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Thresholds for Endocrine Disrupters and Related Uncertainties

Report of the Endocrine Disrupters Expert Advisory Group

Sharon Munn
Marina Goumenou

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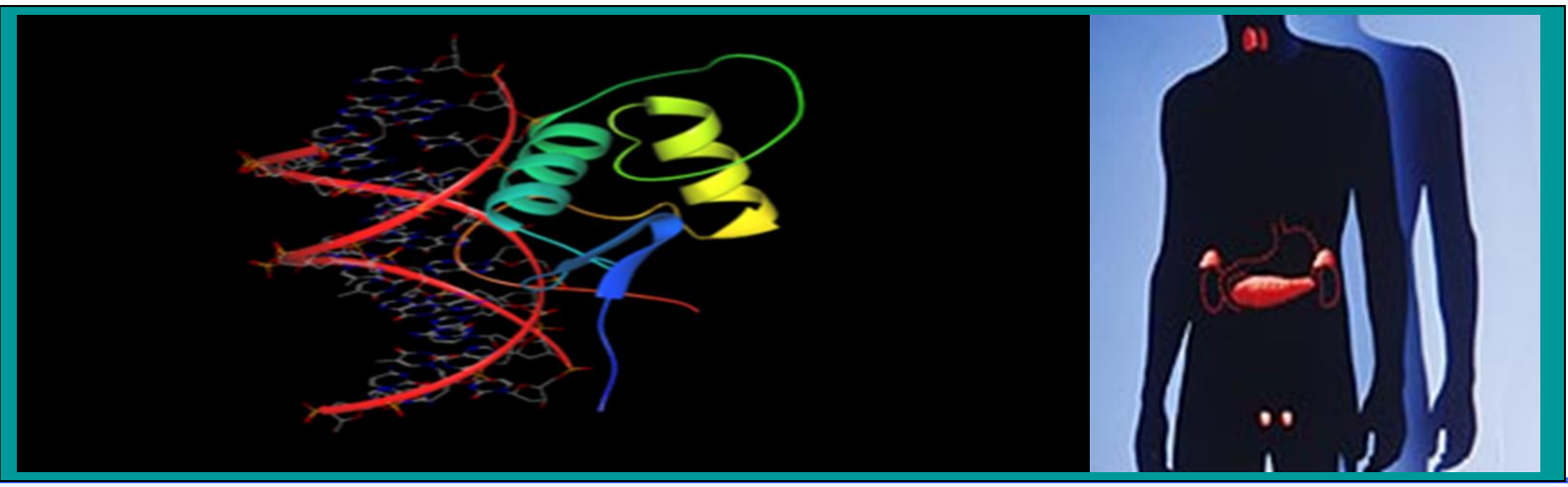

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Contact information
Sharon Munn
Address: Joint Research Centre, Via Enrico Fermi 2749, TP 202, 21027 Ispra (VA), Italy

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Sharon Munn, Marina Goumenou

Contributors

Planning Committee

Jukka Ahtiainen, Finnish Safety and Chemicals Agency, Finland; **Yvonne Andersson,** Swedish Chemicals Agency (KemI), Sweden; **Johanna Barthelemy-Berneron,** ANSES, France; **Susy Brescia,** Health & Safety Executive, United Kingdom; **Elise Grignard,** European Commission, DG JRC**; Marina Goumenou**, European Commission, DG JRC; **Ulla Hass,** Technical University of Denmark, Denmark**; Karen Hirsch-Ernst,** Federal Institute for Risk Assessment (BfR), Germany; **Sharon Munn**, European Commission, DG JRC**; Ing-Marie Olsson,** Swedish Chemicals Agency (KemI), Sweden; **Frauke Stock,** Federal Environment Agency, Germany

