VERBAND DER CHEMISCHEN INDUSTRIE e.V.
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VCI Overview on “Endocrine Active Substances”

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Introduction

Endocrine active (hormone active) substances are given considerable attention by the scientific community, public authorities, industry, and non-governmental organizations. For more than two decades much effort has been put into research in this area, however without consensus regarding the relevance and existence of new hazards.

In respect of regulation, there is a stronger focus on endocrine disrupting substances in the EU Regulatory Framework. For this purpose, criteria how to identify endocrine disruptors are needed. At present criteria and a guidance document are being developed and discussed.

The German Chemical Industry is actively involved in the onging scientific and political debate about endocrine disruptors. The VCI overview on “endocrine active substances” provides a concise picture of the topic from the VCI’s perspective. The document starts off with basic concepts and definitions. Furthermore test methods and the current situation are explained as well as proposed criteria and guidance for regulation. The VCI overview on “endocrine active substances” was first published for wider audiences in 2002 and has been regularly updated ever since.

This document serves as a scientific backbone to various short communications by VCI on specific issues.

Content

1. Endocrine effects

1.1. What is the endocrine system?

Alongside the nervous system, the endocrine system is the “management system” of multicellular organisms, responsible for the coordination and integration of the functions of various organs and tissues. The endocrine system permits flexible adaptation of the organism (e.g. to prevailing external influences) and makes use of the circulation to bridge larger distances within the body. Hormones are the chemical messengers of the endocrine system: they are produced in specialised cells or tissues in the body (e.g. pancreas – insulin). Hormones (from the Greek, hormein: to stimulate) and hormone-producing cells or tissues count as parts of the endocrine system in the narrow sense. In the wider sense, organs that react to or are the targets of the various hormones count also as part of the endocrine system (e.g. the uterus as the target organ of sex hormones). To achieve hormonal control, both messenger substances - the hormones and hormone receptors, are necessary. The latter are proteins that usually bind hormones very specifically and are either located on the cellular surface and transmit secondary signals to the inside of the cell or are located inside the cell and act as ligand (= hormone)-activated transcription factors. As a result of the hormone/receptor interaction, cellular or physiological functions are stimulated, e.g. the production of second messengers or specific proteins, or as feedback signals for the regulation of hormone levels.

Working closely with the control centers in the brain and the autonomic nervous system, the endocrine system regulates feeding, metabolism, growth, physical and mental development and maturation, reproductive mechanisms, modulation of performance, and the “internal environment”.

Fauna and flora

It is now known that endocrine systems with hormonal messengers are widespread in the animal kingdom; they are found in simple forms even in the lowest organized multicellular organisms. In the course of phylogenesis, these messengers have proven to be very “conservative” in respect of evolutionary change, and thus maintain their significance beyond the boundaries of systematic groups. However, this does not necessarily mean that they have the same function or serve the same mechanisms in all these groups of animals.

In addition, we know that certain groups of animals, e.g. arthropods, have indeed developed their own endocrine messengers and perfected them in the course of phylogenesis for a great variety of physiological control processes.

Signal transmission with the aid of chemical messengers is not limited exclusively to multicellular animals, but is also found – in completely different forms – in plants, bacteria, and single-celled animals. Since, to the best of our knowledge, none of these cases has any recognizable relevance to the question of “endocrine disruption” and industrial chemicals, this specific subject will not be discussed further here.

1.2. Definitions: difference between endocrine active substances and “endocrine disruptors”

What is an endocrine active substance?

An endocrine active substance has a hormonal or hormone-like activity, i.e. because of its structural similarity it acts e.g. like an estrogen. This definition does not include any information about the potency, i.e. the strength of the action and whether the hormonal action has any adverse effects. An endocrine activity is not necessarily equivalent to an adverse effect (Bolt & Degen, 2000; BfR, 2010; DE-UK, 2011, EFSA 2013). Weak endocrine activity can be advantageous, detrimental or neutral for the organism. To illustrate this: a change in room temperature, a meal or daylight may induce changes in circulating levels of hormones, such as thyroid hormones, insulin or melatonin, respectively.

The effects of a substance with hormone-like action can be expressed as an action which is the same as that of the hormone (agonistic effect) or an action counteracting that of the hormone (antagonistic effect) (DGPT, 1999). In principle, an effect on the hormone balance can occur via many different mechanisms, including changes in hormone synthesis or metabolism, changes in receptor concentrations, changes in the concentrations of the steroid-binding proteins or changes in the higher-level feedback mechanisms and their control centers (so-called indirect mechanisms). The mechanism is direct if a substance in the body cell triggers the same specific reactions as the corresponding hormone or suppresses the hormone signals (by specific actions), for example by binding to a receptor.

Examples of endocrine active substances include:

- natural hormones from animals and humans (e.g. estradiol, testosterone, insulin, epinephrine)
- natural substances, e.g. plant constituents (e.g. phytoestrogens, goitrogens in cabbage, genistein, caffeine)
- hormone drugs, i.e. synthetic substances specifically developed to obtain a hormonal action

- some synthetic substances (industrial chemicals)
- physiologic metabolites of ingested dietary or pharmaceutical substances
- environmental (spontaneous or bacterial, etc.) degradation products or metabolites of synthetic chemical substances.

What is an "endocrine disrupter"?

In the English-speaking world, the term "endocrine disruption" was coined for influences from outside the organism (exogenous influences) that change the hormone balance or the action of hormones in the body, and thus lead to adverse effects. In human and animal medicine, certain therapeutic and diagnostic procedures – some of them introduced many years ago – deliberately influence the hormone balance. Changes in the hormone balance due to physical and emotional factors have been described for many years. By contrast, the term "endocrine disruption" in the current discussion on chemical substances refers to unintentional interference in the hormone balance caused by chemical substances.

The experts of the 'Societa Italiana di Tossicologica' published a position paper in 2013, highlighting that the distinction between endocrine activity and endocrine disruption is linked to the degree of biological impact, indicating that disruption occurs beyond the biological threshold. A more in depth assessment is available in the published literature (Testai, 2013) and concluded: *"The present paper attempts to discuss that perturbation of normal endocrine homeostasis in itself may not be an adverse effect, since the endocrine system is naturally dynamic and responsive to various stimuli as part of its normal function and it is modulated according to the characteristic trend of the dose–response curve. EDs should be evaluated using a weight-of-evidence (WoE) approach. If a chemical meets the criteria to be defined as an ED in experimental animals, the relevance of observed effects to the human then needs to be addressed. Hazard-based risk management is therefore not justified since does not meet the criteria for a sound scientifically based assessment."* (Testai, 2013).

Experts from the scientific community, regulatory affairs, and industry from throughout Europe took part in a workshop organized by DG XII Research of the European Commission in Weybridge in December 1996 with the participation of OECD and some

EU Member States (e.g. the German Ministry for the Environment (BMU) and the German Federal Environmental Agency (UBA)). The following definition was agreed at that workshop:

"*An endocrine disrupter is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary (consequent) to changes in endocrine function.*

A potential endocrine disrupter is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism" (Weybridge, 1996).

This so-called Weybridge definition is clearly focused on adverse effects observed in the intact organism, and the hormonal disruption is considered to be the toxic mechanism for this harmful effect. This enabled at least the scientific debate in Europe to be brought back to a more objective level.

In accordance with the Weybridge definition, in 2002 the WHO defined an endocrine disrupter as follows:

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

In an ECETOC evaluation (ECETOC, 2009, Bars et al. 2011, Bars et al. 2012) the Weybridge definition was considered to be the most appropriate one, as it considers the biological plausibility between the whole organism and the Mode of Action which results in the adverse effect. It is also stated in the report that - within the context of ecological risk assessment and in terms of the ECETOC report - the Weybridge definition can be expanded to include adverse population-relevant effects that are mediated through the endocrine system of individual organisms. This reflects the differences in protection goals between human health assessments (individuals) and environmental assessments (populations of species).

In current discussions – starting from the State-of-the-Art Report on Endocrine Disrupters (Kortenkamp 2011) to the Commissions draft criteria - there is however general consensus to use the WHO IPCS definition. This was also re-confimed numerously, e.g. by an international workshop in 2011 (Rousselle et al. 2013), by the Endocrine Disrupter Expert Advisory Group at the Joint Research Centre (Munn & Goumenou 2013) and by EFSA (2013).

As for the Weybridge Definition, it needs to be outlined that adverse effects on other species than humans are usually defined as population-relevant effects.

Adverse influences on endocrine functions have long been observed, characterized and evaluated as effects in toxicology and ecotoxicology studies and assessment of adversity in not unique to endocrine related effects. Endocrine disruptions are thus not previously unknown or unconsidered effects, but one of many possible modes of action of natural or synthetic substances. So-called “threshold values”, i.e. doses at which effects of this type just do not occur, are used to evaluate and extrapolate adverse effects mediated by hormonal mechanisms, as they are for other mechanisms. Recently the term “threshold for adversity” was introduced to describe the limit of overloading homeostasis, whereas *“homeostasis provides a level of protection that is essential for the maintainance of life”* (Piersma et al. 2011).

The Endocrine Society published an alternative definition (Zoeller et al. 2012) *“An ED is an exogenous chemical, or mixture of chemicals, that interferes with any aspects of hormone action”*. As a response EFSA in 2013 stated that *“a positive signal for endocrine activity obtained from in vitro or in vivo testing does not automatically imply that an adverse effect will be observed in an intact organism”* and therefore did not agree with this definition.

Overall, despite some alternative minority views, there is a broad agreement that the WHO IPCS definition is the best available definition and should be the basis for e. g. regulatory concepts.

1.3. Basic principles of the evaluation of endocrine active substances and Weight of Evidence

It is generally accepted that the endocrine activity of a substance can provide a mode of action that forms the basis for an effect to be evaluated, but not an independent “endpoint” (EU TGD, 2002; Harvey & Johnson, 2002; SCTEE, 1999; Crisp, 1998; WHO IPCS, 2002; SCHER, 2005; ECHA, 2007, ECETOC, 2009; EFSA 2010; Bars et al. 2011, Bars et al. 2012, SCCS 2012, EFSA 2013). Endocrine activity can have an influence on various endpoints. The relevance of an endocrine activity to a particular endpoint (e.g. survival, growth, fertility) must be evaluated on the basis of all the available data using the weight of evidence. Potential sensitive life stages need to be considered in the assessment. Suitable tools (test methods) already exist to support this evaluation e. g. and these are evaluated by specific OECD guidelines (i. e. OECD 414 and 443).

Decisive factors for the evaluation are (Harvey & Johnson, 2002; Nilsson, 2000; ECETOC, 2009; Bars et al. 2011; Bars et al. 2012, BfR 2010; DE-UK 2011, EFSA 2013, Fegert 2013, SCCS 2014):

- Adversity (according to Weybridge/WHO definitions): Should an observed effect be considered harmful, or simply a physiological reaction as a result of functional flexibility? Is the observed effect the result of an endocrine mechanism?
- Potency: Effect threshold and degree of effect compared to the dose.
- Sensitivity: Strength of the endocrine activity compared with the general toxicity of the substance. Are the endocrine-mediated effects observed at a dose that already leads to other toxic effects or are the endocrine-mediated effects the most sensitive changes?
- (Ir)reversibility and severity
- Possible exposure: What is the relationship between the threshold dose for endocrine activity in an intact organism and the exposure? What is the relevant route of exposure?

Whereas adversity is part of the hazard identification, aspects such as severity, (ir)reversibility and potency are part of the hazard characterisation while exposure is the second component of risk assessment.

Thus, evaluating the relevance of endocrine activity of a substance to the particular endpoint and to its hazard and risk potential is complex. The judgement whether a change of a particular parameter is within the normal range of variation requires furthermore appropriate expertise, and must always be carried out on the basis of all available data (EFSA, 2010).

An ECETOC working group (ECETOC, 2009; Bars et al. 2011; Bars et al. 2012; Fegert 2013) developed scientific criteria for the determination of endocrine disrupting properties that consider information from both regulatory (eco)toxicity studies and mechanistic/screening studies. These scientific criteria rely on the nature of the adverse effects detected in regulatory (eco)toxicity study(ies) that give concern for endocrine toxicity and the description/understanding of the mode of action of toxicity which scientifically support and explain the adverse effects. Since chemicals having endocrine disrupting properties may not all represent the same hazard, an element or assessment of potency was proposed to discriminate chemicals of high concern from those of lower concern. Other important aspects are relevance to humans as well as lead toxicity and specificity. In 2017 a further ECETOC-Report has been published (ECETOC, 2017) in response to the Commissions draft criteria describing seven steps for the identification of endocrine disrupting properties (see Chapter 6.6).

The importance of aspects as lead toxicity and potency is confirmed by a publication that e. g. examined the endocrine activity of caffeine (Tinwell et al. 2013). The

publication showed that caffeine is a substance with endocrine activity, but based on hazard characterization, especially potency and lead toxicity consideration, caffeine is not a substance of regulatory concern related to that endocrine activity. The importance of considering potency and severity for the identification of endocrine disruptors was reassured in a recent case study, which examined the potential impact of the Commission's proposal for criteria on natural substances (Schuhmacher-Wolz, 2017; see Chapter 5.4). Other examples of substances with endocrine activity are the phytoestrogens (natural plant products as part of our daily diet, e.g. in soy products) that have estrogen like activity in vitro and in vivo, but that would need very high doses to lead to adverse effects due to that activity. In 2015 the European Food Safety Authority published a risk assessment for peri- and post-menopausal women taking food supplements containing isoflavones and concluded: *"A comprehensive review of the available scientific evidence has revealed no indication that isoflavones at levels typically found in food supplements cause harm to post-menopausal women. Isoflavones are naturally occurring substances which are found, among other sources, in soy, red clover and kudzu root. Their extracts are often used as ingredients in nutritional supplements"* (EFSA 2015a).

Regarding the method of weight of evidence – that is considered to be very important also in the context of an assessment of the endocrine activity of substances - SCENIHR in 2012 provided an overview on what to consider (SCENIHR 2012). EFSA in 2013 in addition to SCENIHR 2012 pointed on the weight of evidence guidance as provided by WHO and published by Boobis et al, in 2006 and 2008 (EFSA 2013). The National Toxicology Program Office of Health Assessment and Translation (OHAT) developed an approach for systematic review and evidence integration for literature-based health assessments (Rooney et al. 2014). A framework for systematic review and evidence integration for reaching hazard identification conclusions is proposed covering *"1) problem formulation and protocol development, 2) search for and select studies for inclusion, 3) extract data from studies, 4) assess the quality or risk of bias of individual studies, 5) rate the confidence in the body of evidence, 6) translate the confidence ratings into levels of evidence, and 7) integrate the information from different evidence streams (human, animal, and "other relevant data" including mechanistic or in vitro studies) to develop hazard identification conclusions"* (Rooney et al. 2014). The respective tool is currently in the process of refinement to assess potential bias for in vitro mechanistic studies (NTP 2015)[1].

1.4. What hormone systems are discussed in relation to endocrine activity?

The main emphasis of the debate about endocrine active substances continues to be on sex hormones and mechanisms associated with the thyroid. Recently novel

[1] https://ntp.niehs.nih.gov/pubhealth/hat/noms/index-2.html

endpoints or “issues” are also an area of scientific discussion (e. g. obesity, diabetes). However the link between these diseases and endocrine disrupting substances is still under scientific consideration. Other factors discussed in the context of obesity are e.g. early life nutrition (Velkoska & Morris 2011; BfR 2012).

Because of the large numbers of hormones and their specific receptors, direct and indirect action mechanisms, and qualitative and quantitative differences in the effects of different hormones at different times of life and in different organs, it is possible to think of or construct numerous pathways for an endocrine effect or even adverse effect. In connection with the hormonal activity of exogenous substances, there is a theory that human reproduction could be permanently disrupted by substances with estrogen-like activity, so the debate about endocrine active substances concentrated on estrogen-like activity in the past. However, other examples are androgenic/anti-androgenic activity and effects on the thyroid.

2. Example: estrogenic activity

2.1. What are estrogens?

The term “estrogens” in the narrow sense is used for endogenous female sex hormones of the class of steroid hormones. They are produced in females, particularly in the ovary and placenta, and in smaller quantities in males, particularly in the testes and adrenals. The most active natural estrogen is estradiol. In a broader sense, synthetic substances are also called estrogens, e.g. substances used in human medicine in contraceptives, or natural substances with estrogenic activity (e.g. phytoestrogens).

2.2. What substances have estrogen-like activity?

There are natural substances, such as the so-called phytoestrogens, that have estrogen-like activity and are found in large numbers in plants eaten by humans, e.g. soybeans, wheat, carrots, and potatoes. In addition, it is known for hundreds of years that grazing sheep or cattle on clover may result in infertility, the so-called “clover disease”. This is due to coumestrol and daidzein, which are natural constituents of clover (Nohynek et al., 2013). Health-promoting effects have been attributed to phytoestrogens (DGPT, 1999; Przyrembel, 1998) and they are also used in alternative medicine for the treatment of illnesses (Bolt & Degen, 2000). In addition, estrogen-like compounds and natural hormones are found in milk, eggs and meat (Borgert et al., 2003; Jouan et al., 2006, Hartmann et al., 1998; Courant et al., 2008). In vitro test

models have shown that numerous foodstuffs and beverages (e.g. tofu, soy sauce, beer, coffee) exhibit estrogen-like activity under selected test conditions (Takamure-Enya et al. 2003). Public debate since the middle of the 1990s, however, has essentially been concerned only with synthetic chemicals introduced into the environment by humans, irrespective of the smaller quantities involved. Because adequate information on the harmful effects of high doses of phytoestrogens was not available (BUA, 2000; Przyrembel, 1998; Greim, 1998; Whitten, 2001), extensive research projects into this subject have been started in the late 1990s and data has become increasingly available (Jefferson et al., 2012). However, mammalian organisms do not differentiate between natural and synthetic substances with respect to potential adverse effects (Nohynek et al., 2013).

2.3. How potent are substances with estrogen-like activity?

The potency of estrogen-like substances is a key topic of the safety assessment and depends on the degree of affinity for the receptor or the extent of its influence on an indirect mechanism. To obtain the same effect as estradiol in test systems, hormone-like substances often have to reach substantially higher concentrations. For instance, genistein - a phytoestrogen in soy protein - has relatively high structural similarity to natural estradiol and, despite this, is two to three orders of magnitude less potent. Common industrial chemicals, tested in corresponding test systems, have potencies that are three to six orders of magnitude lower than that of the natural hormone (Nielsson, 2000; Bolt & Degen, 2000; Witorsch, 2002a; Greim, 2004; Safe, 2004; Golden et al., 2005; Witorsch & Thomas, 2010; Nohynek et al., 2013).

For example, butylparaben, although falsely branded to be an “endocrine disrupter”, only showed extremely weak potential for estrogenic activity (rat uterotrophic test) when administered subcutaneously (injected under the skin) at doses of 800 mg/kg and higher (Routledge et al., 1998). This would correspond to a human subcutaneous dose of 48 grams in a 60 kg human being in order to produce a potential activity (corresponding to 50 kg of a cream, containing 0.2% of butylparaben), not to mention that humans are less sensitive to some hormonal effects than rats (Borgert et al., 2012; Witorsch, 2002b). When butylparaben was given orally to rats or applied to the skin of rats it had no estrogenic activity (Routledge et al., 1998). In a reproductive study in young male rats, butylparaben produced no adverse changes in reproductive organs or reproductive hormone levels at oral doses exceeding 1000 mg/kg/day (Hoberman et al., 2008). Considering this, it is biologically implausible how butylparaben, when included at 0.2% in a cream, could have any effect at all or even pose a human health risk. Overall, when taking into account their limited skin penetration, their metabolism in the skin and their minute estrogenic potency of substances used as cosmetic ingredients, a risk to human health may be excluded altogether (Golden et al., 2005; Witorsch & Thomas, 2010). This view was also supported by the results of biomonitoring studies on actual blood levels of parabens in human consumers. Given

that detected levels were absent or negligible, a hormonal effect, not to mention a human reproductive risk, may be clearly excluded (Sandanger et al., 2011).

