIFRA Sub-division E, Guideline 71-4; OECD 206)	2250 (HDT)	-	No effects observed on eggs laid, eggs damaged/laid, eggs set, viable embryos, live 3 week embryos, hatchlings, 14-day survivors, eggs laid/female, eggs laid/female/day, 14-day survivors/female or egg shell thickness

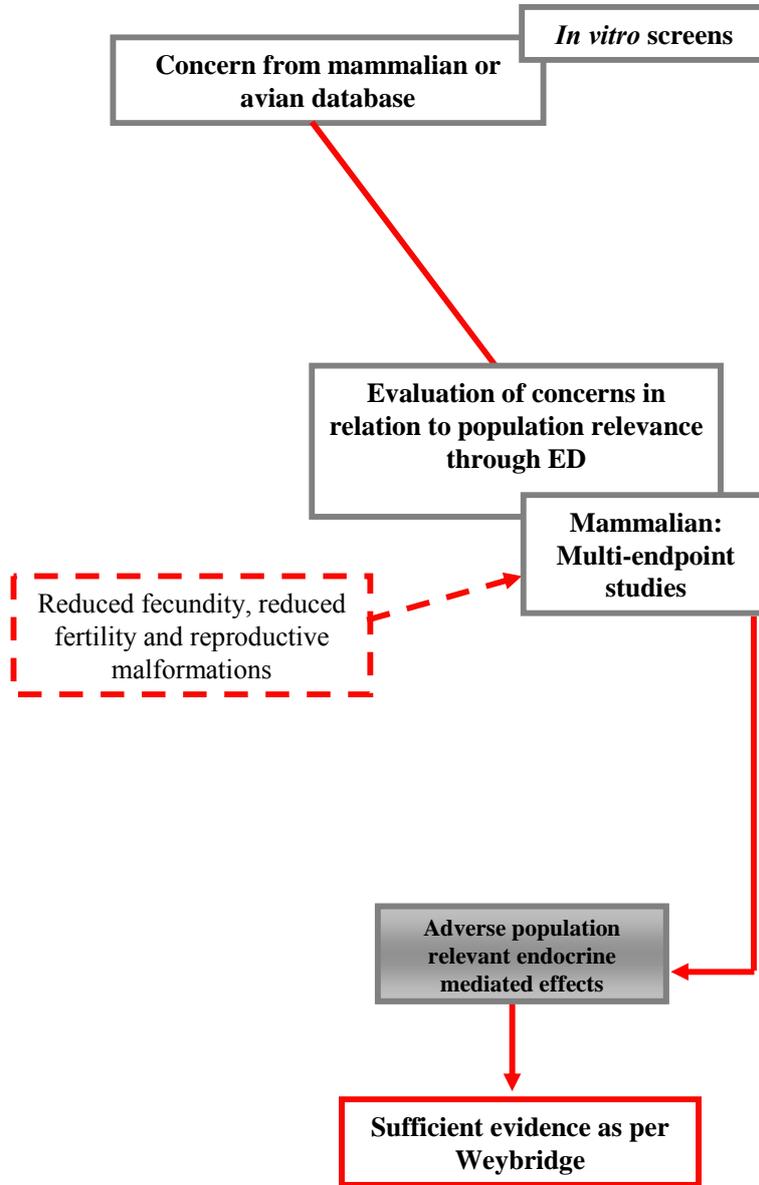
HDT = highest dose tested; NOAEL = no observed adverse effect level; LOAEL = Lowest observed adverse effect level

5.2.2 Dibutyl phthalate

- Flow chart for effects in fish and amphibians



- Flow chart for effects in birds and mammals



- Potency assessment

Endocrine disruption is established for the mammalian risk assessment. For ecotoxicology, in the absence of a full regulatory package of data and considering the effects observed in toxicological studies DBP should be considered an endocrine disrupter and the risk assessment performed accordingly. However, it is likely additional regulatory ecotoxicological studies would be required (particularly in fish) to fully address the effects observed in the non-standard studies.

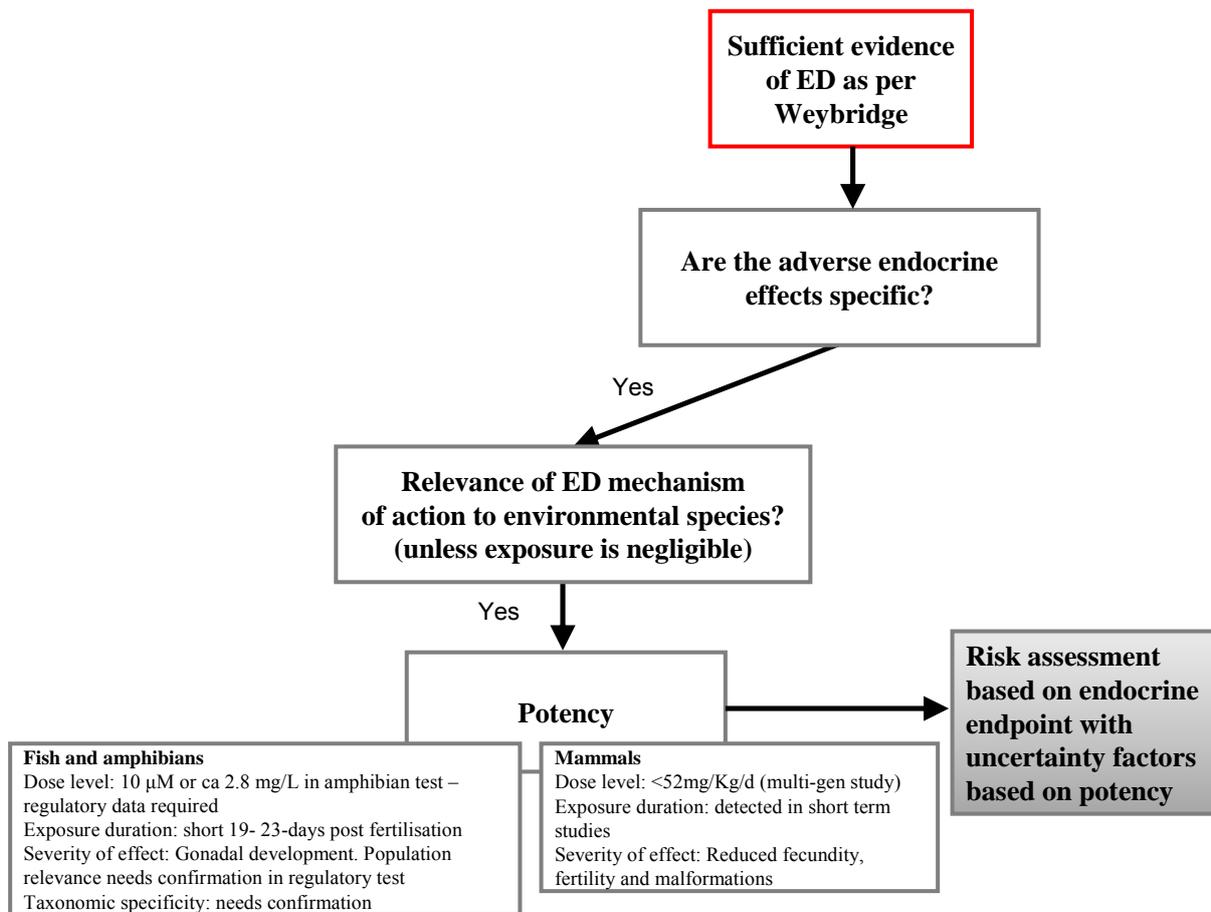
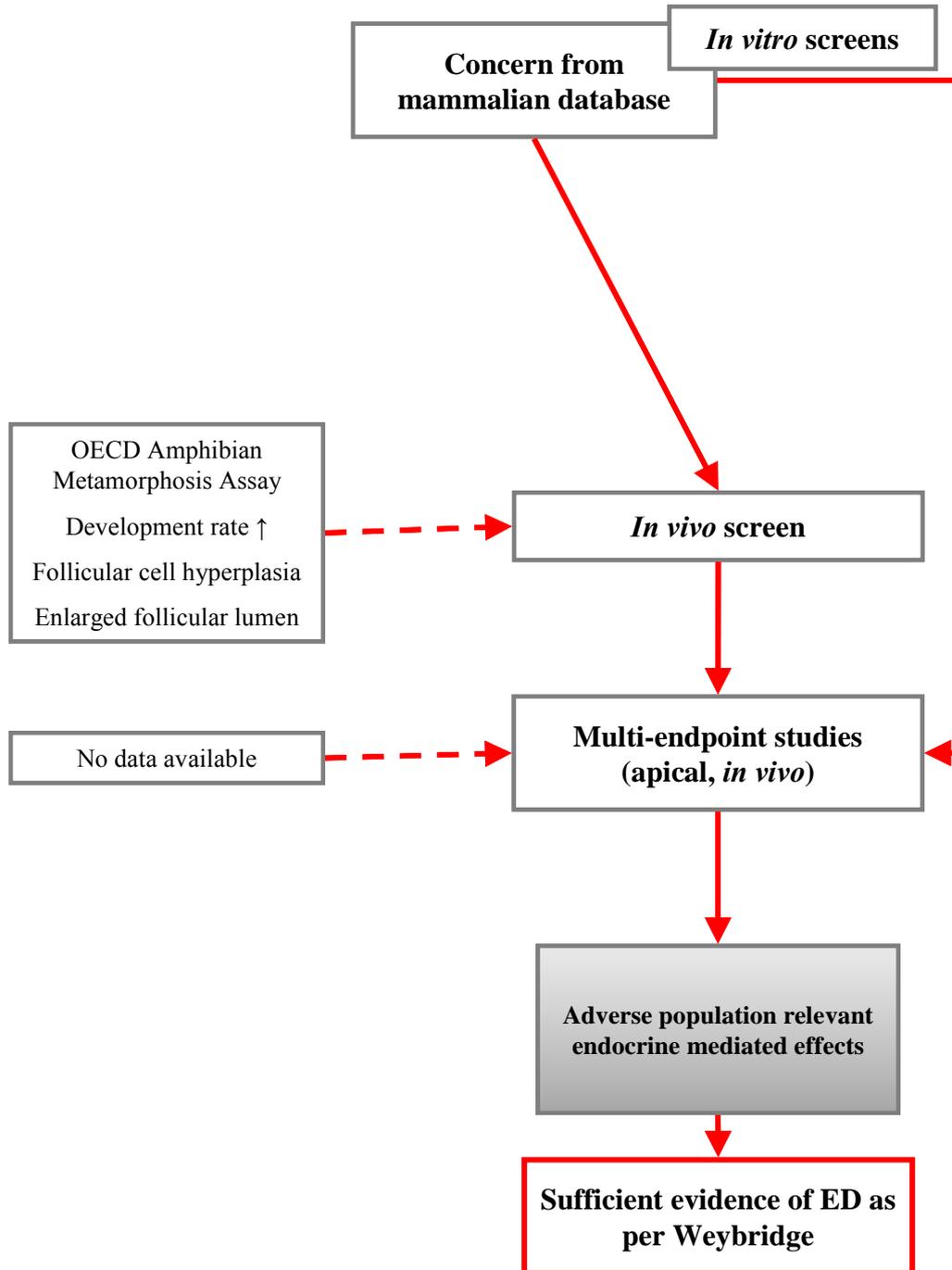


