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Key scientific issues relevant to the identification and characterisation of endocrine disrupting substances

Report of the Endocrine Disrupters Expert Advisory Group

Sharon Munn
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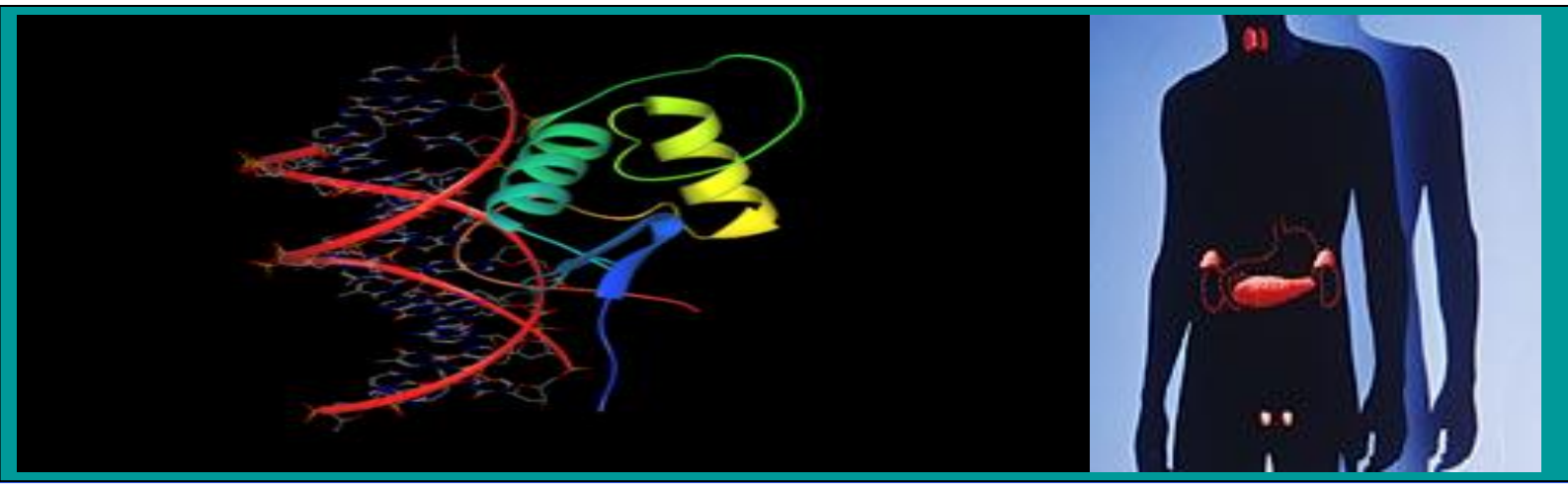

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Sharon Munn, Marina Goumenou

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EXECUTIVE SUMMARY

The Endocrine Disrupters Expert Advisory Group (ED EAG) was established in November 2011 as a sub-group of the ad hoc group of Commission Services, EU Agencies and Member States for the Community Strategy on Endocrine Disrupters. The main role of the ED EAG is to provide detailed reflections on scientific issues relevant to endocrine disrupting substances, not specific to any regulatory framework, including advice/orientation on scientific criteria for the identification of endocrine disrupting substances.

The ED EAG is composed of toxicologists and ecotoxicologists with regulatory and/or endocrinology backgrounds, nominated by Member State Competent Authorities for REACH (CARACAL) and the Plant Protection Products Regulation (PPPR) (Standing Committee), relevant industry associations and non-governmental consumer/environmental protection organisations. The European Commission's Joint Research Centre facilitates and chairs the meetings of the sub-group and prepared this report. Representatives of other relevant Commission services and EU Agencies are invited to attend the meetings as observers.

The scope of the present report is to capture the experts' opinions on key scientific issues relevant to the identification of endocrine disrupting substances (EDs) in order to support the ad-hoc group discussion and the Commission's decisions on the establishment of horizontal criteria for the identification of EDs for use in different regulatory contexts. It was agreed that the ED EAG was not required to reach consensus and may present differing opinions and options for consideration by the ad hoc group.

The ED EAG identified a number of key scientific reference documents as a basis for the discussion including the State of the Art Assessment of Endocrine Disrupters from Kortenkamp et al 2011 (SoA report), the IPCS/WHO State of the Science Report from 2002 (2012 update not being available at the time of the discussions) and two OECD documents from 2012, these being a guidance on use of standardised test guidelines for evaluating endocrine disrupters and a review of the state of the science of novel *in vitro* and *in vivo* assays for evaluating endocrine disrupters.

The Expert Group accepted the use of the current international definition of an endocrine disrupter from IPCS/WHO as a working definition to stimulate discussion by analysing each of the elements within the definition. In addition, it was agreed to take as a starting point for discussion the factors or elements (described below) identified in the SoA report under decision criteria for the regulation of endocrine disrupting properties. During the discussion it became obvious that some of the proposed factors were connected to the identification of EDs, while other factors were more connected to a characterisation of the hazard posed.

The ED EAG agreed that the elements for identification of an endocrine disrupter were demonstration of an adverse effect for which there was convincing evidence of a biologically plausible causal link to an endocrine disrupting mode of action and for which disruption of the endocrine system was not a secondary consequence of other non endocrine-mediated systemic toxicity. Relevance of the data to humans should be assumed in the absence of appropriate data demonstrating non-relevance. In relation to wildlife populations, data on all species are generally considered relevant. Relevance is instead applied in the context of identified adverse effects being relevant at population rather than individual level.

Factors such as potency, severity, irreversibility and lead toxicity were considered not part of the identification but rather inform on characterization of the hazard of EDs. The human health experts agreed that all the factors were relevant for hazard characterisation and could play a role in ranking and priority setting. Some experts supported using the information for differentiating EDs into

classes or categories of lower or higher concern (acknowledging that deciding on where to place a potency cut-off would be a policy decision) and some experts did not support the use of the information for this purpose but considered that the information could only be used within a risk assessment context. With respect to ecotoxicological assessment, there was no agreement on how to consider these factors with respect to hazard characterisation of EDs outside the context of risk assessment.

The ED EAG discussed a basic scheme for the consideration of available evidence and for building evidence on endocrine disrupting properties of substances. The ED EAG supported consideration of mode of action and adversity in parallel applying weight-of-evidence approaches, weighing all available evidence, both positive and negative, including human epidemiology data, field data, animal experimental (eco)toxicology studies, *in vitro* data, (Q)SAR, analogue and category approaches to reach a conclusion.

The ED EAG was not able to provide a full evaluation of the adequacy of the currently available test methods in the time available. However, it was observed that currently available OECD standardised tests within the OECD Conceptual Framework for the testing and assessment of endocrine disrupters are mostly focused on the identification of substances acting by interference with estrogen, androgen or thyroid hormone pathways, including steroid hormone production, for mammals, fish and possibly amphibians, but not for birds or invertebrates. Existing standardised assays might miss some endpoints sensitive to endocrine disruption and it was acknowledged that there was no standardised assay currently available in mammals that allows the investigation of early life/*in utero* exposure on effects which may appear in later life stages, such as cancer incidence, impact on menopause, senescence etc. Further work to identify relevant *in vivo* biomarkers indicative of endocrine activity to augment existing assays was recommended. It was also recommended that priority areas for further development of assays to investigate specific endocrine pathways should be informed by emerging human health issues or observed negative impacts on wildlife populations and hypothesised link to endocrine-related causes.