Members of ED EAG

Jukka Ahtiainen, Finnish Safety and Chemicals Agency, Finland; **Yvonne Andersson,** Swedish Chemicals Agency (KemI), Sweden; **Johanna Barthelemy-Berneron,** ANSES, France; **Remi Bars,** ECETOC; **Poul Bjerregaard,** University of Southern Denmark, Denmark; **Marie-Noëlle Blaude,** WIV-ISP Scientific Institute of Public Health, Belgium; **Els Boel,** Federal Public Service, Health, Food Chain Security and Environment, FOD VVVL, Belgium; **Teresa Borges,** General Directorate of Health, Portugal; **Martine Bourqui,** Federal Office of Public Health, Bern, Switzerland; **Alan Breen,** Pesticide Control Service, Ireland; **Susy Brescia,** Health & Safety Executive, United Kingdom; **Jana Budašova,** Health Board, Estonia; **Annamaria Colacci,** Center for Environmental Carcinogenesis and Risk Assessment, Italy; **Zhichao Dang,** RIVM, Netherlands; **Anne-Laure Demierre,** Federal Office of Public Health, Switzerland; **Mariana Fernandez,** University of Granada, Spain; **Ulla Hass,** Technical University of Denmark, Denmark; **Karen Hirsch-Ernst,** Federal Institute for Risk Assessment (BfR), Germany; **Bettina Hrdina-Zödl,** Austrian Agency for Health and Food Safety, Institute for Plant Protection Products, Department of Toxicology, Austria; **Agn Janonyt,** Environmental Protection Agency, Lithuania; **Efrosini Katsanou,** Directorate of Laboratory Controls, Benaki Phytopathological Institute, Greece; **Katerina Kyriakopoulou,** Directorate of Laboratory Controls, Benaki Phytopathological Institute, Greece; **Petra Kunz,** Swiss Centre for Applied Ecotoxicology, Eawag/EPFL, Switzerland; **Cinzia La Rocca,** Istituto Superiore di Sanità, Italy; **Birgitte Lindeman,** Norwegian Institute of Public Health, Norway; **Gwynne Lyons,** Chemicals, Health and Environment Monitoring Trust **(**CHEM Trust); **Ailbhe Macken,** NIVA, Norway; **Zoltán Marcsek,** National Institute of Chemical Safety, Hungary; **Hans Meijer,** RIVM, Netherlands; **Angel Nadal,** Miguel Hernandez University, Spain; **Dimitra Nikolopoulou,** Directorate of Laboratory Controls, Benaki Phytopathological Institute, Greece; **Daniela Oggier,** Bundesamt fur Gesundheit, Switzerland; **Ing-Marie Olsson,** Swedish Chemicals Agency (KemI), Sweden; **Catherine Pepper,** Health & Safety Executive, United Kingdom; **Jean-Marc Porcher,** INERIS, France; **Lucija Perharič,** National Institute of Public Health, Slovenia; **Ninja Reineke,** Health & Environment Alliance (HEAL); **Martine Rohl,** Federal Public Service, Health, Food Chain Security and Environment, FOD VVVL, Belgium; **Christophe Rousselle,** ANSES, France; **Wilfried Sanchez,** INERIS, France; **Frauke Stock,** Federal Environment Agency, Germany; **Knut Erik Tollefsen,** NIVA, Norway; **James Wheeler,** ECETOC.

Observers

Niklas Andersson, ECHA; **Bernard Bottex,** EFSA; **Julien Burton,** European Commission, DG JRC**; Jorge Manuel Costa-David,** European Commission; **Vladimir Garkov,** European Commission, DG SANCO; **Anne Giral-Roebeling,** European Commission, DG ENTR; **Elise Grignard,** European Commission, DG JRC**; Marina Goumenou**, European Commission, DG JRC; **Karola Grodzki,** European Commission, DG ENTR; **Peter Korytar,** European Commission, DG ENV; **Teresa Lettieri,** European Commission, DG JRC; **Alfonso Lostia,** European Commission, DG JRC; **Sharon Munn**, European Commission, DG JRC**; Bram Versonnen,** ECHA**; Maurice Whelan,** European Commission, DG JRC.

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LIST OF ABBREVIATIONS

BMD	Benchmark Dose
CMR	Carcinogenic, Mutagenic, toxic to Reproduction
DNEL	Derived No Effect Level
EAG	Expert Advisory Group
ED(s)	Endocrine Disrupter(s) or Endocrine Disrupting Substance(s)
ENTIS	European Network of Teratology Information Services
HBM	Human Biomonitoring
HPA	Hypothalamic-Pituitary-Adrenal
MoA	Mode of Action
NOAEL	No Observed Adverse Effect Level
NOAEC	No Observed Adverse Effect Concentration
NMDR (s)	Non-monotonic Dose Response(s)
PBT	Persistent, Bioaccumulative and Toxic
PCOS	Polycystic Ovary Syndrome
PNEC	Predicted No Effect Concentration
ppb	part per billion
ppt	part per trillion
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals Regulation
TG	Test Guideline
vPvB	Very Persistent, very Bioaccumulative

1. INTRODUCTION

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 identifying and assessing endocrine disrupting substances, not specific to any regulatory framework.

The ED EAG is composed of toxicologists and ecotoxicologists with regulatory and/or endocrinology backgrounds, nominated by Member State Competent Authorities for both REACH and the Plant Protection Products Regulation (PPPR), 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 first task of the ED EAG was to provide scientific advice on key scientific issues relevant to the identification and characterisation of endocrine disrupting substances[1]. The scope of the present report is to capture the experts' opinions on scientific issues relevant to the likelihood of the existence of thresholds for a biological response of an organism to an ED, in particular considering thresholds of adversity and the uncertainties associated with reliably estimating such thresholds from experimental data. The question was posed to the ED EAG by the ad hoc group in relation to a review of the REACH regulation concerning the treatment of EDs under authorisation (Article 138 (7)), however the issue of thresholds was considered to be also of general relevance to the evaluation of EDs and hence the ED EAG provided a useful forum for discussion.

This report captures the range of views expressed during a one day session devoted to the topic at the 5th meeting of the ED EAG on the 4 & 5th February 2013. The ED EAG's Planning Committee (a small working group of volunteering experts assigned to assist in the preparation of the meetings) prepared a background paper as a basis for the discussion including reference to a number of pertinent papers from the open scientific literature [1-18] and posing a number of questions related to different types of thresholds, low dose response, non-monotonic dose responses (NMDRs), critical windows of exposure, limitations of experimental approaches, characterising uncertainties and impact of mixture effects in relation to the evaluation of EDs [See Appendix 1].

Discussion in 3 break-out groups on the second day of the meeting was preceded by four scene-setting presentations at the end of the first day [19-22], 2 of which were given by invited speakers external to the ED EAG. Discussion of the background paper and reference material took place in break-out groups followed by reporting back and further discussion in plenum. The various views and opinions expressed were then captured in this report that was drafted by the JRC in consultation with the ED EAG.