Table 22: Dibutyl phthalate: Amphibian and fish toxicity database

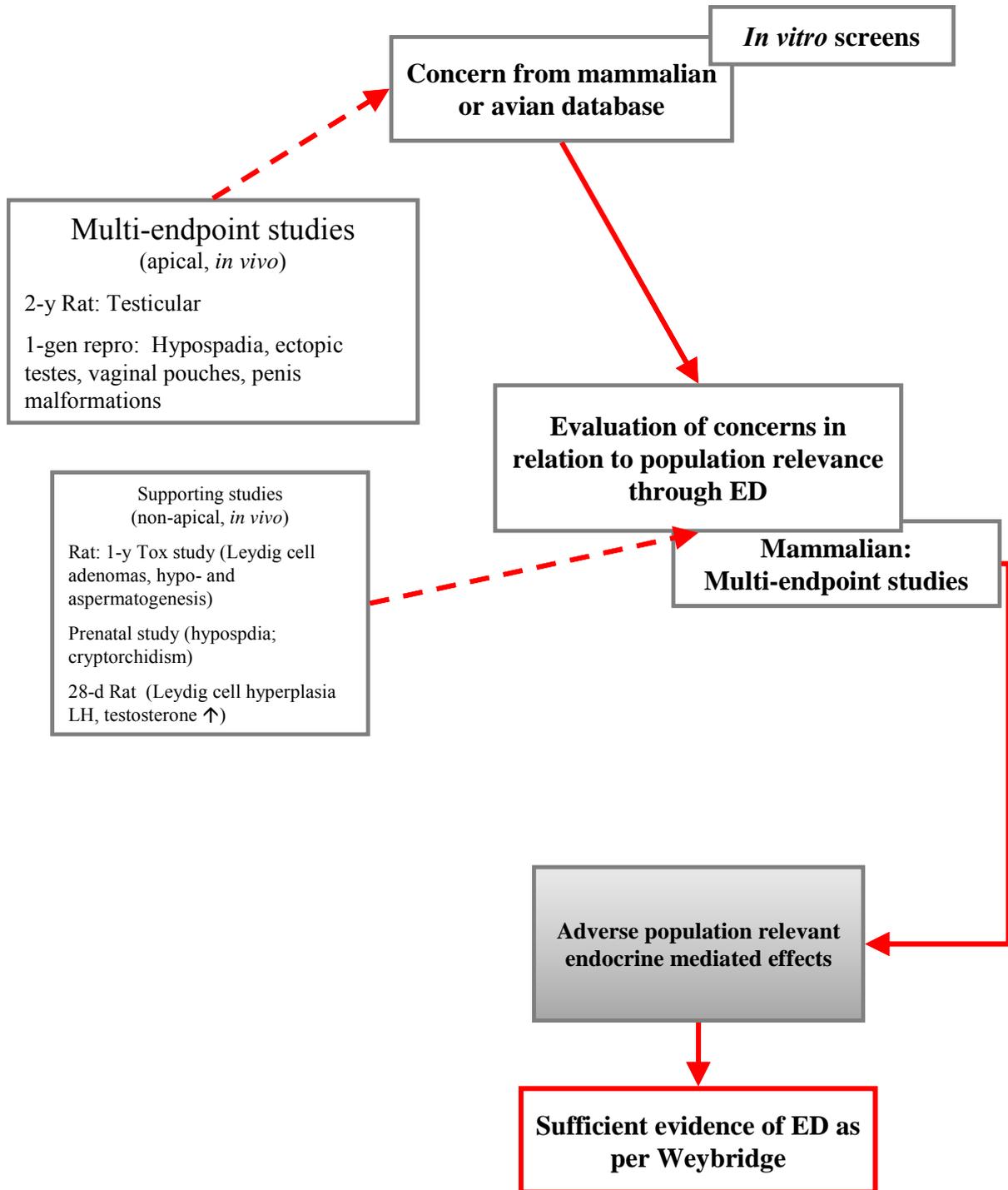
Study	Species	Adverse effect or endocrine activity giving concern for endocrine toxicity	NO(A)EL	Other effects (no endocrine activity or toxicity)	NO(A)EL	Reference
Apical / definitive multi-endpoint	Exposure of male tadpoles during days 19-23 post-fertilisation	Male tadpoles (<i>Rana rugosa</i>)	Development of gonads of complete or partial ovarian structure	Up to 17% of tadpoles at 10 µM DBP, cf. 100% of tadpoles at 1 µM of 17B-oestradiol	-	Ohtani <i>et al</i> , 2000
Supporting studies	<i>In vitro</i> screening assays	<i>Xenopus laevis</i> tadpoles	T3-antagonistic activity: IC ₅₀ : 39 ± 1 µM		-	Sugiyama <i>et al</i> , 2005
	<i>In vivo</i> screening assays	<i>Xenopus laevis</i> tadpoles		No significant effect at 4 µM	-	Sugiyama <i>et al</i> , 2005
<i>In vitro</i> (targeted)	Fish oestrogen receptor binding assay	Rainbow trout	Binding to fish oestrogen receptor	Reduced binding in an assay with cytosolic liver extracts at concentrations of approximately 10 ⁻⁵ to 10 ⁻⁷ M	-	Jobling <i>et al</i> , 1995
Concern from mammalian database	See toxicology					

5.2.3 Phenobarbital

- Flow chart for effects in fish and amphibians



- Flow chart for effects in birds and mammals



- Potency assessment

Endocrine disruption is established for the mammalian risk assessment. However, for the risk assessment based on endocrine endpoints definitive apical data from regulatory aquatic ecotoxicology studies would be required.

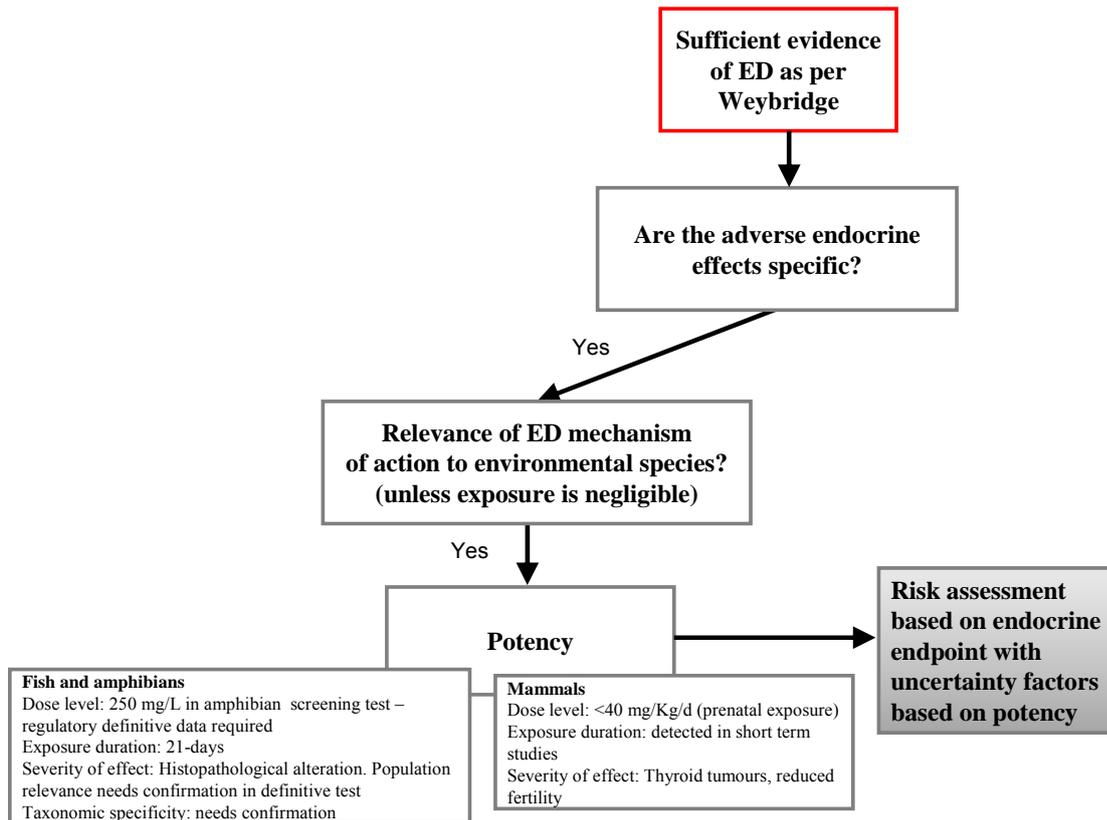


Table 23: Phenobarbital - Amphibian metamorphosis database

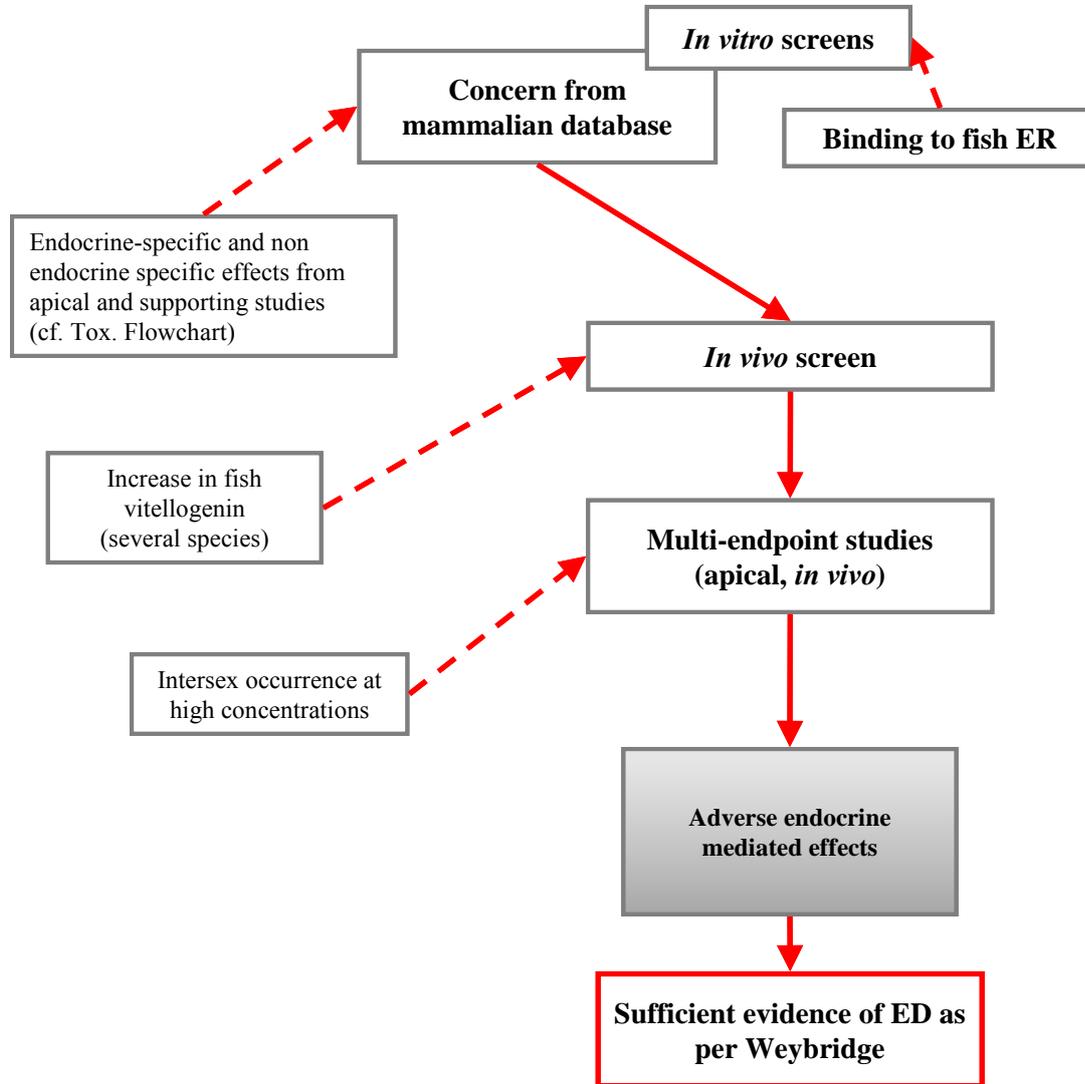
	Study	Species	Adverse effect or endocrine activity giving concern for endocrine toxicity	NOEC	General findings	NOEC	Reference
Screening	Amphibian metamorphosis assay	<i>Xenopus laevis</i>	Accelerated development rate, follicular cell hyperplasia and enlarged follicular lumen	N/A ^a	N/A ^a	N/A ^a	US EPA, 2007
Multi-endpoint apical study	N/D ^b						
Concern from mammalian database	See toxicology database						

^a N/A: Not applicable.

