DEFINITION OF AN ECOTOXICOLOGICAL ENDOCRINE DISRUPTER FOR REGULATORY PURPOSES

Contents

1. Background

1. The prompt for this work was the introduction into the new European Union Plant Protection Products Regulation, PPPR (1107/2009) of an exclusion criterion for authorization which explicitly indicates that any active substance, safener and synergist with endocrine disrupting properties that may cause adverse effects on non-target (environmental) organisms cannot be approved for marketing and use unless the exposure of non-target organisms under realistic proposed conditions of use is negligible (see Appendix 1).

2. A similar approval exclusion criterion is proposed in the draft new EU Biocidal Products Regulation (COM(2009)267) that is currently under negotiation.

3. Substances with endocrine disrupting properties are also targeted within the REACH Regulation (1907/2006). Identification of substances as endocrine disrupters (EDs) may lead to their inclusion in the list of substances subject to the Authorisation requirements of REACH (see Appendix 1).

4. Despite these stipulations, at the present time there is no definition and/or set of criteria within these pieces of legislation, by which to identify substances that are endocrine disrupters (EDs), in relation to potential effects on human health and/or

other species in the environment. The aim of this paper is to propose a definition and associated interpretative criteria that can be applied to identify EDs; this paper specifically focuses on ecotoxicological EDs – we have also produced a counterpart paper dealing with EDs in relation to human health.

5. The proposal aims at identifying EDs of concern for which regulatory action can be taken within the provisions of the current legislative framework. The proposal has been developed in the context of the needs and characteristics of EU Plant Protection Products (pesticides) legislation, in terms of availability of data and regulatory consequences.

6. The proposals could also be relevant to the way in which endocrine disruption is intended to be a focus of attention under forthcoming EU biocides legislation; and to the requirements of identifying industrial chemicals as EDS and thereby potentially subject to Authorisation under REACH. In these cases it might be that some adjustment in the criteria by which EDs are identified is necessary to accommodate the characteristics of these pieces of legislation and the substances and situations they cover.

2. Outline of document

7. This paper will first consider a scientific definition for an endocrine disrupter. A consideration will then be given to what studies and other information is usually available to regulatory authorities to use to identify endocrine-disrupting substances; and what is the regulatory context for their identification. From these considerations, a definition and associated criteria for identification of a substance as an ecotoxicological ED for regulatory purposes will be proposed. Then, consideration is given to the implementation of the regulatory definition/criteria to key groups of wildlife.

3. Scientific definition of an endocrine disrupter

8. A number of definitions for EDs have been proposed (Kavlock, 1996; NRDC, 1998; Weybridge, 1996, WHO/IPCS, 2002 – see Appendix 2). Some of these definitions (e.g. Kavlock, 1996; NRDC, 1998) are ambiguous and, for regulatory purposes, are overly inclusive, in that they fail to discriminate between alterations of the endocrine system which fall within the physiological balance/homeostatic capabilities of an organism and adverse effects that disturb an organism's endocrine system to an extent beyond that compatible with normal function. This has led to the development of more restrictive definitions (e.g. Weybridge, 1996, WHO/IPCS, 2002).

9. Still, even the more restrictive definitions remain quite general, which is acceptable as a scientific definition for EDs but requires further development and elaboration to produce a basis for identifying EDs for particular regulatory attention and potentially stringent regulatory measures.

10. The widely accepted scientific definition of an endocrine disrupter by WHO/IPCS is proposed as a starting point for characterising an ED for regulatory

purposes. This is a well-established and widely recognised definition produced by a global, authoritative organisation through a world-wide initiative of highly scientific rigour (WHO/IPCS, 2002). In addition, it is supported by a number of organisations and regulatory bodies around the world, including the US EPA, the Canadian Centre for Occupational Health and Safety (CCHOS) and the International Union of Pure and Applied Chemistry (IUPAC).

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

12. This definition embodies two key elements on which one can build criteria for identifying an ED for regulatory purposes: endocrine-mediated adversity and intact organism observations.

13. With regard to adversity, it is proposed that the global and widely accepted definition produced by WHO/IPCS in 2004 is used to determine whether effects caused by exposure to a chemical are adverse:

14. "A change in morphology, physiology, growth, reproduction, development or lifespan of an organism which results in impairment of functional capacity or impairment of capacity to compensate for additional stress or increased susceptibility to the harmful effects of other environmental influences (WHO/IPCS 2004)."