It has been argued that the potency of most active environmental estrogens would need to be at least 1000-times higher in order to present human reproductive risks (Borgert et al., 2012). Estrogen-containing drugs (e.g. the contraceptive pill) or the synthetic estrogen DES possess potencies that are by 6 or 7 orders of magnitude higher than that of long-chain parabens or UV filters with “estrogenic activity”. Yet, prenatal in utero exposure of men to estrogen drugs or DES did not adversely affect their fertility (Hemminki et al., 1998; Wilcox et al., 1995; Leary et al.,1984) or sperm parameters (Schumacher et al., 1981). Human in utero exposure to maternal doses of a total of 1.4 g DES over 101 days (approximately 0.25 mg/kg/day), a huge dose in terms of estrogenic potency, did not produce urogenital abnormalities or abnormal sperm parameters in male offspring (Fish, 2000). Ethinyl estradiol (an active ingredient of the contraceptive pill) when injected in adult men at high doses (60 µg/day), affected sperm motility and density; in contrast, 20 µg/day, still a huge dose in terms of hormonal potency, had no effect on sperm motility and density (Lübbert et al., 1992).

The possibility of weak actions having consequences in the human body is hotly debated in the scientific community. However, taking the above-mentioned data into account, the hypothesis that the low exposure of humans to chemicals of weak hormonal potency could have an effect on human fertility is without scientific basis.

In the public debate, references to the weak potency and the low concentrations of suspected endocrine active substances present in the environment are often countered by the possibility of accumulation on repeated exposure, assuming much higher potency in combination with various estrogen-like acting substances, and references to unusual dose-effect relationships (non monotonic dose relationships). These arguments will be discussed in the later sections (see below).

3. Endocrine effects – human aspects

3.1. What is the main concern for human health?

In a publication from 1993, the authors Sharpe and Skakkebaek (Sharpe and Skakkebaek, 1993) developed a working hypothesis. According to this, the increase in diseases or functional disorders of the male reproductive organs - which they had observed - might have been associated with the presence of estrogen-like acting compounds in the environment.

The diseases and functional disorders discussed were testicular cancer, reduced sperm counts or quality, and malformation of the male genital tract (undescended testes, hypospadias), which some scientists now regard as parts of a syndrome – so-called testicular dysgenesis syndrome (TCD). It is suspected that these abnormalities could have a common origin during the fetal period (from week 9 of pregnancy until

birth). However, there is no scientific evidence for a general increase in the incidence of cryptorchidism or hypospadias in male infants, and statistically, only 5% of testicular cancers are caused by cryptorchidism. Thus, due to the complexity of pathogenic and epidemiologic features of each component it is difficult to ascribe them to a single unifying process, such as TDS, particularly when so little is known of the actual mechanisms of disease (Thorup et al., 2010).

The absolute number of cases of testicular cancer was indeed increasing in many western countries at least until the 1990s, though there were clear geographical differences with regard to the time course and extent of this increase.

Although a large number of studies have been published in the last twenty years, it is still controversial whether the evidence does - or does not - support a general decrease in sperm count or quality. Most studies relating to this are based on historical data, are inconsistent in their findings, show strong regional variability, and display the major methodological problems of such studies (National Research Council, 2000; Jorgensen et al., 2001; Damstra et al., 2002; Safe, 2004; Fish 2008). Particularly flawed are retrospective case-control studies which try to associate serum or urinary levels of suspected "endocrine disruptors" with serum hormone levels (e.g. De Hond et al, 2015), thus claiming evidence for disturbed fertility without being able to prove real exposure, and without taking into account kinetics and potency of the measured substances. An evaluation from 2010 concluded that "*the claims that population fecundity is declining and that environmental pollutants are involved, can neither be confirmed nor rejected"* (te Velde et al., 2010). A special issue of the Asian Journal of Andrology in 2013 published reflections after 20 years of controversial discussion of the issue of an alleged decline in human sperm count related to an exposure to industrial chemicals. In the respective editorial it is mentioned, that virtually all the authors agree that the meta-analysis that triggered the discussion was *"seriously flawed and untenable in its conclusion on world-wide falling sperm output"*. An aspect highlighted to resolve the still ongoing discussion is the need of thorough examination in well-designed and controlled large-scale prospective studies (Cooper & Hendelsman 2013). This view is shared by other authors in that special issue (e.g. Nieschlag & Lerchl 2013, Ford 2013).

Sharpe pointed out in a review article (Sharpe, 2003) that reliable information on the subject can be obtained only if, in future, standardised methods are used and various influences (such as time of year, age, duration of sexual abstinence, and geographical location) are taken into account and clearly defined sperm parameters are determined. Until such standardised prospective studies are designed and conducted, it will not be possible to find out whether, for example, the lower sperm values reported for Danish recruits compared with older men (Andersen et al., 2000) are not perfectly normal for 18-20 year-olds.

Recent data on trends in sperm count from Denmark and Sweden were described as showing no change over time (but continuing to be low in Denmark), whereas from Finland and France a decline in various semen parameters was recorded (Skakkebaek et al., 2011; Bonde et al., 2011; Jorgensen, 2010, Rolland et al., 2012). A prospective study in Denmark with a fifteen year of monitoring of semen quality in men indicated a slight increase in sperm concentration and total sperm count. This study has defined considerable strength as it is a large prospective study among men of the general population unselected with regard to fertility and a standardised procedure was used; the lack of historical directly comparable data is a limitation (Jorgensen et al., 2012).

Regarding the endocrine disrupter hypothesis, Safe (Safe, 2013) noted the lack of correlation between sperm count changes and in utero exposure to estrogenic compounds. Other factors that might have an influence on the sperm count are e.g. alcohol intake, dietary fat, smoking, stress or sleep disturbances (Hansen et al. 2012, Olsen & Ramlau-Hansen 2012, Pastuzak & Lamb 2013, Jensen et al. 2013). Finally, the notion that prenatal estrogen exposure has adverse effects on male fertility has been refuted by studies on boys born to women exposed to high oral DES doses during pregnancy. Neither fertility nor sperm output were adversely affected despite massive in utero estrogen exposure, although minor urogenital malformations did occur in this population. There was an approximate 14-fold difference between the highest and the lowest clinical dose of DES; reproductive malformations were observed only among the offspring of women who received high-dose regimens (Nohynek et al., 2013).

In addition, a general increase in malformations of the male genital tract (undescended testes, hypospadias) was discussed. However, at present it is not possible to make any statements about trends, because until recently there were no studies that applied clearly defined diagnostic criteria to record virtually all such malformations in a population group (Dolk et al., 2004). Results of such more differentiated investigations are now available: According to data from a research group in Rotterdam, the incidence of hypospadias was about 0.7% of all male neonates, whereas undescended testes were found in about 1.2% at an age of approx. 1 month. While the incidence of undescended testes was comparable to earlier studies, the number of hypospadias was rather larger than expected from earlier studies. The authors emphasise that this was most probably due to the previously incomplete detection/registration of cases (Pierik et al., 2002).

In a further pulication, the same research group (Pierik et al., 2004) reported on parental risk factors for undescended testes and hypospadias. Suboptimal maternal health, low maternal educational level, and Turkish ancestry were associated with both observations (undescended testes and hypospadia), paternal smoking with hypospadias and paternal exposure to crop protection agents with undescended

testes. However, the purely qualitative data in relation to exposure to crop protection agents was taken mainly from retrospective statements by the mother or father, so misclassification is possible. Identification of specific crop protection agents was not possible.

Maternal and gestational risk factors for hypospadias are discussed by Akre et al. (2008). Factors, such as diet lacking in meat and fish and placental insufficiency, were mentioned. Another study came to the conclusion that no evidence was found that low intensity maternal periconceptional occupational pesticide exposure was a risk for hypospadias (Rocheleau et a., 2011). Overall, the assumed rise in hypospadia rates is still controversial and, according to Fish et al. (Fish, 2010) lacks clinical support. In a review on the epidemiologic evidence it was concluded that the observational epidemiologic literature falls short regarding whether (or which) environmental exposures contribute to hypospadia etiology. Overall, there is no scientific evidence for a general increase in the incidence of cryptorchidism or hypospadias in male infants; in addition, it has been argued that these two pathologies are caused by different mechanisms, which cast even more doubt on a common origin or a common causal agent (Thorup et al., 2010). Another factor discussed, but also with no firm evidence yet is the contribution of genetic variation to hypospadias (Carmichael et al., 2012).

Swan et al. (2005) reported results from a cross sectional study in the United States on 85 boys (age 2-36 months) or in the extension of the study on 106 boys (Swan, 2008) the reduction of the anogenital index (AGI = anogenital distance (AGD) corrected for body weight) was correlated to the level of Monobutylphthalate (MBP) or Monoethylphthalate (MEP) in maternal urine during pregnancy. The extended study found also a negative correlation of AGI for the content of MEHP and its two secondary metabolites Monoethyl-hydroxyhexyl- and Monoethyloxohexylphthalate. An association between exposure to MEHP, monobenzyl phthalate, MEP, and MBP during pregnancy and AGD in male newborns was reported for a cohort of 73 Mexican women (Bustamente-Montes, 2008). These associations between phthalate exposure during pregnancy and reduced anogenital distance/index in humans can not be taken as causal relation due to several reasons. Phthalate exposure is measured by single spot urine analysis during pregnancy, however, phthalate exposure is highly variable and phthalates have short eliminiation half-lives. In the published human studies, urinary MEP was associated with a reduction of AGI, however, in several studies where rats were exposed in utero, even at very high dose levels, there is no effect of MEP on fetal testosterone or on AGD/AGI (Gray,2000; Howdeshell, 2008; Fujii, 2005). The studies by Swan et al. have been criticised based on methodological aspects. A major point of concern is related to the fact that the mean age of boys in the study was 15.9 ± 8.6 months. In the study by Salazar-Martinez et. al (2004), where the method for AGD measurement in human males was described, the authors measured the AGD within 6 hours after birth. This procedure is very similar to the AGD measurements in animal

studies, where AGD is recorded at a specific time after birth, e.g. on postnatal day (PND) 1 or 3. Salazar-Martinez et al. were not sure whether the method could be used as marker of in utero exposure to hormonally active substances. Further, the statistical methodoloy used by Swan et al. has been criticised. In addition, a similar study found the complete opposite (Huang et al., 2009), i.e., no association between demonstrated exposure and effect.

A connection between the real (e.g. testicular cancer) or suspected increase in diseases/functional disorders of the male reproductive organs described above and the hormonal effects of some chemicals has not been proven scientifically (National Research Council, 2000; Damstra et al., 2002; Vrijheid et al., 2003; Breithaupt, 2004).

In a review article from 2006, Storgaard, Bonde and Olsen conclude: *"With the possible exception of testicular cancer there is no strong epidemiological evidence to indicate that prenatal exposure to estrogen are linked to disturbed development of the male reproductive organs"* (Storgaard et al., 2006). A more recent systematic literature review and meta analysis by Bonde et al. (Bonde, 2017) showed an overall risk estimate for male reproductive disorders marginally above equity (OR 1.11). It was concluded that the view that ubiquitous endocrine disrupting chemicals would play a substantial role in the development of male reproductive disorders is to some extent challenged by their review and the evidence is limited.

In another meta-analysis, Martin et al. found an association only for diethylstilbestrol (DES) after oral ingestion of pharmacologically active doses during pregnancy, but neither for environmental nor for other pharmacological exposures:
"While it is clear that hypospadias, cryptorchidism and testicular cancer are all positively associated with prenatal exposure to DES, this meta-analysis was unable to produce evidence that such effects were associated with environmental estrogens, or even accidental use of oral contraceptives during pregnancy" (Martin et al., 2007).

Furthermore, in recent decades girls have been observed to enter puberty earlier in many, particularly western, countries (De Muinck Keizer-Schrama and Mul, 2001; Karlberg, 2002). The possible causes are unknown, but demographic and socioeconomic influences and lifestyle are considered relevant factors (Persson et al., 1999; Kaplowitz et al., 2001). Also smoking by the mother during pregnancy has increasingly been discussed as a possible relevant factor – not just for an earlier onset of menarche (Windham et al., 2004), but also, in particular, as regards an association with testicular cancer and sperm parameters in sons. For instance, Kaijser et al. (2003) described an increased risk of testicular cancer compared to the general population in the sons of Swedish mothers who later developed lung cancer, i.e. the majority of whom had smoked; Pettersson et al. (2004) observed a clear correlation between the

smoking habits of women and testicular cancer in their sons' generation in four Scandinavian countries. Other authors report a reduced number or quality of sperm in men whose mothers had smoked during pregnancy, compared with men whose mothers' had not (Storgaard et al., 2003; Jensen et al., 2004).

In a further study of semen parameters of 347 sons of mothers belonging to a Danish pregnancy cohort established in 1984-1987, a significant inverse association between maternal smoking during pregnancy and total sperm count was found, but no associations for sperm motility or morphology (Ramlau-Hansen et al., 2007). The authors conclude that these results indicate that prenatal exposure to tobacco smoke may have an adverse effect on semen quality and, if these associations were causal, they could explain some of the reported differences between populations and secular changes in semen quality.

Particularly in the last two decades the prevalence of obesity has risen considerably – not only in developed, but also in poorer countries. Some authors suggest that, as an example of the growing scientific field termed "the developmental origins of adult disease", abnormal programming by endocrine disrupting chemicals might contribute to this rise in obesity (Newbold et al., 2007). However, there is no indication from human data that this might indeed be the case. WHO Europe confirms that the prevalence of excess body weight in 13-and 15-year-olds is unacceptably high in the region, ranging from 5% to almost 35% in some countries. They make clear that *"unhealthy diets and physical inactivity are the main contributors to excess body weight and obesity"* (WHO Europe, 2007). Nevertheless, there are epidemiological studies which *"suggest that the fetus adapts to an adverse intrauterine environment with metabolic, endocrine and hemodynamic changes which, if they persist, predispose to hypertension and type 2 diabetes mellitus"* (Beinder, 2007). According to Beinder an adverse intrauterine environment in Western countries is often due to placental insufficiency. It may be associated with psychosocial stress during pregnancy, unfavourable working conditions, unbalanced nutrition, maternal diseases, drug intake or smoking.

Novel endpoints under discussion e.g. obesity are addressed in research programs like OBELIX[2] (under the EU 7th framework programme; finished in 2013) or on OECD level.

Overall, causality between exposure to endocrine active substances and endocrine disruptive effects in the exposed or the offspring have not ben conclusively reported so far, excluding those endocrine active substances specifically applied in high doses to combat cancer or other diseases (Dietrich, 2010).

In addition, there is a broad discussion on the use and value of epidemiological studies, e.g. for the characterisation of hazards related to exposure to environmental stressors. A Guideline for Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) was proposed (van Elm et al. 2007, Vandenbroucke et al.

[2] http://www.theobelixproject.org/

2007). This was taken up by Money et al. (2013) in a proposal for a systematic approach for evaluating human data. EFSA pointed on confounding factors that make the assessment in epidemiological studies challenging (EFSA 2013).

3.2. What is the current estimate of the possible risk to humans from endocrine active substances?

Assumptions about the harmful effects of estrogenic compounds in humans are based mainly on observations made after the administration of massive amounts of highly potent synthetic hormones. In addition, effects observed in aquatic organisms are cited as evidence of similar effects in humans, and the fundamental principles of hormonal action are often not given sufficient attention. According to the information currently available, natural variations in the physiological concentrations of estradiol - resulting from the dynamic feedback mechanisms of the hormonal system and variations in the quantities and metabolism of phytoestrogens ingested daily with food - are orders of magnitude higher than the expected exposure to synthetic chemicals with hormonal effects. Against this background, the proposed causal relationship between exposure to endocrine active compounds and the phenomena cited above remains speculative. This is particularly true when, in a rash manner, so-called environmentally hazardous substances are held solely or partly responsible for the overall exposure of humans to endocrine active substances, although they contribute only to a very small extent.

The German Research Foundation's Senate Committee for the Assessment of the Safety of Foods came to similar conclusions in 1998 (DFG, 1998): *"In summary, it can be stated that, among the substances with hormonal potential that occur in foodstuffs, phytoestrogens play the greatest role. (...). Substances of anthropogenic origin that possess estrogenic properties are ingested with foodstuffs in much smaller quantities. According to the available information, the low concentrations of these substances found in foodstuffs do not pose any risk to health."*

The Global Assessment of the State-of-the-Science of Endocrine Disrupters Report issued by the International Programme on Chemical Safety (IPCS) (Damstra et al., 2002) also stated that the human data currently available are not sufficiently strong to support the conclusion that human reproductive health is adversely affected by exposure to endocrine active substances. In a further review article Storgaard, Bonde and Olsen conclude: *"With the possible exception of testicular cancer there is no strong epidemiological evidence to indicate that prenatal exposure to estrogen are linked to disturbed development of the male reproductive organs"* (Storgaard et al., 2006). Others stated that *"Right now, environmental endocrine disruption in humans is more hypothetical than demonstrated fact"* and link that to the difficulty, especially with the available epidemiologic tools (Rogan & Ragan, 2007).

An update of the IPCS State of the Science report was published in 2013 (Bergman 2013) that adressed a series of key concerns. This WHO/UNEP report was published together with a “Summary for decision-makers”. Both documents were discussed and highly critizised. Arguments why certain parts of the report seem to be biased or give the impression that the report did not take into account all available scientific evidence have been published by ICCA[3]. A more detailed criticism that explains why the WHO-UNEP report 2013 fails to be a “state-of-the-art report” was published by Lamb et. Al (2014). The publication triggered a response by Bergman et al. (Bergman, 2015). In Lamb et al. (2015) the different view are explained in more detail.

The publication of the WHO/UNEP-Report and the subsequent discussions as well as statement of the Endocrine Society in the USA (Gore, 2015) lead to a more principle discussion. It became once more obvious hat there are contradicotry scientific views.

Autrup et al (Autrup, 2015) evaluated the principles of pharmacology and toxicology in the light of the ED debate and came to the conclusion that these principles are still valid.

A number of well known scientists wrote an open letter[4] to the European Commissions president’s scientific advisor Anne Glover that was flanked by additional publications in scientific papers. The scientists are mainly driven by the concern that the political process could rewrite well-accepted scientific and regulatory principles. Topics like ED being a mode of Action (MoA) and the ignored advise to evaluate ED via a scientific sound risk assessment approach are some examples (Dietrich 2013).

3.3. What information about hormone-like acting substances can be derived from studies examining classical toxicological endpoints ?

In toxicity tests generally specific endpoints are investigated. The result of such tests is a conclusion that a certain substance in a defined test system has a harmful effect above an observed dose or does not have a harmful effect below a certain dose. The question of the underlying mechanism is of secondary importance (in apical or classical studies), since the question of whether a particular mechanism is involved, is not crucial for the protection of humans and the environment but rather, whether an adverse effect occurs (irrespective of particular mechanisms).

Clarification of the mechanism that leads to adverse effects can help to refine the extrapolation of experimental results to humans. In the case of estrogen-like effects and other hormone-receptor-mediated effects, this widely accepted procedure has long

[3] http://www.cefic.org/Documents/Other/ICCA%20Council%20Secretary%20letter%20to%20A%20%20Steiner%20re%20Endocrine%20Disruptors%2022%2005%202013.pdf

[4] https://kops.uni-konstanz.de/bitstream/handle/123456789/26321/Dietrich_263219.pdf?sequence=2

been turned on its head. A receptor interaction is demonstrated and, on that basis, a hypothetical harmful effect is assumed, albeit without any experimental evidence of this harmful effect. However, a hormonal action should initially be considered a neutral observation. It is necessary to test whether an interaction of this kind is causally responsible for a hazard on a case-by-case basis, and this depends, like other effects, on the dose, the toxicokinetics, and the potency. This viewpoint is also becoming established in the field of effects due to endocrine activity (SCHER, 2005).

