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WORKSHOP REPORT ON OECD COUNTRIES ACTIVITIES REGARDING TESTING, ASSESSMENT AND MANAGEMENT OF ENDOCRINE DISRUPTERS

APPENDICES 1-10
PART II

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

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FOREWORD

This document includes the Appendices 1-10 to the Workshop Report on OECD Countries Activities Regarding Testing, Assessment and Management. Appendices 1-9 are contributions from individual countries/regions and stakeholders to the case study report prepared for the workshop. Appendice 10 includes presentations made at the workshop by countries/regions and stakeholders.

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Appendix 1

Contribution from Denmark

OECD Case Study Document on Endocrine Disrupters

Danish Contribution

1. Background

In May 2008 a letter from the OECD was sent to the Working Group of National Co-ordinators of the TG (WNT) and the Endocrine Disrupters Testing and Assessment Advisory Group (EDTA AG) requesting contributions for the development of a "case study" document that is expected to lead to a "Report on Endocrine Disrupters Assessment in OECD Member Countries".

By this paper Denmark is submitting its contribution by providing an overview of different governmental programmes and a description of various initiatives listed according to the different levels in the conceptual framework, where possible.

2. Introduction

During the last 15 years there has been a growing concern about endocrine disrupting substances in Denmark. In the beginning of the 90'es, it attracted attention that some industrial chemicals were able to mimic the effects of intrinsic oestrogenic compounds and in 1993 the Danish scientist, Niels Erik Skakkebæk in cooperation with Richard Sharpe launched "the oestrogen hypothesis", arguing that the increasing incidence of reproductive abnormalities in the human male may be related to increased exposure to oestrogenic compounds in utero and this may be a result of exposure to industrial chemicals [1]. In the following years it was realised that some chemicals were able to interfere with several intrinsic hormonal systems and affect the whole endocrine system and chemicals with these properties were referred to as endocrine disrupters. Endocrine disrupters have been on the political agenda in Denmark since the oestrogen hypothesis was launched. The constant political focus on the endocrine disrupters problem have led to several political initiatives, including research initiatives.

3. Governmental Programmes

As a follow-up to intense public debate after the launch of the oestrogen hypothesis the Environmental Protection Agency (DK EPA) published a report in 1995 summarising the current knowledge on male reproductive disorders and environmental chemicals with oestrogenic effects [38].

In 1996, the DK EPA prepared a status on "Chemical Substances with Estrogen-hormone-like Effects" [14].

In 1997-2000 the Danish Council for Strategic Environmental Research supported 8 projects under the programme "Hormone-mimicking Substances".

In 1996-2000, the Research Centre for Oestrogenic Substances was established under the Strategic Environmental Research Programme, a programme co-funded by 7 ministries in Denmark.

<u>Strategy for Human Health and the Environment (2003)</u>

In 2003 the Danish government published this strategy, which sets up aims and initiatives to prevent and limit negative environmental effects on human health. A cross-ministerial working group for health and environment was formed, and 10 focus areas were pointed out. One focus point was that the efforts towards endocrine disrupting substances should be strengthened [18] [1].

The Danish National Strategy for the work with Endocrine Disrupting Chemicals (EDC)

A special grant on the state budget is dedicated the work on strengthening the scientific basis for managing the endocrine disrupter problem. In 2002 the first report on the Danish National Strategy for the work with EDCs was presented in Parliament, and status for the work has been reported in 2003, 2004 and 2007 [2]. Actions are prioritised in accordance with a 3-pronged strategy and the prioritised activities are very much in line with the EU strategy on Endocrine Disrupters from 1999.

The 3 focus areas are:
1) Knowledge building and development of test methods
2) Investigations of cause and effect and preventive efforts
3) Regulation

In order to use resources most efficiently the strategy is – as far as possible – to link and coordinate all activities and thereby obtaining synergy. The scientists involved in projects under this strategy are organised in an endocrine disrupters network which meet twice a year to report progress of the work to the Danish EPA. This informal discussion of research progress between regulators, toxicologists, ecotoxicologists, endocrinologists, paediatricians and epidemiologists working with endocrine disruption in different organisms (fish, rats, mice and humans) has turned to be very fruitful both from a scientific and a regulatory point of view. Not only new evidence on likely causes and effects of exposure to individual EDCs both in wildlife and humans is being discussed at these meetings but considerable discussion also take place regarding issues like testguideline development within the OECD TGP & EDTA, effects of combined exposure to EDCs, exposure analysis concerning EDCs etc [1].

Strategic Research 2003-2005

The Danish government started a strengthened research initiative in the area of endocrine disrupters in 2003. This initiative supported a number of research projects in 2003-2005, which were administered by the Ministry of Science, Technology and Innovation. The themes for the research projects were: Breast cancer, diseases in the male reproductive organs and reduced semen quality, diverging pubertal patterns, influences of chemicals, deformities of sexual organs in newborns and the effects of endocrine disrupters in the environment and in humans [1].

Pesticides Research Programme

With the aim to ensure a solid scientific basis for the administration of pesticides, a governmental programme for pesticides research has been running for the last 2 decades. Several projects related to endocrine disrupting effects of pesticides have been carried out through the years [2].

Nordic co-operation, Nord-Utte

In 1994 the Nordic Group for the Development for Test Methods (Nord-Utte) was established under the Nordic Chemicals Group under the auspices of the Nordic Council of Ministers. Swedish, Norwegian, Finnish and Danish representatives from the environmental protection agencies participate in the work. Nord-Utte aims at integrating the scientific and regulatory work on testing of chemicals in the Nordic countries. Beyond contributions to the OECD test guideline programme Nord-Utte organises network meetings and support projects involved in the development of test methods. One of the main priorities of the group has been the development of test methods for detection of endocrine disrupting substances [17] [1].

EU Co-operation

One of the short-term activities in the European Strategy on Endocrine Disrupters is establishment of a priority list of substances for further evaluation of their role in endocrine disruption. DK EPA has prepared a proposal for ensuring a dynamic process for the EU priority list of substances with potential endocrine disrupting effects covering inclusion and exclusion of substances when new data are available. The proposal was presented at a Commission stakeholder meeting in October 2003.

Centre for Endocrine Disrupters

A Danish Centre for Endocrine Disrupters is established as a result of the negociations of the state budget for 2008. After an EU tender inviting for the management of the centre and co-ordination of research projects the Centre opened 1 December 2008. The Centre is formed as a network of scientists and relevant institutions working with knowledge building focusing on authorities preventive work and with a formal centre management placed at the Department of Growth and Reproduction at the Copenhagen University Hospital.

4. Danish initiatives in relation to the OECD conceptual framework for testing and assessment of endocrine disrupting substances

Criteria for assessment of endocrine disrupters have not yet been established within the chemicals regulation due to the lack of proper test methods. Therefore, one of the focus areas in the national strategy is development of test methods. Until test methods have been developed endocrine disrupters are identified case-by-case and in this work decisions based on preliminary indications are necessary. The above mentioned strategies and activities have resulted in a number of initiatives and projects related to the identification of endocrine disrupters and preventive measures. Until now these activities have mainly concentrated on knowledge building, however, as more and more evidence for harmful effects is obtained the work is turning towards regulatory initiatives. In the following these activities will be described in line with the national strategy and when possible presented according to the 5 levels in the OECD conceptual framework.

From a general perspective it is important to stress that the OECD conceptual framework for testing and assessment of endocrine disrupters may be used differently with regard to testing for toxicological effects than for testing for ecotoxicological effects. In principle, toxicological and ecotoxicological test methods are designed with particular reference to different protection purposes, as the use of ecotoxicological tests is aimed at protection on an ecosystem level (i.e. protection of a fish population) whereas toxicological tests are aimed at protection of a single species (man).

Level 1 - Sorting and prioritisation based upon existing information

Knowledge building and development of test methods:

Inter-ministerial network

In 2007 an inter-ministerial endocrine disrupter's network was established in order to co-ordinate the work on endocrine disrupters among relevant ministries. The Ministry of the Environment is responsible for the national strategy on endocrine disrupters and therefore, one important task for the network is to ensure that knowledge and new data is transferred and shared among authorities so relevant precautionary measures can be taken and furthermore, to set up an alert system between ministries in case immediate action is necessary. The network consists of representatives from the Ministry of the Environment, the Ministry for Food, Agriculture and Fisheries, the Ministry of Health and Prevention, the Ministry of Employment and the Ministry of Science, Technology and Innovation[1].

Workshops

A number of workshops with focus on endocrine disrupters and their effects have been arranged during the years that are funded or co-funded by the Ministries of the Environment and Health and Prevention. An international three day workshop about testicular cancer was held in August 2002. The ministries have also funded several three-days international conferences named "Copenhagen Workshops on Endocrine disrupters, the 2nd: A possible role of mixed exposures for reproductive failures and malignancies" in December 2002, the 3rd: on Environment, Reproductive Health and Fertility in January 2004, the 4th: Endocrine disrupters and consumer products: possible effects on human populations in May 2007 and the 5th : Ubiquitous endocrine disrupters and possible human health effects will take place in May 2009. Finally, a one-day national workshop: "EDC's – how far are we?" was arranged in December 2006 and a national workshop: "Focus on recent Research in Endocrine Disruption" was arranged in May 2008 and in addition a half-day workshop took place in December 2008 focusing on new Danish data on endocrine disrupters[1].

Analyses of Substance Flows of Resorcinol and 4-nitrotoluene

After publication of the 1st step of the work with establishment of an EU priority list of substances for further evaluation of their role in endocrine disruption, substance flow analyses were conducted for two substances on the list, namely resorcinol and 4-nitrotoluene, in order to investigate if there is a risk related to consumer exposure. The analyses included an investigation of possible alternatives and a screening of effects on environment and health. No exposure of consumers or the environment were detected for 4-nitrotoluene. For resorcinol the use in hair colours was found to be potentially problematic and the report was forwarded to the European Commission as input to future evaluations of the use of the substance in hair colours [4, 5] [3].

Survey of the use of 22 active ingredients in pesticides with endocrine disrupting properties in other products than pesticides

As another follow-up to the publication of the 1st step of the work with establishment of an EU priority list for potentially EDCs, it was investigated whether 22 active ingredients used as pesticides and listed on the priority list were used in other chemical products than pesticides. There was no widespread use of these substances in other chemical products than pesticides in Denmark. Other uses were already regulated or the ingredients were used in very small quantities [13] [2].

Investigations of causes and effects and preventive efforts:

Investigation of endocrine disruption in freshwater fish

Endocrine disruption was observed in freshwater fish in Danish water courses in 2001 [3]. These findings resulted in the establishment of a series of projects aimed at investigating the cause and extent of these findings [10, 11, 12, 24, 25]. Based on these studies, it was concluded that the observed endocrine disruption in freshwater fish most probably was caused by exposure to natural oestrogens and not synthetic oestrogens or oestrogenic substances. Further, it was concluded that the majority of Danish wastewater is treated sufficiently to reduce the amount of oestrogens released the environment to a level which is considered of low significance. The projects have been followed up by more research-oriented activities aimed at studying the importance of oestrogens released to the environment when manure is us as fertiliser on farmland[1].

Surveillance of the semen quality in young Danish men

Surveillance of the semen quality of young healthy Danish men was initiated in 1996, and since 2001 the project has been supported by the Danish EPA and the Ministry of Health. The investigations have shown that young Danish men living in Copenhagen have a relatively low semen quality, also when compared to similar countries as e.g. Finland. The research activity has also focused on the reason for the geographical differences[1].

Surveys of chemicals in consumer products

Since 2001 the Danish EPA has surveyed the exposure of consumers to chemicals from consumer products. The surveys have mainly included chemicals in toys, cosmetics, jewellery, furniture, household chemicals and hobby products, whereas pharmaceuticals, medical devices, foods and products which are in contact with food have not been included. The main focus has been on chemicals which could be sensitising, carcinogenic, persistent, bioaccumulative or toxic, but substances with endocrine disruptive effects have also been surveyed. Examples of surveys of exposure to endocrine disrupters are: "phthalates in products with PVC", "TBT and DBT in consumer products" and "use of PVC and phthalates in Denmark in 2000 and 2001". Furthermore, an investigation of 2-year old infants' exposure to chemicals is ongoing, and the result will be published in 2009. This project focuses especially on exposure to endocrine disrupters and sensitising substances [3].

***Regulation**:*

Input to the EU priority list of substances for further evaluation

The Danish EPA has requested the EU Commission to include parabens on the EU priority list on substances for further evaluation in their role of endocrine disruption, and has continuously pointed out the importance of updating the list. (Regulation of several classes of chemicals)

Input to REACH

In the REACH-process Denmark worked for inclusion of endocrine disrupters in the REACH regulation in the best possible way. One of the inputs to the REACH process was the Nordic discussion paper on if and how the OECD conceptual framework can be used for regulation of endocrine disrupting substances, which is described below under level 3. In the final REACH text, endocrine disrupters are covered by the authorization procedure by a case-by-case assessment. This means that if it can be justified that a substance may have endocrine disrupting properties leading to adverse effects, the substance can be banned generally, however, approval for certain uses can be granted if safe use can be documented.
(Regulation of several classes of chemicals)

National list of Undesired Substances

In 1998 the Danish EPA published an advisory list of undesirable substances, that gave priority to 26 substances for which the authorities indicated interest in restricting use or completely banning in future. The list serves as guidance to companies that wish to engage proactively in voluntary phasing out undesired substances. The list has been updated three times, most recently in 2004. In 2004 those category 1 substances on the EU priority list of substances for further evaluation that were not already subjects to regulation or covered by an authorisation system for plant-protection products [6] were included in the list of undesired substances. In 2008 it was announced that all category 1 substances on the EU priority list will be included in the revised list of undesirable substances which is expected in 2009. (Regulation of several classes of chemicals)

International regulation of endocrine disrupting compounds

A number of endocrine disrupting compounds have been subjects for international regulation - regionally as well as globally. Examples are regulation of certain brominated flame retardants (EU), organic tin compounds (EU, International Maritime Organization), nonylphenol and nonylphenolethoxylates (EU) and persistent organics pollutants like PCBs, DDT, dioxin and furans (EU and the Stockholm Convention). It is not possible to attribute information from certain levels of the OECD conceptual framework for these substances as documentation for these regulations. Furthermore, these regulations have also referred to other inherent hazardous properties of the chemicals than endocrine disruptive properties.
(Regulation of several classes of chemicals)

Guidance to consumers

Food for thought – facts about endocrine disrupting substances

In 2002 the booklet "Stof til eftertanke – fakta om hormonforstyrrende stoffer" (in English: "Food for thought - facts about endocrine disrupting substances", only available in Danish) was published in a co-operation between the Danish Food Directorate, the Danish EPA and the National Board of Health. The booklet informs about endocrine disrupters, which effects they might cause and how and where one can be exposed to them. It aims at all Danish consumers, but has a special focus on pregnant and parents with small children [7]. (Guidance mainly related to industrial existing chemicals)

Good chemistry to pregnant and nursing mothers - 9 good habits

In September 2006 the Ministry of the Environment launched the campaign "Good chemistry to pregnant and nursing mothers - 9 good habits". The campaign pointed out 9 easy ways to reduce the exposure of the mother and the baby to chemicals, including endocrine disrupters, in cosmetics, toys and baby products. It was a network campaign with a network of midwifes, doctors and nurses, who distributed the material and used it for dialogue with the pregnant and nursing mothers and new parents. The campaign was very successful. An evaluation showed that 2/3 of the respondents in the target group were aware of the campaign, 2/3 of these had obtained new knowledge. Fifty percent of the respondents who were aware about the campaign followed the advise beforehand, while 30-35% had changed their behaviour as a result of the campaign [8, 9]. (Guidance mainly related to industrial existing chemicals)

Voluntary agreements

- Voluntary agreement since 1987 between the Danish EPA and the trade organization SPT (soap, perfume and technical-chemical articles) about substitution of nonylphenol-ethoxylates in washing- and cleaning agents. (Voluntary programme for industrial existing chemicals)

- Voluntary agreement from 1995 between the Danish EPA and the "Dansk Planteværn" (Danish Association of manufacturers and importers of pesticides) about removal of oestrogen-like additives in pesticide formulations. (Voluntary programme for pesticides)

Level 2: In vitro assays providing data about endocrine mechanisms

Knowledge building and development of test methods:

Development of computer models ((Q)SARs) for endocrine disrupting effects

The (Q)SAR-group at the National Food Institute have developed three computer models ((Q)SARs) for endocrine disrupting effects in *in vitro* assays.

1) Oestrogen α-receptor binding (in vitro). The training set uses data from METI (Japan). Data on 595 chemicals constitute the training set - the domain of the model within about 47,000 EINECS substances was 52%. Cross-validation results for the ((Q)SAR) model showed sensitivity, specificity and concordance to be 77 %, 87 % and 83 %, respectively. In the experimental method, human oestrogen receptor produced from *Escherichia coli* was used. The chemical substance is added to a system where RI-labelled oestrogen as reference hormone binds to the human oestrogen receptor. The chemical concentration that inhibits 50% of the binding of the reference hormone to the receptor is measured and defined as IC_{50}. As endpoint units, RBA (Relative Binding Affinity) between the IC_{50} values of the chemical and a natural hormone (E2, etc.) when the IC_{50} concentration of natural hormone is set at 100 was used [41-43, 45].

2) Oestrogen reporter gene (in vitro). The training set was derived from METI (Japan). Data on 481 chemicals constitute the training set - the domain of the model within about 47,000 EINECS substances was 61%. Cross-validation results for the (Q)SAR- model showed sensitivity, specificity and concordance to be 46 %, 95 % and 81 %, respectively In the experimental method, the oestrogenic effect of chemicals was measured as an increase of the luminescence response induced by the synthetic oestrogen E2 in harvested MCF-7 cells. As endpoint units the increase in luminescence response was used as active/not active intersection point. The test identifies chemicals, which have influence on oestrogen receptor binding and transactivation of the receptor followed by oestrogen-dependent gene expression [41-43, 45].

3) Androgen receptor antagonism (in vitro). The training set contains data from own experimental testing and data from the literature. Data on 523 chemicals constitute the training set - the domain of the model within about 47,000 EINECS substances was 56%. Cross-validation results for the ((Q)SAR) model showed sensitivity, specificity and concordance to be 64 %, 84 % and 76 %, respectively. In the experimental method, the androgen receptor antagonism of chemicals was measured as the inhibition of the luminescence response induced by the synthetic androgen R1881 in harvested Chinese Hamster Ovary (CHO) cells. As endpoint units, inhibition of luminescence response as active/not active intersection point was used. The activity is observed as an inhibition of the progress of androgen receptor binding and transactivation of the receptor followed by gene expression visualized by enzyme response [43-45] [1].

Participation in OECD validation of an in vitro steroid synthesis assay (H295R)

Some compounds can modulate steroid hormone production or breakdown and cause endocrine disruption without acting as direct hormone mimics. The H295R cell line is a human female adrenocortical carcinoma that produces many steroid hormones including progestins, androgens & oestrogens, glucocorticoids & mineral corticoids and expresses most of the important steroidogenic enzymes (CYP11A, CYP11B, CYP17, CYP19, CYP21). The National Food Institute has participated in the two rounds of the pre-validation and the validation of this assay aimed to detect substances that influence the steroid synthesis. The results showed that absolute hormone levels may differ, but relative dose-response profiles are comparable for all model compounds tested across laboratories, that the assay can discern between strong, medium and weak inducers and inhibitors of T and E2 production as well as negative responses and that the H295R Steroidogenesis Assay is a rapid, economic and cost effective screen of chemicals for their potential to alter steroidogenesis. Thus, the work with this assay has taken important steps forward towards the development and adoption of a test guideline for the method in the OECD Test Guideline Programme. This project was supported by the Danish EPA and the Nordic co-operation Nord-Utte [17] [1].

Development of in vitro assay to screen for effects on the thyroid receptor (T-screen assay)

The T-screen assay has been established at the National Food Institute and many chemicals have been tested for effects on the proliferation of murine GH3 cells. The current experience shows difficulties by identification of positive chemicals in this assay and the future regulatory use of this assay seems to be limited[1].

Development of androgen receptor reporter gene assay (AR-assay)

The androgen receptor reporter gene assay has been established at the National Food Institute and used with much success in order to detect anti-androgenic activity. Many of the potential anti-androgens found in this in vitro assay were subsequently shown to be anti-androgens in vivo[1].

Development of method to include metabolism in in vitro assays

The National Food Institute has begun research and development concerning the metabolic capacity of commonly used in vitro assays as well as how to incorporate external metabolism in cellular assays[1].

***Regulation**:*

The OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters as a basis for regulation of substances with endocrine disrupting properties

Denmark initiated and led this Nordic (Nord-Utte) project, investigating if and how the OECD conceptual framework can be used for regulation of endocrine disrupting substances. A number of proposals of how to use test results from the different levels of the conceptual framework were put forward together with a number of proposals of how to enhance the existing test guidelines in order to assess endocrine disrupting effects [15]. The results were presented at EDTA8 in January 2005. Specific proposals for the use of the test results from level 2-5 in the conceptual framework as regulatory instruments in the EU can be found in the report, however, the conclusions for the regulatory use of test results from each level will be presented for each level below.

The conclusion for the use of level 2 tests for regulatory purposes was that: "positive *in vitro* test results indicate potential ED activity and a potential for ED effects *in vivo*. *In vitro* data can provide valuable mechanistic data that is useful for the design of further *in vivo* studies. The *in vitro* tests are relevant for effects in humans because many of these tests are based on human hormone receptors. Chemicals that bind to these receptors are therefore likely to cause effects in *in vivo* studies and on reproductive function in humans. Negative *in vitro* test results cannot be used to exclude potential EDC activity because of limitations such as inability or unknown capacity to metabolically activate toxicants and because EDC activity can occur through mechanism other than those tested in the *in vitro* test system. (Q)SAR models for ED activity and reproductive toxicity effects are under development but at present the use for priority setting and risk assessment is undecided". (Regulation of several classes of chemicals)

Level 3 - In vivo assays providing data about single endocrine mechanisms and effects

Knowledge building and development of test methods:

- Participation in the development of the Uterotrophic assay

The Uterotrophic assay is an *in vivo* test method for detection of chemicals that have the potential to act like and interfere with the endogenous female sex hormone. The National Food Institute participated in the initial work towards validation of the uterotrophic assay in 1999-01 (phase one), the validation using weak oestrogens and a negative control in 2000-2 (phase two) and the OECD discussion on the progress of the work and the draft OECD test guideline in 2003-7. This work was supported by the Danish EPA[1].

Participation in the development of the Hershberger assay

In 2000-2001, the National Food Institute participated in all three phases of the validation of the Hershberger assay, where two potent chemicals were tested, i.e. the AR-agonist testosterone propionate and the AR-antagonist Flutamide. In 2002-3, the Institute participated in phase two, where several weak agonists and antagonists were tested. In 2004-5, the Institute participated in phase thee of the OECD validation, where the purposes were to investigate the reproducibility of results by comparisons to the results in phase one and 2, the specificity by testing of negative compounds and compare data from Hershberger assay using adult castrated rats and immature male rats. During 2000-8, experts from the Institute also participated in the OECD discussion on the progress of the work and the various versions of the ring test reports and draft OECD test guideline. This work was supported by the Danish EPA[1].

Assays to detect chemicals with effects on the thyroid

To test whether a chemical disrupts the hypothalamus-pituitary-thyroid axis (HPT-axis), a limited number of screening assays are available. EDSTAC recommends the frog metamorphosis assay, the adult male assay (to detect anti-androgenic effects, full thyroid and reproductive hormone screen) and the Japanese quail has been proposed as an *in vivo* model for action on TH-binding proteins (Ishihara et al., 2003).
None of these tests have yet been validated for guideline purposes, and there is only one vertebrate-based assay currently being evaluated namely the "frog metamorphosis assay" (Gray et al., 2002, EPA), but this assay in the OECD TGP do not address human health. The National Food Institute has worked with development of an *in vivo* thyroid assay in pregnant rats since 2004. The main endpoints are thyroxine (T4) levels in dams during pregnancy and lactation, T4 in pups on postnatal day 13 and growth and development of the pups before weaning. The main purpose with the work is to study if NOAELs from this model gives sufficient protection in relation to developmental neurotoxicity effects later in life (see further details under level 5). This work is supported by the Danish EPA[1].

Participation in the Fish Screening Assay ring test

In 2003 and 2004 Danish laboratories participated in the ring test of the Fish Screening Assay (FSA). The FSA utilizes adult fish (preferably zebra fish, fathead minnow and Japanese medaka). The induction of vitellogenin is the major endpoint in the test. Thus, the test is mainly sensitive towards endocrine disrupting chemicals with an oestrogenic mode of action. University of Southern Denmark (SDU) and DHI have contributed with research on the development of methods for immunochemical determination of vitellogenin concentrations in plasma of various fish species and whole body of zebra fish and dose-response relationships for various chemicals (i.e. parabens, alkylphenols, bisphenols A, chemical UV-filters, natural and synthetic oestrogens). Furthermore, different exposure routes (water, food, injection) in different fish species have been investigated and the results have been published. Both SDU and DHI have been actively involved during the experimental validation phase of the test proposal. This project was supported by the Danish EPA and the Nordic co-operation Nord-Utte [17] [1].

Investigations of causes and effects and preventive efforts:

The National Food Institute has investigated 5 currently used pesticides (prochloraz, simazine, deltamethrin, tribenuronmethyl, methiocarb) as single chemicals and as a mixture in the Hershberger Assay [46, 77, 81]. The results showed anti-androgenic effects and the mixture effects appeared additive. This work was supported by the Danish EPA. The test results for prochloraz have been used as part of the documentation for banning the use of prochloraz. (Governmental programme for pesticides and to be used for several classes of chemicals)

Regulation:

- Nordic project on the possible use of the OECD Conceptual Framework as a basis for regulation of substances with endocrine disrupting properties, see level 2. The conclusion for the use of level 3 tests for regulatory purposes was: "that the assays at level 3 provide an *in vivo* screening of potential endocrine disrupting activity of a substance. Except for the frog metamorphosis assay, the assays at level 3 provide information about the potency of the compound *in vivo*. Furthermore, the outcome of the assays indicates potential for adverse effects in the reproductive developmental studies at level 5. At present, it is uncertain to what extent the frog metamorphosis assay can be used for screening in relation to effects on humans".

Level 4 - In vivo assays providing data about multiple endocrine mechanisms and effects

Knowledge building and development of test methods:

Active commenting of the work for updating the 28-days study with regards to EDC properties (enhanced/updated TG407)

The National Food Institute has participated in the OECD discussions on the validation results in relation to the revision of the 28-days repeated dose toxicity study (OECD TG 407) since 2005. The intention was to enhance the sensitivity of this assay for detection of endocrine disrupting chemicals. The validation results showed that the revised assay detected only potent EDCs, whereas weak EDCs, i.e. those causing effect only during development, were not detected. In addition, only one chemical with potent effects on thyroid hormones were included in the validation. Consequently, the National Food Institute found that the sensitivity of the enhanced/updated assay was problematic and hesitated to recommend further development into a guideline. As the updated assay, however, seems to detect potent anti-androgens and oestrogens further development was accepted if the first paragraphs in the revised guideline clearly states that a negative result in this assay cannot exclude endocrine effects. During 2007-8, the National Food Institute has commented on the OECD Guidance Document on histopathological effects. This work was supported by the Danish EPA[1].

Enhancement of the prenatal developmental toxicity study (OECD TG 414) in order to detect effects on foetal steroidogenesis and anogenital distance

The purpose of the project performed at the National Food Institute was to investigate whether implementation of assessment of testosterone levels and anogenital distance (AGD) in male foetuses would be useful for investigating endocrine disruptive effects in the OECD Test Guideline 414: "Prenatal Developmental Toxicity Study". In 2004-2006 samples from male foetuses exposed to fourteen different substances were analysed [47-50, 54-55]. The results showed that it is feasible to include assessment of testosterone synthesis and anogenital distance (AGD) in the male foetuses in a TG 414 study design. This project was supported by the Danish EPA and the Nordic co-operation Nord-Utte [17] [1].

Lead for development and validation of the Fish Sexual Development Test (FSDT)

The FSDT utilizes the fact that the sexual development in fish is very sensitive to exposure to chemicals that interfere with the effect of the normal sex hormones. This makes the FSDT very sensitive to oestrogenic, androgenic, anti-oestrogenic, anti-androgenic and aromatase-inhibiting chemicals. The FSDT (earlier named the Fish Partial Life-Cycle Test) is a modified version of the OECD guideline 210 adopted in 1992 - the Fish Early-Life Stage Toxicity Test with added end-points for the detection of endocrine disrupters (secondary sex characteristics, vitellogenin (VTG) concentration and sex ratio) (OECD, 2005). The main idea behind this assay is that exposure to endocrine disrupters during the sensitive window during sexual development of the fish will alter the VTG concentration and sexual development based on the property of the hormone they are exposed to.

The FSDT - with Denmark as lead country - was included in the work plan of the OECD test guideline program in 2003. During autumn 2005 and early spring 2006 in-depth statistical evaluation of potential test designs was performed. The outcome was presented at the EDTA9 in 2006. The OECD fish drafting group agreed then on the statistical design and to carry through experimental validation of the test method. First round of the validation took place until summer 2007 and has successfully been completed. Phase 2 of the validation exercise is taking place during 2008. This project has been supported by the Danish EPA and the Nordic co-operation Nord-Utte [17] [1].

Participation in the development of the Copepod Development and Reproduction Test

At the moment there are no internationally harmonized (i.e. OECD) test methods for chronic effects on marine invertebrates even though they are an ecologically important and large group of organisms. These invertebrates serve as food for fish larvae and they need to be protected to secure transfer of energy from the primary producers to higher levels in the ecosystems. Therefore, the development of a test for reproduction and development of marine copepods was started in 2005, and has been included in the work plan of the OECD Test Guideline Programme. A couple of Danish labs have been involved in pre-validation work. This work was supported by the Danish EPA and the Nordic co-operation Nord-Utte [17].

Lead for development of an OECD TG on reproduction of springtails

Springtails are important in soil, especially in temperate soils, where they are thought to be the most important group of arthropods. An ISO test guideline already exists for one of the springtail species, *Folsomia candida*. This test is already in use for pesticide evaluation within e.g. Europe and USA, and in certain cases for evaluating the potential problems related to application of sewage-sludge containing industrial chemicals on agricultural land. The springtail test includes an option between two test species: One asexually reproducing species and one sexually reproducing species. It also covers the reproductive capacity of the animals, will enable a detection of damage to the reproductive system which may be caused by compounds that modulate the arthropod sex hormone system. A draft OECD Springtail test guideline was pre-validated in 2004, and accepted for the work plan of the OECD TGP at the WNT-meeting in 2005. A draft TG proposal (with a ring test report annexed) is shortly being circulated to OECD NCs for comments. This project has been supported by the Danish EPA and the Nordic co-operation Nord-Utte [17, 20] [1].

Investigations of causes and effects and preventive efforts:

Investigation of endocrine disruptive effects of nitrate and nitrite in vitro and of nitrate in vivo (after exposure in utero)

The National Food Institute has studied in vitro effects of nitrite and nitrate in the steroid synthesis assay (H295R) and effects on anogenital distance and testosterone production in male rat foetuses from dams exposed to nitrate via drinking water (not published yet). This project has been supported by the Danish EPA [3].

Regulation:

Nordic project on the possible use of the OECD Conceptual Framework as a basis for regulation of substances with endocrine disrupting properties, see level 2. The conclusion for the use of level 4 tests for regulatory purposes was that: “the assays at level 4 provide a thorough assessment of the potential endocrine disrupting effects of a substance in pubertal and young adults. Furthermore, level 4 provides information about the potency of a compound to be investigated at level 5. Effects on various endpoints included in the assays can either be considered as adverse or represent an effect on a mechanism relevant

for human e.g. changes in hormone levels. Therefore, these assays can be used to provide NO(A)ELs/LO(A)ELs to be used in human risk assessment until further studies are available. The intact male assay and the TG 407 may be more capable for detecting aromatase inhibitors and compounds affecting the steroid synthesis compared to the pubertal male assay. On the other hand, the two assays in intact young males may be less sensitive compared to the Uterotrophic and the Hershberger assay as well as the male and female pubertal assay".

Level 5 - In vivo assays providing adverse effects data from endocrine and other mechanisms

Knowledge building and development of test methods:

Enhancement of existing OECD guidelines for reproductive toxicity testing

The National Food Institute has during many years studied a number of EDCs and their effects with the purpose of providing proposals for enhancing generation studies with respect to detection of endocrine disrupting effects. Input for this has been given at OECDs EDTA meetings and in the OECD VMG Mammalian[1].

Participation in the work for extending the 1-generation study

The National Food Institute participates in the work on enhancing generation studies with respect to detection of endocrine disrupting effects, incl. the ongoing development of the draft OECD test guideline for the Extended One-Generation study. There are indications that female mammary gland development is a sensitive endpoint for effects of EDCs and consequently, it has been proposed to include a placeholder for assessment of this effect in the draft OECD test guideline for the Extended One-Generation study[1].

Effects on thyroid hormones during development and behavioural effects

The National Food Institute studies the relationship between effects on thyroid hormones (thyroxine, T4) during pregnancy and lactation and behavioural effects in the offspring. In 2005-2006, propyl thiouracil was studied and the results showed that the effects on T4 during pregnancy and lactation were good and sufficiently sensitive predictors for effects on motor activity and learning effects in adult offspring [54]. In 2007-2008 mancozeb was studied. Mancozeb caused unexpected marked acute neurotoxicity in the dams at the doses used and consequently, the knowledge on the relationship between effects on thyroid hormones and behaviour in the offspring could not be expanded based on this study. In 2008, a study on the UV-filter octyl methoxycinnamate (OMC) was started. OMC caused marked effects on thyroid hormones in both dams and offspring and decreased testes and prostate weight as well testosterone levels in the male offspring [52-53]. The behavioural testing is ongoing. This project is supported by the Danish EPA[1].

Participation in the development of the OECD Test Guideline 426 - Developmental Neurotoxicity Study

A research group has since around 1993 (at the Institute for Occupational Health and since 1999 at the National Food Institute) developed methods for developmental neurotoxicity testing and participated in the development of the OECD Test Guideline 426 - Developmental Neurotoxicity Study [60-74]. This guideline is relevant for EDCs, because hormones organize the sexual dimorphic development of the brain (sex hormones) and the general development of the brain (thyroid hormones).
This project was supported by the Danish EPA, the Nordic co-operation Nord-Utte and others[1].

Participation in the development of the Fish Full Life Cycle Test
In 1999 and 2000 the potential usefulness of zebra fish as test species in the 2-generation test in fish for detection of endocrine disrupting properties was investigated. The results supported the development of an

OECD DRP (lead by US) concerning a full life cycle test on fish. This project was supported by the Danish EPA and the Nordic co-operation Nord-Utte [17, 19] [1].

Investigations of causes and effects and preventive efforts:

Mixture effects of similarly and dissimilarly acting EDCs

Researchers at the National Food Institute have participated in the EU Strategic Research Framework programme project EDEN. During 2003-7, the Institute has investigated the effects of nine anti-androgens on sexual development and behaviour both as single chemicals and in combinations. Two mixture studies of respectively similarly and dissimilarly acting anti-androgens has been performed [56-59]. The aim of the work was to investigate if mixture effects occur at NOAELS for the single chemicals and to assess whether such effects can be predicted based on dose-response studies for the single chemicals using dose-additivity modelling. From 2008, the National Food Institute participates in a new EU project CONTAMED where the aim is to study mixture effects after developmental exposure to 10-12 environmentally relevant EDCs at low doses. This project was supported by the Danish EPA and the EU[1].

Effects of endocrine disrupting pesticides, phthalates and others

The National Food Institute has investigated the effects of several endocrine disrupting pesticides, phthalates and others chemicals (e.g nonylphenol, di(2-ethylhexyl) adipate) on sexual differentiation of the reproductive system and the brain [56-59, 75-83].

Regulation:

- Nordic project on the possible use of the OECD Conceptual Framework as a basis for regulation of substances with endocrine disrupting properties, see level 2. The conclusion for the use of level 5 tests for regulatory purposes was that: "The reproductive toxicity studies provide adverse effect data and are especially useful for risk assessment as they indicate potential for effects in humans. The effects observed in reproductive toxicity studies may be due to other mechanism that endocrine effects, but the pattern of effects, e.g. decreased anogenital distance and malformations of reproductive organs in males, may indicate that endocrine effects are involved. Among the OECD test Guidelines for reproductive toxicity, exposure during all vulnerable periods of development is only performed in the two-generation study design. Late effects becoming manifest after weaning of the animals are partly covered in young adults, especially in relation to reproductive function and developmental neurotoxicity, but potentially important late effects are not assessed. Effects becoming manifest during ageing are not included in any guidelines for reproductive toxicity. A number of enhancements of the OECD Test Guidelines for reproductive toxicity for the detection of effects of EDCs seem relevant and lack of effects in reproductive toxicity studies can therefore at present not fully exclude the possibility for ED effects caused by chemicals tested negative".

Attachment

Cases

The following cases illustrate that the regulatory initiatives towards endocrine disrupting compounds in many cases are influenced by exposure and not only effect or activity considerations. The cases also illustrate that assessments of the substances usually are based on weight of evidence, including information obtained by using non-test and test methods from several levels in the conceptual framework.
One important outcome of all of the following cases has been contributions to the development of new or enhancement of relevant existing OECD test guidelines.

Case 1: Azol fungicides

The National Food Institute has investigated the effects of several commonly used azole fungicides (prochloraz, tebuconazole, propiconazole, and epoxiconazole) in various *in vitro* assays, the Hershberger assay and pre-and postnatal developmental toxicity studies [48, 77, 81, 83]. The studies showed that epoxiconazole, propiconazole, and tebuconazole, possess similar properties as the imidazole fungicide prochloraz regarding interactions with the ER, AR, and AhR, as well as effects on steroid hormone synthesis and the activity of the enzyme aromatase that converts testosterone to oestrogen. The three triazoles (tebuconazole, propiconazole, and epoxiconazole) turned out to be less potent than prochloraz in some of the assays. In order to investigate the effects on steroid synthesis, the H295R assay was established – a spinoff of this work was the participation in the validation of the assay for development in the OECD TGP. Furthermore, a bioassay (T-screen) for detecting binding and activation of the thyroid receptor (TR) was established and used to investigate the ability of prochloraz, epoxiconazole, propiconazole, and tebuconazole to be thyroid hormone agonists or antagonists. Convincing interactions with the TR were not found for either prochloraz or any of the triazole fungicides.
Anti-androgenic effects of prochloraz, propiconazole and tebuconazole in vivo in young adult rats were studied using the Hershberger assay. None of the triazole fungicides, propiconazole or tebuconazole, had any androgen receptor blocking effect in vivo in the Hershberger assay at doses at or below 150 mg/kg bw/day. This is in contrast to prochloraz that induced anti-androgenic effects in this assay at doses between 50 and 150 mg/kg bw/day.

Developmental effects of prochloraz, epoxiconazole and tebuconazole were investigated in rat offspring after exposure during pregnancy and lactation. Both prochloraz, epoxiconazole and tebuconazole caused an increased gestational length. This effect is probably related to the observed marked increases in plasma concentrations of progesterone in the mothers induced by these fungicides. Tebuconazole and epoxiconazole induced a high frequency of post-implantation loss, and epoxiconazole caused a marked increase in late and very late resorptions. Prochloraz and tebuconazole decreased the testosterone level in testis from the male fetuses and increased the number of nipples in the male pups, i.e anti-androgenic effects.

Overall the results indicate that azole fungicides in general have a similar profile of action in vitro but that the profile of action in vivo may differ. The common features for the tested azole fungicides are that they all increased gestational length and increased progesterone levels in the dams. Prochloraz and tebuconazol caused anti-androgenic effects in male offspring. The effects on steroid hormone synthesis in vitro, the lack of effect in the Hershberger assay (where effects on steroid synthesis is omitted by using testosterone supplemented castrated male rats) combined with the effects on reproductive developmental after perinatal exposure strongly indicate that one of the main responsible mechanisms may be disturbance of key-enzymes involved in the synthesis of steroid hormones.

The use of azoles within greenhouse production of ornamental plants where the workers are highly exposed to this group of chemicals have been investigated during the last years. An epidemiological study showed elevated foreign oestrogen activity in the blood from female greenhouse workers compared to non-exposed women [39]. Also, preliminary results have shown increased incidences of impaired reproductive development in sons of female greenhouse workers from the island Funen [40]. The study was performed some years ago, and many of the investigated pesticides are not commercially available any longer. However, as azole fungicides are still approved for use in green houses these observations are concerning in the light of the endocrine disruptive properties of azole fungicides that have been shown in animal studies. As a consequence the DK EPA has now initiated several activities – first of all new risk assessments for the azole fungicides approved for green house use has been carried out, including new methods for exposure calculation at re-entry and application of an extra safety factor. The DK EPA is also supporting a follow-up study that will include the same children now at their age of 7-10 years.

Based on existing information and the outcome of the research mentioned above, stricter guidelines for handling azole fungicides in greenhouses have been established.
(Governmental Programme and regulation of pesticides)

Case 2: Mixture effects/combined exposure

Studies indicate that incidences of disorders in the male reproductive system, including hypospadias in newborn boys, have risen during the last 50 years, but the impact of human exposure to endocrine disrupting chemicals is largely unknown at present. Chemicals risk assessment is currently based on the no-observed-adverse-effect-levels (NOAELs) for effects of single compounds. Using this approach, single endocrine disrupting chemicals alone may appear to be present in human tissues at levels too low to cause concern for adverse reproductive effects. However, as several anti-androgenic chemicals have been found as mixtures in humans, including children, the National Food Institute have investigated the ability of mixtures of anti-androgens to induce disruption of male sexual differentiation after perinatal exposure in extensive dose-response studies.

In the first mixture study on similarly acting anti-androgens (three AR-antagonists, vinclozolin, flutamide, and procymidone), the joint effects were essentially dose-additive for all endpoints. A combination of doses of each chemical that on its own did not produce significant effects induced marked mixture effects on anogenital distance (AGD,) nipple retention, the weight and histopathology of the seminal vesicles, and *PBP C3* gene expression in the prostate, and caused marked dysgenesis of external reproductive organs on PND 16 [56-57]. Major events in the development of male external sex organs occur after postnatal day 16 during the pubertal period. Consequently, the frequencies of hypospadia and other external sexual malformations were recorded also in the young adult male rats. An additional aim was to examine if the antiandrogenic effects observed in the male pups were predictive of the external malformations (hypospadia) observable in the young adult male animals later in life. The mixture of 24.5 mg/kg/day vinclozolin, 0.77 mg/kg/day flutamide and 14.1 mg/kg/day procymidone induced hypospadias in 60% of the male offspring, whereas the frequency after exposure to the chemicals alone appeared similar to the untreated controls, i.e. 0% [58].

The second mixture study focused on the effects of four dissimilarly acting anti-androgens, i.e. vinclozolin (androgen receptor antagonist), DEHP (testosterone synthesis inhibitor), finasteride (inhibitor of steroid type II 5-reductase) and prochloraz (multiple mechanisms incl. androgen receptor antagonism). AGD, nipple retention, and reproductive organ weights at PND 16 were clearly affected in the mixture groups at dose levels where the individual chemicals alone caused no or only minor effects. The joint effects were equally well predicted by dose-addition or independent action. In addition, a very high frequency of external malformations such as hypospadias was seen for a mixture for which the individual compound

caused no significant effects. This mixture effect was worse than predicted based on dose-addition or independent action [59].

In conclusion, in vivo studies indicate that the joint effects of combined exposure to more anti-androgens in many cases can be predicted by dose-addition models and that marked effects can occur at doses below NOAELs for the single chemicals. The significance of these findings for human and environmental risk assessment should be emphasised, because they clearly indicate that risk assessment based on NOAELs for single antiandrogens alone might severely underestimate the risk for hypospadias and other adverse antiandrogenic effects.

This work was part of the EU EDEN-project *"Endocrine Disrupters: Exploring Novel Endpoints, Exposure, Low Dose- and Mixture-Effects in Humans, Aquatic Wildlife and Laboratory Animal"* and was financially supported by the European Commission and the Danish EPA. As a follow-up to this work the DK EPA is hosting an expert workshop on combination effects of endocrine disrupters with focus on regulatory aspects in January 2009. Furthermore, various Danish scientists continue research in this field e.g. in a new EU Cluster Project and also sponsored by the Danish EPA and the Nordic Council of Ministers.

In 2006 the indications from animal studies regarding effects on the unborn child after combined exposures to endocrine disrupters together with a rising public awareness about these substances, lead to the campaign: "Good chemistry to pregnant and nursing mothers – 9 good habits". The campaign pointed out 9 easy ways to reduce the exposure of the mother and the baby to chemicals, including endocrine disrupters, in cosmetics, toys and baby products. Because of the indications and the awareness, Denmark is also working on the inclusion of combination effects of endocrine disrupters as an area of priority at the 5th WHO Ministerial Conference on Environment and Health to be held in 2010. Furthermore, in the new European pesticides regulation, Denmark has promoted acceptance of establishment of cut-off criteria for non-inclusion of pesticides with certain especially concerning dangerous inherent properties, including endocrine disrupting properties.

Case 3: Endocrine disruption in freshwater fish

Endocrine disruption in fish was already in the late 1990'ies rather well investigated and the causal mechanisms were in contrast to other endocrine related effects rather well understood. This was the basis for establishing the Nordic work on developing an OECD test guideline for detection of endocrine disrupting effects of chemicals in Zebra fish in 1998. Since that time the work has been supported by the Danish EPA and the Nordic co-operation Nord-Utte [17, 19], and resulted in the development of the OECD test guideline proposal "Fish Sexual Development Test" (FSDT). In the FSDT, the endpoints are vitellogenin in male fish and change of the sex-ratio. The latter endpoint is directly relevant for risk assessment, whereas the former indicates a biological response to exposure. These endocrine related effects have been observed in exposed wild fish populations in Danish watercourses. After public debate a series of projects aiming at investigation of the cause and extent of these findings were initiated [3]. The first projects reviewed the methodologies available for measuring oestrogenic activity *in vitro* and for measuring the chemical substances causing the effects [10, 11]. Since the substances could originate from sewage treatment plants, a project was dedicated to investigate the degradation of oestrogens in sewage treatment plants [12]. These initial follow up projects were followed by a major project where the oestrogenic activity in effluents and surface water was monitored [24, 25]. Based on the data obtained in all these projects, it was concluded that the observed endocrine disruption in freshwater fish by far and most probably was caused by exposure to natural oestrogens (excreted to sewage by the female part of the population and to a minor extent by farm animals to manure and via agricultural run off to water courses) and not by exposure to synthetic oestrogens or other chemicals with oestrogenic activity. Furthermore, it

was concluded that the majority of Danish wastewater is treated sufficiently to reduce the amount of oestrogens released to the environment to a level which is considered of low significance.

The projects has been followed up by more research-oriented activities aimed at studying the importance of oestrogens released to the environment when manure is used as fertiliser on farmland[1]. These studies have shown that oestrogens in cases of heavy rain may leach to drain-water from farm-land after up to 7 months after fertilising the agricultural fields with manure. The studies focused on the conditions in geographical regions with intense pig-farming (oestrogen rich manure from pregnant sows were used) and with soils where leaching have been shown to be influenced by preferential transport through soil-pores. The results may explain the findings of oestrogens in the previously mentioned surveys in cases where release of wastewater to the recipients can be excluded [86].

Case 4: Phthalates

Use of phthalates, exposure of consumers and effects of phthalates in the environment in Denmark was investigated by the Danish EPA for the first time in 1984. The background for the investigation was indications of DEHP being carcinogenic and other phthalates being toxic to reproduction. Also, phthalates had been measured in the environment and had been shown to accumulate in aquatic organisms and sediments [37]. In 1993, phthalates were pointed out as a new group of problematic substances from waste treatment. Both the exposure and the effects contributed to the concern. Especially, DEHP had been observed in several environmental compartments, and human exposure was assumed, even though this had not been investigated. BBP and DBP were shown to be weak oestrogens in breast cancer cell lines, DEHP had been shown to be toxic to reproduction and induce damage to testes in animal studies and also DBP had been assessed as toxic to reproduction. Furthermore, DEHP was suspected to be carcinogenic, and DEP, BBP, DnOP, DINP, DIDP were proposed to be classified as dangerous for the environment. The investigations of phthalates were closely connected to discussions of the use of PVC [32].

In the EU programme for risk assessment of existing substances, a number of phthalates were prioritised for risk assessment (DEHP, DOP, DBP, DINP, DIDP and BBP) [27]. The participation, completion and follow-up of these risk assessments have been of high priority in Denmark due to the concern arising from suspected exposure and effects.

In 1996 the Danish EPA evaluated the human health effects of DEHP and other phthalates based on the available literature [26]. Also a review of environmental fate and effects of DEHP was performed [36]. Furthermore, environmental releases of DEHP from various types of polymers used for different purposes were monitored and estimated [84, 85]. Due to a number of effects of DEHP on human health and the environment (chronic effects in the aquatic environment, decreased fertility and toxic effects on testes, oestrogenic and possible anti-androgenic effects in vitro and carcinogenicity), combined with the diffuse and widespread use of phthalates, an action plan to reduce and phase out the use of phthalates was published in 1999 [30] together with a strategy for decreasing use of PVC in Denmark [31]. The action plan focussed on the use of phthalates in soft PVC, which was estimated to cover 90% of the use of phthalates in Denmark. The aim was to reduce the use of phthalates with 50% during 10 years through a number of means: bans, taxes, grants, public green procurement and ecolabelling. Also increased knowledge about the effects and dispersal of phthalates and their alternatives was highly prioritised. At the same time the use of all phthalates in toys intended for children up to 3 years of age was banned. During the following years a number of projects focussing on alternatives to phthalates were published by the Danish EPA (Alternatives to soft PVC in the building industry, in flexible PVC, in printing inks, paint and lacquer, glues, silicone products, rubber, molten metal) [33, 34, 35].

Also several surveys of the use of phthalates in consumer products in Denmark were performed. Examples are: “phthalates in products with PVC” and “Use of PVC and phthalates in Denmark year 2000 and 2001”

[28, 29]. In 2003 the action plan was followed up by a status on the use of phthalates [21]. At that time DEHP and DBP had been classified in the EU as toxic to reproduction, and the status showed that the former use of DEHP consequently had decreased, whereas the consumption of DINP used as an alternative substance had increased. Also BBP and DIBP have in the EU been classified as toxic to reproduction. In 2007 a harmonized EU regulation banned 6 phthalates in toys intended for children up to 14 years of age (DEHP, DBP and BBP in all toys and DINP, DIDP and DNOP in toys if they can be put in the mouth). Denmark has maintained the ban of all phthalates in toys intended for children up to 3 years of age [22]. During the years the National Food Institute has conducted several experimental studies on different phthalates [47, 49, 50, 78, 80, 82] showing their endocrine activity and their endocrine disrupting properties.

The DK EPA has used these studies as documentation for the reproductive effects of phthalates both within the EU programme for risk assessment of existing substances and in the subsequent restrictions of use of phthalates in consumer products.

These studies have also contributed with important information in relation to the use of anogenital distance and nipple retention as new end-points for detection of endocrine disrupting effects and input for improvement of the TG 414 and the one-generation study.

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Appendix 2

Contribution from the European Commission

COMMISSION STAFF WORKING DOCUMENT

On the implementation of the "Community Strategy for Endocrine Disrupters" - a range of substances suspected of interfering with the hormone systems of humans and wildlife (COM (1999) 706), (COM (2001) 262) and (SEC (2004) 1372).

SUMMARY

Following the adoption by the Commission of a Communication to the Council and European Parliament on a "Community Strategy for Endocrine Disrupters" in December 1999 (COM (1999)706), the Council invited the Commission to report regularly on the progress of the work. The first progress report was adopted in June 2001. A second progress report summarising the implementation of the Strategy during the period 2001-2003 was adopted in October 2004 (SEC (2004)1372). This is the third progress report on the implementation of the Strategy during the period 2004–2006. It describes the developments that have been made in terms of activities on prioritising substances for further investigation, stimulating research, agreeing test methods or adapting legislation.

The "Community Strategy for Endocrine Disrupters" contains activities in the short, medium and long term. The short and medium term actions focus on gathering scientific data on "candidate substances" with a view to prioritising testing, guide research and monitoring efforts and to identify specific cases of consumer use and ecosystem exposure. The long-term actions focus on review and possible adaptation of policy and Community legislation. Considering that the "endocrine disruption" is not a toxicological endpoint per se, but it is a class of many mechanisms of action that may lead in different species to various types of effects which may result in adverse consequences on humans and ecosystems, the key shortterm action is the establishment of a priority list of substances for further evaluation of their endocrine disrupting effects. This prioritisation work started in the year 2000. A number of some 600 chemical substances ("candidates") have been screened, evaluated and a preliminary priority list was established. This work was completed at the end of 2006.

The preliminary priority list of substances for further evaluation is not a negative list of substances but it is meant to provide a basis for gathering further data on endocrine disrupting effects of those substances and for their subsequent evaluation. The list was elaborated in a stepwise approach. Between 2000 and 2006 the Commission has contracted three studies on identification and evaluation of substances.
In total 575 substances were investigated over the past six years as to their endocrine disrupting (ED) effects. In terms of prioritisation, it was found that, out of this number, 320 substances showed evidence or potential evidence for ED effects, while in total, 109 substances were not retained in the priority list, either due to insufficient data on ED effects or insufficient scientific evidence. 147 substances have been excluded from the evaluation during the process as they were identified as double entries, mixtures or of doubtful relevance. An assessment of the legal status of the substances with evidence or potential evidence of endocrine disrupting effects showed, that the majority of them are already subject to a ban or restriction or are addressed under existing Community legislation, although for reasons not necessarily related to endocrine disruption.

As regards medium-term actions, the Commission and Member States continue to participate in the OECD - Endocrine Disrupter Testing and Assessment Task Force (EDTA), which was set up in 1998 with the goal of developing agreed test methods for endocrine disrupters. The latest estimates are that agreed test methods for some environmental and human health effects will be finalised in 2007. Furthermore, addressing the medium term research and development objectives, endocrine disrupters were addressed under the 5th (FP5 – 1998-2001) and 6th (FP6 – 2002-2006) EU Research Framework Programmes and

will also be addressed under the 7th Framework Programme of the European Community for Research, Technological Development and Demonstration Activities (FP7 - 2007–2013).
Regarding long term actions, relevant developments since 2004 were the adoption of the regulation concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), formally adopted on 18 December 2006, the proposal for a directive setting environmental quality standards for priority substances under the water framework directive (2006) or the proposal for a regulation revising directive 91/414/EC on plant protection products (2006).

1. CONTEXT

Endocrine Disrupters are a group of chemicals (natural, synthetic, industrial chemicals or by-products) present in the environment and suspected to alter the functions of the endocrine system and, consequently, causing adverse health effects in an intact organism, or its offspring, or (sub) population.
In wildlife, endocrine disrupters have clearly been shown to cause abnormalities and impaired reproductive performance in some species, and to be associated with changes in immunity, behaviour and skeletal deformities. In humans, endocrine disrupters have been suggested as being responsible for apparent changes seen in human health patterns over recent decades. These include declining sperm counts in some geographical regions, increased incidences in numbers of male children born with genital malformations and increased incidences of certain types of cancer that are known to be sensitive to hormones. More controversially, links have been suggested with impairment in neural development and sexual behaviour.
In order to address the potential environmental and health impacts of endocrine disruption the Commission adopted a Communication to the Council and European Parliament on a "Community Strategy for Endocrine Disrupters" in December 1999. This Strategy sets out a number of actions relating to, *inter alia*, identification of substances, monitoring, research, international co-ordination and communication to the public. On 26 October 2000, the European Parliament adopted a Resolution on endocrine disrupters, emphasising the application of the precautionary principle and calling on the Commission to identify substances for immediate action.

On 30 March 2000, the Environment Council adopted "Conclusions on the Commission Communication" in which it stressed the precautionary principle, the need to develop quick and effective risk management strategies and the need for consistency with the overall chemicals policy. The Council invited the Commission to report back on the progress of the work at regular intervals, and for the first time in early 2001.

2. PROGRESS ON SHORT-TERM ACTIONS

As it is emphasized in the Opinion of the Scientific Committee on Health and Environmental Risks (SCHER) adopted in November 2005[1], "endocrine disruption is not a toxicological endpoint per se, but is one class of the many mechanisms of action that may lead to various types of effects in different species, which may result in adverse consequences on humans and ecosystems". The short-term actions have therefore focused on the need to gather up-to-date scientific information on endocrine disrupting effects and on the extent to which it is affecting people and wildlife. The work on identification and prioritisation of substances for further evaluation of their endocrine disrupting effects has continued and a preliminary priority list of the substances was established.

2.1. Establishment of a priority list of substances for further evaluation of their endocrine disrupting effects (see Attachment 1, Fig. 1)

2.1.1. Background

Between the years 2000 and 2006 the Commission has contracted three studies on identification and evaluation of substances as to their endocrine disrupting effects. In June 2000, the study towards the establishment of a priority list of substances for further evaluation of their endocrine disrupting effects – preparation of a "candidate list" of substances as a basis for priority setting[2] (BKH-study), established a list

[1] SCHER Opinion on "Endocrine Disrupting Chemicals: a Non-animal Testing Approach" (BUAV report–2004)

[2] Study "towards the establishment of a priority list of substances for further evaluation of their role in endocrinedisruption – preparation of a candidate list of substances as a basis for priority setting" BKH, 2000

of 553 candidate substances. Stakeholders, Member States and the Commission Scientific Committee on Toxicology, Ecotoxicity and the Environment (CSTEE) were consulted on the approach and gave their input. Furthermore, a first list of 118 substances showing endocrine disrupting effects or potential endocrine disrupting effects was established. Out of these 118 substances indicating evidence or potential evidence of endocrine disrupting effects, 109 were already addressed under existing community legislation. In 2002, a follow up study "on gathering information on 435 substances with insufficient data"[3] (RPS-BKH-study) was carried out. It aimed on refinement of the evaluation-methodology and on the investigation of the remaining 435 substances from the "candidate list" and also focused on candidate substances identified as High Production Volume Chemicals (HPVC), persistent in the environment and to which human- or wildlife-exposure could be expected. 204 substances were identified according to these criteria and their endocrine disrupting effects were evaluated. 147 of them were identified, showing either a clear evidence of endocrine disrupting effects or potential endocrine disrupting effects. For the substances that showed a clear evidence of endocrine disrupting effects, it was investigated if there is exposure concern for humans or wildlife. 84 of them showed high exposure concern, 5 showed medium and 4 substances showed low exposure concern.