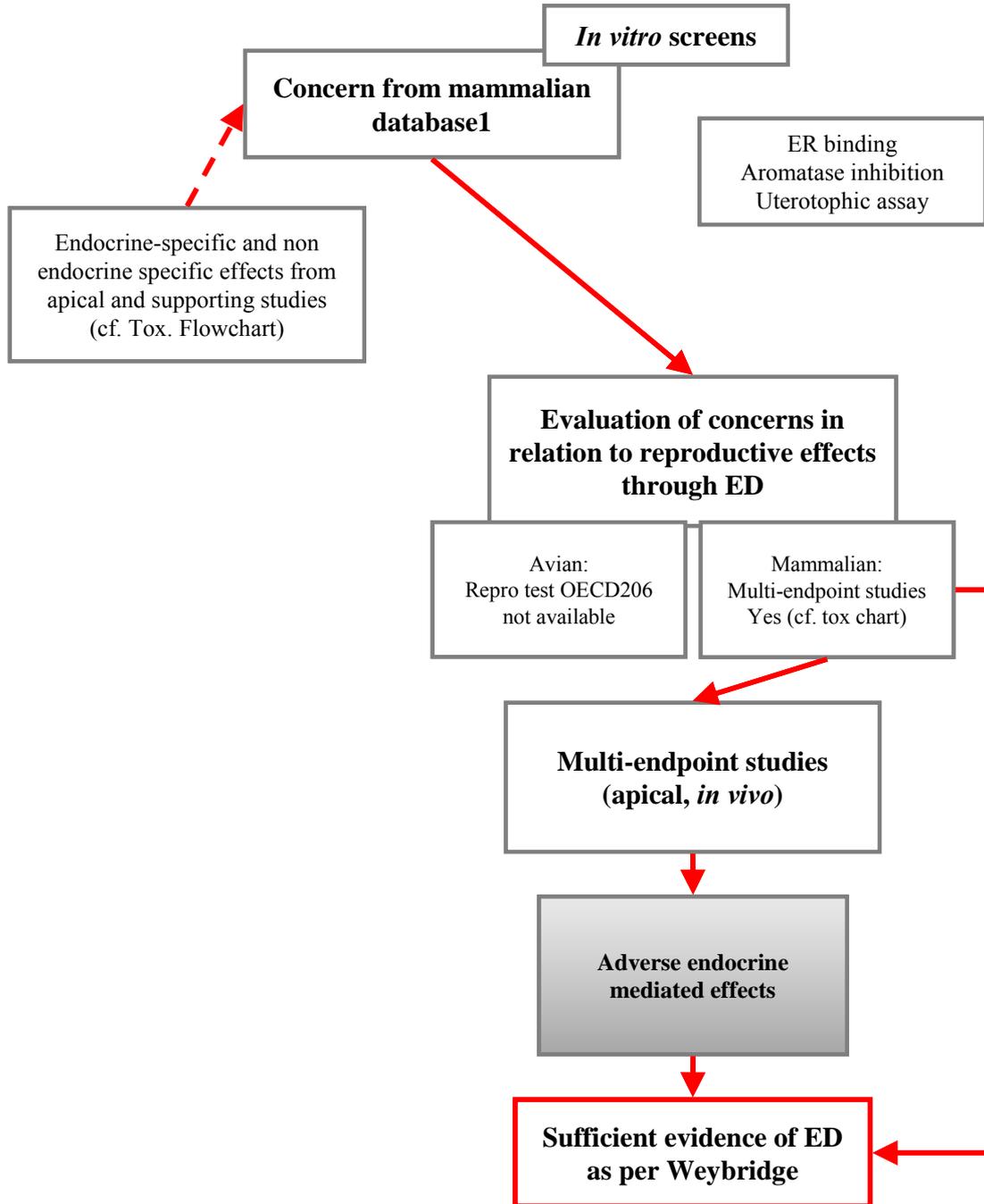
^b N/D: No study available.

5.2.4 Genistein

- Flow chart for effects in fish and amphibians



- Flow chart for effects in birds and mammals



- Potency assessment

Endocrine disruption is established for the mammalian risk assessment. For ecotoxicology, the risk assessment based on endocrine endpoints requires definitive apical data from regulatory aquatic ecotoxicology studies. Note that the population relevance of findings in the fish apical study presented is currently unknown.

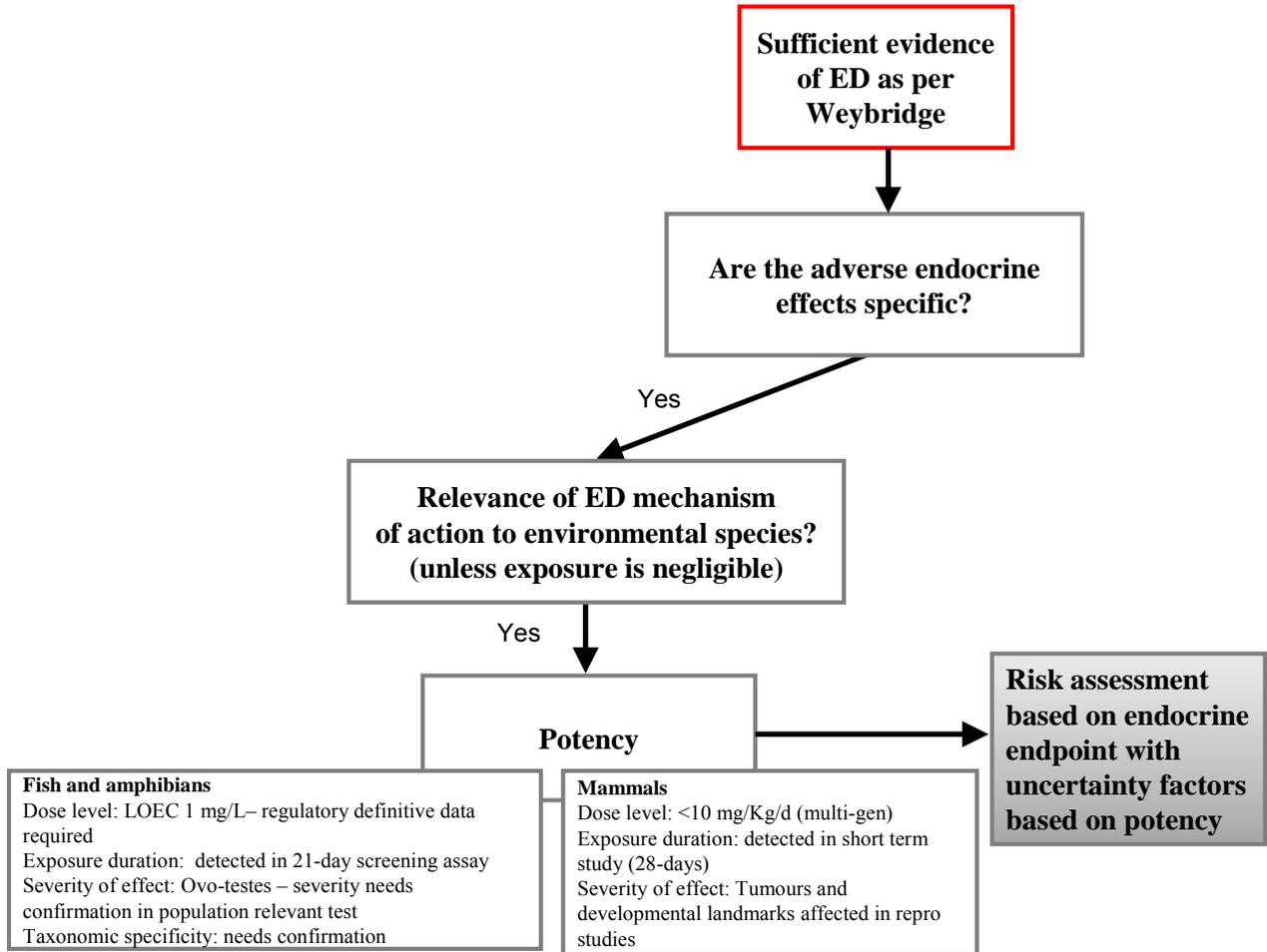
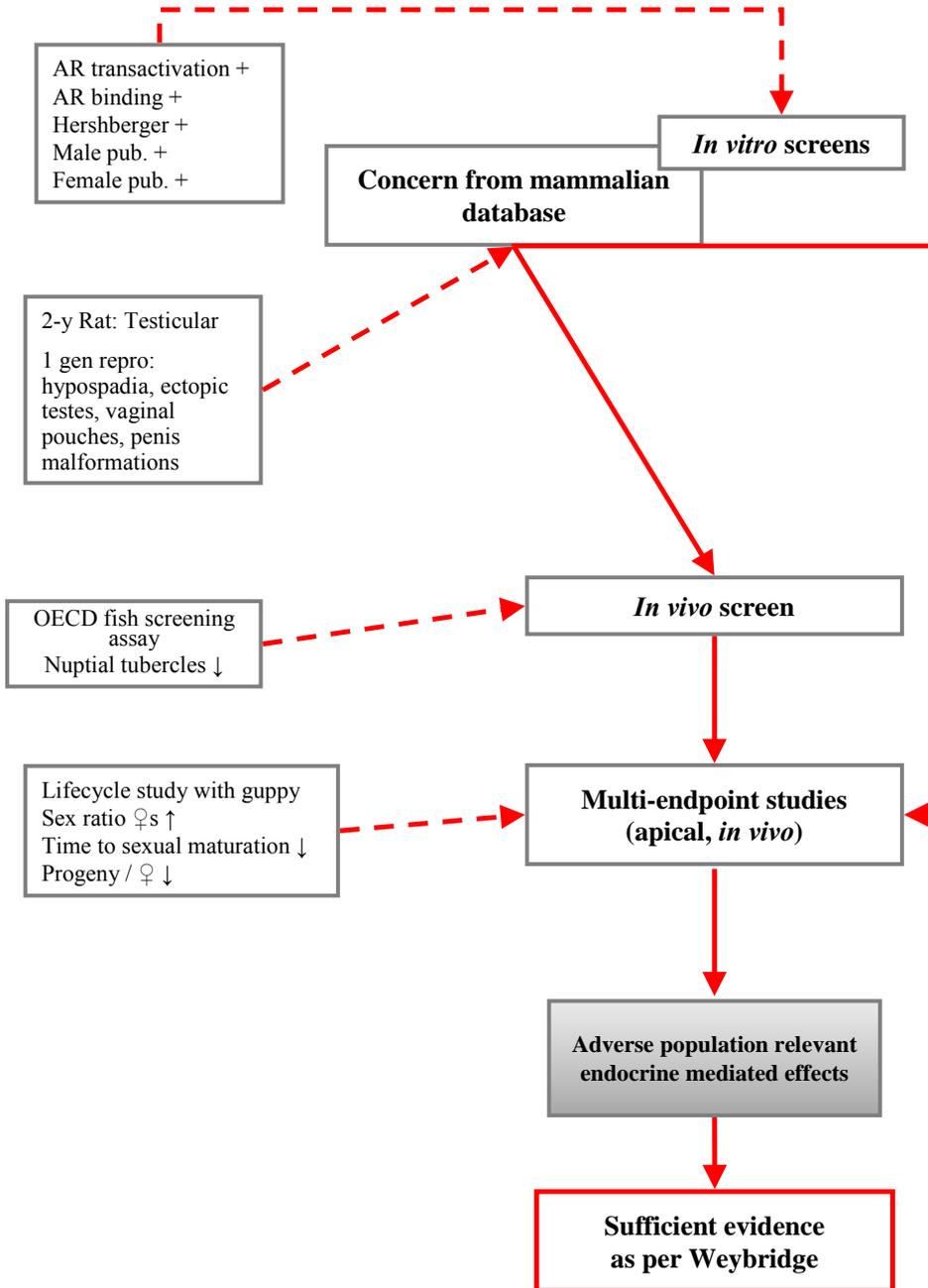