List of Abbreviations

ADME	Absorption, Distribution, Metabolism, Excretion
AGD	Anogenital Distance
AOP	Adverse Outcome Pathway
AR	Androgen Receptor
BPR	Biocidal Products Regulation
CF	Conceptual Framework
CLP	Classification, Labelling and Packaging
CMR	Carcinogenic, Mutagenic, toxic to Reproduction
DG	Directorate General
EAG	Expert Advisory Group
EAS	Endocrine Active Substance
EAT	Estrogen, Androgen and Thyroid
EATS	Estrogen, Androgen, Thyroid and Steroidogenesis
EC	European Commission
ED(s)	Endocrine Disrupter(s) or Endocrine Disrupting Substance(s)
EDSP	Endocrine Disruptors Screening Program
EFSA	European Food Safety Authority
ER	Estrogen Receptor
EU	European Union
GLP	Good Laboratory Practice
HPA	Hypothalamic-Pituitary-Adrenal
HPG	Hypothalamus-Pituitary-Gonad
HPT	Hypothalamic-Pituitary-Thyroid
IPCS	International Programme on Chemical Safety
MoA	Mode of Action
MS	Member State
NOAEL	No Observed Adverse Effect Level
NOEC	No Observed Effect Concentration
OECD	Organization for Economic Co-operation and Development
PPPR	Plant Protection Products Regulation
(Q)SAR	(Quantitative) Structure-Activity Relationship
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals Regulation
SoA	State of the Art
TG	Test Guideline*
EOGRTS	Extended One-Generation Reproductive Toxicity Study
ToR	Terms of Reference
EPA	Environmental Protection Agency
WHO	World Health Organization

http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm

1. INTRODUCTION

The European Commission, under Directorate-General for Environment (DG ENV), has established in 2010 an ad hoc group of Commission Services, EU Agencies and Member States under the Community Strategy for Endocrine Disrupters (COM(1999)706) to exchange information on endocrine disrupters, to assist the Commission in shaping future policy in this area and to promote coordination with the view to ensure an integrated, coherent and consistent approach to dealing with endocrine disrupters across the different regulatory frameworks.

The ad hoc group works at the interface between science and policy providing an appropriate forum for information exchange on endocrine disrupters, for bringing science on endocrine disrupters and chemicals' policy together, for discussing horizontal aspects of regulation of endocrine disrupters and for providing orientation to the European Commission on development and implementation of EU policy in this field. At its 2nd meeting held in May 2011 the ad hoc group agreed to establish an expert sub-group tasked with making detailed reflections on the scientific issues and in particular on providing advice/orientation to the ad hoc group on the scientific criteria for the identification of endocrine disrupters.

1.1 Terms of Reference of the Endocrine Disrupters Expert Advisory Group (ED EAG)

Although no formal terms of reference were adopted the main purpose of the ED EAG was described and agreed at the 3rd ad hoc group meeting and 1st meeting of the ED EAG held back-to-back on 16 & 17 November 2011 to be as follows:

- To provide detailed reflections on scientific issues including advice/orientation on scientific criteria for the identification of EDs.
- To provide a forum for information exchange and discussing scientific aspects to facilitate consistent, coherent approaches to identification and assessment of EDs.
- To provide generic advice not specific to any regulatory framework.
- To feed output into the ad hoc group where science and policy issues interface.

In addition it was recommended that participants to the ED EAG should have an (eco)toxicology background, preferably with a regulatory background and/or specific experience in endocrinology.
It was clarified and agreed that the ED EAG was not required to reach consensus and may present differing opinions and options for consideration by the ad hoc group.

In relation to the ED EAG composition Member State Competent Authorities for REACH[1] (CARACAL) and PPPR[2] (Standing Committee), relevant Industry Associations and NGOs could nominate up to 2 experts each. The European Commission's Joint Research Centre facilitated and chaired the meetings and prepared this report. Representatives of other relevant Commission services and EU Agencies were invited to attend the meetings as observers. Other experts could be invited (by the Commission) as appropriate.

Additional terms were that:

- The ED EAG would work by e-mail and have face-to-face meetings
- The activities of this group would be reported to every meeting of the ad hoc group.

[1] Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) Regulation (EC) No.1906/2007, OJ L136, 3-280

[2] Regulation (EC) No. 1107/2009 concerning the placing of plant protection products (PPP) on the market, OJ L309, 1-50

The ED EAG agreed to take the State of the Art report by Kortenkamp A, et al., 2011 (commissioned by the European Commission) (from now on referred as the SoA report) as the basis for the further work taking the first 3 stages of the decision criteria as a starting point for organising the discussions at the following meetings. These stages were:

- Stage 1: Adversity and Mode of Action
- Stage 2: Human and wildlife relevance
- Stage 3: Toxicological Evaluation (potency, lead toxicity, specificity, irreversibility and severity)

A small working group of volunteering experts within the ED EAG was assigned to act as a planning committee to assist in the preparation of the meetings.
Although the value of discussing human health and wildlife issues together was recognised, it was agreed that the most practical and efficient way of working was to have separate parallel sessions at the meetings for the human health and the environmental aspects with a common plenary session to discuss overarching issues.
With respect to the specific deliverable of an advisory report on scientific criteria for the identification of EDs for further regulatory use, June 2013 was proposed as the target date (later revised to March 2013), with December 2012 proposed as a target for a draft report.
The participants of the ED EAG agreed with the objectives as outlined.

1.2 Scope of the report

The scope of the present report is to capture the experts' opinions on key scientific issues relevant to the identification of endocrine disrupting substances (EDs) in order to support the ad hoc group discussion and the Commission's decisions on the establishment of horizontal criteria for the identification of EDs for use in different regulatory contexts. Factors considered relevant to characterisation of identified EDs with respect to level of concern were also discussed.
In order to identify key scientific issues, the state of the art report (Kortenkamp et al, 2011) and the factors identified in the report were used as a basis. During the discussion it became obvious, that some of the factors are connected to the identification of EDs, while other factors are more connected to characterisation of the hazard posed. The report also captures this discussion.

1.3 Structure of the report

The structure of the present report was designed to support its scope, meaning to facilitate further the discussion of the ad hoc group and the Commission's decisions. In order to achieve this aim it was considered appropriate to focus on the factors most important to identification and characterisation of EDs, and represent the experts' views in a transparent and unbiased way.

The first two chapters are dedicated to the terms of reference, and the presentation of the terms and definitions used to guide and underpin the further discussions. Chapter 3 starts with factors relevant for the identification of an endocrine disrupting substance followed by a discussion about other factors which might be relevant for hazard characterization of such EDs. Chapter 4 presents the discussion in relation to the SoA Report's suggested scheme for the evaluation of EDs and weight of evidence approaches to assessment of available data. In chapter 5 the currently available tools and methods for assessing EDs are briefly discussed. This is followed by a brief discussion of which additional test methods might be needed, as well as the need for further research into other possible endocrine-related adverse effects and modes of action.

It was considered more appropriate to combine the different discussions for the human health and the environment together, providing exemplifications from each of the disciplines of the points discussed, where appropriate, and highlighting differences where they existed.

1.4 Background material

As agreed the "State of the Art Assessment of EDs" report (Kortenkamp A, et al., 2011) was used as one of the main reference documents for the discussion.

The following literature, amongst others, was also used as reference material:

- The Detailed Review Paper (DRP) on the State of the Science on Novel *In Vitro* and *In Vivo* Screening and Testing Methods and Endpoints for Evaluating Endocrine Disruptors, Series on Testing & Assessment No. 178 (OECD 2012a)
- The OECD Conceptual Framework and Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption, Series on Testing & Assessment No. 150 (OECD 2012b)
- The IPCS mode of action and human relevancy framework (Boobis, et al, 2009)
- The IPCS/WHO "Global assessment of the State-of-the-Science of Endocrine Disruptors report" (IPCS/WHO 2002)[3]
- The article from Klimisch et al. for evaluating the quality of experimental toxicological and ecotoxicological data (Klimisch H.J., et al., 1997)

1.5 Case Studies

The use of substance-specific case studies as a tool for exemplifying the critical issues within each of the topics was generally accepted. The choice of appropriate substances to reveal data gaps and relevant issues was emphasised. It was considered that the application of discussed factors for ED identification to real data sets, as provided by the case studies, would help inform the discussion and might serve to exemplify where guidance would be required in the future application of criteria.
In order to facilitate the discussion on the adequacy of data and the strength of evidence both data-rich and data-poor case studies were selected. For some case studies clear evidence for adversity and/or mode of action was available while the data for other cases were considered inconclusive. The suite of case studies covered both human health and environment. During the discussion of the factors for stages 1 to 3 (as described in the SoA report) the identities of the substances were masked in order to avoid possible bias.

2. DEFINITIONS AND UNDERSTANDING OF BASIC TERMS

2.1 Endocrine Disrupter

The ED EAG accepted the use of the following current international definition of an endocrine disrupter from IPCS/WHO as a working definition to stimulate discussion by analysing each of the elements within the definition.