Although the interaction of substances with the estrogen receptors is not directly detected in the classical toxicity test, tests for endpoints and harmful effects that could result from such interactions certainly are carried out. For example, significant effects that could be due to disruption of the hormonal system are routinely recorded in subacute (28-day) and subchronic (90-day) tests. Examples include effects on the uterus, adrenals and testes, which are examined for macroscopic and microscopic changes. Pre-, peri-, and postnatal toxicity tests establish whether a substance can induce structural or other damage to the progeny. Single generation and multi-generation studies normally investigate the effects of a substance on reproduction, including the fertility of the males and effects on offspring. All these in vivo test systems with a broad range of parameters (e.g. including histopathology) cover sex hormone related effects as well as other hormone and non-hormone related effects. In summary, it can be assumed that classical toxicity tests provide sufficient evidence of harmful effects, irrespective of whether or not an effect is due to an interaction with an estrogen receptor, another hormone receptor, or another endocrine (or non endocrine) effect or a not yet defined mechanism. The view on the relevance of the apical tests was also shared by a working group of the OECD, which at that time identified a long-used method (OECD Guideline 416, the so-called two-generation study) as the definitive test for effects that could be due to interference with the sex-hormone balance (Gelbke et al., 2004). The above view was shared as well as by Germany and the UK in their position paper on regulatory definition of an endocrine disrupter in relation to a potential threat to human health (DE-UK, 2011).

Extensive experimental studies carried out under the auspices of the OECD investigated how far the parameters currently used in subacute studies and the potential additional parameters that have been identified are able to detect endocrine-mediated effects. The evaluation of these studies shows that existing parameters effectively detect such effects. Some of the additional parameters were also found to be useful for the detection of endocrine effects; in particular, they often provided hints as to the underlying action mechanism. Integration of these additional parameters identified as effective in the subacute study protocol (Gelbke et al., 2004; SCHER, 2005) was done in 2008 by modifying the study protocol (see also Chapter 3.4).

3.4. (Screening) methods to detect endocrine effects

In 2002, the OECD - through its Task Force EDTA - adopted a conceptual framework for the testing and assessment of potential endocrine disrupting chemicals. The framework was developed as a tool box in which the various tests that can contribute information for the detection of hazards of endocrine disruption are placed. The tool box is organised in five levels. It includes the use of existing data, in vitro and in vivo screening assays to identify substances with potential activity, and definitive tests to evaluate dose-response relationships and adverse effects. An update of this framework was published in 2012 and considered the recent developments. The most recent version of the framework is outlined in Appendix 2 and further details can be obtained from the OECD website[5] and in the OECD Guidance Document on Standardized Test Guidelines for Evaluating Chemicals for Endocrine Disruption (OECD 2012a). Currently a further update of the OECD GD 150 is in progress[6].

A *"Detailed review paper on the state of the science on novel in vitro and in vivo screening and testing methods and endpoints for evaluating endocrine disruptors"* was finalized by OECD in 2012 (OECD 2012b). The OECD also highlights a *"number of existing Test Guidelines may also provide useful information for the assessment of endocrine disrupters"* and mentioned in this context test methods allocated to level 4 or 5 of the framework, e.g. the Reproduction/Developmental Toxicity Screening Test (TG 421), the Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test (TG 422), the Two-Generation Reproduction Toxicity (TG 416), the Repeated Dose 90-Day Oral Toxicity Study in Rodents (TG 408) and the Extended One –Generation Reproductive Toxicity Study (TG 443) (OECD 2016). The Reproduction/Developmental Toxicity Screening Test (TG 421) and the Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test (TG 422) both were updated in July 2015 specifically with additional parameters to also cover endocrine disruptor aspects.

For a better evaluation of potentially endocrine active substances by manufacturers and the authorities, in the 1990s there was a need to re-evaluate the earlier test strategies for chemicals and other substances relevant to humans and the environment, such as crop protection agents or biocides[7]. Furthermore, test methods needed to be modified or new test methods needed to be further developed. This process has been initiated by the OECD. The Working Group of the National Coordinators of the Test Guidelines Programme (WNT) agreed to establish a special

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5 http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono%282012%2922&doclanguage=en

6 http://www.oecd.org/env/ehs/testing/GD150_Jul2017_clean_v2.pdf

7 http://www.oecd.org/env/ehs/testing/oecdworkrelatedtoendocrinedisrupters.htm

activity to address the issue of endocrine active compounds and develop new OECD test guidelines as appropriate. The responsible body is the Task Force on Endocrine Disruptors Testing and Assessment (EDTA). The EDTA agreed to initially pursue efforts to develop and validate test guidelines for the uterotrophic assay and the Hershberger assay, and to evaluate enhancements to the current OECD Test Guideline 407. In order to manage these projects, the Validation Management Group on mammalian tests (VMG-mammalian) was established. In the meantime this activity is largely finalised.

In-vitro-sceening tests were established in different mammalian cells. They can be used for identification of possible mechanisms of action, prediction of adverse outcome pathways and priority-settings.

In order to complete the conceptual framework, OECD is also engaging in the field of in-vitro screening assays. The first OECD TG in that respect – the stably transfected human estrogen receptor-α transcriptional activation assay for the detection of estrogenic agonist-activity of chemicals (TG 455) - was released in 2009. In 2012 this test guideline was developed into a performance based test guideline now supporting the use of two different cell lines (hERα-HeLa-9903 cells and BG1Luc-4E2 cells) for the detection of estrogenic agonist activity. In addition, the use of BG1Luc-4E2 cells for the detection of estrogen agonist and estrogen antagonist activity is described in the newly (2012) released TG 457. Most recently, the content of TG 457 was merged into TG 455. In the revised performance based TG 455 (July 2015) the use of both cell lines for the detection of estrogen agonist and estrogen antagonist activity is described, whereas TG 457 will be deleted at the next opportunity (2016). A third cellular system, the ERalpha CALUX based on genetically engineered U2-OS cells may be added to this TG provided that the submitted validation is appoved by OECD, wheras TG457 will be deleted at the next opportunity. An OECD protocol for the assessment of effects on steroidogenesis based on the H295R cell line is already available since 2011 (TG 456). In all these cell-based tests not only effects on transactivation and steroidogenesis, respectively, but also cytotoxicity of the test item is assessed. Recently (July 2015), a performance based test guideline (TG 493) for human recombinant estrogen receptor (full length and ligand binding domain) binding assays to detect chemicals with ER binding affinity became available. A stably transfected human androgen receptor transcriptional activation assay (AR EcoScreen™) for the detection of androgenic agonist and antagonist activity of chemicals has undergone full validation including additional validation work requested by OECD. Draft validation report and draft TG are currently in the commenting round at OECD. The final TG may be expected at the end of 2016. Two further human AR transcriptional activation assays based on the genetically engineered U2-OS cell line (AR CALUX assay) and on the human prostate cancer cell line 22RV1, respectively, are currently undergoing validation and may form a performance-based TG together with the AR EcoScreen™ later on. The OECD evaluation of in vitro/ex vivo assays for the detection of thyroid effects with regard to

their readiness to undergo validation has been published in 2014 (OECD 2014). It can be expected that OECD will focus on the validation of in vitro thyroid assays with proven readiness (e.g., thyroid peroxidase inhibition) in the near future.

The following table gives an overview of OECD adopted in vitro test guidelines for detection of endocrine activity:

OECD #	Test	Year of adoption/ Update	Endpoints
455	Stably Transfected Transactivation In Vitro Assays in hERα-HeLa-9906 or BG1Luc-4E2 cells	2009/ 2012/ 2015	Identifying Estrogen Receptor Agonists and Antagonists
456	H295R Steroidogenesis Assay	2011	Enzyme mediated production of Testosterone and estradiol
457	BG1Luc Estrogen Receptor Transactivation Test Method for	2012	Identifying Estrogen Receptor Agonists and Antagonists
493	Performance-Based Test Guideline for Human Recombinant Estrogen Receptor In Vitro Assays to Detect Chemicals with ER Binding Affinity	2015	ER binding affinity

In all these cell-based tests not only effects on transactivation and steroidogenesis, respectively, but also cytotoxicity of the test item is assessed. In addition, recently OECD started to evaluate in vitro assays for the detection of thyroid effects with regard to their readiness to undergo validation.

The **uterotrophic assay** - as an in vivo screening assay for estrogenicity - was validated using two different animals models: the juvenile intact female rat and the adult ovariectomized rat (Kanno et al., 2001; Kanno et al., 2003). The draft protocol was thoroughly discussed, modified and finally accepted by EDTA and WNT and formed the basis of the official test guideline (OECD TG 440).

In principle, the use of the uterotrophic assay as an in vivo screen for anti-estrogenicity was demonstrated as well, however, no full validation was performed. Detailed instructions for this approach are given in an OECD guidance document (OECD, 2007a).

Similarly, the **Hershberger assay** - as an in vivo screening assay for (anti-)androgenicity - was validated using the surgically castrated peripubertal rat as

experimental model. Furthermore, validation of a second animal model, the juvenile intact male rat, was undertaken. (Owens et al., 2006; Yamasaki et al., 2003; OECD, 2003; OECD, 2007b). Following the evaluation and thorough discussion of all validation data, an official test guideline (OECD TG 441) for the Hershberger assay was released recently. It is based on the castrated rat model, since the juvenile intact rat was considered to be less sensitive. The use and limitations of the juvenile intact rat are layed down in a detailed OECD guidance document (OECD 2009).

The **OECD Test Guideline 407** - a 28-day repeated dose toxicity study on young adult rats - provides information on the possible health hazards likely to arise from repeated exposure. This test guideline is frequently part of regulatory data requirements of chemicals and is commonly performed for most high production volume chemicals. With regard to the central role of this test, efforts were made to update this test guideline with parameters considered to effectively detect endocrine-mediated changes. The validation indicated that not all of the proposed additional parameters were effective. It also demonstrated 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. However, the updated test guideline 407 represents the most comprehensive study type to evaluate both general toxic and endocrine mediated effects. Furthermore, when compared to higher tier studies, with regard to the observed overall (i.e. irrespective of the type of toxicity) no adverse effect level the difference in sensitivity is usually not more than a factor of 10 (Gelbke et al., 2007). Thus, besides the detection of substances with strong and moderate endocrine activity, the updated test guideline 407 could be used – in combination with appropriate exposure assessment - to reduce the need for further studies on endocrine-mediated effects. The updated test guideline 407 was adopted in 2008. In 2015, OECD revised the protocols of **TG 421, the reprotoxicity and developmental scenting assay** and **TG 422, the combined 28d and reprotoxicity sceening assay** and added some endocrine parameters covering some sensitive life stages.

The **extended one generation reproductive toxicity study (EOGRTS)** was adopted firstly in 2011. It combines the apical testing of the traditional generation study with the determination of a number of endocrine parameters. The study design can be vaired to also investigate effects in the second generation or with expansion of cohorts to detect immunodevelopmental and neurodevelopmental effects.

EFSA in 2013 stated that *“despite the fact that the existing internationally standardized assays might miss some endocrine-sensitive endpoints, this should not necessarily lead to the non-identification of EDs. Given the complexity of the endocrine system with its multiple signaling pathways and cross-talks, an ED is expected to produce a pleiotropic response with a range of effects, some of which are likely to be observed in an appropriate guideline study”* (EFSA 2013).

3.5. Is screening for hormone-like effects reasonable and which studies are relevant for the assessment? Test methods and test strategy for the detection of endocrine effects

As already explained above (section 1.2), hormonal effects can occur as a result of many different molecular mechanisms and these are utilized in the development of so-called screening assays. For example, a large proportion of the frequently cited in vitro screening tests (such as various ligand binding tests, as well as reporter gene assays in yeast or mammalian cells) are based on a direct interaction between a molecule and a hormone receptor, e.g. the estrogen receptor. In vivo screening assays, such as the uterotrophic assay or the Hershberger assay, are ultimately based on the mechanisms described. The available screening assays are thus able to identify an endocrine activity of a substance based on the molecular mechanisms described. However, they cannot provide information about whether the observed effect ultimately leads to an adverse effect in the organism, i.e. any hazard potential of a substance due to endocrine effects can never be identified by screening tests alone. The decisive question as to the extent to which an endocrine active substance represents a hazard to humans can ultimately be answered only by comprehensive animal studies (e.g. subacute/subchronic/chronic toxicity tests; generation studies) and a risk assessment based on them, taking all available exposure data into account. To this end, the VCI has proposed a toxicity test strategy to permit the evaluation of the possible risk to health of an endocrine active substance (see Appendix 1) (Klotz, 2003).

In the US, the Endocrine Disruption screening program (EDSP) is based on a tiered approach. In the first step, designated substances have to be tested in a screening approach using 11 test methods ranging from in vitro to in vivo screening in mammals and environment to cover estrogenic, androgenic and thyroidal pathways. After evaluation of the results, only those substances go through the further test program, when they are considered to have endocrine effects. In the second tier, testing is specially designed based on the endpoints of the first tier. The second tier aims to get informations regarding risk based decision making. In 2013, first results of the testing were published as well an overall evaluation of the test methods used. For further details refer to the EDSP Website[8] or to e. g. Juberg et al, 2014.

EFSA (2011, 2012a) proposed that - within the concept of Threshold of Toxicological Concern (TTC) - the existing TTC values also cover endocrine mediated adverse effects, particularely those involving reproduction, development, and thyroid function. It was concluded that:

[8] https://www.epa.gov/endocrine-disruption/endocrine-disruptor-screening-program-edsp-overview

"a. In most situations where the TTC approach might be applied, there would be no a priori knowledge that a substance has endocrine activity.

b. If there are data showing that a substance has endocrine activity, but the human relevance is unclear, then these data should be taken into consideration, case-by-case, in deciding whether or not to apply the TTC approach.

c. If there are data showing that a substance has endocrine-mediated adverse effects, then, as would be the case for adverse data on any other endpoint, the risk assessment should be based on the data, rather than the TTC approach.

d. In view of the extensive work, currently ongoing, to develop an EU-wide approach for defining and assessing endocrine disrupters, once that approach is finalized it will be necessary to consider any impact it may have on the use of TTC approach.

e. In the meantime, the Scientific Committee recommends that untested substances, other than steroids, can be evaluated using the TTC approach recommended in this opinion."

3.5 Early concept for assessment: Hygiene-based margin of safety (HBMOS) evaluation model from a VCI/UBA joint research project

Early in the discussion it has been argued that exposure to e.g. estrogenic synthetic substances should be evaluated against the background of unavoidable ingestion of naturally occurring, hormonally acting substances (e.g. phytoestrogens) with food and against the physiological variations in endogenous hormone levels (Safe, 1995). To develop a solid basis of data relating to this subject, a research project supported jointly by UBA and VCI was started at the end of the 1990s to carry out comparative studies of the effects of selected synthetic chemicals and naturally occurring estrogens, taking into account combination effects. The research project yielded the following information:

- In vitro test models are suitable for screening assays and for studying mechanisms.
- In vivo test models are suitable for investigating effects ("in vivo veritas").
- Toxicokinetic studies are meaningful as a bridge between in vitro and in vivo data and for comparisons between different species (animal/human).

According to the results of the project, a threshold value and classical dose-effect relationships can also be assumed for estrogenic effects.

An evaluation model, the HBMOS concept, was developed from the results of this research project. The central point of the HBMOS concept is the derivation of a **hygiene-based margin of safety (HBMOS)** by the quantitative comparison of the potencies of chemicals that have hormone-like effects with natural components of the diet (e.g. phytoestrogens). The determination of the relative potencies for the derivation

of a HBMOS is made on the basis of in vivo test results and data on toxicokinetics. It has been shown on the basis of the data obtained in the above research project that, despite maximal doses, the in vivo potency of none of the chemicals reached the maximal value of the positive control, ethinyl estradiol. The HBMOSs for the chemicals investigated in the project suggested at that time that these substances can be assumed not to pose any risk to humans (e.g. Bolt et al., 2001; Bolt et al., 2002; Bolt & Degen, 2000). A more recent study of mixtures based on human dietary uptake and actual ratios in serum came to the same conclusion (van Meeuwen et al., 2007).

For recent discussion on risk assessment see Chapter 6.

4. Endocrine effects – environmental aspects

Also in the environment, endocrine disruption is only one possible mode of action that can lead to adverse effects on the populations of vulnerable species. Thus the general approach to deal with endocrine disruption based on scientific considerations would be a risk assessment that covers specific concerns, as brought up in the debate about endocrine disruptors (Swiss National Science Foundation, 2008, Kime, D.E., 1998):

- delayed effects (e.g. effects that are caused by exposure of an embryo may result in disturbed reproduction long after the exposure has ended. No other apparent effects on health are observed beforehand);
- effects at extremely low concentrations and high ratios between acute and chronic toxicity, which might be missed in standard testing such as change of morphological sex, infertility or abnormal reproductive behavior.

To address this concern screening methods were developed to identify potential endocrine disruptors, and many in vivo test methods have been implemented to identify the safe concentrations in the environment that include endocrine effects (see section 4.4). Further concern, the occurrence of inverse dose-response curves, is discussed in section 4.4 and consequences for chemical mixtures are discussed in section 5.1.

4.1. Endocrine systems in animals

While research on endocrine effects on humans has a sound basis of knowledge on the human endocrine system, endocrine effects in the environment may occur in an enormous number of species with specific characteristics, which are investigated in detail only for some model organisms. Although we know in broad terms about the endocrine systems in the animal kingdom, and in some cases about the actual elements involved, the necessary detailed knowledge (e.g. about molecular structures, receptor interactions, and the biological activity spectrum) is largely lacking. Such

knowledge of the scientific details would, however, be an important prerequisite for an assessment of whether chemicals can cause harmful effects in organisms by means of agonistic or antagonistic action. A summary of the limited knowledge on the endocrine system of different taxonomic groups is provided in Evans et al., 2011. In contrast to the safety considerations for humans, the central issue in the environmental debate is not the wellbeing of the individual but the protection of populations and species. Only in the case of rare, endangered, and long-lived animal species (and specifically top predators), as well as species with low rates of reproduction, the individual animal appears to deserve a correspondingly high value from the viewpoint of animal protection legislation.

Beyond the need to enlarge our knowledge on the hormonal systems of environmental species, we also need to better understand what their **normal** range of reactions under control conditions (= undisturbed conditions) is, **before** we set up complicated test systems to check their response to **abnormalities** (e.g. xenobiotic influences). This key message, raised by Sumpter and Johnson (Sumpter and Johnson, 2005), subsequently received considerable attention within the scientific community. Following up, Grim (Griem et al., 2007) “undermined” one of the paradigms of environmental endocrinology by showing that control Japanese medaka fish from various high quality studies spontaneously exhibited testicular oocytes, or ovarian testicular tissue, respectively. So far, there had been broad consensus among the scientific and regulatory community that this “intersex” generally mirrors adverse influences of xenobiotics on the gonad histology of fish species. Another example for a normal biological response is the impact of freshwater on the prolactin kinetics of the mature Chum salmon Oncorhynchus keta (Ogasawara et al, 1996). Plasma prolactin remained low in the fish kept in seawater. On transfer to fresh water, a significant increase in plasma prolactin concentration was seen in females, indicating that prolactin secretion is affected by reproduction-related changes occurring in mature females in fresh water. However, it would certainly not be appropriate to claim that fresh water is an endocrine disruptor.

Based on these examples, we hypothesise that many more phenomena in environmental toxicology and endocrinology merit to be looked at more thoroughly, in order to distinguish between normal and abnormal responses.

4.2. Adverse effects observed in the environment

There is no dispute whether damage to ecosystems due to endocrine-mediated effects of chemicals is possible in principle: The particular example of triorganotin compounds in ship paints and their effects on the species spectrum and population densities of sensitive marine snail species is well documented (Matthiessen and Gibbs, 1998). Once these results became available, rigorous action was taken (partial bans) which subsequently led to a visible recovery of the affected snail populations with exception

of large harbours, where exposure is continuing (Birchenough et al. 2002, Jörundsdottir et al. 2005).