15. In a regulatory context, the focus of ecotoxicological risk assessment is the avoidance of evident mortality and the protection of ***populations***. It is the latter that is pertinent in relation to endocrine disruption. Therefore it is proposed that consideration of whether or not a substance appears to have ecotoxicological endocrine disrupting properties should focus on *adverse consequences for reproduction, growth/development, disease incidence and survival,* as these are the effects most likely to impact on population recruitment and stability.

16. So, bringing together these considerations, endocrine disruption in its widest sense is a perturbation of the normal endocrine homeostasis, for instance, a change in the circulating levels of a particular hormone. However, such perturbation in itself is not considered to be an adverse effect, as the endocrine system is naturally dynamic and responsive to various stimuli as part of its normal functioning. In this context, endocrine perturbation is considered as a mode of action, potentially on a pathway to other outcomes, rather than an ecotoxicological endpoint in itself. Crucially, to designate a substance as an ecotoxicological ED, any endocrine perturbation must result in, or be plausibly connected with, observed adverse ecotoxicological effects in intact organisms that can impact detrimentally on the population of one or more environmental species.

4. Studies and other information likely to be routinely available to regulatory authorities

17. Studies in experimental mammals performed primarily for the purposes of assessing the potential of a substance to affect human health are clearly also of relevance to wild mammals.

18. In addition, the current draft of Plant Protection Products data requirements refers to three screening assays for ecotoxicological endocrine-disrupting potential. These are the fish short-term reproduction assay (OECD 229), 21-day fish assay (OECD 230) and the amphibian metamorphosis assay (OECD 231). Each study is briefly summarised in Appendix 3.

19. In addition to the above, the current draft of the data requirements for Plant Protection Products states the following:

> *"There needs to be a consideration as to whether the substance is a potential endocrine disrupter in aquatic non-target organisms. Data on the toxicity profile and mode of action should be scrutinised as well as any other additional information. There should be a consideration of all the existing data and guidance as described in OECD Guidance Document on the Assessment of Chemicals for Endocrine Disruption.*[1]*"*

The wording of this requirement is aimed at ensuring that there is a detailed consideration of all available and relevant in vivo and in vitro data in determining whether a substance is (potentially) an ecotoxicological endocrine disrupter. Such additional information is unlikely in itself to confirm definitively that a substance is or is not an ecotoxicological ED, but it might provide valuable supporting evidence in decision-making.

20. There are two reproductive studies in non-mammalian species available that will allow the identification of adverse effects on population recruitment and stability. These are the fish full life cycle study (EPA OPPTS 850.1500) and the avian one-generation study (OECD 206). It is acknowledged that as OECD 206 only investigates reproductive effects on the first generation the study is not sufficient to detect all possible endocrine-mediated adverse effects. Both of these studies are briefly summarised in Appendix 3.

21. It should be noted that there are under development international guidelines for several additional studies[2] which will aid the determination of whether or not a compound is an ecotoxicological endocrine disrupter. This document and the proposals within it should be revisited once these new OECD guidelines are agreed and adopted.

22. Nonetheless, on the basis of data likely to be immediately available (e.g. during 2011) it is clear that the ability to show in the same vertebrate taxonomic group adverse effects towards a population, with evidence indicating a likely relationship to an underlying mode-of-action of endocrine disruption, exists only for mammals and fish. That is, only in these two groups are there international test

[1] Note that this document is still in draft. It is to be considered by OECD in April 2011, but may not be finalised until later in 2011 or 2012. It covers all the tests which are still in validation (see below), as well as those already published as TGs.

[2] Test guidelines that are currently under development in the OECD are:

- a Medaka Multi-Generation Test (MMGT) which includes a suite of endocrine endpoints as well as the traditional apical endpoints available in OPPTS 850.1500
- a Fish Sexual Development Test (FSDT)
- a longer-term Larval Amphibian Growth and Development Assay (LAGDA).
- An Avian 2-generation study that includes mode-of-action endpoints for endocrine disruption.

guidelines available to identify potential endocrine effects (e.g. the screening assays) as well as a study to identify whether there is the potential to produce a related adverse effect at the population level. A screening assay (OECD 231) is available on amphibians and this is aimed at determining whether the substance has an effect on the thyroid. At present there is no higher tier study linked to this assay, either in terms of potential adverse, population-level effects on amphibians or other organisms. In light of this, at present it is not considered appropriate to request this study in pursuit of determining whether or not a substance is an ED from an ecotoxicological perspective. [However, this study might have value in relation to the assessment of endocrine-disrupting potential in relation to human health considerations].