Table 24: Genistein: Toxicity database

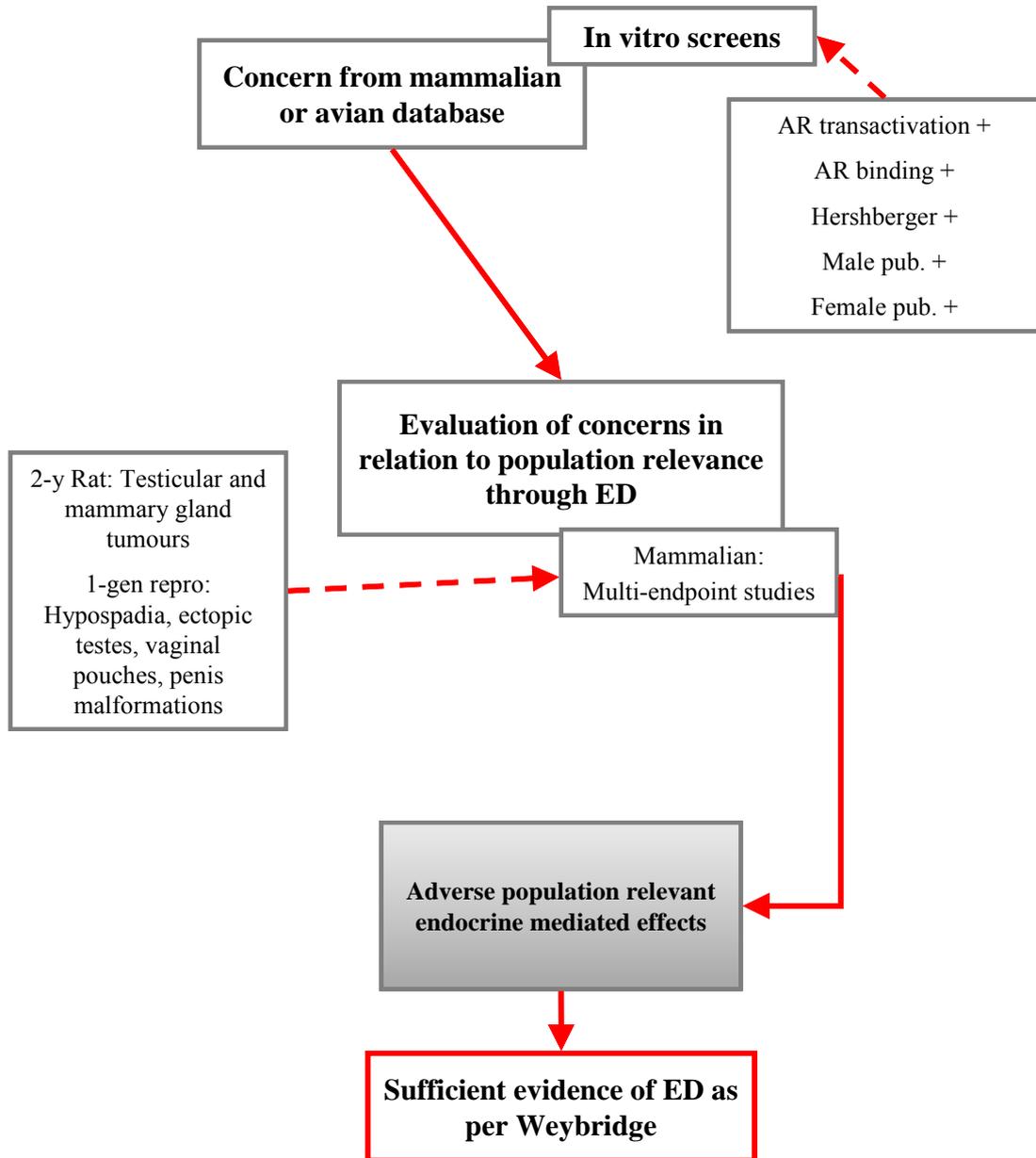
Study	Species	Effect giving concern for endocrine activity or toxicity	NO(A)EL	Other effects (not endocrine activity or toxicity)	NO(A)EL	Reference
Apical / definitive multi-endpoint	Extended fish early life stage test	Medaka	Intersex (12%), SSC, atretic oocytes	LOEC 1 mg/L		Kiparissis <i>et al</i> , 2003
Supporting studies	1-year dietary study	Rainbow trout	Decreased testosterone in males and increased vitellogenin	LOEL 500 mg/kg /diet	None	Bennetau-Pelissero <i>et al</i> , 2001
<i>In vivo</i> (targeted)	Fish endocrine screening assay	Fathead minnow	Vitellogenin	LOEC 70 µg/L	None	Panter <i>et al</i> , 2002
<i>In vitro</i> (targeted)	Fish oestrogen receptor binding assay	Fathead minnow and rainbow trout	Binding to fish oestrogen receptor	Ca. 1/100 compared to oestradiol		Denny <i>et al</i> , 2005
Concern from mammalian database	See toxicology					

5.2.5 Flutamide

- Flow chart for effects in fish and amphibians



- Flow chart for effects in birds and mammals



- Potency assessment

Endocrine disruption is established for the mammalian and fish risk assessment. Population relevant impacts observed in the fish full lifecycle would require clarification in a regulatory study with aqueous exposure.

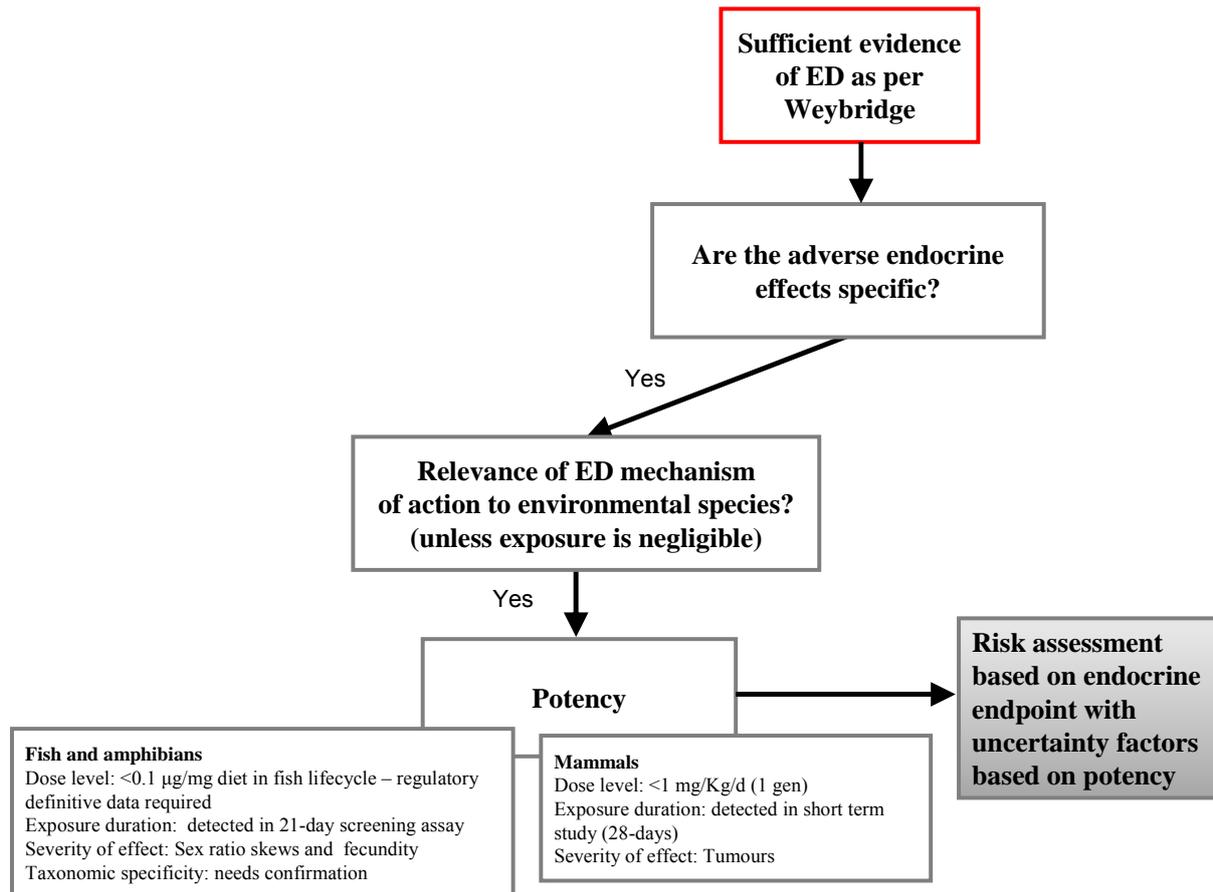


Table 25: Flutamide: Toxicology database

Study	Species	Adverse effect or endocrine activity giving concern for endocrine toxicity	NO(A)EL or NOEC	Other effects (not endocrine activity or toxicity)	NO(A)EL or NOEC	Reference	
Apical / definitive multi-endpoint	Fish life-cycle test (dietary exposure)	Guppy	Sex ratio skew (increase in females), decrease in time to sexual maturity and decrease in number of young/female	<0.1 µg/mg	Length	0.1 µg/mg	Bayley <i>et al</i> , 2002
Supporting assay	Reproduction assay	Fathead minnow	Elevated vitellogenin and E2 in males	62.7 µg/L	Fecundity, hatch success	62.7 µg/L	Jensen <i>et al</i> , 2004
	Adult 30-day exposure (dietary exposure)	Guppy	Decreased sperm count	<1 µg/mg	GSI decrease	<1 µg/mg	Baatrup and Junge, 2001
<i>In vivo</i> (targeted)	21-day fish screening assay	Fathead minnow	Decrease in male nuptial tubercles (secondary sex character)	N/A	-	-	Panter <i>et al</i> , 2004
<i>In vitro</i> (targeted)	See toxicology						
Concern from mammalian database	See toxicology						

5.3 Conclusion

These examples illustrate the applicability of this proposed approach to evaluate a spectrum of different chemicals for their endocrine disrupting potential in either mammalian or environmentally relevant species.

6. RISK ASSESSMENT / CHARACTERISATION

6.1 *Approaches for risk assessment*

Risk assessment, based on safety factors, relies on our current understanding of mechanisms of toxicity and assumes that there is a threshold for most types of toxic effects. The threshold is usually defined as a level of exposure below which no adverse effect is produced either because the substance has had no effect or because the body's homeostatic mechanisms have reversed any changes caused. Risk assessment is, therefore, dependent on the characterisation of the adverse effects observed in apical tests so that No Observed (Adverse) Effect Levels (NO(A)ELs), Lowest Observed Effect Levels (LOELs), No Observed (Adverse) Effect Concentrations (NO(A)ECs) or Lowest Observed Effect Concentrations (LOECs) can be identified. For the environmental assessment the protection goal is not at the individual level (as is the case for the human assessment) but rather at the population and community level. Consequently, effects on the individual are tolerated as long as no adverse effects on the population are predicted. This accepted approach to risk assessment has been, and continues to be applied to all manners of toxicity (neuro-, hepato-, cardiotoxicity) including endocrine toxicity.

However, this widely accepted approach for risk assessment, when applied specifically to endocrine toxicity, is being challenged despite the fact that endocrine toxicity is not a novel concept and endocrine mediated adverse effects and no effect levels are successfully detected and characterised in appropriate (eco)-toxicology tests (Appendix C). As shown in Tables C-1 – C-3, when focussing specifically on endocrine mediated toxicity, LOELs, LOECs, NO(A)ELs, NO(A)ECs can clearly be identified independent of the parameters examined:

- The model system used;
- the species investigated;
- the detailed endocrine molecular mechanism involved;
- the exposure duration or critical life stage under consideration;
- and finally the nature of endocrine disrupter agents under consideration (i.e. chemicals, dietary factors, physical stress) (Table C-4).

Nonetheless, despite evidence to the contrary such as that presented in Tables C-1 – C-4, several alternative concepts for endocrine mediated toxicity have been proposed: 1) there is no threshold for endocrine toxicity (Sheehan, 2006); 2) there are still effects at 'dose levels below the range typically used in toxicity studies' (Wade *et al*, 2003); 3) there are non-adverse or beneficial effects at dose levels below the NO(A)EL or NOEC according to the concept of hormetic dose-response relationship (Calabrese, 2004).

These various concepts are part of an ongoing and controversial debate within the field of pharmacology and toxicology which is not unique to endocrine toxicity but instead embraces all forms of toxicity (e.g. liver, kidney, central nervous system) which are mediated by similar molecular targets (cell receptors, enzymes, transporters, etc) and use similar molecular pathways (e.g. cell proliferation/apoptosis). In any case the ongoing debate on these concepts is far from being resolved (Ashby *et al*, 2004b; Mushak, 2007) and unless the current risk assessment paradigm for chemical toxicity in general is modified due to new and well established scientific evidence it would be inconsistent to change it specifically for the field of endocrine mediated toxicity.

7. CONCLUSION

This ECETOC technical report provides guidance for the identification of endocrine disrupting effects by integrating available sets of (eco)toxicity data, including *in vitro* and *in vivo* targeted studies, supporting studies and multi-endpoint apical studies. A framework is provided, which can be used to determine whether a chemical meets the specific scientific criteria (molecular targets and a physiological response which drive an adverse effect) to be considered an endocrine disrupter and which takes into account the specificity, relevance and potency of the adverse effects. Concepts of human or population relevance are incorporated for the human and environmental assessments, respectively. A number of worked examples illustrate the concepts elaborated by the task force.

The report remit has been limited to hazard considerations whilst paying little attention to exposure considerations. However, the risk that chemicals pose to human health and the environment cannot be based simply on an evaluation of hazard but instead should take into consideration all available scientific data so as to adequately characterise risk based on hazard characteristics, dose-response considerations and exposure data. The issues of chemical classification and risk assessment of endocrine disrupters should be addressed in a subsequent ECETOC task force.