"An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations" (IPCS/WHO, 2002).[4]

2.2 Scope of endocrine system

[3] The WHO/UNEP State of the Science of Endocrine Disrupting Chemicals – 2012 was released too late to be taken into account in this report

[4] The WHO/UNEP State of the Science of Endocrine Disrupting Chemicals 2012 update released February 2013, did not propose any modification of the IPCS/WHO 2002 definition.
http://www.who.int/ceh/publications/endocrine/en/index.html

In order to apply the above mentioned IPCS/WHO definition of endocrine disrupter which refers to *an exogenous substance or mixture that alters function(s) of the endocrine system* it would appear that the scope of the endocrine system would need to be defined. Many descriptions of the endocrine system can be found in authoritative sources such as the IPCS/WHO, 2002, State of the Science review (IPCS/WHO, 2002) and the OECD report on novel endpoints (OECD 2012a) including reference to both the components and the function of the endocrine system. The endocrine system could be considered as regulating all biological processes in the body by synthesising chemical messengers (hormones) in one tissue which are transported (by the circulatory system) to other tissues in which they produce their physiological effects. Thus the endocrine system could be considered to encompass the hormone-producing tissue, the specific target receptors, transport proteins and associated enzymes. According to the SoA report (Section 2.1, p.13) "*The three important endocrine axes are the hypothalamus-pituitary-gonad (HPG) axis, the hypothalamic-pituitary-adrenal (HPA) axis and the hypothalamic-pituitary-thyroid (HPT) axis. These axes describe the boundaries within which the endocrine system and endocrine disruption have been confined from the perspective of classical endocrinology*. *However, the scientific advances in our understanding of receptor signalling and molecular biology are continuously blurring the borders between the nervous system, immune system and endocrine system. …….An implicit understanding of the endocrine system or endocrine signalling can therefore span from the classical definition of the endocrine system to one that encompasses any type of receptor-mediated signalling.*"
Interest has been focused so far primarily in relation to reproductive and developmental toxicity via perturbations of the estrogen, androgen and thyroid hormone (EAT) pathways. Assays have been developed and validated at OECD which mainly address the ability of chemicals to interfere with EAT signalling processes and steroidogenesis. In consideration of other endocrine pathways which may also be susceptible to disruption by environmental chemicals, the OECD recently commissioned a review (OECD 2012a), in which the authors were tasked with identifying novel assays and endpoints that could be used for assessing whether or not chemicals might have endocrine-disrupting activity beyond EAT pathways and steroidogenesis. Evaluations covered not only the HPA, HPG and HPT endocrine axes but also the somatotropic axis and the retinoid, vitamin D and peroxisome proliferator-activated receptor (PPAR) signalling pathways. Whilst acknowledging that the ligands to some of these receptors did not fit the conventional view of a hormone (e.g. retinoids and fatty acids), the authors considered that they did fit into the broad definition of a hormone described by Thomas (1984) as "*a substance, originating in one tissue and conveyed by the bloodstream to another to effect physiological activity*".

The SoA report also raises the question in the ecotoxicological context of whether the term "endocrine system" should be interpreted in the very narrow sense of the hormonal system of vertebrates or whether it should include not only invertebrates, but also microbes and plants.
In answer to this question the ED EAG concluded that even if it were desirable to address endocrine disruption across all taxa, based on current knowledge it is not possible to define the limits of the endocrine system for all environmental species. From a pragmatic point of view there are methods and tools available and known adverse outcomes for EAT pathways for certain species. Thus the definition of the endocrine system must include the existing knowledge (EAT pathways and steroidogenesis), but must be relatively open and flexible in order to cover less known and newly or still to be discovered endocrine system elements in several taxa (e.g. ecdysteroids for moulting and egg maturation in crustaceans and insects, juvenile hormones to regulate metamorphosis and reproduction, neuropeptides to regulate metabolism or diapause in insects and all other invertebrate endocrine systems).

Overall, the Expert Group supported the notion of a broad definition of the endocrine system that would not require constant updating as knowledge develops. However, it was acknowledged

that currently the possibilities for identifying an endocrine disrupting mode of action were largely confined to the EAT pathways as well as interference with steroidogenesis, where mechanistic assays have been developed and validated

2.3 Adversity

Another element of the IPCS/WHO definition of endocrine disrupter is "adverse health effects". The concept of adversity within toxicology and the point at which an observed change should be considered adverse is a topic of continuous debate and the IPCS has adopted a general definition not specific to endocrine disrupters as follows:

"*A change in the morphology, physiology, growth, development, reproduction or lifespan of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences*" (IPCS/WHO, 2009).

This IPCS definition of an adverse effect includes consideration of a population level effect. Since the protection level is set at the population level for environmental assessments, for an effect to be considered adverse it should have the potential to impact at the population level (Suter et al., 1993; EFSA, 2010c). The protection of the population requires the protection of all (sensitive) life stages. The ED EAG accepted the IPCS definition of an adverse effect as a working definition on which to base the further discussions.

2.4 Endocrine Mode of Action

The IPCS/WHO definition of an endocrine disrupter refers to "an alteration of the function of the endocrine system". A substance may cause such an alteration by many different "modes of action". The term "mode of action" has been used by IPCS within the specific context of the mode of action and human relevancy framework to refer to how a substance interacts with a biological system to produce an adverse effect. In this context mode of action is defined as follows:

"The biologically plausible sequence of key events, starting with the interaction of an agent with a cell, through functional and anatomical changes leading to an observed effect supported by robust experimental observations and mechanistic data" (Boobis et al, 2009).
In addition *"Mode of action differs from mechanism in that the latter implies a more detailed understanding of the molecular basis of the toxic effect" (Seed at al, 2005).*

2.5 Proof of causality

In deconstructing and analysing the elements of the IPCS/WHO definition of an endocrine disrupter a causal association between an alteration of the function of the endocrine system and the adverse health effect is provided by the term 'and consequently causes'. Whilst acknowledging that absolute proof of causation might be too high a requirement in establishing a substance as an endocrine disrupter, a biologically plausible linkage between the activity of the chemical in producing the alteration of the endocrine system and an observed adverse effect would need to be accepted as the most likely underlying explanation. Such a causal chain of events from initial interaction of a substance with its target site in the organism through to the adverse outcome was seen to be encapsulated in the definition of mode of action (Boobis et al, 2009) and are key in the development of Adverse Outcome Pathways (Ankley et al., 2010). During the discussion it became obvious, that the type and amount of information needed to

demonstrate a biologically plausible linkage depend on the mode of action and the type of effects observed (see discussion under 3.1.2, 3.1.3 and 4). For example, even where data for several species are available, with *in vitro* data sometimes indicating several different potential modes of action, a causal link and proof of adversity often may only be possible for one, well understood adverse outcome pathway (e.g. ER-mediated impairment of fish reproduction, where ER-binding/VTG-induction/induction of testis-ova/skewed sex-ratio could be a plausible series) and for one species. Sometimes adverse effects are difficult to link to modes of action, even though mode of action as well as adversity point towards endocrine disruption.

2.6 Endocrine Activity

The term endocrine activity is often used to refer to substances capable of interacting with the endocrine system, e.g. by binding to and activating or blocking steroid receptors, but for which evidence linking such activity to an adverse effect is currently lacking, i.e. there is no evidence of endocrine disruption in accordance with the IPCS/WHO definition.
A report of EFSA's Endocrine Active Substances Task Force uses the term endocrine active substance (EAS) "*to describe any chemical that can interact directly or indirectly with the endocrine system and subsequently result in an effect on the endocrine system, target organs and tissues.*" and adds *"Whether the effect is adverse or not will depend on the type of effect, the dose and the background physiological situation"* (EFSA, 2010a).

3. FACTORS CONSIDERED

In the SoA Report (Kortenkamp et al, 2011) it is stated in relation to the IPCS/WHO definition for an endocrine disrupter that *"there are clearly two requirements for a substance to be defined as an endocrine disrupter, namely that of the demonstration of an* ***adverse effect*** *and of an* ***endocrine disrupting mode-of-action".*** The SoA report under Section 7.2, *Decision criteria for the regulation of endocrine disrupting properties* proposes that weight-of-evidence approaches be applied to evaluation of both adversity and endocrine mode of action in parallel and further suggests that other factors (such as relevance, potency, specificity, severity and irreversibility) are considered at subsequent stages of a step wise decision criteria approach. The ED EAG discussed each of the factors and whether they are relevant with regard to the identification of a substance as an ED or for a further hazard characterization of such EDs. The output of the discussions is captured below, grouped according to whether the factor is considered to be part of ED identification or further hazard characterisation.

3.1 Factors relevant for the identification of a substance as an endocrine disrupter

In line with the SoA report the following factors were considered relevant for the decision whether or not a substance is an endocrine disrupter according to the IPCS/WHO definition.