2. LEGISLATIVE BACKGROUND – REACH REVIEW (ARTICLE 138 (7))

As stated above, although the issue of thresholds was considered relevant to assessment of EDs in general, it had been raised in the context of a review of REACH according to Article 138 (7). Under the authorisation title of REACH, an authorisation may be granted if the risk to human health or the environment from the use of a substance arising from the intrinsic properties specified in Annex XIV is adequately controlled in accordance with Section 6.4 of Annex I. This 'adequate control' requires,

[1] http://publications.jrc.ec.europa.eu/repository/bitstream/111111111/28127/1/lbna25919enn.pdf

inter alia, exposure to be below the PNEC or DNEL. Under Article 60(3), the adequate control route for authorisation does not apply to CMRs 1A and 1B (under the CLP Regulation[2]) and EDs (or other substances) meeting the criteria in Art 57 (a-c) and (f) for which it is not possible to determine a threshold, or to PBTs or vPvBs identified under Articles 57 (d), (e), or (f). Authorisation for these substances may be granted via the 'socio-economic' route Article 60(4). In addition Article 138(7) of REACH requires a review by June 2013 to assess whether or not, taking into account latest developments in scientific knowledge, to extend the scope of Article 60 (3) to EDs identified under Art 57(f) in general (independent of whether or not a threshold could be determined).

3. RELEVANCE OF THE PRINCIPLES OF ENDOCRINOLOGY TO THE EXISTENCE OF THRESHOLDS OF ADVERSITY FOR ENDOCRINE DISRUPTING SUBSTANCES

According to Slob et al, 1999, the term "threshold" can be defined in three ways as follows:-

1. *Biological threshold*: The dose below which the organism does not suffer from any [*adverse*] effects from the compound considered.
2. *Experimental threshold*: The dose below which no effects are observed.
3. *Mathematical threshold*: The dose below which the response is zero, and above which it is nonzero.

However the paper also states that the existence of thresholds for any biological response to a treatment cannot be proven or ruled out by experimental approaches owing to the limit of detection of any experiment to detect effects, which may be related to group size and also normal biological variation obscuring a small treatment-related effect. The *biological threshold* may be considered the true threshold that science cannot precisely determine (because it would require an experiment with an infinite number of animals, an infinitesimally sensitive method and an infinitesimally small dose) as opposed to the *experimental or practical threshold* (e.g. the NOAEL/NOEC or BMD05) that can be determined by experimentation.

The current risk assessment paradigm follows one of two approaches; either assuming a biological threshold exists and taking the experimental NOAEL/NOEC or benchmark dose from the critical study as being a dose level at which there is a small response level and incorporating a number of uncertainty or variability factors to derive an acceptable (by society) exposure level; or in the case of genotoxic carcinogens and germ cell mutagens, being unsure about whether or not a biological threshold exists and that even if an experimental NOAEL were to be available, it has to be considered inappropriate to derive an acceptable exposure level by applying the same methodology used for threshold effects. The latter approach leads in many regulatory domains to risk management measures by removing the substance from the market or, if not possible, by reducing exposure to as low as achievable. In the case of genotoxic carcinogens this so-called non-threshold approach has as its historical origins the premise that even one molecule could cause one irreversible mutation which could be the starting point of an eventual malignant tumour. For a genotoxic carcinogen, the NOAEL in a carcinogenicity test is not considered to be a dose level at which there is a small response level and close to the threshold but rather the limit of detection of the assay. The shape of the dose response below the observable range is not known but the response is assumed to decrease with decreasing doses towards zero.

The distinction between the two approaches relates to the mode of action of a chemical and, in the non-threshold approach, the potential to produce adverse effects as a consequence of theoretically

[2] Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging (CLP) of substances and mixtures. OJ L353/1, 31.12.2008

even one molecule. A key question is, therefore, whether EDs exert their effects in a manner similar to the former ("threshold") or the latter ("non-threshold") case.

The Endocrine Society proposes in a position statement from 2012 (Zoeller et al, 2012) [16] that screening and testing for EDs and estimating potency require insight derived from principles of endocrinology according to which (endogenous) hormones:

1. coordinate the development and function of tissues in a highly integrated manner
2. act via receptors
    a. receptors are tissue specific, most sensitive to the hormones at the low end of the dose response curve, and amplify the response;
    b. affinity for receptors is distinct from overall *in vivo* potency;
    c. response is dependent on hormone concentration, receptor affinity, receptor abundance and co-regulatory proteins;
    d. binding to different receptors may occur as dose increases (receptor cross-talk), resulting in different responses from those seen at low doses;
3. act at very low concentrations, in the ppt-ppb range;
4. often result in non-monotonic dose responses (or in different effects at different dose levels) due to multiple mechanisms and
5. produce effects that are life stage dependent, with a greater potential for permanent effects occurring following activity during development

Accepting that true thresholds for any biological response to a treatment cannot be demonstrated experimentally but are inferred/postulated by mechanisms of action and current understanding of biology, the mechanistic basis for assuming either a threshold or non-threshold approach for EDs was discussed considering ED modes of action and general principles of endocrinology, physiology, biology and toxicology.