23. There should also be some consideration of the potential of a substance to produce endocrine disruption-related effects on invertebrates. However, at present no screening studies for endocrine disruption are available for either aquatic or terrestrial invertebrates. Therefore it is not possible to routinely determine if a substance is an endocrine disrupter in invertebrates. However some pesticide active substances are designed to have endocrine-disrupting effects as their mode of action on the target pest species; how such situations should be considered is described later in this document.

24. All studies used in an assessment must have been conducted to an internationally recognised protocol, be of a good standard and have been well reported.

5. Regulatory definition of an ecotoxicological endocrine disrupter

25. Before one starts to consider whether a substance is an ecotoxicological endocrine disrupter for regulatory purposes, one should consider whether or not the substance meets the conditions for being an ED because of human health (toxicological) concerns. If it does, the stringent regulatory consequences that pertain to ED substances in PPPR already apply. Hence, in most such cases, there is no additional value in pursuing the ED issue for ecotoxicity, or put another way, if the substance is considered to be an EDs from a human health perspective it is unlikely to need consideration from an ecotoxicological perspective.

26. Information from standard screening assays for endocrine disrupting activity (e.g. OECD 229 and/or OECD 230), along with any additional information should be used to determine whether the substance has a potential endocrine-disrupting mode-of-action. These studies will not, however, be able to indicate adverse consequences for population recruitment or stability[3]. From a regulatory point of view a positive result in a screening study should signify that a substance is a "*potential* endocrine disrupter".

27. If a positive effect is seen in a screening assay then data are required in order to indicate the potential for *adverse* effects at the population level. For fish the relevant study is the fish full life cycle study (FFLC) (EPA OPPTS 850.1500). It is

[3] TG 229 includes the measurement of fecundity which is an adverse endpoint indicative of endocrine disruption if plausibly associated with changes in vitellogenin or secondary sexual characteristics. However, it is considered necessary to determine whether the effects seen in this study could result in adverse effects at the population level.

acknowledged that it is not possible to directly observe a link between mode-of-action effects seen in a screening study and adverse population effects in higher tier studies (i.e. the FFLC). Therefore there should be a consideration of the likelihood of a relationship between adverse effects seen in the higher tier study and the evidence of endocrine-disrupting potential seen in the screening study. In this context, there should also be a consideration of any other relevant information. In order to conclude that a substance is an ecotoxicological ED there must be a reasonable and coherent line of evidence for a link between adverse population-related effects seen in intact organism studies and an endocrine-disrupting mode-of-action.

28. In contrast to toxicology, where there is a single species (humans) on which all considerations ultimately focus, the scope of ecotoxicology is vast and encompasses organisms in a variety of taxonomic categories. The relationship between any two species in the environment can be much more distant than, for instance, between one "environmental group" (say, rodents) and humans. The marked differences that exist between different categories of organism in the environment impose strong restrictions on the reliability of reading across ecotoxicological findings and considerations from one class/phylum to another.

29. Given these constraints on reading across and the requirements that:

- an ED needs to demonstrate the ability to produce an adverse effect in an intact organism;
- the nature of the effect must pose a threat to population recruitment or stability: and
- there should be a reasonable and coherent line of evidence that the mode-of-action underlying the effect is endocrine disruption

then as indicated above, it is really only possible to determine whether or not a substance is an ecotoxicological ED for mammals and fish.

30. As regards aquatic and terrestrial invertebrates there is currently no internationally accepted screening study available for endocrine-disrupting potential. Where a substance is considered to a potential ED towards invertebrate species due to its mode of action on the target pest species (e.g. substances that change ecdysteroid and juvenile hormone systems), it is proposed that the potential population effects on invertebrates should be determined at the field scale (or equivalent) – see section 6.