ABBREVIATIONS

ACSA	Agricultural chemical safety assessment
AED	Antiepileptic drugs
AGD	Anogenital distance
AR	Androgen receptor
bw	Bodyweight
DBP	Dibutyl phthalate
DNA	Deoxyribonucleic acid
DNB	Dinitrobenzene
dph	Days post hatch
ED	Endocrine disrupter
EDSP	Endocrine Disrupter Screening Program
EDSTAC	Endocrine Disrupters Screening and Testing Advisory Committee
EDTA	Endocrine Disrupters Testing and Assessment Task Force
EE2	Ethinylestradiol
ER	Oestrogen receptor
ESD	Environmental sex determination
F ₀	Parental generation
F ₁	First generation of offspring
F ₂	Second generation of offspring
F ₃	Third generation of offspring
FSA	Fish screening assay
GLP	Good Laboratory Practice
GSD+TE	Genotypic sex determination mechanism influenced by temperature
HDT	Highest dose tested
HPG	Hypothalamic-pituitary-gonadal axis
HPT	Hypothalamic-pituitary-thyroid axis
IPCS	International Programme for Chemical Safety
LO(A)EC	Lowest observed adverse effect concentration
LO(A)EL	Lowest observed adverse effect level
LOEC	Lowest observed effect concentration
LOEL	Lowest observed effect level
MoA	Mode of action

NO(A)EC	No observed adverse effect concentration
NO(A)EL	No observed adverse effect level
NOEC	No observed effect concentration
NP	Nonylphenol
OECD	Organisation for Economic Co-operation and Development
PGC	Primordial germ cell
PND	Post natal day
QSAR	Quantitative structure activity relationship
REACH	Registration, evaluation, authorisation and restriction of chemicals
SD	Sex determination
SPC	Steroid producing cell
SVHC	Substance of very high concern
T ₄	Thyroxine
TSD	Temperature-dependent sex determination
TSH	Thyroid stimulating hormone
TSP	Thermosensitive period
US EPA	United States Environmental Protection Agency
VTG	Vitellogenin

GLOSSARY

Apical study	Highest level regulatory study to identify and characterise the eco/toxicological adverse effect(s) on form and/or function in an organism or population. For example the two-generation rat (OECD 416), chronic carcinogenicity toxicity (OECD 453) or fish full life-cycle studies.
Targeted study (<i>in vitro</i>)	Study designed to identify a potential intrinsic activity at the cellular or molecular target level. For example hormone receptor binding and steroidogenesis assays.
Targeted study (<i>in vivo</i>)	Study designed to identify potential activity at the whole animal level. May provide evidence of endocrine activity <i>in vivo</i> though does not demonstrate endocrine disruption <i>per se</i> . May provide appropriate information of an endocrine mode of action. For example Hershberger and 21-day fish endocrine screening assays.
Adverse effect	A biochemical change, functional impairment, or pathological lesion (in response to a stimulus) that either singly or in combination adversely affects the performance of the whole organism or reduces the organism's ability to respond to an additional environmental challenge. Contrasted to adverse effects, non-adverse effects can be defined as those biological effects which do not cause physical, physiological, behavioural and biochemical changes that affect the general well-being, growth, development or life span of an animal.
Adverse effect(s) giving concern to endocrine toxicity	Adverse effects on endocrine active organs, tissues or processes associated with the HPG or HPT axes.
Endocrine activity	Modulation of endocrine processes that may or may not give rise to adverse endocrine effects.
NO(A)EL	The highest exposure level at which there are no statistically or biologically significant increases in the frequency or severity of adverse effects between the exposed population and its appropriate control. Some effects may be produced at this level, but they are not considered to be adverse or precursors to adverse effects.

LO(A)EL

The lowest exposure level at which there are statistically or biologically significant increases in the frequency or severity of adverse effects between the exposed population and its appropriate control.

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APPENDIX A: TEST METHODS

Details of individual test methods

EDSP TIER 1 ASSAYS: ER BINDING ASSAY SUMMARY OF STUDY DESIGN - DRAFT

NOTE - this assay was due to complete validation in Q2 2008. The detailed protocol is not yet available but is likely to be similar to the AR binding assay.

Purpose of protocol:	This assay evaluates potential of test compounds to bind to the estrogen receptor (ER). It measures the inhibition of estradiol binding to ER using recombinant protein as a biological source of ER. A saturation assay is performed first to characterize the receptor activity followed by competitive binding of test compounds and a positive control compound to ER using radiolabelled estradiol.
Endpoints:	Saturation assay: Affinity of ligand for ER, determined as K_d and maximum specific binding (B_{max}). Competitive assay: Affinity of test compound for ER in competition with estradiol, determined as IC_{50} and relative binding affinity (RBA).
Tissue source:	Recombinant hER alpha from Invitrogen or CER1; or rat uterine cytosol.
Saturation binding assay:	ER saturation binding using [3H]- estradiol at eight concentrations in triplicate. Three independent replicates should be run.
Competitive binding assay:	ER competitive binding for a single concentration of [3H]-estradiol using unlabelled estradiol (standard curve at five concentrations), test substance (eight concentrations) and a positive control (also at eight concentrations). All incubates are done in triplicate. Three independent replicates should be run.
Test substance and analysis:	Chemical purity and stability in vehicle must be known prior to testing so that concentrations are correctly prepared. Analysis of achieved concentrations.
Data analysis:	Calculations and graphical presentation of data as specified on the EPA EDSP website.
Report:	Full regulatory report including individual data and data summaries as specified on the EPA EDSP website.

**EDSP TIER 1 ASSAYS:
ER STABLY TRANSFECTED TRANSCRIPTIONAL ACTIVATION ASSAY
SUMMARY OF STUDY DESIGN – DRAFT**

NOTE - the details of this assay are not yet published but it is listed for inclusion Tier 1.

Purpose of protocol:	This assay provides an <i>in vitro</i> cell-based assay to detect chemicals that bind to the ER and alter gene transcription.
Endpoints:	The assay measures bioluminescence reflecting changes in gene transcription as a result of chemicals binding to ER.
Tissue source:	Cultured HeLa-9903 cells (available from Sumitomo Chemicals Co. and the Japanese Collection of Research Biosources).
Assay method:	HeLa cells are cultured to between passages 1-40. They are then used in three independent replicates of the experiment. Each experiment consists of addition of test substance (at 7 concentrations) to the cells for 24 h. A positive control compound and 4 reference chemicals are tested in parallel (also at 7 concentrations). At the end of the experiment the cells are lysed and expression of reporter gene determined using a standard luciferase assay. Cytotoxicity is determined in a preliminary experiment. Proficiency chemicals (11) must be run prior to the chemical under test to demonstrate the responsiveness of the test system.
Test substance and analysis:	Chemical purity and stability in vehicle must be known prior to testing so that concentrations are correctly prepared. Analysis of achieved concentrations.
Data analysis:	Calculations and graphical presentation of data as specified on the EPA EDSP website.
Report:	Full regulatory report including individual data and data summaries as specified on the EPA EDSP website.

**EDSP TIER 1 ASSAYS: AR BINDING ASSAY
SUMMARY OF STUDY DESIGN**

Purpose of protocol:	This assay evaluates potential of test compounds to bind to the androgen receptor (AR). It measures the inhibition binding of R1881 to AR in rat ventral prostate cytosol as a biological source of AR. A saturation assay is performed first to characterize the receptor activity, followed by competitive binding of test compounds and a weak positive control (dexamethasone) to the AR using radiolabelled R1881 as ligand.
Endpoints:	Saturation assay: Affinity of R1881 for AR, determined as K_d and maximum specific binding (B_{max}). Competitive assay: Affinity of test compound for AR in competition with R1881, determined as IC_{50} and relative binding affinity (RBA).
Animals and tissue source:	Male SD or Wistar rats are castrated at 85-100 days of age and killed 24 h later. Ventral prostates are harvested and cytosol prepared and frozen.
Saturation binding assay:	AR saturation binding using [3 H]-R1881 at eight concentrations in triplicate (0.25-10.0nM). Three independent replicates should be run.
Competitive binding assay:	AR competitive binding for a single concentration of [3 H]-R1881 using unlabelled R1881 (standard curve at five concentrations), test substance (eight concentrations) and a weak positive control (dexamethasone also at eight concentrations). All incubates are done in triplicate. Three independent replicates should be run.
Test substance and analysis:	Chemical purity and stability in vehicle must be known prior to testing so that concentrations are correctly prepared. Analysis of achieved concentrations.
Data analysis:	Calculations and graphical presentation of data as specified on the EPA EDSP website.
Report:	Full regulatory report including individual data and data summaries as specified on the EPA EDSP website.

EDSP TIER 1 ASSAYS: H295R STEROIDOGENESIS ASSAY SUMMARY OF STUDY DESIGN - DRAFT

NOTE - this assay was due to complete validation in Q2 2008. The detailed protocol is not yet available but pre-validation studies have been published.

Purpose of protocol:	This assay evaluates the potential of test compounds to affect steroidogenesis pathways using the H295R adrenocarcinoma cell line. These cells express all the key enzymes for steroidogenesis, show both inhibition and induction of these enzymes in response to test compounds and produce estradiol and testosterone as end products.
Endpoints:	The assay measures production of estradiol and testosterone as end products of steroidogenesis. Cell viability is also determined.
Tissue source:	Cultured H295R adrenocarcinoma cells.
Assay method:	H295R adrenocarcinoma cells are cultured to passages 5-7. They are then used in three independent replicates of the experiment. Each experiment consists of addition of test substance (at 7 concentrations) to the cells for 24 h. Two positive control compounds are tested in parallel (at 2 concentrations) and various quality controls. The end of the experiment the medium is removed and cells are tested for viability using suitable assay. Estradiol and testosterone are determined in the extracted medium.
Test substance and analysis:	Chemical purity and stability in vehicle must be known prior to testing so that concentrations are correctly prepared. Analysis of achieved concentrations.
Data analysis:	Calculations and graphical presentation of data as specified on the EPA EDSP website.
Report:	Full regulatory report including individual data and data summaries as specified on the EPA EDSP website.

EDSP TIER 1 ASSAYS: AROMATASE ASSAY SUMMARY OF STUDY DESIGN

Purpose of protocol:	Aromatase is the enzyme complex responsible for the conversion of androgens to estrogens. This assay evaluates the ability of test compounds to inhibit this enzyme. The known aromatase inhibitor, 4-hydroxyandrostendione (4-OH ASDN), is used as a positive control substance.
Endpoints:	The substrate for the assays is androstenedione (ASDN), which is converted by aromatase to estrone. The endpoint measured is tritiated water formation and a mixture of non-radiolabeled and radiolabeled ASDN is used as the substrate.
Tissue sources:	Human recombinant microsomes are obtained from (Gentest [Human CYP19 + P450 Reductase SUPERSOMES], Woburn, MA).
Assay method:	Microsomes are incubated with [³ H] ASDN as substrate. Inhibition by test substance is determined using 8 concentrations (1×10^{-3} - 1×10^{-10} M). The positive control is tested concurrently also using 8 concentrations (1×10^{-5} - 1×10^{-10} M). All incubates are done in triplicate. Three independent replicates should be run.
Test substance and analysis:	Chemical purity and stability in vehicle must be known prior to testing so that concentrations are correctly prepared. Analysis of achieved concentrations.
Data analysis:	Calculations and graphical presentation of data as specified on the EPA EDSP website.
Report:	Full regulatory report including individual data and data summaries as specified on the EPA EDSP website.

**EDSP TIER 1 ASSAYS: UTEROTROPHIC ASSAY
SUMMARY OF STUDY DESIGN**

Purpose of protocol:	The uterotrophic assay is a short-term screening test that evaluates the ability of a chemical to elicit biological activities consistent with agonists of natural oestrogens. It is based on the increase in uterine weight or uterotrophic response.
Endpoints:	Growth (daily body weight). Uterus weight (blotted).
Animals:	Immature female SD or Wistar rats, weaned on PND 18, 6 rats per group, housed 3 per cage. Rats are PND 18 at start of treatment. The laboratory diet and bedding materials should demonstrably not contain high levels of phytoestrogens. (An adult OVX version of the assay is also possible).
Verification of laboratory proficiency:	Laboratory proficiency must be demonstrated initially and then can be verified by the periodic or concurrent use of positive controls.
Groups:	1 vehicle control and 2 test groups.
Dose administration:	Daily, orally by gavage or by subcutaneous injection, for 3 days, from PND 22 to PND 42, on a mg/kg body weight basis.
Test substance and analysis:	Chemical purity and stability in vehicle must be known prior to testing so that dose levels are correctly prepared. Analysis of achieved concentration at all doses and homogeneity (for suspensions).
Body weights and clinical observations:	Daily body weights and detailed clinical observations.
Necropsy:	Rats are killed 24 h after the final dose. Uterine blotted weight is determined.
Data analysis:	Relevant data will be analysed statistically using ANOVA and ANCOVA followed by appropriate pairwise comparison tests.
Report:	Full regulatory report including individual animal data and data summaries as specified on the OECD website.

**EDSP TIER 1 ASSAYS: HERSHBERGER ASSAY
SUMMARY OF STUDY DESIGN**

Purpose of protocol:	The Hershberger assay is a short-term screening test that evaluates the ability of a chemical to elicit biological activities consistent with androgen agonists or antagonists. It is based on the changes in weight of five androgen-dependent tissues in the castrate-peripubertal male rat.
Endpoints:	Growth (daily body weight). Organ weights: ventral prostate (VP), seminal vesicles (SV) (plus fluids and coagulating glands), levator ani-bulbocavernosus (LABC) muscle, paired Cowper's glands (COW) and the glans penis (GP).
Animals:	Male SD or Wistar rats, 6 rats per group, housed 3 per cage. They should be castrated at approximately PND 42 and allowed a minimum of 7 days recovery before dosing. Dosing may commence as early as pnd 49, but not later than pnd 60. Age at necropsy should not be greater than pnd 70. The laboratory diet and bedding materials should demonstrably not contain high levels of phytoestrogens.
Verification of laboratory proficiency:	Laboratory proficiency is demonstrated by the use of concurrent positive controls.
Groups and dose administration:	Androgens: 1 vehicle control and 2 test groups administered test substance daily by oral gavage for 10 days. Anti-androgens: 1 vehicle control and 2 test groups administered test substance daily by oral gavage for 10 days in concert with daily testosterone propionate doses (0.2 or 0.4 mg/kg/day) by sc injection. All doses are given on a mg/kg body weight basis.
Test substance and analysis:	Chemical purity and stability in vehicle must be known prior to testing so that dose levels are correctly prepared. Analysis of achieved concentration at all doses and homogeneity (for suspensions).
Body weights and clinical observations:	Daily body weights and detailed clinical observations.