Out of 147 substances indicating evidence or potential evidence of endocrine disrupting effects, 129 were already addressed under existing community legislation. In the 2002 study, 172 candidate substances were reported as not yet evaluated, as they were supposed to be mainly Low Production Volume Chemicals (LPVC).

2.1.2 "Study on enhancing the endocrine disrupter's priority list with a focus on low production volume chemicals" (DHI –Study)

In November 2005 the Commission, DG Environment, contracted "DHI Water and Environment" to carry out a further study[4], focusing on the remaining Low Production Volume Chemicals (LPVC). Commissioning this study, the Commission also took into account the recommendation from the former Scientific Committee on Toxicity, Ecotoxicity and Environment (CSTEE)[5], where the committee stated that LPVC with high release in the environment or with high potency were not sufficiently covered in the previous work on the priority setting.

Starting work, it turned out that 173 substances were left from the previous study and 22 substances were newly identified as candidates by stakeholders, therefore, in total 195 substances were identified for evaluation. After a first close look at the substances, it became obvious that many of them were neither LPVC nor HPVC, but they were produced in quantities lower then 10t/year. Furthermore, it turned out that 88 substances were of doubtful relevance as they were not listed in the existing chemicals database "ESIS"[6], an indication that they are not in use anymore, or not identified clearly by a single CAS N°. It was decided to exclude these 88, and thus, 107 substances remained for evaluation.

As in the previous study, the evaluation of endocrine disrupting effects in humans or wildlife was based on the following screening criteria: persistency, production data, consumption/use patterns, environmental concentrations (range), evaluation of endocrine disrupting effects taking into consideration the relevance of the effects parameter, test reliability, dose-response relationship, endocrine disruption potency, endocrine disruption structure-activity relationships, comparison with systemic toxicity and evaluation of exposure concern to human and wildlife. The results of the evaluation were: out of 107 substances, 34 were showing

[3] Study on "gathering information on 435 substances with insufficient data", RPS-BHK, November 2002.
[4] Study on" enhancing the endocrine disrupter priority list with a focus on low production volume chemicals",DHI Water and Environment 2006
[5] Opinion of the CSTEE on "two study reports for endocrine disrupters by WRc-NSF and BKH Consulting Engineers", November 2003
[6] ESIS: European chemical Substances Information System, DG JRC, European Chemicals Bureau

a clear evidence of endocrine disrupting effects in at least one intact organism (Category 1), 21 were showing some evidence, suggesting endocrine disrupting potential (Category 2) and 52 substances showed insufficient data to decide on endocrine disrupting (ED) effects or there was no scientific basis for their inclusion in the priority list (Category 3a or 3b). For results, see Attachment 2, Grouping of substances, Tables 1 – 5. Furthermore, for the 34 substances showing a clear evidence of endocrine disrupting effects, exposure concern to human or wildlife (Category 1) was evaluated. As only very few monitoring data sets were available for these substances, it was decided to base exposure evaluations on EUSES[7] calculations. EUSES is designed to be a decision support system for the evaluation of exposure of chemicals to man and the environment. Out of 34 substances, 12 substances showed high exposure concern, 16 substances showed medium and 6 substances showed low exposure concern.

An assessment of the legal status of the DHI- evaluated substances with an evidence or potential evidence of endocrine disrupting effects showed, that 29 are already subject to a ban or restriction or are addressed under existing Community legislation, whereas 27 are not (see Attachment 2, Grouping of substances, Tables 1 – 3). During the DHI study, stakeholders, experts and Member States were involved at the very beginning, when input in terms of recent scientific data on the candidate substances in question as well as identification of further candidate substances was requested. Later on, they were involved again when the draft final report was subject to internet consultation during November 2006. Then, mainly experts replied, providing input on single substances evaluation as well as on the presentation of the report. Industry expressed its concern that the results of the evaluation of the substances could be misunderstood as full risk assessments. Moreover, industry expressed the concern that the priority list could be seen as a definitive list of substances and not as a list of substances for further evaluation of their endocrine disrupting effects. Industry also provided scientific data on some of the evaluated substances. All received comments were taken into account as far as possible.

The final report of the DHI study is available on DG ENV's Endocrine Disrupters Website[8].

2.1.3. Preliminary priority list of substances for further evaluation of their endocrine disrupting effects

The substances on the priority list are mainly man-made chemicals used in industry, agriculture and consumer products. Substances with a clear evidence of endocrine disrupting effects belong to many different groups of chemicals, e.g., alkylphenols and its derivatives, benzoates, chlorinated paraffines, phthalates, dioxins/furans, triazines or PCBs. The preliminary priority list of substances for further evaluation is not a negative list of substances but it is meant to provide a basis for gathering further data on endocrine disrupting effects of those substances and for their subsequent evaluation. The list now comprises in total 428 substances. Out of them, 194 showed a clear evidence of endocrine disrupting effects (Category 1), 125 showed a potential evidence of endocrine disrupting effects (Category 2), whereas 109 showed either no scientific basis for inclusion in the list or insufficient data to decide about (Category 3a or 3b).

A database comprising all scientific information underlying the priority setting was established. It presents in a transparent manner the scientific data and references on human health, wildlife effects gathered in the three studies, as well as the categories in terms of priority concluded on that scientific basis. Compiling the database was one of the deliveries of the DHI - Study. The database is available on DG ENV's ndocrine Disrupters Website[9].

[7] EUSES: European Union System for the Evaluation of Substances

[8] http://ec.europa.eu/environment/endocrine/index_en.htm

[9] http://ec.europa.eu/environment/endocrine/index_en.htm

For the future, it is planned to work on the database further by developing a methodology that makes the list iterative, meaning that substances can be included or released from it. The methodology to be developed needs to be broadly discussed with experts, Member States and stakeholders. The database will then serve as a "dynamic working list", ensuring that substances, where necessary, can continuously be fed into relevant legislation in order to manage risks properly. The assessment of the legal status of priority substances (Category 1 and Category 2) resulted in total in 269 substances that are already subject to a ban or restriction or addressed under existing Community legislation and 51 substances that are neither restricted nor being addressed.

3. PROGRESS ON MEDIUM-TERM ACTIONS

As part of the medium-term actions, the Commission is supporting the development and validation of test methods by working closely with Member States to coordinate the European Union input to OECD. The medium-term actions also include research and development.

3.1. Identification and assessment of endocrine disrupters

The availability of agreed test strategies/methods to identify and assess endocrine disrupting chemicals is a basic requirement for comprehensive legislative action aimed at protecting people and the environment from the potential dangers posed by these chemicals.

The Commission participates in the OECD - Endocrine Disrupters Testing and Assessment Task Force (EDTA), which was set up in 1998 under the authority of the National Co-ordinators for the Test Guidelines Programme. The aim of the Task Force is to develop an internationally harmonised testing strategy as well as coordinating and overseeing the work of different sub-groups charged with developing new or revising existing test guidelines to assess the potential endocrine disrupting properties of chemicals. The Task Force met for the tenth time in spring 2007.

The latest estimates are that agreed test methods for human health effects will be finalised in 2007. In particular, an in vivo test guideline for screening for estrogenic effects, uterotrophic assay, will be most probably available by the end of 2007. Other draft in vivo test guidelines like the Hershberger assay or revised test guideline 407 are under development and might be available from 2008 onwards. Test methods for environmental effects include fish screening assay, amphibian metamorphosis assay and some invertebrate tests (e.g. copepod test). The first agreed test guidelines are estimated to be available by 2008. Alternative non animal test methods for ED screening are also progressing under the auspices of the OECD. In particular, a draft OECD test guideline for agonist estrogenic assessment using an in vitro estrogen receptor (ER) transcriptional activation assay has been submitted by Japan and was peer reviewed earlier this year. The EC/JRC is collaborating with the US and Japan on the validation of (ER) binding assays, androgen receptor (AR) binding assays and steroidogenesis assays.

An overview of the ED test methods that are currently in (pre)validation under the auspices of the OECD is set out in Attachment 3.

3.2. Research and development

Under the Fifth Framework Programme (FP5, 1999–2001), over 60 million euros were spent on 23 projects dealing with endocrine disrupters. The final reports of most of these projects are available[10]. As a direct

[10] http://ec.europa.eu/research/quality-of-life/ka4/ka4_reports_en.html

response to the call to enhance research efforts by the European Commission's Strategy on endocrine disrupters, the CREDO cluster (Cluster of Research into Endocrine Disruption in Europe) was established[11]. It was launched in April 2003 and will last until 2007. Four projects with a total budget of approximately 20 million EUR participated in the cluster encompassing 63 laboratories in Europe. The cluster was co-ordinated by the EDEN project[12]. CREDO has until now already greatly contributed to improving the knowledge about endocrine disruption.

The projects funded under FP5 focused on a number of issues such as hazard and risk characterisation of various groups of endocrine disrupters; epidemiological approaches to exposure assessment including the use of biomarkers and birth cohorts; development of new methods and tests for analysis of toxicity; the role of genetic susceptibility in disease development; and investigation of mechanisms of disease development in various organs in 'real life' exposure situations (i.e., with low doses and multiple exposures). Endocrine-related reproductive effects were also widely studied, showing correlations especially between exposure to various chemicals and reproductive parameters in a variety of animal models and also in human studies.

Furthermore, the projects observed neurobehavioural effects of some chemicals. In particular, elevated polychlorinated biphenyl (PCB) serum concentration was correlated with poor sensomotor function in children. Finally, work on multiple effects of endocrine disrupters gave contradictory results with some research demonstrating no morphological effects, while others found effects, elaborated mechanisms and proposed risk assessment for non-reproductive organs in humans.

The Sixth Research Framework Programme (FP6, 2002-2006), addressed different topics related to endocrine disruption. By the end of FP6, 10 projects with at least some relevance to the area of endocrine disrupters were funded, with EC contributions of around 50 million EUR in total. The adoption of the European Environment and Health Action Plan in 2004 has served as a stimulus for research, as one of the goals of this Action Plan is to understand health impacts of endocrine disrupters[13] in view of co-ordinating risk reduction measures, which is one of the recommendations of this Action Plan. The recently published mid-term review[14] of the Action Plan has acknowledged significant progress in better identification of the mechanisms for co-ordinating risk reduction measures. It is, however, recognized that "more work needs to be done in linking research on priority diseases to appropriate policy processes and information systems".

The projects supported under FP6 focus on the studies of health outcomes after exposure to chemicals with potential neuroimmune or endocrine mediated effects or develop test methods for their detection. Some projects have also addressed the issue in a context of integrated environment and health risk assessments.
The integrated project REPROTECT[15], led by the Joint Research Centre (JRC) with 32 participating European groups, began in July 2004 with an EC contribution of 9.1 million EUR, and a total budget of 13.2 million EUR over 5 years. The aim is to validate a conceptual framework in the area of reproductive toxicity and to develop a substantial number of alternative test methods making use of advanced technologies. Within this project, six tests for assessing (anti)estrogenic and (anti)androgenic compounds have been optimised and are now being analysed for predictive power. Two of these tests are continuing validation under the auspices of the OECD. These in vitro tests could contribute to substantially reduce the number of animals that are currently required in reproductive toxicity testing. Furthermore, such methods have discrete advantages that go beyond reduced speed, cost and animal use, such as the possibility to use human cells and receptors.

[11] http://www.credocluster.info/
[12] http://www.edenresearch.info

[13] http://ec.europa.eu/environment/health/index_en.htm

[14] http://eur-lex.europa.eu/LexUriServ/site/en/com/2007/com2007_0314en01.pdf
[15] http://www.reprotect.eu

The CASCADE project has started in 2004 with an EC contribution of 14.4 million EUR during five years[16]. CASCADE brings 24 research groups from nine EU member states together in a network for durable coordination and integration of research on chemical residues in food, especially chemicals with endocrine disrupting properties.

In addition, the following projects are of relevance to the field of endocrine disruption: NEWGENERIS[17] focusing on the role of exposure to genotoxic chemicals (including endocrine disrupters) in the development of childhood cancer and immune disorders, PHIME[18] focusing on public health impact of long-term, lowlevel mixed element exposure in susceptible population strata, BIOCOP[19] working on new technologies to screen multiple chemical contaminants in food and NOMIRACLE[20] developing novel methods and tools to better evaluate chemical risks. INTARESE[21] aims at producing a new integrated risk assessment framework, based on the full chain approach (causal chain spanning sources of pollution, releases into various media, dispersion and transport, exposure medium inhalation/dermal contact/ingestion, intake, uptake, dose, health effects and impacts). It includes household chemicals. Another two integrated projects, HEIMTSA and 2-FUN, which started in 2007, aim at further enhancing the full chain methodology and extending it to cover monetary valuation of health impacts from environmental stressors, including endocrine disrupting chemicals, and the health impacts of combined exposure to them and other stressors. These projects are expected to develop computational tools for assessing the health impacts of Community policies linked to chemicals such as endocrine disrupters. Moreover, eight "Specific Targeted Research Projects" with EC contributions between 1-5 million EUR and a duration of up to four years, as well as a few Coordination Actions/Specific Support Actions were financed.

The projects funded by FP6 related to endocrine disrupters are listed in the annex of the mid-term review report of the Environment and Health Action Plan published in June 2007[22]. Funding of research on endocrine disrupters will continue in the Seventh Research Framework Programme (FP7, 2007–2013). Theme 6 'Environment' as an example, will include an "Environment and Health" activity which will fund, among others, research related to health impacts of chemicals including endocrine disrupters. In addition, the JRC has been developing toxicogenomic capabilities for studying the biological basis underlying endocrine disruption in the context of its work on human exposure to environmental stressors and health effects. The JRC also held an expert workshop on molecular modelling approaches for human hazard assessment of chemicals (2006) using endocrine disruption and metabolism as case studies (report in preparation). This workshop identified promising in silico approaches for ED hazard assessment testing batteries which are now being followed up in the JRC led validation programme.

A webpage on endocrine disrupter-related research was created by DG Research[23] and the JRC[24] will soon host the database on endocrine disrupting chemicals developed under the DG ENV's contracted studies (part 2.1.3. above).

[16] www.cascadenet.org
[17] www.newgeneris.org

[18] www.phime.org

[19] www.biocop.org

[20] http://nomiracle.jrc.it/default.aspx

[21] www.intarese.org

[22] http://eur-lex.europa.eu/LexUriServ/site/en/com/2007/com2007_0314en01.pdf

[23] http://ec.europa.eu/research/endocrine/index_en.html

[24] http://ecb.jrc.it

4. PROGRESS ON LONG-TERM ACTIONS

The long-term actions include the review and adaptation of existing legislation, governing testing, assessment and use of chemicals and substances within the EU.

4.1. Legislative actions

4.1.1. Regulation (EC) N° 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)

The Regulation concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)[25] was formally adopted on 18 December 2006 by the Council of Environment Ministers following the vote in second reading of the European Parliament on 13 December 2006. REACH has entered into force on 1 June 2007.

One of the key elements of REACH is an authorisation procedure for substances of very high concern. Substances of very high concern include those that are carcinogenic, mutagenic or toxic to reproduction (CMRs), categories 1 and 2. Furthermore, substances meeting the regulation's criteria for being persistent, bioaccumulative and toxic (PBTs) or very persistent and very bioaccumulative (vPvBs) are also of very high concern. In addition, substances that are not covered by the CMR-, PBT- or vPvB- criteria can be identified on a case-by-case basis when they are causing serious and irreversible effects to humans or the environment, which are equivalent to those of the CMRs, PBTs and vPvBs. These substances are then considered to be "of equivalent concern" and can also be subject to authorisation as far as they are proposed to be put on the "candidate list" for inclusion in Annex XIV of REACH and prioritised for authorisation. Substances of "equivalent concern" include those having endocrine disrupting properties. The identification of the substances of very high concern starts on the initiative of the Commission or Member States and leads to the establishment of the candidate list. The "endocrine disrupter priority list" could be used as a reference material for this process. A guidance document on how Member States should choose and justify their proposals for substances of equivalent concern is currently being prepared by the European Chemicals Bureau[26].

The authorisation procedure requires the Commission to give specific permission before a substance subject to authorisation could be used for a particular purpose, marketed as such or as a component of a product. Given that many of the serious human health effects which have so far been associated with endocrine disrupting chemicals are testicular cancer, breast cancer, prostate cancer, decrease in sperm quality, cryptorchidism and hypospadia, it is likely that many substances will fall under this authorisation procedure directly as a CMR substance. Furthermore, adverse effects on the endocrine system of wildlife species have been causally linked o certain persistent, bioaccumulative and toxic substances. Such substances would be subject to authorisation as a result of their PBT-properties. It should be noted that many of the priority substances are pesticides and by-products formed, for example, during combustion, and therefore they are not within the scope of REACH. The number of substances out of the endocrine disrupter priority list that could be expected to be identified, on a case-by-case basis, as substances of "equivalent concern" as defined in REACH, might potentially be a few dozen.

[25] Regulation concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals, establishing a European Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) N° 793/93 and Commission Regulation (EC) N°1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEX, 93/67/EEC and 2000/21/EC

[26] RIP-4.3: Guidance document on Inclusion of Substances in Annex XIV

4.1.2. Directive 2000/60/EC establishing a framework for Community action in the field of water policy (Water Framework Directive) and Directive 2006/118/EC on the protection of groundwater against pollution and deterioration

The **Water Framework Directive** (WFD) sets environmental objectives of good chemical status for surface waters and for the prevention of pollution of groundwater. For **surface waters**, the Directive provides for a two-tiered approach to control chemical pollution, which includes actions at national level and EU wide action. At the ***national level***, Member States are required to identify chemical pollutants of significance for each of the water bodies (an indicative list of the main pollutants is included in the Annex VIII of the Directive), to set quality standards for the water, to establish emission control measures and to achieve these standards by 2015. A specific category includes those *"substances and preparations, or the breakdown products of such, which have proved to possess carcinogenic or mutagenic properties which may affect steroidgenic, thyroid, reproduction or other endocrinerelated functions in or via the aquatic environment"* (Annex VIII – Group 4). This means that there is an obligation for Member States to take action to prevent human exposure of endocrine disrupting substances via the aquatic environment. This action shall be coordinated in river basins, and a programme of measures shall be in place in 2009 and become operational in 2012.

At the ***Community level***, the WFD sets out a strategy against pollution of surface waters by chemical pollutants (Article 16). This strategy includes the identification of substances of particular concern at Community level, and the adoption of environmental quality standards and emission controls for such substances. The first list of 33 substances was adopted in 2001 (Decision 2455/2001/EC), and the Commission adopted a proposed Directive (COM (2006)397) setting environmental quality standards for these substances in 2006, as well as a related Communication (COM (2006)398). It should be noted that, out of these 33 substances, 21 are candidate endocrine disruptor substances for which an evidence or potential evidence of endocrine disrupting effects was found during the priority setting process in the years 2000–2003.
The list of substances is to be reviewed every four years, and as further knowledge regarding endocrine disrupting properties is gathered, this information could be taken into account in the future prioritisation of substances for action at Community level. From the first priority list, certain substances can also be classified as "priority hazardous" and should be subject to complete phase-out of all emissions, losses and discharges during a 20-year timeframe. Endocrine disrupting effects could become an important factor for sorting substances or groups of substances into this group.

Regarding groundwater, the new **Groundwater Directive** (developed under Article 17 of the WFD) was adopted on 12 December 2006 (Directive 2006/118/EC). It requests Member States to establish threshold values (groundwater standards for defining the groundwater good chemical status) for all pollutants representing a risk to groundwater, taking a minimum list of pollutants (Annex III of the directive) into consideration. Although endocrine disrupters are not explicitly listed, in principle, they could be covered by this clause if Member States identified them and considered that they could represent a risk for the pollution of groundwater. This proposal also includes the requirement to identify and reverse a significant increasing trend in pollutant concentrations, which would implicitly cover endocrine disrupters if they have been identified as representing a pollution risk.

Regarding prevention, direct and indirect inputs of pollutants are regulated both by the Water Framework Directive and the Groundwater Directive proposal (Article 6 of the proposal), thus ensuring a continuity of the protection regime of Directive 80/68/EEC which will be repealed in 2013. Along this principle, inputs of hazardous substances have to be prevented, while inputs of non-hazardous pollutants have to be limited to avoid groundwater pollution.

4.1.3. Directive 91/414/EEC concerning the placing of plant protection products on the market

Directive 91/414/EEC sets out a Community harmonised framework for authorisation, use and control of plant protection products. In 1992, the European Commission started a Community-wide review process for all active ingredients used in plant protection products within the European Union. The review should make sure that active substances can be used safely regarding human health, the environment, ecotoxicology and residues in the food chain. It will be completed in 2008.
The issue of possible endocrine disrupting properties of active substances used in plant protection products is not yet fully incorporated in the risk assessment procedures because of lack of harmonised and internationally-agreed test protocols. But the competent authorities in the EU have identified this gap and have highlighted the need for a test procedure which could confirm whether or not "identified candidates" are real endocrine disrupting substances. Work on this matter is ongoing at the OECD and it is foreseen that as soon as agreed test methodologies are endorsed, these would be integrated into the assessment procedures applied in the Community risk assessment. In the meantime, where substances are currently being evaluated and where there is a suspicion of endocrine disrupting potential of a substance, additional testing has been requested and performed, and the results assessed. Several substances have so far been tested according to a specific protocol called "fish full life cycle test". The results of these tests have allowed competent authorities to resolve doubts about those substances. Apart from technical aspects that are expected to be solved in the near future, consideration of endocrine disrupting properties of active substances would receive greater priority in decision-making. The new proposal for a regulation revising Directive 91/414/EEC, which was adopted by the Commission in July 2006, includes provisions that prohibit the use of active substances that have been identified as endocrine disruptors, unless the exposure of humans to the active substance in a plant protection product, under realistic proposed conditions of use, is negligible.

4.1.4. Directive 98/8/EC concerning the placing of biocidal products on the market

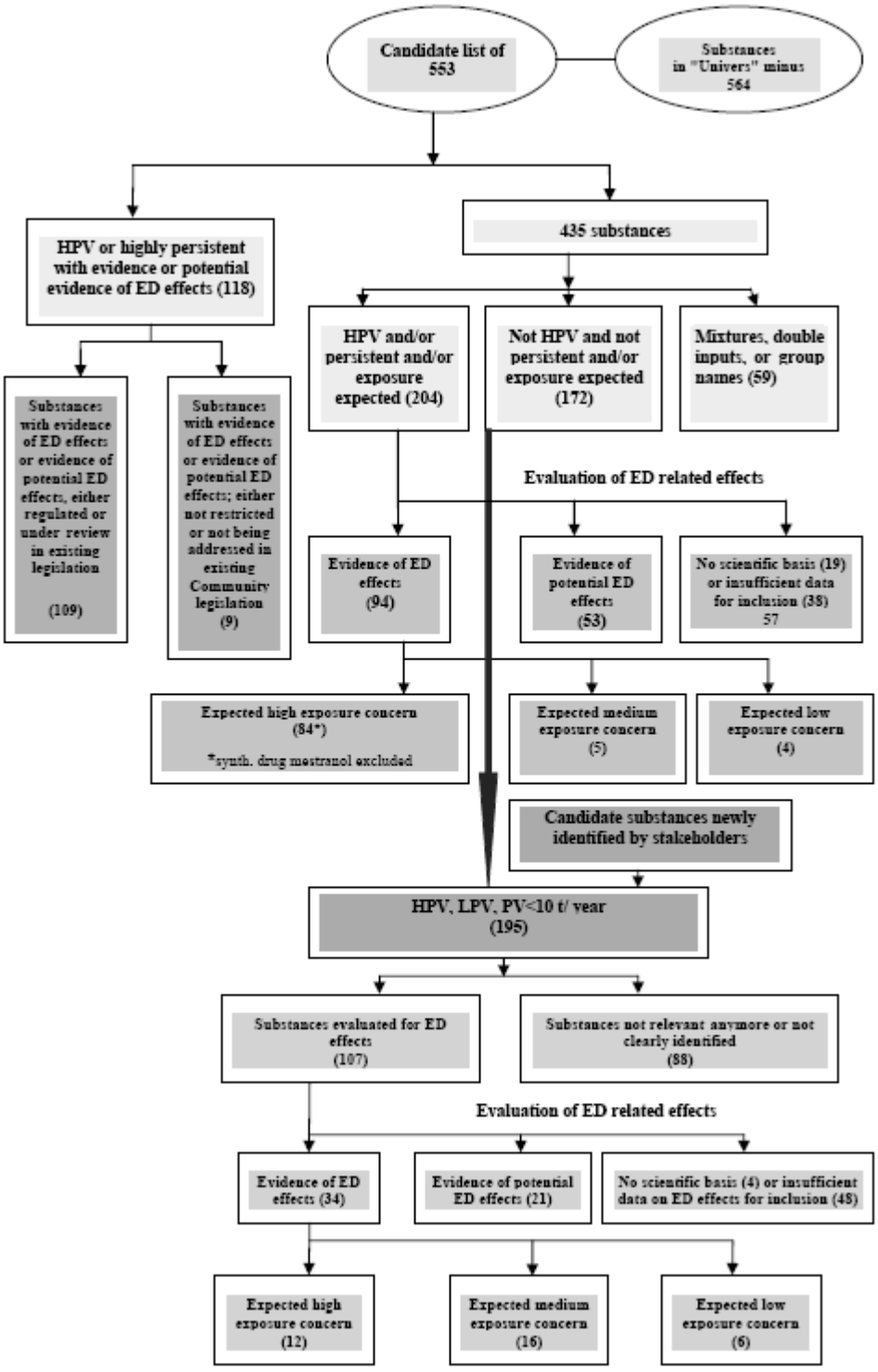
Within this Directive, active substances are being evaluated and authorised for biocidal applications after the risk for man and environment has been assessed. This assessment also takes into consideration the potential adverse effects that rise from exposure to endocrine disrupting chemicals. Although a specific evaluation strategy for the assessment of endocrine disrupting effects is not explicitly described within this Directive, these adverse effects are taken into account when assessing risks associated with the use of the substances. These aspects are also taken into account in the specific "PBT Working Group" assessing the persistence, bioaccumulative and toxic properties of biocides. Like all other legislative actions, also Directive 98/8/EC will benefit from the final acceptance of the standardized testing methods at the OECD level.

4.1.5. Directive 96/22/EC concerning the prohibition on the use in stock-farming of certain substances having a hormonal or thyrostatic action and beta-agonists

The use of substances having an oestrogenic, gestagenic or androgenic effect is restricted under Directive 96/22/EC concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and betaagonists, as amended by Directive 2003/74/EC. The Directive prohibits the use of substances having a hormonal action for growth promotion in farm animals and identifies precise circumstances under which they may be administered to food
producing animals for other purposes. After 14 October 2006, i.e., the date of expiry of the transitional period established in Article 5a, oestradiol 17β or its ester-like derivatives may no longer be used for oestrus induction in cattle, horses, sheep or goats.

Attachment 1: Establishment of the priority list

Figure 1: Establishment of the priority list of substances for further evaluation of their endocrine disrupting (ED) effects



Attachment 2: Grouping of substances evaluated during DHI – Study 2005/2006

Table 1: Substances with evidence or potential evidence of ED effects which are neither restricted nor currently being addressed under existing Community legislation (**27 substances**)[1]

Group name	CAS Number	Substance	Status under Dir 76/769/EEC28 or adaptations to technical progress[2]	Status under Reg 793/93/EEC[3]	Status of review under Dir 91/414/EEC[4]	Other Risk Assessment Instruments	Other Risk Management Instruments	Other Hazard Identification Instruments
Alkylphenols and derivatives	99-71-8	4-sec-Butylphenol = 4-(1-Methylpropyl)phenol						
	1131-60-8 4-	Cyclohexylphenol						
	3115-49-9 4-	Nonylphenoxyaceticacid						
	27193-28-8	Phenol(1,1,3,3-tetramethylbutyl) octylphenol[5]						
Benzophenones	131-55-5	Benzophenone-2,(Bp-2), 2,2',4,4'-tetrahydroxybenzo-phenone						

[1] These substances have not been specifically evaluated in the past because they were considered as having a low priority due to their low production volumes and the fact that, at that point in time, there was no toxicological knowledge on their possible endocrine disrupting effects available.

[2] Directive 76/769/EEC relating to restrictions on marketing and use of certain dangerous substances and preparations, or adaptations to technical progress (ATP) of Dir 76/769/EEC

[3] Regulation (EEC) No.793/93 on the evaluation and control of the risks of existing substances

[4] Directive 91/414/EEC concerning the placing of plant protection products on the market

[5] Despite the fact that this substance is not a priority substance under Reg. 793/93/EC, UK has put this substances on its national priority list because of its similarity to nonylphenol

Group name	CAS Number	Substance	Status under Dir 76/769/EEC28 or adaptations to technical progress	Status under Reg 793/93/EEC	Status of review under Dir 91/414/EEC	Other Risk Assessment Instruments	Other Risk Management Instruments	Other Hazard Identification Instruments
	131-54-4	2,2'-Dihydroxy-4,4'-dimethoxybenzophenon						
Bisphenols	77-40-7 2,2-	Bis(4-hydroxyphenyl)-nbutan= Bisphenol B						
	92-69-3	4-Hydroxybiphenyl = 4-Phenylphenol						
	1806-29-7	2,2'-Dihydroxybiphenyl = 2,2'-Biphenol						
Camphor 3-derivatives	15087-24-8	Benzylidene camphor (3-BC)						
	36861-47-9	3-(4-Methylbenzylidene)-camphor						
Coumaric acid and derivatives	7400-08-0	p-Coumaric acid (PCA)						
	5466-77-3	2-ethyl-hexyl-4-methoxycinnamate						
DDT derivatives and metabolites	83-05-6	p,p'-DDA						
Flavonoids	491-80-5	Biochanin A						
	84-69-5	Diisobutylphthalate						
	4376-20-9	Mono 2 ethyl hexylphthalate						

		(MEHP)						
	131-70-4	Mono-n-butylphthalate						
	131-16-8	Di-n-propylphthalate (DprP) = Dipropylphthalate						
	84-75-3	Di-n-hexylphthalate (DnHP) = Dihexylphthalate (DHP)						
Group name	**CAS Number**	**Substance**	**Status under Dir 76/769/EEC28 or adaptations to technical progress**	**Status under Reg 793/93/EEC**	**Status of review under Dir 91/414/EEC**	**Other Risk Assessment Instruments**	**Other Risk Management Instruments**	**Other Hazard Identification Instruments**
Siloxans	33204-76-1	2,6-cis-Diphenylhexamethylcyclotetrasiloxane - 2,6-cis-[(PhMeSiO)2(Me2SiO)2]						
Other substances	77-09-8	3,3'-Bis(4-hydroxyphenyl)-phthalid = Phenolphthaleine						
	81-92-5	2-[Bis(4-hydroxyphenyl)-methyl]-benzylalkohol = Phenolphthalol						
	14007-30-8	2,2-Bis(4-hydroxyphenyl)-nhexane						
	2581-34-2	3-methyl-4-nitrophenol						
	50-18-0	Cyclophosphamide						
	96-12-8	Dibromochloro-propane (DBCP)						

Table 2: Substances with evidence of ED effects (Category 1), which are already regulated or being addressed under existing legislation (20 substances)

Group name	CAS Number	Substance	Exposure concern	Status under Dir 76/769/EEC28 or adaptations to technical progress	Status under Reg 793/93/EEC	Status of review under Dir 91/414/EEC	Other Risk Assessment Instruments	Other Risk Management Instruments	Other Hazard Identification Instruments
Alkylphenols and derivatives	104-40-5 4-	Nonylphenol (4-NP)	Medium	Dir 2003/53/EC 26th ATP. Dir 76/769/EEC (Restriction)					
Alkylphenol ethoxylates	20427-84-3 4-	Nonylphenoldiethoxylate (NP2EO)		Dir 2003/53/EC 26th ATP. Dir 76/769/EEC (Restriction)					
Benzenic acid and derivatives	94-26-8	n-Butyl p-hydroxybenzoate	Medium					Com Dec 1999/217/EC[6]	
	94-13-3	n-Propyl-phydroxybenzoate	Medium					Dir 2002/72/EC[7]	
	99-76-3	Methyl p-hydroxybenzoate	Medium					Dir 2002/72/EC; Dir 95/2/EC	
	120-47-8	Ethyl-4-hydroxy-benzoate	Medium					Com Dec 1999/217/EC Dir 2002/72/EC;	

[6] Commission Decision 1999/217/EC adopting a register of flavouring substances used in or on foodstuffs drawn up in application of Regulation (EC) No 2232/96.

[7] Directive 2002/72/EC relating to plastic materials and articles intended to come into contact with foodstuffs

Group name	CAS Number	Substance	Exposure concern	Status under Dir 76/769/EEC28 or adaptations to technical progress	Status under Reg 793/93/EEC	Status of review under Dir 91/414/EEC	Other Risk Assessment Instruments	Other Risk Management Instruments	Other Hazard Identification Instruments
	99-96-7	p-Hydroxybenzoic acid	Medium				Dir 2002/72/EC		
Benzo-phenones	131-56-6	2,4-Dihydroxybenzophenon = Resbenzophenone	High				Dir 2002/72/EC		
	611-99-4	4,4'-Dihydroxybenzophenon	Medium				Dir 2002/72/EC		
Biphenol	92-88-6	4,4'-Dihydroxy-biphenyl = 4,4'-Biphenol	High				Dir 2002/72/EC		
Organo-phosphor pesticides	1113-02-6	Omethoate	Low		Phased out in 2003 (Regulation (EC) 2076/2002)	Omethoate is a metabolite of dimethoate. Risk for consumer assessed when setting pesticider residues MRLs			
Organothio phosphor pesticides	13593-03-8	Quinalphos = Chinalphos	Medium		Phased out in 2003 (Regulation (EC) 2076/2002)				
Phthalates	131-18-0	Di-n-pentylphthalate (DPP) = Dipentylphthalate	Medium						Dir 67/548/EEC Annex I

Siloxans	556-67-2	Cyclotetrasiloxane	High						Dir 67/548/EEC ,Annex I
Group name	**CAS Number**	**Substance**	**Exposure concern**	**Status under Dir 76/769/EEC28 or adaptations to technical progress**	**Status under Reg 793/93/EEC**	**Status of review under Dir 91/414/EEC**	**Other Risk Assessment Instruments**	**Other Risk Management Instruments**	**Other Hazard Identification Instruments**
Other substances	25013-16-5	tert.-Butylhydroxy-anisole (BHA)	High				Dir 2002/72/EC; Dir 95/2/EC[8]		
	1634-04-4	Methyl-tert-butyl ether (MTBE)	Medium		Priority substance covered by Reg. (EC) 143/97				
	10043-35-3	Boric Acid	Medium		Priority Substance covered by Reg. (EC) 2364/2000	Covered by Dir 91/414; phased out in 2004 by Decision 129/2004		Dir 2002/72/EC; Dir 95/2/EC	
Other pesticides	6164-98-3	Chlordimeform	Low						PIC substance
	1582-09-8	Trifluralin	High			SCFCAH voted in March 2007 in favour of COM proposal not to authorise the substance;			

[8] Directive 95/2/EC of the European Parliament and of the Council of 20 February 1995 on food additives other than colours and sweeteners

						publication of decision is Pending			
Group name	**CAS Number**	**Substance**	**Exposure concern**	**Status under Dir 76/769/EEC28 or adaptations to technical progress**	**Status under Reg 793/93/EEC**	**Status of review under Dir 91/414/EEC**	**Other Risk Assessment Instruments**	**Other Risk Management Instruments**	**Other Hazard Identification Instruments**
	96-45-7	Ethylene Thiourea (ETU)	Low			ETU is a metabolite of some dithiocarbamates. Risk for consumer assessed hen setting pesticide residues MRLs			

Table 3: Substances with potential evidence of ED effects (Category 2), which are already regulated or being addressed under existing legislation (9 substances)

Group name	CAS Number	Substance	Status under Dir 76/769/EEC or adaptations to technical progress	Status under Reg 793/93/EEC	Status of review under Dir 91/414/EEC	Other Risk Assessment Instruments	Other Risk Management Instruments	Hazard Identification Instruments
Alkylphenols and derivatives	106-44-5	p-cresol					Dir 2002/72/EC Com Dec 1999/217/EC	Dir 67/548/EEC, Annex I
Bisphenols	6807-17-6	2,2-Bis(4-hydroxyphenyl)-4-methyl-n-pentane						Dir 67/548/EEC, Annex I
Benzophenons	131-57-7	2-hydroxy-4-methoxybenzophenone					Dir 2002/72/EC	
Nitrophenols	100-02-7	4-nitrophenol						Dir 67/548/EEC, Annex I
Organophosphor pesticides	2597-03- 7	Elsan = Dimephenthoate						Dir 67/548/EEC, Annex I
PCBs	2051-60-7	PCB 1 (2-Chlorobiphenyl)					Reg 850/2004/EEC[9]	
	2051-61-8	PCB 2 (3-Chlorobiphenyl)					Reg 850/2004/EEC	
	2051-62-9	PCB 3 (4-Chlorobiphenyl)					Reg 850/2004/EEC	
Pyrethrins	121-29-9	Pyrethrin			covered by Dir91/414;			Dir 67/548/EEC,

[9] Regulation (EC) N° 850/2004/EEC of the European Parliament and of the Council of 29 April 2004 on persistent organic pollutants and amending Directive 79/117/EEC

								Annex I

Table 4: Substances with no or insufficient data on ED effects gathered (Category 3a and 3b), for which there is currently no support for their inclusion in the priority list (48 substances)

Group name	CAS Number	Substance
Alkylphenols and derivatives	87-26-3	2-sec-Pentylphenol = 2-(1-Methylbutyl)phenol
	27214-47-7	Phenol, 4-sec-octyl-
	26401-75-2	Phenol, 2-sec-octyl-
	18626-98-7	Phenol, 2-(1-methylheptyl)-
	1818-08-2	Phenol, 4-(1-methylheptyl)-
	17404-44-3	Phenol, 2-(1-ethylhexyl)-
	37631-10-0	Phenol, 2-(1- propylpentyl)-
	3307-01-5	Phenol, 4-(1-propylpentyl)-
	949-13-3	Phenol, 2-octyl-
	27985-70-2	Phenol, (1-methylheptyl)-
	3884-95-5	Phenol, 2-(1,1,3,3-tetramethylbutyl)-
	3307-00-4	Phenol, 4-(1-ethylhexyl)-
	1322-97-0	Ethanol, 2-(octylphenoxy)- = Octylphenolethoxylate
	27986-36-3	Ethanol, 2-(nonylphenoxy)-
	1009-11-6	4-Hydroxy-n-butyrophenone
	70-70-2	Hydroxypropiophenone
	628-17-3	4-vinylphenol (4-VP)
	7786-61-0	4-4-vinylguaiacol (4-VG)2

Group name	CAS Number	Substance
Phenylhydroxyphenylmethanes	28994-41-4	Phenyl-2-hydroxyphenylmethane = 2-Benzylphenol = o-Benzylphenol
Chlorphenole	25167-81-1	Dichlorophenol
Hexachlorocyclohexane and isomers	13171-00-1	4-Acetyl-1,1-dimethyl-6-tert.-butylindane
Naphthalene and derivatives	530-91-6	Tetrahydronaphthol-2
	15231-91-1	6-Bromo-2-naphthol
	1125-78-6	5,6,7,8-Tetrahydro-2-naphthol = 6-Hydroxytetralin
	90-15-3	1-Naphthol(*)
Bisphenols	3373-03-3	1,1-Bis(4-hydroxyphenyl)-n-heptane
	24362-98-9	1,1-Bis(4-hydroxyphenyl)-n-hexane
	620-92-8	Bis(4- hydroxyphenyl)methane
	52479-85-3	2,3,4,3',4',5'-Hexahydroxybenzophenon
Siloxanes	56-33-7	Diphenyltetramethyldisiloxane PhMe2-SiOSiMe2Ph
	10448-09-6	Phenylheptamethylcyclotetrasiloxane [(PhMeSiO)(Me2SiO)3]
Polycyclic Aromatic Hydrocarbons	53-96-3	n-2-Fluorenylacetamide
Methoxychlor and derivatives	14868-03-2	Bis-OH-MDDE
Organophosphor pesticides	682-80-4	Demefion
	2540-82-1	Formothion

	70393-85-0	Glufosinate-ammonium
Polychlorinated Biphenyls and biphenylethers	2050-68-2	PCB 15 (4,4'-Dichlorobiphenyl)(*)
Group name	**CAS Number**	**Substance**
Other substances	303-38-8	2,3-dihydroxybenzoicacid (2,3-DHBA)
	490-79-9	2,5-dihydroxybenzoicacid (2,5-DHBA)
	533-73-3	Hydroxyhydroquinone
	1222-05-5	1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta(g)-2-benzopyrane(*)
	33704-61-9	6,7-dihydro-1,1,2,3,3-pentamethyl-4(5H)indanone
	114369-43-6	Fenbuconazole
	118-56-9	3,3,5-trimethyl-cyclohexyl salicylate
	25550-58-7	Dinitrophenol(*)
	463-56-9	Thiocyanate(*)
	21245-02-3	2-ethyl-hexyl-4-dimethyl-aminobenzoate
	79-44-7	Dimethylcarbamylchloride(*)

(*) Some information and legislative coverage for these substances is already achieved, but there is no data available in relation to endocrine disrupting effects that would support their inclusion into the priority list

Table 5: Substances which are deemed not to be endocrine disrupters, on the basis of available information (4 substances)

Group name	CAS Number	Substance
Alkylbenzenes and Styrenes	104-51-8	n-Butylbenzene
Pyrimidines and Pyridines	314-40-9	Bromacil
Other substances	537-98-4	Ferulic acid (FA)
	545-55-1	TEPA

Attachment 3: Overview of the ED test methods currently in (pre)validation under the auspices of the OECD.

Receptor Binding Assays				
hrERα	The FWA assay protocol utilizes the Pan Vera hrERα full length ER, and the CERI protocol utilizes the CERI-ERα, which contains the ligand binding domain of hrERα.	Binding	Validation starting in February 2007 in 5 labs	US lead international Collaboration study
hrAR	Human recombinant AR assay. Ligand binding domain expressed in E. coli.	binding	Under development. Approximately 900 chemicals have been tested.	METI Japan
hrAR	Human recombinant AR assay.	binding	Prevalidation starting now (initially as part of ReProTect.)	EC:ECVAM Bayer Lead International collaboration study
	Human recombinant TR assay. Full-	binding	Under development.	METI Japan

hrTR	length expressed in E. coli. TRs α1 and β1 binding assays.		Approximately 60 chemicals have been tested using both receptors.	
Transcriptional Activation Assays				
ERα	HeLa-9903 cells with plasmids containing hERα cDNA driven by SV40 promotor and luciferase reporter plasmid.	Stable, ag/antag	The agonist assay was peer reviewed in March- 07. International validation of the antagonist assay is planned for 2007.	CERI/MHLW Japan
ERα .	HeLa-9903 cells: hERα/pcDNA3.1 receptor expressing plasmid and EREAUG- Luc+ reporter plasmid	Transient, ag	Pre-validated and Validated under domestic multi-lab. Validation using same test chemicals as hERa-HeLa-9903 cell line. Should be considered for (preliminary) Peer review.	CERI/MHLW Japan
ERα	MELN. MCF-7 cells with endogenous ERα + luciferase stably transfected	ag/antag	Validation in 2007. Report in late 2007 or early 2008	EC/ECVAM
ERα	ER-CALUX. T47 D (human breast cancer) cells with endogenous ERα + luciferase stably transfected	ag/antag	Going through optimisation. Validation planned for 2008	EC/ECVAM
ERα	LUMI cell, BG1 cells with endogenous ERα + luciferase stably transfected (XDS Inc)	ag/antag	Validation will be initiated in late 2007, done by late 2008. In delay.	US lead (ICCVAM) international collaboration study with ECVAM and JaCVAM
ERβ	HeLa,hERß/pcDNA3.1,ERE-AUG-Luc+	Transient, ag	Completed data collection for 250 compounds	CERI/MHLW Japan

AR	CV-1 cells hAR/pcDNA3.1 receptor expressing plasmid and AREAUG- Luc+ reporter plasmid	Transient, ag/antag	Pre-validated and validated in Japan in 4 labs, with 5 chemicals. Should be considered for (preliminary) Peer review.	CERI/MHLW Japan
AR	AR-Ecoscreen™ stable CHO clone	Stable, ag/antag	Pre-validated and validated in Japan in 4 labs, with 5 chemicals. should be considered for (preliminary) Peer review	CERI/MHLW Japan
AR	PALM. PC-3 (prostate adenocarcinoma) cells stably transfected with hAR and luciferase reporter gene	ag/antag	Validation in 2007	EC/ECVAM
AR	CALUX. U2-OS (bone cell) cells stably transfected with hAR and luciferase reporter construct	ag/antag	Validation in 2007	EC/ECVAM
TRβ	RXR co-transfected CHO cells are used	Transient, ag/antag	Under development, 150 chemicals tested so far	MHLW Japan
Aromatase & Steroidogenesis Assays				
	Aromatase, KGN cells.		Prevalidated	
	Steroidogenesis, H295R		Validation starts March 2007.	US lead international collaboration study

Appendix 3

Contribution from France

1. French regulatory authorities do not currently assess the endocrine disrupters properties yet within any national chemicals regulation. However, France has two main government programmes to assess impact of general environmental factors on workers and general population. These programmes, the so-called PNSE (National Environment and Health action Plan) and PST (Occupational and Health action Plan), are objective programs, which determine the main research axis in a defined time-scale.

2. PNSE (2004-2008)[1] cover the entire field of relationships between the environment and human health. One of the three major goals of this programme is to prevent environmental exposure associated diseases. Among the 45 actions, 2 actions concern the endocrine disrupter as action 11 to limited water and soils pollution caused by pesticides and other potentially dangerous substances including endocrine disrupters compounds and action 34 to reinforce and coordinate the calls for research projects to improve knowledge on the presence and effects of several kind of compounds (endocrine disrupters compounds are included) to facilitate public decision.

3. PST (2005-2009) is specifically dedicated to workers and completes PNSE. PST aims to improve professional risk prevention. Objective 1 of PST is to develop knowledge in professional domain, to coordinate the use of the means assigned to the research finalized in support with the public policies and coordinate the calls for research projects to improve knowledge on the presence and effects of several kinds of compounds to facilitate public decision (i.e. endocrine disrupters compounds, specifically the reprotoxic ones). Objective 4, according to REACH European Regulation, promotes the principle of substitution of the most dangerous chemical substances: CMR, PBT, vPvB and endocrine disrupters.
   The programmes described below are included in these frameworks.

Governmental Programme, Several classes of Chemicals: Industrial Existing Chemicals and Pesticides.

4. According to the previously cited programmes, the French Ministry of Ecology and Sustainable Development (MEEDDAT) develops a programme specifically directed on the assessment of endocrine disrupters compounds: PNRPE.

5. The National Research Program into Endocrine Disrupters (PNRPE) was proposed by the Minister and launched in 2004 following the recommendations of the Prevention and Precaution Committee (CPP) based on an enquiry into endocrine disrupters (conclusions published in December 2003). This program aims to answer calls from public authorities and support fundamental and applied multidisciplinary research into screening methodologies, mechanisms for action, the search for effects biomarkers, the movement and transformation of endocrine disrupters in the organism and the environment, the identification of dangers, the evaluation of risks, surveillance and connected socioeconomic aspects. The first Call for Research Proposals was launched in May 2005 and resulted in the selection of 7 structural projects. Abstracts of these projects are only available in French at http://www.ecologie.gouv.fr/-Projets-retenus,1917-.html, and the restitution of results should occur during autumn 2008. A new Call for Research was launched in June 2008 to continue efforts and respond to questions raised by the previous 7 projects. Finally in the framework of REACH procedure, the installation of the PNRPE is an important tool in the evaluation of the toxicity of several chemical pollutants as endocrine disrupters.

[1] Summary available in English on Afsset website :

http://www.afsset.fr/upload/bibliotheque/422558914315638390228017937553/2004_2008_pnse_abstract_en_260707.pdf

Strategy under development, Industrial Existing Chemicals: Methodology to build Toxicological Reference Values (VTR) based on reproduction and development toxic effects.

6. In France, parallel to the great programs, there are specific questions coming from the ministries to the Health Agencies: the so-called "saisines". According to action 21 of PNSE ("to develop tools for better evaluating biological or chemical substances health hazards"), the French Agency for Environmental and Occupational Health Safety (AFSSET) has taken a "saisine" to evaluate some Glycol Ethers, and in this framework, in order to improve expertise in France in this domain, AFSSET had established a national programme on VTR based on reproduction and development toxic effects.

7. VTR is a value making it possible to establish a quantitative and/or qualitative relationship between an exposure to a chemical substance and an adverse health effect on human (also known as reference doses (RFD), or minimal risk level (MRL)). VTR are used to build qualitative aims of the mediums of the environment, guidelines values, recommendations or standards.

8. AFSSET sets up an inter establishments task force gathering INERIS, INRS, Afssa, InVS, the ENSP, Cnam, Inserm, CNRS, the CAPs and UIC[2] in order to propose a reference methodology for the construction of VTR on reproduction and development for toxic substances and to identify which substances could be the priority in order to build such VTR. This concern, included in a European context, mainly aims at developing a critical analysis on the choice and the construction of VTR for the most alarming reprotoxic chemical substances. Two reports[3] has been published:

    - Identification of a list of toxic substances for reproduction and development, proposition of a hierarchy to analyze VTR, 2005.
    - Reference document to build a VTR based on reprotoxic effects, 2006.

9. Then, within the framework of the development method of construction of reprotoxic VTR, a pilot phase was carried out to "test" the methodology suggested by the task force on some model substances. For this purpose, AFSSET called upon several organizations of expertise to build, according to the reference document of the task force, the reprotoxic VTR of the following substances[4]:

    - Toluene (INERIS)
    - Ethylène Glycol Ethyl Ether ; EGEE (INERIS)
    - Linuron (Vincent Nedellec Consultant)
    - Benzyl butyl phthalate; BBP (Vincent Nedellec Consultant)
    - Nonylphenol (ESMISAB/UBO, Technopôle Brest-Iroise)
    - Di-n-butyl-phtalate ; DBP (ESMISAB/UBO, Technopôle Brest-Iroise)

10. This work, issued from a saisine in the framework of PNSE (action 21 and 24), details the development method of VTR based on reprotoxic effects, when well even a VTR numerically

[2] Afssa : Agence Française de Sécurité Sanitaire des Aliments ; CAP/TV : Centres Anti-poison et de Toxicovigilance ; Cnam : Conservatoire National des Arts et Métiers ; CNRS : Centre National de la Recherche Scientifique ; ENSP : Ecole Nationale de la Santé Publique ; Ineris : Institut National de l'environnement et des risques industriels ; INRS : Institut National de Recherche et de Sécurité ; Inserm : Institut National de la Santé et de la Recherche Médicale ; InVS : Institut de Veille Sanitaire ; UIC : Union des Industries Chimiques.

[3] Reports are available in French only, but a poster is available in English on AFSSET website : www.afsset.fr

[4] Reports are available in French only on AFSSET website : www.afsset.fr

weaker exists or could be developed on non-reprotoxic effects. The construction of a VTR for a specific effect reprotoxic is justified by:

- the description of effects on the development occurring for particular exposure times (short window of exposure);
- the need for having an answer specific to the reprotoxic effects

11. France is aware that reprotoxic compounds are not necessary endocrine disrupters; however, many of the substances tested interfere on endocrine-dependent system, which make them endocrine disrupters suspects.

12. In order to continue these topics, a new PNSE 2 is being established, and a new Reprotoxic VTR task force is being created.

13. In addition, some systems of surveillance concerning the interaction between reproduction and environment exist, with principally, a register (for congenital malformations, stillbirth…), an epidemiologic observatory of fertility in France or a record of health event (by CECOS which is the center of studies and conservation of eggs and sperm).

Appendix 4

Contribution from Germany

German contribution to the development of a Report on Endocrine Disrupters Assessment in OECD Member Countries

HUMAN HEALTH

This section will be provided later

ENVIRONMENT:

Legal Background

2.1.1 REACH

Article 57 (f) of EU Directive 1907/2006 REACH facilitates to submit endocrine disrupters to authorisation. For this, criteria and data necessary for proof are not specified in detail so far. In each individual case a decision is necessary whether the substance in question 'for which there is scientific evidence of probable serious effects to human health or the environment which give rise to an equivalent level of concern to those of other substances listed in the points (a) to (e)' (CMR, PBT, vPvB).

2.1.2 Biocides

Directive 98/8/EC Concerning the Placing of Biocidal Products on the Market addresses endocrine effects in article 38: 'In those cases where the test appropriate to hazard identification in relation to a particular potential effect of an active substance or a substance of concern present in a biocidal product has been conducted but the results have not led to classification of the biocidal product then risk characterisation in relation to that effect shall not be necessary unless there are other reasonable grounds for concern. Such grounds may derive from the properties and effects of any active substance or substance of concern in the biocidal product, in particular:

- any indications of bioaccumulation potential,
- the persistence characteristics,
- the shape of the toxicity/time curve in ecotoxicity testing,
- indications of other adverse effects on the basis of toxicity studies (e.g. classification as a mutagen),
- data on structurally analogous substances,
- endocrine effects'

Still the directive does not include special data requirements to clarify an endocrine mechanism. Therefore any request for studies on endocrine effects in the assessment of biocidal products - even with justified suspicion - is difficult.

2.1.3 Plant Production Products

The current Council Directive 91/414/EEC concerning the placing of plant protection products on the market makes not direct reference on endocrine disruption but indirectly via requirements of chronic studies such as a requirement of a fish full life cycle test in certain cases. In the guidance documents on aquatic testing and terrestrial testing endocrine disruption is addressed without a testing strategy.
The proposed EU regulation on the marketing of plant protection products however is supposed to address among others endocrine disruptors to be candidates for substitution.

2.1.4 Pharmaceuticals

EU Directive 2001/83/EC on the Community Code Relating to Medicinal Products for **Human Use** does not include any special assessment of endocrine effects. In the 'Guideline on the Environmental Risk Assessment of Medicinal Products for Human Use' (EMEA/CHMP/SWP/4447/00 dated 01.06.2006) includes a text passage that certain substances such as lipophilic compounds and potential endocrine

disrupters may need to be addressed irrespective of the quantity released into the environment (action value 10 ng/L predicted environmental concentration (PEC)).

In EU Directive 2001/82/EC on the Community Code Relating to Veterinary Medicinal Products hormonal effects are only directly addressed in Article 68 '1. Member States shall take all measures necessary to ensure that only persons empowered under their national legislation in force possess or have under their control veterinary medicinal products or substances which may be used as veterinary medicinal products that have anabolic, anti-infectious, anti-parasitic, anti-inflammatory, hormonal or psychotropic properties.
In 'Guideline on the Environmental Impact Assessment for Veterinary Medicinal Products (CVMP/VICH/592/98-final dated 30.06.2000) hormones are excluded from an extended environment assessment in phase I when only single animals are treated. In phase II (CVMP/VICH/790/03-final dated October 2004) endocrine disruption is dealt with as other mechanisms and is not mentioned separately.

Existing Concepts and developments

Concerning the environmental assessment endocrine disruption can generally be assessed on a risk basis..
Except for REACH no testing strategy for endocrine disruption in the regulatory substance assessment in Germany (EU) exist. A general screening for endocrine disruption is not intended in any legal framework and case-by-case decisions on preliminary indication are necessary. Depending on the legislative background it is possible to require the respective studies from applicants to assess endocrine effects.

To address this problem in Germany a workshop took place on 'Characterization of endocrine mediated effects in fish' with representatives of academia, industry and administration. The workshop discussed besides others the issues of
1) What constitutes for environmental relevant substances (e.g. PPP, industrial chemicals) a suspicion for endocrine disruption?
2) Weight of evidence: According to which rules a decision for a fish screening assay (FSA) is made?;
3) Function of FSA: Clarifying the mode of action (moa)? Use of results in regulatory decision making?
Definite test: (…)
6) Which test is suitable: full life cycle test (FLCT) or 2-generation-test (2-GT)?

The results were published as 'WORKSHOP-BERICHT Zur Entwicklung einer Prüfstrategie auf sexualendokrine Wirksamkeit einer chemischen Substanz bei Fischen' Christoph Schaefers • Tobias Frische • Hans-Christian Stolzenberg • Arnd Weyers • Sabine Zok • Thomas Knacker (page 229-233 in 'Umweltwissenschaften und Schadstoff-Forschung, Zeitschrift für Umweltchemie und Ökotoxikologie', Bd. 20, Nr. 3, August 2008). So far available in German only.

Funded by the German environmental research programme UFOPLAN, the R+D project 20467454/02 has been conducted to feed into German contributions to the OECD EDTA special activity of the Test Guidelines Programme. The project aimed specifically on further development and (pre)validation of a FLCT with the zebrafish (*Danio rerio*), with particular view on identifying most relevant endpoints, and including a conceptual approach on how the FLCT can be applied as a definitive test for determining the toxicity of endocrine disrupting chemicals in fish. To achieve this objective, (1) existing data on relevant fish tests from literature according to specific ED-related mode-of-actions had been compiled and evaluated, (2) data gaps had been identified that might require the performance of FLCTs with substances showing specific ED-related mode-of-actions; (3) three FLC (2-generation) studies had been conducted with trenbolone, flutamide, and tamoxifene as test substances. The final project report is expected to be published by end of 2008.

REACH

The detailed technical guidance documents to REACH give for the first time in a regulatory relevant document a concrete instructions that can mainly be found as appendix R.7.8-5 page 102 in part R.7b[5] of the guidance on information requirements and chemical safety assessment. The guidance document describes in principle a risk oriented procedure in three steps (1. *Preliminary indication*, 2. *Indication of specific endocrine modes of action in intact aquatic organisms*, 3. *Characterisation of longterm adverse effects*). The third step has a special role when a causal link between endocrine effect and adverse population relevant effects are the basis for bringing a substance in authorization according to art. 57 (f).

Biocides

No concepts exist so far. Decision on testing for endocrine disruption are made on a case-by-case study. Because of missing legal background for data requirements (see 2.1.2) in most cases no further studies can be required even with justified suspicion.