3.1.1 Adversity

A number of questions were posed in consideration of two aspects of adversity, i) when an observed change can be considered as adverse (considering the IPCS definition) and ii) what types of adverse effects may be endocrine-mediated (or even diagnostic of endocrine disruption). The latter is considered relevant with regard to the question whether or not a certain type of effect may be clearly endocrine mediated (i.e. diagnostic) or may rule out endocrine activity as the effect is clearly not endocrine mediated.

With respect to i) when a change can be considered adverse, the IPCS/WHO definition of adverse health effect, refers to changes in morphology or physiology leading to impairment of function. For example, with regard to human health endpoints, it could be discussed whether a change in morphology such as adrenal vacuolation without impairment of the functional capacity of the adrenal could be considered as adverse. On the other hand, it was noted that it might be easier to conclude a change in morphology as adverse compared to a change in physiology which is a dynamic process and deciding when such a change becomes adverse is very difficult. One of the roles of the endocrine system is to maintain homeostasis in response to physiological modulations. Fluctuations within the normal limits of homeostasis may be considered as physiological modulation without adverse consequence. At what point these fluctuations may become significant in the absence of an accompanying observable adverse effect on function could not be defined and would always be a case-by-case decision. For example a fluctuation in thyroid hormones for a short period of time during critical windows of development could lead to serious adverse effects. It was considered helpful to know the normal range of fluctuations in untreated groups. Positive control studies using a known reference chemical could give useful information in this direction in relation to the homeostatic capacity of the system. The sustainability of a fluctuation is also an important parameter in the evaluation. If the stimulus is constant the fluctuation may be maintained (high or low compared to normal) indefinitely. It was considered important to evaluate the possible impacts of such a maintained change of state, particularly with respect to the IPCS definition of adversity which refers to a change which may impair an organism's capacity to compensate for additional stress.
In contrast to the human health assessment, the protection goal of environmental assessment is the protection of populations rather than the individual (Suter et al., 1993; EFSA, 2010b). Thus, for an effect to be considered adverse it should have the potential to impact at the population level, including sensitive life stages. With regard to ecotoxicological assessment, effects on apical endpoints which are likely to affect the population such as growth, reproduction and development are usually considered adverse. Changes in vitellogenin levels in fish can be considered as a physiological modulation as well as a biomarker of endocrine activity, however the effect is not considered as adverse in itself.

With respect to ii) what types of adverse effects may be endocrine-mediated it was agreed that when considering a broad definition of the endocrine system (see Section 2.2) it is difficult to assess which adverse effects are clearly endocrine-mediated or non-endocrine mediated, unless there is prior knowledge of the biological processes leading to the adversity. However, since standard *in vivo* toxicity studies are not designed to demonstrate how a substance produces its toxic effects, the mode of action is unknown for most substances.
In assessment of mammalian toxicity in relation to human health, the experts concluded that specific endpoints where there is already some knowledge of mode of action such as skin, eye and respiratory tract irritation, skin and respiratory sensitisation, mutagenicity and genotoxic carcinogenicity can be regarded as endpoints/effects clearly not endocrine-mediated. On the other hand, toxic effects on endocrine glands or effects on reproduction or development were seen as candidates for adverse effects caused by endocrine disruption. In addition, since every organ and system in the body is under some form of hormonal or neuro-hormonal control, it was considered important to discriminate between endocrine toxicity as a primary effect of an endocrine MoA and endocrine toxicity *secondary* to other toxic effects not mediated by an endocrine MoA [see 3.1.3. Specificity].
In consideration of adversity in relation to ecotoxicological assessment it was stated that many of the population-relevant endpoints measured in standard Test Guidelines related to reproduction, growth and survival are quite generic and it is difficult to exclude any of these adverse effects as not endocrine-mediated without some information on mode of action. However, an understanding of the level of systemic or generalised toxicity can give an indication of whether

effects are specific and therefore more or less likely to have an underlying endocrine disrupting MoA.
There was also a discussion on whether some effects may be *both* adverse as well as indicative of an endocrine disrupting MoA, i.e. whether specific adverse effects or patterns of adverse effects could already be identified on the basis of existing knowledge to be diagnostic for endocrine disruption. Two specific examples were discussed:

Example 1. In ecotoxicological assessment a change in sex ratio of fish was seen as both adverse and, according to the majority of the experts, highly likely to be a marker of endocrine disruption. An example was given of the OECD fish sexual development TG (OECD TG 234) in which consistent co-observation of a change in sex ratio accompanied by a change in vitellogenin level (biomarker of endocrine activity) has been observed in certain fish species (OECD, 2012b). For other fish species than those recommended in the TG the basis for using sex ratio as diagnostic of endocrine disruption was unclear. The degree of change in sex ratio would also be a factor in weighing the strength of evidence as a complete feminization/masculinisation could be considered as diagnostic while only a small change or a delay in sexual differentiation might not.

Example 2. In humans a pattern of effects known as testicular dysgenesis syndrome including hypospadias, cryptorchidism and decreased sperm quality which can also be replicated in laboratory mammals by certain chemicals (including hypo- and a-spermatogenesis, atrophy of the seminal vesicles and prostate, nipple retention, hypospadia, penis malformations, vaginal pouches, ectopic testes and decreased anogenital distance), was seen as highly likely to be mediated by an anti-androgenic mode of action. Such a clear pattern of effects occurring together was considered by most experts as diagnostic of an endocrine disrupting MoA, however others considered that in order to identify a substance as an ED a hypothesised endocrine MoA needed to be supported by some relevant mechanistic data in all cases, not only to identify an ED but also to further knowledge and understanding of how chemicals alter the function of the endocrine system (e.g. the need to populate databases with *in vitro/in vivo* screens and apical studies to understand the correlation between data from these various studies for a better prediction when dealing with data poor chemicals).

A substance disrupting the function of the endocrine system might be expected to lead to a spectrum of effects since the endocrine system has many interactions, feedback mechanisms and cross-talks with other systems and the possibility exists that several organs and several functions in the body might be affected. Such spectrum of effects all contribute to a weight-of-evidence assessment of endocrine disruption. An isolated finding may be less convincing but may nevertheless be concluded to be adverse.

3.1.2 Endocrine mode of action and causal link to adversity

An exogenous substance may alter the function of the endocrine system through many different modes of action including interference with production, transport and metabolism of hormones thus altering circulating hormone levels or by disrupting the target receptor function by inappropriately activating the receptor (hormone receptor agonist) or by inhibiting the action of the receptor (hormone receptor antagonist). A substance may be shown to be endocrinally active usually through *in vitro* mechanistic assays demonstrating for example receptor binding/(in)activation or interference with hormone production, however, such activity may not be expressed *in vivo* and the link to an adverse outcome is not provided by evidence of such endocrine activity alone. In the context of the IPCS mode of action and human relevancy framework (Boobis et al, 2008 & 2009) whereby mode of action is defined as "*The biologically plausible sequence of key events, starting with the interaction of an agent with a cell, through functional and anatomical changes leading to an observed effect supported by robust experimental observations and mechanistic data*" the causal link is embedded in the definition.

The IPCS MoA framework describes a process for laying out the strength of evidence in a transparent manner supporting a particular hypothesised mode of action applying modified Bradford Hill considerations of association such as biological plausibility and coherence, consistency of findings, concordance of dose response relationships and temporal associations and most importantly characterisation of uncertainties. The ED EAG considered that the level of evidence required by the framework in supporting the sequence of key events leading to adversity might be too high a requirement for the identification of an ED for regulatory purposes since it was developed within the context of relevancy of adverse effects in animals to humans where a high degree of confidence is required that a particular MoA is operative in the animal as well as confidence that this MoA is not relevant for humans. Nevertheless it was considered that the framework could be adapted to the demonstration of an endocrine disrupting MoA. The ED EAG considered that evidence of endocrine activity *in vitro*, along with evidence of an *in vivo* biomarker of endocrine activity and adverse effect coupled with a biologically plausible relationship between the measured parameters should be sufficient to conclude on endocrine disruption. The type and amount of information needed at the different levels depend on the mode of action considered as well as the type of effect observed, e.g. for an estrogen agonist mode of action an increased *in vivo* vitellogenin level together with *in vitro* information and decreased reproductive success in the absence of systemic toxicity might be enough to conclude that the adverse effects are caused by an endocrine (estrogenic) mode of action

3.1.3 Specificity

Specificity in terms of an adverse effect that manifests itself as a consequence of an endocrine disrupting mode of action, and not indirectly as a result of other non endocrine mediated systemic toxicity was considered to be an integral part of ED identification with respect to whether or not the primary MoA for the adverse effect is endocrine-related.