Since hormones act via receptors and the response is dependent on hormone (and receptor) concentration it follows that a certain level of receptor occupancy is required before a response is produced. For exogenous substances acting as receptor agonists it was generally agreed that theoretically one molecule could activate a receptor when adding to a background level of endogenous hormone and so it could be considered that there is no threshold on this level. However some experts noted that this does not imply that one molecule can lead to adversity by changing a normal response to an abnormal response. Others maintained that this could, in principle, produce an adverse effect if it occurred early in development by triggering a process leading to a premature change in cell differentiation. An example given was that the entire cerebral cortex is produced by only 11 rounds of cell division of the founder population. Triggering premature differentiation of even a single cell early on could reduce the number of cells that would make up a particular region of the cortex[3].

Most experts of the ED EAG considered that thresholds of adversity are likely to exist for EDs but may be very low for individual EDs depending on the mode of action, potency and toxicokinetics, and may be particularly low during foetal development due to the immaturity of homeostatic mechanisms and absence of endocrine feed-back loops or immaturity of toxicokinetic defence/detoxification mechanisms as compared to adult life stages. For these reasons some experts considered it uncertain whether there is a threshold during development. Several experts also expressed the view that, although thresholds may exist, it might be difficult to estimate with any confidence the biological thresholds of adversity based on currently available standard tests. Other experts considered that a threshold of adversity for EDs might be lower in the developing organism than in the adult and the

[3] Caviness VS Jr, Takahashi T, Nowakowski RS. 1995. Numbers, time and neocortical neuronogenesis: A general developmental and evolutionary model. *Trends Neurosci*., 18(9):379-83

nature of the effect might be different (severe, permanent change in the foetus versus a less severe effect in the adult), but a threshold of adversity must exist and can be estimated with appropriate testing (involving exposure during development and other sensitive life stages). See section 7 "Critical window of sensitivity".

4. LOW DOSE EFFECTS

As it is described by Zoeller et al (2012) [16] according to the principles of endocrinology the endogenous hormones act at very low concentrations, in the ppt-ppb range. Strictly controlled hormone action is possible because receptors are tissue specific, have a high affinity for the ligand (endogenous hormone), and that receptor signals are amplified intracellularly which result in biologically significant cell responses (e.g. increased gene transcription) even at relatively low receptor occupancy. In addition, the same ligand can bind to and activate different receptors at different concentrations (receptor cross-talk), resulting in partly different cellular and/or tissue responses at low compared to high ligand concentrations. At high concentrations of the ligand, receptor saturation can occur, resulting in a plateau or even some diminution in the response.

Since endogenous hormones are active at very low doses, a key question is whether or not it is to be expected that substances which interfere with hormone action may also be active at low doses. All experts considered that effects from low doses of chemicals may exist depending on the chemical (similarity with endogenous hormones may increase this possibility), its potency and MoA. It was generally accepted that there is sufficient evidence that potent chemicals can cause adversity at low doses. However, the issue central to the so-called "low dose response" in toxicology is characterized by effects (potentially adverse) being reported at doses well below the NOAEL/NOECs established in conventional regulatory guideline toxicity studies and often at doses within the magnitude of actual or predicted levels of the chemical in the environment. Those who argue for the "low dose response" argue that one of the reasons for this could be that the specific endpoints are not measured in the guideline studies or that the dose response is non-monotonic such that adverse effects can arise both above and below the NOAEL/NOEC in guideline studies (see NMDRs below).

It was acknowledged that there is a lack of scientific consensus on the evidence for such low dose responses and this was reflected in the lack of consensus in the ED EAG. Some experts considered that there was sufficient evidence to conclude that adverse effects could be caused by low doses of some chemicals with endocrine disrupting properties since during the last years there has been an increase of publications that demonstrated low dose effects, and while some findings at low doses have not been reproducible in several laboratories, others have been reproduced. Other experts, not convinced by the available data, proposed that "low dose response", in particular "low dose adversity", needs to be further studied by performing inter-laboratory comparisons using sensitive test methods before drawing any firm conclusions or incorporating such considerations into regulatory decision-making. If substantiated there was a general agreement that the use of the NOAEL/NOECs from conventional toxicity studies as the point of departure for risk assessments could be problematic and flawed. It was also highlighted that the experimental NOAEL/NOEC is not equivalent to the true biological threshold but rather reflects the limit of detection of the method for that endpoint, regarding statistical power to detect an effect as well as the inclusion of relevant sensitive endpoints. A study with greater statistical power and inclusion of more sensitive endpoints may lead to a lower NOAEL/NOEC. This would not indicate the lack of existence of a threshold but may indicate a lower experimental threshold than previously measured. Thus it was agreed that the sensitivity of the methods used in combination with adequately sensitive endpoints was of great importance. Although, it was also noted that these experimental limitations are not specific to

endocrine disruption, but apply to all hazards, some considered the experimental limitations with regards to EDs larger than for other types of toxicity, since in the latter case development of testing methods (including discussion on sensitive endpoints and sensitivity of the methods etc.) has been on-going for a much longer time.

With respect to protection of wildlife populations, some experts considered that the current standard tests do not adequately cover sensitive endpoints and that it could be considered to include non-adverse biological responses for hazard assessment, as the response might not result in adverse effects in the organism tested but might do so in more susceptible organisms or during more susceptible life stages.