31. ***Dose/concentration level/potency considerations:*** OECD 229 and OECD 230 indicate that three concentrations should be tested. Deciding the appropriate concentrations to be investigated is not simple. However, guidance is provided in the OECD guidelines regarding the highest concentration, i.e. it should be at maximum tolerated concentration (MTC)[4]. The guidelines recommend a further two concentrations are tested with a range of spacing factors between 3.2 and 10. It is proposed that relatively wide spacing is used to ensure that the potential for effects at lower concentration are covered. The lowest treatment group should be close to the limit of detection.

[4] The MTC is defined as the highest test concentration of the chemical which results in less than 10% mortality

32. If a full fish life cycle study is required, then it is proposed that the test concentrations investigated in the FFLC study should encompass concentrations which cause an effect in the associated screening study.

33. ***Decision-making:*** In determining whether a compound is an ecotoxicological endocrine disruptor or not there should be a consideration of the concentration or dose causing ED effects. For example, if the key endpoint from fish assays and the full fish life cycle study are several orders of magnitude greater than other key endpoints then the ED effect can be considered to be of limited regulatory relevance. This is due to the fact that any regulatory decision, for example no authorisation, implementation of buffer zones or other risk mitigation measures will be based on a significantly lower endpoint. This is illustrated by the following example – a new herbicide has an EC50 for *Lemna* of 1.0 µg a.s./L. This is the lowest endpoint and is 'driving' the risk assessment whereas the NOEC from the full fish life cycle study is 10 mg a.s./L. In this situation, it is proposed that the results of the FFLC are of limited regulatory relevance.

34. Therefore overall, in relation to potential ecotoxicity concerns, it is proposed that a substance is regarded as an ecotoxicological ED for regulatory purposes when it satisfies the following definition and associated criteria:

- it should be an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse effects in an intact organism, or its progeny, or (sub)populations.

and in doing so satisfies the following criteria:

a. the nature of the effect must pose a threat to population recruitment or stability: and
b. there should be a reasonable and coherent line of evidence from within the same taxonomic group that the mode-of-action underlying the effect observed is endocrine disruption
c. there should be a consideration of the concentration/dose causing adverse endocrine effects as the example described in paragraph 33.

6. Implementation of the proposed regulatory definition of an ecotoxicological endocrine disrupter

35. Outlined below is a discussion for each taxonomic group that is routinely considered in ecotoxicological risk assessment.

a. Mammals in the environment

36. In relation to studies in mammals, if a substance is identified as an endocrine disrupter in relation to human health concerns, in most cases there is no regulatory purpose in pursuing the issue of whether or not it is also an endocrine disrupter in relation to other environmental species.

37. However, one possible situation arises where a substance exhibits endocrine-disrupting properties in experimental mammals (rodents), but the effects are judged not to be relevant to human health. In such circumstances:

- if there are clear adverse effects in experimental mammals that in all probability have an underlying mode-of-action of endocrine disruption; and
- this is a prominent feature of the (eco)toxicological profile of the substance in the experimental animals,

then it might be appropriate to designate the substance as an ecotoxicological ED for regulatory purposes, in respect of its threat to mammals in the environment. However, there needs to be a judgement made about the relevance of the experimental species used in the testing in relation to the species occurring in the environment, e.g. the relevance of thyroid clearance in rats to focal species of concern - if there is uncertainty then the effect should be considered to be relevant.

b. Fish

38. If either general data (see paragraph 19) or data from OECD 229 and/or 230 indicate that the substance in question might have endocrine-disrupting potential, then further testing should be performed, i.e. a fish full life cycle study (USEPA 850, 1500) should be undertaken. If in such a study an effect is observed for which there is a coherent line of evidence that it could be related to an endocrine-disrupting mode-of-action, and there are considerations of the concentration causing at which these effects are occurring (see paragraph 33) then the substance should be regarded for regulatory purposes as an ED in fish.

c. Birds

39. Currently there are no internationally recognised standard assays for endocrine-disrupting properties in birds. It is possible that there might be initial indications from non-standard studies (e.g. in vivo or in vitro studies done for research purposes) of endocrine-disrupting potential and the results of a standard avian one-generation study might suggest a potential endocrine-disrupting mode-of-action. However, because of uncertainties about the reliability of reading across to birds the results from assays in other vertebrate classes, it is as yet unlikely that there will be a sufficiently clear and strong set of experimental evidence that can be generated, sufficient for a substance to be regarded for regulatory purposes as an ED in birds. This situation may change in the future as new international test guidelines are introduced.