Plant Production Products

According to the legal background chronic studies can be required and therefore studies to verify an existing endocrine effect of a plant production product (PPP). Still no general testing strategy or screening for endocrine disrupters in the PPP assessment exists. Therefore decisions on testing for endocrine disruption are made on a case-by-case basis (see case study on DMI-Fungizide point 2.3) taking into account mechanisms of the active agents, chemical structure and others.
The status and concept so far developed to assess endocrine disruptors under the pesticide's regime is summarized in the paper "Identification, Characterization and Environmental Risk Assessment of Endocrine Mediated Adverse Effects of Plant Protection Products and their Active Ingredients in the German Authorization Procedure (Responsible authority: UBA, Federal Environment Agency)", attached as Attachment 1.

Pharmaceuticals

No concepts exist. Because of available data on mechanisms of the active agent endocrine effects are more easily detected and further testing can be demanded than according to the legal background (see 2.1.4)

Case Studies

During the last 2 – 3 years the endocrine effects of DMI-fungicides were assessed in the framework of the plant protection product authorization. From that work resulted a project report on 'Characterisation of endocrine mediated impacts on fish. Relevant parameters for the development of a new OECD test method and the application in regulatory environmental risk assessment'. The report is available in German only but an English abstract exist:

'Scope of the study was the collection and comparison of existing experience with fish full life cycle studies on endocrine disrupting chemicals to derive general conclusions about endpoint sensitivity and help reducing uncertainties in the assessment. Characteristic population relevant and indicative endpoints for relevant direct sexual endocrine Modes of Action (MoA) were identified and compared for sensitivity. The data base consisted of Full Life Cycle Tests (FLCT) and two-generation tests by the Fraunhofer IME and from literature. Based on the results, test data from the UBA regulatory data base were tried to classify. In the second part, biomarker data from Fish Screening Assays (FSA) were compared with the effect data from population relevant FLCT endpoints and assessed according to sensitivity and predictive potential to

[5] URL: http://reach.jrc.it/docs/guidance_document/information_requirements_r7b_en.pdf?vers=30_07_08

derive a proposal for a tiered test strategy for potentially endocrine disrupting chemicals. The most sensitive exposure period for interactions with the estrogen receptor (ER) is the sexual development phase. The most sensitive manifestation endpoint is the reduction of the fertilization rate, the sensitivity of juvenile growth and sex manifestation being very close. The vitellogenin (VTG) measurement in a FSA is comparably sensitive. When using it as a lower tier test, a false negative result is not expected. Androgen receptor-(AR-) interactions have to be differentiated in the agonistic and antagonistic MoA. For both, VTG is not always a powerful biomarker. Fecundity is the most sensitive population relevant endpoint for the investigated AR antagonist; the most sensitive indicative endpoint in the FSA and the two-generation test is the enhanced sexual steroid 11-keto-testosterone. Regarding the AR agonists, sexual development is the most sensitive exposure period, the sex ratio being the most sensitive endpoint. Sufficiently sensitive biomarkers were not identified. Sexual development is also the most sensitive exposure period for aromatase inhibitors, becoming manifest in different endpoints with close sensitivity: the shift towards males in sex ratio, growth retardation or fecundity. VTG reduction was sensitive in the definitive tests as well as in the FSA. The investigated fish species were principally comparably sensitive towards hormone receptor interaction. The manifestation of effects may differ, i.e., in Medaka and Fathead Minnow, ER agonists cause femalization, whereas Zebrafish are arrested in their male protogyne development phase; zebrafish seem to be more sensitive to masculinisation by aromatase inhibition, whereas Fathead minnow seems to be more sensitive in a reduction of juvenile growth. Whether a two-generation test should be performed instead of a FLCT depends on the relevance of maternal effect transfer and could not be clarified due to a lack of comparable data. For using a shortened test procedure instead of a FLCT or two-generation test, precise information about the MoA is necessary as well as evidence on whether the shortened protocol is appropriate to cover the most sensitive exposure period and the most sensitive population relevant endpoint. Partial life cycle tests, such as short-term reproduction tests, thus can never be appropriate. The Fish Sexual Development Test (FSDT) could be the adequate test for AR agonists and aromatase inhibitors. The Early Life Stage or Juvenile Growth Test can be used for DMI-fungicides for a sufficiently safe extrapolation to FLCT results to perform a preliminary regulatory risk assessment.'

Another expert report on the issue of the DMI-fungicides was commissioned by the 'Industrieverband Agrar – IVA (Industrial Association Agriculture) under the title 'Assessment of the safety of an extrapolation from growth data of Early Life Stage- and Juvenile Growth Tests (OECD 210, 204, 215) to the NOEC of Fish Full Life Cycle Tests in the risk assessment of DMI-fungicides'. The expert report is attached as Attachment 2 to this document.

GERMAN INDUSTRY

The Verband der Chemischen Industrie e. V. (VCI - the German chemical industry association) is engaged in the political and technical discussion on endocrine disruption. For the proposals of the VCI for testing strategies (toxicological and ecotoxicological) it is referred to the documentation in the 'VCI overview on endocrine active substances' which is published on the VCI website (http://www.vci.de/default2~rub~0~tma~0~cmd~shd~docnr~116782~nd~.htm).

VCI also participated at the German workshop on the 'Characterization of endocrine mediated effects in fish'.

Attachment 1

IDENTIFICATION, CHARACTERIZATION AND ENVIRONMENTAL RISK ASSESSMENT OF ENDOCRINE MEDIATED ADVERSE EFFECTS OF PLANT PROTECTION PRODUCTS AND THEIR ACTIVE INGREDIENTS IN THE GERMAN AUTHORIZATION PROCEDURE (RESPONSIBLE AUTHORITY: UBA, FEDERAL ENVIRONMENT AGENCY)

1. Basically, UBA considers the risk-based procedure as established by EU Directive 91/414/EEG also applicable to those plant protection products and their active ingredients which provoke adverse effects in non-target organisms by a disturbance of the endocrine system (so called "endocrine disrupters").
2. This perception is in line with the state of the science in ecotoxicology and compliant to e.g. the guidance document SANCO/4145/2000 associated to Directive 91/414/EEG: "Endocrine disruption is to be viewed as one of the many existing modes of action of chemicals and thus can be assessed in the normal conceptual framework. The environmental assessment is based on the ecological relevance of the observed effects, independently on the mechanisms of action responsible for such effects. Therefore, the general procedure for risk assessment can also be used for endocrine disrupters."
3. However, the availability of adequate (i.e. endocrine specific) strategies for the testing and assessment of endocrine mediated effects in non-target organisms including the respective biological test procedures is an essential pre-requisite for any reliable risk assessment. At present, internationally agreed endocrine specific testing and assessment strategies are lacking for all relevant groups of non-target organisms in order (i) to systematically identify potential endocrine disrupters, (ii) to mechanistically characterize endocrine mediated effects and (iii) to reliably derive thresholds of no-concern and regulatory acceptable concentrations, respectively for endocrine mediated adverse effects of ecological relevance. The development and international validation of endocrine specific testing procedures for the derivation of qualitative and mechanistic information (screening assays) as well as testing protocols for a quantitative assessment of endocrine mediated effects on apical endpoints (e.g. growth, reproduction, sexual development) are just underway (cf. OECD activities). Moreover, endocrine specific data requirements are not explicitly laid down in Directive 91/414/EEG and the respective national legislation, too.
4. This shortcomings cause serious concern whether endocrine mediated adverse effects in non-target organisms can be adequately assessed based on the standard data requirements according to Directive 91/414/EEG. This uncertainty is especially critical for those groups of organism where only limited knowledge about the significance and functioning of endocrine systems is at hand and/or for which adequate testing protocols are not existing (terrestrial and aquatic arthropods, terrestrial and aquatic mollusks, amphibian, birds).
5. As a consequence, an all-encompassing and systematic endocrine specific assessment of plant protection products and their active ingredients is not feasible today, and supposedly will be not in the near future, too. Extrapolation from toxicological and clinical data derived for human health risk assessment is also of restricted relevance when assessing endocrine effects in other non-target organisms.
6. Therefore, the assessment of endocrine mediated effects within the national authorization procedure for plant protection products is case-by-case and based on a weight-of-evidence approach, respectively. If there is any indication for an endocrine disrupting potential of a particular substance (e.g. from toxicological data provided by a notifier or the open scientific literature), all available information are evaluated by UBA regarding their reproducibility, plausibility, significance and relevance. If the endocrine potential of the particular substance is established by weight-of-evidence and there is concern about non-acceptable harm to populations

of non-target organisms, the agency is asking the notifier for additional information. The information requested must be adequate to reliably derive thresholds of no-concern and regulatory acceptable concentrations, respectively for endocrine mediated adverse effects of ecological relevance. This may necessitate the notifier to conduct an endocrine specific non-standard study (e.g. with aquatic mollusks).

7. The development of endocrine-specific testing and assessment today is – on both national and international level - most advanced for fish. In December 2007 a workshop took place entitled „Characterization of endocrine mediated effects in fish“ (translation from German). Experts from Germany representing industry, regulatory authorities and academia agreed on the basic design of an (national) testing and assessment strategy for endocrine effects in fish. The workshop protocol was just recently made available to the public (Sorry, accompanying paper in German language only. An English publication is underway.). However, further work is necessary for consolidation and refinement of the agreed strategy. For this reason, the implementation into national legislation is not realizable to date.
8. If high-quality data suitable for a quantitative ecological risk assessment for the potentially affected non-target organisms are at hand for a specific plant protection product or the active ingredient(s) identified to be an endocrine disrupter, the standard assessment factors according to Directive 91/414/EEG are appropriate for the risk assessment. Moreover, all risk mitigation measures generally available in Germany may be taken into account in such cases, too.

Attachment 2

Expert Report

Assessment of the safety of an extrapolation from growth data of Early Life Stage- and Juvenile Growth Tests (OECD 210, 204, 215) to the NOEC of Fish Full Life Cycle Tests in the risk assessment of DMI-fungicides

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Schmallenberg, October 10, 2007

1. Introduction

Active ingredients of plant protection products of the demethylase-inhibitor (DMI)-fungicide group show a potential for endocrine disruption based on the intended effect mechanism (inhibition of the ergosterol-biosynthesis in target fungus), because the inhibited target enzyme is a cytochrome P-450 enzyme showing a high structure analogy to aromatase among others. Aromatase is a central enzyme of the steroid-biosynthesis in vertebrates and molluscs, which converts testosterone to 17-beta-estradiol, thus playing a determining role in the sex regulation and the expression of sexual physiological characteristics. The assumption that these substances may have an effect on the endocrine system of vertebrates was confirmed in long-term studies on mammals, birds and fish for several representatives of this group.
For this reason, the German Registration Authorities regards all representatives of the DMI fungicide group as potential endocrine disruptors. Until this "initial suspicion" is addressed over the expected environmental concentration range using suitable studies, registrations have been blocked. During the last discussions between the authorities and IVA, it was agreed that, under certain conditions registrations may continue until appropriate data have been submitted and reviewed, e.g. Full Life Cycle Test (FLCT). For this purpose, it is necessary to determine a criterion to allow for an interim registration. Therefore a suitable extrapolation factor is under discussion allowing the extrapolation of a FLCT NOEC from existing fish chronic studies (ELS etc). Considering such a factor would allow a preliminary assessment of the acceptable risk and take into account all relevant risk mitigation measures (minimum distance requirements). In an assessment by the German Federal Environment Agency (UBA), a factor of 5 was proposed.
The robustness of this extrapolation factor shall be evaluated by the Fraunhofer IME as neutral instance based on all available data, and a modified factor shall be proposed, if sensible.

2. Available data and definition of task

UBA confidentially provided study reports of FLCTs that had already been assessed. Furthermore, company data were collected on a confidential basis consisting of data on acute and chronic fish toxicity (OECD 203, 204, 210, 215), on potential endocrine effects (Fish Screening Assay (FSA); Fish Sexual Development Test (FSDT)) and on the bioconcentration potential (OECD 305), besides FLCT-data. In addition, companies provided a literature evaluation on aromatase inhibition of various DMI-fungicides. The aim was to compare available endpoints from different studies for a large number of DMI-fungicides with as far as possible different physico-chemical properties and potencies, to ensure that the discussed extrapolation factor would cover a broad range of properties. In particular, the available fish studies were evaluated to:

1. identify the sensitive endpoints in the FLCT and to:
    - show the ratios between the sensitive population relevant endpoints (e.g. sex ratio, growth, reproduction) and the ratios to the indicative biomarker endpoints, such as secondary sex characteristics or vitellogenin (VTG),
    - discuss the robustness of different endpoints for endocrine mediated risks.
2. to compare the sensitive FLCT-data (cf. 1) with Early Life Stage- (ELS), Juvenile Growth- (JG), FSA- and FSDT-data in order to:
    - clarify, which factor would be sufficiently safe from a regulatory perspective to extrapolate from the NOEC of an ELS- or JG-test to the NOEC of a FLCT.
3. assess the influence of the tested fish species on the respective finding (cf. points 1 and 2). Trends are shown for endpoint specific differences and common features in sensitivity.
4. compare these data with the most sensitive ones of the other relevant ecotoxicological data (e.g. algae) to clarify, if the observed endocrine mediated effects are ultimately relevant in the risk assessment.

Options, limits and minimal requirements to extrapolate to copounds with less information are evaluated. In addition to assessing the safety of the extrapolation factor applied to ELSNOECs in order to decide on the required studies concomitant to the authorization, further information is derived that could contribute to the development of a definitive test strategy for DMI-fungicides.
Relations between sensitivities in in-vitro tests and FLCTs as well as between sensitivities and compound properties, like bioconcentration, are established in order to provide contributions to a clearer mechanistic overall picture and to increase the safety of future regulatory decisions.
Whether a FSA can be a safe trigger or range-finder for a FLCT, or whether a FSDT can be a sufficient alternative to the FLCT for a given mode of action (MoA), cannot clearly be answered based on the data base available for this investigation.

3. Data handling

For the compilation of the data in the following figures, NOEC- and ECx-values of the various reported. FLCT data and some strongly deviating NOECs from ELS- or JG-tests were evaluated in detail. Not only the statistical significance, but also the dose-response relationship has been considered, as the evaluation was targeted towards a comparative evaluation of endpoint sensitivity and not towards a regulatory risk assessment. Where a reevaluation by UBA or the company existed, the more conservative value was used. In the following, figures are presented without the names of the substance. If substances have been ranked, this was done with decreasing sensitivity of the relevant endpoints.

4. Results

4.1 Comparison of sensitive endpoints in the FLCT

Data for 16 substances were submitted. Nine FLCT for 7 substances were considered. One study was excluded as there were too many uncertainties about the reliability of the NOECs. To compare intrinsic toxicities, time weighted average (TWA) concentrations were used for the studied life phase of the static FLCT with sediment. In all tests, reproduction and growth were assessed. Where data on sex ratio were missing, these were either assessed from the raw data (partly only stated as „no evidence of...“), or from a separate FSDT. Sex ratio was the most sensitive endpoint in 4 tests, growth in 3, and reproduction in 2 tests (Fig. 1). In both tests with reproduction as the most sensitive endpoint (different fish species), sex ratio has not been assessed directly. However, the NOEC for sex ratio determined at a later stage based on the raw data is identical to the NOEC for growth.

The sensitivity of the population relevant endpoints sex ratio, growth and reproduction is essentially comparable; the differences in sensitivity of significant effects were within one concentration step in 6 out of 9 tests for all 3 endpoints.

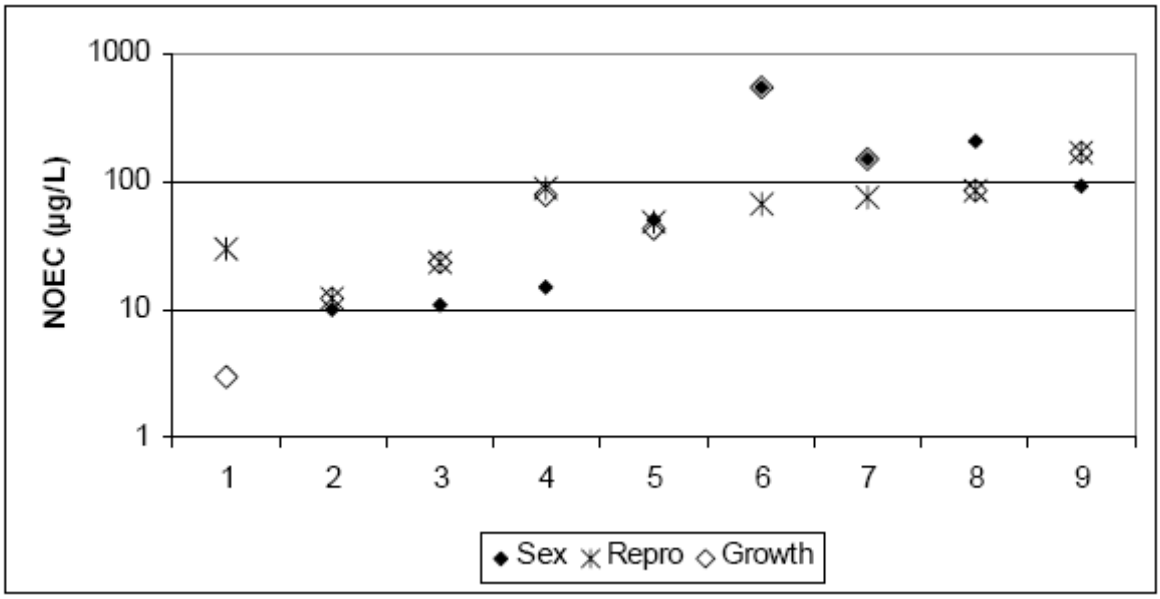


Figure 1: Comparison of the sensitivity of the NOECs of various population relevant endpoints in Fish Full Life Cycle Tests. Ranking of the tests (1-9) by the degree of sensitivity Assessment of the Safety of an extrapolation from growth data of Early Life Stage- and Juvenile Growth Tests to Fish Full Life Cycle Tests of DMI-fungicides

There are 3 exceptions:
In a static study with sediment (Nr. 4) to assess effects on different life stages after an initial peak exposure, the sex ratio was 5 times more sensitive than growth and 6 times more sensitive than reproduction. This ranking of sex ratio > growth > reproduction reflects the sustainability of effects set by a peak exposure, because with decreasing concentrations, the endpoint reproduction recovers first (because of the duration of involved physiological processes), followed by growth. A recovery of sex reversal, once induced, seems not possible in the studied species. In so far, these results don’t contradict the situation in the 6 studies above; for a comparison of the intrinsic toxicities, only the sex ratio should be used.

In study 1, the sex ratio in the controls was strongly biased towards females, while there were normal sex ratios in the test item concentrations and no concentration-responserelationship was visible. Effects on the control regarding growth and reproduction cannot be excluded. In so far, the study should be treated with caution.
In study 6, reproduction was 8 times more sensitive than growth and sex ratio. A closer look at the study revealed that the low reproduction rate at the higher test concentrations did not allow a replication of the F1 and hence no statistical evaluation was possible. Consequently, the growth NOEC is only based on the adult F0; the study was not designed to evaluate sex ratio anyhow. For this reason, the differences in sensitivity of the endpoints appear less meaningful.
The indicative endpoint VTG was only assessed in 3 studies, and its sensitivity always corresponded to the sex ratio, the most sensitive population relevant parameter. For another substance, a literature reference was considered, in which the VTG decrease was as sensitive as sex ratio, but 2 times less sensitive than the most sensitive endpoint growth. Overall, the FLCT data set is weak with regard to the determination of the sex ratio. A determination of VTG is not important for the determination of a decisive regulatory NOEC, but would be helpful for the identification of the relevant toxicological MoA.

4.2 Comparison of NOECs from FLCT with ELS- and JG-tests

For all 16 substances, there are ELS studies (OECD 210) and/or growth data from prolonged fish or JG-tests available (OECD 204, OECD 215; not distinguished as JG any longer in the following). A comparison of these NOECs with those of existing FLCTs for the 7 substances should elucidate, whether the extrapolation to the NOEC of the FLCT by the application of a factor of 5 would have been sufficiently safe for a regulatory decision.

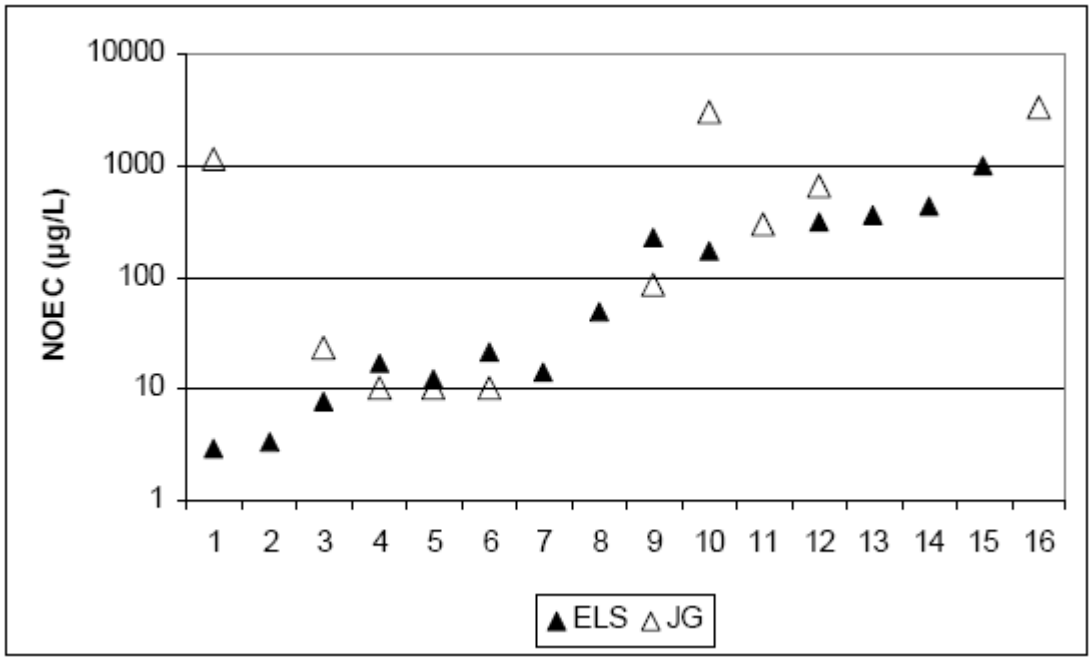


Figure 2: NOEC-data for growth from ELS- and JG-tests. Ranking of the substances (1-9) according to sensitivity.

For 8 substances, growth-NOECs were determined in ELS- and prolonged- or JG-tests. In 6 cases, the results were almost similar (Fig. 2) and allow the conclusion that for the given MoA, both test methods are equivalent (with respect to the endpoint growth) for the derivation of a regulatory NOEC. In the 2

remaining cases (substance 1, 8 and 10 in Fig. 2), the JG-test was remarkably less sensitive. Both cases represent older studies, in which the growth data were assessed in a 28 days OECD 204-test with rainbow trout. No information is given about the initial size and the increase in fish size, only the size at the end of exposure was compared resulting in no effect up to the NOEC in regard to lethal effects. These tests are not regarded as an adequate alternative to the OECD 215- or OECD 210-tests.

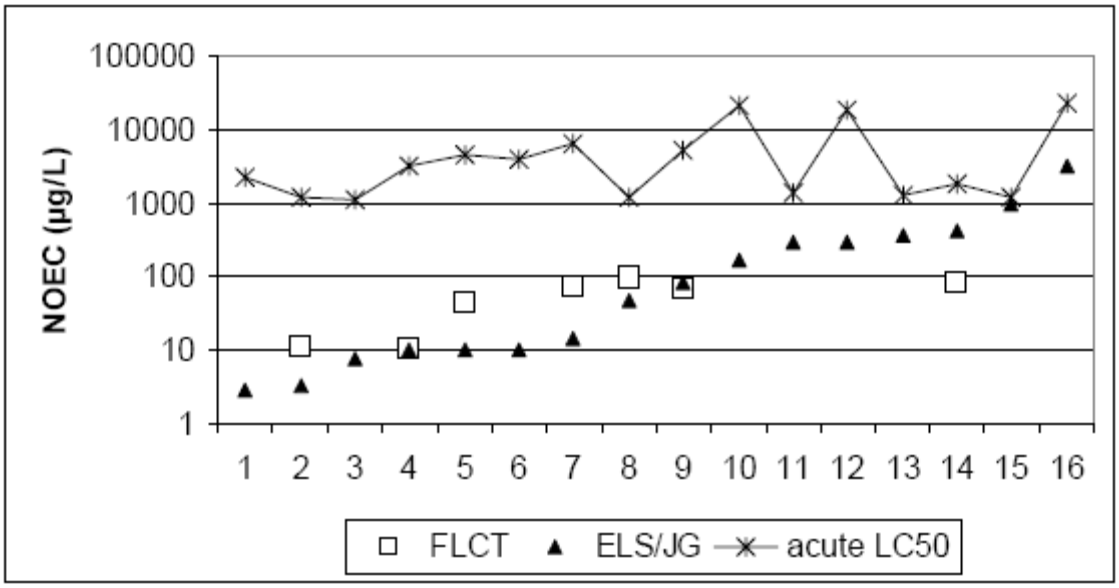


Figure 3: Most sensitive NOECs from ELS- or JG-tests in comparison to the lowest endpoints of valid FLCTs- and the acute toxicity data. Ranking of substances according to the sensitivity in ELS/JG-tests.

It is remarkable that the acute toxicities of the 16 substances studied are less scattered (1.5 orders of magnitude) than the chronic effects (3 orders of magnitude; Fig. 3). This means that the substances under investigation have very different acute to chronic ratios (ACR). In the context of substance regulation for unspecific acting substances, an extrapolation from the acute LC50 to the chronic NOEC by one order of magnitude is applied. A high ACR suggests a wide span between the sensitivity of the chronic endpoint and the general basal toxicity, and indicates a specific MoA. For the most sensitive substances 1-10, the ACRs are in the range of 125 to 760 (exception: substance 8 with 25) indicating a clear specific effect on fish, while for the substances 11-16, the ACR are never higher than 100, and in 5 cases even lower than 10. In this segment of the sequence, unspecific basal toxicity may possibly be expected in the concentration range of chronic effects.

The comparison of the most sensitive population relevant NOECs from FLCTs and the NOECs from ELS- and JG-tests show that for all substances in the more sensitive half of the sequence, the ELS/JG-NOEC is lower than or identical to the FLCT-NOEC. For substances with higher NOECs, there are less FLCT-data. Here is the only value with a higher sensitivity of the FLCT-NOEC: substance 14 with a factor of 5.1 has the only critical value (in regard to the aim of the present evaluation). A closer examination of the test results gave no indication of irregularities. However, it should be considered that the ACR is only 4 in relation to the ELS and about 20 to the FLCT. Because the most sensitive endpoint in the FLCT is growth, a more general MoA than aromatase inhibition might be the cause.

In order to assess the safety of the extrapolation from ELS- or JG-NOECs to FLCT-NOECs, however, it is not sufficient to base it on the lowest value of the growth data only. The extrapolation should be possible with high statistical safety from each submitted ELS- or JGtest. For the present evaluation, all available NOECs from ELS- or JG-tests of the 7 substances with valid FLCTs were related to the most sensitive NOECs from FLCTs (Table 1). For substance 14 discussed above, it gets clear that the ratios for the

maximum and minimum values (11,5 and 5,1) both lie outside the 99,9%-confidence limit of the mean and therefore represent a special case also statistically. Even for this substance, the upper 99,9%-confidence limit still lies within the discussed factor of 5. Without this substance, a factor of 3 would be possible; although the extreme value which is based on a reliable study lies between 4 and 5.

Table 1: Statistics of all NOEC-ratios of ELS- or JG-Tests in relation to FLCTs NOEC ELS or JG / NOEC FLCT

NOEC ELS or JG / NOEC FLCT	All seven substances	Without substance
Number of comparisons	16	14
Mean	2,2	1,4
Standard deviation of the population mean	2,8	1,3
Maximum	11,5	4,6
Minimum	0,2	0,2
Upper 95% confidence limit of mean	3,6	2,12
Upper 99% confidence limit of mean	4,1	2,3
Upper 99,9% confidence limit of mean	4,6	2,5

Based on the data available so far, a factor of 5 applied to the most sensitive NOEC of ELS- or JG-tests is protective for a preliminary extrapolation to the NOEC of a FLCT.

Especially for substances with a clear dominance of endocrine mediated effects, there is a high safety in this respect.

Because a FLCT is regarded as the only test that covers all potential MoAs, the necessity to run such a test increases with increasing ACR. For the present MoA, the safety of the estimate seems to increase also by the use of the endpoint "juvenile growth". If the MoA „aromatase inhibition" is clearly dominant, it should be discussed whether the final risk assessment without the availability of an FLCT can be performed by applying an additional safety factor on a suitable growth-NOEC. If required by the submitter, the necessary risk mitigation measures might probably be reduced by submitting a higher-tier test (FLCT), provided the test results do allow this.

4.3 Comparison of NOECs from FLCT with FSA- and FSDT-data

Fish Screening Assays (FSA, on the verge of implementation as OECD test method) are performed to confirm a suspicion derived from preliminary information (MoA, toxicology, in vitro data) by an in vivo test. If this is the case, further test-tiers shall elucidate the potential for population relevant effects. This implies that the FSA allows to exit the test scheme in case of negative results. Therefore, false negative results should be excluded in view of the precautionary principle. From the viewpoint of risk assessment, it is also not desirable to have a too strong sensitivity (false positives); the FSA must have a predictive potential for effects in the FLCT. This can be elucidated by an effect specific comparison of the sensitivities between FSA and FLCT.
Fish Sexual Development Tests (FSDT, at the time in the validation process of the OECD) are extended ELS-tests that expose the fish until sex development is completed, assessing sex ratio, histological endpoints and mostly also VTG, apart from the ELS endpoints. The feasibility of such tests in the risk assessment of endocrine active substances is being discussed controversially. The author regards the FSDT as not suitable for an intermediate tier between FSA and FLCT, because • on the one hand it does not

contain the essential population relevant endpoints (e.g. reproduction), therefore, it is not sensitive and cannot trigger a FLCT • on the other hand it covers the most sensitive phase (e.g. sexual development) as part of the FLCT and can, therefore, replace a FLCT. There remains only to be discussed whether the shortened test method in comparison to the FLCT can be used as a final-tier test or not. Up to now the data base is not sufficient to allow a solid comparison of the most sensitive endpoints from FLCTs with those of FSAs (partly with reproduction endpoints) or FSDTs. Only for 4 substances there is information on endocrine mediated effects from these specific tests. For 3 substances, VTG was assessed, sex ratio for 2 and reproduction for one substance. For 3 additional substances there exist FSA-results, but no FLCT-data. All NOECs or EC10s for VTG were within a factor of 2 to the NOECs of the sex ratio from FLCTs and matched the VTG-NOECs from FLCT, where available. Reproduction was included in one case yielding an identical NOEC consistent with the respective FLCT-NOEC (177 µg/L versus > 85 µg/L). In one FSDT, the NOEC of the sex ratio was the most sensitive endpoint as was the NOEC of the VTG. For another substance, the sex ratio had not been assessed in the FLCT. The FSDT resulted in a similar NOEC as the population relevant endpoints in the FLCT, but additionally it provided a remarkably more sensitive histological NOEC based on liver toxicity. This value was more sensitive than the FLCT-data, but close to the lowest NOEC for growth derived from ELS/JG-studies.

Overall, it can be concluded that the existing FSA- and FSDT-data do not provide any evidence that they would not be predictive for a FLCT (FSA) or that they would not deliver equally sensitive results (FSDT). An extension of the data base is desirable. An exact comparison of FSA- and FLCT-data is not possible because FSAs normally were performed with only 3 concentrations using a wider spacing, and no NOEC or ECx was determined. It is regrettable that the classification of the FSA as a pure screening test makes it difficult for this type of test to generate data useful for the purpose of a risk assessment. Provided that sufficient sensitivity of the FSA can be confirmed, it should be discussed if the FSA can be used to either trigger a further test (which in the opinion of the author would represent the final tier) or to exit the testing scheme. The exit criterion could be the determination of a NOEC in the concentration range of e.g. 10 times below the acute LC50.

4.4 Comparison of different fish species

Acute Tests

If the substances are ranked according to their sensitivity in the ELS/JG-test, then rainbow trout is always the most sensitive species for the substances in the more sensitive half of the ranking. In the less sensitive half, it was 3 times the trout, 3 times the bluegill, once the golden orfe and once the sheepshead minnow.

ELS/JG-Tests

In the prolonged fish- or JG-tests, rainbow trout was used exclusively. ELS-tests were conducted with trout (7x) or fathead minnow (7x). There is one ELS-test with the sheepshead minnow, which showed only a slightly lower sensitivity than the trout for the same substance and test.

FLCT

Of the 9 FLCTs, four were conducted using fathead minnow, 3 using zebrafish, and 2 sheepshead minnow. Only for 2 substances, there are comparable data for fathead minnow and zebrafish basically indicating comparable sensitivity for both species. It seems that there is a difference in the expression of the effects between zebrafish and fathead minnow due to the sex development mechanism of zebrafish (conversion of the protogyn gonad in developing males, probably triggered by the ratio of the hormone titer). Zebrafish therefore react more sensitive to an aromatase inhibition than fathead minnow, and fathead evidently appears to dispose of a higher compensation potential for counter regulation. In turn fathead minnow, because of its bigger absolute growth potential, allows a higher accuracy of statistical discrimination of growth efects. This again applies for zebrafish in regard to reproduction parameters, due to its more homogeneous and proliferous egg production. These conclusions are based on test and assessment

experience of many years, but cannot be drawn from the limited number of studies in this evaluation. However, they don't contradict the findings of the present data. Most FLCTs with fathead minnow were performed in the US and represent older data, performed without endocrine focus. Therefore, a comparison is difficult. Because of the nature of the MoA under discussion, it appears that all population relevant endpoints are of comparable sensitivity. Therefore, no recommendation regarding the potential sensitivity of the species can be deduced. Practicability, effort and usability for modified test protocols are to be discussed elsewhere. The author does not regard himself as neutral in this respect.

4.5 Consideration of aromatase inhibition activity and BCF

Due to its conservative evolution, the structure of the steroid biosynthesis enzymes can be regarded as being relatively similar within vertebrates. For this reason, the most sensitive (molar) population relevant data from FLCTs were plotted against the IC50 of human aromatase (Trösken et al., 2004) to elucidate the mechanistic background of the effects. In one case, the missing IC50-data could be estimated roughly from other published data. A significant correlation between the IC50 of human aromatase and relevant endpoints for fish populations is resulting *($r=0.858$; $p<0.05$),* if the substance with the most sensitive aromatase inhibition is regarded as an outlier (Fig. 4). Because this substance has been described as being very potent also in rainbow trout microsoms, its relatively insensitive endocrine effect in the chronic fish test must be due to other reasons. Because the effects of a substance not only depend on its affinity towards the target but also on its availability, the lowest NOECs of endocrine mediated effects in long-term fish studies were plotted against the BCFs from studies according to OECD 305 (Fig. 5). The relation between high bioconcentration and sensitive effect is not significant, even if the substance discussed in the last paragraph is regarded as an outlier (a particularly high BCF with only medium effect).

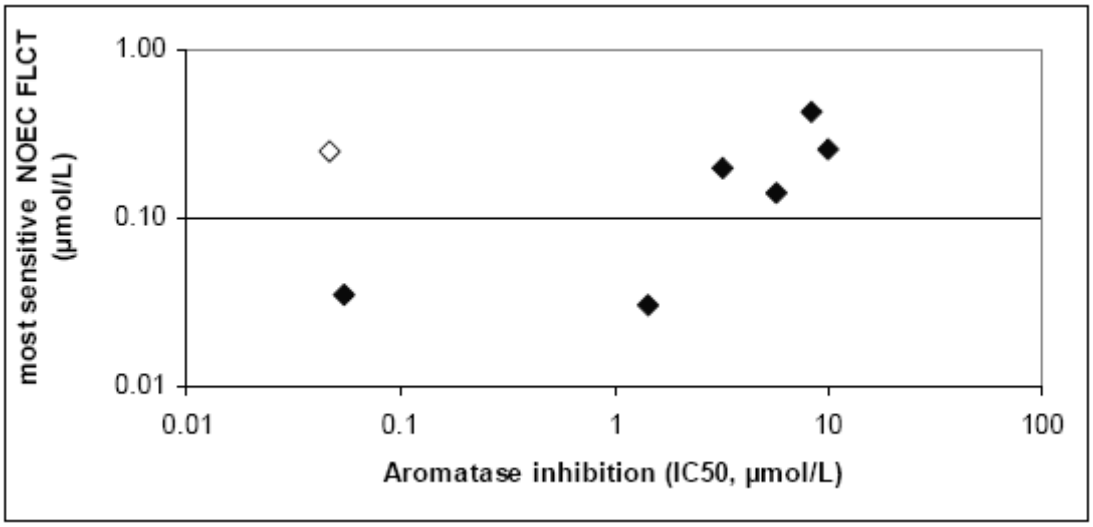


Figure 4: Lowest NOECs from Fish Full Life Cycle Tests compared to the inhibition of human aromatase. Open rhombus: Potential outlier.

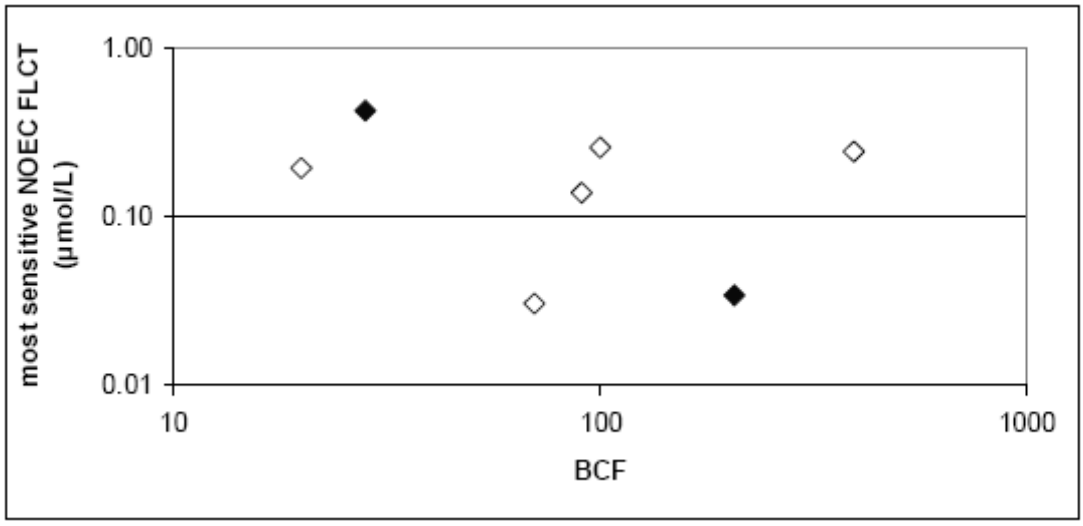


Figure 5: Lowest NOECs from Fish Full Life Cycle Tests compared to BCF from OECD 305 studies. Open rhombi: BCF only determined for total radioactivity

The possible reason for the weak correlation between BCF and NOEC may be due the fact that the BCF of 5 out of 7 substances was only given as total radioactivity. The true BCFvalue is lower, as the total radioactivity also includes all the breakdown products. Depending on the depuration rate in fish, the reduction of the BCF is substance-specific, resulting from the relation of uptake and depuration rate, which cannot be determined, if only the total radioactivity is given. This caveat applies also to the relation between NOECs of endocrine effects in fish long-term tests and the rectified accounting of the BCF and IC50 (Fig. 6). Between the highest and lowest NOEC belonging to the two „true" BCFs, the NOECs are in a clear dependency to the calculation factor particularly due to the aromatase inhibition, again with the exception of the outlier mentioned in the two previous chapters.

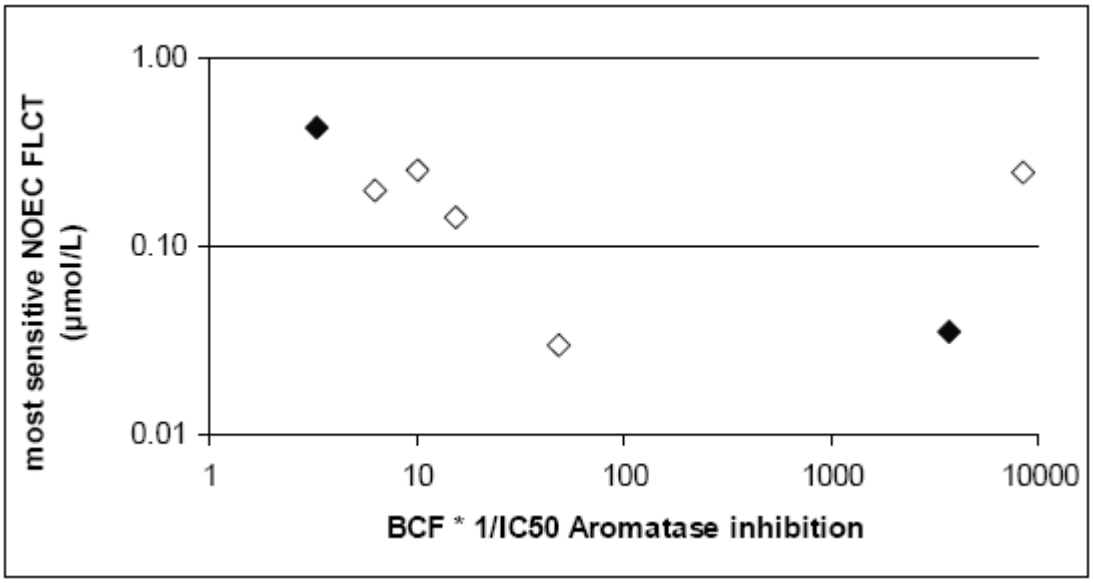


Figure 6: Lowest NOECs from endocrine long-term tests with fish compared to the product of BCF and 1/IC50 aromatase inhibition. Open rhombi : BCF only determined for total radioactivity.

4.6 Comparison with other relevant ecotoxicological endpoints

A comparison with the other most sensitive ecotoxicological endpoints relevant for the assessment (algae, Daphnia, Lemna, Chironomus) should clarify, whether the observed endocrine mediated effects in fish are relevant for the regulatory effect assessment. For this purpose, data were provided for 11 of 16 substances (Fig. 7).

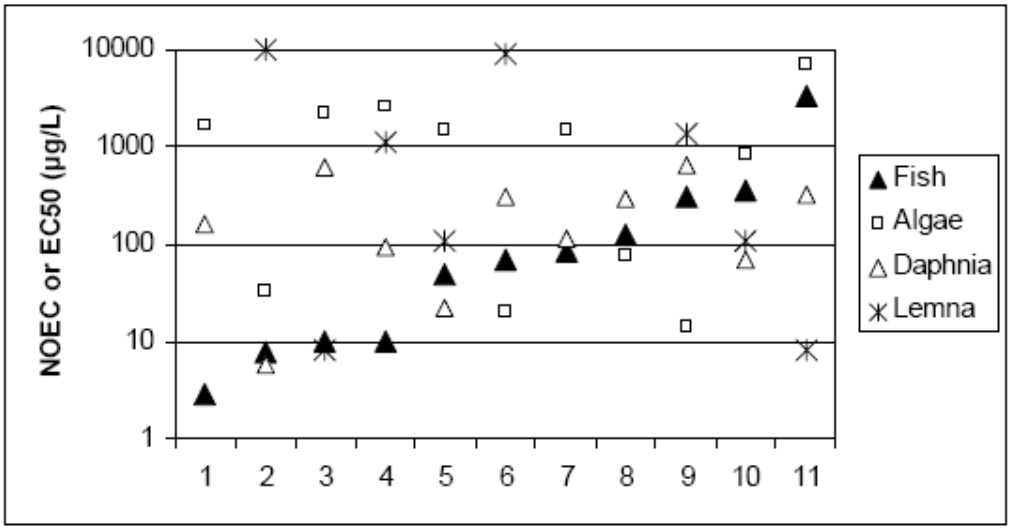


Figure 7: Most sensitive regulative endpoints for the assessment of the chronic toxicity in aquatic organisms. Ranking of the substances (1-11) according to the sensitivity in the fish test. Chironomus endpoints always less sensitive than fish endpoints, therefore not indicated.

For this MoA, no characteristic regularity of the relation between chronic fish toxicity and chronic endpoints for other aquatic organisms is apparent. If the different sensitivities of the fish NOECs of the individual substances are taken into account (Fig. 8), it gets obvious that with NOECs higher than 100 µg/L, other endpoints clearly react more sensitive and thus the effects on fish are less relevant for the assessment. In the range 10 to 100 µg/L, in most cases a NOEC of another aquatic organism with comparable sensitivity exists. In this situation, the fish NOEC may represent the critical value due to the long generation time of fish and its reproductive potential to recover. Both fish NOECs, which are clearly more sensitive than all other aquatic endpoints, are lower than 15 µg/L.

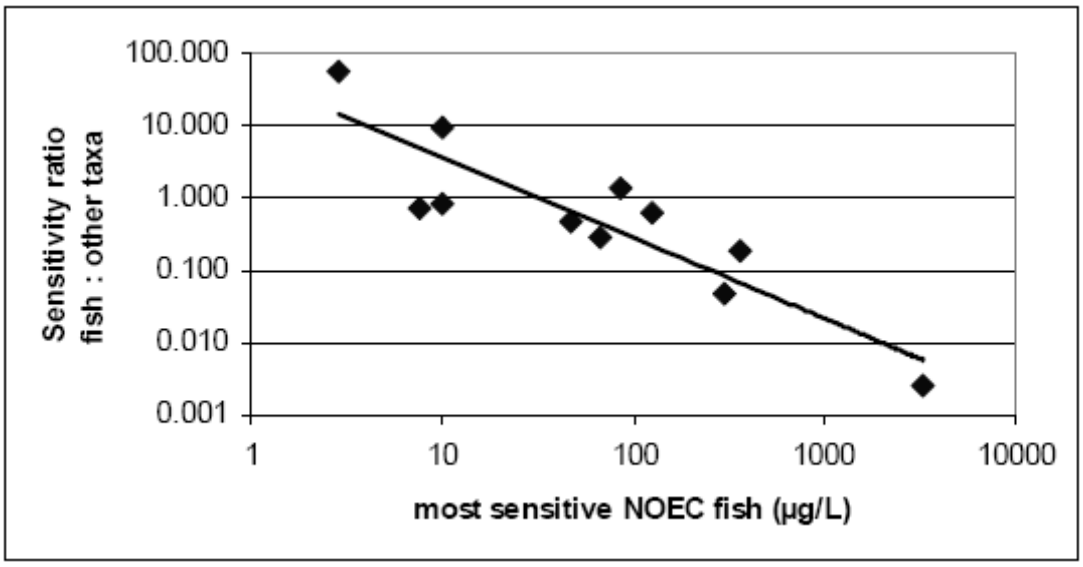


Figure 8: Factor of sensitivity between the most sensitive chronic NOECs from fish tests and those from tests with other aquatic organisms.

4.7 Recommendation for data requirements to assess the risk of DMI-fungicides to fish

Preliminary risk assessment to clarify if the definitive risk assessment can be done as a post registration requirement

Based on the available data, an extrapolation applying a factor of 1/5 to the NOECs of ELSor JG-data is sufficiently safe to estimate the NOEC of a FLCT for effects caused by aromatase inhibition. In the future, it should be granted that growth data are elaborated in prolonged fish test according to OECD 215.

Definitive risk assessment

A FLCT is still necessary for DMI-fungicides as final-tier test method, because different endpoints were identified as most sensitive ones in the available FLCT. An extrapolation from growth-NOECs to the NOECs of the final-tier tests by adding an additional safety factor is to be discussed. Indicative endpoints (e.g. VTG or hormone titer) may explain the mode of action and thus may be helpful to the reduction of the uncertainty of the regulation, but are not needed for the purpose of the deduction of the decisive NOECs for the regulation.

The evidently close correlations between the endpoint sensitivities should allow to replace the FLCT by the FSDT in the mid-term, provided future studies will confirm the comparable sensitivity of data derived from FSDT. To decide whether it will be possible in the future to assess the risk based on IC50- and BCF-data would require a re-assessment of the BCFs based on the parent compound.

Appendix 5

Contribution from Japan

Ministry of Economy, Trade and Industry

1. The advisory body

Ministry of Economy, Trade and Industry (METI) established the Endocrine Disruptive Effect Subcommittee, an advisory body of the Minister, under the Chemical Substances Council in 1999. The Subcommittee consists of specialists of biology, ecology, medical science and pharmacology, and has been held three or four times a year.
In 2000, the Subcommittee publicized an interim report which collects information to develop testing methods of endocrine disrupting properties as well as existing data of chemical substances which had been concerned as substances having endocrine disrupting properties.
In 2002, the Subcommittee addressed assessment result of hazard information on fifteen priority chemical substances.
In 2006, the Subcommittee publicized an interim report on hazard assessment on human health effect of endocrine disrupters and action plan for future researches and studies.

2. Researches and studies

METI, along with Chemicals Evaluation and Research Institute (CERI), has started researches and studies regarding human health effect of endocrine disrupters since 2000. In early stage of the studies, hazard assessment was conducted to fifteen chemical substances which had been concerned as substances having endocrine disrupting properties, and it was concluded in above Subcommittee that no significant risk to human health was fund at Japan's present situation.
METI has shifted main target of studies to contribution to the OECD Test Guideline Programme and has been conducting non animal and animal testing as below under the technical advice of the Subcommittee.

(1) Non animal testing
- i. Receptor binding assay
- ii. Reporter gene assay
- iii. Steroidgenesis assay
- iv. QSAR

(2) Animal testing
- i. Uterotrophic assay
- ii. Hershberger assay
- iii. Enhanced TG407
- iv. in utero and lactationaly exposure study
- v. Two generation study

Ministry of Health Labour and Welfare

1. The advisory committee

The Advisory Committee on Health Effects of Endocrine disruptive chemicals has been established since 1998. The committee is a private panel under the Director-General of the Pharmaceutical and Food Safety Bureau in the Ministry of Health Labour and Welfare (MHLW). The committee has consisted of epidemiologist, toxicologist, analyst, members of consumer representative and so force.
Since 1998, the committee has been held 20 sessions with open to the public. The committee has compiled and published the interim report (1998), the 1st supplemental report of the interim report (2002) and the 2nd supplemental report of the interim report (2005).
The committee addresses evaluation of risk of endocrine disrupters toward human health, necessity of prompt action to protect public health, risk communication with the general public and so force.
The committee has mapped out the action plan regularly. The first action plan was established in 2002, and the second one was established in 2005. Various researches and studies have been conducted in accordance with these plans.
The committee has described the framework of testing scheme on possible chemicals as endocrine disrupters. The framework consists two parts; one is *in silico*, *in vitro* and *in vivo* assay, the other is definitive test. The committee has drawn up the list of chemicals according to the prioritized by the result of the former part.
To improve risk communication, MHLW has established the web site regarding endocrine disrupter chemicals in cooperate with the committee. In this homepage, MHLW has prepared FAQs, explanation about the committee's discussion and various statements submitted by the committee.

2. Researches and studies

MHLW has provided Health and Labour Sciences Research Grants. Since 1996, MHLW has started new study field concerning health effect on human of endocrine disrupters. In this new study field, study regarding epidemiological research, exposure and effects of endocrine disrupters to the human body and test method to detect endocrine disruptors have been implemented.
MHLW has also conducted screening test projects as a contracted research entrusted with National Institute of Health Sciences. In the project, Hershberger Bioassay and rodent Uterotrophic Bioassay has been conducted. MHLW has conducted these *in vivo* assays in order of priority by the result of *in silico* and *in vitro* assay.

Ministry of the Environment

1. The advisory body

(1) History
The Japan Environment Agency (former Ministry of the Environment (MOE) (since 2000)) established "The Exogenous Endocrine Disrupting Chemical Working Group" to discuss the endocrine disrupting effects in the environment in March 1997. The WG was consisted with experts of biology, ecology, medical science and pharmacology. This WG released an interim report in July 1997. MOE published the "Strategic Programs on Environmental Endocrine Disruptors98" (SPEED '98.) in May 1998, based on the report from WG.

For the further scientific discussion and implementation of SPEED '98, the "Commission on Endocrine Disruptors" was established in June 1998.

MOE established a SPEED '98 Revision Working Group in 2003, composed of specialists, experts in the field, and representatives from consumer groups etc. This revision WG held meetings for two years. It compared the activities and results of SPEED '98 thus far to the issues that have been indicated internationally, and identified issues for the future. The revision WG also held hearings from local governments on this matter.

(2) Current advisory body
A Committee composed with experts from academia, media and civil society has worked on the evaluation of research results and improvement of public awareness. This Committee has four Sub Committees on i) effects of endocrine disrupters, ii) basic researches on the mechanism of endocrine disrupters, iii) monitoring of wild life, and iv) risk communication.

2. Research and studies

(1) Program Related to Endocrine Disrupting Chemicals: ExTEND 2005
i. Background
MOE published the "MOE's Perspectives on Endocrine Disruption-ExTEND 2005-" (hereafter called "ExTEND 2005", The ExTEND 2005 subtitle is an acronym for Enhanced Tack on Endocrine Disruption) in March 2005. In accordance with this program, research on the mechanisms of endocrine disruption has progressed, along with environmental monitoring, development of test methods, annual international symposia and collaborative researches with related countries.

ii. Research areas
The following are the main pillars of the activities to deal with the endocrine disruptor matters in ExTEND 2005: 1) Observation of wildlife, 2) Evaluation of the environmental concentrations and exposure level, 3) Promotion of fundamental research, 4) Hazard assessment, 5) Risk assessment, 6) Risk management, and 7) Promotion of information sharing and risk communication.

1) Observation of Wildlife
   a. Continuous observation of wildlife at the local level
   b. Evaluation of the results by experts
2) Evaluation of the Environmental Concentrations and Exposure Level
   a. Environmental survey and wildlife observation
   b. Estimation of concentration levels of substances in the environment
   c. Environmental sample preservation
   d. Development of more sensitive analysis methods

3) Promotion of Fundamental Research
   a. Accumulation of biological knowledge in wildlife
   b. In-vivo research
   c. In-vitro research
   d. Basic research contributing to test methods development
4) Hazard Assessment
   a. The selection and assessment procedures of test substances concerning the endocrine disrupting effects
   b. Implementation of experiments based on selected procedures
5) Risk Assessment
6) Risk Management
7) Promotion of Information Sharing and Risk Communication
   a. Information sharing
   b. Risk communication
   c. Environmental education

(3) Bilateral Cooperation

Bilateral cooperation of scientific studies on endocrine disrupting effects have been promoted with UK since 1999, and US since 2004.

i. UK-Japan

The 10th Workshop UK-Japan joint research in North Bovey (UK) was slated for October 5-7, 2008.

ii. US-Japan

The 4th US-Japan Workshop was held in San Francisco (USA) in February 14-15, 2008. The 5th US-Japan Workshop in Tokyo (Japan) is slated for December, 2008.

(4) Promotion of sharing information

MOE has implemented various measures to scientifically evaluate, and reduce environmental risks. As part of such measures, the Ministry has held international symposiums once a year with the aim of bringing together members of the public, industry and government to share accurate information on endocrine disruptors acquired in Japan and other countries.

Appendix 6

Contribution from Korea

National Plan for Endocrine Disruptors
(Revised in 2007)

March 2007

Republic of Korea

Ministry of Land, Transport and Maritime Affairs

Ministry for Food, Agriculture, Forestry and Fisheries

Ministry of Environment

Rural Development Administration

Korea Food and Drug Administration

1. Introduction

1-1. Background

Ever since the endocrine disruptors (EDs) loomed large as an international issue in the late 1990s, the relevant government ministries including the Ministry of Environment has established "Mid/Long Term Research Plan on Endocrine Disruptors" and each ministry has conducted various research projects thereafter.
Ministry of Land, Transport and Maritime Affairs (marine ecosystem), Ministry for Food, Agriculture, Forestry and Fisheries (risk assessment & management, animal drugs, livestock products, seafood) Ministry of Environment (air, water, sediments, soil, biota etc.), Rural Development Administration (agricultural pesticides & farmland), Korea Food and Drug Administration (food and drug etc.)
Projects have been promoted primarily focusing on the monitoring of endocrine disruptors in various environmental media due to the ambiguity of endocrine disruption and the international delay in the preparation of test guidelines on endocrine disruptors, and thus, there are limitations in the policy application. In particular, the individual "Mid/Long Term Research Plan on Endocrine Disruptors" among relevant ministries was insufficient in sharing research results and promoting joint research projects.
As the various projects progressed on, the necessity for the revised plan that reflects the research achievement on endocrine disruptors as well as the necessity for the cooperation between the ministries was proposed.
When the inter-ministerial conference held to discuss in details the cooperation plans among the relevant ministries, the ministries agreed to jointly revise 5-Year (2007-2011) Plan on Endocrine Disruptors (August 2005).
Furthermore, considering the ambiguity of the effects of endocrine disruption and the international delay in the preparation of test guidelines on endocrine disruptors, it is necessary to review and revise the current mid-long term research plan of each ministry.
Accordingly, the ministries made the next five year (2007~2011) research plan harmonized on endocrine disruptors by reviewing the results of research projects that have been conducted up to now. The plan will contribute to lead to the preparation of appropriate plans for the safety management of endocrine disruptors of each ministry.

2. Major Achievement of Mid-Long Term Research Projects

2-1. Field of Terrestrial Environment

2-1-1 Overall Summary

The Ministry of Environment established "Mid-Long Term Research Plan on Endocrine Disruptors" in 1999 and has been promoting various research projects in accordance with this plan. Through the actual execution of research projects from 1999 to 2005, the Ministry of Environment had invested 11.5 million US dollars on 34 projects based on its Research Plan on Endocrine Disruptors. It contributed to satisfying the public's right to know by annually disseminating the survey results to the public and continuously providing information to the public through the Ministry's website (http://www.ncis.nier.go.kr), specifically designated for the chemical information service.

2-1-2 Achievement Evaluation by Projects

Conducted environmental monitoring and ecological effects from 1999 to 2005 on the total of 228 sites and provided about 1 million US dollars to these projects every year.

Media Subjected to Inspection		No. of Survey Sites
Environmental media	Air (Ambient)	42
	Water	73
	Soil	82
	Sediment	27
Biota monitoring	Fish	32
	Amphibians	32
Total		288

For the preparation of screening methodologies, the ministry has carried out some research projects related to tool development for measuring vitellogenin in bullfrog in 2002 and environmental fate estimation model from 2001 to 2004.
As a part of international cooperation, the Ministry of Environment has been holding symposium and carrying out joint research projects between the governments of Korea and Japan since 2001.