Another example are the often-cited harmful effects on the reproductive behavior of alligators and turtles in Lake Apopka in Florida (Guillette et al., 1997) after increased contamination with pesticide residues such as DDT, which, beyond being endocrine-mediated effects, represent evidence of an acute chemical accident with massive systemic effects.

The chronic DDT intoxication of populations of birds of prey in the second half of the 20th century can be cited as a further example of the involvement of endocrine effects. It led to disturbances of reproduction and a marked thinning of the eggshells of certain species, such as the peregrine falcon, which are “top predators” at the end of long food chains. The associated high embryonic mortality led the species concerned to the brink of extinction, at least regionally. The example of DDT does, however, prove very effectively how concerted action – in this case the prohibition of manufacture and use – led to rapid and clear improvements in this difficult situation: The concentrations of contaminants in bird eggs decreased since the 1980s (Landesanstalt für Umwelt Baden-Württemberg, 2003) and in remedied locations, the peregrine falcon is no longer a rare species in Central Europe (Website “Global raptor information network”; Verdejo et al., 2008).

Several reports are available on endocrine disruption of sewage effluents. Evans et al. 2011 claim in their literature overview that intersex in invertebrates is increased in Scotland near sewage treatment outfalls, citing Moore and Stevenson 1991. However, intersex in invertebrates as well as in fish, is a common phenomenon in many species and may be caused by several environmental factors. Pastorinho et al. 2009 interpreted the results of Moore and Stevenson as no clear relationship between exposure to sewage effluent and intersex. Also other investigations have failed to demonstrate a relationship between intersex and pollution in invertebrates, as in Gammarus fossarum (Jungmann et al., 2004) and in Gammarus pulex (Gross et al., 2001). For the effects of sewage effluents on fish, Evans et al. (2011) provide a very comprehensive overview on the available literature, which demonstrates that sewage effluents can cause specific effects in fish - like vitellogenin induction or sex reversal, especially in partly artificial situations like laboratory studies with effluents or fish caged close to sewage treatment outlets. However, the effect on real fish populations is less conclusive. Monitoring of fish populations over time (as described below in section 4.3.1) point to a clear recovery of populations in the last decades rather than to a decline.

In conclusion the available data demonstrate that endocrine disruption caused by massive chemical contamination of the environment was a severe problem in the

1970s for several animal populations. Since then, regulatory measures have led to considerable improvements and a recovery of the endangered populations, given no other causes that impeded a recovery of the population, so that today in Europe and North America endocrine disruption is no longer a general phenomenon, but is restricted to local hot spots.

As also outlined below by further examples, it is easy to draw premature conclusions on potential endocrine effects of chemicals. Many reports may well attract the attention of the media, but from our point of view they do not fulfill the minimum quality requirements for sound field studies (e.g. targeted study design; representative sampling; study population(s) large enough to be statistically meaningful).

4.3. The aquatic environment

4.3.1. Central-European rivers – a well-documented “case”

The claimed extreme sensitivity of various freshwater species to industrial chemicals with estrogenic or androgenic potential (e.g. Schulte-Oehlmann et al., 2001), should have had a major impact on wild populations in German rivers. In fact the opposite trend has been observed over the past 30 years. The Rhine and the Elbe river are well-documented examples that aquatic communities, including sensitive fish and invertebrate species, have successfully recovered from severe depressions in the early 70s of the last century. This continued, and still ongoing, improvement of ecosystem structure is well documented on the web pages of various governmental, federal, and scientific institutions, a representative selection of which is listed under the references (BMUB = Federal Ministery for the Environment, Nature Conservation and Nuclear Safety; BfG = Federal Institute of Hydrology; LANUV NRW = North-Rhine-Westphalia State Environment Agency; FGG Elbe = River Basin Community Elbe; ICPR = International Commission for the Protection of the Rhine; University of Cologne, Faculty of Mathematics and Natural Sciences). Aquatic key species like the salmon and the sea trout which had gradually disappeared decades ago, due to structural and chemical deficiencies of their natural habitats, have successfully recolonized Central European rivers and have even become symbols for the recovery process of formerly deteriorated aquatic ecosystems (IKRS 2001, IKRS 2015).

Because this recolonization of previously contaminated river ecosystems ultimately occurs through the migration of species into the river and subsequent reproduction, the hypothesis of a latent danger to reproduction or pending extinction of wildlife species due to endocrine effects of industrial chemicals is not supported by the evidence.

These achievements are largely due to improved waste water management measures and purification technologies in municipal and industrial waste water treatment plants which ultimately reduced considerably emissions of industrial chemicals, including those regarded as endocrine disrupters. Also, the ecological management of river habitat has supported the recovery of fish populations along the river Rhine (IKRS, 2015). On the other hand, it has not been (and is not) possible to eliminate highly active natural and synthetic hormones originating from the human metabolism (e.g. estrogens, androgens) to the same extent even with the most modern purification techniques. It is thus not surprising that British scientists were able to demonstrate repeatedly (Allen et al., 1999; Desbrow et al., 1998; Routledge et al., 1998; Jobling et al., 2006; 2009; Purdom et al., 1994) that observed effects on local fish species (increase of vitellogenin level; intersex forms, changes of the sex ratio) in receiving streams could be explained exclusively, or partially at least, by the introduction of natural/synthetic hormones (e.g. steroidal estrogens) after passage through waste water treatment plants. An open question still is whether or not this “sexual disruption” in feral fish causes concomitant adverse effects on reproductive health of whole fish populations and, more generally, how widespread these phenomena are in the aquatic environment. The ongoing debate within the scientific community also covers the question of effects caused by single vs. multiple substances, or mixture toxicity, respectively (Brian et al., 2005).

4.3.2. The sex ratio of freshwater fish – VCI-funded field biology research project

Field-oriented research work which tries to confirm laboratory-based findings under real-world conditions, faces daunting challenges, related to the complex mix of environmental factors (e.g. temporal and spatial discontinuities; representativeness of samples; severity vs. reversibility of effects). In view of this difficult, but at the same time promising task, the scientific community has strongly focused its interest on the endocrine system and endocrine-mediated harmful effects due to xenobiotics and natural hormones, mainly in fish. This has led to an ever-increasing number of scientific publications over the past 10 years, but the details of this information are sometimes contradictory, leading to heightened uncertainty about potential environmental effects, both in the public and the scientific community (e.g. Harris et al, 2011; An et. al. 2009; Bjerregaard 2006; Blazer et al. 2007; Grist et al. 2003; Jobling et al. 1998, 2006; Kidd et al. 2007; Lange et al. 2008; Sumpter & Johnson 2008; Thomas et al. 2006; Werner et al. 2006). The majority of studies carried out, however, were limited to in vitro tests (of a screening nature) or in vivo tests, as preferred for regulatory purposes, especially for risk assessments in environmental compartments (e.g. Länge et al. 2001; Sohoni et al. 2001; Yokota et al. 2001) .

In view of the scarcity of reliable and well-conducted epidemiological field studies in the aquatic environment, the VCI has sponsored a research project „Freilanduntersuchungen zur Geschlechterverteilung einheimischer Fischpopulationen“ (Field studies on the sex ratio in indigenous fish populations) that has been published in a peer reviewed journal (Allner et al. 2010). This project determined whether and to what extent the endpoint “sex ratio of fish populations” varied under uncontaminated field conditions. The study was carried out in two species of freshwater fish (roach and perch) that differ in their feeding and habitat preferences. Moreover, to obtain a clearer idea of the informative value of the sex ratio as an endpoint for endocrine disruption, the situation in uncontaminated places was compared with contaminated areas.

The project produced the following results:

In all locations classified as **uncontaminated** the sex ratio of the two fish species – over the entire study period from 2001 to 2003 was balanced. However, individual (seasonal) fishing exercises did reveal significant deviations in sex ratios for individual locations, illustrating the extent to which the results of limited data bases can provoke misinterpretation.

In the evaluation of gonadal development, the project showed that, for roach in general, no intersex forms occurred, whereas, in the perch, female gonadal cell types existed in 7% of the testes. This applied equally to uncontaminated and contaminated locations. This is evidently a species-specific peculiarity that, quite apart from any speculation about endocrine mediated effects, expresses itself in a certain “plasticity” in the sexual development of the perch (probably similar to the medaka; see Grim et al. 2007). Further details of this interesting phenomenon can be obtained from the discussion in the final report of this research project, published on the VCI website[9] or from the published paper already mentioned above (Allner et al., 2010).

The three locations classified as **contaminated** in this study displayed clear adverse effects on one or more of the biological criteria listed above, which was no real surprise – and expected within the context of this study – because the local fish populations were exposed to a variety of stressors throughout the year.

In detail, the following “deviations from the norm” in relation to the biological endpoints under consideration were observed at the three contaminated locations:

- In one, but not in the other two locations, the sex ratio of the perch differed slightly, but not statistically significantly, from balance (60% males; 40% females).
- In all three locations there was evidence of a delay in sexual development. According to the authors of this research project, the increased incidence of

[9] https://www.vci.de/vci/downloads-vci/114736-abschlb.pdf

nonspecific histopathological findings in the gonads is more likely to be due to general stress, and not correlated with specific, endocrine-mediated effects.

- Vitellogenin, the precursor of the characteristic yolk protein of reproductively active female fish, was also detected in some adult male roach at concentrations > 10 µg/ml.

Often new questions arise at the end of well planned and executed field studies, which cannot be answered with the methodological approach selected. That was also the case with the contaminated sites of this study, since the question of the relevance of the observed harmful effects to the population biology remained unanswered, especially as its numerical basis (i.e. the numbers of adult males and females caught) was extremely low and detailed statistical confirmation could not be obtained. There were interesting follow-up questions, but they went far beyond the conceptual approach of the present study: Do the deviations of the biological markers from the norm indicate impairment of reproduction in the roach and perch? If so, where do the adult and juvenile fish sampled come from? To what extent can the two species migrate? Should the reliability of the capture method (electrofishing) be challenged because of the increased electrolyte content at the contaminated locations? These are questions unavoidable in relation to the present study; questions which however also cast doubt on the validity of many field studies on subjects of a similar nature, but with inadequate study planning and fewer data.

Accordingly, this is the major conclusion that the VCI draws from the results of the study: In the present case, the sex ratio, as an informative parameter for the reproductive health of fish populations, was found to be fairly stable under both uncontaminated and contaminated conditions. To obtain a reliable answer to such an apparently trivial question requires a series of fishing exercises over a long period of time, since the results of individual fishing exercises are impaired by seasonal fluctuations.

4.3.3. Evaluation of findings from field studies

When evaluating the results of field studies, the following points should be borne in mind:

What is the general state of health of the animal populations under investigation? Reproductive impairment or changes in organs and even changes in internal levels of hormones or vitellogenin, which are generally regarded as evidence for a specific effect, can often be the result of a poor state of health and are not necessarily caused by substances with endocrine effects. For example, the parasite Ligula intestinalis can influence the pituitary-gonadal axis and affect the gonadal growth of fishes which are infected with this tapeworm species (Arme 1968, 1997). Furthermore, field studies show that Ligula intestinalis is capable to significantly alter e.g. the gonadosomatic

index, plasma vitellogenin level, and plasma concentrations of some sex steroid hormones, respectively, in host fishes (Minier et al., 2000; Hecker & Karbe, 2005; Schabuss et al., 2005; Trubiroha 2010). This example shows that a meaningful study should always keep the entire organism in view and not restrict itself to a few parameters. The general condition of the environment of the organisms under investigation should also be taken into account in the evaluation.

Is the environmental situation favourable for the investigated organism?

In studies with caged organisms in a stream the general health in such an artificial situation needs specific attention and it should be ensured that the animals do not experience stress, which is likely to change the function of the endocrine system.

Effects that resemble endocrine disruption may not only be caused by parasites, but also by other unfavourable conditions like hypoxia, which has caused suppression of gonadal growth and the neuroendocrine function (Thomas et al., 2007, Thomas et al. 2006). Furthermore it has to be considered, whether the investigated population occurs naturally in the habitat in question, or whether it was artificially introduced and may be not specifically adapted to the environment.

Where were abnormalities observed?

Are the observations limited to local, contaminated, areas or are they widespread? For example, the disturbances in the reproduction of alligators and turtles in Lake Apopka (mentioned above) was the result of a chemical accident in 1980. The lake is still relatively heavily contaminated (sediments). Normalisation can take a long time, particularly in long-living animal species.

4.3.4. Informative significance of the biomarker vitellogenin

There are now a large number of field studies that have examined changes in certain parameters in organs, body fluids or the morphology of the organisms that could be associated with the influence of exposure to endocrine active substances. For example, the detection of vitellogenin (a precursor of the yolk protein, which is used by females to produce the egg) may be an evidence of exposure to estrogenic substances (when it is found) in male animals.

Vitellogenins belong to a class of yolk proteins that are widespread in the animal kingdom. In invertebrates (worms, mollusks, insects, crustaceans, echinoderms), they are synthesized in various organs. All egg-laying vertebrates (cyclostomes, fish, amphibians, reptiles, birds) synthesise it as the main component of the yolk of the egg. In vertebrates, vitellogenin is synthesized in the liver after estrogen receptor activation by endogenous sex hormones (17β-estradiol). In sexually mature female salmonids,

blood plasma concentrations can rise to more than 10 mg/ml. Vitellogenin is transported in the blood - in the form of a lipophosphoprotein - to the ripening egg follicles in the female sex organs, transported into the egg cell, and converted into lipovitellin or phosphitin. All the individual steps in this complex process are under hormonal control (gonadotropins). Due to the hormone status of males and sexually immature females, vitellogenin biosynthesis is not induced. However, deliberate exogenous administration of 17β-estradiol or substances with estrogenic activity leads to the stimulation of vitellogenin synthesis in the livers of male and immature female fish as well, since the estrogen receptor - i.e. the docking station for the sex hormone 17β-estradiol - is always present, irrespective of the maturity and sex of the fish. These observations gave rise to the idea of using the synthesis of vitellogenin in male fish as a sensitive signal (biomarker) when testing estrogen-like activity of chemicals.

However, a changed vitellogenin concentration in the blood plasma does not allow conclusions about possible harmful effects in the population concerned. As yet, there are no data that permit the prediction of an effect on the population concerned on the basis of increased vitellogenin concentrations in the blood of male or juvenile fish (Bergt-Schrenk and Steinberg, 2001). Rather, such biomarkers can help to identify the substance(s) that may be responsible and then, by means of more detailed laboratory investigations with appropriate test organisms, to evaluate a concentration-effect relationship for ecotoxicologically relevant endpoints (e.g. fertility or sex ratio). These investigations can then be used in a risk assessment of whether substance concentrations in the environment could trigger a population-relevant harmful effect.

4.3.5. Informative significance of the Gonadosomatic Index (GSI), gonad histology, and sex ratio

Because there are similar problems in their evaluation, the GSI, sex ratio, and gonad morphology will be discussed together here.

In-situ determination of the sex of fish is often problematic, as many species have little, if any secondary sex characteristics or such characteristics occur only after many years of development. In many cases it is necessary to sacrifice the fish in order to dissect the sex organs and, subsequently, determine the sex. This is often possible macroscopically, but in juvenile fish it may be necessary to carry out histological examinations. When doing so, the gonads are also examined for the occurrence of intersex forms. In many fish species the original genetic sex can be changed during development to the phenotype of the other sex, as a result of a variety of natural and chemical influences (which is used in various ways in modern fish farming). There are even species in which an individual fish can change sex several times during its life-

time. Taking this into account, it is still unclear if an unbalanced sex ratio indicates an adverse effect on fish populations in the field.

By determining the weight of the animals and their sex organs, it is possible to calculate the GSI, which is defined as the ratio of the weight of the sex organs to the weight of the whole body.

For various reasons, the informative value of this parameter for the detection of endocrine active substances in the wild is very limited.

GSI, sex ratio and gonad morphology, which are under discussion for the detection of endocrine effects may be influenced by many different factors, which may occur alone or in combination. The cause for an observed change in one of these parameters is not necessarily an endocrine-mediated effect of a chemical.

Important factors that affect the observed parameters include, among others, the age of the animals, their state of health and reproduction, the water temperature, fish density, and the time of the year.

Here are some examples:

- A long, cold winter with low water temperatures can delay sexual maturation and thus change the size of the sex organs. Comparing such catches with those from other climate zones, may result in misinterpretations.
- In a number of fish species, the gonadal disposition is originally female. When male sex organs develop in genetically male animals during sexual maturation, transitional forms (intersex forms) may occur, leading to misinterpretations.
- The seasonal growth and regression of the sexual organs in various fish species makes the analysis even more difficult.

Because of the large degree of variability in the results, it is essential to examine samples of sufficient size to rule out misinterpretation. This requirement is often not fulfilled, in which case the findings can be regarded merely as random results.

Thus, very exact analysis of the data is required to distinguish between random results (artifacts) and real effects. Can it be ruled out that the observed effects were caused by other factors than by endocrine-disrupting chemicals? The next question is: Do the observed effects - e.g. a change in the sex ratio - have an influence on the reproductive health of fish populations?

4.4. Informative significance of experimental laboratory studies

Experimental studies are designed to identify the substance-related hazard and to establish the no-observed effect concentration (NOEC).

Experimental laboratory and field studies can identify substance-related effects. However, the crucial factor for the environmental relevance of laboratory findings is whether the exposure conditions in the experiment (mode of administration, concentration or long-term bioavailability) reflect the actual exposure conditions in the field. Short-term exposure may cause measurable effects in individual animals in targeted experiments, but does not necessarily cause population-relevant impairment under field conditions.

The possibility of the existence of non-monotonic concentration-effect relationships which differ from classical concentration-effect curves, is still under discussion. Such observations were made for individual parameters, e.g. if a substance initially increases the activity of an enzyme at low doses, until the toxic effect leads to inhibition of the activity at higher doses. However, usually in this scenario also other parameters would be affected in the toxic concentration range. For detection of an effect threshold in an ecotoxicological test, the sum of all observed adverse effects is considered not just an individual parameter. According to present knowledge, it is unlikely that harmful effects can be expected in concentration ranges well below the identified NOEC (no observed effect concentration), if the NOEC considers all relevant effects. If unusual concentration-effect relationships occur, it should be reviewed if the study design covers all relevant adverse effects.

The direct relevance of effects measured in laboratory studies is rarely investigated in field populations. Therefore, it is common agreement that (sublethal) experimental endpoints - such as growth retardation, changes in sex ratio and sexual development, time to spawn, fecundity and fertility parameters, and hatching of off-spring - are relevant for the evaluation of an (endocrine disrupting) effect which has an influence on the sustainability of populations. However, there are a few studies reported in context with the effects of endocrine active substances on fish populations in the field. One study, for instance, was a field experiment, in which an experimental lake in Canada was treated with the synthetic hormone 17α-ethinylestradiol over three consecutive seasons and the result of this treatment was compared to a neighbouring untreated lake (Kidd et al, 2007). The treatment resulted in a decline and eventual extinction of the fish species fathead minnow (Pimephales promelas) after the third treatment season. This study was of particular relevance, because fathead minnow were used in a number of long-term laboratory studies to investigate the effects of low concentrations of the hormone 17α-ethinylestradiol. Consequently, it could be established that concentrations in a low ng/L range have the ability to extinguish fish

populations in the field, which was in agreement with the evaluation of long-term laboratory studies.

Other cases, which provide information on the relevance of single parameters observed in laboratory and field studies, are the occurrence of fish with mixed gonads which have been exposed to estrogenic acting chemicals. The first reports of such intersex fish in context with potential exposure to estrogens came from English rivers in the 90's (Jobling et al., 1998). Similar findings are common in laboratory studies with estrogenic acting substances. However, it is rarely investigated how far male fish with mixed gonads are able to produce viable gametes, in order to provide for a sustainable population. Jobling et al. (2002) reported that wild intersex fish from English rivers (roach; Rutilus rutilus) had reduced semen quality when moderately or severely feminized, compared to normal or less severely feminized fish. This indicates that the feminization effect of estrogenic chemicals, leading to intersex fish may affect reproduction. However, a proof for this hypothesis is not yet available, because it is not known whether a reduced semen quality decreases the affected fish population.