Apart from identifying EDs as those targeting the endocrine system and circulating hormone levels, EDs may also be identified as those affecting hormone targets (i.e. hormone receptors).

An adverse effect, arising from selective cytotoxicity to hormone producing cells can be considered as having arisen via an endocrine disrupting MoA and adverse thyroid effects secondary to liver enzyme induction leading to enhanced hormone metabolism was also considered to represent adversity arising via an endocrine disrupting MoA. However, non-specific, marked systemic toxicity where effects on the endocrine system might be observed along with other toxic effects should not be considered to be the result of an endocrine disrupting MoA in the absence of any other specific information that might be indicative of a plausible endocrine disrupting MoA.

Some guideline studies for both human health and ecotoxicology may not allow a distinction to be made between primary and secondary effects. Therefore when considering an endocrine disrupting MoA it is important to consider the onset and the time-line of the endocrine-mediated chain of events together with the limitations of the available standardised tests (e.g. the conclusions related to endocrine disruption and adversity may be very different in a short term compared with that of a long term study).

3.1.4 Human and wildlife relevance

It was agreed that it was often difficult to demonstrate convincingly the non-relevance to humans of adverse effects observed in the animal models and that the usual approach was to assume relevance unless non-relevance to humans could be convincingly demonstrated by, for example, applying the guidance provided by the IPCS mode of action and human relevancy framework (Boobis et al, 2008).Thus relevance to humans should be assumed by default in the absence of appropriate scientific data demonstrating non relevance.

In relation to ecotoxicology, data on all species, including mammalian data generated to assess human toxicity, are generally considered relevant to the assessment of effects on ecosystems. In addition, since ecotoxicological assessment relates to impact at the population level rather than the individual level, relevance is applied in the context of identified adverse effects being relevant for the population.

3.1.5 Summary

The elements for identification of an endocrine disrupter were agreed to be demonstration of an adverse effect for which there was convincing evidence of a biologically plausible causal link to an endocrine disrupting mode of action and for which disruption of the endocrine system was not a secondary consequence of other non endocrine-mediated systemic toxicity. Relevance of the data to humans and wildlife populations should be assumed in the absence of appropriate data demonstrating non-relevance.

3.2 Factors possibly relevant for the characterization of Endocrine Disrupters

The following factors suggested by the SoA report were considered not relevant for the identification of substance as ED but could provide information with regard to the further characterization of the hazard of such substances.

3.2.1 Severity of Effect

Both seriousness (in the qualitative sense of the nature of effect) and magnitude (quantitative) was considered to be covered by the term severity.
From the perspective of human health assessment, seriousness should consider consequences on quality of life (e.g. infertility, developmental effects or shift in IQ of population) and was also considered to be linked to irreversibility in relation to the nature of effect. It was considered that EDs once identified based on adverse effect and endocrine MoA may be further differentiated in relation to the severity of the adverse effect. The experts agreed that severity is not part of identification but rather informs on hazard characterisation. The magnitude of the effect at a given dose was linked to potency (discussed below) whereas the seriousness of the effect was linked to level of concern regarding impact on environment and society and therefore more relevant to risk management considerations.

With respect to ecotoxicological assessment, most of the experts considered that severity (magnitude and nature of effect) was properly addressed by the concept of adversity (any effects considered adverse at population level being by definition severe), and that further grading was not required. One expert thought that the magnitude of an effect could be a measure of severity and thus could be used for further characterizing an ED (e.g. a small reduction in reproduction compared to zero reproduction, where both are considered adverse but differentiation on the basis of the magnitude of the adverse effect can be made). To characterize an ED based on the nature of the effect was considered difficult as the test and endpoints measured are indicative of effects on the population level and it is thus difficult to correlate nature of effect with potential impact at population level. The experts also discussed whether or not with regard to the environment the number of species affected might be a factor to characterize an ED. However the experts concluded that such a differentiation would be difficult as even if only a small proportion of species is affected they might be of high relevance for the ecosystem.

3.2.2 Irreversibility

In terms of the hazard characterisation, irreversibility was considered to be part of severity in relation to the nature of the effect. It was considered that even where an adverse effect might be reversible following cessation of exposure, in considering a continuous emission exposure scenario the possibility for recovery was not provided thus the issue of determining within an assessment whether an effect was reversible or not was not so important. However in terms of the possibility of treatment and recovery of an individual or population following identification and removal of the causative factor, or in the case of persistent chemicals the lack of ability to remove the causative factor, knowledge of the irreversibility of the effect would be extremely important information in relation to level of concern regarding impact on environment and society and therefore more relevant to risk management considerations. In addition, for known and well described non-continuous exposure scenarios (e.g. plant protection products) knowledge on potential reversibility of an effect may be important in relation to level of concern. However, it was emphasised that current (eco)toxicity test guidelines are often not designed for determining whether effects are reversible or not, thus limiting the possibility to address this issue. In addition the reversibility/irreversibility of an effect may depend on the timing of exposure (e.g. in adult life or during a critical time of development such as during pregnancy when hormones are triggering organ differentiation).

3.2.3 Lead toxicity

Substances may produce different effects at different dose levels by different modes of action in the same species. Lead toxicity is the effect that occurs at a lower dose than other toxic effects, i.e. the most sensitive or dominant feature of the hazard profile of a substance. Lead toxicity is generally used in risk assessment to identify the most sensitive endpoint (i.e. critical effect) as a point of departure for risk assessment. The premise is that when conducting a risk assessment on the effect seen at the lowest dose level this will also protect the individual or population from effects occurring at higher dose levels. However this would only be applicable to endpoints for which a threshold approach was considered appropriate and where there is confidence that the toxicity studies have covered critical windows of exposure and relevant endpoints for endocrine disruption. The experts agreed that lead toxicity is not relevant for hazard identification (i.e. if adverse effects are observed which are clearly endocrine mediated, the substance should be identified as ED irrespective of whether or not the endocrine-mediated effects are the most sensitive effects), but some human health experts considered it may have a role in hazard characterisation. For example, if non endocrine-mediated toxicity was expressed at concentrations orders of magnitude lower than the dose producing the endocrine-mediated effect this may inform under hazard characterisation on the potential importance of this endocrine-mediated effect to the overall toxicity profile of the substance but this would depend on the type of effect at the lowest dose and the dose spacing.

Other human health experts argued that it can be difficult to identify the lowest effect level caused by endocrine disruption, because of the possibility of non-monotonic dose responses. In addition, it was noted that even endocrine-mediated effects not arising at the lowest adverse effect dose level can contribute to additive effects on co-exposure to other substances acting via similar modes of action or causing common adverse outcomes.

In ecotoxicological assessment it may be difficult to separate endocrine-mediated adverse effects from non endocrine-mediated adverse effects within the same species, since different types of toxicity in different target organs, which could help to conclude that one adverse endpoint is endocrine-mediated and another is not, are generally not assessed. Hence the majority of the experts concluded that it is difficult to use lead toxicity for further characterisation of the hazard profile within a particular species. However, where diagnostic measures of endocrine activity are also measured in higher tier tests it may be possible distinguish between specific effects having

an underlying endocrine mode of action from those effects caused by other modes of action. As such lead toxicity, was considered by some experts, as a tool to inform hazard characterisation. Lead toxicity might also be interpreted in the ecotoxicological context in the sense of sensitivity across species, however, such taxonomic specificity was not discussed by the experts.

3.2.4 Potency

From a scientific point of view it was agreed that potency considerations were not part of the identification of a substance as an ED but rather play an important role in hazard characterisation, by characterising the dose/concentration-response. The experts discussed whether or not such considerations were relevant, along with other factors such as severity and irreversibility, in identifying the level of concern for regulatory purposes.

In the human health sub-group, it was agreed that although potency was useful for characterising EDs and ranking them in order of concern, an appropriate potency cut-off value between higher concern and lower concern EDs could not be scientifically determined and it would be primarily a policy decision on where to place the cut off.
In relation to defining such categories of lower and higher concern, approximately half of the human health sub-group considered that potency couldn't be used for categorization while the other half considered that potency should be taken into account for categorization in combination with other factors such as irreversibility and severity.

In the environment sub-group, there was no consensus on the use of potency for hazard characterization. In line with the human health sub-group, it was agreed that an appropriate potency-based cut-off between higher concern and lower concern EDs could not be scientifically determined. The sub-group further agreed that potency alone should not be used for the characterization of EDs. Some of the experts felt that it cannot be used at all while other experts considered potency to be an extremely important factor in characterisation together with other factors such as severity and irreversibility.