2-2. Field of Marine Environment

2-2-1 Overall Summary
The Ministry Land, Transport and Maritime Affairs dedicated 3.3 million dollars as project expenses on the total of 9 projects from 2002 to 2005 based on the Research Plan on Endocrine Disruptors within the Marine Ecosystem.

2-2-2 Achievement Evaluation by Projects

Monitoring
- Performed the environmental monitoring in 25 representative sites from 2002 to 2005 (dedicated about 0.3~0.4 billion every year). The concentration of endocrine disruptors in overall was very similar every year and lower than or similar to that of foreign countries' deeply polluted areas.
- Conducted the continuous monitoring the investigation of dioxins in marine organisms.

Assessment of ecological effects
- Promoted the following researches: Development of vitellogenin assay, investigation into detoxifying enzymes, antioxidative system and sex ratio and verification of genetic sequence of TTR and RBP, usability of CYP1A1 biomarker, and molecular biological toxicity
- Study on bio-indicators using the seashore fish and research on the community structure of benthos

Development of predictive model on fate of EDs in the marine ecosystem
- Research on input load of dioxins in Ulsan Bay, the mouth of Nakdong River, Busan Bay and Jinhae Harbor, and substance transportation among environmental media

2-3. Field of Livestock

2-3-1 Overall Summary
Executed screening and assessment of suspected endocrine disruptors, and investigation into pollution level in order to (1) prevent the contamination by endocrine disruptors during the production process of food for the security of food safety and to (2) prepare risk management policies on pesticides, feed

additives, animal drugs and environmental contaminants that are used or polluted unavoidably during the production process.

- With the goal of securing the safety of animal drugs, livestock products, and feed, National Veterinary Research and Quarantine Service received about 2.8 million US dollars as research fund from 1998 to 2005 to prepare the risk assessment methods on residual substances that might cause harm to human health and livestock and to provide scientific foundation for pre-protection from such harmful residues.
- Established and developed 15 techniques including screening tools for thyroid hormone disruptors using transgenic cells and evaluated the risk of 14 substances in the viewpoint of endocrine disrupting effects.

2-3-2 Achievements Evaluation by Projects

Research on development of assays

- The total of 15 assays have been established and developed from 1998 through 2005 and include the following projects: Development of Transgenic Yeast for Screening Sex Hormone (Estrogen, Androgen, Progesterone) Disruption, Development of Transgenic Cells for Screening Thyroid Hormone Disruption, Establishment of in vivo Tier I and II Assay (Uterotrophic Assay, Hershberger Assay, One generation reproduction study, Multi-generation reproductive study, Andrological assay), Development of Screening Technique for Dioxin-like Substances using Transgenic Cells, Development of Biomarkers for the Screening of Exposure Level of PAH Compounds, Polybrominated Compounds and Heavy Metals, and Development of a Unique Intoxication Parameters for Mycotoxins.
- The originality and applicability of the developed techniques have been verified both domestically and internationally through the publication of research papers, the acquisition of patents and the utilization as standard official technology.
- In reality, these techniques have been contributing to the improvement of rapidity and accuracy in the assessment of endocrine disrupting chemicals including pesticides, environmental pollutants and animal drugs, which can reside in animal originated food or animal feed.

2-4. Field of Pesticide (Agricultural Products & Farmland)

2-4-1 Overall Summary

Among the 67 endocrine disruptors enlisted by WWF, 17 pesticides are still used for agricultural plant protection in Korea. Rural Development Administration (RDA) carried out several cooperative researches on 17 pesticides suspected as endocrine disruptors from 2000 to 2002 with ca. 4.5 million dollars of research fund.

Major goals of the projects were to establish preliminary screening methods for possible endocrine disrupting pesticides and monitor the pollution level of the pesticides in agricultural products and the agricultural environment e.g. surface water and farmland soil. In addition, the investigation of endocrine disrupting effects of several pesticides on environmental organism (fish) and human health risk assessment were conducted.

2-4-2 Major Achievements

Monitoring of residues of 17 pesticides suspected of being endocrine disruptors in the crop and soil in nationwide plastic house facilities

- Analyzed pesticide residues in 133 samples from 6 types of fruit vegetables and 170 samples of arable soil.

Assessment of human exposure to pesticides suspected of being endocrine disruptors

- Investigated exposure amounts of pesticide of the workers after spraying pesticides in a pear orchard.

Investigation into the endocrine disrupting effects of pesticides on environmental organisms

- Conducted a research on the effects of 3 pesticides that are suspected of being endocrine disruptors on sword fish and established an assay method for endocrine effects.

Investigation into the endocrine disrupting effects of pesticides on mammals

- Evaluated the effects on reproductive organs in animals for 5 pesticides and tested for dose-response relationships.

2-5. Field of Food & Drug

2-5-1 Monitoring of Pollution Level in Food

The results of monitoring conducted between 1999 to 2005, on pollution level of endocrine disruptors from various materials such as food, containers, drugs, herbal medicine, cosmetic products and dish detergents demonstrated a lower or similar level compared to that of the foreign countries.

2-5-2 Human Health Effects

Performed dynamic researches on reproductive structures (sperm count and male reproductive structure, female infertility), congenital malformation, breast cancer and thyroid malfunction in order to investigation into the effects of endocrine disruptors on human health.

2-5-3 Establishment of Screening Assays

Research projects conducted from 1999 to 2005 on screening and testing methods were consisted of 30 projects for endocrine disruptors.

- 30 projects executed up to 2005 corresponded to Level 2 ~ Level 4 assays that have been recommended in OECD EDTA conferences and developed countries.

Establishment the internationally standardized screening assays

- E-screen assay, enzyme activation assay, estrogen/androgen receptor binding assay

2-5-4 Risk Assessment

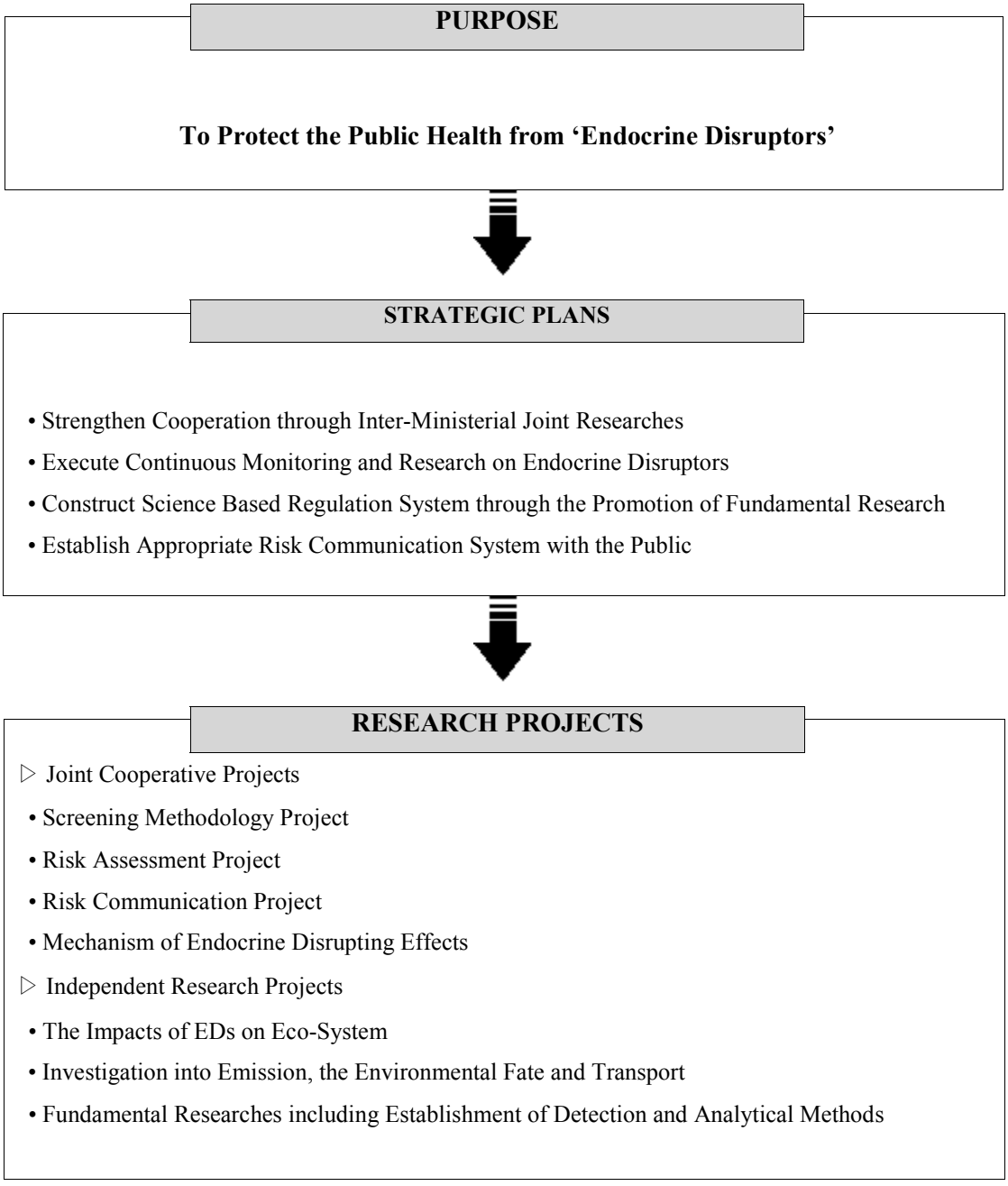
Researches on the methodology of risk assessment have been carried out annually as an independent project of National Institute of Toxicological Research.

- Establishment of Tolerable Daily Intake (TDI) of Dioxin (January 2001): 4 pg TEQ/kg b.w. /day and provided the results for resetting TDI for dioxin (2005 and 2006), etc.
- Tolerable Daily Intake (TDI): Concentration level that is considered to be safe from the lifelong exposure to dioxin

Education & Publicity

- Development of customized education and publicity material by classes

3. 5-Year Plan for Endocrine Disruptors



3-1 Direction & Major Outline of the Plan

3-1-1 Project Direction

Projects will be focused on establishment of safety management policies based on the results of researches performed up to now.

- Monitoring of endocrine disruptors in the terrestrial/marine environment and food present the potential effects on human health to secure the primary data for the future risk assessment.
- Conduct ecological effects on the pollution route and health effects of endocrine disruptors and figure out the exposure related variables.
- Select the hot spots for investigation based on the results of the monitoring on the terrestrial environment and conduct inter-ministerial joint monitoring that takes the food chain of organism species living in the wild into account.

3-1-2 Project Strategy

Classification of research projects into Joint Research Projects and Independent Research Projects

- Joint research projects will be selected through the inter-ministerial policy committee and the research priority will be given to the substances of high public interest or the substances that has become social issues, and risk assessment will be conducted on these substances
- Search for the projects (Inquiry into Endocrine Disruption, Production and Supply of Joint Publicity Materials, Risk Assessment, etc.) among the detailed research projects that can benefit more from inter-ministry networking or joint project plans promote such projects as joint cooperation projects.
- As a strategic approach for the risk assessment of endocrine disruptors, divide the roles based on each ministry's area of work, and share information produced from research projects in risk assessment and management.

Independent research projects will be conducted by establishing independent plans of each ministry.

Preparation of Project Strategy

- Projects of 2007 were performed according to the research plans of each ministry and the details of the projects were discussed among the relevant ministries.
- Starting 2008, research projects shall be conducted more systematically through the selection of joint and independent projects based on the 5-Year Plan.

Projects will be evaluated twice: self-evaluation by each ministry and evaluation on the achievements of inter-ministerial cooperation through the inter-ministry policy committee. The comprehensive evaluation of the ministries will be conducted once every three years through the policy committee and project results will be shared through workshops and symposiums.

Researches on the clarification of ambiguity of endocrine disruption must carry out. The project strategy aims at the establishment of infra-structure for initial risk assessment and plans on advancing into the risk assessment and management by understanding the trends of international organizations such as OECD and the developed countries.

On the specific chemicals that need risk assessment, the ministries such as Ministry of Land, Transport and Maritime Affairs, Ministry of Environment, Ministry for Food, Agriculture, Forestry and Fisheries and Korea Food and Drug Administration come together to agree on the comprehensive research project for carrying out the risk assessment on the whole process including exposure assessment and dose-response

relationship. The Ministry of Environment takes charge of inter-ministerial adjustment and arrangement on research details for the effective and efficient implementation of the project.

Financial resource investment for the next five years (2007~2011) for the implementation actively secure the estimated budget according to the priorities in order to prevent the setback in the research plan from the reduction in research expenses.

3-1-3 Classification into Inter-Ministerial Collaboration Projects and Independent Projects

Subjects such as risk assessment that can gain from the synergistic effects of inter-ministerial cooperation will be selected as the topics for joint projects and cooperative research (task force team formed if necessary) will be promoted.

< Joint Projects & Independent Projects >

Classification	Details	Research Subjects
Joint Projects	**Selection of Joint Project through the Inter-Ministerial of Policy Committee**	**Action Mechanism** **Risk Assessment,** **Education & Publicity**
Independent Projects	Projects that are Suitable for the Responsibilities of Each Ministry but Avoids Duplication through Prior Consultation and Review	**Ministry of Environment**: air, water, soil, sediment, biota **Ministry of Health and Welfares**: food and drug **Ministry of Land, Transport and Maritime Affairs**: marine ecosystem, etc. **Ministry for Food, Agriculture, Forestry and Fisheries**: farmlands, livestock products, etc.

- Data sharing and strengthening of network system through the organization of relevant ministerial policy committee.
- Organization of Endocrine Disruptor Policy Committee: Ministry of Environment act as the national coordinator.
- The construction of cooperative system such as data sharing through the joint symposiums and seminars.

3-1-4 Major Outline of the Plan

Four ministries will invest a total of 51.3 million US dollar on matching /research projects until 2011.

Classification	Field & Projects	Est. Budget (Mil. US dollar)
Ministry of Environment (National Institute of Environmental Research)	20 Projects in 6 Fields	13.1
Ministry of Land, Transport and Martime Affairs (National Fisheries Research and Development Institute)	8 Projects in 3 Fields	13.9
Ministry for Food, Agriculture, Forestry and Fisheries (National Veterinary Research & Quarantine Service)	13 Projects in 5 Fields	2.0
Rural Development Administration	9 projects in 4 fields	4.5
Ministry of Health and Welfare	105 Projects in 5 Fields	10.8

(Korea Food and Drug Administration)		

Considering the ambiguity of endocrine disrupting effects and the international trends, fundamental research and monitoring will be carried out continuously followed by risk assessment and management. Ministries must jointly contemplate on the desirable solution for relieving public's anxiety through systematic and effective risk communication to the public.

<Investment Scale and Details by Field >

Classification	Details	Est. Budget (Mil. US dollar)
Monitoring	Verification of Concentration Level of Endocrine Disruptors in the Terrestrial & Marine Environment, and Food	21.65 (42%)
Risk Assessment	Risk Assessment on Humans & Eco-system	7 (14%)
Fundamental Research such as Action Mechanism	Development of Analytical Methodology & Toxicity Research	22.65 (44%)

3-1-5 Joint Projects and Independent Projects

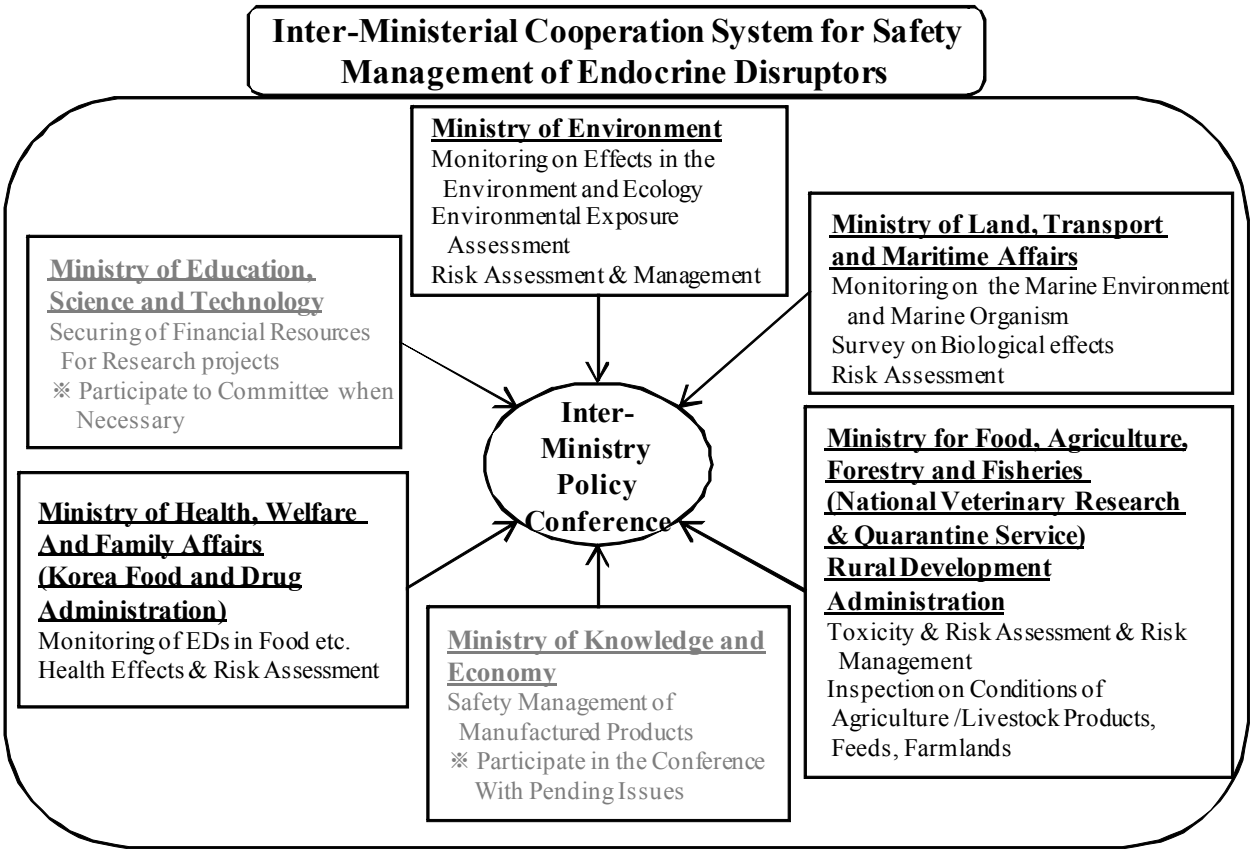
Joint Projects

- Monitoring research **(Joint & Independent)** (Ministry of Land, Transport and Maritime Affairs, Ministry for Food, Agriculture, Forestry and Fisheries, Ministry of Environment, Rural Development Administration, and Korea Food and Drug Administration)
  - Basically, each ministry promotes independent researches but must promote joint monitoring of the areas concerned of contamination (integration of place and substance)
- Development of screening and testing methods **(Joint & Independent)** (Ministry of Land, Transport and Maritime Affairs, Ministry for Food, Agriculture, Forestry and Fisheries, Ministry of Environment, Rural Development Administration, and Korea Food and Drug Administration)
  - Each ministry establishes and develops screening methods that take its responsibilities into consideration, but must promote joint research on some subjects if necessary.
- Inquiry into the mechanisms of endocrine disruption (**Joint & Independent**) (Ministry of Land, Transport and Maritime Affairs, Ministry for Food, Agriculture, Forestry and Fisheries, Ministry of Environment,, Rural Development Administration, and Korea Food and Drug Administration)
  - Each ministry conducts independent projects, such as a research on action mechanism, according to the responsibilities of each ministry, and also conducts joint research by distributing screening tasks of suspected endocrine disruptors that have yet been assessed among the ministries based on the testing methods that have been internationally recognized up to now.
- Risk assessment research **(Joint)** (Ministry of Land, Transport and Maritime Affairs, Ministry for Food, Agriculture, Forestry and Fisheries, Ministry of Environment, Rural Development Administration, and Korea Food and Drug Administration)
  - Ministries promote joint risk assessment research project based on the basic monitoring results of each ministry.

- International Cooperative Project **(Joint & Independent)** (Ministry of Land, Transport and Maritime Affairs, Ministry for Food, Agriculture, Forestry and Fisheries, Ministry of Environment, Rural Development Administration, and Korea Food and Drug Administration)
  - Ministries will prepare joint cooperative plan for the areas that allow joint participation such as the participation in OECD Screening and testing method development, and the projects that will be performed independently by each ministry will remain independent. (e.g.. Korea-Japan Joint Research by the Ministry of Environment)
- Publicity and information sharing projects for the public (**Joint & Independent**) (Ministry of Environment, Ministry for Food, Agriculture, Forestry and Fisheries, Rural Development Administration, Korea Food and Drug Administration)
  - Publicity projects for the public will be conducted independently based on the work distribution among the ministries, and as for the joint projects, the ministries jointly will search for and carry out large-scale projects such as television program development and risk communication program development.

Independent Projects

- Research on ecological effects will be conducted independently due to the differences in the living organisms (ex. Ministry of Environment → inland fish species marine fish species Ministry for Food, Agriculture, Forestry and Fisheries → livestock products).
- Survey of emission source & toxicity reduction technology development
  - Survey of emission source and the development of toxicity reduction technology on substances will be conducted independently since these projects will require specialized responsibilities of each ministry.
- Research on the environmental fate and transport
  - Research on the fate and transport of endocrine disruptors in the environment will be conducted independently due to the differences in the interests and subjected media of each ministry.
- Research on analysis methods
  - This type of research will be also conducted independently due to the difference in the media that are subjected to the research analysis.



3-2. Detailed Project Planning of Each Ministry

3-2-1 Ministry of Land, Transport and Maritime Affairs (National Fisheries Research and Development Institute)

- **Goal**
  Formation of clean marine environment, maintenance of sound marine ecosystem and supply safe fisheries products
- **Overall outline**
  Ministry plans to input 12.8 million US dollars from 2007 to 2011 (5 year period) for the promotion of 8 research projects in 8 fields.

Major research projects

1) Foundation research
   - Establishment of analysis method
   - Establishment of standard methods for EDs in marine environment
   - Development of inter-calibration program for quality assessment / quality control
   - Production of standard reference materials for quality assessment / quality control
2) Monitoring of EDs within the marine ecosystem
   - Monitoring of pollution level in each environmental medium
   - Development of monitoring technique

3) Research on international cooperation & strategic response to domestic/international regulations
- Strengthening foundation for international cooperation
- Propose policies on securing safety of marine organism
- Construction of inter-ministry network

Assessment of ecological effects

1) Research on ecological effects
- Inspection on ecology of marine life in the polluted areas
- Assessment of ecological toxicity

2) Research on toxicity assessment technique
- Development of biomarkers
- Development of screening assay using genetic recombinant microorganism
- Development of kits for screening and bioassay on Eds

Research for the prediction model on the fate of Eds

1) Research on input load within the marine ecosystem
- Identification of inflow pathway of pollution sources and their contribution ratio
- Research on the characteristics of movement and distribution of EDs
- Survey input parameters required in the model

2) Development and application of prediction model on the fate of EDs within the marine ecosystem
- Development of model for predicting EDs' fate
- Construction of cyber monitoring system within the marine ecosystem

3-2-2 Ministry for Food, Agriculture, Forestry and Fisheries (National Veterinary Research and Quarantine Service)

- **Goal**

- Safety management of production of agriculture, forestry and livestock products
- Production and distribution of safe agricultural and livestock products

- **Overall Outline**

- Construction of foundation for toxicity assessment on EDs used or polluted during the production process of agriculture, forestry and livestock products
- Construction of risk assessment and safety management plans for EDs
- Inquiry into the pollution level of EDs in agriculture, forestry, and livestock products and feeds
- Strengthening research on risk reduction
- Activation of risk information exchange and advancement in prevention management system
- Ministry for Food, Agriculture, Forestry and Fisheries plans to input 1.8 million US dollars from 2007 to 2011 (5 year plan) for the promotion of 10 research projects in 4 fields.

- **Major Project Research**

- Development & application of screening assays on EDs
- Risk assessment and establishment of risk management policies on residues of animal drugs, pesticides and environmental contaminants in agricultural or livestock products
- Monitoring and human exposure assessment of EDs
- Research on toxicity prevention or reduction and development of substitute substances
- Construction of prevention management system on EDs in livestock products

Research Details by Field

Development of screening assays

1) Development and establishment of screening technique for EDs with high throughput and high sensitivity
- Development of unique biomarkers for searching endocrine disruption
- Development of endocrine disruption assessment technology using the proteomics and/or genomics

Toxicity & risk assessment

1) Assessment of endocrine disrupting effects of animal drugs and Etc.
- Assessment of thyroid disrupting effects of antimicrobials such as sulfa drugs
  - Research on receptor mediated action mechanism in transgenic cells
  - Research on endocrine disruption through pituitary glands
- Risk reassessment on sulfa drugs and more
  - Re-assessment of 'No Observed Adverse Effect Level (NOAEL)'
  - Re-assessment of 'Acceptable Daily Intake (ADI)'

2) Assessment on carcinogenic Potential of EDs
- Research on the correlation between endocrine disruption and carcinogenicity
  - Research on the correlation between endocrine disruption and carcinogenicity using functional genes
  - Research on time courses of endocrine disturbance and carcinogenicity at the proteomic level
- Research on the creation of appropriate safety factors on EDs
  - Creation of appropriate safety factors by expression mechanism of toxicity and by correlation with carcinogenicity
  -

Monitoring (exposure assessment)

1) Monitoring of EDs in Livestock, livestock products, and feed
- Monitoring of EDs in livestock
- Monitoring of EDs in livestock products
- Monitoring of EDs in feed

2) Monitoring of EDs in the livestock environment

Toxicity prevention & development of substitute substances

1) Development of techniques for toxicity reduction and substitute substances
- Development of cell and animal model for human diseases
- Development of remedy inducing substance with no observed adverse effect level for hormonal receptors using the high sensitivity model
- Detection of toxicity reducing biodegradable substance originating from natural products

Establishment of prevention management system

1) Establishment of information network on the risk of EDs in livestock products
2) Publication of publicity material on prevention measures for EDs in livestock products
3) Joint Research on international cooperation & strategic response to domestic/international regulations

3-2-3 Ministry of Environment (National Institute of Environmental Research)

- **The goal** of ministry of environment is "establishment of infrastructure for sound management of endocrine disruptors"
  - Establishment of screening and risk assessment methodology
  - Preparation of infrastructure for risk assessment based environmental management and policy decision
  - Strengthening domestic and international cooperation for EDs research.

For the accomplishment of the goal, the Ministry of Environment plans to input 13.1 million US dollars from 2007 to 2011 for the performance of 15 research projects in 6 fields classified as below:

- Environmental monitoring (assessment of the environmental contaminated level of EDs)
- Survey on their effect to ecosystem (does-response relationship)
- Investigation of emission sources and affected areas (evaluation of exposure sources)
- Risk assessment and management (risk characterization)
- International cooperation (strengthening of joint research projects)
- Foundation research (examination of endocrine disruption)

- **Each research field consist of some details**

1) Environmental monitoring

Environmental monitoring in a variety of media
- Focusing on the priority chemicals, investigation will be classified based on the background and industrial areas

Environmental biota monitoring
- Biota monitoring will be taken focused on the freshwater fish and amphibians.
- Accumulation pattern of living organisms, concentration level of vitellogenin, and malfunction of reproductive organ are key data of biota monitoring

Environmental sample conservation project
- The ministry of environment makes a building for long term conservation for environmental samples such as soil, sediment, biota including fish and amphibians.

2) Survey on their effect to ecosystem

In-depth Investigation of ecological effects
- Survey on life cycle of indicator organism and ecological test of laboratory scale
- Close examination for the hot spot to identify the effect of EDs

Inquiry into ecological effects
- Research on action mechanism of EDs in the wildlife
- Inspection of species sensitivity and development of indicators of ecological effects

3) Investigation of emission sources and affected areas

Survey of emission sources
- Monitoring of pollutant transport and discharged route

- Preparation of discharge list and emission inventory for priority chemicals

Research on environmental fate

- Development and validation of regional scale fate estimation models

4) Risk assessment & management

Establishment of screening methodology

- Level 1 list of substances of concern
- Level 2 In vitro screening methodology
- Level 3 In vivo biomarker measurement
- Level 4 reproduction test
- Level 5 life cycle assay

Establishment of methodology of risk assessment
Strengthening of risk communication and information service system
Technology for source management

5) Strengthening international cooperation

Strengthening of Korea-Japan joint research and participation of OECD testing program

6) Fundamental research

Strengthening fundamental research on wild life

- Investigation into life cycle of wild life including fish and amphibians
- Investigation into the effects of various environmental factors on living organisms
- Examination on the fate of chemicals within living body

Development of high sensitivity analysis method

- Examination and development of analysis method that is equipped with sufficient sensitivity for risk assessment

Development of testing method

- Search for biomarker using toxicogenomics
- Examination of correlation between the results of in vitro and in vivo test

3.2.4. Rural Development Administration (Mainly conducted by National Academy of Agricultural Science)

- **Goal**

- Construction of risk management system on EDs in agriculture products and agricultural environment

- **Overall outlines**

- Understanding the pollution level of endocrine disrupting pesticides in agricultural products, farmland soil and non-target organisms, fish dwelling in agricultural reservoirs.
- Establishment of standard toxicity and exposure analysis system on pesticides and/or EDs used during the production process
- Global harmonization of national risk assessment and risk management systems for EDs
- Strengthening research on risk reduction and remediation of polluted farm land.
- Activation of industry/academic joint research and international cooperation.
- Consisting of task force team for stable execution of the research plans

- **Major research projects**
- Pollution monitoring (agricultural water, soil, agricultural products) and prediction of behaviors in crop cultivation (study on the fate and mobility of pesticides, trace on pollution routes in crops)
- Investigation of endocrine disrupting effects on agricultural ecosystem (dose-response assessment, biomonitoring of endocrine disrupting pesticides in agricultural reservoirs)
- Establishment of assessment system on human risks (risk assessment of pesticides for farmland workers) Basic research (establishment of analytical methods, risk communication etc)

- **Projects details**

1) Monitoring of pollution level and fate study

Pollution monitoring
- Sampling site: farms, orchards, paddy field, etc.
- Sampling matrices: soil of farmland, agricultural crops, agricultural water
- Analytes: dioxins, PCBs, PAHs, organochlorine, etc.

Research on fate of pesticides in the agricultural environment
- Research on the source of pollutants
- Research on the characteristics of mobility and distribution of pesticides
- Research on residual properties in various conditions

Study on mobility of pesticides into crops
- Research on the mobility path of pollutants in crops
- Study on mobility assessment using the typical regional models
- Investigation of the mobility characteristics by major crops and target pesticides

2) Biological effect assessment of pesticides suspected as EDs in agricultural ecosystem

Inspection of harmful effects on agricultural ecosystem
- Monitoring of pollution level in fish living in the major agricultural reservoirs
- Study of adverse effects on the indicator organisms

Research on test methods for the assessment of ecological effects
- Research on the mechanism of endocrine effects in the environmental organisms
- Development of exposure and effect indicators
- Development of screening methods, study of species sensitivity, etc.

3) Research on the human risk assessment of pesticidews suspected as Eds

Research on the exposure assessment of agricultural workers and risk reduction
- Survey of the pesticide use
- Bio-monitoring on agricultural workers
- Establishment of risk assessment system for agricultural workers
- Research on risk reduction from pesticides for agricultural workers

Risk analysis of polluted agricultural products
- Monitoring and risk assessment of the pollution level in agricultural products
- Determination of NOAEL
- Development of risk mitigation measures

4) Basic research on pesticide suspected as Eds

Establishment of analysis methods on EDs
- Establishment of standard test methods on EDs
- Development of mutual verification program for QA and QC management
- Production of standard substance for QA and QC management

Strengthening of international cooperation and risk communication system
- Strengthening of foundation for international cooperation
- Proposal of policies and plans for the securing of safety in agricultural products
- Construction of joint information network on inter-ministry strategic plans

3.2.5. Korea Food and Drug Administration (National Institute of Toxicological Research)

- **Goal**
- Preparation of scientific and rational management plans on EDs

- **Overall outline**
- Strengthening research base on EDs
- Extending research on the health effects of Eds
- Information sharing from industry – academic relation and domestic/international network
- Strengthening research on risk reduction & safety management
- Korea Food and Drug Administration plans to input 9.9 million US dollars from 2007 to 2011 into the promotion of 105 research projects in 5 fields.

- **Major research projects**
- Monitoring of food, food packagings, cosmetics, etc.
- Establishment of screening & testing method and development of assessment technology
- Human health effects
- Toxic effects and action mechanism
- Safety management methodology
- Publicity and public education

- **Detailed research fields**

1) Monitoring

Monitoring of EDs in food and food containers, etc.

2) Development of on screening & testing method through international cooperation

Establishment of screening and testing method on EDs
Assessment of EDs using high throughout technology such as tanscriptomics

3) Toxic effects

Research on toxicity and action mechanism
- Study at the individual level
- Study at the cellular and molecular level
- Study on the next generation effects

4) Human health effects

Monitoring of EDs in the human biological samples

- Investigation of the exposure amount of EDs contained in the human biological samples such as breast milk
- Investigation of the relativity between the number and motility of sperm and EDs

Research on the correlation between the endocrine diseases and EDs

5) Research on safety management methodology

Information system on EDs

- Construction of endocrine disruptor related database

Risk assessment and management system

- Risk assessment and risk communication system

Publicity and public education

Safety management methodology

Appendix 7

Contribution from the United Kingdom

UK Contribution to the Development of a Report on Endocrine Disrupters Assessment in OECD Member Countries.

Introductory Note

This paper reflects opinions across the community in the UK involved in the development of OECD validated tests for endocrine disrupting chemicals, rather than presenting solely a UK Government view.

General Approach

1. Where the UK has strong suspicions about the endocrine disrupting activities of specific chemicals, our basic approach is to put them forward for a full risk assessment at the European level – so that where risks are identified, European-wide measures will be taken to control them. The UK has led such risk assessments for several chemicals that have featured prominently in discussions about endocrine disruption, including nonylphenol and bisphenol A.

Human Health

2. The use of specific tests for endocrine disruption in the assessment of human health risks is quite different from that in the environmental arena.

3. For the assessment of the potential risks to human health, UK regulators do not generally recommend that specific tests are carried out for substances known or suspected to have endocrine disrupting potential. UK regulatory authorities require new chemicals to be tested using the standard toxicology test requirements established by the relevant UK or European authority for that class of chemical (i.e. high production volume chemical, pesticide, pharmaceutical, food additive etc). If standard toxicity tests reveal effects on an endocrine system, then the significance for human health would be assessed before a decision on approval or risk reduction measures is taken. Similarly, specific tests for endocrine disruption have not been used in the UK to assess whether chemical contaminants may have an endocrine disrupting effect, although this remains a possibility for the future. Specific tests for effects on the endocrine system may be useful for clarifying a mechanism of an effect observed in a standard toxicology test. In practice, it is rare for the UK to commission toxicological testing of specific contaminants. Government research funds are limited and it is more likely for programmes of research on specific themes to be commissioned, rather than toxicity tests on specific chemicals.

4. The most likely use of specific tests for endocrine disruption is within industry, for screening of new candidate chemicals for endocrine testing effects prior to making a decision on whether to take them forward for standard toxicological testing.

Plant Protection Products

5. Basically, the UK does not have a set procedure for testing for endocrine disrupting activity for chemicals in this category, but assessments conducted under Directive 91/414/EEC are based on information in the EU aquatic and terrestrial guidance documents SANCO/3268/2001 (rev 4) and SANCO/10329/2002 (rev2).

6. When considering a pesticide that has potential endocrine disrupting effects, the lead is usually provided by the mammalian toxicological assessment – that is, if the assessment for human health indicates that the active substance may have endocrine disrupting properties, then further ecotoxicological data may be requested. For example, when considering aquatic life we would consider requesting either a full fish life cycle study or a partial fish life cycle study. As regards other

areas – such as aquatic invertebrates, birds and terrestrial invertebrates - there is currently a lack of study protocols or guidelines to enable the potential to be determined appropriately. When interpreting any data that indicate a potential effect, it is necessary to consider the exposure profile; i.e. is the potential for endocrine disruption due to short or prolonged exposure? There is currently a lack of information on this and hence a precautionary approach tends to be taken that assumes that effects are the result of short exposures.

Environmental Assessments

7. With regard to environmental assessments, the UK tends to take a pragmatic approach to the testing of potential endocrine disrupting chemicals, so that non-standard data on endocrine effects are taken into account - if they can be credibly shown to link with an adverse health effect in an individual, which in turn may have a population level impact and as long as they appear to be scientifically reliable. For example, when setting draft EQSs for certain organophosphates recently under WFD Annex VIII, data on possible interference with fish olfactory membranes and consequent pheromonal triggering of sperm maturation were considered. Equally, experimental data on super-ovulating female gastropods were taken into consideration for the recent risk assessment of bisphenol-A, along with other data, though on a relative 'weight' basis.

8. The UK has tended to be wary of the direct use of biomarkers of endocrine disrupting activity, such as vitellogenin (VTG) induction and hormone imbalances, for risk assessment purposes because the mechanistic links with adverse health effects of population relevance have not yet been demonstrated. It is certainly the case, though, that some of these links are getting stronger[6] and this is an area of work of high priority. Similarly, for ovotestis in fish induced by endocrine disruption, there are data that provide evidence for a link with the production of gametes of poorer quality[7].

9. Whilst it would be unsound to claim that biomarkers of ED are now ready for critical use in risk assessment and as key evidence, they may be used in a supporting role (e.g. to validate apparent outliers); for example, if a fish life cycle test gave a very low NOEC which might otherwise be considered an outlier, VTG data showing effects at a similar concentration could be used as corroborative evidence when setting an EQS (or more strictly, in setting a PNEC). Additionally, biomarkers such as VTG can have animal welfare benefits in the sense of being used to guide test concentration selection and aid efficient test design[8,9]).

[6] See, for example: Miller DH, Jensen KM, Villeneuve DL, Kahl MD, Makynen EA, Durhan EJ, et al. 2007. Linkage of biochemical responses to population-level effects: a case study with vitellogenin in the fathead minnow (*Pimephales promelas*). Environ Toxicol Chem 26: 521-527; and

Folmar LC, Gardner GR, Schreibman MP, Magliulo-Cepriano L, Mills LJ, Zaroogian G, Gutjahr-Gobell R, Haebler R, Horowitz DB, and Denslow ND. 2001. Vitellogenin-induced pathology in male summer flounder (**Paralichthys dentatus**). Aquat Toxicol. 51:431–441; and also

Karen A. Kidd, Paul J. Blanchfield, Kenneth H. Mills, Vince P. Palace, Robert E. Evans, James M. Lazorchak and Robert W. Flick Collapse of a fish population after exposure to a synthetic estrogen (http://www.pnas.org/content/104/21/8897.full).

[7] See, for example: Jobling, S., Coey, S., Whitmore, J. KIme, D.E., vanLook, KL, McAllister BG, Beresford, N., Henshaw, AC, Brighty, G., Tyler, C.R. and Sumpter, J.P. (2002). Wild roach (*Rutilus rutilus*: Cyprinidae) living in effluent contaminated rivers have reduced fertility. Biology of Reproduction 67 (2):515-524

[8] Williams TD, Caunter JE, Lillicrap AD, Hutchinson TH, Gillings EG, Duffell S (2007) Evaluation of the reproductive effects of tamoxifen citrate in partial and full life-cycle studies using fathead minnows (*Pimephales promelas*). *Environmental Toxicology and Chemistry* 26: 695-707

10. In short, the UK supports the view expressed in VMG-eco, that the use of biomarkers in definitive apical tests (e.g. fish lifecycle tests) is likely to be desirable rather than compulsory – e.g. for use in the rare cases when a substance is being tested for ED-related hazards at Level 4 without knowledge of its mode of action from mammalian or *in vitro* studies.

11. However, where data are obtained from tests that have not been validated and internationally standardised, especially where there are difficulties in replicating the results, then disputes over interpretation can easily arise. This is a particular problem in assessments of potential endocrine disrupting chemicals, since there are as yet no EDC-sensitive OECD guidelines (apart from the mammalian uterotrophic assay), despite almost a decade of extensive effort including discussion, provision of extensive data sets, reports and even a series of directed studies to address standardisation issues. A fish life cycle test conducted to USEPA guidelines is the only occasion when we can be reasonably sure that endocrine effects will have been covered. For invertebrates, the only routinely used reproductive test is the Daphnia 21 day guideline, which is certainly not sensitive to several relevant modes of EDC action.

12. Such uncertainty creates costly difficulties for industry and regulators alike; the UK therefore fully supports the OECD in attempting to speed up the test validation process, on the basis of continuing sound science, and sees it as a high priority to work with partners to that end. One way forward may be to truncate the current validation criteria – for example, validation of fish life cycle tests might be limited to a single species and more use could be made of published data. There may also be no need to duplicate the validation of fecundity measurements if these have already been validated in partial lifecycle tests.

13. Further priorities for the UK are:

    a) ethical issues, such as the reduction of the numbers of fish tested via the efficient use of biomarkers, or the replacement of vertebrate test species by the use of mechanistically relevant alternative test species[10];

    b) to extend coverage to animal groups of intrinsic ecological and economic importance which currently lack suitable ED tests, such as molluscs (it being recognised that certain groups may initially require studies of fundamental physiology); and

    c) getting endpoints/tests accepted for EDCs with specific modes of action for the present guideline species.

UK Department for Environment, Food and Rural Affairs
August, 2008

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[9] Hutchinson TH (2007) Small is useful in endocrine disrupter assessment – four key recommendations for aquatic invertebrate research. *Ecotoxicology* 16: 231-238

[10] Hutchinson TH, Caldwell D, Galay-Burgos M, Hartmann A, Holt M, Huggett D, Mastrocco F, Maund S, Oberwalder C, Versteeg D (2007) Intelligent testing strategies in ecotoxicology: mode of action approach for specifically acting chemicals. ECETOC Technical Report number 102, 145 pp

Appendix 8

Contribution from the United States

Overview of U.S. EPA Endocrine Disruptor Screening Program (EDSP) For Contribution to OECD Case Study Document

Final draft: Aug 14, 2008

I. Introduction

In May 2008 a notice was sent from the OECD WNT - EDTA to the working group for a request for contributions for the development of a "case study" document that is expected to lead to a "Report on Endocrine Disruptors Assessment in OECD Member Countries".

In response, the US EPA is submitting its contribution by providing an overview of its Endocrine Disruptor Screening Program (EDSP) which is a two-tiered system for screening and testing the potential interactions of pesticide and non-pesticide chemicals on the estrogen, androgen and thyroid (EAT) hormonal systems with application to human health, animal wildlife and the environment. To address how the EPA expects to screen for potential endocrine disruptors, a discussion is presented herein with emphasis on the EDSP Tier-1 battery. Selective examples are presented to illustrate the potential to detect chemicals with estrogenic, androgenic and thyroidogenic activity. The *in vitro* and mammalian and non-mammalian *in vivo* screening assays were designed to cover agonistic and antagonistic effects involving the estrogen and androgen modes of action and respective steroidogenic pathways as well as the hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-thyroidal (HPT) axis. It will be emphasized how complimentary and corroborating evidence among assays within the Tier-1 battery can be used with a weight-of–evidence approach to determine whether or not a test chemical interacts with the endocrine system. If results from Tier 1 are positive, indicating a substance does exhibit the potential to interact with the E, A or T hormonal pathways, then more complex and definitive dose-response testing would likely be done in Tier 2 to further identify the potential hazard and to assess adversity and risk to the public and the environment.

II. Overview of EDSP

A. Congressional mandate

Passage of the Food Quality Protection Act (FQPA) in 1996 and subsequent amendments to the Safe Drinking Water Act (SDWA) and Federal Food, Drug, and Cosmetic Act (FFDCA) required EPA to:

> *develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or other such endocrine effect as the Administrator may designate [21 U.S.C. 346a(p)].*

In response to this mandate, the Agency established a multi-stakeholder federal advisory committee, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) under the Federal Advisory Committee Act (FACA), 5 U.S.C. App. 2, Section 9(c). This committee was asked to provide advice to the Agency on how to design a screening and testing program for endocrine disrupting chemicals. In 1998, the EDSTAC published their final report, which included three fundamental recommendations as summarized below:

1) Expand the evaluation of additional modes of action beyond estrogen disruption to include test systems that detect androgen and thyroid disruption via the hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-thyroidal (HPT) axes.

2) Expand the target population beyond humans to include animal wildlife.

3) Incorporate a two-tiered approach whereby Tier 1 would consist of a suite of complementary assays designed to be run together as a battery to effectively and efficiently screen substances for interactions with the estrogen, androgen and thyroid (EAT) hormonal systems. If, by weight-of-evidence, the results from the Tier-1 battery indicate that a test substance does exhibit the potential to interact with the E, A or T hormonal systems, then additional testing would be required in Tier 2 with more comprehensive assays to further identify the potential hazard and assess risk through dose-response relationships.

The EPA considered the recommendations from EDSTAC, adopted the two-tiered testing strategy, and expanded the EDSP to include the androgen and thyroid hormonal systems, as well as animal wildlife as reviewed in detail at the EDSP website: http://www.epa.gov/scipoly/oscpendo/

B. Assay validation

The fundamental basis for assay validation is to establish relevance and reliability. In the context of the EDSP Tier-1 screening battery, relevance is the ability of an assay or endpoints within an assay to detect chemicals with the potential to interact with one or more of the EAT hormonal systems, whereas reliability is the reproducibility of those results within and between or among laboratories. Throughout the validation process of individual assays and in accord with the FACA, the EDSP sought guidance (e.g., protocol development, selection of known positive and negative test chemicals, and interpretation of results) from within the EPA (Office of Research and Development, ORD) and federal advisory committees such as the Endocrine Disrupter Methods Validation Sub-committee (EDMVS), Endocrine Disrupter Methods Validation Advisory Committee (EDMVAC) and FIFRA SAP from 2001 through 2007 that were open to the public for comment.

The EPA has followed a five-stage assay validation process that included: 1) Test development, 2) Pre-validation, 3) Inter-laboratory validation, 4) Peer review and 5) regulatory acceptance as reviewed in detail at the EDSP website. http://www.epa.gov/scipoly/oscpendo/pubs/assayvalidation/status.htm

C. Basis for assay selection for the Tier 1 screening battery

The screening battery was designed to work as a whole. The basis for selecting a candidate assay to include in the battery involved: 1) the capacity of that assay to detect estrogenic- and androgenic-mediated effects by various modes of action including receptor binding (agonist and antagonist) and activation/transcription, reproductive steroidogenesis, and hypothalamic-pituitary-gonadal (HPG) feedback, and 2) the degree that *in vitro* and *in vivo* assays complemented one another in the battery as summarized in Table 1 below. In addition, rodent and amphibian *in vivo* assays were selected for the proposed battery based on their capacity to detect direct and indirect effects on thyroid function (hypothalamic-pituitary-thyroidal, HPT, feedback). Thus, the robustness of the proposed Tier-1 Screening Battery is based on the strengths of each individual assay and their complementary nature within the battery to detect effects on EAT hormonal function.

Table 1: Suite of Assays in the EDSP Tier-1 Screening Battery

In vitro
Estrogen receptor (ER) binding – rat uterus or recombinant
Estrogen receptor (hERα) transcriptional activation - Human cell line (HeLa-9903)
Androgen receptor (AR) binding – rat prostate
Steroidogenesis – Human cell line (H295R)
Aromatase – Human recombinant
In vivo
Uterotrophic (rat)
Hershberger (rat)
Pubertal female (rat)
Pubertal male (rat)
Amphibian metamorphosis (frog)
Fish short-term reproduction

The endocrine modes of action and complimentary assays expected to detect effects on the E, A or T hormonal systems are indicated:

1) *Estrogenicity or anti-estrogenicity* – acting agonistically by potentiating or antagonistically by attenuating the estrogen signal as determined by the combined results of the *in vitro* estrogen receptor binding and transcriptional activation assays and *in vivo* uterotrophic, pubertal female and fish short-term reproduction assays.

2) *Androgenicity or anti-androgenicity* - acting agonistically by potentiating or antagonistically by attenuating the androgen signal as determined by the combined results of the *in vitro* androgen receptor binding assay and *in vivo* Hershberger, pubertal male and fish short-term reproduction assays.

3) *Steroidogenic effects* – altering steroidogenic processes by inducing or inhibiting enzymes involved in gonadal steroidogenesis as determined by the combined results of the *in vitro* steroidogenesis and aromastase assays and *in vivo* pubertal female and male and fish short-term reproduction assays.

4) *Hypothalamic/pituitary/gonadal effects (HPG)* – interference with the hypothalamic-pituitary regulation of gonadal steroidogenesis and gametogenesis as determined by the combined results of the *in vivo* pubertal female and male and fish short-term reproduction assays.

5) *Hypothalamic/pituitary/thyroid effects (HPT)* – altering processes directly involved in thyroid hormone receptor interactions and indirectly involved in thyroid function as determined by the combined results of *in vivo* pubertal female and male and amphibian metamorphosis assays.

In vitro and *in vivo* assays are included in the Tier-1 screening battery to cover multiple modes of action and provide corroborating information among assays within the battery that will support a weight-of-evidence approach. The degree of complimentary actions among assays in the battery is summarized in Table 2.

Table 2. Modes of Action and Degree of Complimentary Action among Assays in the EDSP Tier-1 Screening Battery.

Battery of Assays	Modes of Action							
	E[2]	Anti-E	A[2]	Anti-A	Steroidogenesis		HPG[3] Axis	HPT[3] Axis
					E[2]	A[2]		
In vitro								
ER Binding[1]	■	■						
ER α Transcriptional Activation	■							
AR Binding[1]			■	■				
Steroidogenesis H295R					■	■		
Aromatase Recombinant					■			
In vivo								
Uterotrophic	■							
Hershberger			■	■				
Pubertal Male			■	■		■	■	■
Pubertal Female	■	■			■		■	■
Amphibian Metamorphosis								■
Fish Short-term Reproduction (male & female)	■	■	■	■	■	■	■	

[1]Estrogen and Androgen Receptor binding
[2]Estrogen and Androgen
[3]Hypothalamic-pituitary-gonadal or –thyroidal axis

When all Tier-1 assays are performed and all assays are negative within the battery, it may be concluded that the test substance will not likely interact with EAT hormonal processes. If results from Tier 1 indicate that a substance does exhibit the potential to interact with E, A or T function, then more complex and definitive dose-response testing would be done in Tier 2.

D. SAP peer review of EDSP Tier-1 screening battery

EPA's Tier-1 battery was reviewed by the FIFRA SAP. The SAP was charged with commenting on whether the proposed battery composition of assays fulfills its purpose to identify the potential of a test chemical to interact with the E, A or T hormonal systems. The final SAP report to the Agency can be found at the SAP website http://www.epa.gov/scipoly/sap/meetings/2008/march/minutes2008-03-25.pdf .

The SAP discussed assays individually and as a battery and concluded:

1) Chemicals testing positive in the battery of Tier-1 assays would be identified as potential estrogenic, androgenic and thyroid hormone active substances.
2) The ability to identify endocrine active substances is enhanced in the Tier 1 battery because the tests provide adequate replication and redundancy.
3) It was clear that the inclusion of apical assays of amphibian metamorphosis and fish short-term reproduction were important to detect endocrine active substances that may operate by mechanisms of action yet to be discovered.
4) The 15-day adult male assay proposed during some public comments would not be an appropriate substitute for the male and female pubertal assays because the pubertal assays provide for differences between the sexes and provide the only approach to testing for organizational effects during development.

Thus, the SAP found the proposed EDSP Tier-1 battery of assays in Table 1 adequate for screening chemicals to detect effects on the E, A or T hormonal systems.

III. Dynamics of the EDSP Tier-1 Screening Battery

A. Assays for detection of compounds that affect the estrogen signaling pathway

The earliest concern for endocrine disruptors was related to environmental chemicals that could bind to the estrogen receptor and thereby interfere with the estrogenic signaling pathway. Hence, it was this concern that led to the statutory requirement in the FQPA to screen pesticide chemicals for estrogenic effects and is, therefore, the first mode of action that the EPA and EDSTAC considered in designing a battery for the EDSP. Estrogen is important for reproductive function in both males and females, including sexual differentiation of the brain and development of secondary female sex characteristics. In addition, estrogen is involved in the structural and functional development of other bodily systems across genders and for maintaining overall homeostasis.

Five assays within the EDSP Tier-1 screening battery are capable of detecting whether or not a chemical affects estrogen receptor function. Together these assays are expected to detect chemicals with estrogenic and anti-estrogenic activity and include: 1) estrogen receptor (ER) binding, 2) ER transcriptional activation, 3) uterotrophic, 4) pubertal female, and 5) fish short-term reproduction assays. Of the five assays, the two *in vitro* assays (ER binding and transcriptional activation) identify the ability of the test chemical to interact with the estrogen receptor, thus providing mechanistic information about how the chemical interacts at the cellular level. The three *in vivo* assays provide confirmatory evidence for the effects of the chemical following *in vivo* exposure via subcutaneous injection, oral gavage, and aquatic medium, respectively. The uterotrophic assay has been shown to detect weak estrogens with subcutaneous treatment, while the female pubertal assay can detect both estrogen agonists and antagonists by examining the age at vaginal opening (VO) in addition to other estrogen-dependent endpoints. While many chemicals are active via the subcutaneous route, in some instances, oral exposure is more effective because of differences in absorption and metabolism. The different routes of exposure associated with the uterotrophic, female pubertal and fish short-term reproduction assays add robustness to the Tier-1 battery and may offer guidance when designing EDSP Tier-2 tests. Interpreting the results of the subset of estrogenic/anti-estrogenic assays within the battery is accomplished by examining the results of all the assays, as the sum of all of the datasets in a weight-of-evidence approach is far greater than the information provided by any one assay alone. A brief description as well as the value of each of the five assays for detection of compounds that can potentially affect the estrogen signaling pathway is provided. In addition, examples with positive test chemicals involving the following assays and this MOA are presented in Section IV.

1. ER Binding Assay

The ER receptor binding assay utilizing rat uterine cytosol (RUC) is a rapid *in vitro* assay that measures the affinity of a test chemical to bind to the estrogen receptor (or ligand binding domain of the estrogen receptor in the case of the human recombinant ER binding assays). It is a mechanistic assay that measures ligand-receptor interactions. It cannot distinguish between agonists, antagonists, or chemicals that have mixed agonist/antagonist activity or functional consequences of the interaction. Yet, the technical simplicity and high-throughput format are conducive for screening large numbers of chemicals. Thus, the assay is a valuable tool for identifying chemicals that can compete with endogenous estrogen for ER binding. The practical use of this assay and its relevance to *in vivo* effects is well documented in the scientific literature. The assay has been standardized and examined with a number of positive and negative test compounds. Inter-laboratory validation of the ER binding assay will be completed and results submitted for peer review in late 2008. A protocol is expected to be available at the EDSP website in early 2009.

2. ER Transcriptional Activation Assay

The ER transcriptional activation (ERTA) assay is a method to detect the interaction and response of a chemical on the estrogen receptor. ERTA assays are based upon the expression of a reporter gene induced by a chemical following the ligand-receptor binding and subsequent transcriptional activation. As part of the Endocrine Disruption Testing and Assessment Task Force activity under the OECD Test Guidelines Program, Chemicals Evaluation and Research Institute (CERI) of Japan developed and validated a stably transfected transactivation assay with ERα using the hER-HeLa-9903 (HeLa) cell line. This assay complements the ER binding assay as it can identify ER agonists. Thus, this assay is included in the battery as a more contemporary and functional approach to detect ERα agonists.

3. Uterotrophic Assay

The uterotrophic assay is an *in vivo* assay that primarily evaluates the ability of a chemical to elicit uterine changes consistent with the effects of estrogen agonists. The assay is conducted using adult ovariectomized females but may be conducted with sexually immature intact female rats in which endogenous estrogens are minimal. An increase in uterine weight in response to estrogen–induced water imbibition and hypertrophy is the principle endpoint. By using a subcutaneous (sc) route of exposure, the uterotrophic assay contributes information on a specific estrogen-related biological response that precludes any first-pass liver metabolism. Thus, data from the uterotrophic assay can complement the *in vitro* ER assays where metabolic activity is either non-detectable (ER binding) or minimal (ERTA assay) or has first pass through the liver as in the *in vivo* female pubertal assay, which involves oral exposure. For example, chemicals that are estrogenic (*e.g.*, bisphenol A) would be positive in the ER binding, ERTA, and uterotrophic assays, whereas chemicals that need to be metabolized in order to be estrogenic (*e.g.*, methoxychlor) may be weak or likely missed in ER binding, ERTA and uterotrophic assays, but positive in the female pubertal assay.

4. Pubertal Female Assay

The pubertal female assay is an *in vivo* assay that is sensitive to estrogens and anti-estrogens. It is the only bioaassay currently validated that can detect estrogen receptor agonists. For example, chemicals with estrogenic activity (e.g., methoxychlor, nonylphenol, and octylphenol) hasten the age of vaginal opening (VO) and onset of puberty. When tamoxifen a selective estrogen receptor modulator (SERM) with mixed agonist/antagonist activity was examined, VO was hastened in response to the tamoxifen-induced estrogenic effects which was substantiated by increased organ weight changes of the uterus and vagina. Estrogenic effects also accelerate the age at first estrus and can induce vaginal cornification. Although

there is a paucity of data describing the effects of known anti-estrogenic compounds in the pubertal female assay because few, if any, environmental anti-estrogens have been identified, the presumptive response to an anti-estrogen would be a delay in VO and onset of puberty. Since the age of VO can also be delayed by an effect on the HPG-axis, change in the time to VO is not necessarily diagnostic for specific ER effects. Nonetheless, when used in combination with the *in vitro* ER binding, ERTA and uterotrophic assays, the distinction between an ER mechanism and other HPG mechanisms is readily apparent. Although knowing the MOA of a particular chemical-induced response is not a criteria for Tier 1, any information gained in this area may be used in designing a strategy for further testing in the EDSP Tier 2.

The female pubertal assay can contribute information on specific estrogen-related biological responses for which absorption, distribution, metabolism and excretion (ADME) are fully taken into account and is crucial to the identification of anti-estrogens and SERMs (e.g., tamoxifen) since oral gavage is the route of exposure.

5. Fish Short-Term Reproduction Assay

The fish short-term reproduction assay with fathead minnows is designed to detect changes in spawning, morphology, and specific biochemical endpoints that reflect disturbances in the HPG axis in response to estrogen agonists and antagonists. Collectively, the endpoints allow for inferences with regard to possible endocrine disturbances involving the estrogen hormonal pathway and, thus, provide guidance for further testing in the EDSP Tier 2.