5. NON-MONOTONIC DOSE RESPONSES (NMDRS)

NMDRs can be defined as a dose-response which changes direction from ascending to descending or vice versa and can occur at any part of the dose axis, not only at the low dose part. It could also be described as a biphasic dose response. All experts agreed that NMDRs may exist for some substances at pharmacodynamic and molecular level. It was also agreed that possible mechanisms that could explain this phenomenon are known (like receptor saturation) or may be explained by dose-dependent targeting (e.g. dose-dependent activation or inhibition of different receptors or biological target molecules). However, not all experts were convinced of the evidence and/or relevance of NMDRs for adverse effects at the functional level *in vivo.* All experts agreed that NMDRs were not specific to ED-related endpoints and that it was not possible to say whether or not NMDRs can be expected for all EDs as this would be dependent on the specific MoA. However, some experts considered that if hormones generally act with NMDRs it is not unlikely that EDs that mimic hormones also exhibit NMDR behaviour.

It was suggested that an NMDR for a specific endpoint may be the output of more than one underlying mechanism operating at different dose levels, and resulting in the same endpoint being affected in opposite directions. Some of the experts also mentioned that there may be evidence for NMDRs in standardized guidelines studies which are dismissed as not dose-related. It was proposed that these findings should be evaluated more fully in future in attempting to delineate the occurrence of "true" NMDRs from normal experimental variability, outliers and/or inappropriate statistical analysis.

The experts agreed that the implication of NMDRs for conventional testing is the possibility of missing effects at doses lower than NOAEL/NOECs. Thus extrapolation to determine acceptable levels of exposure becomes less secure. It was agreed that, if they arise, NMDRs do not affect *per se* the existence of a biological threshold but may affect the appropriateness and ability of conventional testing to identify where the overall experimental threshold lies and thus the ability to predict no effect concentrations with current standard tests methods.

6. LIMITATION OF EXPERIMENTAL APPROACHES AND POSSIBLE WAYS TO OVERCOME SUCH LIMITATIONS

As indicated above for any biological response to a treatment it is not possible to measure the true threshold. An experimental NOAEL/NOEC gives some indication of where a threshold of adversity might lie (related to a particular species and effects), but is also a function of the sensitivity of a method to detect an effect. Further research investigations may identify additional endocrine-relevant endpoints to be included in bioassays and it was specifically recommended to look at how *in vitro* data (e.g. metabolomic/toxicogenomic data) could inform and focus *in vivo* studies with respect to what might be expected. It was further recommended to give specific attention to sensitive

windows of exposure, e.g. during development, or during puberty. It was agreed that accepting the existence of NMDRs, whilst not affecting the existence of a threshold would affect the appropriateness of conventional testing to identify an experimental threshold. With respect to investigation of low dose effects and NMDRs some experts proposed to re-consider the current experimental test design by incorporating more dose groups over a wider range attempting to cover a more relevant part of the dose/response in relation to anticipated exposures. Other experts noted that the move to reduce animal testing in the EU would in practice preclude the testing across the wide range of dose groups necessary to establish whether thresholds could be determined for each substance. Optimising statistical methods to increase the power of the studies was suggested by some experts. Use of the BMD approach was one specific suggestion where it may be possible to increase the number of dose groups without increasing the number of animals used or at least minimising the additional use of animals implied by extending the dose range. Another specific suggestion was to assess the value and make better use of current high quality human biomonitoring (HBM) studies which measure actual human exposures (typically low dose or at least below the experimentally derived NOAELs) and effects, e.g. human data collated and analysed by the European Network of Teratology Information Services (ENTIS). In summary several aspects to reduce limitations of experimental approaches were considered and there were different opinions on whether or not it would be feasible to overcome the limitations in the near future in a way that the uncertainties discussed would be appropriately addressed.

7. CRITICAL WINDOWS OF EXPOSURE

The key questions posed to the experts was about the implications of potentially greater sensitivity of an organism to hormonal action during critical periods of development (e.g. male and female programming windows) for the likelihood of there being a threshold and how such increased sensitivity might affect the uncertainties for ED assessment.

It was generally accepted that there is an important difference in maturity and functionality of the endocrine system between the pre- and post- natal life. The major issue is related to the immaturity of the homeostatic mechanisms, the immature metabolism as well as the absence of some endocrine axes during sensitive periods of foetal life (e.g. the HPA axis is not developed during the sensitive window of sexual differentiation). These facts increase significantly the concerns in relation to the existence of a threshold of adversity and the possibility, if it exists, to be determined with sufficient confidence. In addition, a small change in hormone levels during development could have permanent serious consequences for the organism.

Other experts expressed the view that a threshold of adversity for EDs might be lower in the developing organism than in the adult and the nature of the effect might be different (severe, permanent change in the foetus versus a less severe effect in the adult), but a threshold of adversity must exist and can be estimated with appropriate testing (involving developmental exposure). Finally other possibly sensitive life stages were mentioned like puberty, pregnancy and menopause for which there is a considerable lack of knowledge. Non-consideration of these life stages in testing protocols would be expected to increase the uncertainty in relation to threshold existence and/or reliable approximation of a threshold.

8. MIXTURES

There is evidence that EDs with similar MoAs can act together in an additive manner to produce effects. In these cases, although experimental ED NOAEL/NOECs may approximate a threshold for the individual ED in the presence of other EDs toxicity may still be expressed at the level of individual NOAEL/NOECs.