3.2.5 Summary

Factors such as potency, severity, irreversibility and lead toxicity were considered not part of the identification but could inform on characterization of the hazard of EDs. For the human health experts it was considered that these hazard characterisation factors may be used in combination to rank EDs according to "level of concern", which could be used by risk managers for prioritisation purposes, however, it was not discussed how these factors could be used together to define different categories of EDs. Half of the human health sub-group were in support of using these factors for creating categories of EDs of higher or lower concern with the prospect of different regulatory consequences applied to each category. These experts referred to the hazard classification criteria under Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures as a good model to follow. In the CLP Regulation, differentiation between Categories 1a/b and 2 for substances classified as carcinogenic, mutagenic or toxic for reproduction (CMR) is primarily related to levels of evidence for the probability of the effect occurring in humans, but there are also elements related to severity of effect (ECHA 2012). Notably the CLP Regulation uses guidance values for sub-categorisation of specific target organ toxicity – repeated exposure (STOT-RE) but not for CMRs.

With respect to ecotoxicological assessment, there was no agreement on how to consider lead toxicity, severity or potency with respect to hazard characterisation of EDs outside the context of risk assessment. Some experts considered that severity could be used in a weight of evidence approach, while the majority considered that it was not useful. For potency some felt it could not

be used at all while others considered it could be used along with the other factors in a weight-of-evidence approach on a case by case basis.

Hazard characterisation, following hazard identification, is a critically important step in risk assessment in the context of derivation of acceptable exposure levels, however, as risk assessment is not carried out for identified EDs under the PPPR and BPR[5] then some experts had difficulties to see how the discussed factors relevant for hazard characterisation could be applied. According to some of the experts, not considering hazard characterisation for EDs under the PPPR and BPR would mean to ignore available scientific data.
Some of the experts proposed that although the elements of hazard identification (ED MoA, adversity, specificity, relevance) are the basis for the scientific definition of an ED, the elements of hazard characterisation should be taken into account in relation to regulating EDs. These elements of hazard characterisation represent considerations of the science; however, their application in differentiating between EDs of higher or lower concern outside a risk assessment context goes beyond science. Therefore, it is for risk managers and policy makers to decide to which extent to apply these elements of hazard characterisation in developing regulatory criteria for the identification and categorisation of EDs of regulatory concern. If it were decided to include these elements in regulatory criteria clear guidance on their application would need to be developed. Some experts considered that although prioritisation on the basis of hazard characterisation is often accepted, "level of concern" should be ultimately based on a risk assessment, i.e. including exposure considerations, since the risk from a low potency chemical can be higher than from a highly potent chemical if exposure to the low potency chemical is high enough to provoke an effect and exposure to the highly potent chemical is so low that no effect is produced.

4. SUGGESTED SCHEME FOR THE EVALUATION OF EDs

4.1 Basic Scheme

4.1.1 ED identification

The ED EAG supported a decision scheme which captures both demonstration of an adverse effect linked to convincing evidence for an associated endocrine disrupting mode-of-action as being the 2 main elements for the identification of an ED. It was also considered that specificity was part of ED identification, since it was agreed that the primary mode of action of a substance should be disruption of the endocrine system rather than the disturbance of the endocrine system being a secondary consequence of other non endocrine-mediated systemic toxicity.
Relevance of the adverse effect and related mode of action to humans or relevance of the endocrine-mediated adverse effect at population level with respect to wildlife was also considered to be a decision criterion for ED identification.
These elements were considered sufficient for the scientific definition of an ED, however, some experts expressed the view that the elements of hazard identification alone (and in particular adversity and endocrine disrupting MoA alone) were insufficient to define a confirmed ED for regulatory purposes, respecting that it is for risk managers and policy makers to decide to which extent to apply elements of hazard characterisation in developing regulatory criteria for the identification of EDs of regulatory concern.

[5] Regulation (EU) No.528/2012 concerning the making available on the market and use of biocidal products (BPR), OJ L167, 1-123

The matrix from the SoA report graphically representing the level of evidence for adverse effects on one axis and level of evidence for endocrine disrupting MoA on the other was considered to be a potentially useful visualisation tool to show how MoA and adversity should be considered in parallel and not in sequence (see Figure 1).

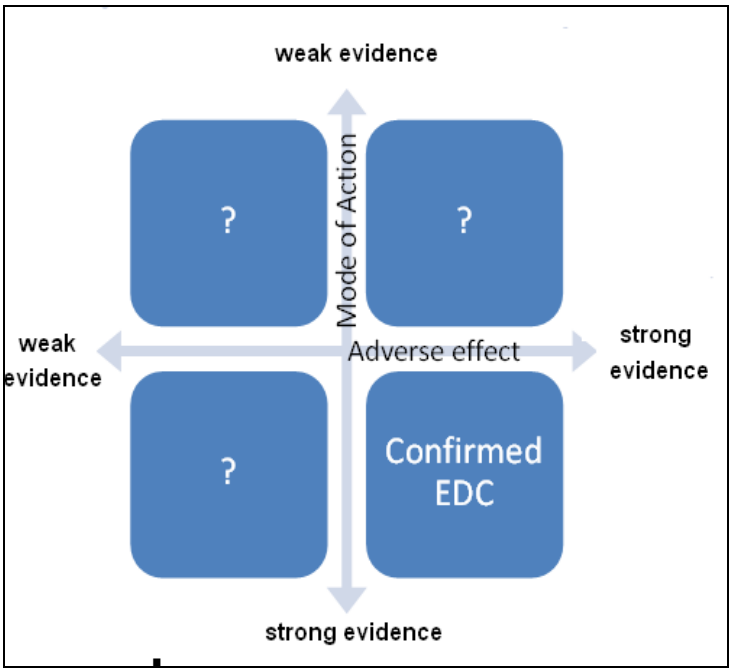

Figure 1: Matrix for evaluating evidence for adversity and endocrine mode of action in parallel (from Kortenkamp et al, 2011. Section 7.2.2, p.127)

It was accepted that the 4 scenarios represented by the 4 quadrants in the matrix, respectively, *strong evidence for adverse effects/strong evidence for endocrine disrupting MoA; strong evidence for adverse effects/weak evidence for endocrine disrupting MoA, weak evidence for adverse effects/strong evidence of endocrine disrupting MoA; weak evidence for adverse effects/weak evidence for endocrine disrupting MoA* may arise and the ED EAG considered the type of evidence likely to lead to inclusion in each quadrant and how to proceed or conclude in each case as described below. It was assumed that the starting point for using the matrix would normally be either some evidence of endocrine activity or some evidence for an adverse effect hypothesised to be caused by an endocrine disrupting mode of action. The scheme could be used not only to evaluate available data but also to identify data gaps and build evidence. The ED EAG stressed that in order to consider a substance as a confirmed ED (i.e. to be placed in lower right quadrant of the matrix) the evidence for both adverse effect and endocrine disrupting mode of action must incorporate the evidence for a biologically plausible causal relationship between the endocrine activity and the observed adverse effect(s). Some experts also preferred the terms *insufficient evidence* in place of *weak evidence* and *sufficient evidence* in place of *strong evidence*.

Confirmed ED – strong (sufficient) evidence for adverse effect(s)/strong (sufficient) evidence for endocrine disrupting MoA (lower right quadrant).

A substance for which there is sufficient evidence of an adverse effect or pattern of effects for which human or ecotoxicological relevance could not be ruled out and for which there was sufficient evidence that the adverse effect arose from an alteration of the function of the endocrine system, rather than arising as a secondary consequence of marked generalised non-

specific toxicity at high dose levels, was considered sufficient to conclude that a substance is an ED.

Strong evidence for adverse effect(s)/weak (no) evidence for endocrine disrupting MoA (upper right quadrant)

Substances for which there was clear evidence of adverse effects but for which the evidence linking the adverse effect or pattern of effects to an endocrine disrupting mode of action was considered insufficient to conclude, would reside in this quadrant. In these cases there may be evidence of endocrine activity *in vitro* but no plausible link to the pattern of adverse effects observed. Further investigations specifically linked to determining the hypothesised mode of action would normally be required in such cases.

Possibilities for further testing may include conducting the battery of available *in vitro* assays covering EATS modalities within the OECD Conceptual Framework (OECD 2012b). Substances showing activity in *in vitro* assays could be supported by ADME data or further investigated in appropriate *in vivo* assays evaluating relevant biomarkers for the type of endocrine activity identified.