d. Amphibians

40. The amphibian metamorphosis assay (OECD 231) is a screening assay that can reveal changes reflecting disruption of the hypothalamus-pituitary-thyroid axis in amphibians. However, the absence of a standard international guideline for a study in amphibians investigating adverse consequences on reproduction or population stability and uncertainties about the reliability of reading across to amphibians the results from assays in other vertebrate classes, it will not yet be possible to identify a

substance for regulatory purposes as an ED in amphibians. This situation may change in the future as new international test guidelines are introduced.

e. Reptiles

41. Currently there are no regulatory studies on reptiles and therefore it is not possible to determine if a substance is an ED in reptiles. This is a research need and this area should be reconsidered once studies or an approach based on available data is available.

f. Invertebrates

42. Currently several aquatic and terrestrial invertebrates are considered as part of the regulatory process. However, the current absence of relevant international test guidelines means that in most cases it is not possible to pursue the question of endocrine disruption capability in relation to invertebrates. There is a research requirement to develop appropriate screening tools as well as higher tier studies.

43 However, it should be noted that some pesticidal or biocidal substances (e.g. insect growth regulators) are designed to interfere directly with the hormonal system of some invertebrates. It is proposed that for such compounds, investigations should be undertaken to explore whether or not there is an adverse effect at the population level and at the field scale. Where such findings arise, then it might be appropriate to conclude that a substance is an ED in relation to non-target invertebrates in the environment.

7. Conclusion

44. In relation to potential ecotoxicity concerns, it is proposed that a substance is regarded as an ED for regulatory purposes when it satisfies the following definition and associated criteria:

- It should be an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse effects in an intact organism, or its progeny, or (sub)populations.

and in doing so satisfies the following criteria:

a. the nature of the effect must pose a threat to population recruitment or stability: and
b. there should be a reasonable and coherent line of evidence from within the same taxonomic group that the mode-of-action underlying the effect observed is endocrine disruption
c. there should be a consideration of the concentration/dose causing adverse endocrine effects as the example described in paragraph 33..

45. In general, the issue of whether or not a substance should be regarded for regulatory purposes as an ED on ecotoxicity grounds needs to be pursued separately for each major category of animals in the environment. At present in most cases it is

only possible to ascribe "ED" status in respect of the effects of a substance in environmental mammals and/or in fish.

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Appendix 1

Regulation 1107/2009 for placing plant protection products on the market – substance approval criteria

Human health

3.6.5 An active substance, safener or synergist shall only be approved if, on the basis of the assessment of Community or internationally agreed test guidelines or other available data and information, including a review of the scientific literature, reviewed by the Authority, it is not considered to have endocrine disrupting properties that may cause adverse effect in humans, unless the exposure of humans to that active substance, safener or synergist in a plant protection product, under realistic proposed conditions of use, is negligible, i.e. the product is used in closed systems or in other conditions excluding contact with humans and where residues of the active substance, safener or synergist concerned on food and feed do not exceed the default value set in accordance with point (b) of Article 18(1) of Regulation (EC) No 396/2005.

Environment

3.8.2 An active substance, safener or synergist shall only be approved if, on the basis of the assessment of Community or internationally agreed test guidelines, it is not considered to have endocrine disrupting properties that may cause adverse effects on non-target organisms unless the exposure of non-target organisms to that active substance in a plant protection product under realistic proposed conditions of use is negligible.

REACH (Regulation 1907/2006) – substances to be included in Annex XIV (substances subject to Authorisation)

Article 57 (f) : substances – such as those having endocrine disrupting properties or those having . . . . . - for which there is scientific evidence of probable serious effects to human health or the environment which give rise to an equivalent level of concern to those other substances listed in points (a) to (e) and which are identified on a case-by-case basis in accordance with the procedure set out in Article 59

[points (a) to (e) cover category 1A and 1B carcinogens, mutagens, and/or substances toxic to reproduction; and/or (very) persistent, (very) bioaccumulative, toxic (PBT or vPvB) substances]

Appendix 2

Definitions of EDs

Kavlock, 1996:

"An ED is an exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes."