Experts acknowledged that such combinational effects were not specific to EDs. Some experts noted that knowledge of MoA was necessary to be able to predict mixture toxicity but others said that the more appropriate way is to base the prediction on common adverse outcomes. There was a general agreement that the estimation of an experimental threshold in the case of mixed exposures, where there is the possibility of combination effects, is even more challenging and that information in relation to the MoAs (e.g. common or different MoAs of the ingredients of a mixture) is important for scientific understanding and for performing the appropriate risk assessment approach. In addition there is not an adequate amount of scientific research to disregard other possibilities for combination effects of mixed exposures (e.g. synergistic, antagonistic action). For example toxicokinetic and toxicodynamic interactions between chemicals may cause deviations on the shape of the dose response curves of individual chemicals (e.g., inhibition of metabolism if substances are sharing the same metabolic pathway). It was also pointed out that mixture toxicity would be difficult to assess in case of NMDRs and that the potential for combinational effects adds to the overall uncertainties of a risk assessment. In addition, some experts noted that in view of the adverse trends in reproductive health and hormone-related cancers in the population at large, the potential for combination effects due to simultaneous exposure to several endocrine disrupters may be particularly relevant to the assessment of EDs.

Some experts considered that since humans and wildlife may be exposed to multiple substances at the same time, and there is evidence that different EDs can act together this may result in an increased risk of adverse effects on human and wildlife health. However as most of the information is still related to individual compounds these experts considered that more comprehensive assessments of human and wildlife exposures to diverse mixtures of EDs are needed, as well as an increased understanding and new approaches to examine the effects of these mixtures. Assessment of combination effects of chemicals in general, not just EDs, is already the subject of a separate parallel initiative in the EU [See Commission Communication to the Council on the combination effects of chemicals - COM(2012) 252 final].

9. OVERALL UNCERTAINTIES

The following uncertainties in carrying out a (eco)toxicological evaluation and risk assessment for EDs were highlighted by some experts, albeit that several of the points relate also to other manifestations of toxicity and their underlying modes of action:

a. The possibility of delay between the exposure and the appearance of the adverse effect especially exposures during the sensitive windows (it is well known that in some cases exposure to EDs in foetal life can lead to adverse effect during adulthood). In this case it is difficult to relate the adverse effect to exposure (e.g. in human epidemiology studies) or it is possible that the adverse effect is not detected because of the experimental design.
b. The possibility of binding of a substance above a certain dose level to different receptors, potentially leading to different adverse effects at different dose levels.
c. The uncertainty related to inter-species and intra-species extrapolation. This issue is important both for human health (e.g. extrapolation from rodents to human) and ecotoxicology (extrapolation between different taxa).
d. The limitation on the methods' sensitivity as well as the possible lack of inclusion of sensitive endpoints relevant to ED.
e. The existence of additional uncertainties in the field of ecotoxicology (e.g. uncertainties introduced by temperature which for some species is an important factor influencing sexual development and sex-reversal).
f. The uncertainties in relation to human populations where it is not possible even with extensive epidemiological studies to demonstrate a population threshold, attributed mainly

to the big inter-individual population variability due to variations in genetics, background levels of endogenous hormones (and possibly xenobiotics) and pre-existing disease or disease predisposition [15].

It was generally agreed that there are many uncertainties in carrying out a risk assessment. Many are not specific to EDs but some factors may be particularly relevant to some EDs, e.g. NMDRs and low dose extrapolation, lack of appropriate animal models/sensitive strains for some known effects in humans, and lack of sensitive endpoints and life stages in current ecotoxicological test methods.

Some experts considered uncertainties were higher for EDs, other experts considered that the uncertainties were not specific for EDs and not necessarily higher than for other types of toxicants acting via non ED MoAs. Some experts pointed out although the discussion was contained within the field of endocrine disruption many of the issues were not unique to endocrine disruption and posed a serious challenge to the principles of both pharmacology and toxicology since the molecular basis (enzyme inhibition/induction/activation, receptor mediated events) underlying endocrine disruption is very similar to the modes of action underlying other types of toxicity which are not of an endocrine nature.

10. SUMMARY

Most experts considered that thresholds of adversity are likely to exist for EDs but may be very low for individual EDs, depending on the mode of action, potency and toxicokinetics and that these thresholds may be particularly low during foetal development (i.e. critical windows of sensitivity) due to the immaturity of homeostatic mechanisms, the immature metabolism as well as the absence of some endocrine axes during sensitive periods of foetal life as compared to adult life stages. For these reasons some experts considered it uncertain whether there is a threshold during development. Several experts also expressed the view that, although thresholds may exist, it might be difficult to estimate with any confidence the biological thresholds of adversity based on currently available standard tests. In addition, small changes in hormone levels during development could have permanent serious consequences for the organism.

Other experts expressed the view that a threshold of adversity for EDs might be lower in the developing organism than in the adult and the nature of the effect might be different (severe, permanent change in the foetus versus a less severe effect in the adult), but a threshold of adversity must exist and can be estimated with appropriate testing (involving developmental exposure).