Vitellogenin is an egg yolk protein in which production is primarily controlled through estrogen-receptor interactions. There are commercially available immunoassay kits specific to the fathead minnow that have made vitellogenin production readily measurable; hence, it is a well-established endpoint. Induction of vitellogenin in male fish is an extremely sensitive and specific indication of ER agonists since males have very low circulating concentrations of endogenous estrogen. Reproductively active females have moderate circulating concentrations of vitellogenin, which can be decreased by ER antagonists. Estrogens and anti-estrogens can also affect egg production in the fish assay. Changes in fecundity combined with alterations in gonadal histopathology provide a good indication of reproductive health and have been demonstrated to be sensitive to estrogenic and anti-estrogenic exposures.

B. Assays for detection of compounds that affect the androgen signaling pathway.

Androgens are critical for sexual differentiation and development of secondary sex characteristics in the male, as well as for a wide variety of functions in both males and females. To date, a number of environmental chemicals have been shown to act as androgens or anti-androgens. Four assays within the EDSP Tier-1 screening battery are capable of detecting whether or not a chemical affects androgen receptor function. Together these assays are expected to detect chemicals with androgenic and anti-androgenic activity and include: 1) AR binding, 2) Hershberger, 3) pubertal male and 4) fish short-term reproduction assays.

The *in vitro* AR binding assay provides mechanistic information on the cellular (nuclear) mode of action. The three *in vivo* assays provide confirmatory evidence for the effects of a chemical on the reproductive system. Specifically, the Hershberger assay is diagnostic for both androgenic and anti-androgenic activity. The male pubertal and fish reproduction assays reflect changes in AR regulation but, due to their apical nature, are also sensitive to chemicals that may affect other modes of action involved in HPG function. Again, interpreting results of these assays within the battery is accomplished by examining the results of all assays, as the sum of all of the datasets in a weight-of-evidence approach is far greater than the information provided by any one assay alone. A brief description as well as the value of each of the four assays for detection of compounds that can potentially affect the androgen signaling pathway is

provided. In addition, an example with a positive test chemical involving the following assays and this MOA is presented in Section IV.

1. AR Binding Assay

The androgen receptor binding assay (AR binding), utilizing rat prostate cytosol, is a rapid *in vitro* assay that measures the affinity of a test chemical to bind to the androgen receptor. It is a mechanistic assay that measures only ligand-receptor interactions. As with the ER binding assay, its technical simplicity and high-throughput format are conducive for screening large numbers of chemicals. Thus, the assay is a valuable tool for identifying chemicals that can compete with the endogenous ligand.

While the AR binding assay detects both agonists and antagonists, it cannot distinguish between the two. Thus, it can be used in conjunction with the Hershberger assay which can distinguish agonists from antagonists.

2. Hershberger Assay

The Hershberger assay is a short-term *in vivo* screen that evaluates the ability of a chemical to elicit biological activities consistent with either androgen agonists or antagonists by utilizing changes in the weights of five androgen-dependent tissues: 1) ventral prostate, 2) seminal vesicle, 3) levator ani-bulbocavernosus (LABC) muscle, 4) Cowper's glands, and 5) glans penis. Specifically, an increase in tissue weights is diagnostic of androgenic activity. In contrast, an anti-androgenic chemical will block any increase in tissue weights when co-administered with an androgen such as testosterone propionate. The Hershberger contributes to the battery by providing information on a specific androgen-related biological response and, being an *in vivo* assay, integrates ADME into the responses. The assay has been used to identify the anti-androgenic effects of several chemicals including vinclozolin and flutamide.

3. Pubertal Male Assay

The male pubertal assay is an *in vivo* test sensitive to disruptions by chemicals that act as androgens or anti-androgens or interfere with androgen synthesis. Importantly, as an *in vivo* assay, it can detect chemicals which require metabolism in order to interact with the AR. For example, chemicals such as vinclozolin delay the age of preputial separation (i.e., onset of puberty) and decrease the growth of androgen dependent tissues. The male pubertal assay is reproducible and sensitive for chemicals which alter androgenic hormone action and provides useful confirmatory information for AR agonists and antagonists which are detected in the *in vitro* AR receptor assay.

4. Fish Short-Term Reproduction Assay

Secondary sex characteristics of fathead minnows are endpoints that are affected by androgenic/anti-androgenic substances. Specifically, females will develop external male secondary sex characteristics (nuptial tubercles) when exposed to an AR agonist. This endpoint is not only specific for this mode of action, but very sensitive in that females typically do not express these characteristics. In contrast, AR antagonists decrease the expression of male secondary sex characteristics in male fathead minnows. Changes in secondary sex characteristics in fathead minnows are biologically relevant, unique and robust. Inter-laboratory comparisons of these endpoints have been reproducible. Androgens and anti-androgens also effectively inhibit egg production in the fish assay with concurrent alterations in gonadal histopathology.

C. Assays for detection of compounds that affect reproductive steroidogenesis

A number of environmental compounds have been shown to interfere with the synthesis of estrogens (*e.g.*, estradiol) and androgens (*e.g.*, testosterone). In this regard, a number of *in vitro* assays for steroidogenesis were considered for the battery with the decision to include the H295R cell line as it offers the potential to identify chemicals that induce or inhibit estradiol and testosterone synthesis. In addition, since many environmental compounds are known to inhibit aromatase and the conversion of androgen substrates to estrogen, the decision was made to validate a human recombinant aromatase assay. A combination of *in vitro* and *in vivo* assays is expected to provide sufficient information for making informed decisions as to whether or not a compound interferes with the production of estrogens and androgens and include: 1) steroidogenesis, 2) aromatase 3) pubertal female, 4) pubertal male and 5) fish short-term reproduction assays. A brief description as well as the value of each of the five assays for detection of compounds that can potentially affect steroidogenesis is provided. In addition, an example with a positive test chemical involving the following assays and this MOA is presented in Section IV.

1. Steroidogenesis (H295R) Assay

H295R is a human adrenocortical carcinoma cell line that possesses all of the key enzymes throughout the steroidogenic pathways. Several studies have shown that these enzymes and their mRNA and intermediate and end products can all be readily measured in a high-throughput format. For the purposes of the EDSP Tier-1 screening battery, the measurement of estradiol and testosterone produced with or without the test compound are the key endpoints. Considering that the H295R cell's ability to metabolize xenobiotics is apparently low, the assay provides a straightforward, inexpensive and specific way to detect chemicals that affect steroid hormone synthesis either by inhibiting the enzymes in the pathway, leading to decreased production of one or more of the endpoints hormones, or inducing the production of enzymes, leading to increased production of one or more of the endpoint hormones. While other assays in the battery can detect the adverse effects of chemicals that interfere with steroid hormone synthesis, they can not identify the specific component of the pathway that was altered. The H295R steroidogenesis assay has been standardized and examined with a number of positive and negative test compounds. Inter-laboratory validation has been completed and results have been submitted for peer review. The protocol is expected to be available at the EDSP website in late 2008.

2. Aromatase (human recombinant) Assay

The human recombinant aromatase assay is an inexpensive, rapid method to detect chemicals that inhibit aromatase activity and thus block the conversion of androgens to estrogens. Together, the aromatase steroidogeneisis assays are complimentary within the Tier-1 battery and are the only assays that have been shown to be sensitive enough to detect the activity of xenobiotics that weakly inhibit aromatase and estrogen synthesis.

3. Pubertal Female and Pubertal Male Assays

Changes in the numerous hormone-dependent endpoints in the male and female pubertal assays will detect the effects of a chemical that interferes with endogenous steroid hormone production by the testes and ovaries, respectively. Together, the pubertal male and steroidogenesis assays provide diagnostic information necessary to discern impaired estrogen and androgen production. The male pubertal assay has also been shown to detect chemicals that affect steroidogenesis prior to the formation of estrogen (*e.g.*, ketoconazole). The female pubertal assay will detect effects of altered aromatase activity. For example, inhibition of aromatase activity by fadrazole altered estrogen-dependent endpoints. Although both the male and female pubertal protocols have the capacity to readily detect compounds that affect steroidogenesis, the proper diagnosis for a steroidogenic mode of action can only be made with supporting

in vitro data. It has also been shown that measurement of androgen hormone levels enhances the sensitivity of the male pubertal assay to detect chemicals that block androgen synthesis by the testes.

4. Fish Short-Term Reproduction Assay

Interference in the steroid synthesis pathways is detected by several endpoints in the fish assay. Proliferation of interstitial cells (Leydig cells) in the male testes, reduction of circulating concentrations of steroids, decreased plasma vitellogenin in females, and impaired reproduction would all signal potential steroid synthesis modulation.

D. Assays for detection of chemicals that affect the HPG axis

The EDSTAC determined that evaluating the effect of environmental chemicals on the hypothalamic-pituitary-gonadal axis (HPG) was also important. To address this issue, the battery includes the male and female pubertal assays and the fish short-term reproduction assay, which includes both male and female fish. The hypothalamic-pituitary regulation of reproductive development and function is sensitive to a number of environmental compounds, as there are a variety of target mechanisms that can be affected. It is well known that many pharmaceuticals can interfere with hypothalamic regulation of gonadal function and ultimately gonadal hormone and gamete production. Similarly, environmental compounds such as dithiocarbamates, formamidines, chlorotriazines, among others, have been found to interfere with endocrine function by altering the hypothalamic regulation of pituitary hormone synthesis and secretion. By this mode of action, it has been shown that many of these same chemicals can interfere with reproductive development and aging.

Although it is not necessary to know the underlying MOA of a particular response affecting EAT during the screening process, the EDSP Tier-1 battery is designed to use the combined results of the *in vivo* tests included in the battery to determine deductively that the HPG axis was altered. For example, if a chemical is found to delay preputial separation and vaginal opening in both male and female rats, respectively, but that ER or AR binding or steroidogenesis were not altered, it would be concluded that the delay in puberty is attributed to impaired hypothalamic-pituitary function. This is the profile produced by compounds that act on the central nervous system such as dithiocarbamate thiram (impairs norepinephrine synthesis and GnRH release) and atrazine (impairs LH secretion) when assessed in the male and female pubertal assays. Pharmaceuticals such as bromocriptine, pimozide and haloperidol, which alter dopaminergic receptor function, have also been evaluated in the pubertal assays. In every case, these compounds that act on the central nervous system were found to alter normal pubertal development.

The fish short-term reproduction assay with fathead minnows is designed to detect changes in spawning, morphology and specific biochemical endpoints that reflect alterations in the HPG axis, including (anti-) estrogen and (anti-) androgen pathways. Again, the combined results of the *in vivo* assay included in the battery are to determine deductively that the HPG axis was altered. An example of the type of exercise that will be needed to differentiate an effect on the HPG axis versus other potential modes of action is shown in Table 4.

Table 4: Profiles Diagnostic for Various Modes of Action (MOA) in the Tier-1 Screening Battery Following Chemical Exposure.

Assay	**Was an effect detected?**			
ER Binding	Yes	No	No	No
AR Binding	No	Yes	No	No
Steroidogenesis	No	No	Yes	No
Male Pubertal	No	Yes	Yes	Yes
Female Pubertal	Yes	No	Yes	Yes
Fish	Yes	Yes	Yes	Yes
Likely MOA:	**ER**	**AR**	**Steroidogenesis**	**HPG**

E. Assays for detection of chemicals that affect the HPT axis

In addition to identifying environmental compounds that have the potential to alter the hormonal regulation of reproductive function involving the estrogen and androgen hormonal pathways, certain assays included in the proposed Tier-1 screening battery will also provide relevant information about the potential of a chemical to interfere with thyroid function. Thyroid hormones are essential for normal development and maintenance of physiological functions in vertebrates. Delivery of thyroid hormones to tissues and cells is highly regulated during early development and in the adult and is governed by complex physiological processes involving the hypothalamic-pituitary–thyroid (HPT) axis, including peripheral organs/tissues. Environmental factors, such as the presence of specific toxicants, can perturb this system at various points of regulation, inducing a variety of responses that can be detected with thyroid-related endpoints in the *in vivo* assays. Three assays have been designed for this purpose: 1) pubertal female, 2) pubertal male and 3) amphibian metamorphosis. A brief description as well as the value of each of the three assays for detection of compounds that can potentially interfere with thyroid development and function is provided. In addition, an example with a positive test chemical involving the following assays and this MOA is presented in Section IV.

1. Pubertal Male and Female Assays

The pubertal male and female assays include multiple endpoints that can detect an interaction of a test chemical with the thyroid hormone system, including serum TSH and T4concentrations, thyroid organ weight and histology, and liver weight. Both the male and the female assays have been shown to detect thyrotoxicants that act by various mechanisms that interfere with the synthesis and elimination of thyroid hormones. While the male and female pubertal assays include the same thyroid endpoints, examining the thyroid axis in both sexes provides the opportunity to detect gender differences in response to treatment. It is not clear whether the male or the female is more sensitive to toxicants that interfere with the thyroid axis at this early age. However, the male pubertal assay may be more robust than the female because the male is treated slightly longer. It has been shown in prevalidation studies that the male may be more sensitive to chemicals that induce hepatic clearance of thyroid hormones based on the response to lower dose levels. In the male assay, a food restriction study showed that a reduction in terminal weight of 9% or greater relative to controls could result in a decrease in circulating T4 concentrations. This effect will need to be considered when interpreting the battery for the thyroid MOA. Together, the complimentary results among the *in vivo* assays are expected to assist with interpretation of the data, as one change in a single endpoint will not be interpreted as a positive result if the other assays find no effect involving the HPT axis.

2. Amphibian Metamorphosis Assay

The amphibian metamorphosis assay (AMA) is a screening assay intended to identify substances which interfere with the normal function of the HPT axis. The AMA represents a generalized vertebrate model to the extent that it is based on the conserved structure and function of thyroid systems among species. The AMA provides a well studied, thyroid dependent process which responds to substances active along the HPT axis, and it is the only assay for the Tier-1 battery that assesses thyroid activity in a species undergoing morphological development.

The AMA is based on the principle that the dramatic morphological changes that occur during post-embryonic development are dependent upon the normal functioning of the HPT axis, and that interference with these processes leads to measurable effects. During tadpole metamorphosis, thyroid hormone (TH) influences virtually every tissue in the animal's body initiating diverse morphological, physiological and biochemical changes that include cell proliferation, differentiation and death. The result is *de novo* organ formation, organ loss, and extensive tissue remodeling. Given the dependence of metamorphosis on TH, and the strict biochemical control under which these processes occur, the timing and character of these processes can serve as endpoints representative of thyroid axis function and, as such, are exploited in the AMA. Additionally, although post-embryonic development appears quite different in mammals and most amphibians (direct development versus metamorphosis), there is a high level of evolutionary conservation of the thyroid system among vertebrates and the underlying cellular and molecular pathways that control these processes are similar, if not identical. The evolutionarily conserved nature of the vertebrate thyroid system enhances the ability to use an amphibian, particularly *Anurans*, as a general model for evaluating HPT axis interference such that the results can be extrapolated to other vertebrate species.

The primary endpoints in the AMA are developmental stage, hindlimb length, and thyroid histology. Each endpoint can be affected by chemicals that interact with the HPT axis. For example, antagonists of thyroid production, iodination and action, such as perchlorate and methimazole, will delay development and induce diagnostic lesions in the thyroid gland. Thyroid agonists (*e.g.,* native thyroid hormone) will accelerate development. Additionally, unlike the mammalian assays that have been developed to detect interactions along the HPT axis, the AMA has the ability to detect chemicals that act on peripheral tissues. For example, inhibition of monodeiodinases that transform T4 to T3 can cause asynchronous development, detected by an inability to assign a developmental stage to a tadpole. Knowledge of this mechanism is important because, in this case, development can be affected without concomitant effects on thyroid histology or circulating thyroid hormone.

IV. Case examples with known test chemicals that interact with the EAT hormonal systems

This section illustrates how the dynamics of the EDSP Tier-1 screening battery can perform through the use of selective examples with test chemicals known to interact with the E, A or T hormonal pathways. The examples comprise a group of individual assays that were designed to cover specific modes of action (MOA) within the battery as discussed in Section III. An overview of the results is presented as part of a validation process that was conduct by the EPA and OECD for individual screening assays. A detailed account of the EPA validation process and results for each assay can be found at the EDSP website. http://www.epa.gov/scipoly/oscpendo/pubs/assayvalidation/status.htm

A. Estrogen MOA and complimentary Tier-1 assays: 1) estrogen receptor (ER) binding, 2) ER transcriptional activation (ER TA), 3) uterotrophic, 4) pubertal female, and 5) fish short-term reproduction.

1. Methoxychlor - a pesticide in which the parent compound is a weak binder compared to the metabolites

ER Binding assay	**K_i = 65 ± 9 uM**
ER TA assay (hER-HeLa-9903)	**PC_{max} = 33.4 ± 0.2%**

Table 1. Methoxychlor binds in a positive manner to the estrogen receptor as shown by the inhibition constant (K_i). Methoxychlor failed to achieve a PC_{50}; therefore, PC_{max} was given. The PC_{max} indicates that methoxychlor was approximately 33% as effective as the positive control in activating transcription.

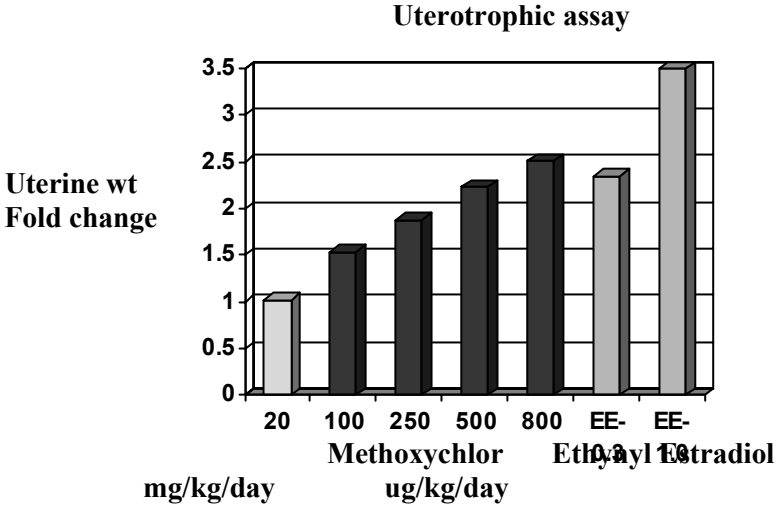

Figure 1. Results averaged over three contract laboratories using ovariectomized rats following subcutaneous injection with methoxychlor. Relative to the vehicle control (0 mg/kg/day) which equals 1 fold change in uterine weight methoxychlor is not different ($P>0.05$) at 20 mg/kg/day (gray bar) but is different ($P<0.05$) at 100 to 800 mg/kg/day (red bars). Similarly, the positive control is different ($P<0.05$) from vehicle control at 0.3 and 1.0 ug/kg/day (green bars).

Pubertal female assay

End point	Methoxychlor dose level (mg/kg/day)			
	0	12.5	25	50
Age at VO (d)	31.9 ± 0.3	27.9 ± 0.2*	27.0 ± 0.2*	26.5 ± 0.1*
BW at VO (g)	117.58 ± 2.5	92.41 ± 2.3*	87.48 ± 2.2*	82.02 ± 1.2*
BW Gain (g)	111.0 ± 1.8	110.8 ± 2.4	113.5 ± 3.1	106.8 ± 1.7
Final BW (% Ctl)	100	97.6	100.7	96.3
Age at first estrus (d)	33.4	30.9*	30.7*	28.6*
Regular cycles (%)	80	80	27*	20*

*Different ($P<0.05$) from vehicle control.

Table 2. Methoxychlor exposure via oral gavage induced early onset of puberty as indicated by a significant reduction in age and body weight (BW) at the time of vaginal opening (VO) beginning at 12.5 mg/kg/day. In addition, age at first estrus and percent of regular cycles were significantly reduced beginning at 12.5 mg/kg/day and 25 mg/kg/day, respectively.

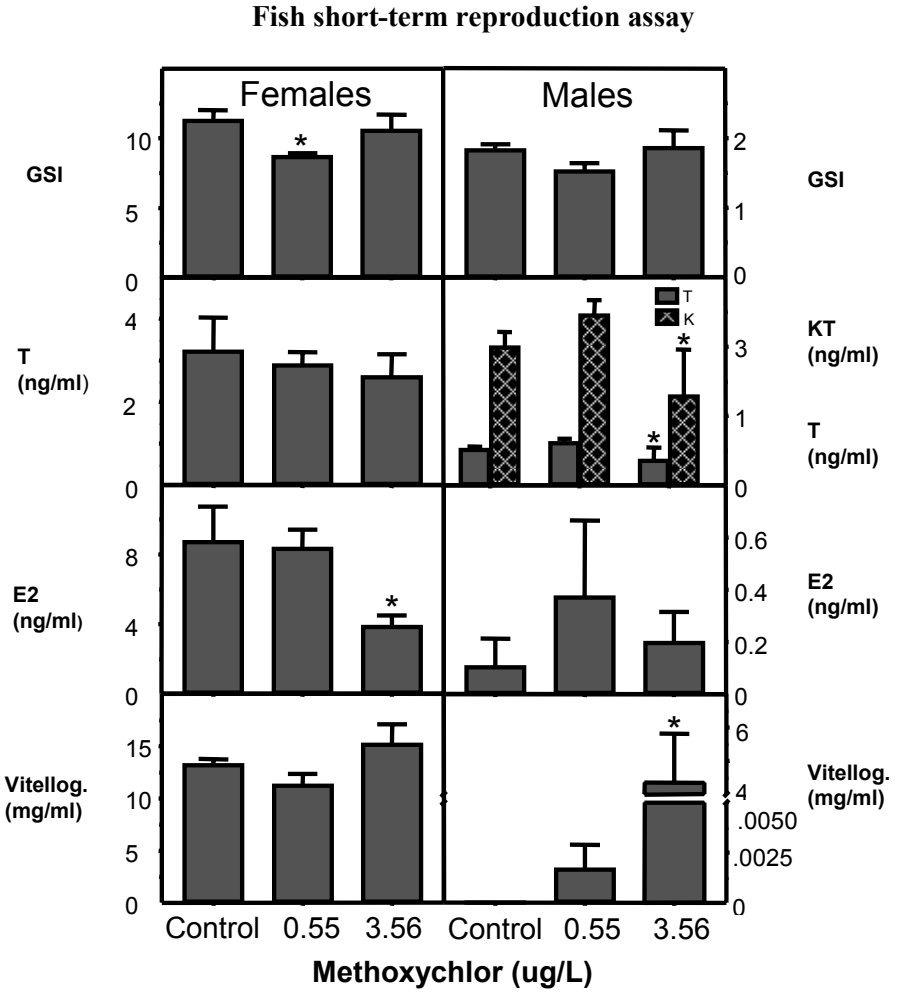

Figure 2. Methoxychlor induced vitellogenin production and inhibited testosterone production in males and estradiol production in females at 3.56 µg/L.

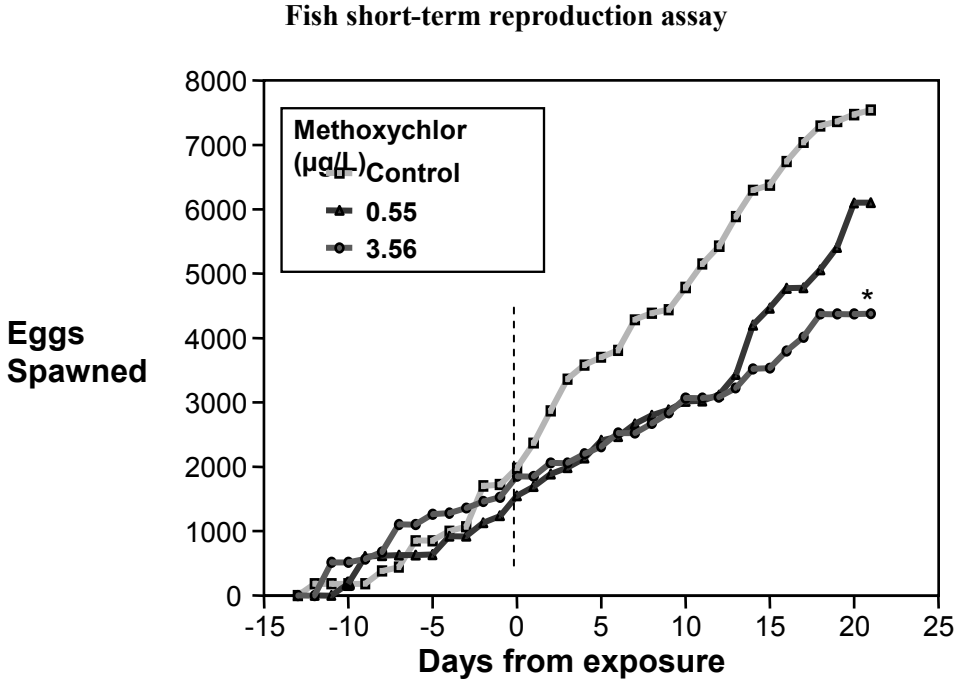

Figure 3. Methoxychlor significantly reduced egg production at 3.56 µg/L from 20.5 to 8.3 eggs/female/day versus control.

Methoxychlor summary – The relatively weak but positive response of native methoxychlor in the *in vitro* ER assays and *in vivo* uterotrophic assay was corroborated by the relatively strong positive response of apparent methoxychlor metabolite (s) in the *in vivo* female pubertal and fish reproduction assays. Hence, the combined positive responses among all assays provides a strong weight of evidence that methoxychlor interacts with the estrogen hormonal pathway in an agonistic way. Consequently, further testing would be triggered in the EDSP Tier 2.

2. Bisphenol A - an industrial compound primarily used in the production of polycarbonate plastics which is estrogenic *in vitro* with mixed results *in vivo*.

ER binding assay	**Full binding curve, %RBA = 0.2**
ER TA assay (hER-HeLa-9903)	**Log PC_{50} = -6.14**

Table 3. Bisphenol A yielded full inhibition curve with a relative affinity of 0.2% (%RBA= IC_{50} reference/IC50 test substance x100) and a Log PC_{50} of -6.14.

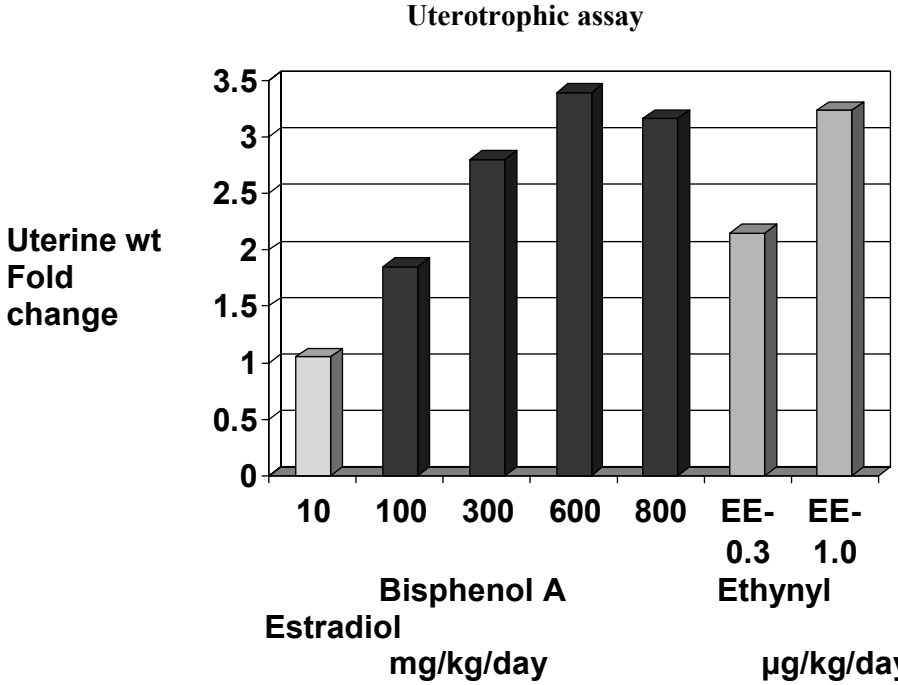

Figure 4. Results averaged over five contract laboratories using ovariectomized rats following subcuntaneous injection with bisphenol A. Relative to the vehicle control (0 mg/kg/day) which equals 1 fold change in uterine weight bisphenol A is not different (P>0.05) at 10 mg/kg/day (gray bar) but is different (P<0.05) at 100 to 800 mg/kg/day (red bars). Similarly, the positive control is different (P<0.05) from vehicle control at 0.3 and 1.0 ug/kg/day (green bars).

Pubertal female assay

End point	Bisphenol A dose level (mg/kg/day)		
	0	400	600
Age at VO (d)	32.3 ± 0.4	33.0 ± 0.6	32.3 ± 0.7
BW at VO (g)	122.70 ± 3.2	116.36 ± 3.5	108.42 ± 4.0*
BW Gain (g)	121.11 ± 2.8	101.82 ± 1.4*	95.86 ± 2.5*
Final BW (% ctl)	100	88.7	85.8
Age of first estrus	33.5	34.4	34.3

*Different (P<0.05) from vehicle control.

Table 4. Results following bisphenol A exposure via oral gavage were considered negative. The only effect observed was a significant reduction in body weight (BW) beginning at 400 mg/kg/day and BW at the time of vaginal opening (VO) at 600 mg/kg/day.

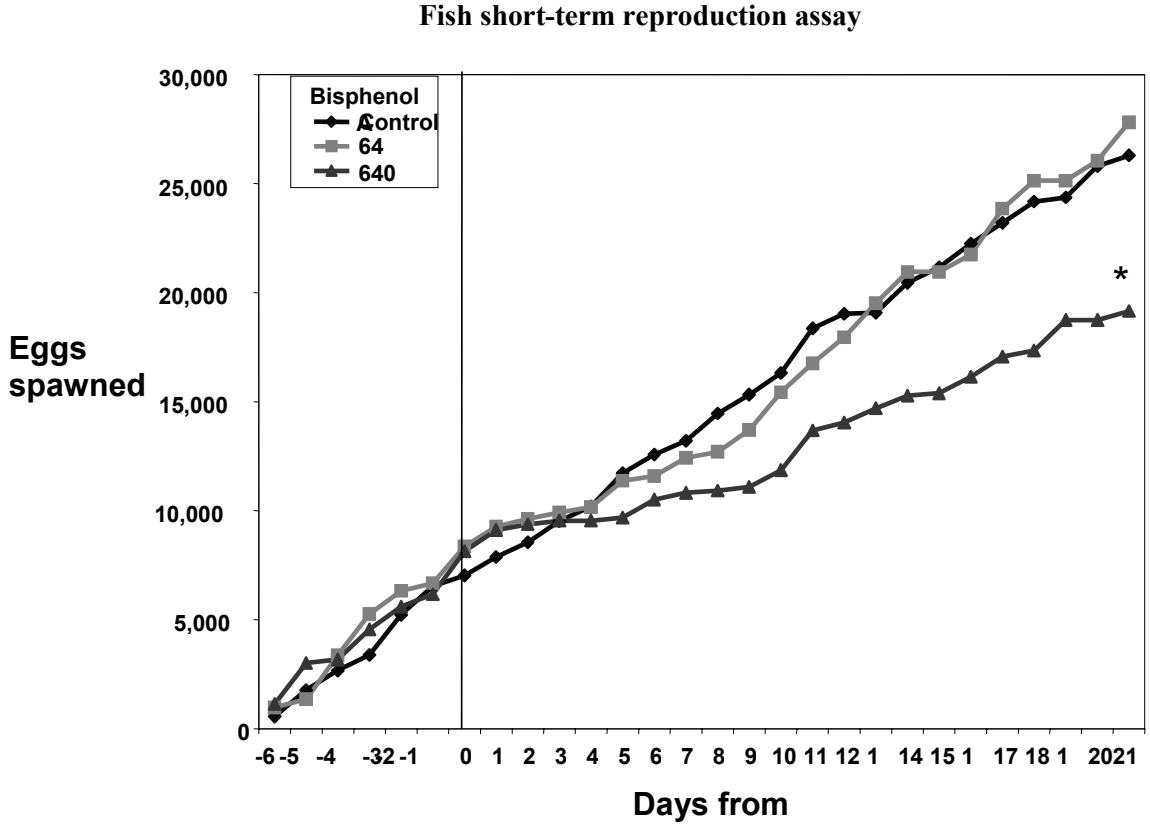

Figure 5. Bisphenol A (BPA) impaired egg production at the high dose and induced vitellogenin in males at both dose levels.

Bisphenol A summary - The effects of BPA in the *in vitro* ER assays and *in vivo* uterotrophic and fish reproduction assays were complimentarily positive. Unlike the subcutaneous and aquatic routes of exposure for the uterotrophic and fish assays, respectively, the oral route of exposure and first-pass metabolism of BPA in the pubertal female assay apparently led to a negative response. The combined results demonstrate the strength of the EDSP Tier 1 screening battery by incorporating both *in vitro* and *in vivo* assays with different routes of exposure and taxa to minimize false negatives. Thus, a positive response in a majority of the assays provides sufficient weight of evidence to indicate BPA interacts with the estrogen hormonal pathway in an agonistic way. Consequently, further testing would be triggered in the EDSP Tier 2.

B. Androgen MOA and complimentary Tier-1 assays: 1) AR binding, 2) Hershberger, 3) pubertal male and 4) fish short-term reproduction.

1. Vinclozolin - a pesticide that has anti-androgenic effects.

AR binding assay	**Equivocal**

Table 5. Vinclozolin was listed as equivocal in the AR binding assay using rat prostate cytosol because it gave a partial binding curve that did not cross the 50% binding threshold. The partial curve may have been observed because the highest concentration at which it was tested was 10^{-4} M, rather than the limit dose of 10^{-3} M.

Hershberger assay

	Vinclozolin dose level (mg/kg/day)				
End point	**0**	**3**	**10**	**30**	**100**
VP (mg)	**131 (34)**	**120 (21)***	**104 (25)***	**78 (21)***	**69 (34)***
SV (mg)	**348 (29)**	**344 (30)**	**271 (30)***	**187 (36)***	**69 (34)***
LABC (mg)	**506 (21)**	**485 (25)**	**445 (20)***	**374 (20)***	**256 (23)***
GP (mg)	**80 (13)**	**78 (12)**	**75 (9)***	**70 (10)***	**57 (12)***
Cow (mg)	**30 (27)**	**29 (29)**	**25 (30)***	**20 (25)**	**12 (38)***

*Different (P<0.05) from vehicle control.

Table 6. Vinclozolin exposure via oral gavage using gonadoectomized rats resulted in a significant reduction in weight of the ventral prostrate at 3 to 100 mg/kg/day and, for all other end points, a significant reduction in organ weight began at 10 mg/kg/day.

Pubertal male assay

End point	**Vinclozolin dose level (mg/kg/day)**		
	0	**30**	**100**
Age at PPS	**41.4 ± 0.7**	**43.8 ± 0.3***	**46.8 ± 0.3***
BW at PPS (g)	**219.5 ± 5.8**	**242.9.15 ± 5.0***	**259.6 ± 6.0***
VP (mg)	**240.7 ± 11.3**	**258.2 ± 16.8**	**206.4 ± 11.7**
SV (mg)	**552.9 ± 33.2**	**465.4 ± 35.5**	**304.4 ± 20.0***
LABC (mg)	**706.0 ± 25.9**	**685.7 ± 44.1**	**544.2 ± 27.3***
Paired Testes (mg)	**2740.0 ± 49.7**	**2960.0 ± 68.7***	**2935.0 ± 77.7***
Paired Epidid (mg)	**485.6 ± 12.9**	**456.1 ± 14.4**	**400.1 ± 15.7***

*Different (P<0.05) from vehicle control.

Table 7. Vinclozolin exposure via oral gavage delayed onset of puberty as indicated by a significant increase in age and body weight (BW) at the time of preputial separation (PPS) beginning at 30 mg/kg/day. In addition, seminal vesicles (SV), levator ani/bulbocavernosus muscle (LABC) and epididymides were significantly decreased at 100 mg/kg/day. Testes weight was significantly increased at 30 mg/kg/day.

Fish short-term reproduction assay

Dose levels (ug/L)	60, 255, 450
Fecundity	decreased at ≥60 ug/L
Gonad Histology (oocyte atresia)	increased at ≥60 ug/L
GSI	increased at≥255 ug/L
VTG	increased at ≥255 ug/L
Tubercles	decreased at 450 ug/L
Reference	Martinovic et al. 2008. ET&C 27(2):478-488

Table 8. Vinclozolin reduced egg production and increased oocyte atresia at all test concentrations (60, 255, and 450 μg/L) and reduced male SSC at the highest test concentration.

Vinclozolin summary – The positive response among the *in vivo* Hershberger, male pubertal and fish reproduction assays compliment one another despite a lack of a corroborating response in the *in vitro* AR assay. Unlike the *in vivo* assays, the AR *in vitro* assay does not have the capacity for metabolism, which apparently led to the equivocal response. The combined results again demonstrate the strength of the EDSP Tier-1 screening battery by incorporating both *in vitro* and *in vivo* assays to minimize false negatives. Thus, positive responses among the *in vivo* assays provides a greater weight of evidence over the equivocal response in the *in vitro* assay to indicate vinclozolin interacts with the androgen hormonal pathway in an antagonistic way. Consequently, further testing would be triggered in the EDSP Tier 2.

C. Steroidogenesis MOA and complimentary Tier-1 assays: 1) steroidogenesis, 2) aromatase 3) pubertal female, 4) pubertal male and 5) fish short-term reproduction.

1. Ketoconazole - a pesticide and pharmaceutical that alters steroidogenic enzymes resulting in enhanced progesterone and reduced estrogen and androgen production.

Steroidogenesis assay (H295R)	**>90% reduction of estradiol and testosterone production**
Aromatase assay (Recombinant human aromatase)	**Full binding curve, Log IC_{50} = -7.16**

Table 9. Ketoconzaole inhibited the production of both testosterone and estradiol production >90% relative to vehicle control in the H295R assay. It also produced a full inhibition curve in the recombinant aromatase assay. This is consistent with a chemical that is a non-specific P450 inhibitor as it inhibits enzymes upstream and downstream of testosterone.

Pubertal female assay

End point	Ketoconazole dose level (mg/kg/day)		
	0	50	100
Age at VO (d)	32.3 ± 0.4	33.0 ± 0.4	33.7 ± 0.6
BW at VO (g)	122.70 ± 3.2	126.54 ± 2.5	122.6 ± 3.6
BW Gain (g)	121.11 ± 2.8	121.11 ± 2.1	107.42 ± 2.5*
Final BW (% ctl)	100	100	92
Age at first estrus (d)	33.5 ± 0.8	33.3 ± 0.6	35.1 ± 0.8
Regular cycles (%)	93	87	85
Adrenal wt (mg)	47.8 ± 2.9	79.9 ± 3.9	78.5 ± 3.7
CL vacuolization	0/15	12/15**	9/15**
CL absence	0/15	0/15	5/15*

*Different (P<0.05) from vehicle control and **numerically different from vehicle control.

Table 10. Results of ketoconazole exposure via oral gavage were considered positive based on apparent reduction in number of ovulations and histopathology associated with development of corpora lutea (CL), especially at 100 mg/kg/day.

Pubertal male assay

End point	Ketoconazole dose level (mg/kg/day)		
	0	50	100
Age at PPS	39.6 ± 0.4	42.4 ± 0.4*	44.1 ± 0.2*
BW at PPS (g)	207 ± 4.2	227.15 ± 7.3*	234.76 ± 5.0*
VP (mg)	265.7 ± 16.8	237.2 ± 11.6	205.8 ± 24.3
SV (mg)	639.6 ± 46.2	478.9 ± 21.5*	419.3 ± 37.2*
LABC (mg)	638.4 ± 27.9	575.7 ± 26.2	542.5 ± 30.6
Paired Testes (mg)	2859.1 ± 28.7	2801.9 ± 53.7	2733 ± 23.8
Paired Epidid (mg)	455.9 ± 12.5	428.4 ± 12.1	413.4 ± 12.5

*Different (P<0.05) from vehicle control.

Table 11. Ketoconazole exposure via oral gavage delayed onset of puberty as indicated by a significant increase in age and body weight (BW) at the time of preputial separation (PPS) beginning at 50 mg/kg/day. In addition, seminal vesicles (SV) were significantly decreased beginning at 50 mg/kg/day.

Fish short-term reproduction assay

End point	Ketoconazole dose level (µg/L)	
	0	400
Testicular histopathology	Interstitial areas contain small aggregates of Leydig cells which have wispy, pale cytoplasm.	Relative to controls, aggregates of Leydig cells are larger, tending to fill and expand the interstitial space. Grade 2 (mild) severity.

Table 12. Ketoconazole induces Leydig cell proliferation in male testes at 400 µg/L.

Ketoconazole summary – Alterations in ovarian and testicular morphology in the *in vivo* female and male pubertal and fish reproduction assays as well as delayed puberty in the male pubertal assay were corroborated by results in the *in vitro* steroidogenesis and aromatase assays. Hence, the positive responses among all *in vitro* and *in vivo* assays provide a strong weight of evidence to indicate ketoconazole likely interferes in the steroidogenic pathway. Consequently, further testing would be triggered in the EDSP Tier 2.

D. Thyroid MOA and complimentary Tier-1 assays: 1) pubertal female, 2) pubertal male and 3) amphibian metamorphosis.

1. Perchlorate - a pesticide and pharmaceutical that alters steroidogenic enzymes resulting in enhanced progesterone and reduced estrogen and androgen production.

Pubertal male assay

Perchlorate dose levels (mg/kd/day)	Thyroid gland histology*	T4*	T3*	TSH*
0	-	-	-	-
62.5	↑ Follicular cell height ↓ Colloid area	-	-	-
125	↑ Follicular cell height ↓ Colloid area	↓	-	↑
250	↑ Follicular cell height ↓ Colloid area	↓	-	↑
500	↑ Follicular cell height ↓ Colloid area	↓	-	↑

*Difference ($P<0.05$) between control and treated groups indicated by arrows for T4 and TSH. Negative sign indicates no significant difference from vehicle control.

Table 13. Perchlorate exposure via oral gavage induced histopathology of the thyroid gland at all dose levels and respective alterations in T4 and TSH beginning at 125 mg/kg/day.

Pubertal female assay

Perchlorate Dose levels (mg/kd/day)	Thyroid gland histology *	T4*	T3*	TSH*
0	**-**	**-**	**-**	**-**
62.5	**↑ Follicular cell height ↓ Colloid area**	↓	**-**	**-**
125	**↑ Follicular cell height ↓ Colloid area**	↓	↓	**-**
250	**↑ Follicular cell height ↓ Colloid area**	↓	↓	↑
500	**↑ Follicular cell height ↓ Colloid area**	↓	↓	↑

*Difference ($P<0.05$) between control and treated groups indicated by arrows for T4 and TSH. Negative sign indicates no significant difference from vehicle control.

Table 14. Perchlorate exposure via oral gavage induced histopathology of the thyroid gland at all dose levels and respective alterations in T4 beginning at 62.5 mg/kg/day, T3 beginning at 125 mg/kg/day and TSH beginning at 250 mg/kg/day.

Amphibian metamorphosis assay

Perchlorate Dose levels (µg/l)	Thyroid gland histology *	Developmental Stage*	Hind limb Length*	Glandular wet wt*	Body Length*
0	**-**	**-**	**-**	**-**	**-**
62.5	**↑ Follicular cell height ↓ Colloid area Follicular cell hyperplasia**	**-**	**-**	**-**	**-**
125	**↑ Follicular cell height ↓ Colloid area Follicular cell hyperplasia**	**-**	**-**	**-**	**-**
250	**↑ Follicular cell height ↓ Colloid area Follicular cell hyperplasia**	**-**	↓	↑	↑
500	**↑ Follicular cell height ↓ Colloid area Follicular cell hyperplasia**	**Delayed**	↓	↑	↑

*Difference ($P<0.05$) between control and treated groups indicated by arrows and negative sign indicated no significant difference from control.

Table 15. Perchlorate at concentrations of $\geq$ 62.5 µg/L increased follicular cell height and reduced colloid area in the thyroid gland. 500 µg/L delayed developmental stage progression.

Perchlorate summary – Alterations in thyroid gland weight, histomorphology and associated hormones in the *in vivo* female and male pubertal and amphibian metamorphosis assays provides a strong weight of evidence that perchlorate interferes in thyroid development and function. Consequently, further testing would be triggered in the EDSP Tier 2.

E. Interpretation of the results of the EDSP Tier-1 screening battery

A science-based approach to interpretation of the results of the battery will generally follow the principles recommended by EDSTAC. The major principles include:

1. Interpretation of the battery will be considered in light of the results of all assays in the battery, using a weight-of-evidence approach, taking into consideration *in vitro/in vivo* discrepancies (if any), metabolism, and route of exposure.

2. When all screening assays are performed and all assays are negative, it may be concluded that the chemical will not likely interact with EAT hormonal processes included in the battery.

United States Environmental Protection Agency's Research Program on Endocrine Disruptors

In 1994, the US Environmental Protection Agency (USEPA) made the decision, as a result of increasing public health and ecological concerns, to integrate and expand its ongoing efforts into a consolidated Endocrine Disruptors Research Program (EDRP). To better understand the state of the science at the time and to identify the key science questions that needed to be addressed, USEPA held two international workshops in 1995 and efforts began to develop a national research plan. In 1996, several events took place that shaped the USEPA's priorities from both a research and a programmatic perspective: 1) USEPA's Office of Research and Development (ORD) identified endocrine disruptors as one of its top six research priorities. This was based upon recognition of: *a) the potential scope of the problem*, *b) the possibility of serious effects on the health of populations*, *c) the persistence of some endocrine-disrupting agents in the environment*, *and d) the widespread global concern about the fate and transport over national borders.* 2) Through enactment of the Food Quality Protection Act and amendments to the Safe Drinking Water Act, the US Congress directed USEPA to screen pesticides and drinking water contaminants, respectively, for their potential estrogenic or other endocrine activity in humans, using validated studies. In order to meet the Congressional mandates, a number of scientific questions needed to be addressed through research. USEPA's research program and the development and implementation of the mandated Endocrine Disruptor Screening Program (EDSP), which is described elsewhere in this document, therefore, have been on parallel highly interactive tracks for almost 13 years.

ORD's Endocrine Disruptors Research Plan was published in 1998[11]. Since then, ORD has developed a *Multi-Year Plan for Endocrine Disruptors* that identifies the specific science questions that will be addressed over the next five to ten years. This document is updated every few years to take into account the current state of the science and the updated strategic directions of the program (www.epa.gov/ord/npd/pdfs/Draft-EDCs-MYP-091407.pdf). In the *Multi-Year Plan*, ORD has identified three Long-Term Goals (LTGs) that will provide the Agency: 1) the methods it needs to implement EDSP, 2) scientific underpinnings to be able to interpret data from EDSP and incorporate them into risk assessments and Agency decisions, and 3) an improved understanding of the impacts of endocrine disruptors on humans and wildlife.

The highest priority for the USEPA EDRP has been the development of protocols for the assays critical to the Agency's EDSP. Over the last thirteen years the program has conducted much of the underlying research, developed and standardized protocols, prepared background materials for transfer, briefed Agency advisory committees, participated on international committees on harmonization of protocols, and/or participated in validation of approximately 19 different *in vitro* and *in vivo* assays for the development and implementation of the Agency's two tiered EDSP. The focus of these activities has been on estrogenic-, androgenic-, and thyroid-mediated mechanisms using mammalian, fish, amphibian, and invertebrate models. Collectively this part of the EDRP has lead to the development of protocols critical to the success of the Agency in fulfilling its Congressional mandates to develop and implement a screening and testing program. (See section on Overview of EDSP elsewhere in this document for details).

As data begin to be submitted to the Agency through the EDSP, the USEPA needs to be able to interpret the findings and integrate them into assessments. There are a number of scientific uncertainties for which research is still needed, and, thus, is continually being addressed through the EDRP. For example, research has been addressing the following science areas and is continuing to focus on:

Understanding of how endocrine disrupting chemicals (EDCs) elicit toxicity through receptor-based interactions, membrane receptors, enzyme alterations, and other non-nuclear receptor-based pathways, **particularly at the low end of the dose-response curve** which is especially relevant to evaluating effects

[11] www.epa.gov/ord/htm/documents/ORD-EDR-Feb1998.pdf

at ambient environmental levels of exposure. This body of research will lead to improved methods to interpret data and, thus, develop improved risk assessments.

Determining the degree to which the effects of EDCs with defined mechanisms/modes of action (MOAs) can be extrapolated across classes of vertebrates. This research is needed to: 1) reduce the uncertainty associated with extrapolating effects of chemicals across species, and 2) understand the degree to which quantitative extrapolation is defensible/possible and comparative toxicological studies using chemicals with well-defined MOAs are necessary. Of significance, the development of approaches to evaluate and conduct inter-species extrapolation research should ultimately help reduce uncertainties in both human health and ecological risk assessments and reduce the number of animals needed for testing.

Developing approaches to assess exposures to mixtures of EDCs. The current Agency default for predicting the effects of mixtures is to assume dose addition. There is a critical need to determine if this assumption accurately predicts the empirical effects of mixtures of endocrine disruptors, with similar and with different mechanisms of action. Furthermore, it is critical to develop approaches to facilitate incorporation of these data into risk assessments.

Determining the critical factors that account for exposures during development resulting in toxicities occurring later in life (e.g., windows of vulnerability, developmental tissue dosimetry, modes of action). Development is a period when hormone-mediated changes in gene expression can have permanent consequences that may not be apparent until later in life because functional changes do not occur until puberty or adulthood and during which extraordinary changes occur.

Developing biomarkers and the next generation of assays for screening chemicals for their potential endocrine disruption. There is a need to take advantage of the tremendous growth in the development of newer molecular approaches and develop predictive biomarkers and the next generation of assays for possible use in subsequent rounds of EDSP. The main advantage of these assays is that they often take less time to evaluate chemicals for their ability to interact with the endocrine system, cost less than other more conventional assays and test, and reduce (and in some cases eliminate) the use of whole animals. These latter elements are consistent with the recently issued National Academy of Sciences report on recommendations for a new testing paradigm in *Toxicity Testing in the 21st Century* (www.nap.edu/catalog.php?record_id=11970#toc).

What are the major sources and environmental fates of EDCs? How can unreasonable risks be managed? There is a need to develop chemical and molecular indicators of exposure on the highest priority endocrine-active chemicals. There are a number of existing risk management tools that possibly could be applied to reduce exposures to EDCs. If technologies exist that can be applied to major sources of exposure, the impact could potentially be a major reduction of EDCs released to the environment. The USEPA is focusing on applying its efforts to identify the key factors that influence human exposures to EDCs and major sources of EDCs entering the environment, such as from wastewater treatment plants (WWTPs), concentrated animal feeding operations (CAFOs), and drinking water treatment plants and is developing tools for risk reduction and mitigation strategies related to these sources.

One of the biggest unanswered questions regarding EDCs is to what extent do they impact humans, wildlife and the environment. USEPA research, therefore, is:

Determining the extent to which human development/reproduction is being adversely affected by exposure to EDCs. Given that development and reproduction appear to be highly sensitive endpoints in laboratory animal and wildlife studies and that there are reported alterations in particular endpoints (e.g., hypospadias, cryptorchidism, sperm quality), if any adverse effects are to be found, then evaluating these

endpoints in humans appears to be logical. Through a multi-US Federal Agency solicitation for proposals, 12 epidemiologic studies were funded, collectively.

Characterizing the occurrence and effects of endocrine active compounds in complex environmental media and developing management approaches to mitigate unreasonable risks. It is important to understand the extent of EDC exposures and the factors influencing the source-to-exposure-to-dose relationships in order to develop effective risk management strategies. Gaining improved understanding regarding the fate and transport processes, the interactions of EDCs from the source to the receptor, and collecting high quality exposure data for the development of multimedia, multi-pathway models are critical for ecological and human health risk assessments. Application of biological indicators of exposure to the study of components of mixtures offers the potential to validate and refine these models. The USEPA is applying the methods, models, and tools that it and other organizations have been developing to characterize releases from WWTPs, drinking water plants, and CAFOs and to develop management approaches that would mitigate any unreasonable risks.

Over the last thirteen years, the USEPA EDRP has provided critical data that have improved risk assessments within EPA and other organizations, have resulted in a suite of assays that have been validated for use in implementing EDRP, and are producing information that is helping the scientific community better understand the impact that EDCs are having on the environment.

The USEPA EDRP has been reviewed by external peer review panels composed of experts in the field (www.epa.gov/osp/bosc/pdf/edc0504rpt.pdr and www.epa.gov.osp/bosc/pdf.edcmc0804rpt.pdf) who found the program to have "achieved significant leadership in EDC research."

USEPA's EDRP and EDSP have integrated their efforts to help the Agency achieve a long term outcome of reducing or preventing risk to humans and wildlife from exposure to endocrine disruptors.

United States Environmental Protection Agency's Research Program on Computational Toxicology As Related to Endocrine Disruptors and Other Chemicals

As part of its Computational Toxicology Research Program, USEPA is carrying out a large-scale experiment, called ToxCast™, to test a high throughput screening (HTS) *in vitro* approach to identify potential toxicity. The aims of ToxCast are to screen the backlog of thousands of untested environmental contaminants using a battery of *in vitro* assays, and to prioritize chemicals for further testing based on bioactivity associated with pathways leading to toxicity. Phase I of ToxCast is testing 309 unique chemicals (320 including replicates), most of which had extensive animal toxicity testing, in order to develop initial predictive approaches that can be used in later large-scale screening phases. These Phase I chemicals are mostly pesticidal active ingredients, plus a number of high production volume industrial chemicals. The toxicity data for these chemicals is contained within the ToxRefDB relational database, facilitating the building of predictive models and comparisons within and across chemical classes. Information on ToxCast and ToxRef can be found at www.epa.gov/ncct. ToxCast Phase I chemical library is being run against 470 separate assays derived from 9 separate assay technologies, all run in concentration response format and, in some cases, with multiple time points. These include cell-free biochemical assays of receptor binding and enzyme inhibition, gene expression assays, transcription factor activity assays, cell-imaging assays and real-time cellular impedance measurements. Among the assays are four that evaluate the androgen signalling pathway, five that relate to estrogen signalling, four to thyroid signalling, and one to aromatase activity. Comparisons between ToxCast endocrine profiling and the results from Tier 1 EDSP battery will be made as those results are obtained, as there are approximately 55 of the EDSP priority chemicals contained with the ToxCast™ Phase 1 chemical library. ToxCast is part of a research collaboration between the USEPA, the National Toxicology Program, the National Institute of Health (NIH) Chemical Genomic Center, and a number of other organizations, screening thousands of environmental contaminants and pharmaceutical compounds to identify mechanisms and pathways leading to human toxicity. In addition, USEPA is working through the OECD's Toxicogenomics Advisory Committee to facilitate international cooperation related to high throughput molecular screening.

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Risk Assessments of Endocrine Disrupters

Features of the Atrazine Case Study

(Key Reference: US EPA, Office of Pesticide Programs, April 2006, Reregistration Eligibility Decision for Atrazine, at http://www.epa.gov/oppsrrd1/REDs/atrazine_combined_docs.pdf)

Human Health Risk Assessment

- Well designed studies were available to sufficiently establish atrazine's neuroendocrine mode of action.
- Atrazine inhibits the pulsatile release of gonadotrophin releasing hormone (GnRH) from the hypothalamus which in turn suppresses the release of luteinizing hormone (LH) from the pituitary.
- Understanding of atrazine's neuroendocrine mode of action (MoA) in laboratory animals (rats) was used to address issues about human relevance that had consequences on risk management decisions about public health**.**
  - MoA for the rat mammary gland tumors was considered not to be relevant to humans given that the LH suppression was affecting a reproductive aging process unique to the rat.
  - MoA and atrazine's effect on LH and consequent effects on development and reproduction in the rat was assumed to be relevant to humans given the homology of the hypothalamic-pituitary control on normal reproductive development and function.

    References:
    1. FIFRA Scientific Advisory Panel Meeting June, 2000, Atrazine: Hazard and Dose-Response Assessment and Characterization, at http://www.epa.gov/scipoly/sap/meetings/2000/june27/finalatrazine.pdf
    2. A MoA case study on atrazine can be found in Meek B. *et al,* (2003) A framework for human relevance analysis of information on carcinogenic modes of action *Crit Rev Toxicol.* 2003;33(6):591-653.
  - WHO's International Programme for Chemical Safety's Human Relevance Framework provides a valuable analytical and harmonized approach to establish the human relevance of animal MoAs. (http://www.who.int/ipcs/methods/harmonization/areas/cancer_mode.pdf)

- An endocrine key event in atrazine's MoA, LH suppression, was used as the basis of the dose response assessment (NOAEL = 1.8 mg/kg bw per day; LOAEL 3.65 mg/kg bw per day from a 6 month dietary rat study). Basing the point of departure and derivation of the reference dose (RfD) on LH suppression would protect for the down stream LH dependent effects on reproduction and development.

- The understanding of atrazine's neuroendocrine mode of action served as the basis for grouping certain chloro-s-triazine pesticides by a common mechanism of toxicity and served as the basis for a cumulative risk assessment.

  References:
  1. US EPA, Office of Pesticide Programs, March 2002, The Grouping of a Series of Triazine Pesticides Based on a Common Mechanism of Toxicity, at http://www.regulations.gov/search/search_results.jsp?css=0&&Ntk=All&Ntx=mode+matchall&Ne=2+8+11+8053+8054+8098+8074+8066+8084+8055&N=0&Ntt=EPA-HQ-OPP-2005-0481-0011&sid=11FDBE85D076
  2. US EPA, Office of Pesticide Programs, March 28, 2006, Triazine Cumulative Risk Assessment**,** at http://epa.gov/oppsrrd1/REDs/triazine_cumulative_risk.pdf

Ecological Risk Assessment

- Potential endocrine effects were considered but were determined inconclusive on aquatic organisms and wildlife.
  - **Amphibians**
    - In particular, the overall weight-of-evidence does not show that atrazine produces consistent, reproducible effects across the range of exposure concentrations and amphibian species tested.
    - Illustrates the importance of well designed studies across taxa and careful interpretation of literature studies.
  - **Fish**
    - Four fish life-cycle tests are available on freshwater fish; two of the studies resulted in reduced growth, one resulted in loss of equilibrium (behavioral effect), and one resulted in reduced survival. None of these effects were interpreted as indicative of effects on endocrine-mediated processes.
    - For example, in a 20-day exposure of largemouth bass (*Micropterus salmoides*) to formulated atrazine at 100 ug/L, female bass exhibited significantly higher plasma estradiol and vitellogenin concentrations compared to controls while male bass exhibited significantly lower plasma 11-ketotestosterone concentrations. Results from this study were highly variable and their utility in risk assessment is uncertain.
    - All of the regulatory endpoints used for evaluating risk of atrazine to aquatic animals were below the 100 ug/L value from the largemouth bass study and the 2003 IRED noted that potential adverse effects on non-target aquatic organisms including their populations and communties are likely to be greatest when atrazine concentrations in water equal or exceed approximately 10 to 20 ug/L on a recurrent basis or over a prolonged period of time. As such, the use of these more sensitive endpoints is likely protective of any potential effect of atrazine on fish at higher concentrations.
  - **Avians**
    - Two avian reproduction studies are available on atrazine. In both bobwhite quail (*Colinus virginianus*) and mallard ducks (*Anas platyrhynchos*), exposure to atrazine resulted in reduced egg production (NOAEC=225 mg/kg diet). In quail, there was wan increase in the number of defective eggs and a reduction in embryo viability while in mallards there was a reduction in egg hatchability. These results can be due to a number of factors and can not be clearly concluded as endocrine related.