NRDC, 1998:

"EDs are synthetic chemicals that when absorbed into the body either mimic or block hormones and disrupt the body's normal functions through altering hormone levels, halting or stimulating the production of hormones, or changing the way hormones travel through the body."

Weybridge, 1996:

"An ED is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function. A potential ED is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism."

WHO/IPCS, 2002:

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

Outlined below are summaries of the key studies that are currently available to assist in determining whether a substance is an endocrine disrupter.

Avian single generation reproduction test (OECD 206, adopted 1984):

Test birds (mallard duck (*Anas platyrhynchos*), bobwhite quail (*Colinus virginianus*) or Japanese quail (*Coturnix coturnix japonica*)) are fed a diet containing the test substance in various concentrations for a period of not less than 20 weeks. Birds are induced, by photoperiod manipulation, to lay eggs. Eggs are collected over a ten-week period, artificially incubated and hatched, and the young maintained for 14 days. Results of the test birds are compared with that of the control group that receive the basal diet only. Parameters considered are as follows:

Mortality of adults;
Signs of toxicity, along with severity, numbers
Food consumption
Egg production;
Eggs set
Cracked eggs;
Egg shell thickness;
Viability;
Hatchability (including normal hatchlings)
Survival of chicks
Results from gross pathological examinations

The endpoint derived from the study is a No Observable Effect Concentration (NOEC) in terms of mg test substance/kg diet. For regulatory purposes the endpoint is converted to a daily dietary dose taking in to account food consumption (mg test substance/kg bw/day).

21-day Fish Assay: A Short-Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition (OECD 230, adopted September, 2009)

This bioassay serves as an *in vivo* screening assay for certain endocrine modes of action. Sexually mature male and spawning female fish are held together and exposed to a chemical during a limited part of their life-cycle (21 days). At termination of the 21-day exposure period, depending on the species used, one or two biomarker endpoint(s) are measured in males and females as indicators of oestrogenic, aromatase inhibition or androgenic activity of the test chemical; these endpoints are vitellogenin and secondary sexual characteristics. Vitellogenin is measured in fathead minnow, Japanese medaka and zebrafish, whereas it is only possible to measure secondary sex characteristics in fathead minnow and Japanese medaka.

Observations
A number of general (e.g. survival) and core biological responses (e.g. vitellogenin levels) are assessed over the course of the assay or at termination of the assay. These are listed below:

Survival is examined on a daily basis. Sex of fish that die during the test should is determined by macroscopic evaluation of the gonads.

Any abnormal behaviour (relative to controls) should be noted; signs of general toxicity including hyperventilation, uncoordinated swimming, loss of equilibrium, and atypical quiescence or feeding. Additionally external abnormalities (such as haemorrhage, discoloration) should be noted.

Physical appearance in adult fathead minnows including body colour (i.e., light/dark), coloration patterns (i.e., presence or absence of vertical bands), body shape (i.e., shape of head and pectoral region, distension of abdomen), and specialized secondary sex characteristics (i.e., number and size of nuptial tubercles, size of dorsal pad and ovipositor).

Biological observations of gross morphology, including secondary sex characteristics and vitellogenin. Vitellogenin measurements are considered positive if there is a statistically significant increase in VTG in males ($p<0.05$), or a statistically significant decrease in females ($p<0.05$) at least at the highest dose tested compared to the control group, and in the absence of signs of general toxicity.

The range of test concentrations, care should be taken not to exceed the maximum tolerated concentration to allow a meaningful interpretation of the data. It is important to have at least one treatment where there are no signs of toxic effects. Signs of disease and signs of toxic effects should be thoroughly assessed and reported. For example, it is possible that production of VTG in females can also be affected by general toxicity and non-endocrine toxic modes of action, e.g. hepatotoxicity. However, interpretation of effects may be strengthened by other treatment levels that are not confounded by systemic toxicity.

Fish short-term reproduction assay (OECD 229, adopted September 2009)

The 21-day fish assay includes the evaluation of quantitative egg production and preservation of gonads for optional histopathology examination. The test Guideline describes an *in vivo* screening assay where sexually mature male and spawning female fish are held together and exposed to a chemical during a limited part of their life-cycle (21 days). At termination of the 21-day exposure period, two biomarker endpoints are measured in males and females as indicators of endocrine activity of the test chemical; these endpoints are vitellogenin and secondary sexual characteristics. Vitellogenin is measured in fathead minnow, Japanese medaka and zebrafish, whereas secondary sex characteristics are measured in fathead minnow and Japanese medaka.