Based on these data it can be concluded that the endpoints studied in long-term laboratory experiments in fish with hormonal active compounds have a relevance for the environment. A direct extrapolation of observations on certain parameters to the field situation and the sustainability of populations is, however, not possible in the absence of longterm field studies.

4.5. Test strategy for detecting endocrine effects

As outlined in the introduction of section 4, the existing testing strategy had to be extended to sufficiently cover endocrine effects. Since tests in fish already play an important role in the environmental risk assessment and since the hormonal system of fish is well developed, test methods in fish are central for most test strategies.

In the OECD Detailed Review Paper 21 (2002) the use of available standard methods to detect endocrine effects was investigated and other more specific non-standard test methods were discussed. Since then, many additional test methods have been developed or are currently under investigation to enhance the tool box to detect and to evaluate endocrine-mediated toxicity. The OECD testing framework (OECD 2012a; see also see Appendix 2) grouped the test methods into 5 levels (in vitro /in vivo, single or multiple mechanisms). The OECD testing framework was, however, not intended to be a tiered testing strategy with clear-cut decision criteria.

A screening test should identify an endocrine disruptor by giving a yes/no answer and characterize the mode of action while higher tiered tests should provide a no effect threshold which also includes endocrine effects. Generally, an effect threshold for risk assessment should consider only population-relevant endpoints like reproduction,

growth and mortality. An impairment of these endpoints can be endocrine-mediated; however, if such an effect is observed in a definitive test, the mode of action could be completely different. On the other hand, most definitions for an endocrine disruptor require a relevant adverse effect as a consequence of a specfic endocrine mode of action. If there is a need to determine whether the chemical is an endocrine disruptor beyond the normal risk assessment then higher tiered tests need to include specific biomarker endpoints that allow the determination of the mode of action, even if these endpoints would not be generally necessary for a sound risk assessment.

A further issue to be considered is the statistical power for the detection of effects. Tests like the fish full life cycle test or the fish two generation test examine many endpoints. The number of replicates is, however, limited by technical considerations. For some of the endpoints with a general high natural variability, e.g. fecundity, the statistical power is thus necessarily low. Partial life cycles can be easier designed to increase the power for a specific endpoint. Taking these requirements into account , it is understandable that most testing schemes are not straightforward, but open up many options how to combine different test methods and to come to a final evaluation - depending on the available knowledge, the mode of action and the regulatory needs.

A comprehensive Guidance Document on standard test guidelines for evaluating chemicals for endocrine disruption was published by OECD in 2012 (OECD 2012a), which provides further guidance which test method could answer which question. All OECD test methods, that are meanwhile validated or are currently in development as standard methods, are discussed therein. A further OECD document summarises the information on in vitro and in vivo screening tests (OECD 2012b).

Other proposals go further by providing guidance for a tiered testing strategy for endocrine effects in the environment (ECETOC 2009, Bars et al. 2011, Bars et al. 2012, Knacker et al. 2010, Länge et al. 2003). Many elements in these test strategies are similar. Matter of debate are generally the following questions:

- When is the weight of evidence sufficiently high to enter a specific testing scheme on endocrine effects?
- Which information is regarded as sufficient to disprove the suspicion of endocrine disruption?
- What is convincing evidence for endocrine disruption?

Generally, a tiered testing strategy is proposed, starting with an evaluation of all available data, followed by in vitro and in vivo screening and ending with definitive tests that include population-relevant endpoints.

In 2017 a further ECETOC Report (TR 130: The ECETOC Seven Steps for the Identification of Endocrine Disrupting Properties) was published as a response to the

announcement of the ECHA/EFSA guidance document on the Commission's draft criteria which is currently under preparation (see Chapter 6.6).

Screening tests

The endocrine system was conserved during the evolution process and thus the same mechanisms are often relevant for many taxonomic groups. Estrogens and androgens may have specific effects at least in all vertebrates and in several invertebrate groups as well. Therefore in vitro screening tests - designed to detect qualitatively the potential of a substance to react with a specific endocrine receptor - are generally not very specific. Differences, however, will be seen more likely at a later stage of in vivo testing, when different exposure routes, metabolic capacity and receptor mediated reactions play a larger role and when the effect is correlated with exposure. Nevertheless, specific in vitro tests were developed to detect endocrine disruption in different species (OECD July 2011).

The specific testing strategy for endocrine effects in fish developed by VCI and IVA (Industrieverband Agrar e.V.) 2007, which is applicable only if relevant exposure occurs, can be still regarded as a general recommendation (see Appendix 3).

The criteria for entering the endocrine specific testing scheme include indications from:

- in vitro testing,
- mammalian testing,
- reproduction toxicity,
- literature data and
- structure-activity relationships.

A weight of evidence evaluation of these initial observations helps to decide on entering the specific endocrine testing scheme or the standard risk assessment approach for fish toxicity. If uncertainty persists regarding the endocrine potential, an additional step of targeted testing is needed for clarification and consideration in the context of all existing data.

The first tier of the specific endocrine fish testing scheme are fish screening assays, which have been adopted by OECD incorporating two different approaches: OECD test guideline 229 (Fish Short Term Reproduction Assay) includes reproductive output and histopathological changes in reproductive organs, while OECD testguideline 230 (21-day Fish Assay: A Short-Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition) focusses primarily on abnormal secondary sex characteristics and vitellogenin production as markers for exposure to hormonal compounds. If no indication for an endocrine effect is seen in the screening test, the specific testing is ended and the test substance will be evaluated in a standard risk assessment for fish.

In all other cases a definite test is needed, which can be either a fish sexual development test (e. g. OECD 234) or a Fish Full Life Cycle test, both of them providing data for the derivation of valid NOECs for risk assessment.

Several designs are discussed for testing over several generations (2-generation fish test, Fish Full Life Cycle Test). While these test designs cover effects that are visible only in later developmental states or even in following generations, they may lack sufficient statistical power due to technical limitations. Thus it needs to be decided based on the available information on the mode of action, what is the most relevant testing method (Teigeler et al., 2007). The test guideline OECD 240 for an extended one generation test has been adopted for the Japanese rice fish (medaka).

In summary, it can be said that the identification of potential hormonal effects of a substance by toxicological or ecotoxicological screening assays - as part of an appropriate test strategy - appears perfectly reasonable. However, the results of such screens cannot be used to identify potential harmful effects, adverse effects or even risks. Such an evaluation can be made only on the basis of large-scale test strategies, covering population relevant endpoints and taking into account available exposure data.

Subsequently to the activities on mammalian testing at OECD level (see 3.4), EDTA established the Validation Management Group on ecotoxicity tests (VMG-eco), in order to manage the activities on modification of current and development of new ecotoxicological test methods. The scope of VMG-eco is much broader than the one of VMG-mammalian, reflecting the whole spectrum of taxonomic groups, ecological types, and hormonal systems encountered in environmental species. Until recently, in-depth knowledge of vertebrate and invertebrate test systems was confined to a handful of key species, primarily from freshwater habitats (e.g. various fish species, the microcrustacean Daphnia magna, and a few others more). Even in these species, the underlying mechanisms of endocrine-mediated effects were not fully understood, but more advanced knowledge has been gathered over the past couple of years. The VMG-eco is currently pursuing various paths:

Firstly, to validate a tiered testing approach with freshwater fish, using well established OECD species like the fathead minnow, the zebrafish, the medaka and the three-spined stickleback, for detection of (anti-)oestrogen and (anti-)androgen substances:

The fish screening assays (tier 1) are intended for screening of endocrine activity and for priority setting for further testing. Core endpoints are sex ratio, secondary sex characteristics, vitellogenin and mortality as well as reproductive output and histopathological changes of reproductive organs. The fish screening assays have been adopted by the OECD.

Further method development for screening has been conducted under OECD auspices with the stickleback, which is intended particularly to detect the activity of anti-androgenic substances in androgenised females, where a reduced production of spiggin-protein, a material produced normally by males for nest-building, is investigated as a response to exposure to those chemicals. Most probably, this rather specific test approach may finally become incorporated into the OECD 230 guideline.

- The fish sexual development test (tier 2a) is a core element in the environmental risk assessment of compounds with endocrine activity. The duration of this trial is much longer. At the current stage of discussion, 60 to 120 days post hatch is being proposed. Essential endpoints are gonad histopathology, vitellogenin, secondary sex characteristics, sex ratio, and overall mortality. The validation process is ongoing.
- The fish full life-cycle (tier 2b) is considered to represent the ultimate “gold standard” fish test. According to current thinking, it is intended for use in exceptional cases only where tier 2a testing, in environmentally relevant compounds, does not provide clear guidance for risk assessment. There is an ongoing debate, yet, if this test is really needed, as the endpoints are largely the same ones as in the fish sexual development test - apart from the specific reproductive output at maturation. In parallel to this discussion of the underlying principles, several OECD countries have initiated experimental work with this test. Additionally or alternatively, a two generation fish test is developed by the OECD countries USA and Japan.
- A fish reproduction test to evaluate effects on fecundity is seen as a gap that should be closed and was proposed as new OECD project by the US in 2011. This partial Life Cycle Test could be designed to maximise the statistical power to detect effects on fecundity, which are difficult to detect in a full life cycle test for technical reasons.

A second path, which the VMG-eco is currently pursuing is to strengthen existing OECD test guidelines for invertebrates by adding additional endpoints - to make them suitable for the detection of endocrine effects:

- The OECD guideline 211 (Daphnia magna reproduction test) has been used as a starting document for an “enhanced Daphnia reproduction test” to detect adverse effects of juvenile hormone mimicks. These compounds, which are used to control insect pests in agricultural crops, specifically impact the sex ratio of the Daphnia offspring. An inter-laboratory ringtest has been performed successfully. Further validation work has recently been stopped at the OECD level, or has been modified, respectively, into a national activity of those countries which are more specifically interested in the rather restricted field of application of the enhanced Daphnia test (juvenile hormone like substances).
- The Chironomid life-cycle test (OECD Guideline 233) adds reproduction-related endpoints to the design of the previous chronic toxicity guideline with Chironomus

spec. (which is based on growth and development) and allows for assessment of transgenerational effects.

Thirdly, the VMG-eco pursues the path to develop new vertebrate and invertebrate test models, encompassing other environmental compartments like soil, sediment and marine waters. Activities related to this target comprise test systems with key representatives from many taxonomic groups, e.g. oligochaet worms, molluscs, mites, springtails, flies, copepods, amphibians, and birds. Some of these test systems are being developed for use in special cases only and not, for example, as a general test approach for industrial chemicals. Not all of these activities are pursued under the framework of the EDTA VMG-eco, but rather within the general Test Guidelines Programme to develop new guidelines for the assessment of biotic effects. As long as reproduction endpoints are covered, endocrine effects may, or may not, be involved.

Those OECD guidelines include the protocols for the snails Potamopyrgus (OECD 242), Lymnea (OECD 243), and for Amphibia (OECD 241), to name a few.

The main aim of all these activities is to develop robust and reliable test systems which are suitable to quantify adverse effects on environmentally relevant endpoints (e.g. reproduction); the focus is not primarily on the clarification of underlying mechanisms.

Most of the above-mentioned activities are ongoing, and more test proposals are on the “waiting list”. Limiting factors for further validation activities will be the availability of expert laboratories willing to participate, as well as funding of their work. In view of the time need for a complete validation procedure, the OECD is looking for ways to shorten the validation processes.

5. Specific discussions on the subject of endocrine activity

5.1. Combination effects

Toxicology:

In toxicology it is generally assumed that substances, which are harmful to the same target organ especially via the same mode of action, can act additively on combined exposure, but do not multiply (potentiate) their effects. In contradiction of this, in 1996 an American group - writing in the prestigious scientific journal “Science” - published an article that reported over-additive (synergistic) effects on simultaneous exposure to combinations of estrogenic substances (Arnold et al., 1996). Later on, the authors of this paper had to officially withdraw the work, as many other scientific teams were unable to confirm the results and even the authors themselves could not reproduce their work (McLachlan, 1997). In 2001, it became known that the data published in

1996 had been falsified and that the experiments described were never carried out in the form described (US Public Health Service, 2001).

An additive effect for endocrine active substances has been demonstrated in in vitro experiments (e.g. Payne et al., 2000; Payne et al., 2001; Silva et al., 2002; Rajapakse et al., 2002; Charles et al. 2002, Charles et al. 2007, Birkhoj et al. 2004; Ghisari et al. 2009). Because of the sigmoid nature of the dose-effect relationship, such addition is linear only in the effective range. If the mechanisms of action are different, or if there are interactions between components of a mixture or differences in pharmacokinetics, a less than additive effect is more likely. In vivo studies - that are particularly relevant to this question, since they cover the wide spectrum of different mechanisms - confirmed the additive or less than additive action for effects on the same target organ system (e.g. Eroschenko et al., 2000; Ashby et al., 1997; Charles et al., 2003, 2004 and 2007; Diel et al., 2001; Takagi et al., 2004; Tinwell & Ashby, 2004; Crofton et al., 2005; van Meeuwen et al., 2007; Howdeshell et al. 2007, Metzdorff et al. 2007, Takagi et al. 2004; Eustache et al. 2009; Taxvig et al. 2013). The potency of the constituents of the mixtures as well as the selected dose relative to the effective dose (dose-response relationship) and pharmacokinetic aspects are assumed to be of major importance for the overall effect of a mixture. Moreover, on the basis of the current scientific evidence a combination-specific adverse effect is typically triggerd by only one or two substances (Price & Han, 2011).

More recent studies examined mixtures of test substances in the lower dose range, starting at the NOAEL for single substances, but also at higher doses, and confirmed an additive action (Christiansen et al. 2009, Blystone et al. 2009, Hass et al. 2007, Rider et al. 2008); new aspects are that additivity was described for target organ system effects due to a shared overall mode of action (e.g. antiandrogenicity), but irrespective of the specific cellular mechanisms of toxicity (Rider et al. 2008, Christiansen et al. 2009). Some of the observations were named synergistic, as the observed responses were greater than would be assumed from the toxicities of the individual chemicals (Christiansen et al. 2009), but in the evaluation of these observations the following aspects need to be considered:

- The fact that the studies available typically used high dose mixtures only (exceeding the single substance NOAEL or less than an order of magnitude below the NOAEL).
- It is stated by the authors that careful consideration is required whether additive or even synergistic effects are likely to occur at low, environmentally relevant exposure levels.

Overall, the dose-response curve of each single substance would need to be available and considered in planning mixture studies. However, typical *in-vivo* study designs do

not yield such detailed dose-response information. A recent review of literature revealed only a very limited number of studies availabe today to be evaluated in that context (Boobis et al. 2011).

ECETOC initiated in 2010 a review of the scientific literature on interactions between chemicals at low doses, particularly those occurring below a toxicological point of departure of the individual chemicals. A report was published in 2012 (ECETOC 2012). It was found that deviations from additivity did not seem to be any more prevalent for endocrine active substances compared to other mode of actions. The overall conclusion was that *"There was no convincing evidence of toxicity for combined exposures to substances present at concentrations that are acceptable for single chemicals"* and the final conclusion *"Based on our evaluation, there is no evidence that exposure to complex mixtures of components, each well regulated according established risk assessment approaches, would pose a health risk to humans."*

In 2012, the three independent non food EU Scientific Committees currently in place (SCCS; SCHER; SCENIHR) issued an opinion on the toxicity and assessment of chemical mixtures and concluded that common modes of action may act jointly by dose/concentration addition - and that interactions usually occur at medium or high dose levels (relative to the lowest effect levels). At low exposure level, interactions are either not occurring or toxicologically insignificant (EU 2012).

EFSA in 2013 announced that the issue of combined toxicity resulting from combined exposure to multiple substances will be adressed by EFSA in a separate activity.

Ecotoxicology:

While in mammalians toxicologial effect levels are generally based on specific findings in organs or on comparably low level metabolic changes, the endpoints in ecotoxicology are generally less specific and do usually consider more general findings like mortality, growth and reproduction. Thus the likelihood to detect additive effects of chemicals is much higher in ecotoxicological studies, since an unspecific endpoint may be affected by nearly all modes of action. Therefore, it is not surprising that most convincing examples for mixture effects are provided in ecotox studies (Kortenkamp et al. 2009, Sumpter 2005).

In particular mixtures, where the concentrations of all single components are close to the no-effect level, are likely to have a visible effect. However, these findings are relevant for risk assessment only in very few limited cases, since the probability of a combination of exactly these threshold concentrations in the environment is highly

unlikely. In the overwhelming majority of real exposure scenarios, only one substance will determine the toxicity of the mixture (Price and Han 2011).

A compilation of theoretical bases and experimental results of combination effects in ecotoxicology can be found in ECETOC (2001), Kortenkamp (2009), and Sumpter (2005).

Since additivity can be expected to be a more general principle in ecotoxicology than in toxicology, it needs to be addressed in risk assessment irrespectively of the mode of action and is no specific concern for endocrine disruptors. However, the actual relevance is limited by the low likelihood of more than one substance of a mixture contributing to the overall toxicity of a mixture under realistic environmental conditions. Appropriate models are described in Price & Han (2011).

Whereas the above mentioned reports reflect mainly the experiences from acute and chronic laboratory studies, “eco”-toxicology provides an alternative approach to address the mixure issue phenomenologically. This means that, beside the chemical and biochemical interactions, biological interactions of different kinds are considered, such as symbiosis, parasitism, or predator-prey-relationship. Bioindicator models are increasingly applied in the area of water quality policies (such as in the European Water Framework Directive) which illustrate the state of the habitat, for instance by species abundance and density. This field-oriented approach includes the advantage of reflecting the interactions in an ecosystem adequately. In the light of the vast efforts needed to study the combination effects in ecotoxicology, the field oriented approach seems to be more pragmatic and eventually, also more reliable.

5.2. Do endocrine active substances have adverse effects at low doses and is it possible to determine thresholds and limit values for hormone active substances?

On the basis of the available evidence adverse effects mediated via the endocrine system, like a large number of other mechanisms leading to toxic effects, have so-called “threshold values”. This means that not every dose, however small, has an effect. On the contrary, an adverse effect is triggered only if a certain dose or concentration is exceeded: The “threshold” is the concentration above which the noticeable effect is triggered. The threshold phenomenon has been confirmed by numerous biological, toxicological, and pharmacological experiments. As the experimental basis for the threshold, toxicity studies usually determine the no observed effect level (NOEL), or the no observed adverse effect level (NOAEL).

As published in 1999 and reconfirmed in 2013 the term threshold may be defined in different ways (Slob 1999, KEMI 2013):

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

These differences in definition and terminology might be one reason for the still ongoing debate for or against a threshold for so called endocrine disrupters. Each definition has a logical applicability. In the context of toxicology, ecotoxicology and medicine the biological definition is the most appropriate and therefore also the basis of this overview report. The biological definition covers different levels of biological responses, e.g. for an endocrine active substance there is a biological threshold of activity, which is lower than the biological threshold of adversity. For chemicals risk assessment purposes, the biological definition related to the biological adversity should be used, which is in-line with the WHO/IPCS definition for endocrine disruptors which in turn includes two key elements: 1) observations of adverse effects and 2) in an intact organism.