- Necropsy:** Rats are killed 24 h after the final dose. Weights of the five androgen-dependent tissues: ventral prostate (VP), seminal vesicles (SV) (plus fluids and coagulating glands), levator ani-bulbocavernosus (LABC) muscle, paired Cowper's glands (COW) and the glans penis (GP) are determined. Liver, paired kidney, and paired adrenal weights may be required.
- Data analysis:** Relevant data will be analysed statistically using ANOVA and ANCOVA followed by appropriate pairwise comparison tests.
- Report:** Full regulatory report including individual animal data and data summaries as specified on the OECD website.

EDSP TIER 1 ASSAYS: MALE PUBERTAL RAT ASSAY SUMMARY OF STUDY DESIGN

Purpose of protocol:	To quantify the effects of chemicals on pubertal development and thyroid function in the intact juvenile/peripubertal male rat. This assay detects chemicals that display anti-thyroid, or androgenic/anti-androgenic activity (e.g. alterations in receptor binding or steroidogenesis), or alter hypothalamic function or gonadotropin or prolactin secretion.
Endpoints:	Growth (daily body weight) Age and body weight at preputial separation Organ weights: Seminal vesicle plus coagulating glands (with and without fluid) Ventral prostate Dorsolateral prostate Levator ani/bulbocavernosus muscle complex Epididymides (left and right separately) Testes (left and right separately) Thyroid Liver Kidneys (paired) Pituitary Adrenals (paired) Histology: Epididymis Testis Thyroid (colloid area and follicular cell height) Kidney Hormones: Serum testosterone, total Serum thyroxine (T4), total Serum thyroid stimulating hormone (TSH).
Animals:	Male SD or Wistar rats, weaned on PND 21, 15 rats per group, housed 2 or 3 per cage. Rats are PND 23 at start of treatment.
Groups:	1 vehicle control and 2 test groups.

Dose administration:	Daily, orally by gavage using a metal gavage needle, from PND 23 to PND 53, on a mg/kg body weight basis.
Test substance and analysis:	Chemical purity and stability in vehicle must be known prior to testing so that dose levels are correctly prepared. Corn oil is the preferred vehicle for all treatment groups. Analysis of achieved concentration at all doses and homogeneity (for suspensions).
Body weights and clinical observations:	Daily body weights and detailed clinical observations.
Preputial Separation:	Beginning on PND 30, rats are examined daily for preputial separation (PPS).
Necropsy:	Rats are killed on PND 53. Blood is collected and serum prepared and frozen for subsequent hormone assays. The testes, epididymides, ventral prostate, dorsolateral prostate, seminal vesicle with coagulating glands and fluid, levator ani plus bulbocavernosus muscles, thyroid (with attached portion of trachea), liver, kidneys, pituitary, and adrenals are removed and the weights of each except the thyroid/trachea recorded. The thyroid (with attached portion of the trachea), a single testis and epididymis and a kidney from each animal are prepared for histological examination. The thyroid is also weighed after fixation and dissection.
Hormonal Assays:	Testosterone (total), T4 (total) and TSH by RIA, IRMA, ELISA, or time-resolved immunofluorescent procedures. Multiple quality control samples must be included and appropriate values for control rats at the laboratory and criteria for evaluating the kit's performance must be included in the study report.
Histology:	Testis, epididymis, thyroid, and kidney.
Data analysis:	Relevant data will be analysed statistically using ANOVA and ANCOVA followed by appropriate pairwise comparison tests.
Report:	Full regulatory report including individual animal data and data summaries as specified on the EPA EDSP website.

EDSP TIER 1 ASSAYS: FEMALE PUBERTAL RAT ASSAY SUMMARY OF STUDY DESIGN

Purpose of protocol:	To quantify the effects of chemicals on pubertal development and thyroid function in the intact juvenile/peripubertal female rat. This assay detects chemicals that display anti-thyroid, or androgenic/anti-androgenic activity (e.g. alterations in receptor binding or steroidogenesis), or alter hypothalamic function or gonadotropin or prolactin secretion.
Endpoints:	Growth (daily body weight) Age and body weight at vaginal opening Organ weights: Uterus (blotted) Ovaries (paired) Thyroid Liver Kidneys (paired) Pituitary Adrenals (paired) Histology: Uterus Ovaries Thyroid (colloid area and follicular cell height) Kidney Hormones: Serum thyroxine (T4), total Serum thyroid stimulating hormone (TSH) Estrus cyclicity: Age at first estrus after vaginal opening Length of cycle Percent of animals cycling Percent of animals cycling regularly.
Animals:	Female SD or Wistar rats, weaned on PND 21, 15 rats per group, housed 2 or 3 per cage. Rats are PND 22 at start of treatment.
Groups:	1 vehicle control and 2 test groups.

Dose administration:	Daily, orally by gavage using a metal gavage needle, from PND 22 to PND 42, on a mg/kg body weight basis.
Test substance and analysis:	Chemical purity and stability in vehicle must be known prior to testing so that dose levels are correctly prepared. Corn oil is the preferred vehicle for all treatment groups. Analysis of achieved concentration at all doses and homogeneity (for suspensions).
Body weights and clinical observations:	Daily body weights and detailed clinical observations.
Vaginal opening:	Beginning on PND 22, rats are examined daily for vaginal opening (VO).
Estrous cyclicity:	Vaginal smears are taken from all rats from the day of vaginal opening until the day of necropsy and estrous cycle patterns characterized.
Necropsy:	Rats are killed on PND 42. Blood is collected and serum prepared and frozen for subsequent hormone assays. The ovaries (without oviducts), uterus, thyroid (with attached portion of trachea), liver, kidneys, pituitary, and adrenals are removed and the weights of each except the thyroid/trachea recorded. The thyroid (with attached portion of the trachea), ovaries, uterus and a kidney from each animal are prepared for histological examination. The thyroid is also weighed after fixation and dissection.
Hormonal Assays:	T4 (total) and TSH by RIA, IRMA, ELISA, or time-resolved immunofluorescent procedures. Multiple quality control samples must be included and appropriate values for control rats at the laboratory and criteria for evaluating the kit's performance must be included in the study report.
Histology:	Ovary, uterus, thyroid, and kidney.
Data analysis:	Relevant data will be analysed statistically using ANOVA and ANCOVA followed by appropriate pairwise comparison tests.
Report:	Full regulatory report including individual animal data and data summaries as specified on the EPA EDSP website.

SINGLE GENERATION REPRODUCTION STUDY IN THE RAT (OECD 415)

Groups:	1 control and 3 tests.
Number of animals:	15 males and 15 females per group.
Analysis of diet preparations:	Achieved concentration at all dietary levels prior to the start of the study and at approximately 2 monthly intervals. Homogeneity and stability at high and low doses.
Dose administration:	Continuous dietary administration throughout the study i.e. for 10 weeks pre-mating and then through mating, gestation, lactation and up to weaning of the next generation.
Clinical observations:	Daily cageside observation, detailed observations recorded at the same time as bodyweight recorded.
Bodyweights:	Weekly during pre-mating period; days 1, 8, 15 and 22 of gestation; days 1, 5, 8, 15 and 22 of lactation.
Food consumption:	Monitored continuously during pre-mating period. [*Gestation/Lactation as option.]
Mating:	One male with one female from the same dose group. Date of mating checked by vaginal smearing.
Litters:	Each pup examined, sexed and weighed on days 1, 5, 8, 15 and 22 <i>post partum</i> . Litters will not be culled.
Pathology:	Reproductive organs from all F ₀ animals preserved. Pups found dead or killed intercurrently examined macroscopically (evisceration).
Data evaluation:	Relevant data will be analysed statistically using the SAS (1999) package or similar.
Report	Regulatory.

MULTI-GENERATION REPRODUCTION STUDY IN THE RAT (OECD 416)

Design:	Two generations, one litter per generation.
Groups:	1 control and 3 tests.
Number of animals:	26 males and 26 females per group for each generation.
Analysis of diet preparations:	Achieved concentration all dietary levels at approximately 2 month intervals; stability and homogeneity at low and high levels.
Dose administration:	Continuous in the diet throughout the study i.e.: for 10 weeks prior to mating and then during mating, gestation, lactation through to scheduled termination.
Clinical observations:	Cageside observation each day. Detailed observations recorded at the same time as bodyweight recorded.
Bodyweights:	Weekly during pre-mating period; days 1, 8, 15 and 22 of gestation; days 1, 5, 8, 15 and 22 of lactation. For F ₁ animals daily from selection until day 50 or the time of sexual maturation.
Food consumption:	Weekly during pre-mating period, gestation.
Oestrus cycle:	Evaluated for all females for three weeks, prior to mating.
Mating:	One male with one female from the same dose group. Mating confirmed by vaginal smearing.
Litters:	Each pup examined, sexed and weighed on days 1, 5, 8, 15, 22 and 29 <i>post partum</i> . Litters will not be culled.
Developmental landmarks:	For selected F ₁ weanlings, age of vaginal opening and preputial separation determined.
Pathology (parents):	Specified organs weighed and tissues stored. Histopathology for 10 males and 10 females in F ₀ and F ₁ generations, control and high dose groups.

- Pathology (offspring):** Up to 3 pups per sex per litter given a macroscopic examination *post mortem*. Specified organs weighed from 1 pup per sex per litter.
- Sperm:** At termination of all F₀ and F₁ males; an assessment of sperm motility, sperm morphology and caudal and testicular sperm number.
- Data evaluation:** Relevant data will be analysed statistically using the SAS (1996) package or similar.
- Report:** Full regulatory standard including individual animal data.

OECD 21-DAY *IN VIVO* FISH ENDOCRINE SCREENING ASSAY SUMMARY OF STUDY DESIGN

Adoption anticipated April 2009

- Purpose of protocol:** *In vivo* screening assay for identifying endocrine active chemicals in sexually dimorphic fish. The assay is intended to detect chemicals that affect HPG axis in fish exposed during a limited part of their life-cycle in which they are reproductively active.
- Endpoints:** Core endpoints: Vitellogenin and secondary sexual characters.
Optional endpoints: Fecundity and gonadal histopathology (currently under debate).
- Validity criteria:** Control mortality < 10% at the end of the exposure period; as well, signs of disease are visible < 10% of control animals during the course of the test.
Dissolved oxygen concentration > 60% of the air saturation value (ASV) throughout the exposure period.
Water temperature must not differ by more than ± 1 °C between test vessels at any one time during the exposure period and be maintained within a range of 2°C within the temperature ranges specified for the test species.
- Test species:** Fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). Suitable size ranges for the different species recommended for use in this test are stipulated. For the whole batch of fish used in the test, the range in individual weights at the start of the test should be kept, if possible, within $\pm 20\%$ of the arithmetic mean weight. It is recommended to weigh a subsample of fish before the test in order to estimate the mean weight.
- Test conditions:** The photoperiod and water temperature should be appropriate for the test species.

Test replicates and controls: The assay is conducted using a water control and three chemical exposure concentrations for each test substance. A solvent carrier control is not preferred, but, in case a solvent would be used to dissolve a test substance, a solvent control must be included, using the same solvent concentration as in the chemical treatments. A positive reference control may be included (1 concentration). Two vessels (replicates) per treatment will be used (each vessel containing 5 males and 5 females) for zebrafish and medaka. For fathead minnow four vessels (replicates) each containing four females and two males. The exposure is conducted for 21-days. Daily observations of the spawning status for each test vessel qualitatively confirm that the fish are in spawning condition. On day-21 of the experiment fish are sampled for the measurement of the core endpoints.

Data analysis: To identify potential endocrine activity by a chemical, responses are compared between treatments versus controls groups using analysis of variance (ANOVA) or regression analysis. Statistically significant effects from control(s) treatment with any concentration-response data and curves if available.

US EPA FISH SHORT-TERM REPRODUCTION ASSAY SUMMARY OF STUDY DESIGN

Purpose of protocol:	As OECD assay.
Endpoints:	Survival, behaviour, growth, fecundity. fertilization success, gonadal histopathology, gonadosomatic Index (GSI), appearance and secondary sexual characteristics, vitellogenin, blood plasma oestradiol and testosterone.
Validity criteria:	As OECD assay.
Test species:	Fathead minnow (<i>Pimephales promelas</i>).
Test conditions:	As OECD assay.
Test replicates and controls:	Four vessels (replicates) per treatment will be used (each vessel containing 2 males and 4 females). Successful spawning is established in a 14-day pre-exposure period followed by 21-days exposure.
Data analysis:	As OECD assay.