It was acknowledged that there is still a lack of scientific consensus on the evidence for "low dose responses' and this was reflected in the lack of consensus in the ED EAG. Substantiation of occurrence and relevance of NMDRs and low dose effects would not impact *per se* on existence of thresholds but would indicate thresholds may be lower than currently assumed and that current experimental approaches may need to be modified to increase sensitivity to identify relevant effects occurring unexpectedly at low doses.

Given that most experts considered that thresholds were likely to exist for EDs the focus moved to the characterisation of the uncertainties in estimating the thresholds of adversity from currently available standard tests, whether these uncertainties were likely to be higher for EDs than for other types of (non-genotoxic) toxicants and the impact of these uncertainties on the appropriate approach, 'threshold' or 'non-threshold', to ED assessment.

Members of the ED EAG expressed divergent views on the key issue of whether or not a "threshold" approach should be taken in assessing EDs. Some argued that the lack of inclusion of some sensitive

endocrine-relevant endpoints or life stages in many of the current standard Test Guidelines, the issue of non-monotonic dose responses for endocrine disrupters and consequent possibility of adverse effects below the conventional NOAEL/NOEC and the potential combination effects of co-exposure to chemicals acting via similar modes of action in an additive manner imply a lack of confidence in following a threshold approach to risk assessment for EDs. Furthermore, some experts noted that even if animal testing utilizing additional dose groups and examining many endpoints sensitive to endocrine disruption could, in some cases, lead to a more robust identification of experimental thresholds for adverse effects, this would be incompatible with the aim of reducing animal testing and moreover, regulation based on such thresholds may still not be adequately protective as it would not address the issue of potential mixture effects. Other experts considered that on a case-by-case basis, a "threshold" may be applied where critical effects according to specific windows of exposure lead to adversity and respective MoA (s) are well understood following the principles of endocrinology.

Other experts considered that a threshold approach is appropriate and justified. They also commented that some of these issues could be addressed further by the appropriate update of Test Guidelines with relevant sensitive endpoints. For example, information generated from updated methods (e.g. post 1998 two-generation reproduction study, extended one generation reproduction study) coupled with data from newly validated *in vivo* endocrine specific assays (e.g. pubertal assays) could clarify uncertainties arising from incomplete or old study designs and address the concerns raised. In addition appropriate dose spacing in updated and newly validated assays could bring confidence in the characterization of the dose-response for sensitive parameters and the identification and use of NOAEL/NOECs.

In conclusion, the ED EAG was asked to consider key aspects related to the appropriateness of following either a threshold or non-threshold approach to the evaluation of EDs. As reflected in this report, there were both points of agreement as well as diverging views between the members of the group on key scientific issues. Therefore no consensus was reached on which approach to follow.

11. APPENDIX 1

QUESTIONS TABLED FOR DISCUSSION AT 5TH ED EAG MEETING

A. Presence or absence of different types of thresholds on the basis of mechanism of action

Q1: Accepting that thresholds cannot be demonstrated experimentally but inferred/postulated by mechanisms, what is the mechanistic basis of assuming a threshold/no threshold for EDs and does this apply to all EDs irrespective of MoA?

Q2: Does there need to be a distinction made between absolute/mathematical thresholds, thresholds of adversity and thresholds for biological response?

Q3: What impact does the background level of endogenous hormone have on the likelihood of a threshold for either receptor agonists or antagonists?

Q4: On a theoretical basis is it possible that one hormone molecule can trigger the cascade of the physiological events leading to gene expression, protein synthesis, etc?

Q5: What is the role of mechanisms that amplify the signal from one molecule with respect to likelihood of a threshold?

Q6: What is the role of signal integration processes occurring on the cellular, tissue, organ and systems level (involving e. g. cross-talk between different cellular signal transduction pathways) for the likelihood of a biological threshold?

Q7: EDs may act by interfering with circulating levels of endogenous hormones rather than through competing with the receptor. What is the impact of such modes of action on likelihood of a threshold?

Low dose effects

'Low dose effects' can be defined as either effects occurring at doses lower than those typically used in standard testing protocols, or doses in the range of human exposure.

Q8: Since hormones are active at very low doses, is it to be expected that substances which interfere with hormone action may also be active at low doses?

Q9: What implications does this have for use of the NOAEL/NOEC from conventional toxicity studies as the point of departure for risk assessments?

Q10: What is the evidence for low dose effects and are the effects reported at low doses really adverse or do they represent biological changes still within normal variation, or changes at the molecular level which do not translate at the tissue- organ or organism-level?

Q11. What is the mechanistic basis underlying the low dose effects reported for some EDs andhow could mode of action considerations be informative in relation to low dose effect (e.g. role of feedback loops, repair mechanisms etc.)?

Q12: How does the discussion on low dose influence the question whether or not thresholds (mathematical, biological or adverse) can be derived for EDs?

NMDRs

A non-monotonic dose response (NMDR) can be defined mathematically as a dose-response that changes slope from one direction to another (from ascending to descending or *vice versa)* and can occur in any part of the dose response, not only at low dose. It could also be described as a biphasic dose response (Conolly & Lutz, 2004; Vandenberg et al., 2012)

Q13: What are the underlying mechanisms of NMDRs and their biological plausibility?

Q14: Could NMDRs also be referred to as dose transitions whereby at different dose levels, different mechanisms operate producing different effects, therefore are NMDRs a result of merging or superimposing monotonic dose-responses for different effects?