References:

1. FIFRA Scientific Advisory Panel Meeting, June 17 - 20, 2003: Potential Developmental Effects of Atrazine on Amphibians, at http://www.epa.gov/scipoly/sap/meetings/2003/061703_mtg.htm
2. FIFRA Scientific Advisory Panel Meeting, October 9 - 12, 2007: The Potential for Atrazine to Affect Amphibian Gonadal Development, at http://www.epa.gov/scipoly/sap/meetings/2007/100907_mtg.htm
3. Potential Risks of Atrazine Use to Federally Threatened California Red-legged Frog (Rana aurora draytonii) and Delta Smelt (Hypomesus transpacificus) Pesticide Effects Determinations, at http://www.epa.gov/espp/litstatus/effects/redleg-frog/atrazine/analysis.pdf

Results from the US Endocrine Disruptor Screening Program (EDSP) Assay Development and Validation Program

Results observed in the EDSP Tier 1 studies (http://www.epa.gov/scipoly/oscpendo/pubs/assayvalidation/status.htm.) are consistent with the EPA risk assessment and the understanding of atrazine's neuroendocrine mode of action. Negative findings were observed in the *in vitro* ER and AR binding assays and for the rat uterotrophic assay (except for a high dose result). However, a delay in female and male puberty was found in the EDSP Tier 1 rat pubertal assays which would be expected given atrazine's ability to suppress the pituitary LH surge which plays a role in sexual maturation. Positive results were also found in the *in vitro* steroidogenesis H295R assay. Although it has been reported that atrazine induces CYP19 or aromatase activity (an enzyme that converts androgens to estrogens) in certain human cell lines (Sanderson, 2000), *in vivo* results indicated no support for any atrazine-induced change in this enzyme's activity or gene expression in the rat's brain, testes or adipose tissue (Modic, 2004a, 2004b, abstract). It was found that atrazine disrupts adrenal steroidgenesis in rats which may be related to the small increase in serum estrone and estradiol observed in the juvenile male (Stoker et al., 2000; Laws, 2006 and 2007 abstracts). This atrazine-induced adrenal response and possible subsequent increase in adrenal steroidogenesis, and peripheral conversion of androgen to estrone occurs at higher doses of atrazine than that required to decrease pituitary LH secretion, the regulatory endpoint in the EPA human health risk assessment. Thus, the suppression of LH represents the most sensitive and biologically plausible end point to protect human health from the potential neuroendocrine effects of atrazine. The overall results from the EDSP Tier 1 fish assay indicated that atrazine exposure had no statistically significant effects on assay endpoints. The only noteworthy findings were subtle changes in testicular histology, which included a significant decrease in the semeniferous tubule diameter in the high exposure group and an increased number of sperm at developmental stage 2A. Other noteworthy but not statistically significant trends were slight decreases in fecundity and E2 levels in females and T and 11-KT in males.

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Risk Assessments of Endocrine Disrupters

Features of the Mancozeb Case Study

(USEPA, September 2005 Reregistration Eligibility Decision for Mancozeb
http://www.epa.gov/oppsrrd1/REDs/mancozeb_red.pdf)

Human Health Risk Assessment

Recognizes the thyroid as a target organ for Mancozeb

- Thyroid toxicity was manifested as alterations in thyroid hormones, increased thyroid weight, and microscopic thyroid lesions (mainly thyroid follicular cell hyperplasia), and thyroid tumors.

Considers the role of metabolites in the risk assessment.

- Ethylene thiourea (ETU) is metabolite and environmental degradate of mancozeb.
- Some of the thyroid toxicity of the parent compound may be attributed to ETU, since it is also known to disrupt thyroid hormone homeostasis.

Thyroid endpoints were used for deriving the chronic RfD as well as endpoints for non-dietary exposures (incidental oral, dermal, and inhalation) (EPA, 2005). For example,

- The chronic dietary assessment is based on thyroid toxicity (LOAEL = 30.9 mg/kg/day based on thyroid toxicity).
- Incidental Oral exposure assessment (any duration; 1 day to 6 months) is based on decreased thyroxine (LOAEL = 17.82 mg/kg/day) from a subchronic rat toxicity
- Inhalation Exposures(any duration; 1 day to 6 months) is based on thyroid hyperplasia and decreased thyroxine (females) (LOAEL = 0.326 mg/L) based from a subchronic rat inhalation, study on thyroid hyperplasia and decreased thyroxine (females)

Understanding the mode of action of thyroid disruption can be used to address issues about human relevance during different lifestages which can have consequences on risk management decisions about public health**.**

- Mancozeb produces rat thyroid follicular cell tumors.
- Rats are known to be substantially more sensitive than humans to the development of thyroid follicular cell tumors in response to thyroid hormone imbalance (e.g., IARC, 1999) Meek et al., 2003). Compounds that act by this mode of action are not likely to be carcinogenic to humans at doses that do not alter thyroid hormone homeostasis.

Altered thyroid hormones may lead to neurodevelopmental effects in the young.

- Critical data need would be a comparative thyroid toxicity study in young and adult rats due to concern for thyroid imbalance and neurodevelopmental effects.

- WHO's International Programme for Chemical Safety's Human Relevance Framework provides a valuable analytical and harmonized approach to establish the human relevance of animal MoAs. Examples of other case studies on a thyroid disrupting mode of action for cancer and noncancer outcomes can be found at:

  (http://www.who.int/ipcs/methods/harmonization/areas/cancer_mode.pdf), Meek B. *et al,* (2003), Crofton and Zoeller (2005), Zoeller and Crofton (2005).

Ecological Risk Assessment

Birds

- Three avian reproduction studies are available on mancozeb. In the two bobwhite quail (*Colinus virginianus*) studies and the one mallard duck (*Anas platyrhynchos*) study, the weight of 14-day old surviving birds was reduced at the lowest adverse effect concentration. In one of the quail studies, additional adverse affects were observed, including hatchling weight reduction and % decline in the 14-day old survivors. In the duck study, additional adverse affects observed at the LOAEC exposure concentration of 125 ppm ai included reductions in the following: egg production; early and late embryo viability; hatchability; and offspring weight at hatch. These results can be due to a number of factors and can not be clearly concluded as endocrine related.

Amphibians

- The role of a major metabolite considered in the endangered species risk assessment. In the California Red Legged Frog (CRLF) Risk Assessment for mancozeb, it was assumed that chronic exposure EECs and toxicity are mainly related to ethylenethiourea (ETU), the major degradate of mancozeb.

- The endpoint determined from a chronic freshwater invertebrate toxicity test conducted with ETU was used to assess potential indirect effects to the CRL via reduction of prey items (freshwater invertebrates).

- ETU adversely affected growth and reproduction of *Daphnia magna* at 4.1 ppm. Adult length, survival, and fecundity (mean number of young per adult per reproductive day) were significantly reduced at the 4.1ppm treatment level.

- The thyroid effects found in the mammalian (rat) studies indicate a potential endocrine effect for both human and ecological systems.

- A critical data need would be the amphibian morphogenesis assay to evaluate the sensitivity of wildlife to thyroid disruption. Mancozeb" metabolite ETU has been evaluated in the EDSP Tier 1 Amphibian Metamorphosis Assay (see below).

Fish

- A freshwater fish early life-stage study conducted on fathead minnow (*Pimephales promelas*) is available on mancozeb. Survival and lack of growth effects were observed at a LOAEC of 2.19 ppb. This was not the endpoint used in the CRLF risk assessment.

Results from the US Endocrine Disruptor Screening Program (EDSP) Assay Development and Validation Program

ETU, which is a thyroid synthesis inhibitor, was found to alter metamorphic development and thyroid gland histology in the EDSP Tier 1 amphibian assay using *X laevis* (see http://www.epa.gov/scipoly/oscpendo/pubs/assayvalidation/status.htm.).

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Risk Assessments of Endocrine Disrupters

Features of the Vinclozolin Case Study

(Key Reference: US EPA, Office of Pesticide Programs, October 2000, Reregistration Eligibility Decision (RED) Vinclozolin, at http://www.epa.gov/oppsrrd1/REDs/2740red.pdf)

Human Health Dietary Risk Assessment

- Recognizes that the principal toxic effects induced by vinclozolin are related to its anti-androgenic activity.
- Considers the role of metabolites in the risk assessment including the establishment of food tolerances.
  - Vinclozolin and two of its metabolites bind and inhibit the function of the androgen receptor.
  - The two metabolites occur in mammals, plants, and soil and are responsible for much of the anti-androgenic activity attributable to vinclozolin.
- Differentiates life stage responses to vinclozolin's anti-androgen effect.
  - Chronic exposure of adult animals leads to Leydig cell tumors and short term exposures during critical developmental periods could potentially lead to, for example, male reproductive tract malformations.
    - Acute dietary pesticide risk assessment was based on the most sensitive developmental effect noted following *in utero* exposure, (*e.g.,* decreased prostate weight, weight reduction in other sex organs, nipple/areolas development, and decreased ano-genital distance in male rats).
- Evaluates the human relevance of the laboratory animal findings.
  - Antiandrogenic effects are expected in humans because the androgen receptor is widely conserved across species.
  - The IPCS/ILSI Human Relevance Mode of Action/Human Relevance Framework Approach is a valuable tool for establishing modes of action and evaluating species differences. See http://www.who.int/ipcs/methods/harmonization/areas/cancer_mode.pdf
    - As discussed in Kavlock and Cummings, (Critical Reviews in Toxicology, 35:721-726, 2005) there are data that show it is likely the vinclozolin will be metabolized in humans to its two more active metabolites, and as demonstrated *in vitro*, and that the two metabolites can bind to recombinant human androgen receptor (AR) and block the AR binding to the DNA response element.

Ecological Risk Assessment

- Illustrates the importance of considering multiple lines of evidence across taxa.
  - Adverse reproductive effects to mammalian and avian species resulting from exposure to vinclozolin and/or its metabolites appear related to its anti-androgenic activity found *in vitro* and *in vivo* studies.
- Identifies important information gaps in better characterizing the adverse consequences of vinclozolin's anti-androgenic activity in certain taxa . The lack of a full life cycle reproductive study in fish and an aquatic invertebrate life cycle study are considered important data needs because of reproductive effects found in other taxa.
  - For example, in a modified fish life-cycle toxicity test, fathead minnow (*Pimephales promelas*) were exposed for 112 days under flow-through conditions to 0.12 mg total vinclozolin residues/L [vinclozolin, metabolite B (acid), and metabolite E (amide)]. In the F_0-generation the number of spawns/female was statistically-reduced and the number of eggs/spawn was notably higher in the vinclozolin treatment group. In the F_1-generation hatch survival was statistically-reduced both at

the start and end of hatch compared to controls. Because only one concentration was tested, the study could not be used to define a NOAEC and/or LOAEC.

Results from the US Endocrine Disruptor Screening Program (EDSP) Assay Development and Validation Program

Results observed in the EDSP Tier 1 studies (found at http://www.epa.gov/scipoly/oscpendo/pubs/assayvalidation/status.htm.) confirm vinclozolin's anti-androgen activity. The EPA risk assessment described above is consistent with the findings from the EDSP Tier 1 assays. In the EDSP Tier 1 fish study, male secondary sex characteristics, testicular degeneration, and male gonad weight and GSI were the most robust and sensitive endpoints which would be consistent with vinclozolin's activity as an AR antagonist. Vinclozolin was also positive in the rat Hershberger assay which is designed to detect androgen antagonists and agonists. In the rat male pubertal assay, vinclozolin showed a profile expected for an anti-androgen (*e.g.,* delayed puberty, testicular histopathology, and epididymis weight as well as increases testosterone at higher doses).

Appendix 9

Contribution from BIAC

BIAC Perspective on a Globally Harmonized Approach for the Evaluation of Endocrine Activity of Chemical Substances

December 2008

1. The chemical industry is committed to providing chemicals that can be manufactured, transported, used and disposed of safely. The industry is also committed to making health, safety, environment and resource conservation critical considerations for all new and existing products and processes. In alignment with Responsible Care®, the member companies of chemical industry associations seek to understand risks posed by products throughout their life-cycle and to have systems in place to manage those risks effectively and appropriately in line with the shared responsibility with downstream users. Consistent with that commitment to Responsible Care®, the chemical industry recognizes the public's concerns that exposure to small levels of man-made and naturally occurring chemicals in the environment may pose a risk to humans and wildlife.

2. The chemical industry will continue to work together with government, the scientific community and other stakeholders to better understand the science related to the endocrine disruption issue. Through ongoing participation in the OECD EDTA, industry has contributed, and will continue to contribute, to the development, standardization and validation of new and revised OECD endocrine screens and tests. Internationally harmonized OECD Test Guidelines based on scientifically validated test methods must form the core foundation for screening substances for the potential to interact with components of the endocrine system and for such substances which have such a potential, for testing these materials to understand the dose-response relationship for production of adverse effects.

3. The chemical industry supports the development of internationally harmonised procedures to prioritise substances efficiently, to screen for endocrine activity, to test for adverse effects and to evaluate substances in the framework of a coherent chemicals policy. These procedures should provide for a tiered, hierarchical scientific framework in which validated screening assays are used to identify substances with endocrine activity and prioritise substances for further, more definitive testing. Definitive testing, using validated, harmonised protocols, is necessary to identify adverse effects caused by alterations to endocrine system function. Using a tiered approach, results from definitive tests must outweigh or supersede results from screening assays in guiding policy and management in both the public and private sectors. Definitive tests are necessary for hazard and risk characterization.

4. For hazard characterization, the chemical industry supports the development of a "weight of evidence" evaluation process that consists of a comprehensive, objective, transparent and balanced interpretation of the totality of scientific evidence regarding hormonal activity and adverse effects that might result from an endocrine mechanism. A defensible hazard characterization for hormonally active chemicals requires not only summarizing toxicological screening and testing data (hazard identification), but also requires an objective weight of evidence evaluation of whether the effects produced are adverse and whether adverse effects are due to a hormonal activity of the chemical.

5. The chemical industry supports the development of a globally harmonized approach concerning the interpretation of endocrine screening data, integration of screening data with definitive testing data and use of such data in prioritization, hazard characterization and risk assessment. Screening assays are to be used to prioritize substances for subsequent definitive testing. The results of definitive tests will provide data on adverse effects and dose response for use in hazard characterization and risk assessment. To perform risk assessments based on screening results would be an inappropriate use of the data. As the OECD finalizes test method validation and adopts endocrine Test Guidelines, there is a pressing need for a globally

harmonized evaluative processes and framework because such assays will soon be deployed in the U.S. as part of the EPA's Endocrine Disruptor Screening Program and in Europe as part of REACH. Consistent interpretations of the same data from the same endocrine-specific Test Guidelines and consistent use of such information in hazard assessment are needed to uphold OECD's Mutual Acceptance of Data.

6. The chemical industry supports the development of overarching guidance, applicable to all OECD member countries to ensure uniform and consistent description of the results of endocrine screening assays as well as the appropriate application of a weight of evidence framework for use of screening and definitive testing results in hazard characterization and risk assessment. (For specifics on weight of evidence see Attachment 1 CEFIC EMSG weight of evidence paper).

7. A tiered, hierarchical scientific framework for Endocrine screening and testing provides the most efficient and effective approach to evaluating substances. In this hierarchical framework, OECD validated, internationally harmonized test guidelines are used to screen substances identifying those with the potential to interact with one or more components of the endocrine system, and prioritizing for further definitive testing. Definitive testing, using validated, harmonized protocols, is necessary to identify adverse effects caused by alterations to endocrine system function. Using a tiered approach, results from definitive tests must outweigh or supersede results from screening assays in policy and risk management. Results from *in vitro* and *in vivo* screening assays only indicate that a substance interacts with a component of the endocrine system through one mechanism. Such screening results do not provide evidence on whether that substance will cause adverse biological effects. The *in vitro* and *in vivo* screening assays do not represent either the biological complexity of the intact endocrine system of an organism or evaluate adverse effects. If results from screening assays suggest the potential for endocrine activity, longer term *in vivo* studies are necessary. In this case exposures will be evaluated in the complete and intact endocrine system encompassing critical life stages and processes. In addition, assessments will include measurements of adverse effects, in order to fully characterize dose response of the potential endocrine mediated adverse health effects upon the endocrine system and in other tissues or organs.

8. Experience has shown that arraying toxicity tests in a tiered framework, provides scientific rigour and flexibility to account for differing chemical toxicities and to address specific concerns associated with existing or anticipated exposures to specific chemicals. In this manner tiered testing focuses efforts to collect data where it is most needed, to promote screening of the greatest number of prioritized chemicals (or classes of chemicals) and, where indicated by specific toxicity results, to indicate which substances pose a particular concern and points to the specific, more complex, test that should be considered. Where scientifically supported, the use of chemical categories can also increase efficiencies in providing knowledge of potential biological activities of chemical substances.

9. The chemical industry agrees that chemicals should continue to be tested for adverse health effects of concern under chemical regulation laws, the OECD high production volume chemical testing programme, REACH and pursuant to product stewardship activities of individual companies even as the development, standardization and validation of harmonized OECD test guidelines of screens and tests for endocrine activity proceeds.

10. The chemical industry has and will continue to contributing funding and expertise to national and international efforts to develop and validate *in vitro* and *in vivo* screening assays necessary to identify and prioritize chemicals that may interact with the endocrine system in humans and wildlife. The chemical industry has been and will continue to participate in the Organisation for Economic Cooperation and Development (OECD) Endocrine Disrupter Testing and Assessment (EDTA) efforts to develop and validate endocrine screens and tests. The chemical industry members urge governments to take advantage of the OECD Task Force on EDTA's on-going efforts to standardise and validate the globally harmonised test guidelines prior to initiating new, routine screening and testing procedures for endocrine disruption.

11. The chemical industry recognizes the benefits of international cooperation among all stakeholders in the validation of screening and testing methods to most efficiently use existing resources, to avoid unnecessary duplication of effort, to minimize the use of laboratory animals, and to help ensure mutual acceptance of data that is essential for international harmonization.

12. The chemical industry agrees that further research and understanding provide a sound basis for more effective public policy. Additional research is necessary to increase our scientific understanding and determine if there are possible adverse effects in humans and wildlife via hormone-mediated processes from exposure to chemicals in the environment. As has been stated in the comprehensive International Union of Pure and Applied Chemistry publication on endocrine disruption, J. Miyamoto and J. Burger (2003), "It is too early to reach firm conclusions about whether human populations are seriously at risk from potential exposures to EAS, and further vigilance is clearly required. However, it is somewhat reassuring that after substantial research in the past decade, there have been no conclusive findings of low level environmental exposures to EAS causing human disease."

13. The chemical industry, on a global basis, coordinates the research of its members to maximize efficiency and effectiveness and prevent duplication of effort, in part through the Long-range Research Initiative (LRI). Working with academic and research institutions and governments, LRI members in Europe, North America and Asia sponsor basic research on the potential of chemicals to interact with and affect the hormone system and cause adverse effects. LRI members have pledged to conduct research through an open and transparent process at institutions selected through a competitive peer-reviewed process. The results of this research will be made available to the public and acted upon by industry in a timely manner.

14. A tiered, hierarchical scientific framework is needed for screening and testing (Table 1). Validated screening assays are used to identify substances with endocrine activity and prioritise substances for further, more definitive testing. Definitive testing, using validated, harmonized protocols, is necessary to identify adverse effects caused by alterations to endocrine system function. Using a tiered approach, results from definitive tests must outweigh or supersede results from screening assays in guiding policy and management in both the public and private sectors. For discussion, three tiers are suggested.

Table 1. Proposed OECD Endocrine Screening and Testing Hierarchical Framework

STAGE	**DESCRIPTION**	**ASSAYS FOR MAMMALIAN TOXICITY**	**ASSAYS FOR ECOTOXICITY**
OECD Stage 1 **Initial Assessment to Set Priorities for Further Evaluation**	Available data (for example) Production volume and pattern of use Available exposure information Predicted environmental properties, e.g., fate Toxicological data, especially endocrine-relevant data (i.e., results of histopathology on reproductive organs from repeat dose studies, developmental or reproductive toxicological information.)	All relevant studies	It is presumed that the data review will incorporate both mammalian and ecotoxicological issues.
	Structure activity relationship	It is presumed that structure activity relationships for receptor mediated modes of action will be applicable across ~~mammalian~~ orders.	It is presumed that structure activity relationships will be applicable across vertebrate classes. ~~Invertebrates may have~~ unique receptors.
	Molecular screening results	All relevant studies	All relevant studies
OECD Stage 2 Screening Assays **(mode of action)** ***OECD 2002 Framework***	*In vitro* assays providing mechanistic information / data ***OECD 2002 Framework Level 2***	Oestrogen and androgen and receptor binding assays Transfected mammalian cell assays (ER and AR and TR)	It is presumed that receptor binding will in principle be applicable across vertebrate classes and to any invertebrates expressing similar

Level 2-4		*In Vitro* Aromatase *In Vitro* Steroidogenesis	receptors.
	In vivo assays providing mechanistic information / data on single endocrine mechanisms ***OECD 2002 Framework Level 3***	Uterotrophic screening assay (estrogen and anti-oestrogen) Hershberger screening assay (androgen and anti-androgen)	Fish screening assay (vitellogenin and secondary sex characteristics) Frog metamortophosis assay
	In vivo assays providing mechanistic information / data on multiple endocrine mechanisms ***OECD 2002 Framework Level 4***	Enhanced TG407* Adult Intact Male assay Male Pubertal assay Female Pubertal assay	OECD Fish Screening Assay (VTG and secondary sex characteristics as mandatory endpoints; other endpoints are optional)
OECD Stage 3 Definitive Testing (evaluation of apical endpoints, adverse effects and dose response for **hazard identification and characterization)** ***OECD 2002 Framework Level 5***	Reproduction/developmental tests –shorter scope - includes *in utero* exposure, developmental, and reproductive capacity endpoints	(TG407* (as adopted on October 03,2008) depending on the exposure situation as the method does not include the reproductive phase) Reproductive /developmental screening test (TG 421) Combined repeat dose with reproduction / developmental screening (TG 422) One generation reproductive toxicity (TG 415) Two generation reproductive toxicity (TG 416) [Enhanced one generation reproductive toxicity -if and when a final OECD TG is developed]	Partial and full life cycle assays in fish, birds, amphibians and invertebrates (developmental and reproduction) Fish full life cycle

** Remark: Depending on the situation a TG-407 would suffice to establish a NOAEL in selected instances. Such instances could include, for example, substances with low potency, minimal human exposure likely intermediates or substances manufactured in closed system, and limited potential for environmental*

release. This would serve to focus the more extensive testing only on substances that have high exposure potential.

15. Proposed OECD Stage 1: Initial Assessment

The purpose of the proposed OECD Stage 1: Initial Assessment (Table 2) is to rapidly assess the universe of substances in order to recognize those substances where scientifically relevant data exist to permit more rapid prioritization and assessment.

The OECD Stage 1: Initial Assessment will permit one to:

- Recognize those substances (a) where pertinent data do not exist and (b) have some likelihood of exhibiting the mode(s) of action, e.g., binding to the oestrogen or androgen receptors, that lead to the particular hazard(s), e.g., reproductive and developmental effects, targeted by the assessment. These substances should then be prioritized.

- Recognize those substances which do not take part or are very unlikely to take part in the mode(s) of action(s) that lead to the particular hazard(s) targeted by the assessment. This reduces the overall effort and size of the assessment program. These substances can be reconsidered should new and compelling information emerge

Table 2. Proposed OECD Stage 1: Initial Assessment to Set Priorities for Further Evaluation

OECD Stage 1 **Initial Assessment to Set Priorities for Further Evaluation**	Available data (for example) Production volume and pattern of use Available exposure information Predicted environmental properties, e.g., fate Toxicological data, especially endocrine-relevant data (i.e., results of histopathology on reproductive organs from repeat dose studies, developmental or reproductive toxicological information.)	All relevant studies	It is presumed that the data review will incorporate both mammalian and ecotoxicological issues.
	Structure activity relationship	It is presumed that structure activity relationships for receptor mediated modes of action will be applicable across mammalian orders.	It is presumed that structure activity relationships will be applicable across vertebrate classes. Invertebrates may have unique receptors.
	Molecular screening results	All relevant studies	All relevant studies

16. Review of available toxicological information is an important step. Current toxicology testing protocols provide much information about potential endocrine mediated toxicity. Endpoints indicating endocrine-mediated toxicities are present in current testing guidelines or are being developed:

- Endocrine-mediated cancer mechanisms are well known, and endpoints for these mechanisms are included in current cancer bioassays,
- Multigenerational and reproductive studies are designed to examine all relevant windows of sensitivity; *in utero*, lactational and pubertal as well as periods of functional reproductive capacity in adults,
- Work is in progress via the OECD Endocrine Disruptor Testing and Assessment Task Force to review and where appropriate change/introduce screening and testing protocols.

17. For priority setting purposes, the totality of scientific evidence regarding hormonal activity and adverse effects that might result from an endocrine mechanism should be considered. This should include: assessment of existing regulatory actions; existing data on adverse effects; the presence or absence of response in different taxa; the nature and magnitude of positive responses in relation to the relevance & reliability of the assays; dose response (or lack thereof); repeatability; relative potency; and coherence of responses across assays in relation to the postulated mode of action. In cases where the weight of evidence indicates no, or at most very low, hormonal potency and there is little or no likelihood of release to the environment or potential for exposure, then such substances should be given a very low priority for further investigations.

18. Structure activity relationships ((Q)SARs) and molecular screening methods, where the model predictions can be validated, would permit the rapid assessment of specific, targeted mode(s) of action. For example, a relevant property for an oestrogenic mode of action is to bind to an oestrogen receptor. The volume and nature of the oestrogen-binding site determines a substance's ability to bind to the receptor and the actual binding affinity. (Q)SARs and molecular screening assays can then assist in the prioritization of substances with predictions of binding affinity and can also indicate a large number of substances where binding to an oestrogen receptor is highly unlikely.

19. Prioritization should include an integrated evaluation of several attributes such as:

- the possible potency of the substance as indicated by initial structural activity assessments;
- production volumes;
- the amount of environmental releases and the environmental levels indicated by monitoring;
- environmental properties such as long-range transport, persistence and bioaccumulation;
- use as related to the number of persons exposed, the relevance of the route of exposure, the intensity and duration of exposure, and possibility for disproportionate exposure in subpopulations; and
- weight of evidence evaluation of existing data.

20. Proposed OECD Stage 2: Screening.

The purpose of the Proposed OECD Stage 2 Screening is to efficiently and effectively develop information as to whether a substance has the potential to interact with one or more components of the endocrine system. Most often OECD Stage 2: Screening assays are based on a particular mode of action especially

leading to relevant biological responses. A positive mechanistic response warrants further evaluation and, if necessary, testing to characterize relevant hazard(s), if any, connected with the mode of action.

21. The proposed OECD Stage 2: Screening (Table 3) is subdivided into *in vitro* assays and *in vivo* assays as these assays are not equivalent. Both economy and animal welfare considerations argue that *in vitro* assays be employed both to accommodate a significant number of compounds and to avoid undue delays in assessing substances. For flexibility, the option to proceed directly to *in vivo* assays should not be precluded, nor should *in vitro* assay results be required if *in vivo* results are available. *In vivo* assays incorporate i) substance-specific complexities that cannot be obtained from *in vitro* assays, including absorption, distribution, metabolism and excretion, and ii) reflect the complex and dynamic homeostasis and operation of the intact endocrine system. Therefore, *in vivo* results would supersede *in vitro* results as noted by the Veldhoven workshop (SETAC Europe 1997). Examples of *in vitro* assays for an oestrogen mode of action include: oestrogen receptor-binding assays, yeast strains containing a hormone receptor and a hormone responsive reporter gene, or mammalian cell lines naturally expressing the oestrogen receptor that are transfected with a reporter gene controlled by an oestrogen receptor.[1] An example of an *in vivo* screening assay is the uterotrophic assay for an oestrogen mode of action. The assay is based on the growth and weight increase response of the uterine target organ, can be conducted in 3 days, and uses a minimum number of animals. The response of the uterine target-organ is highly relevant for the oestrogen mode of action.

[1] *Mammalian cell lines transfected with both a receptor and a reporter gene may have patent protection and their use therefore may be restricted.*

Table 3. Proposed OECD Stage 2: Screening Assays to Evaluate the Potential of Substances to Interact with One or More Components of the Endocrine System

LEVEL	DESCRIPTION	**ASSAYS FOR MAMMALIAN TOXICITY**	**ASSAYS FOR ECOTOXICITY**
Stage 2: Screening **(mode of action)**	*In vitro* assays providing mechanistic information / data ***OECD 2002 Framework Level 2***	Oestrogen and androgen and receptor binding assays Transfected mammalian cell assays (ER and AR and TR) *In Vitro* Aromatase *In Vitro* Steroidogenesis	It is presumed that receptor binding will in principle be applicable across vertebrate classes and to any invertebrates expressing similar receptors.
	In vivo assays providing mechanistic information / data on single endocrine mechanisms ***OECD 2002 Framework Level 3***	Uterotrophic screening assay (estrogen and anti-oestrogen) Hershberger screening assay (androgen and anti-androgen)	Fish screening assay (vitellogenin and secondary sex characteristics) Frog metamortophosis assay
	In vivo assays providing mechanistic information / data on multiple endocrine mechanisms ***OECD 2002 Framework Level 4***	TG407* Adult Intact Male assay Male Pubertal assay Female Pubertal assay	

** Remark: Depending on the situation a TG-407 would suffice to establish a NOAEL in selected instances. Such instances could include, for example, substances with low potency, minimal human exposure likely intermediates or substances manufactured in closed system, and limited potential for environmental release. This would serve to focus the more extensive testing only on substances that have high exposure potential.*

22. The principles for the consideration of assays into the screening level of the tiered framework are:

- Individual assays should be selected and the protocols designed to provide the necessary information in a resource efficient manner. That is, in addition to having the capacity to address the anticipated number of substances, the assays themselves should be simple, rapid, and economical.

- Highly specialized and technically demanding techniques should be avoided unless essential. The assay technique(s) should be within the competence and qualifications of the necessary laboratory resources.

- As the results from assays comprising the proposed OECD Stage 2: Screening provide only mechanistic information and not evidence for adverse effects, such results should not be used for classification or regulation. In accordance with the Weybridge definitions (EC 1996), OECD Stage 2: Screening stage results do not indicate that a compound is an 'endocrine disruptor.'

- The TG407 has some utility as both a screening assay and definitive test. The TG407 has played a major role in the OECD SIDS battery as one of the leading repeat dose toxicity studies for commodity chemicals. The validation of the TG407 showed that only substances with strong or moderate endocrine activity were detected, especially as for weakly active substances, the overall study outcome was governed by general toxicity. In this respect, the TG 407 is a method evaluating some endocrine specific endpoints as well as apical endpoints, adverse effects and dose response, and can therefore be used in certain instances for hazard identification and characterization. As such, the TG407 would fall within the proposed OECD Stage3: Definitive Testing. However, as described in the newly issued TG407 OECD Test Guideline, there are some strengths and limitations inherent in employing the TG407 as either a screening assay in the proposed OECD Stage 2: Screening step or as a test for adverse effects in the proposed OECD Stage3: Definitive Test step. In a comparison of TG407 to higher tier studies, with regard to the observed overall (i.e., irrespective of the type of toxicity) No Adverse Effect Levels, the difference in sensitivity is usually not more than a factor of 10 (Gelbke *et al*., 2007). Depending on the exposure situation a TG 407 may suffice for the proposed OECD Stage3: Definitive Testing for human health hazard characterization in selected instances. Such instances could include, for example, substances with low potency, confined intermediates, minimal human exposure, and limited potential for environmental release. Reliance on the TG407 in such cases for human health hazard characterization would serve to focus the more extensive testing only on substances that have high exposure potential

23. The screening assays comprising Stage 2: Screening focus on detecting estrogenic, androgenic and thyroid modes of action. To effectively screen chemicals with unknown endocrine activity, evaluation would consist of laboratory studies of the chemical in the complete battery of assays that comprise the proposed Stage 2: Screening step. If information from existing assays or functionally equivalent test methods is available that allows one to reach a scientifically sound conclusion regarding the activity of the substance with respect to one or more of the modes of action encompassed in the proposed Stage 2: Screening step, then those assays for such modes of action would not necessarily need to be run for such a substance.

24. A weight of evidence process needs to be implemented in order to integrate results across the complement of assays that comprise the proposed OECD Stage 2: Screening step. . Substances which are positive based on overall consideration of the weight of evidence in OECD Stage 2: Screening are considered to be high priority candidates for further evaluation in definitive tests (OECD Stage 3: Definitive Testing). However, there is not an automatic triggering. Instead, a weight of evidence approach (see Attachment 1, CEFIC EMSG Weight of Evidence paper) should be used to evaluate results of screening battery. Prior to initiating additional work, it is appropriate to consider the potential for human exposure and potential for entrance into the environment. The weight of evidence evaluation should include: the presence or absence of response in different taxa; the nature and magnitude of positive responses in relation to the relevance and reliability of the assays; dose response (or lack thereof); relative potency; and coherence of responses across assays in relation to the postulated mode of action. In addition, as indicated above, in cases where the weight of evidence (derived from consideration of results of

validated screening assays) indicates at most very low potency and there is little or no likelihood of release to the environment or potential for exposure, then such substances should be given a very low priority for further investigation in definitive tests. Existing information and data from standard toxicity studies (e.g., Repeat Dose Study (TG 407 and TG 408), Developmental Toxicity Study (TG 414; TG 421/422), Chronic Toxicity Study (TG 452 and 453)) should be reviewed as part of the weight of evidence evaluation. Results from these studies can provide important information on dose response and adverse effects on endpoints of potential concern. The term 'potential endocrine disruptor' could be easily misinterpreted, and generally use of this term should be avoided. From a scientific perspective, it is important to determine the overall weight of evidence of the performance of a substance in the screening assays/battery, as described above.

25. Proposed OECD Stage 3: Definitive Testing

The purposes of the proposed OECD Stage 3: Definitive Testing are to accurately and effectively identify and characterize the hazard(s) from chemicals. The tests comprising the proposed OECD Stage 3: Definitive Testing has been specifically developed to assess reproductive and developmental toxicity. The results obtained should be assessed relative to target site toxicity, and the endpoints evaluated include both endocrine and non-endocrine toxic endpoints. The array of tests included in Table 4 should be viewed as a matrix of available options, and not as a sequential list of assays and tests. It would not be necessary to conduct all tests, but instead, from this matrix, the appropriate test could be selected. In the interests of flexibility and minimizing animal and resources, for example the enhanced repeat dose study and the shorter-scope reproduction/developmental tests would not be required in cases where a longer scope test is already available or planned.

Table 4. Proposed OECD Stage 3: Definitive Testing for Adverse Effects and Dose Response (for use in hazard identification and risk assessments

LEVEL	DESCRIPTION	**ASSAYS FOR MAMMALIAN TOXICITY**	**ASSAYS FOR ECOTOXICITY**
Stage 3: Definitive Testing (evaluation of apical endpoints, adverse effects and dose response for **hazard identification and characterization)** ***OECD 2002 Framework Level 5***	Reproduction/developmental tests –shorter scope - includes *in utero* exposure, developmental, and reproductive capacity endpoints	(TG407* depending on the exposure situation as the method does not include the reproductive phase) Reproductive /developmental screening test (TG 421) Combined repeat dose with reproduction / developmental screening (TG 422) One generation reproductive toxicity (TG 415) Two generation reproductive toxicity (TG 416) [Enhanced one generation reproductive toxicity -if and when a final OECD TG is developed]	Partial and full life cycle assays in fish, birds, amphibians and invertebrates (developmental and reproduction)

** Remark: Depending on the situation a TG-407 would suffice to establish a NOAEL in selected instances. Such instances could include, for example, substances with low potency, minimal human exposure likely intermediates or substances manufactured in closed system, and limited potential for environmental release. This would serve to focus the more extensive testing only on substances that have high exposure potential.*

26. A defensible hazard characterization for hormonally active chemicals requires not only summarizing toxicological screening and testing data (hazard identification), but also requires an objective evaluation of whether the effects produced are adverse and whether adverse effects are due to a hormonal activity of the chemical. Hazard characterization is based on overall consideration of the weight of evidence. This includes consideration of the proposed OECD Stage 2 Screens and proposed OECD Stage 3 Definitive Tests, and results from standard toxicity studies (e.g., Repeat Dose Study (TG 407/408), Reproduction/Developmental Toxicity Study (TG 414/421/422), Chronic Toxicity Study (TG 452/453) etc). Such an evaluation provides the context for considering adverse effects across organ systems and modes of action and for comparing NOAELs. However, hazard identification is insufficient to characterize risks.

27. Risk characterization requires integration of scientific data and knowledge of hazard, dose-response and exposure, as well as an evaluation of the foundations of the hazard data, inferences drawn from the data, and inherent uncertainties. In cases of low potency and low or negligible actual and potential exposures, test methods such as the Repeat Dose Study (TG 407) or the Reproduction/Developmental Toxicity Screening Tests (TG 421/422) could be used to provide dose-response data of effects on apical endpoints. This would serve to focus the more extensive testing only on substances that have high production volume and the highest potential for human and ecological exposures. In all cases, results from Stage 3: Definitive Testing outweighs or supersedes results from Stage 2: Screening.

28. In developing the OECD framework for evaluating endocrine activity, all have recognized the need to employ the OECD must use standardized, validated and internationally harmonized test methods, and this has been the foundation of the OECD EDTA and WNT work over the past 10 years. In considering the screens and tests for evaluating endocrine activity, the OECD EDTA determined that certain types of studies were not standardized and lacked adequate data for a validation determination. Hence, the OECD EDTA formed the validation management groups to coordinate the necessary laboratory studies to achieve standardized and validated test methods. Considerable progress has been made in this regard, due to the efforts of industry, government, academia and laboratories.

29. Research laboratory studies using novel test methods, non-standardized, and not yet validated methods and / or non-standard test species generally lack the quality criteria that typically encompassed in studies employed using OECD Test Guidelines and Good Laboratory Practices, and thus such novel methods or non-standard test species must be evaluated with due caution for regulatory purposes. As basic research of endocrine mechanisms advance, new and novel scientific methods have, and will continue to be reported. These new and novel types of studies are significantly different from laboratory studies using standardized and validated techniques. For example, they may lack appropriate documentation for reliability of the test method performance or unambiguous interpretation of the relevance of the endpoints evaluated. Thus, such novel research studies should not be used for regulatory action, but should trigger further research and/or method validation efforts.

30. Validation of a test method is a required prerequisite for it to be considered for regulatory use. For a new or revised test method to be considered validated for regulatory purposes it should meet the criteria specified by OECD GD 34 (2005) and/or ICCVAM (1997)). For validation, the extent of a test method's variability and reproducibility within and across laboratories must have been demonstrated, and sufficient data provided to permit assessment of the method's range and limitation of application.

31. The issues of GLP, quality assurance and quality control have important international dimensions, as recognized by OECD. Under Mutual Acceptance of Data, regulatory authorities in OECD member countries can rely on safety test data developed abroad, thus eliminating duplicative testing, and this serves to both enhance laboratory animal welfare and to create testing efficiencies while at the same time assuring high quality scientific information is developed for regulatory and product stewardship purposes.

Screening and testing of substances using validated test methods yields greater confidence in results compared to studies performed using non-validated methods. The requirement across OECD member countries of mutual acceptance of data is based on the foundation that evaluations of chemicals are based on test data of sufficient quality, rigour and reproducibility.

32. As scientific methods advance, regulatory bodies will continue to be challenged to review and interpret new and novel methods and studies with non-standard species. The responsible standard when dealing with results from such studies, whose findings may be especially influential, is the standard which is embodied in the scientific method – hypothesis formulation, hypothesis testing through experimentation, and independent replication. The OECD EDTA Framework should endorse and integrate this responsible standard concept into the OECD globally harmonized approach for evaluation of the endocrine activity of chemical substances

33. New and novel methods, and studies with non-standard species, may provide important scientific information. Recognizing this fact, we suggest the following course of action for addressing such study reports. The first order task should be a thorough review of the study report. This would entail independent review of the methods and procedures and review of the data. The study documentation should be sufficient to permit independent verification/calculation of results, and the methods should be sufficiently described to permit replication of the study procedures by other laboratories. Following this review, it is recommended that an additional laboratory replicate the study. Then, if the findings are shown to be reproducible, then two courses of action would be advised:

> A) The new or novel test method could be subjected to standardization and validation within the OECD TG program (EDTA) or within a similar formal program sponsored by a national government or recognized scientific organization (e.g., ISO, ASTM), provided that such a method is viewed as necessary to augment or replace one or more existing test methods in the screening and testing battery. International harmonization should also be an objective, to promote mutual acceptance of data and to optimise utilization of laboratory animals. To the extent possible, in a manner similar to that of the OECD EDTA, it is beneficial for lead organizations to undertake a certain amount of standardization and pre-validation work, and for information to be shared across sectors (government, industry, academic and other scientific researchers). Organization & conduct of the formal test method validation effort should be conducted within the existing processes of the relevant competent authorities; or
>
> B) The substance of concern could be evaluated in one of the wide variety of existing validated test method using standardized OECD TG methods and species (or similarly validated scientific methods, for example those promulgated by ISO, ASTM, US EPA). Results of this study would then be evaluated within the tiered hierarchical OECD EDTA Framework. In general, this would be the preferred course of action.

34. While review of the totality of relevant scientific information is needed for robust regulatory decision making, in regulatory reviews the weight afforded to results from standardized and validated test methods and laboratory studies conducted under GLP generally exceeds the weight given to novel and non-validated, non-GLP studies. Until such time as either course A or B in paragraph 33 is completed, it would be imprudent to initiate extreme risk management actions based upon a report of a new, novel or non-validated test method. Experience has shown that it is inappropriate to automatically accept novel and new research reports for regulatory purposes. At times, even peer review by scientific journals won't suffice to screen out flawed studies, a fact that should counsel against overly enthusiastic acceptance of any single study – especially one whose results are both extraordinary and groundbreaking. If the results of single studies seem especially important special care should be taken to verify the quality of such studies. It is also important to consider the scope of peer review. For the vast majority of scientific journal publications, peer reviewers in fact do not examine the detailed laboratory study records. Instead, this

system relies on the submitting author's summarization of experimental procedures and results. If the results of single studies seem especially important special care should be taken to verify the quality of such studies.

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Attachment 1. CEFIC EMSG Paper: Towards the Establishment of a Weight of Evidence Approach to Prioritizing Action in Relation to Endocrine Disruption

SUMMARY

Large uncertainty still remains about actual risk posed by endocrine disrupting substances and the scientific assessments that should be included when developing risk management options. The position of CEFIC is that a weight of evidence approach using the most credible scientific data should be used in making decisions.

A number of publications describe how to evaluate data quality and their use in hazard and risk assessment (e.g. Ref. 1 & 2). This document builds on this earlier work and summarises the key elements of a procedure to evaluate the balance of scientific evidence in relation to the potential of a substance to cause adverse effects through disruption of the endocrine system. It addresses the issues of data relevance, quality and significance - using a weight of evidence approach to indicate whether, and what action needs to be taken in order to assess the hazards and risks of a substance. It has been developed specifically to enhance the prioritisation process and output of the EU DG Environment project: "Towards a Priority List of Substances for Further Evaluation of their Role in Endocrine Disruption."

The procedure includes:

- A data collection step that covers a search of the published literature and an extension of the search into unpublished literature, particularly to gather data used for regulatory purposes.

- An evaluation step that considers:

    1. What endpoint has been measured and the relevance of that endpoint to the effects of potential endocrine disruption mechanisms.

    2. The repeatability, reliability and quality of a particular study and its protocol, together with the extent of peer review.

    3. The significance (or 'weight') of the data based on the assessments under 1 & 2 above.

    4. Whether there is sufficient coherence of the data to draw conclusions (balance of the 'weight of evidence')

    5. What further evidence is required, including a prioritised action identification step leading to risk assessment in accordance with the existing, or any future coherent chemicals regulatory framework.

INTRODUCTION

The European Commission has completed the initial stages of a project through DG Environment to prepare a "Priority List of Substances for Further Evaluation of their Role in Endocrine Disruption." This exercise required the evaluation of toxicological data in order to achieve a prioritisation rating, but the Chemical Industry believes that the approach taken to create the initial list was too superficial to add meaningfully to the debate and that the list may be misinterpreted.

The process to develop the list used an "evidence of suspicion" approach in which the presumption of endocrine toxicity may be based upon as little as a single data point. Studies showing consistently that there is no evidence of endocrine toxicity have been ignored, irrespective of quality, since they do not add to the strength of suspicion.

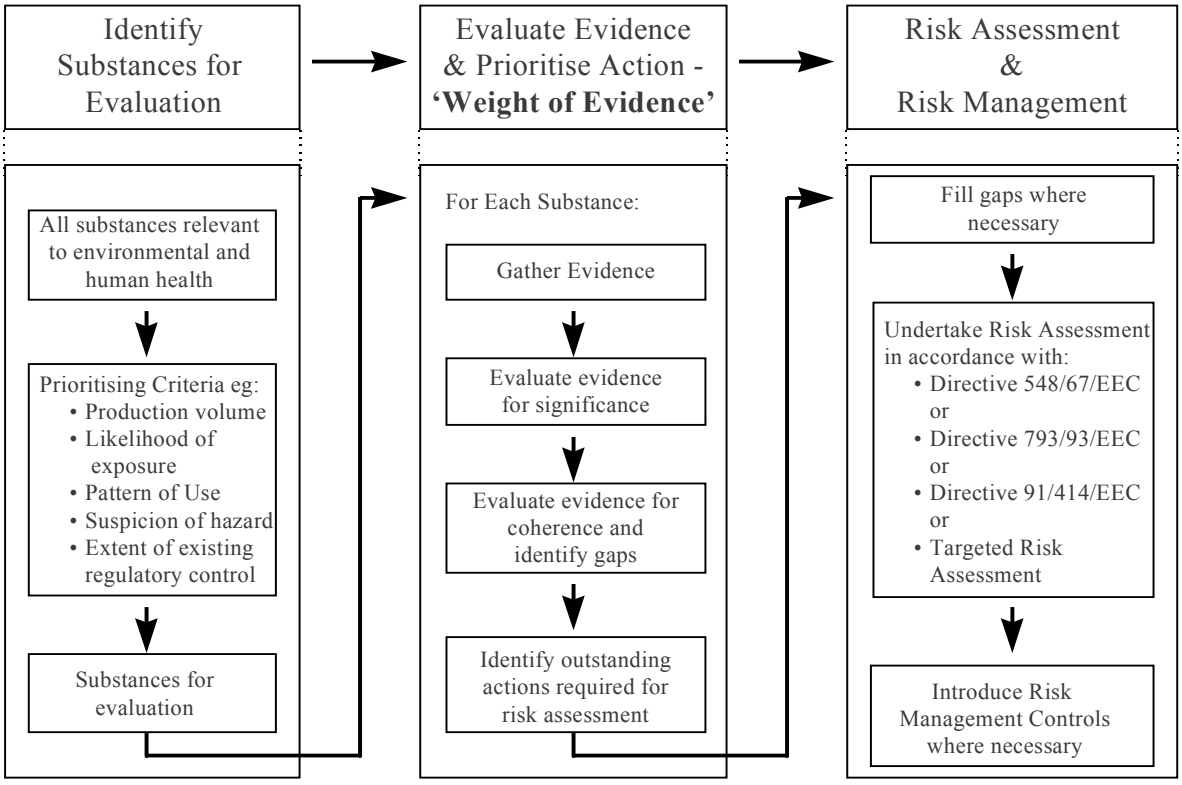
Furthermore, the 'List' adds nothing to the debate because it fails to identify and incorporate the priority actions required to assess ED hazards and risks properly. It also fails to present a strategy for assessing all other substances for which there is little or no data to judge the ED hazard. It is merely another list of often poorly founded suspicions that, because of its apparent 'official' status and pseudo-scientific analysis, may be misinterpreted as a 'Definitive List of Endocrine Disrupters'. Failure to take all available data into consideration could well lead to economically damaging de-selection of products without protecting human health or the environment.

Despite these well founded concerns, the European Chemical Industry accepts the political desire to develop a list of priority substances for further evaluation of their role in endocrine disruption but believes that the interests of both public and environmental health would be better served if the 'List' was to be more 'action' orientated and based on sound scientific principles.
Through this document, CEFIC offers an approach that weighs both the relevance and reliability of evidence in the balance using scientifically based criteria to identify the priority actions required for each substance under suspicion and indeed, any other substance that may come under review - truly providing a priority action plan towards risk assessment within the processes laid down under existing community regulations. We have termed this approach as the 'Weight of Evidence Approach' and it fits between an initial step to define a group of chemicals requiring evaluation and later steps to fill data gaps and undertake risk assessment. This is shown graphically in Figure 1 on the next page.

At a European Union meeting in Weybridge (Ref. 3), the following definitions were agreed:
"An endocrine disrupter is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function."
"A potential endocrine disrupter is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism."
These definitions have gained wide acceptance in the international arena, have been adopted by the International Programme for Chemical Safety (IPCS) and consequently, have been used as the basis for this set of proposals.

In agreement with the aim of the European Commission project, the procedure developed works towards prioritising actions required for the further assessment of substances in relation to their potential endocrine disrupting activity. In this context, the phrase 'consequent to' is interpreted to mean demonstration of a causal link between mechanistic activity and adverse health effects.
It specifically addresses six potential mechanisms - agonistic and antagonistic effects on the oestrogen, androgen and thyroid systems. However, where relevant, it also makes provision for reporting non-endocrine adverse effects so that risks from other sources are not ignored.

Figure 1:The Role of A Weight of Evidence Approach



WEIGHT OF EVIDENCE APPROACH

The following approach was designed to assist in the conduct of a weight of evidence review of available toxicological data in order to enable the identification and prioritisation of chemicals for further assessment in relation to endocrine related activity. It consists of the two basic tasks shown below:

2.1 Collecting the data.

2.2 An evaluation step that considers:

2.21 What endpoint has been measured and the relevance of that endpoint to the effects of potential endocrine disruption mechanisms **(Data Relevance)**.

2.22 The repeatability, reliability and quality of a particular study and its protocol, together with the extent of peer review **(Study Repeatability)**.

2.23 The significance (or 'weight') of a data set based on the assessments under 2.21 & 2.22 above **(Data Significance)**.

2.24 Whether there is sufficient coherence of the data to draw conclusions (balance of the 'weight of evidence'), what further evidence is required to take action and what that action should be. **(Coherence, Gaps and Framework for Further Action)**.

Expert judgement is required at each stage and it is important to record the basis of decisions to aid transparency (See Section 3).
It should be emphasised that none of these proposals are new. Such an approach is well accepted and documented in peer reviewed journals (e.g. Refs. 1, 2 and 4).

2.1 Data Collection

In order to ensure that as many data as possible are included in the assessment, an extensive search of all relevant databases is required. This should capture any data available in SIDS, IUCLID and other relevant databases, as well as in the published literature. Criteria for the search and organisation of the search results should be based on expert judgement, and developed on a case-by-case basis, details of which should be recorded. (See Section 3). The literature search should, as a minimum, include those commercially available databases listed in Annex 1. Consideration should also be given to seeking unpublished literature, particularly data used for regulatory purposes - but only where the quality can be assessed under Section 2.23.

2.2 Data Evaluation

2.21 Data Relevance

There are various assays, measures and toxicological endpoints that are claimed by different sources to be relevant to the assessment of endocrine disruption. In reality, this field is still in an early stage of development and there is uncertainty regarding the significance of many of the findings, especially the relevance of *in vitro* assays and short-term screening assays to toxicological effects.
A weight of evidence approach should be able to differentiate between various toxicological endpoints in relation to their relevance to mechanistic evidence and observed effects. For the approach described here, endpoint relevance has been weighted to enable a hierarchy which can differentiate between:

- Observed adverse health effects with mechanistic support to establish causal linkage
- Observed adverse health effects with limited understanding of mechanism

- Biomarker of exposure
- Mechanistic potential with no observed effect

Substances should only be considered endocrine disrupters if they cause "adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function" (Ref. 3). Hence, it is inappropriate to assess a substance as an endocrine disrupter on the basis of mechanistic *in vitro* assays alone and the approach has been designed to reflect this.
Similarly, many current testing criteria exist for the *in vivo* determination of adverse effects on reproduction and/or development without providing evidence of mechanistic cause. Under these circumstances, a negative result may be sufficient to demonstrate that a substance is not en endocrine disrupter, but a positive result may need further testing to distinguish the mechanistic cause. Nonetheless, those with a financial interest in the substance may feel that it is more prudent and efficient to proceed directly towards risk assessment - rather than undertake additional testing - on the conservative assumption of an endocrine cause.
For non-standard protocol endpoints, the assessment of endpoint relevance would in many instances be a subjective decision which should be based on sound expert judgement. If such a judgement proves impossible, then the data should be treated as being of 'low significance' (See Section 2.23) until such time that additional research is able to clarify the relevance to risk for species known to be exposed to the substance in question.
In all instances, the relevance rating would need to be clearly documented with appropriate justification. Adverse effects identified but thought to be of non-endocrine origin should be reported for further assessment by the relevant Competent Authorities.

Assessing Relevance of *In vivo* Data
The most relevant data for reaching an evaluation of endocrine toxicity is found from repeat dose toxicity and/or reproductive toxicity studies which include measurements and observations associated with endocrine toxicity. Other types of *in vivo* studies, including screening assays such as the uterotrophic and Hersberger assays do provide relevant information, and data from such studies should be included in the any weight of evidence review. It must be remembered that, positive results in screening assays are not conclusive evidence of adverse health effects and are of lower relevance than the repeat dose studies in making a judgement about endocrine disruption. Nevertheless *in vivo* screening assays do serve a useful purpose by indicating potential for harm and should be regarded as "indicative studies" leading to actions as indicated in Figure 2.
A summary of the relevance of *in vivo* studies is shown in Table 1

Table 1: Relevance of In Vivo Assays

High relevance	□ endpoint(s) in a multi-generation test or other repeat dose toxicology test that is specifically controlled by the endocrine system, or □ parallel dose-responsive changes in hormone levels in the presence of consequent toxicological effects (mammalian only) □ negative data from a short term/screening assay specifically controlled by the endocrine system
Medium relevance	□ endpoint in a multi-generation test, or other repeat dose standard toxicology test, which may be influenced by the endocrine system, but is also known to be affected by other factors, e.g. water quality, environmental stress, toxicity etc.; or □ endpoint in a short-term/screening assay specifically controlled by the endocrine system; or

	□ changes in hormone levels in the absence of any toxicological effects (mammalian only)
Low relevance	□ evidence indicates that the endpoint is not controlled by the endocrine system. Positive results of adverse effects should be reported for further risk assessment.

Assessing the Relevance of *In vitro* Data

The purpose of *in vitro* testing is basically to identify intrinsic endocrine modulation potential and determine potency relative to a reference hormone. For example, "can a substance bind to a receptor?" and "what amount is required to produce an equivalent response to a natural hormone such as oestrogen?". As the predictive ability of *in vitro* tests to detect effects in animals is, at best, uncertain it must be recognised that results from *in vivo* assays are more relevant for judging whether or not a substance will cause endocrine toxicity.

Despite such limitations, *in vitro* tests can be reliable for detecting potential endocrine modulating activity *per se* and therefore are a useful tool in the overall context of endocrine toxicity testing.

A number of *in vitro* screening systems are available which involve the interaction of chemicals with vertebrate steroid receptors. Although the number of *in vitro* assays for taxa other than mammals is limited, receptors, such as for oestrogen, androgen, and thyroid, and their essential roles are conserved across vertebrates. The endocrine systems of invertebrates are poorly understood. The role of oestrogen and other vertebrate hormones, if any, in invertebrates is unclear, and will not be further discussed here.

It is recommended that the data review incorporates all available *in vitro* data, and that for the purposes of assessing the relevance of *in vitro* endpoints, attention should be focused on both a hierarchy of information and the quality of the particular measurement system:

- whether the assay is designed to indicate simple receptor binding potential or the more indicative receptor binding coupled with transcriptional activation.
- whether the assay is a cellular or subcellular assay, which would be indicative of whether or not the endocrine receptor was likely to be exposed to metabolites of the parent compound.
- whether the assay examines relevant endocrine parameters such as steroid metabolism.

On the basis of the above discussion, a hierarchy of in vitro endpoint relevance is proposed in Table 2.

Table 2: Relevance of *In Vitro* Assays

High relevance	□ endpoint is based upon receptor binding potential coupled with transcriptional activation, whole cell or subcellular assay; or □ receptor binding potential in a whole cell assay □ assessment of steroid metabolism in a whole cell assay
Medium relevance	□ endpoint is based on receptor binding activity in a subcellular assay, or □ endpoint is based on cell growth or other endpoint not a direct measurement of receptor mediated activity □ endpoint of steroid metabolism in a subcellular assay
Low relevance	□ not applicable

It should be noted that the hierarchy is solely for the relevance of the endpoint, and is not indicative of the final weighting applied to the result. The weight of the evidence procedure is described in Section 2.23.

2.22 Study Repeatability

An assessment of study repeatability takes into account:

- The extent to which protocols have been validated and the limits within which conclusions can be drawn
- The extent to which the toxicological endpoints are understood
- The extent of the historical database and the confidence that this provides
- Basic experimental design - adequacy controls; suitability of concentration range
- Exposure data - purity of test material, verification of exposure concentrations
- Test species - suitability, general health, environmental conditions
- Analysis of results - statistical validity of observed effects
- Transparency of the study report

It is essentially, an assessment of the confidence one might have in being able to repeat the study and reproduce the results.