That the experimental threshold is not identical with the biological threshold has been known for decades and is irrespective of the Mode of Action. This is confirmed by the EU Scientific Committees in 2011 in their Opinion on mixtures stated (Citation from the Opinion, page 32):

"…. It is important to note that NO(A)ELs and NO(A)ECs derived from experimental studies do not always represent zero-effect levels. The NOAEL(C)s and NOECs estimated in toxicity and ecotoxicity studies, respectively, are often associated with effect levels in the range of 5 to 20% and hence no "zero-effect levels". It cannot be assumed that in all cases E(Ci) is equal to zero for exposures at the NOAEL(C) or NOEC of a particular study. As the NOAEL(C) or NOEC do not necessarily represent a value for which E(Ci) = 0, exposures equal to these levels may also contribute to mixture effects for dissimilarly acting substances. The question, therefore, is not if exposures to mixtures of substances at the NOAEL or NOAEC for each component represent a potential risk, but if exposures to mixtures well below these levels, and in particular at the level assumed to be safe for each component (TDI, DNEL, PNEC or equivalent) may produce adverse effects. The answer to this question is different for human health and ecological assessments…."[10]

[10] http://ec.europa.eu/health/scientific_committees/environmental_risks/docs/scher_o_155.pdf

In addition to the above listed thresholds a new term, "**Regulatory threshold**", was introduced in the discussion. The "Regulatory Threshold" is the experimental threshold divided by safety/extrapolation factors. The regulatory threshold should be protective for the biological threshold and related adverse effects. The regulatory threshold can also be thought of as a limit value such as the acceptable daily dose/concentration (e.g. ADI, DNEL, or PNEC) for the respective population.

Such a regulatory threshold has already been applied by ECHA. An ECHA RAC opinion recently on the restriction of four LMW phthalates (DEHP, DBP, DIBP and BBP) with clear anti-androgenic effects included application of a threshold approach as well as use of a risk assessment approach, using a DNEL and exposure information.[11]

EFSA in 2013 stated that compensatory feedback mechanism and homeostatic capacity of the endocrine system needs to be considered and that the threshold of adversity is crossed if the body is unable to compensate for the induced changes. Even if the existence of dose thresholds cannot be proven or ruled out the homeostatic mechanisms would support that a biological threshold between endocrine modulation and adverse effect exists (EFSA 2013). Moreover, thresholds for induction of hormonal effects appear to be a fundamental principle for the hormone system without which normal physiological functions would be impossible due to obligatory ability to discriminate important hormonal signals from background noise. From such thresholds, safe levels of exposure can be estimated (Borgert et al., 2013).

5.2.1. Existence of thresholds of adversity in the context of the REACH Authorisation:

REACH Authorisation (see also Chapter 6.8).

In the context of the REACH-Review 2017, the European Commission examined whether it is possible to determine thresholds for endocrine disruptors. This is a prerequisite to allow REACH Authorisation via the "Adequate Control Route". In its report[12] the Commission concludes:

"The current legislation in Article 60(3)(a) of REACH already lays down that for substances for which it is not possible to determine a threshold, the 'Adequate Control Route' for authorisation is not possible.
Based on the information provided in the previous sections, it is concluded that it is not appropriate to extend a-priori the scope of Article 60(3) to all substances identified under Article 57(f) as substances with endocrine disrupting properties which have an equivalent level of concern.
Consequently, Article 60(3) of REACH will continue to be applicable to those EDs for which

[11] http://echa.europa.eu/documents/10162/77cf7d29-ba63-4901-aded-59cf75536e06

[12] https://ec.europa.eu/transparency/regdoc/rep/1/2016/EN/COM-2016-814-F1-EN-MAIN-PART-1.PDF

it is not possible to determine a threshold. It remains the responsibility of applicants for authorisation to demonstrate that a threshold exists and to determine that threshold in accordance with Annex I to REACH. Even though this might be particularly difficult for EDs, it cannot be excluded on the basis of current knowledge that it will be possible. It is up to RAC to assess the validity of the assessment and ultimately decide on the possible existence or not of this threshold. Furthermore, as for other substances, RAC may on a caseby-case basis set reference DNELs, or reference dose-response curves, which industry can use when applying for authorisation. Therefore, as under REACH as it stands today only the 'Socio-Economic Route' can be used when a threshold cannot be determined, and considering the conclusion of the REACH Review that regulatory stability is desirable, the Commission will not propose a change to the legislation."

5.2.2. History of the "low dose debate":

In the 1990s, some groups published observations that questioned the existence of thresholds for endocrine active substances. A British research group reported effects on the testicular weight of laboratory animals which occurred at doses 1000 times less than the no-effect level (Sharpe, 1995). An American research group observed changes in the prostate weights of laboratory mice at exposure levels 25,000 times less than the established no-effect level (Nagel et al., 1997). These observations received high interest both in the scientific community and among the general public. About 2½ years after their publication and after carrying out new experiments, in May 1998 the British group published further results which, in their opinion and in the opinion of other scientists, suggest that the observed effect on the testicular weight is not caused by administration of the substance (Sharpe et al., 1998). The publication by the British group can thus no longer be cited as evidence of so-called low-dose effects. The investigations by the American research group have been repeated by different working groups, but it has not been possible to replicate the results (Cagen et al., 1999; Ashby et al., 1999; BUA, 2001; Owens & Chaney, 2005), i.e. to date none of the observed low-dose effects has been confirmed by investigations in other research groups. Various factors have been suggested as the reason for the lack of reproducibility, e.g. the animal feed, genetic differences, animal keeping, seasonal fluctuations, and biological variability (Witorsch, 2002a and 2002b; Ashby, 2002; Ashby, 2001; Ashby, 2004; Milman, 2002; Sharpe et al., 1998; Kamrin, 2007). In addition, the ability to extrapolate such results from the mouse to humans has been questioned because of physiological differences. While mouse ovaries are of central importance as a hormone source throughout pregnancy, in humans this is true only in the first trimester of pregnancy. The relationship between male and female sex hormones and the blood hormone levels during pregnancy are also markedly different: In humans, for example, the blood estrogen values are 100 times higher than in the mouse. From this, it was deduced that humans are very much less sensitive to estrogen exposure during pregnancy (Witorsch, 2002b) and effects caused by very small additional doses of estrogenic substances are not plausible (SCHER, 2005).

In October 2000, the NTP (National Toxicology Program), under contract to the US EPA, made a scientific expert assessment of low-dose effects and a comparative assessment of the available data (Melnick 2002). As a consequence, the US EPA declared in 2002 that further research into the so-called low-dose hypothesis was needed and that no routine testing for low-dose effects is required before such scientific clarification becomes available (EPA, 2002).

Even though this statement by the EPA was based primarily on laboratory populations of rodents, it applies equally to representatives of aquatic and terrestrial ecosystems (environmental species), organisms for which the endocrine systems are generally much less well known. Only after such gaps in our knowledge have been filled, will it be possible to show whether assumed effects in the low-dose range (e.g. in snails and arthropods) are not, in fact, an expression of the conceptual and methodological weaknesses of the studies concerned.

Lack of replicability of unexpected low dose effects was also mentioned recently in the assessment[13] of the use of propylparaben in human medical products by the European Medicines Agency (EMA 2015).

5.2.3. Further discussion-points:

More recent discussion:

Biological plausibility: EFSA (2010) stated that there is a lack of scientific consensus whether low-dose effects have biological plausibility. As a key principle in biology and physiology, thresholds enable the body to distinguish vital chemical signals from background biological noise. Given the presence of structurally similar molecules relative to hormones in nature (e.g. phytoestrogens), the challenge is great for the body to maintain a functional and efficient hormone-based communication system. Without a sophisticated ability to clearly distinguish between molecules that convey critical physiological information and structurally similar molecules in the body, the endocrine system would be unable to process specific, vital signals amidst a steady roar of biological noise.

Scientific consensus is that for receptor-mediated processes, thresholded dose–responses are to be assumed and based on that tolerable exposures are derived by

13 http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2015/11/WC500196733.pdf

the application of safety (or uncertainty) factors (Dekant & Colnot 2013). Borgert et al. 2013 highlighted that the fundamental principles of hormonal effects are the basis for the existence of thresholds and that these principles also define how exogenous chemicals can interfere with the endocrine function. By that safe levels of exposure can be set for endocrine active substances. Autrup et al. 2015 confirmed the plausibility of the existence of a threshold.

Homeostasis: EFSA in 2013 stated that compensatory feedback mechanisms and the homeostatic capacity of the endocrine system need to be considered and that the threshold of adversity is only crossed if the body is unable to compensate the induced changes. Even if the existence of dose thresholds cannot be proven or ruled out the well-known homeostatic mechanisms would support that a biological threshold between endocrine modulation and adverse effect exists (EFSA 2013). Also Dekant & Colnot (2013) stated: *"Adaptive responses are part of the normal function of the endocrines system and fall within the physiological balance/homeostatic capabilities of the organism. Adverse effects are only caused when the interferences with the endocrine system cause changes to an extent beyond that compatible with normal function…."*

Sexual development: a potential argument against the role of homeostasis to prevent adversity is that homeostatic control at early life stages would be not fully functional or developed. It is not currently well understood how hormone production is initiated or maintained during sexual development but it should not be assumed that biological thresholds do not apply as these levels and the incidence of related endpoints (such as ano-genital distance) can be manipulated using high doses of known reproductive toxicants under laboratory conditions (Macleod et al., 2010; Welsh et al., 2010).

Further, it is known that during pregnancy and for young children the reference/normal values for e.g. blood hormone values, have a considerable range; examples from the clinical practice are offered.[14]

It should be noted that confirmed developmental toxicants induce their impact during early life stages and yet they are still subject to risk assessment through the establishment of a toxicological threshold.

Reproductive endpoint threshold: A recent review (Piersma et al. 2011) on the threshold for adversity of reproductive toxicants came to the conclusion that "the low-dose issue is highly controversial and has not provided convincing arguments against a threshold approach for reproductive toxicology". Piersma et al. (2011), surveyed the

[14] http://www.medizin1.uk-wuerzburg.de/de/schwerpunkte-und-funktionseinheiten/endokrinologie/hormonlabor.html
http://www.umm.uni-heidelberg.de/inst/ikc/ikc-endokrinologie.html

scientific basis for the current threshold approach for reproductive hazard and risk assessment. This publication concluded that for reproductive toxicants (which include also synthetic hormonal substances) the threshold dose approach remains valid. As an example an important homeostatic mechanism for reproduction is the hypothalamic-pituitary-adrenal-gonadal-axis regulating the level of reproductive hormones via multiple receptor-mediated feedback mechanism. These homeostatic mechanisms provide protection to the organism for coping with xenobiotic exposures. After exposure to a chemical, xenobiotioc activity can be neutralized by homeostatic control mechanisms, preventing adverse effects occurring. This was shown for example in a study using juvenile male rats where the co-administration of the physiological androgenic hormone testosterone was able to largely prevent the adverse effects and abnormalities in the male reproductive system induced by the synthetic estrogen diethylstilbestrol (Rivas et al., 2003).

Pharmaceutical efficacy: the use of human pharmaceutical data can ensure the setting of human relevant thresholds based on potency (Borgert et al. 2012). Certain pharmaceuticals are designed to manipulate the normal endocrine homeostasis to create a desired beneficial outcome e.g. female contraceptive medication. The evidence of thresholds for endocrine activity is particularly strengthened by the mode of action of the contraceptive pill as demonstrated by the occurrence of unwanted pregnancies of women who did not follow the pill regime exactly as prescribed, i.e. typically by “missed pills”. The risk of ovulation and thus, the risk of an unwanted pregnancy may increase when pills are missed at start of the pack and the days without active pills are extended (Wright and Johnson, 2008).

Species differences: may exist regarding the extent of hormonal control during gestation as highlighted by Witorsch (Witorsch 2002 b). He highlighted that gestational levels of oestrogens during pregnancy in humans are very high and by that additional estrogen or xenoestrogen is unlikely to have an effect in humans during pregnancy. Further, emerging research indicates that laboratory test species may be more sensitive to exogenous endocrine disruption than developing humans (van den Driesche et al., 2012; Mitchell et al., 2012; McKinnell et al., 2009). Rats, for example, have a much lower endogenous plasma estrogen level and a lower tolerance to estrogen variability than humans, resulting in a much higher sensitivity of rats to estrogen exposure than humans (Ström et al., 2008). Thus, while rodent models can provide us with valuable information on endocrine-related mechanisms, it is crucial that they be put into context when applied to human risk from low doses in light of the differences between internal doses of weak endocrine-active substances relative to endogenous estrogens in rodents and humans (Goodman et al., 2006).

In 2012 the debate on low-dose effects was again stimulated by a review publication from Vandenberg et al. (2012). A commentary on that review was published

(Rhomberg & Goodman 2012) and workshops on that issue were organized by EFSA and NIEHS/EU Organizations in 2012.

At the workshop organized by the US National Institute for Environmental Health Sciences/NIH and the Joint Research Centre's Institute for Health and Consumer "Protection on low dose effects and non-monotonic dose responses for endocrine active chemicals" in Berlin on 11-13 September 2012 there was a suggestion that a definition of „low dose" would be helpful, as it is currently used with different meanings in different contexts, and that there was also a need to carry out a practical assessment of the type of effects that may be considered adverse, in the context of endocrine disruption (EFSA 2013). A report of the Berlin-Workshop "Low Dose Effects and Non-monotonic Dose Responses for Endocrine Active Chemicals: Science to Practice Workshop: Workshop Summary" has been accepted for publication and was published in the journal "Chemosphere" (Beausoleil et al. 2013).

For the EFSA workshop a report is available (EFSA 2012b) .Whereas there was no consensus at the workshop on the significance of the "low dose effect" or a Non-Monotonic Dose Response Relationship (NMDRC) it was stated that an adequate and generally accepted definition of "low-dose effects" and of NMDRC is needed in order to facilitate discussions and that the amount of evidence needed to decide if in a particular case a "low-dose effect" or an NMDRC has to be taken into account should be defined. The criteria for adversity should be the same for all types of effects. Additional aspects mentioned were the need for well-designed studies covering wide dose ranges.

In 2013 EFSA stated that "*Thus on balance, reviewing the recent colloquia, workshop and WHO/UNEP expert report, the debate is evolving in the scientific community as to the existence and/or relevance of low-dose effects and NMDRCs in (eco)toxicology in relation to endocrine disruption or other endpoints/modes of action, but still lacks consensus. More work needs to be conducted to agree on the definitions of the respective terms, and in practical terms to consider whether or how it could impact upon risk assessment (i.e. assessment of dose response relationships for adverse effects) and testing strategies.*

Therefore, the Scientific Committee cannot conclude whether the current test methods are adequate to fully define dose response relationships. However, the available information is equally insufficient to conclude that current dose response analysis in regulatory (eco)toxicology should be modified on a routine basis. Nevertheless, on a case-by-case basis, if triggered by unusual findings, an extended dose response analysis could be performed in a second tier.

The SC further notes that, as low-dose effects and NMDRCs are not unique to endocrine activity, these subjects merit a follow-up in a broader context."

In 2013 EFSA pointed also on the fact that the term low-dose effect in not synonymous with or equivalent to NMDRC and that the use of the both term interchangeably by many authors creates considerable confusion (EFSA 2013).

In 2015 EFSA (EFSA 2015b) informed about ongoing "*initiatives to develop scientific knowledge in the field of endocrine active substances and also on overlapping issues such as non-monotonic dose-response relationships and biological relevance in risk assessment*"[15] as follow up on the 2013 scientific opinion by EFSA.

A recent published study (Sarrabay et al. 2015) specifically examined the dose-response of endocrine mediated toxicity covering the low-end of the curve after treatment of young adult male rats with the antiandrogen flutamide as reference endocrine active substance for 28 days. The study included a broad variety of parameters and covered reproducibility by performing the study three times. Only monotonic dose-response curves were observed on each individual key event of the Mode of Action and by that build strong confidence of an overall toxicological threshold of toxicity.

Overall, the dose is decisive for the effect of a substance, and this applies to endocrine activity as for any other form of biological activity potentially leading to toxicity. For this reason, limit values can be derived for these substances too. There is no convincing evidence that substances with endocrine disruption properties should be handled differently to chemicals acting with other Mode of Actions in the eco/toxicological risk assessment.

Whereas the minimum level of interaction at specific life stages (e.g. during development) for endocrine active substances might be lower than in adults, and the nature of effects might be different (severe in the fetus vs. less severe in adults) this does not speak against the general principle of a biological threshold of adversity. Special attention needs to be given to particularly sensitive groups of the population (e.g. children or pregnant women) in the form of specific testing or safety factors. It is common practice e.g. unter REACH to use a higher safety/extrapolation factor for the general population compared to working population. The ECHA REACH guidance provides over 100 pages of information on how to scientifically determine appropriate interspecies and intraspecies assessment factors based on toxicokinetic and toxicodynamic factors. Extrapolation factors are defined. If there is relevant data then the assessment factors can be reduced.

[15] http://www.efsa.europa.eu/en/press/news/150520

Overall, there is no scientific agreement to support the low dose hypothesis and there is no evidence of endocrine disruptors having to be assessed as "substances without threshold concentration" generally.

VCI Positionpaper:

www.vci.de/Downloads/PDF/VCI%20Position%20Threshold.pdf

5.3. Non Monotonic Dose response Relationship (NMDRC)

In 2013 EFSA pointed on the fact that the term low-dose effect is not synonymous with or equivalent to NMDRC and that the use of the both term interchangeably by many authors creates considerable confusion (EFSA 2013). Work is ongoing to review non-monotonic dose-responses for substances for human risk assessment (EFSA 2015b).

At a Workshop organized by the US National Institute for Environmental Health Sciences/NIH and the Joint Research Centre's Institute for Health and Consumer Protection on low dose effects and non-monotonic dose responses for endocrine active chemicals in Berlin on 11-13 September 2012 most of the participants were in agreement that non-monotonic dose responses do occur and may be expected at some dose ranges for some substances, but the extent to which they might occur at so-called „low doses" was considered to be a separate issue. (EFSA 2013).

A non-monotonic dose relationship is a dose response that changes the slope of the curve; such a situation can occur at all biological active dose levels. Situations when a non monotonic dose relationship can be expected in vitro as well as in vivo are when different mode of action for a substance overlap , e.g. pharmacological activity (e.g. stimulation) and toxicity (e.g. cytotoxicity) or two distinct pharmacological activities of different potency (e.g. estrogenic activity and anti-androgenic activity). Other reasons might be related to toxicokinetics or toxicodynamics, e.g. saturation of metabolism at the high dose range. The key message here is that all the effects that contribute to the overall dose response curve are already occuring at a biological active dose, which means above the overall effect threshold. The most sensitive effect typically will define the overall biological threshold and by that guide the risk assessment and risk management measures.

There is however extremely little evidence that non-monotonic dose relationships occur frequently. Most of the described alleged "low dose" or "U-shaped"-effects are coming from studies, in which either non-standard parameters (more sensitive or sometimes non-adverse parameters), or different windows of exposure were investigated, or unphysiological routes of exposure were used, or applications were in the pharmacologically active dose range.

An example of a non-monotonic dose relationship is the so-called "Tamoxifen Flare": this phenomenon is observed during therapy of women under treatment for breast cancer, which means at a proven therapeutic/ pharmacologically active dose and above the defined biological threshold.[16]

5.4. Potency of industrial chemicals in comparison with naturally occurring substances

Toxicology:

Exposure to synthetic estrogen-like acting substances needs to be evaluated against the background level of the unavoidable ingestion of phytoestrogens in food and the physiological variation in endogenous hormone levels (DGPT, 1999; Bolt & Degen, 2000). This applies to the situation in humans (Bolt et al., 2001; Safe, 2000; Nilsson, 2000) as well as to data from animal studies (Owens et al., 2003; Nilsson, 2000).

Several research groups have developed evaluation concepts for endocrine active substances - based on a comparison of the potency of industrial chemicals with natural occuring substances with similar mechanisms of action, for example, substances found in food (e.g. phytoestrogens) (e.g. Bolt et al., 2001; Bolt et al., 2002; Bolt & Degen, 2000; Safe, 2000; Safe, 1995; van Meeuwen et al., 2007; van Meeuwen et al., 2008, Becker et al. 2014). The published examples showed that the endocrine active industrial chemicals investigated represent no relevant contribution to the exposure with natural estrogens or estrogen-like substances. In addition, a recent publication on propyl paraben showed again that there was no evidence of any adverse effect on male reproductive organs of a substance considered to have possible effects on endocrine function, based on weak in vitro and in vivo uterotrophic test data (Gazin et al. 2013). Autrup et al. (2015) mentionend that many natural chemicals are more potent than typical industrial chemicals with consumer exposure.