It was discussed that there may be clear cases where an adverse effect, or more likely a pattern of adverse effects, may be considered to be both adverse as well as directly indicative (i.e. diagnostic) of endocrine disruption. Such cases may be moved to the confirmed ED quadrant, indicating sufficient evidence of adversity as well as sufficient evidence of an endocrine disrupting MoA. However, it was considered that apical adverse effect data were rarely able to provide suitable information on the mode of action and in most cases further confirmation of the specific MoA by generation of some appropriate mechanistic data would be required.

Insufficient evidence of an endocrine disrupting MoA may be due to lack of standard assays to probe disruption of specific pathways e.g. non-EAT modalities. Specific *ad hoc* mechanistic studies may need to be designed in such cases.

In standard toxicity studies it is required that the test substance is tested up to maximally tolerated doses, so adverse effects would be observed in almost all cases, at least at the top dose level. Therefore, particular consideration should be given to whether or not these adverse effects are likely to be endocrine-mediated, or just a consequence of marked toxicity. Lack of any evidence of endocrine activity from the available *in vitro* assays investigating EATS modalities would not necessarily rule out an endocrine disrupting MoA since active metabolites may be generated *in vivo* or the substance may be active via other modalities for which assays are not yet available. However, negative results in these assays and the absence of any hypothesised link of the adverse effect to any endocrine mode of action according to current knowledge would be indicative of no evidence for endocrine disruption.

Weak (no) evidence of adverse effects /strong evidence for endocrine disrupting MoA (lower left quadrant)

Substances with endocrine activity demonstrated *in vitro* and confirmed *in vivo* via appropriate biomarkers might be expected to produce adverse effects coherent with the type of endocrine activity observed. If the expected adverse effects are observed this should be sufficient to move the substance into the confirmed ED quadrant, however, if not observed it has to be considered whether the appropriate assay, covering the critical windows of exposure, and appropriate observations, in relation to the apical endpoints relevant for assessment of adversity, have been conducted.

The assessment of adversity may be on the borders of whether recorded observations should be considered as physiological modulations or as truly adverse. For example, an increase in vitellogenin (egg yolk protein) in fish is considered to be a physiological modulation as well as an

in vivo biomarker of an estrogenic mode of action. Although not adverse in itself, according to the IPCS definition for adversity, it can be correlated with a change in sex ratio, which is considered an adverse effect relevant at the population level. Such evidence (i.e. increased vitellogenin) would be strongly suggestive of endocrine disruption but in the absence of adversity (e.g. change in sex ratio) could not be concluded as an ED. It has not yet been demonstrated that changes in vitellogenin are necessarily a population relevant change as they are not always associated with altered sex ratio or significant impacts on fecundity, however, some experts considered that at some future time, knowledge might become sufficient to be able to equate a certain level of vitellogenin reduction or induction with population level effects.
A measure of anogenital distance (AGD) is also considered an ED-sensitive *in vivo* biomarker which is a morphological change indicating effect on sexual differentiation and increased risk for reproductive effects later in life (Bowman et al, 2003; Christiansen, et al, 2008; McIntyre et al, 2002) but with no known direct effect on function and hence may not be considered adverse according to the IPCS definition of adverse effect.
Some experts questioned whether such a clearly undesirable change in morphology as decreased AGD should not be considered as enough evidence to conclude the substance to be an endocrine disrupter, especially since AGD may be used in defining the NOAEL according to OECD TG 443 (EOGRTS). However, this rationale was not supported by the whole of the human health sub-group since AGD is likely to be accompanied by other effects when used to set a NOAEL and AGD as an isolated finding would not be considered as enough evidence to conclude the substance to be an endocrine disrupter.

Weak (no) evidence of endocrine disrupting MoA/weak (no) evidence of adverse effects (upper left quadrant)

In such cases where there are no adverse effects for which there is a hypothesised endocrine disrupting MoA nor any convincing evidence of endocrine activity a decision not to proceed further was supported, however, the term non-ED should not be applied, rather it should be stated that there is no evidence that the substance is an ED. Cases of weak evidence of endocrine activity may warrant further investigation even in the absence of strong evidence of an adverse effect depending on the completeness of the available data.

4.1.2. Further characterisation of identified EDs

Following identification as ED, substances in the lower right quadrant could be further characterised according to factors such as magnitude and nature of the effect (severity and irreversibility) and dose at which the effect or effects were observed (potency), which could also be compared with doses at which other non endocrine-related toxicity occurred. For considerations of how to use these data outside a risk assessment context see section 3.2.

4.2 Weight-of-Evidence Considerations

4.2.1 Weight of evidence

Weight-of-evidence approaches should be applied in the evaluation of both adverse effects and mode of action. Weight-of-evidence approaches generally refer to weighing all available evidence, both positive and negative, including human epidemiology data, field data, animal experimental (eco)toxicology studies, *in vitro* data, (Q)SAR, analogue and category approaches in order to reach a conclusion.
Factors that were identified as important in a weight-of-evidence approach to both ED identification and characterisation and for either adverse effect or endocrine disrupting MoA include the quality, reliability and relevance of the individual studies, as well as consistency and

reproducibility of reported effects, the pattern of effects across and within studies, number of species showing the same or similar effects, time of onset of effects and life stage affected. These factors are not specific to assessment of endocrine disruption, *per se*, but relevant for any toxicity assessment. With respect to reproducibility it was pointed out that such evidence for reproducibility is often difficult to obtain from expensive *in vivo* studies with a high animal consumption that are unlikely to be repeated, like reproductive toxicity studies. Adequacy of the study with respect to sensitivity and observation of relevant endpoints was also highlighted as important.
With respect to study quality it was agreed all studies should be considered but quality criteria were necessary to accept the validity of the reported findings. The SoA report highlights the approach developed by Klimisch (Klimisch et al., 1997) for assessment of the quality of toxicological studies. According to Klimisch, a study is reliable without restrictions if it is generated according to generally valid and/or internationally accepted testing guidelines (preferably performed according to GLP) or in which the test parameters documented are based on a specific (national) testing guideline (preferably performed according to GLP) or in which all parameters described are closely related/comparable to a guideline method. The importance of a) GLP as a worldwide accepted lab accreditation system for assuring the appropriate documentation of results; b) OECD study guidelines as validated, robust, reproducible methods that have been tested in many labs before approval to ensure consistent, valid results; and c) worldwide recognition of Test Guideline data under the OECD Mutual Acceptance of Data (OECD MAD, 1981) was recognised. Nevertheless, it was proposed that non-guideline data (e.g. from academic laboratories) following good scientific principles in design, conduct and reporting and employing appropriate statistics, should be judged on their scientific merit and not automatically considered of lower quality to a Test Guideline conducted by a GLP accredited facility.

The quality of human epidemiology data was acknowledged to be much more difficult to establish because of uncertainties in e.g. exposures, confounding factors, and study size, making their interpretation challenging. Nevertheless taking appropriate account of such factors is critical to the acceptance of the strength of evidence of the associations described. Bradford Hill considerations of causal inference are generally used to help evaluate the reliability and robustness of findings from human observational studies. In the last decade human biomonitoring programmes have been established in many countries across the globe. The modern human biomonitoring studies are well designed with rigorous, scientific approaches to data collection, analysis and interpretation. Many uncertainties characteristic of older epidemiological studies, such as poorly defined exposure and confounding factors, can now be addressed. Therefore these newer epidemiological studies offer scientifically more robust results to which Bradford Hill considerations can be applied (Albertini, et.al., 2006; Knudsen & Merlo, 2012).

For *in vitro* mechanistic assays used for screening some participants considered that the level of validation required for acceptance may not be so critical, provided some quality control could be demonstrated e.g. at a minimum there should be appropriate positive and negative controls, and reproducibility should be adequately demonstrated.

4.2.2 Mode of action analysis methodology

A specific weight of evidence approach providing a methodology for analysing and transparently laying out the evidence for the association of the activities of a chemical with specific adverse effects is provided by the IPCS's Mode of Action framework as already described under Section 3.1.2. The methodology is applicable to the assessment of any mode of action including endocrine disrupting MoAs. The concept has also been taken up recently by the OECD in relation to Adverse Outcome Pathways (AOPs) as an approach to evaluate and integrate many different

types of chemical and biological information following a mode of action-based approach to understanding adverse effects (OECD 2012c). OECD have adopted the term Adverse Outcome Pathways (AOPs) for this activity, a term which originated in ecotoxicology but for which the concept is equally applicable, as is MoA, across both disciplines.