Additionally, quantitative fecundity is monitored daily throughout the test. Gonads are also preserved and histopathology may be evaluated to assess the reproductive fitness of the test animals and to add to the weight of evidence of other endpoints

These observations and parameters measured are listed below:

Survival is examined on a daily basis. Sex of fish that die during the test should is determined by macroscopic evaluation of the gonads.

Any abnormal behaviour (relative to controls) are noted; signs of general toxicity including hyperventilation, uncoordinated swimming, loss of equilibrium, and atypical quiescence or feeding. Additionally external abnormalities (such as haemorrhage, discoloration) should be noted.

Physical appearance in adult fathead minnows is recorded including body colour (i.e., light/dark), coloration patterns (i.e., presence or absence of vertical bands), body shape (i.e., shape of head and pectoral region, distension of abdomen), and specialized secondary sex characteristics (i.e., number and size of nuptial tubercles, size of dorsal pad and ovipositor).

Fecundity is examined by daily quantitative observations of spawning should be recorded. Egg production is recorded as the number of eggs/surviving female/day.

Biological observations of gross morphology, including secondary sex characteristics and vitellogenin. Vitellogenin measurements are considered positive if there is a statistically significant increase in VTG in males ($p<0.05$), or a statistically significant decrease in females ($p<0.05$) at least at the highest dose tested compared to the control group, and in the absence of signs of general toxicity.

Performance of gonadal histopathology is an additional step that can be requested by regulatory authorities to study the target organ on the HPG axis following chemical exposure. In this respect, gonads are fixed either whole body or dissected. Specific endocrine-related responses on the gonads are looked for in the assessment of the endocrine activity of the test substance. These diagnostic responses essentially include the presence of testicular oocytes, Leydig cell hyperplasia, decreased yolk formation, increased spermatogonia and perifollicular hyperplasia. Other gonadal lesions like oocyte atresia, testicular degeneration, and stage changes, may have various causes.

In setting the range of test concentrations, care should be taken not to exceed the maximum tolerated concentration to allow a meaningful interpretation of the data. It is important to have at least one treatment where there are no signs of toxic effects.

Amphibian metamorphosis assay (OECD 231, adopted September 2009)

The Amphibian Metamorphosis Assay (AMA) is a screening assay intended to empirically identify substances that may interfere with the normal function of the hypothalamic-pituitary-thyroid (HPT) axis. The AMA represents a generalized vertebrate model to the extent that it is based on the conserved structures and functions of the HPT axis. It is an important assay because amphibian metamorphosis provides a well-studied, thyroid-dependent process that responds to substances active within the HPT axis, and it is the only existing assay that detects thyroid activity in an animal undergoing morphological development.

The general experimental design entails exposing stage 51 *Xenopus laevis* tadpoles to a minimum of three different concentrations of a test chemical and a dilution water control for 21 days. There are four replicates of each test treatment. Larval density at test initiation is 20 tadpoles per test tank for all treatment groups. The observational endpoints are hind limb length, snout to vent length (SVL), developmental stage, wet weight, thyroid histology, and daily observations of mortality.

v) Fish full life-cycle test (EPA OPPTS 850.1500)

Fish are cultured in the presence of the test substance from one stage of the life cycle to at least the same stage of the next generation (e.g. egg to egg). Testing is performed on a freshwater fish (e.g. fathead minnow).

The following information is obtained:

Reproductive effects;
Detailed records of spawning, egg numbers, fertility, and fecundity;
No-effect level, and mortality data;
Statistical evaluation of effects;
Locomotion, behavioural, physiological, and pathological effects;
Definition of the criteria used to determine effects;
Summary of general observation of signs of intoxication or other effects;
Stage of life cycle in which organisms were tested.

It is noted that the OECD are have produced a detailed review paper of the fish full life cycle study. However, the protocol has not yet been accepted. The detailed review paper can be found at the following web address:

'No 95: Detailed Review Paper on Fish Life-Cycle Tests' http://www.oecd.org/document/30/0,3343,en_2649_34377_1916638_1_1_1_1,00.html