In a publication from the OECD program on the validation of the uterotrophic assay, the influence of the feed (in particular the phytoestrogen content of the feed) on the results of the animal study was evaluated and the "total genistein equivalent" proposed as reference parameter. In a comparison of the intake of natural components of the diet that have estrogen-like activity with an industrial chemical, it was found that e.g. the so-called low doses of the industrial chemical (bisphenol A) correspond to a quantity of 0.002% of the total daily dietary genistein equivalent ingested by these animals, which could explain the lack of reproducibility of the findings described (Owens et al., 2003).

[16] Tamoxifen flare in advanced breast cancer. Plotkin D, et al. JAMA. 1978 Dec 8; 240(24):2644-6.'

The European Food Safety Authority in 2015 published[17] a risk assessment for peri- and post-menopausal women taking food supplements containing isoflavones and concluded (EFSA 2015) *"A comprehensive review of the available scientific evidence has revealed no indication that isoflavones at levels typically found in food supplements cause harm to post-menopausal women. Isoflavones are naturally occurring substances which are found, among other sources, in soy, red clover and kudzu root. Their extracts are often used as ingredients in nutritional supplements."*

In addition to hormonally active substances derived from plants exposure of human also occurs via meet and milk. As mentioned by the BfR (2014) *"A considerable proportion of the daily adult total intake of estrogens (approx. 60%) and progesterones (approx. 80%) via these foods comes from cow's milk." It is further stated that: "The available scientific data do not currently give any reason to assume any relevant health risk."*[18]

Ecotoxicology:

In the assessment of potential ecotoxicological risks of synthetic estrogen-like acting substances the background of natural estrogens - in particular, the presence of estradiol and its metabolites estrone and estriol - in the environment, originating from natural sources such as human or animal excreta, are overlooked. Data on concentrations of such natural estrogens are available for many regions of the world. European waters contain estradiol concentrations between < 0.1 ng/L (typical detection limit) up to 25 ng/L (Johnson and Harvey, 2002). In contrast, synthetic estrogenic chemicals can be measured at concentrations of several µg/L (IPCS, 2002). The risk of these different fractions of estrogenic substances for the environment can be evaluated by analyzing the estrogenic potency of those chemicals in comparison to natural homones. This means that the estrogenic activity of synthetic chemicals - as the source for potential impairment of environmental populations - should be analysed in comparison to the estrogenic activity originating from natural estrogens. This could be achieved by comparing the estrogenic activity of different substances in either in vitro or in vivo laboratory models. This has been done in numerous exercises for example in receptor binding assays such as described in Jobling et al. (1995). or in in vivo assays such as used by Thorpe at al. (2009).

Another commonly used approach is the assessment of estrogenicity of an environmental water sample (e.g. from waste water effluent) in a laboratory model by chemical fractionation using analytical tools, and subsequent biological toxicity assaying in order to identify the influence of different components in the sample to contribute to the overall estrogenicity. This method was initially used by Desbrow et al. (1998) to investigate the causes of estrogenicity of municipal waste water effluents.

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[17] http://www.efsa.europa.eu/de/efsajournal/pub/4246
http://www.efsa.europa.eu/en/press/news/151021

[18] http://www.bfr.bund.de/en/questions_and_answers_on_hormones_in_meat_and_milk-191516.html#topic_190531

In essence, it can be concluded that the main amount of estrogenic activity in the environment originates from natural estrogenic hormones. Although present only in minute concentrations, they are much more potent than synthetic estrogenic active chemicals, because the higher concentration of the latter is outweighed by their lower potency.

5.4.1. Possible implications of the criteria-options proposed by the Commission for natural substances – a case study

In 2014 the European Commission (EC) developed four options for criteria for the identification of endocrine disruptors and performed an impact assessment (for a description see Chapter 6). Natural substances were not considered in this evaluation. In a case study commissioned to FoBiG by ECPA and Cefic, it was examined which potential impact the EC proposals and a proposal from Industry may have on the identification of natural substances as endocrine disruptors (Schuhmacher-Wolz, 2017).

Set-up and results: The following 4 substances were chosen: genistein, caffeine, vitamin D3 and sucrose. The study was not intended to assess the substances. All the substances chosen are considered to be safe in the current use. In the case study, it was examined, whether the options could (erroneously) implicate everyday natural substances as having an adverse effect on the endocrine system. The study results are published in the open literature (Schuhmacher-Wolz, 2017). The study demonstrates that the Commission's proposal assessed in the case study identifies more than the endocrine disruptors that would be of regulatory concern. This evaluation shows that it is of utmost importance to include also potency and severity into the criteria for the identification of endocrine disruptors.

5.5. Validity and quality of scientific studies and Weight of Evidence Approach

Uniform and universally accepted criteria - allowing study results to be taken into account in regulatory measures - are required for the evaluation of the validity and quality of toxicity and ecotoxicity tests and of epidemiology studies. Comprehensibility, plausibility, relevance and reproducibility are given as criteria for the evaluation. Particularly in the case of non-standardised test procedures, consideration of these criteria can help in evaluating the results of the investigations concerned in a uniform and transparent manner (Länge et. al., 2006).

In the meantime there is a broad discussion on the use and value of epidemiological studies, e.g. for the characterisation of hazards related to exposure to environmental stressors. Guideline for Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) are proposed (van Elm et al. 2007, Vandenbroucke et al. 2007). This was taken up by Money et al. (2013) in a proposal for systematic approach for evaluating human data. EFSA pointed on confounding factors that makes the assessment in epidemiological studies challenging (EFSA 2013). LaKind et al. (2014) published a proposal for assessing the study quality of epidemiology studies especially for short-lived chemicals.

Overall assessment should be done using the weight-of-evidence (WoE) approach. The basic principles of the WoE approach are described in a recent guidance document from the US EPA in the context of the Endocrine Disruptor Screening program (EPA 2011). As defined in this document, general assessment factors are soundness, applicability and utility, clarity and completeness, uncertainty and variability as well as evaluation and review. WoE relies on expert judgement, as multiple lines of evidence have to be assessed in an integrated manner. Considerations for the individual studies cover the quality/validity of the method, the reliability of the results, the nature of the observed effects, the consistency and interrelationship among endpoints reported in an individual assay, as well as relevance, specificity, and sensitivity of the endpoints measured. Regarding the method of a weight of evidence approach SCENIHR in 2012 provided an overview on what to consider (SCENIHR 2012). EFSA in 2013 in addition to SCENIHR 2012 pointed on the weight of evidence guidance as provided by WHO and published by Boobis et al, in 2006 and 2008 (EFSA 2013). The National Toxicology Program Office of Health Assessment and Translation (OHAT) developed an approach for systematic review and evidence integration for literature-based health assessments (Rooney et al. 2014). A framework for systematic review and evidence integration for reaching hazard identification conclusions is proposed covering *“1) problem formulation and protocol development, 2) search for and select studies for inclusion, 3) extract data from studies, 4) assess the quality or risk of bias of individual studies, 5) rate the confidence in the body of evidence, 6) translate the confidence ratings into levels of evidence, and 7) integrate the information from different evidence streams (human, animal, and "other relevant data" including mechanistic or in vitro studies) to develop hazard identification conclusions”* (Rooney et al. 2014). The respective tool is currently in the process of refinement to assess potential bias for in vitro mechanistic studies (NTP 2015).

Examples of the use of weight-of-evidence (WoE) approach – using data from multiple animal species and in vitro systems in order to analyse the endocrine disruption potential - are in the meantime published, e.g in Michaich et al, 2017.

The inclusion of all available data is a main aspect of the WoE approach. In this context, all positive and negative data should be available. The tendency of journals not to publish “negative” data leads to a publication bias (Bolt 2011). For sound science evaluations, publication of results - irrespective of positive or negative outcome - is needed and “good scientific practice” is an area of current discussion (see e.g. discussion on publication of sperm count data in 2011; Wilocx, 2011;Bonde et al., 2011; Skakkebaek et al., 2011).

6. Endocrine effects – regulatory significance

6.1. Definitions, risk assessment

The influence of exogenous substances with endocrine-like activity on the hormone system of humans and animals (and of plants) is one of many principles of action that can lead to reversible and/or irreversible effects. Hormone-like activity should not, therefore, be considered adverse per se. All prestigious scientific organizations are in agreement about this (SCHER [= former SCTEE], DGPT, BUA, Sachverständigenrat für Umweltfragen [German Advisory Council on the Environment], IUPAC, ECHA, ECETOC and others).

Although there has been a long lasting discussion about the definition of an endocrine disruptor (see Chapter 1.2), all recent discussions are based on the WHO/IPCS definition as basic, scientific definition.

In accordance with the prior Weybridge definition, in 2002 the WHO defined an endocrine disrupter as follows:

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

An endocrine disrupter can, therefore, be identified only from an animal study (in vivo). However, a potential endocrine disrupter can be identified also by screening procedures (*in vitro*). It is defined as follows:

> *A potential endocrine disrupter is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)population (WHO IPCS,2002)*

Adverse hormonal effects can result from prolonged disruption of the most important hormonal feedback mechanisms, affecting for example the reproductive, thyroid, or adrenal systems. Under existing EU legislation, chemicals are required to undergo a

very specific programme of testing and assessment. Potential adverse hormonal effects can be detected by the toxicology test hierarchy which requires the determination of subacute, subchronic, and reproductive toxicity and of the carcinogenic properties, depending on the production volume of the chemical and already available data. The objective of the test in these cases is to establish whether the substance causes adverse effects in a defined test system above a defined dose. The question of the mode of action – i.e. the mechanism leading to the adverse effect - is of secondary importance.

Only when an adverse effect is detected the mechanism of action is important for the risk assessment, i.e. for the extrapolation of the experimental data to humans. For ecotoxicology, population-relevance is essential.

Ultimately decisive for the risk assessment is the most sensitive adverse endpoint used to determine the no observed adverse effect level (NOAEL).

A substance is an endocrine disrupter in the sense of the WHO definition if NOAEL (endocrine) = NOAEL (systemic), the latter covering the other endpoints for toxic effects. That means a substance is considered an endocrine disrupter only where the endocrine effect is the most sensitive endpoint.

To illustrate the principles for evaluation, ECETOC developed in 2009 guidance flow charts for both human (mammalian) and environmental (fish/amphibians and birds/wild mammals) that outline

1.) a decision tree to decide if a substance should be considered an endocrine disrupter according to the Weybridge definition and

2.) the integration of specificity, relevance and potency assessment in the overall evaluation.

In the last years further concepts how to assess substances with endocrine disrupting properties have been proposed by several EU Member States. As described in Chapter 6.6., rationale for these activities is that endocrine disrupters are referred to in various European regulations. Taking into account some key aspects of these concepts, the EU commission is now developing criteria for the identification of endocrine disruptors (see 6.4.).

Regarding definition of Adversity/Non-Adversity ECETOC in 2012 gave the following definition: "*Adverse effects:…adversely affects the performance of the whole organism and reduces the organism´s ability to respond to additional environmental challenge. In contrast to adverse effects, non-adverse effects can be defined as those biological effects that do not cause biochemical, behavioral, morphological or physiological changes that affect general well-being, growth, development or life-span of an animal. Effects are less likely to be adverse if….*" (ECETOC 2012).

Dekant & Colnot 2013: *"…it is important to recall the widely accepted definition of an "adverse effect" as a "change in the morphology, physiology, growth, development, reproduction, or life span of an organism, system, or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences."* (WHO/IPCS, 2004). Consequently*, "Contrasted to adverse effects, non-adverse effects can be defined as those biological effects that do not cause biochemical, morphological, or physiological changes that affect the general well-being, growth, development or life span of an animal."* (Lewis et al., 2002)."

6.2. Is a separate hazard category required?

In the discussion of the effects of endocrine active substances, a separate hazard category as "endocrine disrupter" is sometimes proposed. However, scientific committees (SCTEE, now SCHER) and the responsible specialist authorities (COM (1999) 706, COM (2001) 262) were of the opinion that endocrine activity is

- neither an independent adverse effect,
- nor a new type of toxic property,
- nor a previously undetected hazard.

Rather, it is a mode of action involving specific mechanisms that could, but do not necessarily, lead to a hazard, i.e. an adverse health effect, particularly after long term exposure. In principle, these adverse effects can be detected by the existing toxicological test strategy. Possible adverse effects of endocrine active substances may affect

- the reproductive system both in terms of fertility and development of the progeny (embryotoxicity),
- the development of cancer, as well as
- functional and morphological changes.

We agree that all these possible adverse effects are covered by the existing classification system of the European chemicals legislation with, for example, the categories for reproductive toxicity (R), carcinogenicity (C) as well as specific target organ toxicitiy (STOT) (EU 1272/2008 as well as EU 67/548/EEC, see also Appendix 4).

6.3. The political context: EU Community Strategy on endocrine disruptors and current developments at EU-level

6.3.1. Community Strategy for Endocrine Disruptors and its revisions

The intensive public debate on endocrine active chemicals led to the adoption of the Community Strategy for Endocrine Disruptors put forward by the European Commission in December 1999: the 'Community Strategy for Endocrine Disrupters - a range of substances suspected of interfering with the hormone systems of humans and wildlife' - COM(1999) 706 - which set out a general framework for studying ED."

The 1999 the Community Strategy was based on the Weybridge definition (see 6.1) and acknowledged the need for further research, both to improve fundamental knowledge and to further develop test methods. It furthermore emphasized the need for international cooperation and more intense public relations work, and an development of an action plan was to be developed that would lead to the comprehensive control of substances suspected of being endocrine disrupters. This policy is based on the instruments of the European chemicals legislation: identification and characterization of hazards, qualitative and quantitative determination of exposure, risk assessment, and risk management.

The European Commission published four Implementation Reports on the Community Strategy for Endocrine Disruptors (June 2001: COM(2001) 262; October 2004: SEC(2004) 1372; November 2007: SEC (2007) 1635; August 2011: SEC (2011) 1001.[19]

For the discussion, the EU commission contracted additionally a report on the State of the Art of the Assessment of Endocrine Disruptors (Kortenkamp et al 2011).The report triggered a controversial discussion based on limitations and conclusions.

The European Commission is currently working on science-based criteria for endocrine disruptors, as required in the Plant Protection Products Regulation and the Biocidal Products Regulation (see 6.3.5ff). For this a roadmap was published in 2014 and a public consultation was carried out in 2015, followed by an impact assessment and the publication of draft criteria in June 2016. Next steps are the approval of final criteria and a guidance document (see 6.3.6 ff.).

6.3.2. European Parliament Initiative Report on ED

On 14 March 2013 the European Parliament adopted an own-initiative report[20] on the Protection of Public Health from Endocrine Disruptors (rapporteur Asa Westlund, Sweden, Socialist Group). In its resolution the European Parliament rather focuses on reduction of exposure and substitution of endocrine disruptors than on an scientific

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19 http://ec.europa.eu/environment/chemicals/endocrine/documents/index_en.htm

20 http://www.europarl.europa.eu/sides/getDoc.do?type=TA&reference=P7-TA-2013-0091&language=EN&ring=A7-2013-0027

based evaluation of this substance group. Moreover, the European Parliament questioned the possibility to identify safe thresholds for endocrine active chemicals. *„unless the manufacturer can show scientific proof that a threshold can be identified, taking into account increased sensitivities durcing critical windows of development, and the effects of mixtures.“*

6.3.3. Preparatory work for defining the criteria in the EU

Two expert groups were established by the European Commission in 2010 to provide information exchange on endocrine disruptors and orientation on various scientific and policy aspects related to this topic. The "Endocrine Disruptors Expert Advisory Group" reflected on scientific issues relevant to endocrine disruptors, not specific to any regulatory framework, including advice/orientation on scientific criteria for the identification of endocrine disrupting chemicals. The outcome of "the Endocrine Disruptors Expert Advisory Group" meetings is summarised in the "JRC Report on key scientific issues relevant to the identification of endocrine disrupting chemicals"[21].

The Commission also asked the European Food Safety Authority (EFSA) to deliver a "Scientific Opinion on the hazard assessment of endocrine disruptors".

6.3.4. EFSA Opinion on ED

On 20 March 2013, EFSA (European Food Safety Authority) published its Scientific Opinion on the hazard assessment of endocrine disruptors: *“Scientific criteria for identification of endocrine disruptors and appropriateness of existing test methods for assessing effects mediated by these substances on human health and the environment”* (EFSA, 2013). Quoted from abstract on EFSA website[22]*: “Upon request of the European Commission, the Scientific Committee (SC) of the European Food Safety Authority reviewed existing information related to the testing and assessment of endocrine active substances (EASs) and endocrine disruptors (EDs). …To distinguish between EDs and other groups of substances with different modes of action, it was concluded that an ED is defined by three criteria: the presence of i) an adverse effect in an intact organism or a (sub)population; ii) an endocrine activity; and iii) a plausible causal relationship between the two. As scientific criteria for adversity have not been generally defined, specific criteria for endocrine disrupting effects could not be identified. Hence, expert judgement is required to assess on a case-by-case basis the (eco)toxicological relevance of changes at the molecular to individual and/or (sub)population level following exposure to an EAS.”*

In 2015 EFSA (EFSA 2015b) informed about ongoing *“initiatives to develop scientific knowledge in the field of endocrine active substances and also on overlapping issues*

[21] http://publications.jrc.ec.europa.eu/repository/handle/JRC79981

[22] http://www.efsa.europa.eu/en/efsajournal/pub/3132.htm

such as non-monotonic dose-response relationships and biological relevance in risk assessment" as follow up on the 2013 scientific opinion by EFSA.

6.3.5. JRC Report

In March 2013 the JRC (Joint Research Centre) published the report of its Expert Advisory Group on "Key scientific issues relevant to the identification and characterisation of endocrine disrupting substances" that can be downloaded from JRC website.[23]

6.3.6. ROADMAP Defining criteria for identifying Endocrine Disruptors in the context of the implementation of the Plant Protection Product Regulation and Biocidal Products Regulation

The European Commission is developing criteria for deciding which substances will be deemed endocrine disruptors in the future. The shaping of these criteria will have considerable effects: Already now, the substances are identified and subject to authorization requirements or use bans under existing legislations (see Chapter 6.7. – ED in different European legislations).

In June 2014 the European Commission published a Roadmap "*Defining criteria for identifying Endocrine Disruptors in the context of he implementation of the Plant Protection Product* Regulation and Biocidal Products Regulation" with four political options for setting criteria:

- Option 1: No policy change. The interim criteria set in Plant Protection Product Regulation and Biocidal Products Regulation continue to apply.
- Option 2: Hazard identification based on the WHO/IPCS definition.
- Option 3: Hazard identification based on the WHO/IPCS definition and introduction of categories based on different strength of evidence (Category 1: ED, Category 2: suspected ED, Category 3: endocrine active substances)
- Option 4: Hazard identification based on the WHO/IPCS definition and hazard characterization by inclusion of potency.

6.3.7. Impact Assessment

In 2014/2015 the EU commission carried out an impact assessment on the four policy options proposed in the roadmap.

[23] http://ihcp.jrc.ec.europa.eu/our_activities/food-cons-prod/endocrine_disrupters/jrc-report-scientific-issues-identification-endocrine-disrupting-substances

- Public consultation:

In a public consultation interested parties were invited to give their comments on the four options of the roadmap until January 2015. Over 2700 responses were gathered. Overall most responses suggest a need to find criteria. Option 1 that proposes no regulatory change was therefore not supported in the consultation. A report on the consultation[24] was published in July 2015. On page 2 the Commission summarises:

"Many respondents raised issues in relation to food safety, the threat that endocrine disrupting substances might pose to human health and/or the environment and the impact of the different options proposed in the roadmap on agriculture, industry, health and environment.
In particular farmers and agri-business highlighted the potential high implications of setting criteria to identify endocrine disruptors on agriculture. Authorities from non-EU countries stressed the potential impact on trade. A risk-based approach for regulating endocrine disruptors was proposed by many respondents who identified themselves as farmers, private companies, industrial or trade organisations, or authorities in non-EU countries. Many respondents supported the use of the WHO/IPC 2002 definition as a starting point for defining an endocrine disruptor. Authorities in non-EU countries noted that any decision on endocrine disruptors must respect the principles of the World Trade Organisation." (EU Commission).