Q15: Is there convincing evidence of NMDRs *in vivo* at cellular, organ or organism level, or could NMDRs be the result of experimental variation and outliers?

Q16: Are NMDRs specific to EDs or could they occur also for other chemicals/effects? Can NMDRs be expected for all EDs?

Q17: What implications do hypothesised NMDRs have for use of a NOAEL/NOEC from conventional toxicity studies as the point of departure for risk assessments?

Q18: How does the discussion on NMDRs influence the question whether or not thresholds (mathematical, biological or adverse) can be derived for EDs?

Limitations of experimental approaches

Q19: Are techniques sensitive enough in order to measure treatment-related effects at low doses?

Q20: How low should a substance be tested in order to exclude the possibility of so-called low dose effects, or existence of an NMDR?

Q21: Can experimental design issues such as low magnitude of effects against normal background variation be overcome?

Q22: Can the issue of the low statistical power due to the small number of animals used be overcome?

Q 23: Are current tests available sufficient to detect endocrine mediated effects (with respect to low dose effects, NMDR, endpoints affected, and (with respect to the environment) to organisms tested.

Critical windows of exposure

Q24: What impact does the specific sensitivity of the organism to hormonal action during critical periods of development (e.g. male and female programming windows) have on the likelihood of a threshold?

B. Characterising uncertainties

In considering uncertainties and levels of concern, one could draw parallels to PBT substances where the uncertainties in the prediction of risk over time led to their identification as substances of very high concern. In the case of endocrine disrupters the serious nature and irreversibility of some effects may impact on the societal acceptance of the magnitude of the uncertainties in risk assessment.

Sources of uncertainties in risk assessments may include:

- Extrapolating from animal data to humans and potential to underestimate human sensitivity;
- Duration of long term animal studies not covering full life cycle;
- Differences in sensitivity between rodent strains (most appropriate animal model);
- No appropriate animal model for some effects in humans (e.g. endometriosis, PCOS);
- Lack of appropriate exposure data (e.g. biomonitoring data);
- Exposures to mixtures of chemicals;
- Genetic and environmental/nutritional variability;
- NMDRs and low dose extrapolation;
- Identifying critical windows of sensitivity;
- Impact of changes in gene pool on population stability

Although not specific to EDs some of these factors may be particularly relevant to EDs.

Q1: In addition to those identified above, what are the uncertainties in identifying an acceptable exposure level for an ED?

Q2: Do we have enough knowledge to perform inter-species and intra-species extrapolation?

Q3: Are uncertainties in extrapolation comparable to those of other non-ED substances, or higher considering aspects such as the modes of action, the conserved nature of the endocrine system and the type of effects expected.

Q4: What are the uncertainties in extrapolating to an acceptable dose/concentration on the basis of NOAEL/NOECs from experimental studies for EDs? Is there a difference between different endocrine modes of action?

Q5: Are these uncertainties larger than for other substances acting by non-ED modes of action?

Q6: What impact does the specific sensitivity of the organism to hormonal action during critical periods of development (e.g. male and female programming windows) have on the uncertainties for ED assessment?

Q7: With regard to the uncertainties discussed, would it be possible to decrease them if more knowledge and test systems become available? If they can be decreased what time scale would be needed to do so?

C. Impact of mixtures on application of threshold or non-threshold approach

Q1: How does the evidence that EDs acting by the same MoA may work together to produce combined effects impact the approach to risk assessment of EDs and the appropriateness of adopting a threshold or non-threshold approach? Are these considerations specific for EDs? How does evidence that mixtures may produce combined effects impact risk assessment in general?

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Presentations delivered prior to break out group discussions at 5TH ED EAG meeting

19. 'Input for REACH Review in 2013 on endocrine disrupters: Threshold, NMDR and uncertainties' Presentation to ED EAG 5th meeting, 4th-5th February 2013 by **Hass, U.,** Division of Toxicology and Risk Assessment, National Food Institute, Technical University of Denmark,

20. 'Thresholds in regulatory tests for endocrine disrupters. Are we satisfied with the estimates?' Presentation to ED EAG 5th meeting, 4th-5th February 2013 by **Morris, I.D.,** Hull York Medical School, UK.

21. 'Uncertainties in risk analysis of endocrine disruptors. Results of a research project funded by the Federal Environment Agency, Germany.' Presentation to ED EAG 5th meeting, 4th-5th February 2013 by **Stocke, F.,** Federal Environment Agency, Germany.

22. 'Principles of endocrinology and relevance to risk assessment of EDs' Presentation to ED EAG 5th meeting, 4th-5th February 2013 by **Zoeller, R.T.,** University of Massachusetts Amherst, USA on behalf of the Endocrine Society.

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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 detailed reflections on scientific issues relevant to identifying and assessing endocrine disrupting substances, not specific to any regulatory framework. The European Commission's Joint Research Centre was tasked with facilitating and chairing meetings of the ED EAG and preparing this report. The scope of the present report is to capture the experts' opinions on scientific issues relevant to the likelihood of the existence of thresholds for a biological response of an organism to an ED, in particular considering thresholds of adversity and the uncertainties associated with reliably estimating such thresholds from experimental data. The question although relevant to the evaluation of EDs *per se* was specifically raised by the ad hoc group in relation to a review of the REACH regulation with respect to treatment of EDs under authorisation (Article 138 (7)).

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