**OECD 21-DAY *IN VIVO* STICKLEBACK ENDOCRINE SCREENING ASSAY
SUMMARY OF STUDY DESIGN**

NOTE - Dropped from inclusion in the OECD fish screening assay but an additional project proposal to further develop the test has been submitted to the OECD.

Purpose of protocol: As OECD fish screening assay though is particularly useful for anti-androgenic mode of action.

Endpoints: Core endpoints: vitellogenin and spiggin. Spiggin is an androgen-specific biomarker, proteinaceous glue normally produced by males for constructing nests during the breeding season, but which is also produced by females exposed to exogenous androgens.

Validity criteria: As OECD assay.

Test species: Three-spined stickleback (*Gasterosteus aculeatus*).

Test conditions: The photoperiod and water temperature should be appropriate for the test species.

Test replicates and controls: Three-week continuous exposure via water.
Two “replicates” per treatment – separate male and female test vessels.
On day-14 and day-21 of the experiment, 10 males and 10 females from each treatment level and from the control are sampled for the measurement of the core endpoints.

Test substance and analysis: See OECD 21-day *in vivo* Fish Endocrine Screening Assay and above.

Data analysis: As OECD assay.

OECD AMPHIBIAN METAMORPHOSIS ASSAY SUMMARY OF STUDY DESIGN

Purpose of protocol:	<i>In vivo</i> screening assay for identifying endocrine active chemicals which may interfere with the normal function of the hypothalamic-pituitary-thyroid (HPT) axis.
Endpoints:	Mortality, whole body length/snout-vent length (d 7 and 21), hind limb length (d 7 and 21), wet weight (d 7 and 21), developmental stage (d 7 and 21), thyroid histopathology (d 21).
Validity criteria:	For any treatment (including controls) mortality <10%, for any replicate mortality must not exceed 3 tadpoles otherwise the replicate is considered compromised, at two treatments should have 4 uncompromised replicates, at least 2 treatments without overt toxicity.
Test species:	<i>Xenopus laevis</i> tadpoles at NF stage 51.
Test conditions:	21-days preferably under flow through conditions.
Test replicates and controls:	Four replicate tanks per treatment. Larval density at test initiation is 20 tadpoles per test tank. Tadpoles are sampled at 7 and 21-days. See OECD 21-day <i>in vivo</i> Fish Endocrine Screening Assay and above.
Data analysis:	Jonckheere- Terpstra or ANOVA for continuous endpoints. For developmental stage step down application of Jonckheere-Terpstra to replicate medians or multi-quantal Jonckheere test from the 20 th to the 80 th percentile.

OECD FISH SEXUAL DEVELOPMENT TEST SUMMARY OF STUDY DESIGN

Phase II validation studies underway

Purpose of protocol:	To assess the impact of putative endocrine disruptors on the sexual development of fish.
Endpoints:	Mortality, hatch success, growth, sex ratio, vitellogenin and behaviour. Gonadal histopathology is included as an optional endpoint.
Validity criteria:	Control mortality < 10% at the end of the exposure period; as well, signs of disease are visible < 10% of control animals during the course of the test. Species specific limits on the control fertilisation success. Dissolved oxygen concentration > 60% of the air saturation value (ASV) throughout the exposure period. Water temperature must not differ by more than ± 1 °C between test vessels at any one time during the exposure period and be maintained within a range of 2°C within the temperature ranges specified for the test species.
Test species:	Fathead minnow (<i>Pimephales promelas</i>), medaka (<i>Oryzias latipes</i>) and zebrafish (<i>Danio rerio</i>). The length of the assay depends on the species selected (time to reach sexual maturation) for medaka and zebrafish 60 days post hatch and fathead minnow between 90 and 120 days post hatch.
Test conditions:	Freshly fertilised embryos are exposed until sexual differentiation.
Test replicates and controls:	Four replicate tanks per treatment (including control). Exact numbers of embryos to be determined. Exposure preferably under flow through conditions.
Data analysis:	ANOVA.

**DRAFT OECD: FISH TWO-GENERATION TEST GUIDELINE
SUMMARY OF STUDY DESIGN**

NOTE – draft guideline dated 8 November 2002; based on an initial zebrafish ring test conducted by Germany

- Purpose of protocol:** Test considers reproductive fitness in parents and offspring of freshwater fish as an integrated measure. It also enables measurement of a suite of histological and biochemical endpoints that allow diagnostic and definitive evaluation of endocrine disrupting chemicals (EDCs) or other types of reproductive toxicant.
- Endpoints:** Survival, behaviour of adults, fecundity, fertilisation success, hatchability, larvae appearance and survival, gonadal-somatic index (GSI), gonadal histology and plasma or whole body concentrations of vitellogenin. Additionally, plasma sex steroids (17 β -estradiol, testosterone, 11-ketotestosterone) and thyroid hormones (T3/T4) could be added to the test depending on purpose.
- Validity criteria:** > 90% survival of control animals in all test phases over the duration of the chemical exposure, and the control fish in each replicate in the two spawning phases should spawn regularly.
> 80% fertility and hatchability of eggs and embryos, respectively, from the control animals.
Dissolved oxygen >60% of saturation.
- Test species:** Fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*), sheepshead minnow (*Cyprinodon variegatus*) and zebrafish (*Danio rerio*).
- Test conditions:** Flow-through or semi-static.

Test replicates and controls:

Parental fish exposure (P) initiated with mature adults with a record of reproductive success as measured both by fecundity (number of eggs) and embryo viability (e.g. hatchability) established during a 7 to 21-d pre-exposure period. Test conducted with min. 5 concentrations, as well as appropriate controls, with min. 4 (3 replicates for zebrafish) experimental units (replicates) per treatment. For fathead minnow each replicate tank contains 4 female and 2 male fish, however, for zebrafish each replicate tank should contain at least 6 individuals (proportionally distributed 1 female to 2 males). Exposure for P fish is conducted for 21-d, during which appearance of the fish, behaviour, and fecundity are assessed daily.

Exposure of F₁ generation - Viability of resultant embryos (e.g. hatching success, developmental rate, occurrence of malformations, etc.) assessed in animals held in the same treatment regime to which the adults were exposed. Between 50 and 100 embryos produced on day 21 transferred to brooding chambers for each replicate under the same treatment regime to which the adults were exposed. After hatching, juvenile F₁ fish are reduced to a min. 25 and max. 50 per brooding chamber. For species with secondary sex characteristics, F₁ fish are thinned to 4 females and 2 males per replicate spawning chamber. For species where sex is difficult to determine by external characteristics (e.g. zebrafish), F₁ fish thinned to > 6 individuals per brooding chamber. Spawning and embryo viability assessed daily until 21 days beyond the mean initial spawn date of controls. At conclusion, blood samples or whole body collected from adults (F₁) for determination of sex steroids and vitellogenin, and gonads sampled for measurement of GSI and histological analyses. Genetic sex ratio and survival of F₂ juveniles assessed.

Exposure of F₂ generation – Viability of resultant embryos from F₁ (i.e. hatching success, developmental rate, occurrence of malformations, etc.) assessed in animals held in the same treatment regime to which the adults were exposed. At test termination (at sexual maturation of the F₂ generation), samples collected for analysis of sex steroids and vitellogenin. Gonads sampled for measurement of the GSI, histological analyses, and phenotypic sex ratios. If possible, genetic sex ratio should be confirmed.

Data analysis:

Determination of a No Observed Effect Concentration (NOEC) or an EC_x. Results must include the mean, standard deviation and range for each test endpoint from the replicates employed in the test. Statistical significance of means should be indicated.

US EPA FISH LIFE-CYCLE TOXICITY TESTS. EPA 540/9-86-137
SUMMARY OF STUDY DESIGN

NOTE – draft guideline dated July 1986. In the absence of a fully validated test methodology this study design has been augmented with endocrine endpoints (vitellogenin, sex ratio and gonadal histopathology). The design has also been extended to allow the measurement of endocrine endpoints in the F₁ generation (effectively a 1.5 generation study).

Purpose of protocol: Evaluate potential long-term (< 1 year) chronic exposure to freshwater fish reproduction and other life-stages.

Endpoints: Embryo and larval survival, time to hatch, hatching success, growth, reproduction, histology.

Validity criteria:

Test species: Fathead minnow (*Pimephales promelas*), sheepshead minnow (*Cyprinodon variegatus*).

Test conditions: Semi-static or flow-through.
 Fathead minnow: 25°C in 16 hr light: 8 hr dark.
 Sheepshead minnow: 30°C in flowing seawater 30°C of > 15% salinity.

Test replicates and controls: Min. 5 concentrations and control (plus solvent control if required) each with min. 2 replicates.
 Based on fathead minnow.
 Embryo exposure (4/5 days): started with embryos < 24 hours old and soaked in dilution water for > 2 hours by randomly distributing 50 embryos to each of 4 replicate larval growth chambers.
 Larval-juvenile exposure (8 weeks): after hatching each group randomly reduced to 25 and survival determined min. weekly. After 4 and 8 weeks after hatching lengths of all fish recorded.
 Juvenile-adult exposure (32-40 weeks): 25 fish transferred into adult spawning tanks at 8 weeks. Fish exhibiting secondary sexual characteristics separate out into 4 males and 4 females assigned to each spawning chamber. Substrates examined daily and embryos removed, counted and recorded.
 Second generation embryo exposure (4/5 days): 50 embryos from each test concentration transferred to incubation cup.
 Second generation larval-juvenile exposure (4/8 weeks): release of two groups of 25 larvae in replicates into growth chambers exposed for 8 weeks after

which 2nd generation fish terminated, weighed and measured.

Test substance and analysis: Analytical determinations made min. of weekly in each test concentration / control.

**US EPA AMPHIBIAN LIFE-CYCLE ASSAY (2-GENERATION)
SUMMARY OF STUDY DESIGN**

This study has been proposed for tier 2 of the Endocrine Disrupters Screening Programme (EDSP). Currently no protocols are available.

OECD 206: AVIAN REPRODUCTION TEST GUIDELINE SUMMARY OF STUDY DESIGN

Purpose of protocol:	To assess the impact of the test substance on avian health and reproduction.
Endpoints:	Mortality, signs of toxicity, body weights of adults, body weights of young at 14-days, food consumption of adults, food consumption of young, gross pathological examination, eggs/female, percentage cracked eggs, viability, hatchability, 14-day survivors, 14-day survivors/female and eggshell thickness.
Validity criteria:	Mortality in the controls should not exceed 10%. Species specific requirements for the average number of 14-day survivors. Species specific requirements for the average eggshell thickness. Evidence test item is satisfactorily maintained in the diet.
Test species:	Mallard duck, Japanese quail and bobwhite quail.
Test conditions:	Adult, egg production, and offspring health are evaluated.
Test replicates and controls:	8-12 breeding pairs/treatment in the parental generation (P). Photoperiod adjusted to induce breeding. With exposure for 8-10 weeks after egg production has begun. Eggs are artificially incubated and hatched. Hatchlings maintained on control diets for 14-days.
Data analysis:	NOEC determination.

DRAFT OECD: PROPOSAL FOR AVIAN TWO-GENERATION TEST GUIDELINE SUMMARY OF STUDY DESIGN

NOTE - draft guideline dated November 2002

Purpose of protocol:	To assess the impact of putative endocrine disruptors upon avian health and reproduction (specifically including the reproductive viability of the F ₁).
Endpoints:	<p>Adult health (Food consumption, body weight, number of eggs per hen per day).</p> <p>Reproductive data (Number of fertile eggs as a percentage of eggs set, number of early viable embryos as a percentage of eggs fertile, number of late viable embryos as a percentage of early viable embryos, number of eggs hatched as a percentage of late viable embryos, number of 14-day old chicks as a percentage of eggs hatched, number of 14-day old chicks as a percentage of eggs set, number of 14-day old chicks per hen per day, number of cracked eggs as a percentage of eggs laid, number of abnormal eggs as a percentage of eggs laid, egg shell thickness and eggshell strength, mean hatchling body weight, mean 14-day old chick body weight, sex ratio of chicks.</p> <p>Endocrine and physiological endpoints (weight of testes, ovaries, thyroid, adrenals, oviduct, cloacal gland, liver; histology of thyroid, adrenals, gonads, brain; testicular spermatid counts and morphology; gross anomalies of the genital tract; feather dimorphism; cloacal gland size, 1st appearance of foam; 1st egg laid; sexual behaviour; faecal/urate steroid hormones (oestradiol, testosterone); egg steroid content (oestradiol, testosterone; tibiotarsus length (F₁).</p>
Validity criteria:	<p>Test substance concentration in the diet to which birds are exposed should be satisfactorily maintained.</p> <p>Parental mortality during the last two weeks of acclimation should not exceed 3%.</p> <p>At least eight breeding F₁ pairs that have produced eggs must be available in the control group at the end of the test period.</p>
Test species:	Japanese quail (<i>Coturnix japonica</i>).
Test conditions:	Adult (P and F ₁), egg production (P and F ₁), and offspring health (F ₁ and F ₂) are evaluated.