Traditionally, toxicity work has been evaluated against compliance with internationally recognised and validated standard protocols (e.g. ASTM, ISO, OECD). Such studies can be repeated with a high level of confidence. Evaluation of protocols for the determination of endocrine disruption is difficult, since standard protocols are not currently available for this specific area. Nonetheless, many of these standard tests shed light on the adverse effects likely to result from endocrine disruption and their results can be relied upon to provide useful evidence.

Other, perhaps more novel protocols may produce endocrine-specific information, but their reliability needs careful evaluation. Proposed criteria for reported data are listed in Annex 2, and have been selected as criteria which are indicative of work which has been undertaken to a good standard of scientific practice. It is proposed that tests carried out in accordance with these criteria form a suitable basis for assessing substances, when combined with a weighting based on the relevance of the endpoint, as previously described in Section 2.21

On this basis, it is proposed that the hierarchy for study repeatability should be ranked as follows in Table 3:

Table 3: Hierarchy of Repeatability

High Confidence of repeatability	All criteria for the experimental design and conditions, and for reporting transparency are met • *full details of experimental method available and these indicate that studies have been carried out to an acceptable standard*
Medium Confidence of repeatability	The main criteria for the experimental design and conditions, and for reporting transparency (see bolded points of attached Annex 2) are met • *some details of the experimental method are available which indicate that studies have been carried out to an acceptable standard*
Low Confidence of repeatability	Insufficient information is available for the experimental design and conditions to determine reporting reliability
Unreliable	Analysis of the experimental design and conditions indicate that the study may be unreliable or not reported transparently.

2.23 Data Significance

The final task in establishing the 'weight' that should be ascribed to any set of data takes into account both the 'Relevance' and 'Repeatability' of the data as evaluated in Sections 2.21 & 2.22. In effect, the 'weight' is measured as the level of significance that can be ascribed to a data set in reaching conclusions about endocrine disruption.

As discussed above *in vivo* data from repeat dose/long term animal studies are the most important in hazard assessment. While *in vitro* information and data from *in vivo* screening studies are useful in making judgments about the presumption of hazard they are not currently linked directly to, or are predictive of adverse/toxicological effects associated with endocrine disruption.

For these reasons *in vivo* data from repeat and chronic* studies examining functional endpoints such as growth, reproduction and development during critical life-stages are considered more significant in assessing the potential for adverse effects and making risk management decision than *in vitro* data. The latter can only provide information about one or two steps in a chain of events that may, or may not lead to health problems. At best, such results can be taken as being only 'Indicative'.

* In this paper, the term 'chronic' is used for all studies of 28 days exposure or longer and reproductive investigations.

This paper proposes 4 levels of significance that might be ascribed to a data set:

High Significance

Indicative Significance

Low Significance

Unusable

These terms are used to calibrate the **level of significance** that can be placed on *in vivo* and *in vitro* data as described below:

Assessing The significance of *In Vivo* Evidence

Table 4 displays the evaluation of 'Significance' for *in vivo* data and is based on the following basic principles:

- As tests for chronic effects are the most relevant, if the effects are of High Relevance, studies of Medium and High Reliability should be considered as of High Significance.
- As the overall significance of screening tests is lower than chronic tests, *in vivo* screening endpoints of High Relevance from studies of Medium and High Reliability should be considered as only of Indicative Significance.
- If the effects from a chronic study are of Medium Relevance, studies of Medium and High Reliability should also be considered as only of Indicative Significance.
- Screening studies of only Medium Relevance, but of Medium and High Reliability should be considered as of Low Significance and used merely as supporting information.
- Data from studies considered as of Low Reliability or as Unreliable should be considered as Unusable.

Table 4: *In vivo* Data Significance

		Study Repeatability			
Endpoint Relevance		**Unreliable**	**Low**	**Medium**	**High**
Chronic	**High**	*Unusable*	*Unusable*	*High*	*High*
test	**Medium**	*Unusable*	*Unusable*	*Indicative*	*Indicative*
Relevance	**Low**	*Unusable*	*Unusable*	*Positive effects to be reported*	*Positive effects to be reported*
Screening test	**High**	*Unusable*	*Unusable*	*Indicative*	*Indicative*
Relevance	**Medium**	*Unusable*	*Unusable*	*Low*	*Low*

Assessing The Significance of *In Vitro* Evidence

Table 5 is based on the following basic principles for assessing the significance of *in vitro* studies;

- No in vitro study can be considered as being of High Significance. At best it can be only 'Indicative' of mechanistic potential. However, a negative result of 'Indicative Significance' would be sufficient to be definitive.
- Only studies meeting both a high reliability and a high relevance should be assessed as being of 'Indicative significance'.
- Studies with a medium reliability and a high relevance, or vice versa should be assigned a 'Low significance' - for support purposes only.
- Data from unreliable studies or those with low reliability should be considered as unusable.

Table 5: *In vitro* Data Significance

		Study Reliability			
Endpoint Relevance		**Unreliable**	**Low**	**Medium**	**High**
Receptor	**High**	*Unusable*	*Unusable*	*Low*	*Indicative*
Relevance	**Medium**	*Unusable*	*Unusable*	*Unusable*	*Low*
Metabolic	**High**	*Unusable*	*Unusable*	*Low*	*Indicative*
Relevance	**Medium**	*Unusable*	*Unusable*	*Unusable*	*Low*

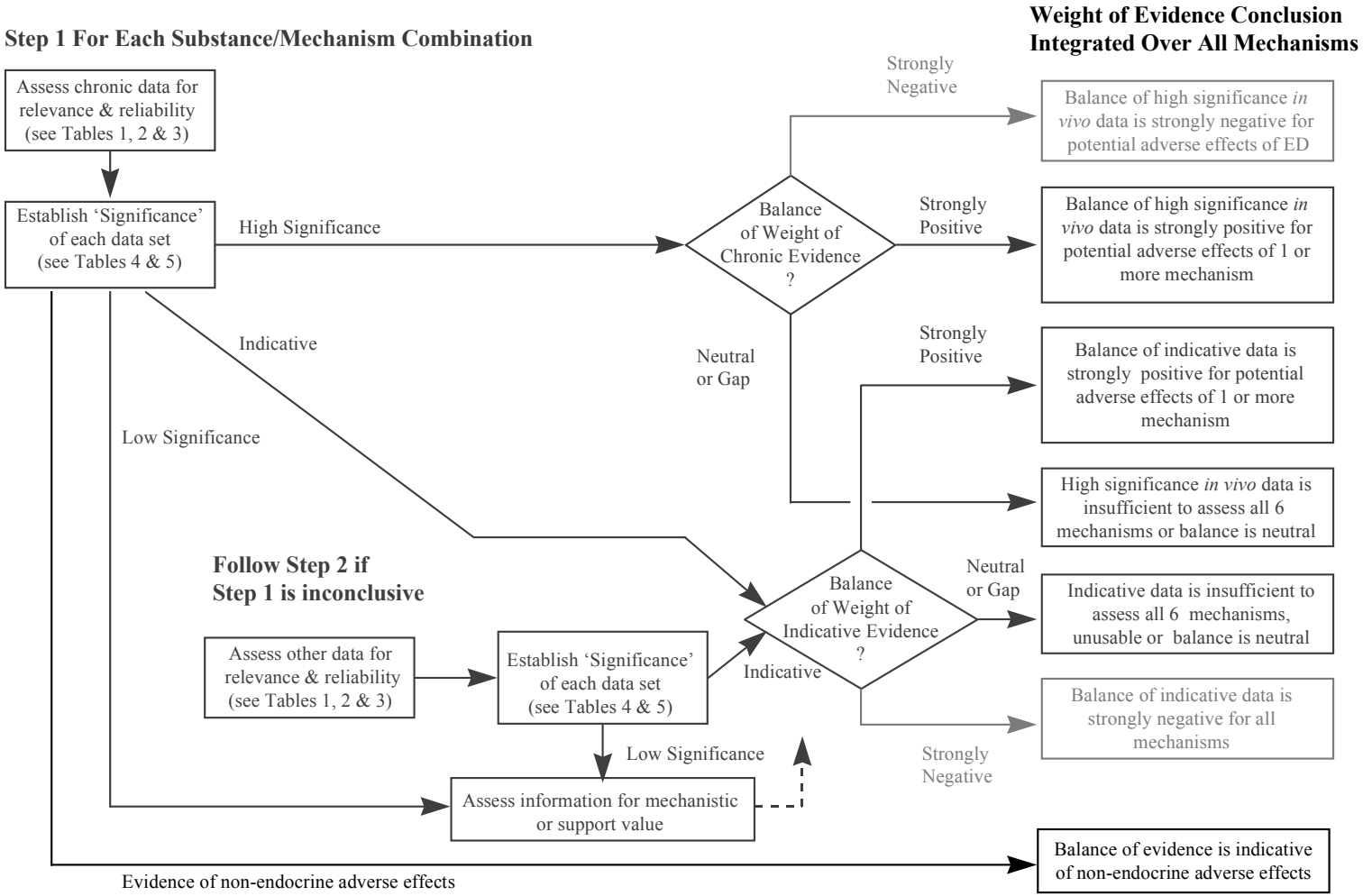
Use of Significance Assessments

Assessments of Significance are used in the process shown in Figure 2 (to be found on the next page*). It shows a 2-step process to be applied to each mechanism and is based on the premise that only evidence of '*In Vivo* High Significance' can be considered as being definitive in the 1st step. Any other *in vivo* data must be considered alongside *in vitro* data in the 2nd step as 'indicative' or as 'supporting' evidence only.

It is only necessary to proceed to the 2nd step if the 1st step is inconclusive.

* Underlying Premises and Assumptions for Figures 2 and 3 can be found in Table 6.

Figure 2: Assessing & Weighing The Balance Of Evidence



Endocrine Disruption
An Approach For Prioritising Action Based on A Weight of Evidence Approach
Table 6: Premises and Assumptions Applied to Figures 2 & 3

- The scheme shown in Figures 2 & 3 is based on a focused evaluation of substances in relation to the adverse effects that may result from 6 mechanisms:
    - Oestrogenic
    - Anti-oestrogenic
    - Androgenic
    - Anti-androgenic
    - Thyroid
    - Anti-thyroid
- Where an adverse effect is identified, but resulting from other mechanisms, then this should be reported and investigated as part of the more general risk assessment of the substance.
- All evidence of less than 'high significance - *in vivo*' is considered as of 'screening value only' or as 'unusable'.
- If sufficient is known about appropriate dose ranges, then dose/response testing could be implemented immediately after screening, thus reducing the overall amount of animal testing required.
- The scheme assumes that OECD Tier 1 tests for the 6 mechanisms above will be available soon for screening and that enhancements for multi-generation testing to cover the relevant end-points will have been agreed and validated as an OECD Tier 2 Test soon after.
- Priority is shown in colour: Red for 'high priority'; Blue for 'medium priority'; Green for 'no further action'.

2.24 Coherence, Gaps and Framework For Further Action

Once all relevant data have been evaluated for significance to all 6 mechanisms in accordance with the procedure outlined in Section 2.23, it should be possible to assign each substance to one of the right hand boxes in Figure 2 and to identify gaps in knowledge that need to be filled.

Simplistically, if all of the data fall into one of the boxes described in Figure 2, the substance could be actioned as proposed in Figure 3 (see next page). For example, if all high significance chronic data fall into the top-right box of Figure 2, then taking this forward into Figure 3, the procedure proposes that there is no need for further action and the substance should be removed from the 'List of Priority Actions'. Alternatively, should all data available fall into the second box, then again, going forward to Figure 3, the recommendation is for urgent risk assessment.

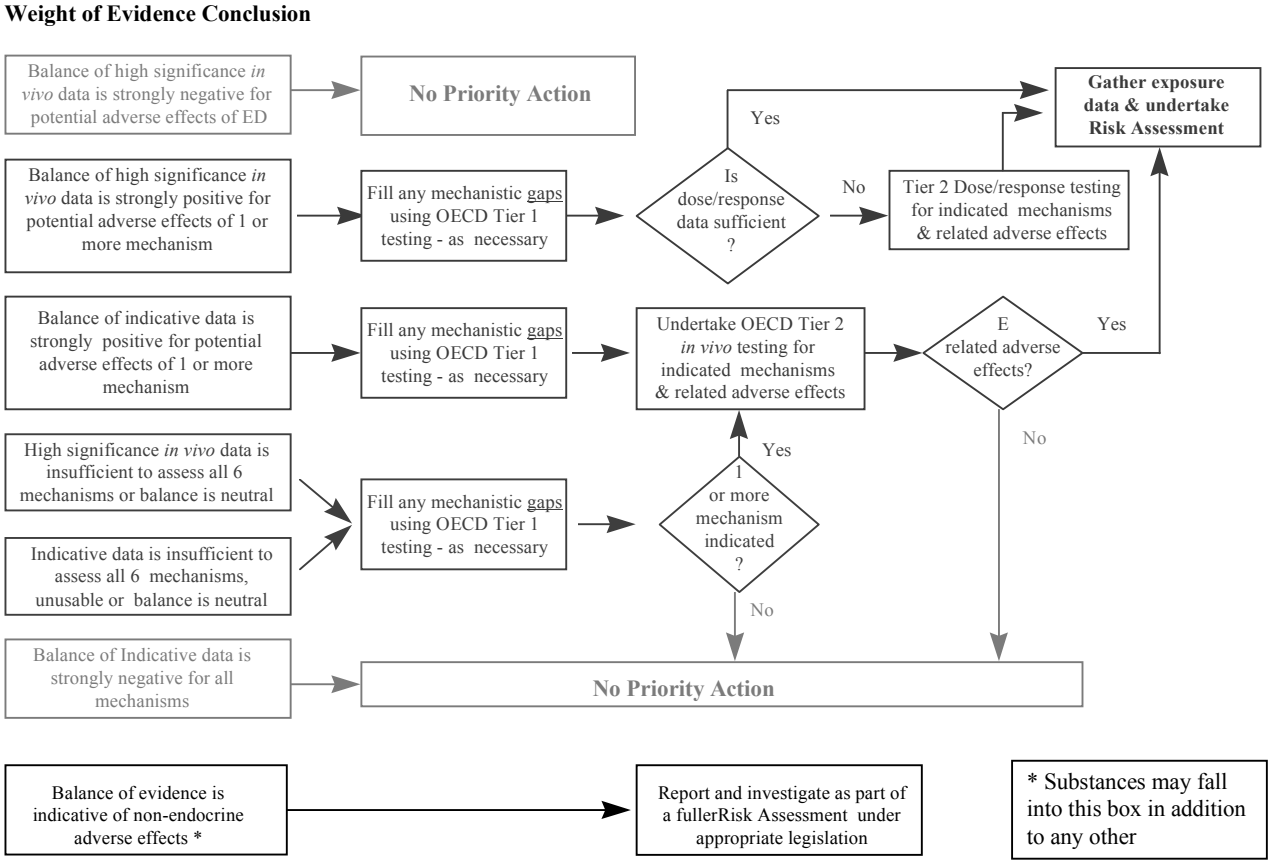
In the event that High Significance - *in vivo* dose response data covering the relevant end-points associated with all six mechanisms exists already, then it would be possible to jump directly to risk assessment without undertaking further testing. However, whilst this may be sufficient to assess the risk, it may leave some ambiguity about the mechanism. Those with an economic interest in the substance should judge whether additional mechanistic evidence can provide added value.

In the event that the available data can only shed light on some of the mechanisms, then it is recommended that gaps are filled initially using the OECD Tier 1 tests. This will then allow the Tier 2 tests to be designed to cover all the mechanisms of concern without unnecessary test complexity.

In practice, many substances may be positive for some mechanisms and negative for others - leading to the actions shown. Furthermore, the data may not be coherent - even for studies that are considered to be of

high significance. Clearly, if there is only one 'odd' study among many of similar significance, then one would be able to draw a conclusion based on the 'balance of the weight of evidence'. However, if the balance is neutral or close to neutral, then it will be necessary to undertake additional high quality studies for one or more mechanism to draw definite conclusions.

Figure 3: Weight of Evidence Derived Action Scheme



REPORTING

Criteria for the information search should be recorded in the report to ensure future duplication of the search is possible. Exclusion criteria should be incorporated into this record to explain why individual references were not considered for further examination.
Decisions taken under Sections 2.21, 2.22 & 2.23 should be recorded and justified.
Actions should be recorded for substances in accordance with Figure 3.
The report would, of necessity, incorporate an extensive list of all data considered, coupled with an explanation, as described in Tables 1-5 and Figures 2 & 3, as to how the final conclusions and recommendations for action were obtained.

CONCLUSIONS

The European Chemical Industry recommends that the weight of evidence approach is included in the process to identify a Priority Action List. This will ensure decisions are made on a complete evaluation of information rather than on a partial assessment, such as the 'evidence of suspicion' approach, which really does little more than count "positive" studies.
The inclusion of the weight of evidence approach introduces scientific rigour into the process for developing a Priority Action List. The actions proposed are specific and truly prioritised in terms of urgency. Furthermore, it provides a platform for evaluating a much larger group of chemicals for which little or nothing is known at the moment.
While the procedure can be applied immediately to existing data, it will quickly become clear from the resulting analysis that there are many data gaps. Furthermore, there is a need to conduct research which will aid better assessment and management of endocrine disrupting chemicals. An important component of this research is in the area of testing methodology and the Commission is most strongly urged to provide much needed support for the OECD testing initiative. While improvements and enhancements of testing protocols will build a better understanding of endocrine toxicity it is nonetheless expected that endocrine disrupting chemicals can be addressed adequately within the current risk assessment/management framework with only minor adjustments to include any new knowledge or enhancements of testing protocols.

REFERENCES

1. Klimisch, H-J., Andreae, M. and Tillmann, U. - A Systematic Approach For Evaluating The Quality of Experimental Toxicological and Ecotoxicological Data. Regulatory Toxicology and Pharmacology **25**, 1-5 (1997)
2. Rosner, G. - Validierung von SDS: Standardbegründungen - Fraunhofer-Institut Für Toxikologie und Aerosolforschung. (1994)
3. European Workshop on the Impact of Endocrine Disrupters on Human Health and Wildlife (Weybridge, UK, 1996). European Union Report EUR 17549
4. Office of Science and Technology Policy. Chemical Carcinogens; A Review of the Science and its Associated Principles. (1985)

Annex 1

DATABASE SEARCH SITES

Aquatic Sci&Fish Abs (c) 1998 FAO (for ASFA Mv Brd)
BioBusiness(R) (c) 1998 BIOSIS
BIOSIS PREVIEWS(R) (c) 1998 BIOSIS
CA SEARCH(R) (c) 1998 American Chemical Society
CAB Abstracts (c) 1998 CAB International
ChemEng & Biotec Abs (c)1998 RoySocChm,DECKEMA,FizChemie
CHEMTOX (R) Online (c) 1998 MDL Info Systems
CHRIS Chemical Hazards response system
Current Contents Search(R) (c) 1998 Inst for Sci Info
Ei Compendex(R) (c) 1998 Engineering Info. Inc.
EMBASE (c) 1998 Elsevier Science B.V.
Energy SciTec (c) 1998 Contains copyrighted material
Env.Bib. (c) 1998 Internl Academy at Santa Barbara
Enviroline(R) (c) 1998 Congressional Information Service
Hazardous Substances Database
Life Sciences Collection (c) 1998 Cambridge Sci Abs
Medline(R) (c) format only 1998 The Dialog Corporation
NTIS Comp&distr 1998 NTIS, Intl Copyright All Righ
Oceanic Abst. (c) 1998 Cambridge Scientific Abstracts
OHMTADS Oil and Hazardous materials/technical assistance data system
Pascal (c) 1998 INIST/CNRS
Pollution Abs (c) 1998 Cambridge Scientific Abstracts
RTECS Comp & dist by NIOSH, Intl Copyright All Rights Res
SciSearch(R) Cited Ref Sci (c) 1998 Inst for Sci Info
The Merck Index Online(SM) (c) 1998,1998 Merck & Co. Inc.
Toxline(R) (c) format only 1998 The Dialog Corporation
Water Resour.Abs. (c) 1998 Cambridge Scientific Abs.
WATERNET(TM) (c) 1998 American Water Works Association
Zoological Record Online(R) (c) 1998 BIOSIS

Endocrine Mechanism	Adverse Effect	Existing Regulations	Classification Category	Indicative Screen	RA Level Test	Existing Research To Fill Test/Screen Gaps
Oestrogenic						
	Carcinogenic (type)	Existing Chemicals				
		Pesticides				
		DSD				
		etc				
	Reduced Sperm Count/Quality					
	Hypospadias					
	Cryptorchidism					
	etc					
Anti-oestrogenic						
Androgenic						
Anti- androgenic						
Thyroidal						
Anti-thyroidal						
Etc						

Annex 2

GENERAL REQUIREMENTS OF RELIABLE in vitro LABORATORY STUDIES

1. **Basic experimental design**
   - There should be a minimum of three (usually five) test concentrations, ideally with one at a concentration expected to cause no response.
   - Intervals between test concentrations should be less than one order of magnitude.
   - Suitable controls should be included as well as the test concentrations, including a carrier control if a carrier solvent is used in the tests.
   - All controls and treatments should be replicated.
   - Top dose should show slight cytotoxicity

2. **Other aspects of test procedure**
   - Source and/or purity of test material should be specified.

3. **Analysis of results**
   - For a positive response, the results should normally show a concentration dependent response.
   - Results should be analysed for confidence limits or statistical significance, and data presented to allow verification.

Tests meeting all the above criteria have a high reliability. Tests meeting the criteria with bolded bullet points have a medium reliability. All other tests merit a low reliability or are unusable.

GENERAL REQUIREMENTS OF RELIABLE *in vivo* LABORATORY STUDIES

1. **Basic experimental design**
   - Top dose should be a maximum tolerated dose level for mammalian tests
   - There should be a minimum of two (usually three) test concentrations for mammalian studies, and typically 3 to 5 concentrations in non-mammalian studies, ideally with one at a concentration expected to cause no effects.
   - Suitable controls should be included as well as the test concentrations, including a carrier control if a carrier solvent is used in the tests.
   - All controls and treatments should preferably be replicated for screening assays (necessity of this requirement may be assessed based upon complexity of the experiment, and may be considered extraneous, based upon expert judgement).
   - Toxicity to the intact organism (animal) and any organ being used as an endpoint should be assessed
2. **Measured concentrations**
   - Exposure concentrations should be analysed
3. **Maintenance of test concentrations (non-mammalian studies only)**
   - Test concentrations should be maintained at reasonably constant levels.
   - Flow-through aquatic studies are usually better at maintaining test concentrations than static studies due to the regular replenishment of test substance(s).
4. **Other aspects of test procedure**
   - The stocking density, or animal numbers, should be appropriate.
   - The test should incorporate an appropriate feeding regime (where necessary).
   - Extraneous sources of stress should be minimised ie. noise, lighting, vibrations.
   - The test organism should be defined, and of a suitable age, sex and health.
   - Use of incompatible materials in the test apparatus should be avoided. (If concentrations are analysed and control mortalities reported, this becomes less important).
   - Purity and source of test material should be specified.
5. **Peripheral data**
   - Peripheral test data should be measured and reported ie. for aquatic studies, pH, dissolved oxygen, temperature and preferably hardness, type of water.
   - Analysis of diet(s) for potentially relevant contaminants (eg. PCBs).
6. **Analysis of results**
   - Results should be analysed in the context of both concurrent and historical control data.

- Ideally the results should show a concentration dependent effect and the results should be analysed for confidence limits or statistical significance.

Tests meeting all the above criteria have a high reliability. Tests meeting the criteria with bolded bullet points have a medium reliability. All other tests merit a low reliability or are unusable.

Appendix 10

Presentations made by reprentatives of countries/region and stakeholders at the workshop

Status of the US Endocrine Disruptor Screening Program (EDSP)

Steven Bradbury
US Environmental Protection Agency
Office of Prevention, Pesticides and Toxic Substances

1

EPA's Statutory Authority

Federal Food, Drug, & Cosmetic Act (FFDCA)

- Requires EPA to:
  - Develop a screening program using validated assays to identify chemicals that may have estrogenic effects in humans.
  - Test all pesticide chemicals (both active & inert ingredients).
- Authorizes EPA to obtain testing on:
  - Other endocrine effects, as designated by EPA Administrator (e.g., androgen & thyroid; endocrine effects in species other than humans).
  - Other chemicals (non-pesticides) that:
    - May have "an effect cumulative to that of a pesticide," if a "substantial human population may be exposed" to the chemical.

Safe Drinking Water Act (SDWA) Amendments

- Allow EPA to require testing of chemical substances that may be found in sources of drinking water if a substantial human population may be exposed to the substance.

2

US EPA EDSP

- Established the following recommendations:
  - The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) of 1996-1998.
- Estrogen, androgen, and thyroid.
- Human health, fish, and wildlife.
- Pesticides, commercial chemicals, and environmental contaminants.

3

US EPA EDSP

Based on EDSTAC Recommendation (1998):

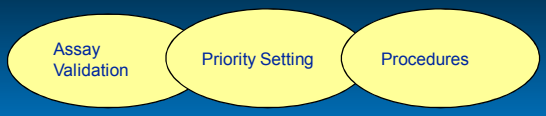
- Sorting and Prioritizing Chemicals.
- Tier 1 Screening.
  - Data to determine if a chemical has the potential to interact with the estrogen, androgen or thyroid systems.
- Tier 2 Testing.
  - Data to determine if endocrine-mediated adverse effects occur and quantify dose-response.
- Hazard and Risk Assessment.

OECD Endocrine Testing & Assessment Conceptual Framework

- Level 1 - Sorting and prioritizing with existing data and/or (Q)SARs.
- Level 2 - *In vitro* assays to provide mechanistic data.
- Level 3 - *In vivo* assays providing data about single endocrine mechanisms and effects.
- Level 4 - *In vivo* assays providing data about multiple endocrine mechanisms and effects.
- Level 5 - *In vivo* assays providing data about endocrine and other effects.

(OECD, 2004)

US EPA EDSP Implementation



- **Assay Validation**
  - Development and validation of test assays.
  - Tier 1 screening and Tier 2 testing.
- **Procedures**
  - Developing procedures to require and evaluate data.
- **Priority Setting**
  - Selecting chemicals to be screened.

6

US EPA EDSP Implementation

- **Assay Validation**
  - Development and validation of test assays.
  - Tier 1 screening and Tier 2 testing.

7

EDSP Assay Validation

Two-Tiered Approach

- Tier 1:
  - *In vitro* & *in vivo* screens.
  - Detect potential to interact with endocrine system.
  - OECD workshop case studies illustrate responses of assays to specific chemicals.
    - Methoxychlor/metabolites and Bisphenol A agonists in ER assays.
    - Vinclozolin/metabolites antagonist in AR assays.
    - Ketoconazole & Perchlorate positive in steroidogenesis assays.
    - Perchlorate positive in thyroid sensitive assays.
- Tier 2:
  - Tier 2 data called in only after review of Tier 1 data.
  - Multi-generation studies covering broad range of taxa.
  - Provide data for hazard assessment.

8

US EPA EDSP Tier 1 Screening Battery

In vitro
Estrogen receptor (ER) binding – rat uterus
Estrogen receptor α (hERα) transcriptional activation - Human cell line (HeLa-9903) [OECD Test Guideline 455]
Androgen receptor (AR) binding – rat prostate
Steroidogenesis – Human cell line (H295R) [US lead, validated in OECD program]
Aromatase – Human recombinant
In vivo
Uterotrophic (rat) [OECD TG 440]
Hershberger (rat) [OECD TG 441]
Pubertal female (rat)
Pubertal male (rat)
Amphibian metamorphosis (frog) [OECD TG 231]
Fish short-term reproduction [OECD TG 229]

US EPA EDSP Tier 2 Tests

Mammalian two-generation rat
(may be replaced by Extended F1-Generation)

Avian two-generation (Japanese quail)
[US lead, OECD validation program]

Amphibian growth/reproduction (S. tropicalis)
[US/Japan lead, OECD validation program]

Fish multigeneration (medaka)
[US/Japan lead, OECD validation program]

Mysid multigeneration
[US lead, OECD validation program]

US EPA EDSP Implementation

- **Priority Setting.**
  - Selecting chemicals to be screened.

11

Sorting & Prioritizing Chemicals

EDSTAC Recommended Four Categories:

1. Chemicals unlikely to interact with hormone systems (*e.g.,* certain polymers, strong mineral acids/bases).
2. Chemicals without sufficient existing data to determine if Tier 2 testing required.
3. Chemicals with sufficient existing data to determine if Tier 2 testing required.
4. Chemicals with sufficient data to support a hazard assessment.

Sorting Chemicals for Screening

Category 3 & 4 Chemicals:

- EDSTAC and SAB/SAP indicated some chemicals, potentially pesticide active ingredients with existing chronic reproductive/developmental assays in rodents, fish and birds, may fall in these categories.
- OECD workshop risk assessment case studies integrate a range of test data related to adverse effects mediated through interactions with endocrine systems.

Standard Toxicity Guideline Studies

- Provides results across different study designs for:
  - Different species (fish, birds, invertebrates, rats, mice, rabbits, dogs).
  - Different time course and durations of exposure (acute, short-term, chronic).
  - Different critical life stages (pre-conception, in utero, young, adults, old) over a broad range of doses.
  - Different routes of exposure (oral, dermal, inhalation).
  - Redundancy in reproductive parameters and histopathology.
- Endocrine disrupting compounds typically cause multiple effects depending on life stage & duration of exposure.

Risk Assessment

- Hazard Assessment/Characterization.
  - Lines of evidence across taxa -- Coherence of observations in the broader data base.
  - Mode of action and human relevance of animal findings.
  - Toxicokinetics/metabolites/degradates.
- Dose Response Assessment/Characterization.
- Exposure Assessment/Characterization.
  - Life stage exposure -- Endpoint selection to match exposure scenarios.
- Risk Characterization -- Information gaps, uncertainties, consistency, etc.
- Importance of well designed studies across and careful interpretation of literature studies.
- Cumulative risk.

Case Studies for Both Human Health & Ecological Assessment

- Atrazine - A triazine herbicide that in rats inhibits pulsatile release of gonadotrophin releasing hormone from hypothalamus, which in turn suppresses release of luteinizing hormone from pituitary.
- Vinclozolin - A dicarboximide fungicide whose metabolites are androgen receptor antagonists.
- Mancozeb – A fungicide, with its metabolite, ethylene thiourea (ETU), alters thyroid hormones, increases thyroid weight, and causes microscopic thyroid lesions and thyroid tumors in rats.

Prioritizing Chemicals for Screening

- Category 2 Chemicals (those without sufficient existing data):
  - Considered by the EDSTAC to have the largest number of chemicals and the greatest need for prioritization.
  - EDSTAC, EPA's Science Advisory Board, and EPA's Scientific Advisory Panel strongly recommended a prioritization scheme that included an effects and exposure component.

Prioritizing Chemicals for Endocrine Disruptor Tier 1 Screening: Effects

- EDSTAC recommended the use of measured or predicted receptor binding and/or transcriptional activation data derived through in vitro assays/High Throughput Screening (HTPS) and (Q)SARs, respectively.
- SAB/SAP in 1999 concurred, however, concluded that HTPS and (Q)SARs were not sufficiently developed at that time – encouraged continued research.
- As part of US EPA's computational toxicology and endocrine disruptor research programs, the Office of Research and Development (ORD), in collaboration with OPP and OSCP, has been developing in vitro assays, HTPS applications, and (Q)SARs.

Priority Setting: First Chemicals to be Screened

- Selection based on potential human exposure.
  - Pesticide active ingredients: presence in food and water, residential use, and occupational contact.
  - High Production Volume inert ingredients detected in human and environmental monitoring.
- Selected chemicals found in multiple exposure pathways.
- Issued the final list of chemicals for initial screening 15 April 2009.
  - 58 Pesticide active ingredients.
  - 9 High Production Volume / pesticide inert ingredients.
- **NOT a list of "known" or "likely" endocrine disruptors.**

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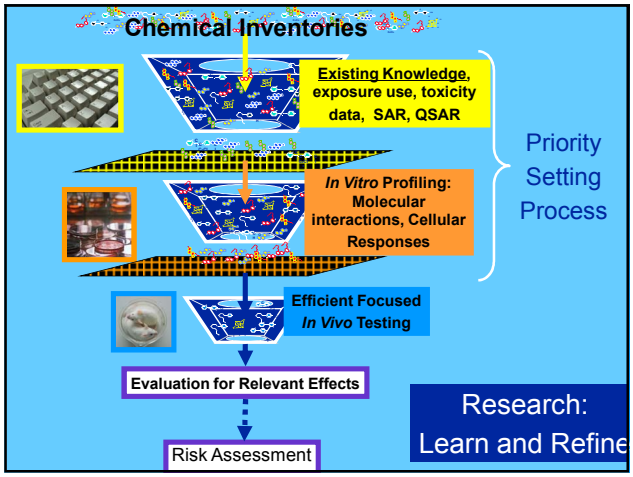
Future Prioritization for EDSP Tier 1 Screening

- Pesticide active ingredients – current plan is to use EPA's schedule for re-evaluating registered active ingredients in the Registration Review program, consistent with EDSTAC and SAB/SAP recommendations (http://www.epa.gov/oppsrrd1/registration_review/)
- Inert ingredients and other chemicals – develop *in vitro* and *in silico* tools that are integrated with exposure-based metrics



A (Q)SAR-Based System to Predict ER Binding Affinity

- Application for use in a prioritization scheme in the context of EDSTAC and SAB/SAP recommendations.
- Development focused on chemicals without sufficient existing data to determine if Tier 2 testing required.
- Model's applicability domain – Structures associated with inert ingredients and antimicrobial pesticides.
- External peer-review by USEPA SAP, August 2009. (www.epa.gov/scipoly/sap/meetings/2009/082509meeting.html)
- Development benefitted from EDTA VMG-NA and two OECD peer consultations.
  - May, 2008 Structural Alert Workshop. (www.olis.oecd.org/olis/2009doc.nsf/linkto/env-jm-mono(2009)4)
  - February, 2009 Expert Consultation to Evaluate an Estrogen Receptor Binding Affinity Model for Hazard Identification. (www.olis.oecd.org/olis/2009doc.nsf/linkto/env-jm-mono(2009)33)

Partnerships

- Collaboration on development and application of predictive approaches models:
  - OECD's Integrative Approaches to Testing and Assessment (OECD Workshop December 2007).
  - EPA's Pesticide Program Federal Advisory Committee Workgroup on Integrated Testing Strategies.
    - Purpose is to advise on communication and transition.

EPA Public Website on Integrative Testing and Assessment
www.epa.gov/pesticides/science/policies.htm

US EPA EDSP Implementation

- **Procedures**
  - Developing procedures to require and evaluate data

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US EPA EDSP Policies and Procedures

- Publication of final notice of Tier 1 Screening Battery – September, 2009.
- Posting of final Test Method Protocols – September, 2009.
- Issuance of Test Orders for 67 chemicals – Begins Fall, 2009.

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EDSP Tier 1Test Orders

- Approximately 750 Test Orders will be issued to the manufactures and importers of the 9 inert ingredients and the manufacturers of the 58 pesticide active ingredients.
- EPA anticipates issuing Test Orders over several months.
- Test Orders for chemicals will require all the assays in the Tier 1 battery.
- Responses to Test Orders due in 90 days.
- Data due 24 months from Test Order issuance.
- EPA will publically post decisions.

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Possible Responses to Test Orders

- I will generate new data.
- I am citing existing data.
- I am entering into an agreement to form a consortium to provide the data.
- I am not subject to the Test Order.
- I intend to voluntarily cancel the pesticide registration.
- I intend to reformulate the product(s) to exclude this chemical from the formulation.
- I am claiming a formulators' exemption.
- I have/am in the process of discontinuing the manufacture/import of this chemical.
- I do not and will not sell my chemical for use as an inert ingredient to the pesticide market.

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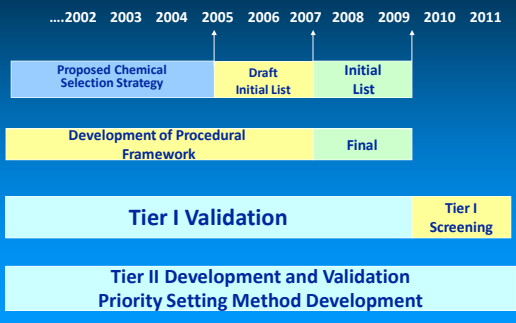
US EPA EDSP Timeline



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Supplemental Information

EPA Strategic Direction

Transition toward new integrative and predictive techniques, to increase efficiency and effectiveness of testing and assessment.

Animal Testing: Reduce, Refine, Replace

- 2005 EPA White Paper on Pesticides and Industrial Chemicals.
- 2007 NRC Report on Testing in the 21st Century.
- 2009 EPA's Strategic Plan for Evaluating the Toxicity of Chemicals.

Continue to advance computational tools to evaluate and establish priorities.

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Testing, Assessment and Management of Endocrine Disrupters in European Union

Peter Korytár
DG Environment
Unit D1: Chemicals

European Union

Strategy on Endocrine Disrupters

- December 1999 - the European Commission adopted a Community Strategy for Endocrine Disrupters (COM (1999) 706)
- Objectives of the paper were:
  - To identify problem of endocrine disruption, its causes and consequences
  - To identify appropriate policy action on the basis of the precautionary principle
- Four key elements/needs were identified
  - The need for further research
  - The need for international cooperation
  - The need for communication to the public
  - The need for policy action

Establishment of priority list of substances

- 575 substances were nominated by stakeholders as suspected endocrine disrupters in 1999
- 4 contracts (one in 2000, two in 2002 and one in 2007) were commissioned by the Commission to gather scientific evidence on the endocrine disruption of these chemicals
- The reports are available on the DG ENV website
  - http://ec.europa.eu/environment/endocrine/documents/index_en.htm

Database of substances having endocrine properties for further testing

- **Substances were categorized into 3 classes:**
  - 1 – At least one study published providing **evidence of endocrine disrupting effects in an intact organism**. Not a formal weight of evidence approach. On the basis of the precautionary approach, substances with insufficient evidence, but chemically closely related to category 1 substances, have been categorized as category 1
  - 2 – **Potential for endocrine disrupting effects**. *In vitro* data indicating potential for endocrine disruption in intact organisms. Also includes effects *in-vivo* that may, or may not, be ED-mediated. May include structural analyses and metabolic considerations.
  - 3a – **substances with no scientific basis for inclusion in list** (ED studies available but no indications on ED effects)
  - 3b – **substances with no or insufficient data gathered.**

Database of substances having endocrine properties for further testing

- **Database currently contain 428 substances**
  - Category 1 – 194 substances
  - Category 2 – 125 substances
  - Category 3a and 3b – 109 substances

Identification and assessment of endocrine disruptors

- Development of testing methods for endocrine disruptors
  - DG JRC – Institute of Health and Consumer Protection
  - Support of the action for harmonization and validation of test methods under auspices of OECD – Working group 'National Co-ordinators for Test Guidelines'
  - Development of alternative non-animal testing methods for screening and testing substances for endocrine disrupting properties by JRC

Research and development

- More than 80 projects funded via the Community Framework Programme for R&D
- The support started under FP4 programme
- Continued under FP5 and FP6
- Support of research focused on effects, identification and assessment of endocrine disrupting chemicals continues under the 7th Framework Programme
- http://ec.europa.eu/research/endocrine/index_en.html

Legislative action

- **REACH (1907/2006) – Regulation concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals**
  - Most of the provisions currently in force
  - Implementation period
- **Regulation on Plant Protection Products**
  - Passed the second reading in Parliament
  - Formal adoption expected in summer 2009
- **Regulation on biocides**
  - Commission proposal in 2009

Registration under REACH (1907/2006)

- no requirement in REACH Annexes VII to X to provide information on the endocrine activity of a substance or on a substance's reproductive or specific developmental toxicity in aquatic organisms

- However, according to Article 12, the information specified in Annexes VII-X is to be seen as a minimum requirement.

Evaluation under REACH

- Community rolling action plan for evaluation will be established

- Prioritization shall be on a risk-based approach (hazard, exposure, tonnage, …)

- If competent authority considers that further information is required, including information not required in Annexes VII to X, it can require from the registrants to submit further information
  - Information on endocrine disrupters can be included

Authorisation under REACH

- Substances to be included in Annex XIV (Article 57)
  - CMR cat. 1 or 2
  - PBT (criteria in Annex XIII)
  - vPvB (criteria in Annex XIII)
  - Substances – such as those **having endocrine disrupting properties** or those having persistent, bioaccumulative and toxic properties or very persistent and very bioaccumulative properties, which do not fulfil the criteria of points (d) or (e) – **for which there is scientific evidence of probable serious effects to human health or the environment which give rise to an equivalent level of concern as CMR, PBT and vPvB** and which are identified on a case-by-case basis in accordance with the procedure set out in Article 59.

Authorisation under REACH

- **Identification of substance as Substance of Very High Concern (SVHC)**
- **Listing of substance in Annex XIV**
- **Application for authorisation**
- **Granting the authorisation**
  - If risks are adequately controlled (not applicable for PBT, vPvB and non-threshold CMs)
  - Socio-economic benefits outweigh the risks and there are no alternatives available

Authorisation under REACH (Review)

- **By 1 June 2013 the Commission shall carry out a review to assess whether or not, taking into account latest developments in scientific knowledge, to extend the scope of Article 60 (3) (socio-economic route) to substances identified under Article 57 (f) as having endocrine disrupting properties. On the basis of that review the Commission may, if appropriate, present legislative proposals**

Restriction under REACH

- **Restriction on any condition for or prohibition of the manufacture, use or placing on the market**
- **May be imposed when**
  - an unacceptable risk to human health or the environment is not adequately controlled
  - this risk needs to be addressed on a Community-wide basis

Restriction vs. Authorisation

- **Authorisation: industry is not allowed to place on the market or use a substance included in Annex XIV unless industry has an authorisation granted by the Commission**
- **Restriction: industry has to comply with the conditions of the restriction in Annex XVII for the substance, no specific dossier submitted**

REACH Guidance

- **Guidance on Information Requirements and Chemical Safety Assessment**
- **Chapter 7.b: Endpoint specific guidance**
- **Appendix 7.8-5 Assessment of available information on endocrine and other related effects (ca 20 pp)**
  - Appended to main guidance on aquatic toxicity testing
  - Evaluation of information

    Not part of standard requirements => available info

REACH Guidance – Content

- **Non-testing**
- ***In vitro***
- ***In vivo*: vertebrates, invertebrates**
- **Use of the data in relation to Article 57.f**
- **Integrated assessment of potential endocrine activity**
  1. *Preliminary indication of potential endocrine activity in aquatic organism*
  2. *Indication of specific mode of action in intact aquatic organisms*
  3. *Characterisation of long-term adverse effects*

REACH Guidance – Integrated Assessment

1) Preliminary indication of potential endocrine activity in aquatic organisms		
Estrogen/androgen axis: - molecular structure - mammalian toxicity - *in vitro* screening	*Thyroid:* - molecular structure - mammalian toxicity	*Invertebrate systems:* - molecular structure
-> determine concern of potential endocrine mode of action of the substance using WoE of all available information, including environmental fate and exposure *-> strong concern may prompt a proposal by the Competent Authority to include the substance in the Community rolling action plan in order to perform a substance evaluation*		
2) Indication of specific endocrine modes of action in intact aquatic organisms		
Estrogen/androgen axis: - biochemical markers - morphological changes (- gonad histopathology)	*Thyroid:* - thyroid histopathology	*Invertebrate systems:* - rare individual cases
Study type: Fish Screening Assay Fish Sexual Develpt. Test Fish Reproduction Test Fish Full Life-Cycle Test	*Study type:* Amphibian Metamorphosis Assay	

REACH Guidance – Integrated Assessment

-> determine concern of potential endocrine mode of action in intact aquatic organisms using WoE of all available information, including environmental fate and exposure *-> strong concern may prompt a proposal by the Competent Authority to include the substance in the Community rolling action plan in order to perform a substance evaluation* 3) Characterisation of long-term adverse effects[#]		
Estrogen/androgen axis: - fish (sexual) development - fish reproduction	*Thyroid:* - amphibian development	*Invertebrate systems:* - development - reproduction
Study type: Fish Sexual Develpt. Test Fish Reproduction Test Fish Full Life-Cycle Test	*Study type:* Amphibian Metamorphosis Assay	*Study type:* Invertebrate Reproduction or Life-Cycle Tests
-> consider use of chronic NOEC for PBT assessment and Chemical Safety Assessment *-> consider classification and labelling according to safety net categories (R52, R53)* *-> causal link of adverse effect with an endocrine mode of action may prompt consideration for Annex XV by CA*		

Regulation on Plant Protection Products

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

Regulation on Plant Protection Products

- Provisions in relation to ecotoxicology (Annex II, point 3.8.2.):
  - An active substance, safener or synergist **shall only be approved if,** on the basis of the assessment of Community or internationally agreed test guidelines or other available data and information, **it is not considered to have endocrine disrupting properties that may cause adverse effect on non-target organism** unless the exposure of non-target organism to that active substance in a plant protection product under realistic proposed conditions of use is negligible.

Regulation on Plant Protection Products

- Requirements on the Commission
  - Within four year from the entry into force of this Regulation, the Commission shall present to the Committee referred to in Article 79 (1) a draft of the measures concerning specific scientific criteria for the determination of endocrine disrupting properties to be adopted in accordance with the regulatory procedure with scrutiny referred to in Article 79 (4)

Next steps

- Development of criteria and assessment methodology for identification of endocrine disrupters
  - **Review of the scientific state of the art of the assessment of endocrine disrupters**
  - **The results will be communicated to EDTA AG**
- Further development of "Endocrine-Active Compounds Database and Web Portal" to become living database
- Continuation in development of alternative testing methods (ECVAM)
- Continue to support OECD work on test methods development

Thank you very much for your attention

Peter Korytár
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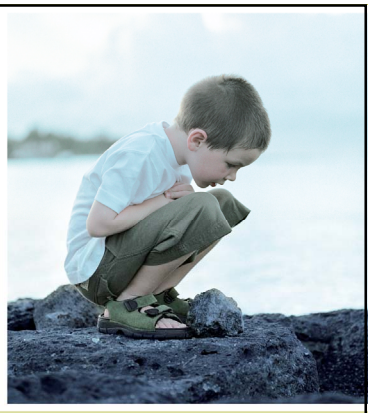
Testing, Assessment and Management of Endocrine Disrupters in Denmark

Pia Juul Nielsen
Chemicals Division
Danish EPA

OECD Workshop 22 September 2009



TESTING, incl. development of assessment tools and research

- **Cause-effect relationship**
- **Increases knowledge about specific substances or specific problems**
- **Development of assessment tools**

- investigation of tools for assessment of combination effects
- development of (Q)SAR models
- development, validation and standardisation of OECD TG's & drafting of DRP's and GD's

DK contribution to OECD TGP

- Fish Sexual Development Test (FSDT)
- Reproduction of springtails (TG232)
- GD on reproductive toxicity *(co-operation with U.S.)*
- Uterothrophic assay (TG440)
- Hershberger assay (TG441)
- Fish short term reproduction assay (TG230)
- Fish screening assay (TG229)
- In vitro steroid synthesis assay (H295R)
- Extended one-generation reproduction toxicity
- Revision of 2-generation study (TG416)
- Developmental neurotoxicity (TG426)
- Repeated dose 28-day study(TG 407)

Research/development

- *In vivo* thyroid assay
- *In vitro* screen for effects on the thyroid receptor
- Method to include metabolism in *in vitro* ED assays with cell lines

Co-operation

- **ED network**
  fruitful integration of research and test method development
  - important new findings
  - new regulatory interventions
- **Centre for Endocrine Disrupters**
  applied research directed towards the preventive work, including regulation

Assessment

General approach in relation to testing, assessment and management of substances with especially concerning hazardous properties

Discriminate between

- **Confirmed/regarded** as
- **Suspected** for
- **Potential** for

2 categories for EDC's

1. **ED *in vivo* (confirmed)**
2. a. **Suspected ED *(in vivo)***
   b. **Potential ED *(in vitro/in silico)***

Assessment and decision making framework on EDC's

Nordic report as input to OECD EDTA

- **EDC *in vivo***

  If risk ⇨ ***risk reduction*** *(restriction of production/use or authorisation)*

- **Suspected EDC *in vivo***
  - preliminary risk assessment – additional assessment factor

  If risk ⇨ ***soft regulatory intervention*** *and/or* ***definitive testing, evidence*** *required from industry*

- **Incentives to industry to provide more confirmatory evidence**
- ***while waiting*** ⇨ **voluntary interventions / advice to the public**

Assessment and decision making framework on EDC's

- **Potential EDC - *in vitro/in silico***

  - prioritisation for further investigations
  - supporting evidence and WoE expert judgements

  Other types of information (exposure, potential for uptake & metabolism etc. are used + other considerations)

Management

- **"Strict"**
  level of evidence high
  severity of the effect/concern large

  ⟹ **strong regulation at the EU level preferably**

  (restrictions or authorisation, e.g. phthalates in toys or phthalates in general)

- **"Soft"**
  Consideration of the level of evidence and severity and nature of hazard and risk

  suspicion substantiated
  severity of the effect/concern large

  ⟹ **regulatory intervention at the national level**
  – make incentives for generation of confirmatory evidence

Soft regulatory interventions

General principles

- Avoid unnecessary use of /minimize exposure to the chemical
- Promote generation of definitive evidence

How to make this operational

- Communication / advice to the public
  - information campaigns www.babykemi.dk
- Incentives for industry
  - development of alternative substances
  - promote voluntary risk reduction agreements
- Proposal for/promotion of regulatory action at the EU level
  - generation of definitive data
  - regulation based on the precautionary principle

DANISH MINISTRY OF THE ENVIRONMENT

The end

- Thanks for your attention!

DANISH MINISTRY OF THE ENVIRONMENT

1

Screening and Testing Scheme for Endocrine Disrupting Chemicals (MHLW) updated for OECD EDTA Workshop, *September 22-24, 2009 @ Copenhagen*

Jun Kanno, MD, PhD,
Division of Cellular and Molecular Toxicology,
Biological Safety Research Center,
National Institute of Health Sciences, Tokyo.

Note: This presentation is partly endorsed by the MHLW Endocrine Disruptor Committee and partly based on personal opinion of the presenter who is a member of the Committee and of a member of the MHLW-funded research groups.

Do not cite, ror the OECD EDTA Workshop only, contains unpublished data

2

- Hormonally Active Chemicals (HACs)
  =candidate for EDC
- Endocrine Disrupting Chemicals (EDCs)
  =HACs that induce "adverse effects"

- Hormonal Effects
  =Receptor Mediated Effects
- Endocrine Disruption
  =Receptor Mediated Toxicity

3

Receptor Mediated Toxicity

- Examples:
  - TCDD : AhR KO* mice → virtually no toxic symptom.
    - Symptoms = results of gene expression through AhR
    - *Wild type mice die of their own gene products!*
  - Estrogens : ERKO** mice → no DES effect.
    No uterotrophic response.
    - Intrinsic ligand (estradiol) is present for physiological function.

Physiological responses ←→ adverse effects

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Traditional Toxicity

Toxic Substance(●)

Target Sites
- Normal function
  - Proteins(Enzyme)
  - Membrane
  - Etc.

Receptor-Mediated Toxicity

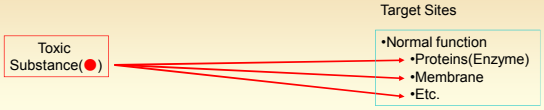
Toxic substance (■)

Receptor

Target Sites
- Protein Expression
  - Normal Timing
  - Right Kind
  - Right Amount

5

Traditional Toxicity



Receptor-Mediated Toxicity

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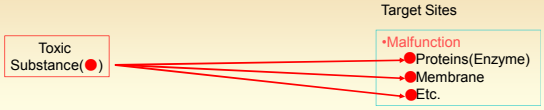
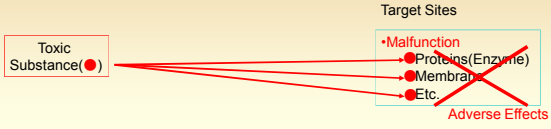
Traditional Toxicity



Receptor-Mediated Toxicity

7

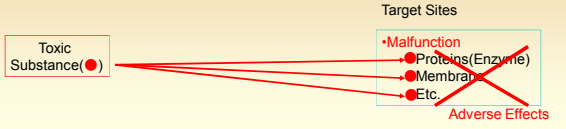
Traditional Toxicity



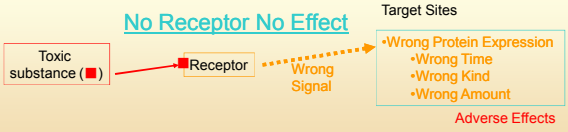
Receptor-Mediated Toxicity

8

Traditional Toxicity



Receptor-Mediated Toxicity



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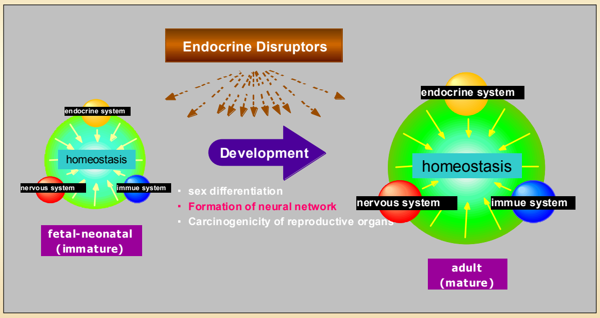
A Paradigm-Shift in Toxicology

Traditional (Regular) Toxicology	Receptor-Mediated Toxicology		Dose-Range
Regular Toxicity (Membrane damage, Enzyme damage, etc.)			
NOEL of Trad Tox	AR system (antagonist)	H	10^{-6} - 10^{-7} M
	ER system (agonist)	UT	10^{-9} - 10^{-10} M

Reference : Oral contraceptive -- EE = ca. 0.5μg/kg/day (P=1.0mg/tab EE=0.035mg/tab)

10

Sensitive time window and adverse effects



11

THE POSTNATAL DEVELOPMENT OF THE VISUAL CORTEX AND THE INFLUENCE OF ENVIRONMENT

Nobel lecture, 8 December 1981

by

TORSTEN N. WIESEL

Harvard Medical School, Department of Neurobiology, Boston, Massachusetts, U.S.A.

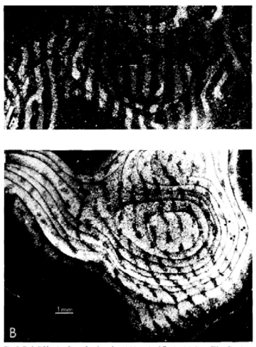


Fig. 3. Dark field autoradiographs of monkey striate cortex following injection of ³H-proline in the vitreous of one eye 2 weeks before.

A: Normal monkey, a montage of a series of tangential sections through layer IVC. The light stripes, representing the labelled eye columns, are separated by gaps of the same width representing the other eye. B: Monocularly deprived monkey, again a montage from a series of tangential sections through layer IVC. Same monkey as in Fig. 1, right, and Fig. 2, which had the right eye closed at 2 weeks for 18 months. The input from the normal eye is in form of expanded bands which in places coalesce, obliterating the narrow gaps which represent the columns connected to the closed eye.

・・・. The design of these experiments was undoubtedly influenced by the observation that children with congenital cataract still have substantial ……..

12

The Developing Synapse: Construction and Modulation of Synaptic Structures and Circuits
Susana Cohen-Cory, *et al.*
Science **298**, 770 (2002);

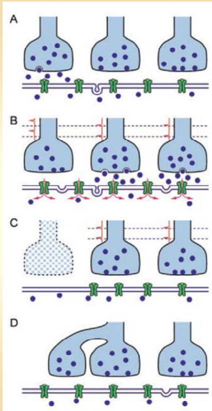


Fig. 4. Activity-dependent refinement of synaptic connections. (**A**) Synapses made by terminals of different axons co-innervating the same postsynaptic dendrite are initially maintained by low-level constitutive secretion of neurotransmitters. (**B**) Synchronous firing of two axon terminals (middle and right) leads to increased postsynaptic depolarization and neurotransmitter receptor activation (red arrows). (**C**) The unsynchronized axon terminal (left) does not experience postsynaptic spiking at the time of synaptic activation, therefore potentially reducing its retrograde neurotrophic support and ultimately resulting in the weakening of the synapse and the eventual withdrawal of the synaptic terminal. (**D**) Terminals that are active receive neurotrophic support and sprout (left) to establish new synapses.

Hebbian theory: Hebb, D.O. (1949), The organization of behavior, New York: Wiley

13

The Journal of Toxicological Sciences (J. Toxicol. Sci.)
Vol.34, Special Issue II, SP279-SP286, 2009

Intrauterine environment-genome interaction and Children's development (2): Brain structure impairment and behavioral disturbance induced in male mice offspring by a single intraperitoneal administration of domoic acid (DA) to their dams

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(Received February 17, 2009)

ABSTRACT — To demonstrate induction of delayed central nervous toxicity by disturbing neuronal activities in the developing brain, we administered a single intraperitoneal dose of domoic acid (DA; 1 mg/kg), a potent glutamate receptor agonist, to pregnant female mice at the gestational day of 11.5, 14.5 or 17.5. The dams had recovered from acute symptoms within 24 hr, followed by normal delivery, feeding and weaning. All male offspring mice after weaning were apparently normal in response to handlers during cage maintenance, body weight measurement and to mate mice in group housing conditions. At the age of 11 weeks, our neurobehavior testing battery revealed severe impairment of learning and memory with serious deviances of anxiety-related behaviors. The developed brain of prenatally exposed mice showed myelination failure and the overgrowth of neuronal processes of the limbic cortex neurons. This study indicates that the temporal disturbance of neurotransmission of the developing brain induces irreversible structural and functional damage to offspring which becomes monitorable in their adulthood by a proper battery of neurobehavioral tests.