4.2.3 Summary

Weight-of-evidence approaches need to be applied in both evaluating adverse effects and endocrine activity, particularly in capturing the weight-of-evidence establishing the relationship between endocrine activity and adverse outcomes. There are a number of examples in the scientific literature describing weight-of-evidence approaches in general and two specific examples describing the application of weight-of-evidence approaches to assessment of EDs is highlighted in the SoA report under section 4.1.4.
Of particular value in the context of evaluating strength of association of an endocrine disrupting MoA to an adverse effect is the IPCS mode of action and human relevancy framework, although the ED EAG considered that the level of evidence required by the framework in supporting the sequence of key events leading to adversity might be too high a requirement for the identification of an ED for regulatory purposes (See 3.1.2). The OECD AOP activity also provides a structured framework to integrate evidence laying out the sequential progression of events from a molecular initiating event to the *in vivo* adverse outcome of either human or ecotoxicological relevance.
Following adoption of criteria the development of guidance for the application of the criteria could use as a basis such weight-of-evidence approaches with appropriate modifications.
It was noted that for a number of the case studies discussed within the human health sub-group, different conclusions might be drawn concerning allocation to any of the 4 quadrants within the suggested evaluation scheme, depending on interpretation of the available data sets in relation to the assessment criteria. This underscores the need to develop further guidance for application of the criteria.

5. TESTING FOR EDs

5.1 Availability and adequacy of current tools and methods for assessment of EDs

The adequacy of current test methods is an issue which needs to be addressed in future in detail, as the ED EAG was not able to provide the necessary detailed advice on this in the time available. The SoA report could form the initial basis of an evaluation of existing regulatory test methods. Discussions focused on the OECD Conceptual Framework (CF) for Testing and Assessment of Endocrine Disruptors as revised in 2012 (OECD 2012b, pp385-387) which includes the OECD TGs available, or under development that can be used to evaluate chemicals for endocrine disruption. The CF is composed of five levels; Level 1 (existing information and non-test information); Level 2 (*In vitro* assays providing data about selected endocrine mechanism(s) / pathways(s); Level 3 (*In vivo* assays providing data about selected endocrine mechanism(s) / pathway(s)); Level 4 (*In vivo* assays providing data on adverse effects on endocrine relevant endpoints); Level 5 (*In vivo* assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism).

5.1.1 Adverse Effects

It was agreed that levels 4 and 5 of the updated 2012 version of the OECD Conceptual Framework (CF) incorporates the *in vivo* guideline studies relevant for the identification of several adverse effects relevant to endocrine disruption, accepting that in these assays effects

can be sensitive to more than one mechanism including non-endocrine disrupting mechanisms. A number of OECD Test Guidelines have been updated recently to include additional parameters to enhance the sensitivity of the assays to identify endocrine-related effects and hence studies conducted according to the old versions of the OECD Test Guidelines may, in some cases, be considered inadequate according to current standards. It was also pointed out that non guideline data which might be generated in academic labs should not be discounted, provided they follow good scientific principles in design, conduct and reporting and employ appropriate statistics.
Some of the endpoints included in level 3 non-mammalian assays are also considered adverse (especially fecundity in the fish short term reproduction assay (TG 229)) but due to the high variability and low statistical power it may be difficult to conclude on a NOEC. If effects are pronounced enough they may be considered as adverse in a case-by-case decision in a weight-of-evidence approach. In relation to ecotoxicological assessment, mammalian data should also be considered.

5.1.2 Endocrine mode of action and causal link to adversity

In vitro and *in vivo* assays included within OECD CF levels 2 and 3, respectively, were considered to be the currently available validated assays for use in determining an endocrine mode of action. It was also accepted that case-by-case *ad hoc* mechanistic studies may also be required, especially for possible endocrine modes of action where validated assays were not yet available.

Some *in vivo* biomarkers indicative of endocrine activity, within OECD CF levels 4 and 5 assays could be also informative in relation to the endocrine mode of action. Read-across from mammalian to non-mammalian mechanistic studies and *vice versa* was also indicated as contributing to the building of evidence for an endocrine mode of action, when appropriate.
Data generated under the US EPA Endocrine Disruptors Screening Program (USEPA EDSP) was suggested as potentially providing a valuable resource in relation to mode of action which may inform on further testing and testing strategies for other chemicals.

5.1.3 Summary

Currently available OECD tests within the OECD Conceptual Framework for the testing and assessment of endocrine disrupters are mostly focused on EAT modalities as well as identification of substances acting by interference with steroidogenesis, for mammals, fish and possibly amphibians, but not for birds or invertebrates. With regard to birds and invertebrates no OECD tests are available which include biomarkers for endocrine activity and the limited knowledge makes it difficult to conclude whether or not effects are endocrine-mediated. Nevertheless, some non-OECD study findings might be sufficient to reach a positive conclusion for endocrine disruption in these species. In some cases it may be possible to reach a conclusion based on a single test (*e.g.* TG234 for fish, TG416 (two generation reproduction assay) or TG443 (EOGRTS) for mammals).

5.2 Further research needs

With regard to adverse effects/apical endpoints relevant to human health assessment, some participants considered that the currently available standardised guideline studies are adequate in most cases, especially for EATS modalities, since, despite the fact that the existing assays might miss some endocrine-sensitive endpoints, substances with endocrine-disrupting activity are likely to produce a range of adverse effects (many of which will be observed in an appropriate guideline study) because of the complexity of the endocrine system with its multiple signalling pathways, feedback mechanisms and cross-talks.

Nevertheless it was acknowledged that there are gaps including in particular the lack of an assay that covers the full lifecycle from *in utero* to old age, to allow investigation of early life exposure on cancer incidence, impact on menopause, senescence etc. manifested in later life stages.
Other experts considered that the currently available guideline studies are inadequate in most cases.

With respect to ecotoxicological assessments, further work to identify relevant *in vivo* biomarkers indicative of endocrine activity to augment existing assays was recommended (also relevant in context of human health assessments). In addition, general knowledge of the endocrine system in invertebrates, birds, amphibians, as well as plants and microbes is currently limited.

The OECD Detailed Review Paper No.178 (OECD 2012a) points to the lack of certain mechanistic assays for the investigation of non EATS modalities relevant to the development of, for example, metabolic syndrome.
Annex III of the SoA report was considered to provide a comprehensive compilation of human health and wildlife adverse effects for which there is evidence or suspicion of an endocrine disrupting mode of action. These effects are matched against the existence of equivalent endpoints or markers in available animal models, specifying which OECD TGs include the endpoints as well as whether they cover the critical windows of exposure. Relevant biomarkers and available assays for endocrine modes of action are also described.

It was recommended that priority areas for further development of assays to investigate specific endocrine pathways should be informed by emerging human health issues or observed negative impacts on wildlife populations and hypothesised link to endocrine-related causes.

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Addendum

One expert (Susy Brescia, Health & Safety Executive, United Kingdom) stated that the final report is not a true reflection of all her views although she did provide comments, many of which were included in the report. She stated:-

"This expert disagrees that hazard identification is equivalent to identification of an endocrine disrupter (as implied by the report) and hence that hazard characterisation cannot be used, on a scientific basis, for identification of EDs. The elements of hazard characterisation are considerations of the science and play an important role in the identification of EDs (especially if these EDs are going to be regulated in a number of contexts). Hazard characterisation is a critical step of hazard assessment; elements of hazard characterisation have been applied for decades and are still applied in the toxicological and ecotoxicological hazard identification system of the CLP Regulations."

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Abstract

The European Commission, under Directorate-General for Environment (DG ENV) created an ad hoc group of Commission Services and Member States to serve the EU Community Strategy on Endocrine Disrupters. The ad hoc group created the Endocrine Disrupters Expert Advisory Group (ED EAG) in November 2011 to provide scientific advice on the development of criteria for identification of endocrine disrupting substances (EDs). The European Commission's Joint Research Centre was tasked with facilitating and chairing meetings of the ED EAG and preparing this report. This report captures the experts' opinions on key scientific issues relevant to the identification and characterisation of EDs. It provides one input to the establishment by the European Commission of horizontal criteria for the identification of EDs to be applied as appropriate across all relevant pieces of legislation concerning the control and risk management of chemicals substances (including pesticides, biocides, pharmaceuticals, industrial chemicals, controls on water quality, occupational exposure, etc).

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