Test replicates and controls: 8 breeding pairs in the parental generation (P). Brood from the P generation are used to establish the breeding first generation offspring (F₁) group. Optimally, 6 eggs are set for each parental pair. The F₁) generation breeding birds are maintained until 6 weeks post-fertility. The test is terminated when observations of the 14-day survival of the 6th week offspring (F₂) are made.

Data analysis: NOEC determination.

APPENDIX B: CONCERN FROM THE MAMMALIAN DATABASE AND *IN VITRO* DATA – RELEVANCE TO ENDOCRINE DISRUPTION IN ENVIRONMENTAL SPECIES

The evaluation of the toxicology database (see Chapter 4) can be used with all other available information, such as structural relationships and results from *in vitro* tests, in a weight of evidence approach (see Chapter 3) to determine if further testing is appropriate for environmental species. Only if the result of such an evaluation gives clear evidence of a potential for endocrine disruption should *in vivo* fish or amphibian testing be triggered. However, it is important to note that it is often difficult to distinguish effects from systemic toxicity. There are also considerable differences to fish that may need accounting for. These differences include exposure routes, targets/receptors, biotransformation pathways and intrinsic differences in endocrine function.

Effects for consideration

The following lists are tentative indicators to give an idea of the kind of effects one might consider. A full evaluation would require the analysis outlined in Chapter 4.

Observations in mammals:

- decreases in sperm function;
- changes in male or female sex organs;
- decreases in reproductive capability;
- premature or delayed puberty;
- changed hormone levels;
- changes in oestrous cycle length;
- carcinogenicity in endocrine organs and mammary glands;
- changes in developmental landmarks.

The majority of these effects can be induced / influenced by general toxicity as well as by an endocrine mode of action. Therefore, effects from the toxicology database should not be used in isolation. Such data sets represent a complex mix of data that will require a weight of evidence evaluation to ascertain its value in relation to endocrine disruption.

***In vitro* data, mode of action information and (Q)SARs**

The necessity for specific *in vivo* endocrine testing may also be based on an evaluation of:

- ***In vitro* data.** These data must be used cautiously since *in vitro* tests do not consider a fully integrated metabolic whole organism response and do not fulfil the Weybridge definition for endocrine disruption. Therefore, precedence should be given to the mammalian toxicology database.

- **Mode of action data.** Information of the substance's intended action on target species and 'read across' within classes of compounds will often indicate whether it is likely to have an adverse endocrine action in non-target species.
- **(Q)SAR data.** If available may be used to predict receptor interactions.

The evaluation of the avian database (see Chapter 4) can be used with all other available information, such as mammalian findings, structural relationships and results from *in vitro* tests, in a weight of evidence approach (see Chapter 3) to determine if a relevant effect operates in environmental species. There are also considerable differences between birds and mammals that need accounting for (EFSA, 2008). These differences include exposure routes, targets/receptors, biotransformation pathways and intrinsic differences in endocrine function.

Effects for consideration

The following lists are tentative indicators to give an idea of the kind of effects one might consider:

Observations in birds:

- Egg shell strength;
- influenced reproduction;
- influenced fertility;
- influenced hatch.

The majority of these effects can be induced / influenced by general toxicity as well as by an endocrine mode of action. Therefore, effects from the toxicology database should not be used in isolation. Such data sets represent a complex mix of data that will require a weight of evidence evaluation to ascertain its value in relation to endocrine disruption.

APPENDIX C: EVIDENCE FOR NO(A)ELs AND NO(A)ECs IN (ECO)TOXICITY STUDIES

Table C-1: Chemicals – rodent toxicity studies

Species	Test	Parameters	Compound	LOEL (C)	NOEL (C)
Rodent	Uterotrophic assay	Uterine weight; vaginal opening; histopathology; gene expression	EE; 0.001 → 10µg/kg/d ^{17; 32; 37}	0.1µg/kg/d	0.01µg/kg/d
			Tamoxifen 1µg → 100mg/kg/d ^{7; 29}	30µg/kg/d	10µg/kg/d
			BPA 1.5 → 600mg/kg/d ^{32; 37}	30mg/kg/d	15mg/kg/d
			NP 1.5 → 200mg/kg/d ^{12; 29; 32; 37}	50mg/kg/d	25mg/kg/d
			Methoxychlor 1µg → 1g/kg/d ^{12; 29; 32}	50mg/kg/d	10mg/kg/d
			E2 0.02µg → 200mg/kg/d ^{29; 32; 37}	0.1µg/kg/d	0.02µg/kg/d
			DES 0.005µg → 1mg/kg/d; ^{29; 32}	0.25µg/kg/d	0.05µg/kg/d
			GEN 0.2 → 200mg/kg/d ^{32; 37}	2.5mg/kg/d	1mg/kg/d
			OP 2 → 400mg/kg/d ^{12; 37}	100mg/kg/d	50mg/kg/d
			Equol 50 and 400mg/kg diet ²²	400mg/kg	50mg/kg
Soy extracts 125 → 4150mg/kg/d ³⁵	720mg/kg/d	300mg/kg/d			
Rodent	Hershberger assay	Sex accessory tissue weights; preputial separation, hormone measurements	DDE 3 → 160mg/kg/d ^{2; 19; 33}	30mg/kg/d	16mg/kg/d depending on system
			LIN 3 → 100mg/kg/d ^{2; 19; 33}	30mg/kg/d	100mg/kg/d
			FIN 0.08 → 25mg/kg/d ^{2; 19}	0.08mg/kg/d	0.2mg/kg/d NOEL not found Depending on system
			PRO 3 → 100mg/kg/d ^{2; 19}	100mg/kg/d	30mg/kg/d NOEL not found Depending on system
			TREN 0.3 → 40mg/kg/d ^{2; 19}	8mg/kg/d	0.3mg/kg/d
			VIN 3 → 100mg/kg/d ^{2; 19}	10mg/kg/d	3mg/kg/d
			Prochloraz 3.9 → 125mg/kg/d ⁴	15.6mg/kg/d	7.8mg/kg/d
Rodent	<i>In utero</i> exposure	Anogenital distance; sex accessory tissue weights; histopathology; hormone measurements	FIN 0.01 → 100 mg/kg/d ⁵	0.1 mg/kg/d	0.001mg/kg/d
			DBP 0.1 → 1000 mg/kg/d ¹³	50 mg/kg/d	10mg/kg/d
			FLU 0.15 → 1000 mg/kg/d ¹⁶	10 mg/kg/d	2.5mg/kg/d
			Fenitrothion 5 → 25 mg/kg/d ³⁴	20 mg/kg/d	15mg/kg/d
	Neonatal exposure	Sex accessory tissue weights; histopathology?	DES 1ng → 10 µg ⁸	100ng	10ng/kg/d
			EB 0.1 and 2500µg/kg/d ⁹	2500µg/kg/d	0.1µg/kg/d

Table C-2: Chemicals – Ecotoxicology studies

Species	Test	Parameters	Compound	LOEL (C)	NOEL (C)
Fish	Fish sexual development test	Survival, hatch, growth, vitellogenin, secondary sexual characteristics, sex ratio and gonadal histopathology	4- <i>tert</i> -pentylphenol 56 →560 µg/L ²⁰	180 µg/L	56 µg/L
			Prochloraz 16 →202 µg/L ¹⁰	202 µg/L	65 µg/L*
Fish	Full life-cycle studies with endocrine endpoints	Survival, development, hatching, growth, sexual differentiation (F ₀ and F ₁), reproduction, and vitellogenin (F ₀ only); histopathology	4- <i>tert</i> -pentylphenol 51 →931 µg/L ²⁶	224 µg/L	100 µg/L**
			Tamoxifen citrate 0.11 →18.2 µg/L ³⁶	5.97 µg/L	1.65 µg/L
			17α-ethinylestradiol 0.2 →64 ng/L ¹¹	4 ng/L	1.0 ng/L
			17α-ethinylestradiol 0.32 →23 ng/L ²¹	0.32 ng/L	<0.32 ng/L
			17α-ethinylestradiol 0.2 →10 ng/L ³	10 ng/L	2 ng/L
			17β-estradiol 0.939 →92.4 ng/L ²⁸	2.86 ng/L	0.939 ng/L
			Methyltestosterone 0.35 →27.75 ng/L ²⁷	9.98 ng/L	3.29 ng/L
4-nonylphenol 4.2 →183 µg/L ³⁸	17.7 µg/L	8.2 µg/L			
Amphibian	Amphibian meta-morphosis assay	General toxicity, sexual development and thyroid histopathology	PTU 2.5 →20 mg/L ⁶	5.0 mg/L	2.5 mg/L
			Methimazole 6.25 →50 mg/L ⁶	6.25 mg/L	<6.25 mg/L
			T4 0.5 →4.0 µg/L ⁶	0.5 µg/L	<0.5 µg/L
			Phenobarbital 125 →1500 mg/L ⁶	250 mg/L	125 mg/L
			Perchlorate 62.5 →500 µg/L ⁶	62.5 mg/L	<62.5 mg/L
			IOP 0.75 →6.0 mg/L ⁶	0.75 mg/L	<0.75 mg/L

Table C-3: Chemicals – In vitro studies

Species	Test	Parameters	Compound	LOEL (C)	NOEL (C)
	Oestrogen binding and transcriptional activation	Oestrogen binding and transcriptional activation	E2, DES, Tamoxifen, 4-hydroxytamoxifen, methoxychlor, HPTE, endosulfan, NP, DDT, kepone ²⁹	Dose response curves established for each parameter with clear LOEL(C)s and NOEL(C)s	
	<i>In vitro</i> E screen	Cell proliferation	21 chemicals tested ³⁰	NOECs established for all compounds	
			E2 ¹⁵	NOECs established	

Table C-4: Dietary factors – Rodent investigations

Species	Test	Parameters	Compound	Comments
Rodent	Uterotrophic	Uterine weight; vaginal opening	20 different test diets ³¹	Increases in uterine weight attributable to metabolisable energy of diet and not just phyto-oestrogen content
			7 different test diets ¹⁸	Increases in uterine weight directly proportional to energy intake VO dependent on cumulative energy intake of ~2300kJ/rat
	Female rat puberty and mammary gland development	Age of puberty (VO?); mammary gland development	Energy restricted diet ¹	Delay in puberty observed
	Food restriction study	Sex accessory weights, hormone measurements, histopath	Diet restriction ²³	Testicular histopathology dependent on duration of diet restriction; hormonal changes dependent on age of animal during food restriction
	Hyperthermia study	Testicular histopathology, gene expression	43°C/20 mins ²⁴	Testicular lesions, increased gene expression
	Hypothermia study	Testicular histopathology	Decreased body temperature through administration of reserpine ²⁵	Body temperature of 26-30°C led to testicular histopathology; body temperature of 33-36°C had no effect

Superscript numbers refer to publications from which data were obtained: 1: Arts *et al*, 1992; 2: Ashby *et al*, 2004b; 3: Balch, 2004; 4: Blystone *et al*, 2007; 5: Bowman *et al*, 2003; 6: US EPA, 2007; 7: Fong *et al*, 2007; 8: Goyal *et al*, 2003; 9: Ho *et al*, 2006; 10: Kinnberg *et al*, 2007; 11: Länge *et al*, 2001; 12: Laws *et al*, 2000; 13: Lehmann *et al*, 2004; 15: Lobenhofer *et al*, 2004; 16: Miyata *et al*, 2002; 17: Naciff *et al*, 2003; 18: Odum *et al*, 2004; 19: Owens *et al*, 2006; 20: Panter *et al*, 2006; 21: Parrot and Blunt, 2005; 22: Rachon *et al*, 2007; 23: Rehm *et al*, 2008; 24: Rockett *et al*, 2001; 25: Sato *et al*, 2007; 26: Seki *et al*, 2002; 27: Seki *et al*, 2004; 28: Seki *et al*, 2005; 29: Shelby *et al*, 1996; 30: Silva *et al*, 2007; 31: Thigpen *et al*, 2002; 32: Tinwell and Ashby, 2004; 33: Tinwell *et al*, 2007; 34: Turner *et al*, 2002; 35: Vieira *et al*, 2008; 36: Williams *et al*, 2007; 37: Yamasaki *et al*, 2002; 38: Yokota *et al*, 2001.

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