Key words: Domoic acid, Prenatal exposure, Brain structure, Behavior

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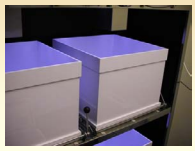
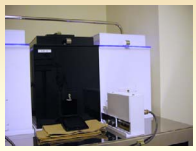
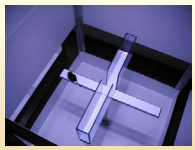
Openfield test



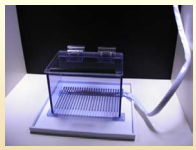
Light/Dark transition test



Elevated Plus maze test



Fear conditioning test



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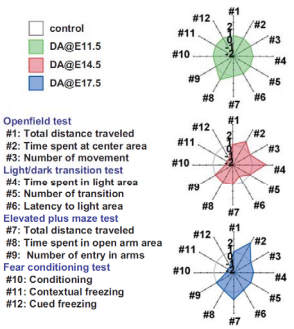

Fig. 7. Summary radar chart of the neurobehavioral battery test results. Radial axis indicates the direction (increase or decrease) of the deviation, and the p value of the endpoints compared to the control (+1 and −1, 0.01 <= p < 0.05, +2 and −2, p < 0.01). Regular dodecagon of radius 0 indicates no deviation from control.

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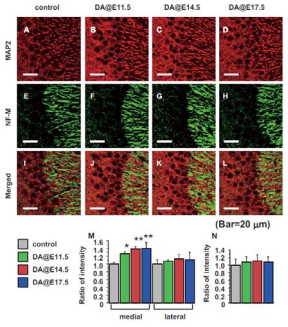

Fig. 2. Delayed effects on hippocampus induced by prenatal exposure of DA. A-D, Immunohistochemical staining against MAP2; E-H, immunohistochemical staining against NF-M; I-L, merged images, of CA3 hippocampus. A, E, I, group A (control), B, F, J, group B (DA@11.5), C, G, K, group C (DA@14.5) and D, H, L, group D (DA@17.5). Scale bar = 200 mm. M, Quantitative analysis of MAP2 expression, and N, NF-M expression among the groups (mean ± S.E.M.). Asterisk (**) and (*) indicated significant difference compared to control ($P < 0.01$) and ($P < 0.05$).

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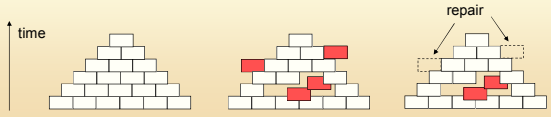
Characteristics of signal toxicity in embryo/fetus, neonate and children

- Target:
  - Neuronal system, Immune system, Endocrine system (under neuro)
- Key
  - Systems that keep MEMORY = Stepwise build up of the system
  - Disruption during building up cannot be perfectly repaired
  - Level of disruption
    - Cell death (loss) level
    - Non-cell death level => Wiring defect / circuit defect



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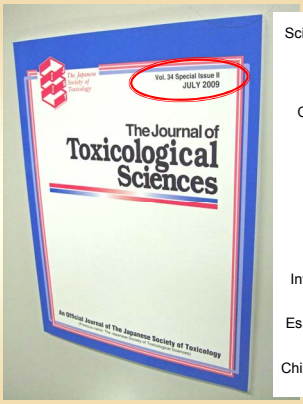

Scientific basis for risk analysis of food-related substances with particular reference to health effects on children
- Yuzo Hayashi

Comparative juvenile safety testing of new therapeutic candidates: Relevance of laboratory animal data to children
- Tim Anderson

Children's toxicology from bench to bed
- Liver Injury x 4

Children's toxicology from bench to bed
- Drug-induced Renal Injury x 4

Intrauterine environment-genome interaction and Children's development x 4

Essentials for starting a pediatric clinical study x 4

Children's Immunology, what can we learn from animal studies x 3

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The Scheme

- Since 1998, multiple Research Groups have been assembled for studying EDCs issue by MHLW*
- MHLW Screening and Testing Scheme for Endocrine Disrupting Chemicals (2002 (ver.1) and 2005 (ver. 2).
- Endorsed by the Advisory Committee on Health Effects of Endocrine Disruptors. http://www.nihs.go.jp/edc/english/edc.html
- Screening: Prioritize tens of thousands of chemicals by hormonal activities.
- Definitive Testing for the risk assessment and following risk management (prioritized chemicals).

*The Japanese Ministry of Health Labour and Welfare (MHLW) Health Science Research Grants.

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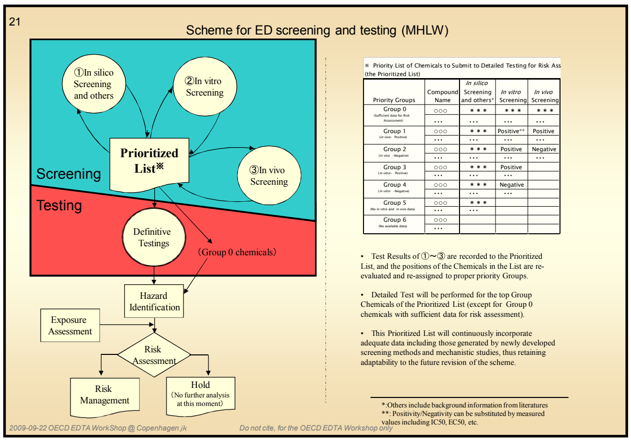
21
Scheme for ED screening and testing (MHLW)
①In silico Screening and others
②In vitro Screening
③In vivo Screening
Prioritized List※
Screening
Testing
Definitive Testings
(Group 0 chemicals)
Hazard Identification
Exposure Assessment
Risk Assessment
Risk Management
Hold (No further analysis at this moment)
※ Priority List of Chemicals to Submit to Detailed Testing for Risk Ass (the Prioritized List)
• Test Results of ①〜③ are recorded to the Prioritized List, and the positions of the Chemicals in the List are re-evaluated and re-assigned to proper priority Groups.
• Detailed Test will be performed for the top Group Chemicals of the Prioritized List (except for Group 0 chemicals with sufficient data for risk assessment).
• This Prioritized List will continuously incorporate adequate data including those generated by newly developed screening methods and mechanistic studies, thus retaining adaptability to the future revision of the scheme.
*:Others include background information from literatures
**: Positivity/Negativity can be substituted by measured values including IC50, EC50, etc.
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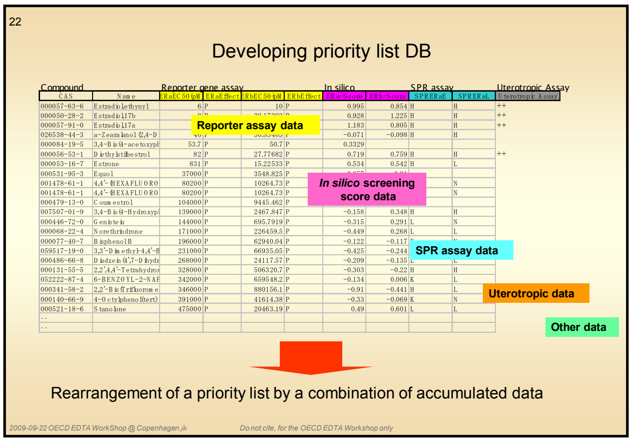
Priority Groups	Compound Name	*In silico* Screening and others*	*In vitro* Screening	*In vivo* Screening
Group 0 (Sufficient data for Risk Assessment)	○○○	＊＊＊	＊＊＊	＊＊＊
	・・・	・・・	・・・	・・・
Group 1 (in vivo- Positive)	○○○	＊＊＊	Positive**	Positive
	・・・	・・・	・・・	・・・
Group 2 (in vivo -Negative)	○○○	＊＊＊	Positive	Negative
	・・・	・・・	・・・	・・・
Group 3 (in vitro- Positive)	○○○	＊＊＊	Positive	
	・・・	・・・	・・・	
Group 4 (in vitro -Negative)	○○○	＊＊＊	Negative	
	・・・	・・・	・・・	
Group 5 (No in vitro and in vivo data)	○○○	＊＊＊		
	・・・	・・・		
Group 6 (No available data)	○○○			
	・・・			


22
Developing priority list DB
Reporter assay data
In silico screening score data
SPR assay data
Uterotropic data
Other data
Rearrangement of a priority list by a combination of accumulated data
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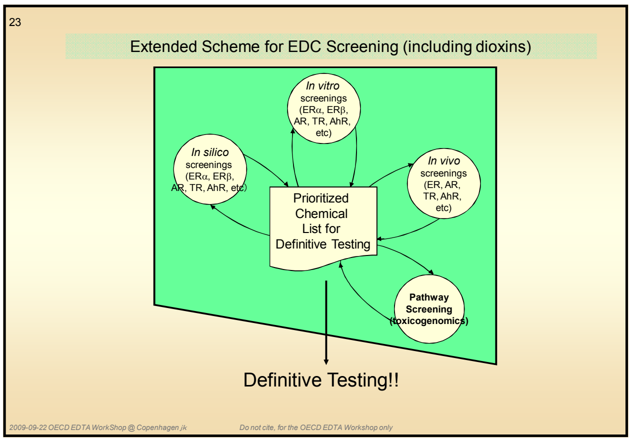
Compound		Reporter gene assay				In silico		SPR assay		Uterotropic Assay
CAS	Name	ERaEC50(pM	ERaEffect	ERbEC50(pM	ERbEffect	ERa-Score	ERb-Score	SPRERaE	SPRERaL	Uterotropic Assay
000057-63-6	Estradiol,ethynyl	6	P	10	P	0.995	0.854	H	H	++
000050-28-2	Estradiol,17b	[illegible]	[illegible]	[illegible]	[illegible]	0.928	1.225	H	H	++
000057-91-0	Estradiol,17a	[illegible]	[illegible]	[illegible]	[illegible]	1.183	0.805	H	H	++
026538-44-3	a-Zearalanol (2,4-D	[illegible]	[illegible]	[illegible]	[illegible]	-0.071	-0.098	H	H	
000084-19-5	3,4-Bis(4-acetoxyph	53.7	P	50.7	P	0.3329				
000056-53-1	Diethylstilbestrol	82	P	27.77682	P	0.719	0.759	H	H	++
000053-16-7	Estrone	831	P	15.22533	P	0.534	0.542	H	L	
000531-95-3	Equol	37000	P	3548.825	P	[illegible]	[illegible]			
001478-61-1	4,4'-(HEXAFLUORO	80200	P	10264.73	P	[illegible]	[illegible]	[illegible]	N	
001478-61-1	4,4'-(HEXAFLUORO	80200	P	10264.73	P	[illegible]	[illegible]	[illegible]	N	
000479-13-0	Coumestrol	104000	P	9445.462	P	[illegible]	[illegible]	[illegible]		
007507-01-9	3,4-Bis(4-Hydroxypl	139000	P	2467.847	P	-0.158	0.348	H	H	
000446-72-0	Genistein	144000	P	695.7919	P	-0.315	0.291	L	N	
000068-22-4	Norethindrone	171000	P	226459.5	P	-0.449	0.268	L	L	
000077-40-7	Bisphenol B	196000	P	62940.04	P	-0.122	-0.117	[illegible]	[illegible]	
059517-19-0	3,3'-Dimethyl-4,4'-B	231000	P	66935.05	P	-0.425	-0.244	[illegible]	[illegible]	
000486-66-8	Daidzein (4',7-Dihydr	268000	P	24117.57	P	-0.209	-0.135	L	[illegible]	
000131-55-5	2,2',4,4'-Tetrahydrox	328000	P	506320.7	P	-0.303	-0.22	H	H	
052222-87-4	6-BENZOYL-2-NAP	342000	P	659548.2	P	-0.134	0.006	K	L	
000341-58-2	2,2'-Bis(Trifluorome	346000	P	880156.1	P	-0.91	-0.441	H	L	
000140-66-9	4-Octylphenol(tert)	391000	P	41614.38	P	-0.33	-0.069	K	N	
000521-18-6	Stanolone	475000	P	20463.19	P	0.49	0.601	L	L	
..										
..										


23
Extended Scheme for EDC Screening (including dioxins)
In vitro screenings (ERα, ERβ, AR, TR, AhR, etc)
In silico screenings (ERα, ERβ, AR, TR, AhR, etc)
In vivo screenings (ER, AR, TR, AhR, etc)
Prioritized Chemical List for Definitive Testing
Pathway Screening (toxicogenomics)
Definitive Testing!!
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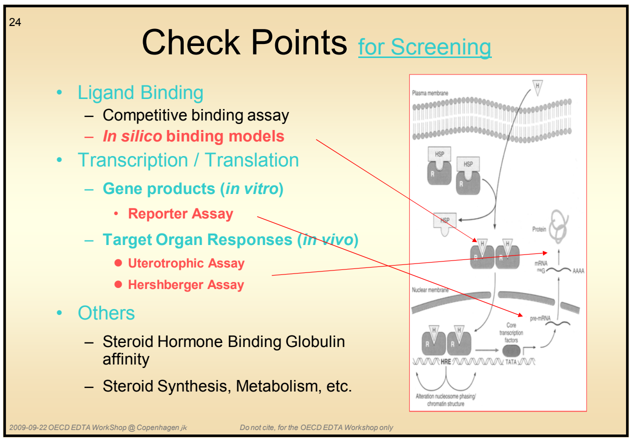
24
Check Points for Screening
• Ligand Binding
– Competitive binding assay
– In silico binding models
• Transcription / Translation
– Gene products (in vitro)
• Reporter Assay
– Target Organ Responses (in vivo)
• Uterotrophic Assay
• Hershberger Assay
• Others
– Steroid Hormone Binding Globulin affinity
– Steroid Synthesis, Metabolism, etc.
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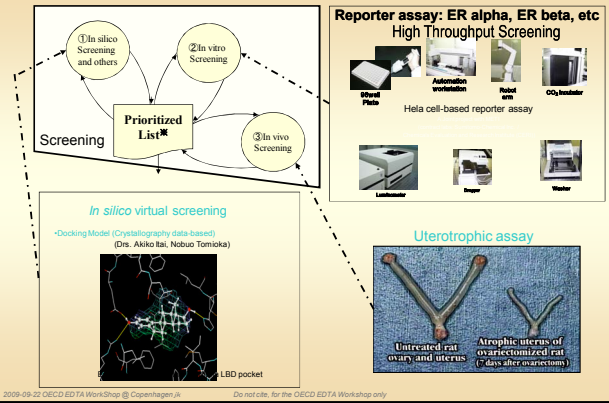
For Estrogenic Compounds



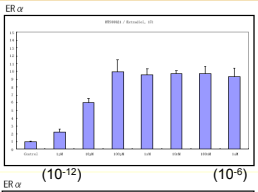
26

Bisphenol A ERα Reporter Assay (Hela cell)

HTS00021 Estradiol, 17b

PC50 (pM): 8 (8×10^{-12}M)

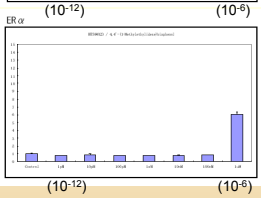
Threshold (pM)



HTS00023 Bisphenol A

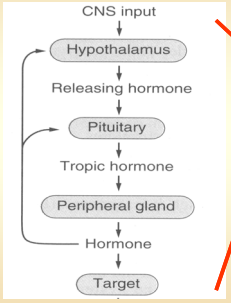
PC50 (pM): 828,081 (8×10^{-7}M)

Threshold (pM) 118,648

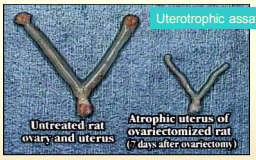


27

Basic Mechanism of Endocrine system; Application to Screening of HAAs



It is a Feedback System :
Hypothalamus and Pituitary Gland Are Monitoring the Serum Levels of Hormones.



No Feedback Loop
= Higher Sensitivity to both Agonists and Antagonists

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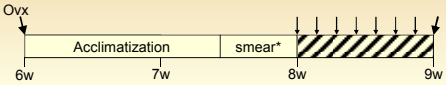
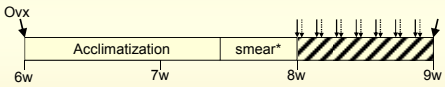
Mouse Uterotrophic Assay

Agonistic activity detection (Groups 1~6)

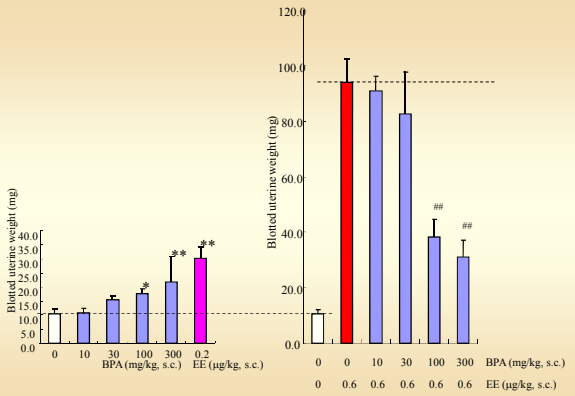


Antagonistic activity detection (Groups 7~11)



↓ : Ovariectomy
smear* : Estrus cycle observation (only uncycling mice were used)
↓ : Test chemical or vehicle (s.c. or p.o. daily administration for 7 days)
⇣ : Ethynylestradiol (0.6 μg/kg) s.c. injection 15 minutes after test chemical administration
↓ : Uterine weight measurement

29



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Data

		In silico Virtual screening	*In vitro* * Reporter Assay]	SPR (cell-free)
Receptor	ERα	◎/antagnist ○ (200,000)	◎ (500)	↓
	ERβ	○ (200,000)	◎ (100)	↓
	AR	△*	◎ (50)	↓
	TRβ	△*	◎ (50)**	↓
Response Elements	ERα+ERE	→		◎ (300)
	ERβ+ERE	→		◎ (30)
	TRβ+DR4	→		△ under dev
	AR+ARE	→		△***
Cofactor	ERα+TIF-2	→		◎ (300)
	ERβ+TIF-2	→		◎ (30)
	ERα+SRC-1	→		○
	ERβ+SRC-1	→		○

In vivo * (UT & Hersh)	
s.c.	p.o.
◎ 35	◎ 35
○ 7	○ 7

* AA sequense-based simulation
** TR beta + RXR system
*** Waiting for AR protein

*Only from MHLW. METI dose some more

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Definitive testing

(under development by MHLW research groups)

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Definitive testing

(under development by MHLW research groups)

Key is to use ordinary strain of rat (or mouse) and combination of ordinary procedures (more precisely "as ordinary as possible").

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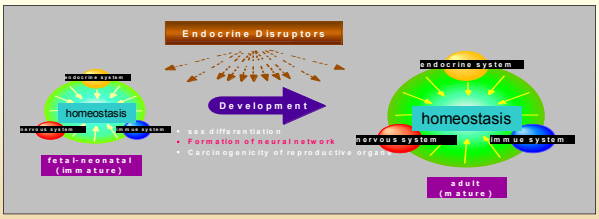
Definitive testing end points:

- Multi-generation study endpoints are not sensitive to known estrogens such as DES and 17beta-estradiol
- "Low-dose effects" = Early Exposure - Late Effect
  - non-reprotox endpoints
  - Neurological endpoints, Immunological endpoints
  - may look like ageing-related phenotypes



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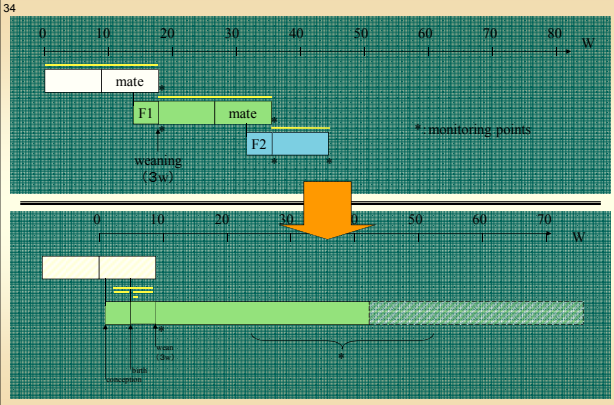

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Bisphenol A perinatal exposure study <1st study>	Bisphenol A perinatal exposure study <2nd study>	Diethylstilbestrol (DES) exposure study <2nd study>
CERI Dr. Shuji NODA	CERI Dr. Shuji NODA	Anpyo Dr. Ryota TANAKA
Bisphenol A(BPA) p.o. to pregnant rat dpc 6 through pnd 20 5, μg/kg/day, 50 μg/kg/day, 40 mg/kg/day, 400 mg/kg/day, and EE 50 μg/kg/day	Bisphenol A(BPA) p.o. to pregnant rat dpc 6 through pnd 20 0.5 μg/kg/day, 5 μg/kg/day, 50 μg/kg/day,	DES p.o. to pregnant rat dpc 6 through pnd 20 0.2 ng/kg/day 2 ng/kg/day 20 ng/kg/day

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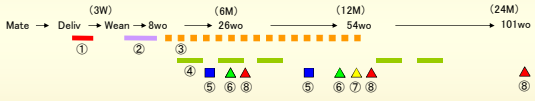
One life span test for rodent by Dr. Ohta, Food and Drug Safety Center

1) Rat strain	CrlCD
2) Route	DES, oral gavage to naonate
3) Dosage	0, 0.05, 0.5, 5 μg/kg/day (cf. 5 μg/kg/day is positive for Uterotropic assay)
4) n	10 dams or more/group (40 or more males and females per group)
5) Exposure period	**PND 1 through 5**
6) End points	



①	exposure	1～5day	gavage
②	Sex mat	3～7weeks	Vaginal opening, preputial separation
③	cyclisity	8～52weeks	Vaginal smear (2w per mo)
④	mating	12,23,34,56,68wo	Max 2w mating
⑤	behavior	24,48wo	Avoidnace test by shuttle box
⑥	immune	26,52wo	Antibody production against SRBC
⑦	ovulation	54週齢wo	Ovulation by hCG
⑧	necropsy	26,52,54,102wo	Sperm tests and histology

37

Schedule Controlled Operant Behavior (SCOB)
by Dr. M. Miyagawa (National Institute of Industrial Health)

0. **Restricted Feeding (Body Weight: 300g(♂)、200g(♀))**
1. **Auto-shaping (7 sessions) (start at 12 weeks of age)**
2. **FR 2 (2 sessions)**
3. **FR 5 (1 session)**
4. **FR 10 (10 sessions)**
5. **Alt Mix FR 10 DRO 10 sec TO 4 sec (25 sessions)**
6. **Alt Mix FR 10 DRO 10 sec TO 4-20 sec (A、25 sessions)**
7. **Alt Mix FR 10 DRO 10 sec TO 4-20 sec (C、25 sessions)**
8. **Pharmacological challenge tests (methamphetamine)**
9. **Pharmacological challenge tests (haloperidol)**

Pregnant rat from dpc 6 to pnd 20

0, 0.33, 3.3, 33 ppm of BPA in diet
(⇒ 0, 0.025, 0.25, 2.5 mg/kg b.w.)

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Percellome Method

- Concept:
  - Obtain mRNA quantity data in a "per one cell" basis (average).
- Merits:
  - Accurate comparison among samples // organs
  - Accurate comparison between studies
  - Accurate comparison between different microarray (different version, different make)

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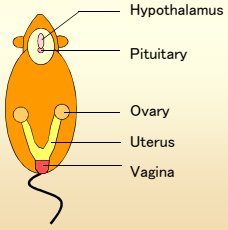
Background Transcriptome Data for Endocrine Disruptor Study: Adult (cycling) Female Mouse

Current Tox Study: Averaging out the cycling females

Mouse : C57BL/6CrSlc♀11wo
cycle : 4 stages (vaginal smear during sampling)
Target : 5 organs→
Percellome
GeneChip Mouse Genome 430 2.0
n=5 per stage



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Percellome Toxicogenomics

- Will shift EDC research from Regression model-based (fingerprint-based) to Signal cascade based (mechanism of action-based)
- Can be used as EDC "Screening backup" and "Testing backup"
- In the future --- may lead to modification of existing methods / protocols

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Receptor-Mediated Toxicity

To cover as many known cascades as possible

- Estrogenic (receptor-mediated)
- Anti-Androgenic (receptor-mediated)
- Anti-Thyroid (TPO inhibitors)

- ER agonists/antagonists
- AR antagonists
- TR antagonists
- RXR agonists
- PPAR agonists
- AhR agonists

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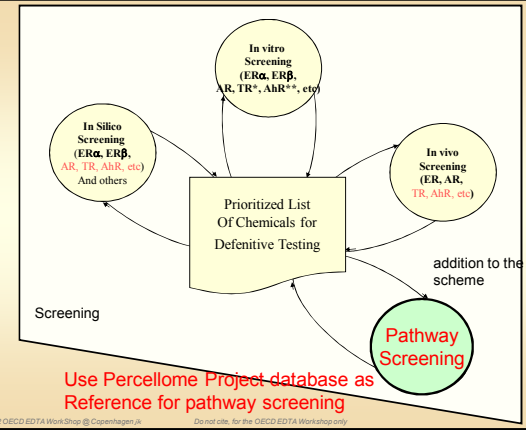

Use Percellome Project database as Reference for pathway screening

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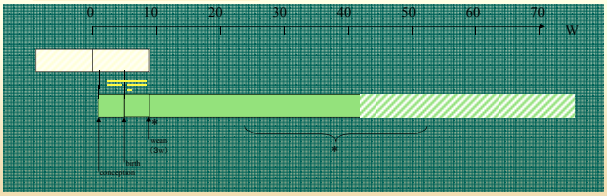
Percellome Project Toxicogenomics Back up

- *in vitro* samples
  - neuronal culture
  - ES culture, etc
- *in vivo* samples
  - brain
  - thymus
  - liver
  - sex organs
  - etc

Neuro-:
behavior (operant experiments)
Immuno-:
autoimmune modulation
Endocrino-:
male
female
development / maturation
aging-related



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Summary

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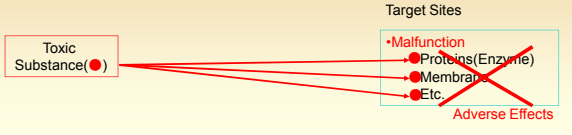
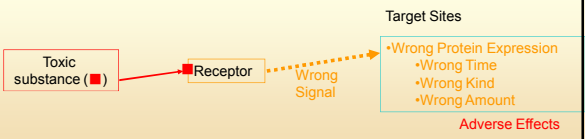
Traditional Toxicity



Receptor-Mediated Toxicity



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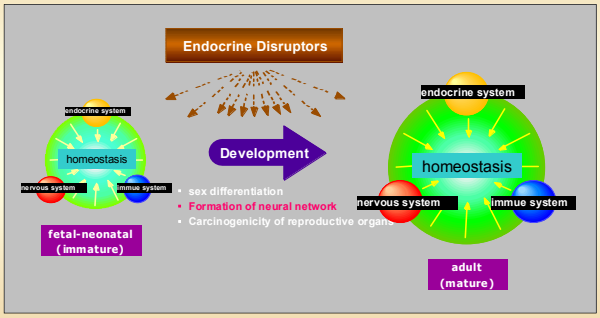
Sensitive time window and adverse effects



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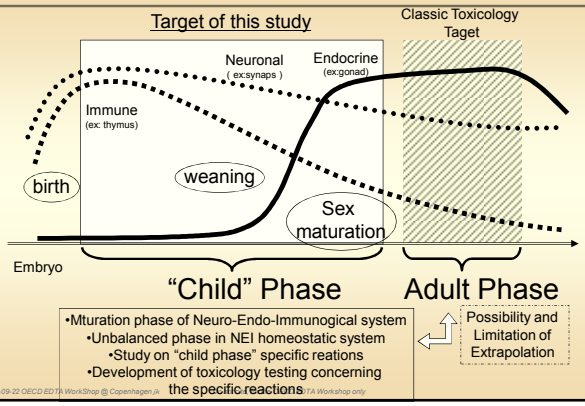
Study Target for Children's Toxicology concerning the maturation of homeostatic regulation systems



•Mturation phase of Neuro-Endo-Immunogical system
•Unbalanced phase in NEI homeostatic system
•Study on "child phase" specific reations
•Development of toxicology testing concerning the specific reactions

Possibility and Limitation of Extrapolation

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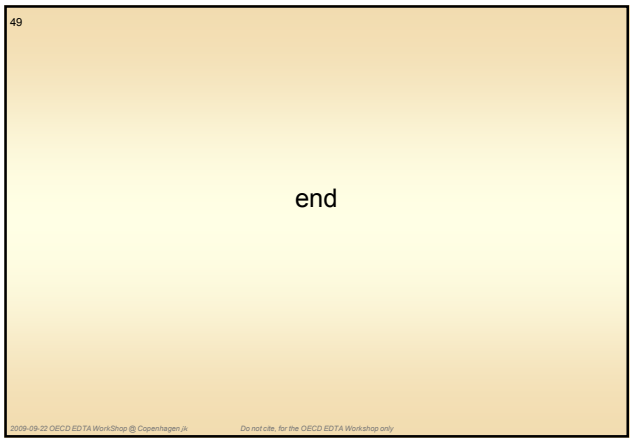
Conclusion

- EDC issue is Receptor-Mediated Toxicity (Wrong Signal)
- Target is Neuro-Immuno-Endocrine Network or Homeostatic system (common/shared signal molecules)
- Modification of NIE network at embryonic, perinatal and/or infantile stages results in Irreversible changes of the fine structures and induces adverse effects that, in some cases, become overt in the later stages of life.
- Screening and Testing protocols should be designed to cover these points.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end
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Activities on Testing and Assessment of ED in Ministry of the Environment, Japan

Taisen Iguchi
National Institutes of Natural Sciences
National Institute for Basic Biology
Okazaki Institute for Integrative Bioscience

Kunihiko Yamazaki
Environmental Health Department
Ministry of the Environment, Japan

1. SPEED '98: Assessment of potential EDCs using medaka
   Monitoring of chemicals in the environment, Literature search,
   In vitro receptor binding,
   Vitellogenin assay, Partial Life Cycle Test, Full Life Cycle Test

2. ExTEND 2005

3. Participation for establishment of TGs in OECD
   Fish: 21-day fish screening assay, Fish sexual development assay,
   (Comparison of 2-generation test vs Full life cycle test)
   Amphibian: Metamorphosis assay, (Partial life cycle test)
   Invertebrates: Daphnia reproduction assay (Annex)
   Non-animal: (*In vitro* screening using receptors of fish, amphibians)

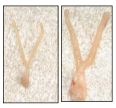
4. Collaboration with USA EPA (US-Japan) and UK DEFRA (UK-Japan)
   Comparison of 2-generation test vs Full life cycle test using medaka
   Amphibian partial life cycle test using *Xenopus tropicalis*
   Use the same chemicals for invertebrates (mysid, copepod and daphnia)
   Establishment of reporter gene assay using fish and amphibian hormone receptors
   Establishing testing methods for stickleback

OECD Science Groups

National Coordinators

Standerdization of Tests For Risk Assessment
- Animal species
- Breeding
- Exposure route
- Dose setting
- Actual concentration
- Animal welfare
- Flow-through system
- Statistics

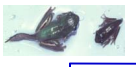
Endocrine Disrupter Testing & Assessment (EDTA) Task Force



Validation Management Group-mammlian

VMG non-animal

VMG-ecotoxicity





Amphibians	Avians	Fish	Invertebrates
Xenopus laevis *Xenopus tropicalis*	Japanese quail	Medaka, Zebrafish, Fathead minnow, Stickleback	Mysid, Daphnia, Copepod, Chironomids, etc.

Framework for the risk assessment of EDCs in SPEED'98

Screening

In vivo testing animal: Japanese medaka *Oryzias latipes*

Selection of priority chemicals based on the monitoring data & published data

Estrogen (Androgen) receptor binding assay

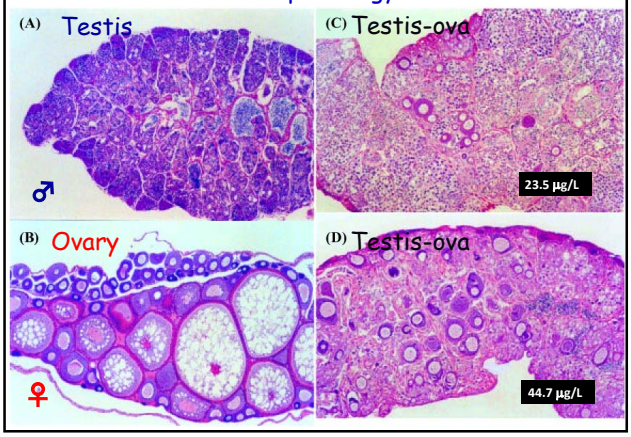
In vivo vitellogenin assay (VTG assay)
21 days: 4 months old, males

Partial life-cycle test (PLCT): Evaluation during 1 generation
Secondary sex characteristics・Hepatic VTG・Gonad histopathology
70 days: Eggs to 2 months old, both sex

Final examination

Full life-cycle test (FLCT): Evaluation during 2 generations
Examining the same analysis as PLCT in both F0 and F1
Breeding test is examined between F0 and F1.
170 days: Eggs to 2nd generation

Histopathology



Medaka Test Results

Methods	Endpoints	LOEC (µg/L) NP	OP	BPA	*op'*-DDT
Estrogen receptor (ER) binding assay	Binding to ER	+	+	+	+
Vitellogenin (VTG) assay	VTG induction	22.5	64.1	334	1.50
Partial life-cycle test	VTG induction	11.6	11.4	470	-
	Testis-ova development	11.6	23.7	890	0.195
Full life-cycle test	VTG induction	-	9.92	>1185	0.522
	Testis-ova development	17.7	30.4	1185	0.522
	Fertility	(17.7)	82.3	>1185	0.522
	NOEC (Testis-ova in Flct)	8.2	9.92	248	0.145

MOE's Perspectives on EDCs —ExTEND 2005—

Observation of wildlife
- ·Continuous observation of wildlife at the local level
- ·Survey and evaluation by monitoring

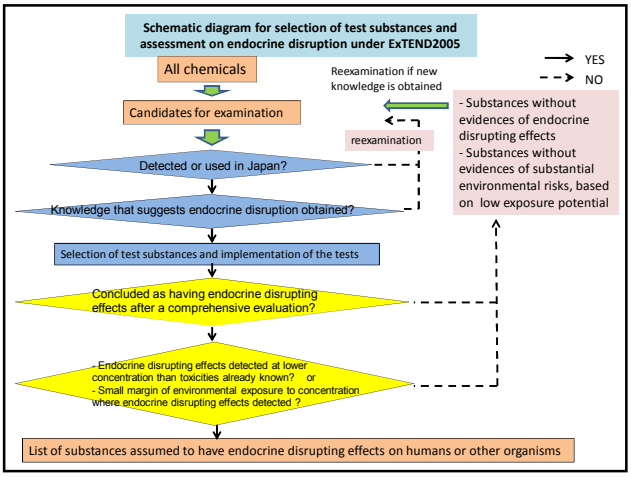
Evaluation of the environmental concentration and exposure level
- ·Environment survey and wildlife monitoring of substance
- ·Estimation of concentration levels of substances in the environment and so on

Promotion of basic research
- · Accumulation of basic biological knowledge of wildlife
- · Species level approach
- ·Cellular/molecular level approach(for example development of DNA micro array)
- · Basic research contributing to test methods development

Assessment the substance which has endocrine disrupting effects
- ▪The selection and assessment procedures of test substances concerning the endocrine disrupting effects(see next figure)
- ▪Implementation of the test(use TGs in OECD and so on)

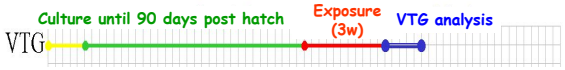
Risk assessment and management
Promotion of information sharing and risk communication

http://www.env.go.jp/en/chemi/ed.html

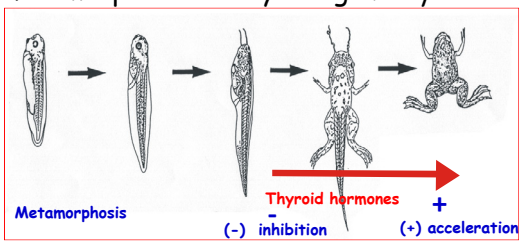


Fish 21-day Screening Assay
Vitellogenin Assay

- Animals : Japanese Medaka (*Oryzias Latipes*)
  age: 2~3 months sex: male keep & breed without EDCs
- Dose: six concentrations including control
- Measuring: pH, DO, temperature (everyday) and actual concentration of chemicals (once a week)
- Feeding : artemia 3 times per day, Gluttony
- Exposure method: Continuous-flow mini-diluter system
- Periods : 1,2,3 weeks, dissect 10 fish at every week
- Endpoint: measure vitellogenin level in liver by ELISA
- Statistical analysis and obtain no-effective-concentration (NOEC)



Metamorphosis Assay using *Xenopus laevis*



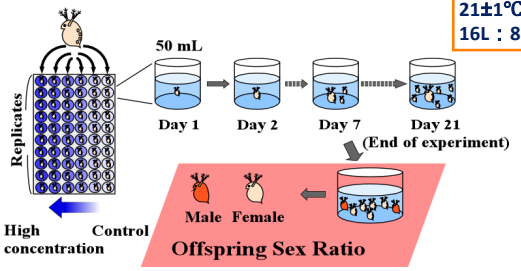
Reproduction Assay using *Xenopus tropicalis* in collaboration with US EPA

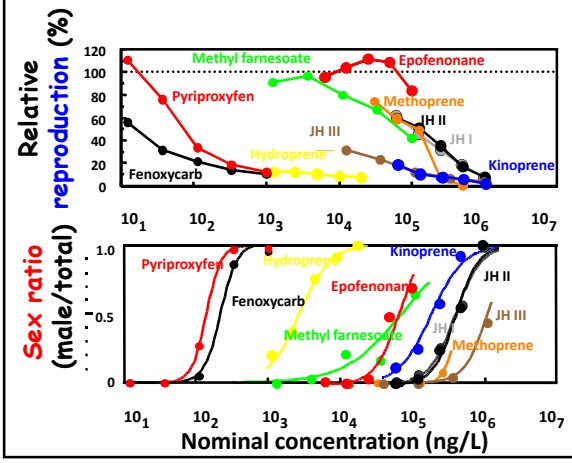
Xenopus tropicalis	2n	life cycle: 8 months
Xenopus laevis	4n	life cycle: 2 years

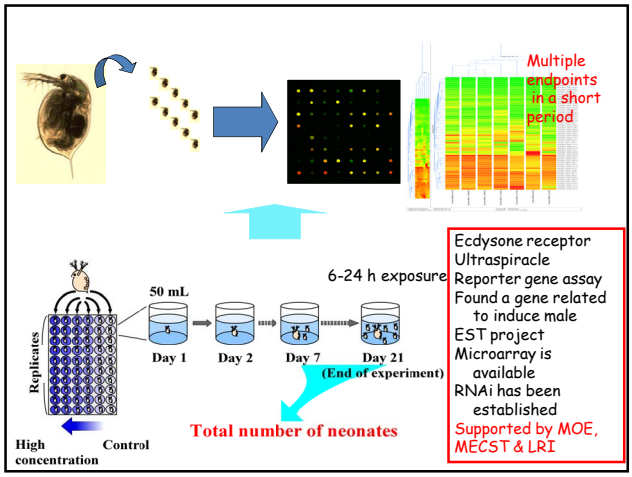
Invertebrate:

Based on OECD TG 211
Daphnia magna reproduction test

M4 medium
21±1℃
16L : 8D







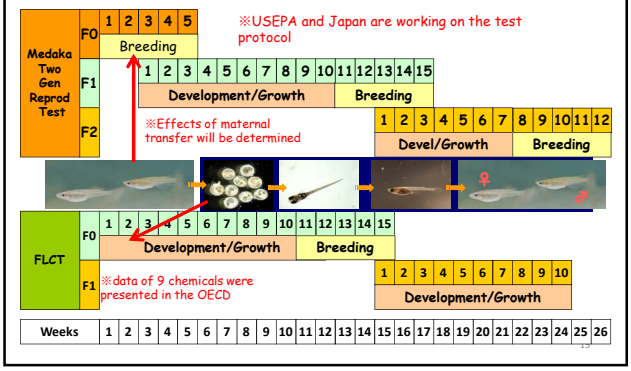
USA-Japan Bilateral Meeting

1.Fish group:
Comparison of Full life cycle test vs Two generation test using medaka

2. Amphibian group:
Establishment of partial life cycle test using *Xenopus tropicalis*

3. Invertebrate group:
Selection of test chemicals for *Daphnia magna*, mysid and copepod.

Comparison of FLCT and Two Generation Test

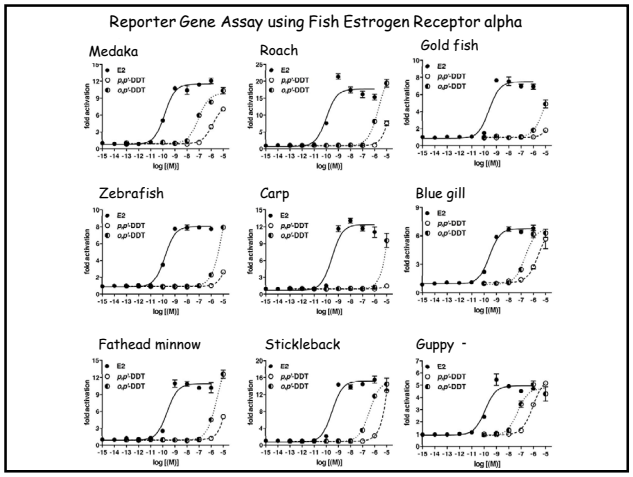


UK-JAPAN Research Cooperation on Endocrine Disruption -Future Aspect-

defra
環境省 MOE

Co-operation will be pursued through several means including, but not limited to:
1. Sharing of research findings and the development of joint research projects;
2. Free exchange of technical information;
3. Joint symposia, workshops and academic discussions ;
4. Exchange of experts.

Themes
1. Study on evaluation of effective reduction of estrogenic activity in wastewater
2. Study on endocrine disrupting potency evaluation method using stickleback
3. Study on ligand-specificity of fish estrogen receptors and marker genes for detection of testis-ova in medaka
4. Study on amphibian ecological impact assessment method and establishment of basic data of *Xenopus tropicalis*


Reporter Gene Assay using Fish Estrogen Receptor alpha
Medaka
Roach
Gold fish
Zebrafish
Carp
Blue gill
Fathead minnow
Stickleback
Guppy
E2
p,p'-DDT
o,p'-DDT
fold activation
log [(M)]

The use of Test Guidelines for assessment of EDCs – views of Environment and Health NGOs

Gwynne Lyons, Director of CHEM Trust

- also on behalf of WWF-EPO and HEAL

OECD Meeting, Copenhagen, Sept 2009

Overview of talk - 8 short topics

- Problems with legal definition of EDCs.
- Non OECD tests must be considered.
- Use sensitive species - OECD mollusc TG.
- Address complexity of endocrine and inter-linked system.
- Ensure best use of sacrificed animals.
- Make explicit - limitations of any proposed battery.
- Take account of new insights in toxicology.
- In risk assessment – consider 'mixtures'

Issue 1: Definition of EDCs

- No need to have an OECD-wide agreed definition, better left to national or regional regulatory regimes.
- IPCS (WHO) definition is NOT acceptable for legislation … "*… alters function(s) of the endocrine system and consequently causes adverse health effects*"
- This requires proof of the mechanism of action – and the ability to show without doubt that the effects seen are a consequence of this mechanism – and also proof of adverse health effects.

Mechanisms of action can take decades or more to elucidate.

- Still debate over how TBT causes imposex.
- Do not want a definition in legal text which would mean regulators having to prove an endocrine disrupting mechanism of action – and that the effects seen were a direct consequence of this.

Inclusion of the term 'adverse' effects in definition is not appropriate

- In EU, REACH regulates chemicals with ED properties if evidence of ***probable*** serious effects.
  - pesticides with ED properties will be regulated if ***may*** **cause adverse** effects.
- Therefore, inappropriate to have a definition which would mean a chemical was only defined as an EDC if a higher level of proof was available to show it definitely caused adverse effects.

Barrier to action if have to prove an EDC causes 'adverse' effects.

- Does it mean adverse effects in humans and wildlife. If so, how to extrapolate with certainty from lab tests?
- If it means adverse effects in lab animal – could preclude regulating on basis of in-vitro tests or on the basis of oestrogenicity identified in a uterotrophic assay – as some suggest increased wt of uterus is not 'adverse'.
- If definition is too restrictive – it will effectively mean that no chemical is ever identified as an EDC.

Issue 2: Use of non-OECD tests

- Scientific knowledge develops all the time. Non-OECD test results must be considered.
- Independent test results should undergo scientific peer review and be published. Regulators should decide how to weight such studies.
- Should not be necessary in all cases to repeat – nor to subject the method to a validation procedure - nor to evaluate the substance in an existing OECD test method.
- Industry should bear responsibility for assessing safety of their chemicals – but may be problems with bias. Need independent scientists & Government laboratories

Issue 3: Identify sensitive species for use in tests

- **Imposex in wild – but still no OECD agreed TG.**
- **OECD now has mollusc draft Detailed Review Paper.**
- **Prioritise partial life cycle test with Potamopyrgus antipodarum. Optimisation and validation of SOP now!**
- **Other mollusc species need to be worked on.**
- **Urge Governments & industry to support this work.**

Issue 4: Address the complexity of the endocrine system

- Could current testing be done better ?
- Need to move beyond attempts to validate a handful of narrowly focused assays.
- Consider a comprehensive assay – looking at all tissues and organs. Examined by experts in that part of the endocrine system.

Issue 5: If test on animals…

.. do not 'waste' the life of these laboratory animals, -

but ensure TGs make best use of the animals that have to be sacrificed to identify harmful chemicals.

If Extended 1– Gen reproductive toxicity test is to be conducted…

- **It should be mandatory to include endpoints for developmental neurotoxicity and immunotoxicity.**
- **Also - include mammary gland development.**
- **Why not? Cost ? Or an unexpressed wish to avoid getting information which could identify chemicals with such worrying effects?**

Issue 6: Proposed testing batteries should make explicit their limitations

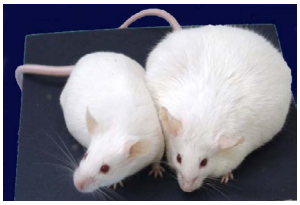
- **Only by making it more explicit where the known gaps are – will there be the necessary impetus to fill such gaps.**
- **Existing screens and tests are sufficient to say some chemicals have endocrine disrupting properties, but not sufficient to ensure a chemical is not an EDC.**

Issue 7: Take account of new insights

- Hormones work at low concentrations
- System already 'off the base-line'
- Low dose effects may not be predicted by high dose testing
- Non monotonic 'inverted-U' or 'U' dose-response curves for EDCs do exist
- LUPRON – for prostate cancer and endometriosis TAMOXIFEN -for breast cancer. Both non-monotonic
- TGs should specify the need for low dose testing

Non-monotonic response to fetal exposure

Control



1 ppb DES

Newbold *et al.* 2005

Mother's exposure during pregnancy to 1 ppb DES causes obesity in offspring, while 100 ppb causes weight loss. High doses did not predict the low dose impacts.

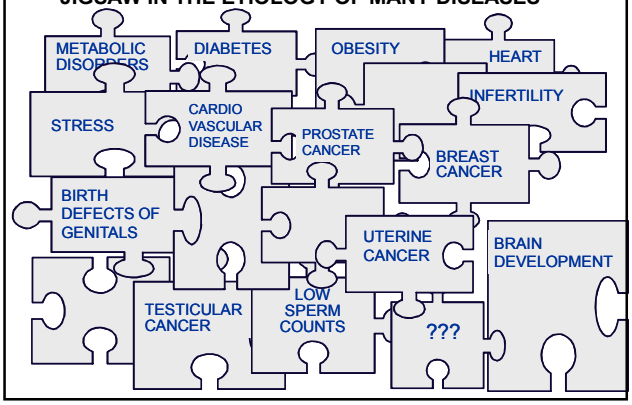
Issue 7: New Insights

- Phytoestrogens in the diet
- TGs need to clearly specify optimum range ?

Issue 8: Mixture effect

- Recent lab work shows that hormone disrupting chemicals can cause effects even if each is individually below its effect level - ' something from nothing'
- Not an issue for test method development – but it is an issue for chemicals assessment
- Must be dealt with – in a simple easy way. Urgently!

Remember: EDCs APPEAR TO BE PART OF THE JIGSAW IN THE ETIOLOGY OF MANY DISEASES



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Conclusions …

- **More work to address complexity of endocrine system.**
- **More resources needed to identify chemicals with ability to cause brain developmental disorders and metabolic disorders.**
- **Urgent need for an OECD TG on mollusc.**
- **Need to find, if possible, alternatives to testing on animals. Improve non-animal tests predictive ability / metabolism.**
- **If lab animal are used in testing, make best use of these.**

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Conclusions….

Progress has been made. TGs have been developed and more in the pipeline. Much has been learned about basic endocrinology in various taxa.

<u>Vital</u> to keep up the momentum.

Governments and industry MUST ensure adequate funding

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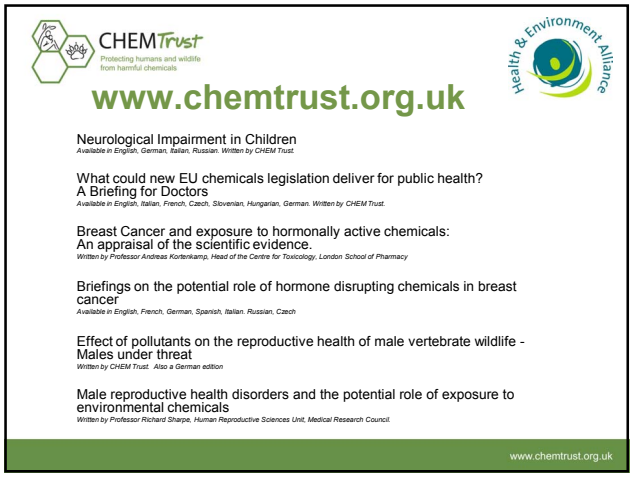
Conclusions cont...

- Environmental NGOs are aware of the many pressures and constraints in your work.
- Whenever consideration of the costs of testing chemicals threaten best practice – we hope you will **ensure these costs are weighed against some of the human and financial costs of ED-linked disorders – such as diabetes, fertility treatments, hormone related cancers, and deficits in intelligence.**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Health & Environment Alliance
www.chemtrust.org.uk
Neurological Impairment in Children
Available in English, German, Italian, Russian. Written by CHEM Trust.
What could new EU chemicals legislation deliver for public health?
A Briefing for Doctors
Available in English, Italian, French, Czech, Slovenian, Hungarian, German. Written by CHEM Trust.
Breast Cancer and exposure to hormonally active chemicals:
An appraisal of the scientific evidence.
Written by Professor Andreas Kortenkamp, Head of the Centre for Toxicology, London School of Pharmacy
Briefings on the potential role of hormone disrupting chemicals in breast cancer
Available in English, French, German, Spanish, Italian, Russian, Czech
Effect of pollutants on the reproductive health of male vertebrate wildlife - Males under threat
Written by CHEM Trust. Also a German edition
Male reproductive health disorders and the potential role of exposure to environmental chemicals
Written by Professor Richard Sharpe, Human Reproductive Sciences Unit, Medical Research Council.
www.chemtrust.org.uk

1

THE USE OF TGS AND OTHER TOOLS FOR THE ASSESSMENT OF ENDOCRINE DISRUPTERS: VIEWS FROM BIAC

Rémi Bars
23 September 2009

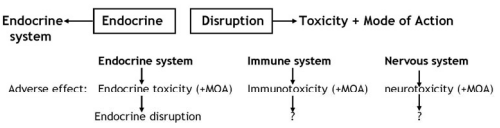
Outline

2

- Endocrine disruption
- Definition
- Assays and testing
- Approaches
  - ECETOC
  - IPCS Mode of action framework
- Hazard identification vs risk assessment
- Challenges
- Suggestions for future OECD activities

Endocrine disruption (toxicology)

3



- Disruption is only used to describe « effects » on the endocrine system. Why?
- Endocrine system is just another physiological system.
- The way endocrine toxicity is determined is no different from the way other forms of toxicity are determined.

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Definition of an Endocrine Disrupter

4

- *"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" (WHO IPCS, 2002)*
- Endocrine activity
- Endocrine disruption / toxicity

Tiered, Hierarchical Screening and Testing

5

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

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

Level 1 Sorting & prioritization based upon existing information	- physical & chemical properties, e.g., MW, reactivity, volatility, biodegradability, - human & environmental exposure, e.g., production volume, release, use patterns - hazard, e.g., available toxicological data	
Level 2 *In vitro* assays providing mechanistic data	- ER, AR, TR receptor binding affinity - Transcriptional activation - Aromatase and steroidogenesis *in vitro* - Aryl hydrocarbon receptor recognition/binding - QSARs -High Through Put Prescreens - Thyroid function - Fish hepatocyte VTG assay - Others (as appropriate)	
Level 3 *In vivo* assays providing data about single endocrine Mechanisms and effects	- Uterotrophic assay (estrogenic related) - Hershberger assay (androgenic related) - Non -receptor mediated hormone function - Others (e.g. thyroid)	- Fish VTG (vitellogenin) assay (estrogenic related)
Level 4 *In vivo* assays providing data about multiple endocrine Mechanisms and effects	- enhanced OECD 407 (endpoints based on endocrine mechanism) - male and female pubertal assays - adult intact male assay	- Fish gonadal histopathology assay - Frog metamorphosis assay
Level 5 *In vivo* assays providing data on effects from endocrine & other mechanisms	- 1-generation assay (TG415 enhanced)[1] - 2- generation assay (TG416 enhanced)[1] - reproductive screening test (TG421 enhanced)/ - combined 28 day/reproduction screening test (TG 422 enhanced)/ [1] Potential enhancements will be considered by VMG meeting	- Partial and full life cycle assays in fish, birds, amphibians & invertebrates (developmental and reproduction)

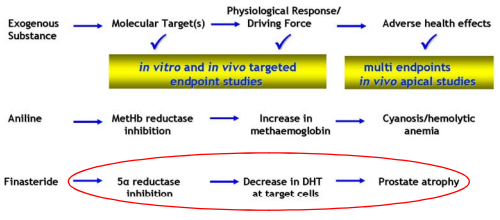
BIAC: Tiered, Hierarchical Screening and Testing

6

- Stage 1: Initial Assessment to set priorities for further evaluation
- Stage 2: Screening Assays focused on mode of action
- Stage 3: Definitive Testing (evaluation of apical endpoints, adverse effects and dose response for hazard identification and use in risk assessment

Tiered, Hierarchical Screening and Testing

7



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Interpreting Results of Screening and Testing

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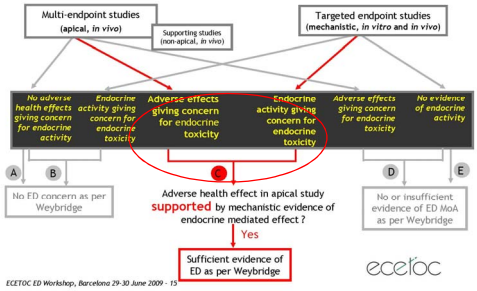

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IPCS Mode of Action Framework

9

- Elements of the IPCS WOE analysis

1/ Description of key events

2/ Dose-response relationship

3/ Temporal association

4/ Strength, consistency and specificity of association of adverse effects with key events

5/ Biological plausibility and coherence

6/ Other modes of action

- OVERALL STRENGTH OF EVIDENCE
  - an evaluation regarding the relationship between an outcome of concern and exposure to a substance and whether or not these associations involve endocrine-mediated mechanisms

Interpreting Results of Testing: Potency Considerations

10

- ✓ **Specificity:** Endocrine adverse effects observed at lower doses than other types of toxicity.
- ✓ **Relevance :** Relevance of ED mechanism of action to human or environmental species
- ✓ **Dose level:** NOAEL for endocrine adverse effects at :

  0.04 – 1 – 10 – 100 – 1000 mg/kg/day (1 ng/l vs ~ 20 000 ng/l)
- ✓ **Exposure duration:** Endocrine adverse effect detected in :

  14/28 days 90 days – 1 year 2 years.
- ✓ **Nature/Severity of adverse effects:** Slight delay in vaginal opening with no consequence on the reproduction function *vs* clear effect on fertility.
- ✓ **Number of species affected from regulatory toxicity studies:** Rat, mouse, dog, fish, amphibian, bird.

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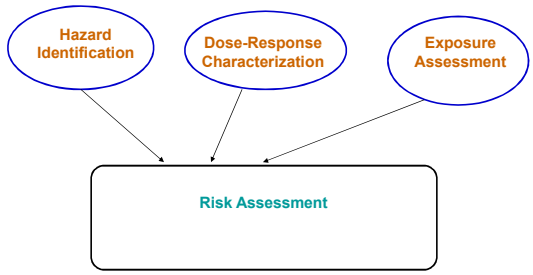
Should Use Components of Risk Assessment



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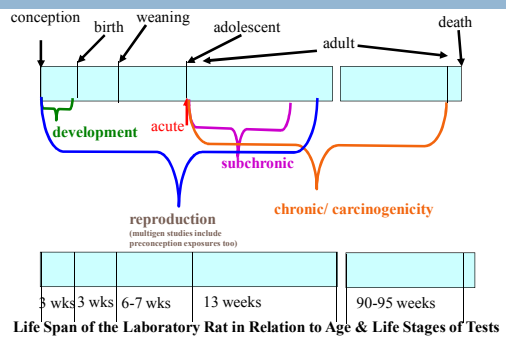
Challenges

12

- Thresholds
  - MoA like others
- Sensitive lifestages

Life-Stages Evaluated by Standard Toxicity Tests: Windows of Vulnerability are Not New nor Unique to the Endocrine Disruption Hypothesis



Life Span of the Laboratory Rat in Relation to Age & Life Stages of Tests

13

Interpreting Results of Screening and Testing

14

- Weight of Evidence: systematic & comprehensive review of ALL relevant studies
- Published studies need careful weighting
- Weight of Evidence requires qualified experts
- Validated appropriate methods conducted to GLP are given greater weight

Path Forward

15

- Need to avoid fragmented approaches to testing and assessment
- Scientific approach must consider mode of action, dose response and exposures and therefore risk
- Must be a formal, systematic Weight of Evidence process applied
- Risk is the basis for determining appropriate product stewardship and management actions
  - with Margin of Exposure using MOA and data derived values for UFs
  - There is a need to have adequate exposure information for EAS substances with thresholds, including exposures of any identifiable susceptible subpopulations.

Consideration for Future OECD EDTA Activities

16

- Work to develop Harmonized Data Interpretation Procedures for endocrine screening assays
  - Organize a workshop on Weight of Evidence approaches integrating results from mechanistic screens (and if available, with results from apical tests)
    - To better understand when additional apical testing would be necessary
- Need for pooling case studies, making these more broadly available: IPCS, OECD, ECETOC, etc.

Consideration for Future OECD EDTA Activities

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- Mutual Acceptance of Data
- Animal welfare

- **Guidance on how to use the TGs in regulatory settings**