VCI took part in the consultation. From our point of view option 4 could be a starting point for an appropriate and effective way to identify EDs of regulatory concern, if it is extended for other elements of hazard characterisation. We feel confident that the development of a single set of criteria for the determination of endocrine disrupting properties, which uses the WHO/IPCS definition as a basis, but which also takes into account the relevance of the adverse effect (that is: severity of effect, (ir)reversibility of effect, potency and lead toxicity) ensures the correct identification of the relevant substances. Without these hazard characterisation elements, substances which pose little or no concern for human health or the environment could be considered to have

Under the lead of Cefic, industry developed an own proposal for criteria for identification of endocrine disruptors (see Chapter 6.6.2).

The impact assessment report consisted of two studies:

- First study – evaluation of options 1 to 4

In a first step about 700 substances were selected for the impact assessment. The method for identifying the substances was presented by the Commission in a workshop in November 2015. The list of substances was published on the Commissions Website in December 2015[25]. The list entailed most crop protection products (i.e. 324

[24] http://ec.europa.eu/health/endocrine_disruptors/docs/2015_public_consultation_report_en.pdf
[25] http://ec.europa.eu/health/endocrine_disruptors/docs/impactassessment_chemicalsubstancesselection_en.pdf

substances), all approved biocidal products (i.e. 95 substances) and selected substance falling under the REACH Regulation (149+52 substances), Cosmetic Products Regulation (45+6 substances) and priority substances under the Water Framework Directive.

The purpose of the Commission's work programme was to examine, across this selection of substances, how well the four policy options can distinguish substances of potential regulatory concern from those of low relevance.

The intention of the exercise was not to evaluate whether a substance should, or should not, be considered an ED. The Commission had clearly stated that the data gathering and screening process using these substances did not replace an in-depth regulatory assessment and had no direct regulatory implications. It is most definitely not a list of recognised EDs and the presence of a substance in the Commission's impact assessment does not require any change to its current uses.

- Second study – Evaluation of socio-economic consequences

After the first part of the impact assessment, a second study evaluated the socio-economic consequences of the four policy options with regards to Plant Protection Products and Biocidal Products. This work shall allow the Commission to then recommend and adopt criteria for identifying EDs.

The results of the impact assessment were published in Summer 2016 on the commissions website[26]. In short "*All options offer the same high level of protection of human health and environment under the current PPPR and BPR because they are all based on the WHO deminition […] and because the Regulations are based on a prior approval system, a high level of data requirements and a regulatory decision making based on thorough risk assessments.*" Concerning the impact, there were differences in the four options, with option 4 having the lowest negative impact.

6.4. Draft proposal for criteria to identify endocrine disruptors and guidance document

On June 15, 2016 the Commission made a draft proposal for criteria to identify endocrine disruptors in the context of plant protection products and biocidal products[27]. The proposal corresponds to Option 2 of the road map (see 6.3.6).

The proposal was being discussed among Member States. After intensive discussions and several postponements, Member States representatives finally agreed on July 4, 2017 on criteria to identify endocrine disruptors in the context of plant protection products[28].

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[26] http://ec.europa.eu/health//sites/health/files/endocrine_disruptors/docs/2016_impact_assessment_en.pdf

[27] http://ec.europa.eu/health//sites/health/files/endocrine_disruptors/docs/com_2016_350_en.pdf

[28] http://europa.eu/rapid/press-release_IP-17-1906_en.htm

Next steps: the agreed criteria (context plant protection products) are now sent to Council and European Parliament for examination before the Commission can finally adopt them.

The setting out of criteria for identifying endocrine disruptors in the context of biocidal products is also expected for 2017. The Commission presented the latest version of the draft Delegated Regulation on the Competent Authorities meeting (CA-Meeting) on July 12, 2017 as a basis for the next steps in procedure as sending to Council and European Parliament. After that the criteria might apply as of mid 2018.[29]

A guidance document how to implement the criteria for endocrine disrupters is in preparation. The guidance is developed by the European Chemicals Agency (ECHA) and the European Food Safety Authority (EFSA). A first outline of the document was published in December 2016[30]. Currently the draft is under revision in the ECHA/EFSA working group. A public consultation is announced for 2017.

6.5. ECETOC Seven Steps for the Identification of Endocrine Disrupting Properties (Technical Report 130)

In March 2017 ECETOC Technical Report 130 was published as response to the announcement of the ECHA/EFSA guidance document on the Commission's draft criteria which is currently under preparation. The ECETOC report gives a guidance on the practical use of the ECHA/EFSA Draft guidance described above. The ECETOC Report is essentially structured as a workflow devided into seven steps which do not have to be applied in series, but can also be used in parallel:

Step I: Gathering of relevant data with regard to adverse effects and endocrine modes-of-action (MoA);

Step II: Evaluation of quality, reliability, reproducibility and consistency of the data;

Step III: Evaluation and summary of the evidence for an adverse effect;

Step IV: Evaluation and summary of evidence for endocrine activity;

Step V: Integration of the evidence and evaluation of biological plausibility that adverse effect and endocrine activity are linked by specific endocrine MoA;

Step VI: Identification of uncertainties;

Step VII: Conclusions on endocrine disrupting properties.

The steps focus only on hazard identification, since this is the given background of the ECHA/EFSA draft. From the point of view of the ECETOC ED Taskforce that developed the Technical Report, such an approach lacks the consideration of risk because "*it is the opinion of the ECETOC ED TF that substances that are identified as possessing*

[29] https://ec.europa.eu/health/sites/health/files/endocrine_disruptors/docs/ev_20170712_mi_en.pdf

[30] https://echa.europa.eu/documents/10162/22879598/Endocrine+guidance+outline/64736dc0-0549-9fc8-d031-151aa76f2137

endocrine disrupting properties should undergo a comprehensive hazard and risk assessment. This entails the determination of safety thresholds, exposure assessment, potency assessment and the determination whether acceptable risk can be demonstrated" (ECETOC TR 130, p.2).

6.6. Background information - starting points for the development of criteria

Endocrine disrupters are referred to in different regulations (REACH, PPPR, BPR). One aim of the Community Strategy is to provide an assessment concept and criteria for ED that can be used in the different regulations.

In the last years, several Member States have proposed various assessment concepts for human health and for the environment with the objective to propose a definition and criteria for endocrine disrupters in the different regulations. Assessment concepts were proposed by Denmark, France, Germany (BfR, 2010; Marx-Stoelting, 2011, Frische et al., 2013), UK (Ewence et al.,2015) and ECETOC.

Another starting point for discussion were options 1 to 4 of the Commission's roadmap and option 4 "b", an extended option, presented by Germany at a Stakeholder conference in June 2015 (see 6.6.1).

Currently these assessment-concepts and different options are not actively followed up. For the ongoing criteria-discussion see 6.4.

6.6.1. German contributions

In the last years Germany has contributed to the ongoing discussion by developing various proposals for assessment concepts. BfR (BfR, 2010; Marx-Stoelting, 2011), BAuA and UBA (Frische, 2013) proposed different concepts. At the EU Commissions stakeholder workshop in June 2015 a German proposal – an extension of option 4 was presented as option 4 "b"[31]:

The BfR proposed to start with the WHO/IPCS definition. They advocate for weight of evidence considerations and that expert judgement should be used case-by-case to decide on the grouping. The current testing and assessment methodologies are seen to be generally suitable to derive dose/concentration levels which can be considered safe.

Based on considerations on specificity, severity, reversibility, potency and consistency of all effects in a decision matrix, substances falling under the WHO/IPCS definition can be identified as ED.

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31 http://www.bfr.bund.de/de/presseinformation/2015/17/bfr_schlaegt_erweiterte_eu_kriterien_zur_identifizierung_endokriner_disruptoren_vor-194549.html

In 2016 the BfR organized a workshop with 23 invited scientists and wanted to help to solve the controversy around the assessment of endocine disruptors. Industry experts were not invited to this event. But although the outcome of the workshop was published as a "consensus statement" (Solecki, 2017), the scientists could only agree on very basic positions on the identification of endocrine-disrupting chemicals. On many aspects no consensus could be found, but knowledge gaps and research needs were mentioned.

6.6.2. Cefic/Industry Proposal for criteria for identification of endocrine disruptors

From the VCI's point of view, we appreciate the attempts to develop clear, objective and science-based criteria to identify substances with ED properties of concern for regulation. Industry engages in the development of criteria. Under the lead of Cefic and the European Crop Protection Association, a proposal for identification of endocrine disruptors was developed. The proposed decision matrix can be found in Appendix 5.

The proposed criteria to to identify substances of regulatory concern take into account both human health and environmental concerns. They are protective, clear, and based on the views expressed in EFSA's Scientific Opinion and the report of the ED Expert Advisory Group (hosted by JRC)[32]. The criteria apply a weight of evidence approach – using all available data – to ensure a balanced, transparent and scientifically robust approach. The main goal is to identify Endocrine Disruptors (EDs) of concern such that appropriate regulatory action can be taken continuing to ensure a high level of human and environmental health.

The concept for the ED criteria is based on the WHO/IPCS definition for an ED. It considers the weight of evidence and the relevance of the adverse effect resulting from an endocrine mode of action.

6.7. Endocrine disrupters in different European regulations

Endocrine disrupting substances are addressed under several European legislations as there are the Plant Protection Products Regulation (1107/2009), Biocides Product Regulation (528/2012), the Regulation on Cosmetic Products (1223/2009), the Water Framework Directive (2000/60/EC) and the REACH-Regulation (1907/2006). The focus of this paper is on industrial chemicals, but as the discussion of defining criteria for identifiying endocrine disruptors is currently lead primarily in the context of the implementation of the Plant Protection Products Regulation and the Biocidal Products Regulation a short summary for all areas concerned is given here, before explaining the REACH-Regulation process in some detail.

[32] http://publications.jrc.ec.europa.eu/repository/bitstream/JRC83204/lb-na-26-068-en-n.pdf

6.7.1. Plant Protection Products Regulation 1107/2009

Regulation (EU) No 1107/2009 relative to the placing of plant protection products on the market entered into force on 14 December 2009, repealing Directive 91/414/EEC and applicable as of 14 June 2011. Unlike the former Directive, the Regulation provides the possibility to reject active substances on the basis of their intrinsic properties. Such 'cut-off' (exclusion) criteria are specified in Annex II point 3, including active substances considered to be endocrine disruptors for human health (3.6.5) or non-target species (3.8.2).

Substances identified as having ED properties that may cause adverse effects in humans cannot be authorised under the new PPP Regulation, unless the exposure under realistic proposed conditions of use is negligible[33]. Currently there is no commonly agreed definition and criteria for endocrine disruption. Due to the intensive discussions the deadline 2013 could not be held by which the Commission was required to present (to the Standing Committee on the Food Chain and Animal Health) a draft of the measures concerning specific scientific criteria for the determination of endocrine disrupting properties (in relation to human health impacts) to be adopted in accordance with the regulatory procedure with scrutiny referred to in Article 79(4). The adoption of criteria is expected soon. The current status is described in Chapter 6.4.. With regard to the ecological impacts, substances having endocrine disrupting properties that may have adverse effects on non-target organisms, cannot be authorised under the PPP Regulation, either. Despite absence of a formal obligation of the Commission to develop such criteria for non-target organisms, it is expected that these will be developed as well.

Pending the adoption of the scientific criteria for the identification of endocrine disruptors, the PPP Regulation requires that substances that are, or have to be classified as Carcinogenic (Category 2) and Toxic for Reproduction (Category 2) shall be considered as having ED properties and accordingly shall not be authorised. In addition, substances, such as those that are or have to be classified as toxic for reproduction category 2 and which have toxic effects on the endocrine organs, may be considered to have such endocrine disrupting properties.

A number of scientific definitions of endocrine disruption exist, of which the WHO/IPCS one is broadly accepted. However, the definition is quite general and requires further development and elaboration for regulatory use and application. The scientific definition cannot discriminate between substances meriting real concern for their ability

[33] * negligible (defined in human area only): 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 synergistic concerned on food and feed do not exceed the default value of <0,01 mg/kg set in accorcance with point (b) of Article 18(1) of Regulation (EC) No 396/2005.

to disrupt the endocrine system and substances that merit little concern in relation to any endocrine-disrupting ability and for which regulatory action is not justified or is of a low priority. Further criteria, on potency, specificity, adversity, population relevance are needed from a regulatory point of view.

Assessment of the endocrine potential will be made on the basis of Community or internationally agreed test guidelines or other available data and information, including a review of scientific literature, reviewed by the Authority. Again, quality criteria for such published studies, which unlike regulatory studies are typically not generated according to GLP and often without analytical determination of doses or concentrations, still need to be defined.

6.7.2. Biocides Product Regulation (BPR)

Regulation (EU) No 528/2012 relative to the placing of biocide products applies from September 1, 2013.

In Article 5 endocrine disrupting properties are addressed as exclusion criteria. The European Commission has to adopt delegated acts in order to establish scientific criteria for the definition and determination of endocrine disrupting effects. Due to the intensive discussions the deadline 2013 could not be held. From the current point of view, criteria might be applicable mid 2018 (see Chapter 6.4).

For interim decisions substances that are classified as carcinogen category 2 and toxic for reproduction category 2, shall be considered to be endocrine disruptors. Substances that are classified toxic for reproduction category 2 and which have toxic effects on the endocrine organs, may be considered to have such endocrine disrupting properties.

6.7.3. Regulation on Cosmetic Products (1223/2009)

In the Regulation on cosmetic products (1223/2009) endocrine disruptors are referred to in Article 15.4. where a review of the Regulation with regard to endocrine disruptors is announced. This review was originally planned for January 2015, but the timeline was not kept, as this point is linked to other EU-Regulations due to the overarching objective to define horizontal criteria.

It has to be noted that according to the EU Scientific Committee on Consumer Safety (SCCS) a conventional risk assessment approach is appropriate for endocrine-active cosmetic ingredients. The SCCS Memorandum on Endocrine Disruptors (SCCS/1544/14) states: “…EDs can therefore be treated like most other substances of concern for human health and the environment, i.e. be subject to risk assessment and not only to hazard assessment.”

One specific aspect for cosmetics is that the internationally agreed WHO/IPCS definition defines an endocrine disruptor on the basis of adverse health effects in an intact organism. However, due to the animal testing and marketing ban for cosmetics since March 2013, *in vivo* tests can no longer be carried out.

OECD has designed a framework for testing and assessment of endocrine disrupters which foresees a combination of initial *in vitro* screening and various tiers of *in vivo* testing (see Chapter 4.5). *In-vitro* tests currently do not allow a definite differentiation between biological activity and adverse health effects, which poses a significant problem for the assessment of cosmetic ingredients. The above-mentioned SCCS Memorandum in 2014 describes this problematic point, i.e. *"With regard to substances with endocrine activity (potential endocrine disruptor), the assessment of their impact to human health without animal data remains a challenge"* (SCCS/1544/14)[34]. As for other sectors, it is of paramount importance to conduct a proper risk assessment which does not only rely on hazard identification and characterization including adversity, potency, sensitivity, (ir)reversibility and severity, but also takes into account the relevant exposure, which in the case of cosmetics is the consumer exposure.

The Environmental Safety of cosmetic ingredients is covered by REACH-Regulation 1907/2006.

6.7.4. Water Framework Directive (2000/60/EC)

Endocrine Disruptors are refered to in Annex VIII, the indicative list of the main pollutants.

Annex VIII, 4. *"Substances and preparations, or the breakdown products of such, which have been proved to possess carcinogenic or mutagenic properties or properties which may affect steroidogenic, thyroid, reproduction or other endocrine-related functions in or via the aquatic environment."*

6.7.5. Endocrine disrupters in the authorisation procedure of the REACH Regulation (EC) No 1907/2006

Under the REACH Regulation there is a general registration requirement for all chemicals produced in or imported into the Community in quantities greater than 1 t/a (COM (2003) 644). The registration requires, inter alia, toxicological and ecotoxicological data and, for quantities over 10 t/a, a chemical safety report.

The REACH Regulation further intends to identify substances of very high concern (SVHC) and subjects them to the authorisation procedure. The aim of the authorisation system under the REACH Regulation is that the risks from substances of very high concern are properly controlled and that these substances are progressively replaced

[34] http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_s_009.pdf

by suitable alternative substances or technologies where these are economically and technically viable.

In REACH Article 57, criteria are listed that may lead to a nomination of a substance as SVHC, e.g. substances classified as causing cancer (carcinogens), substances that alter the genetic material (mutagens) or endanger reproduction (reproductive toxins) in the EU categories 1A and 1B (so-called CMR substances) according to regulation EU 1272/2008 may be subject to authorisation. The same may also apply to substances , for which there is scientific evidence of probable serious effects to human health or the environment which give rise to an equivalent level of very high concern as CMR, PBT, or vPvB substances. This “opening clause” is referred to in Art. 57 f and substances that have endocrine disrupting properties are listed as one example.

Substances with the above described properties are not automatically subject to authorisation. Instead they need to be selected on a case-by-case basis and included in Annex XIV “List of substances subject to authorisation” of the REACH Regulation. Only if this has been done, certain uses of the substance need to be authorised.

Authorisation can be granted via two different routes: via adequate route of control, or socioeconomic. For ED an authorization via adequate route of control is possible (see Chapter 5.2.1).

As described in section 6.2 and elsewhere, endocrine activity is not a separate harmful property, a new type of toxic effect, or a previously undetected hazard. Rather, endocrine activity comprises specific mechanisms of action which can lead to a hazard to health or to the environment, particularly after long-term exposure. These adverse effects can be addressed by existing testing strategies and classification regulations (CMR substances, PBT substances).

In its "Guidance for the preparation of an Annex XV dossier on the identification of substances of very high concern" the European Chemicals Agency states (ECHA, 2007):

- *"…the decision on whether or not this mechanism of action is a relevant consideration for the given chemical will need to be taken on a case-by-case basis…"*
- *"Given the complexities of the possible mechanisms and effects of endocrine active substances it is unlikely that the results from isolated screening assays will be sufficient to confirm that the substance has potential to cause endocrine disrupting effects in humans or wildlife. Therefore a weight of evidence approach is needed."*
- *"If a substance is identified as having endocrine disrupting effects, it should be confirmed that a traditional hazard assessment approach could not be used or would be insufficiently protective of such effects."*

ECHA Expert-Group for endocrine disruptors

In 2014 ECHA has set up an working-group consisting of experts from EU member states, EU commission, EFSA and stakeholders. Aim of the group is to discuss and provide non-binding scientific advise on questions related to the identification of endocrined disrupters, namely on

- "*Questions related to screening methods/activities to identify potential endocrine disruptors (e.g. for the CoRAP and/or Candidate List)*
- *Questions related to the development of integrated approaches to testing and assessment of endocrine disrupting properties*
- *Feedback and recommendations on complex (specific/generic) scientific issues related to information and (tiered) testing needs for potential endocrine disruptors (e.g. under dossier or substance evaluation or under the evaluation by the evaluating competent authority of a biocides active substance application)*
- *Specific questions on the interpretation of test data as well as other relevant information in relation to the identification of endocrine disrupting properties (e.g. during the development of an SVHC dossier or a biocides active substance evaluation).*"
  (source: ECHA Website)[35]

This support can be used by ECHA and by EU member state competent authorities under the REACH Regulation and the Biocides Regulation.

6.8. Characterisation of endocrine active substances outside Europe

Scientists from the US FDA (Division of Bioinformatics and Biostatistics, National Center for Toxicological Research) have recently developed an Estrogenic Activity Database (EADB) and made it freely available to the public[36]. EADB contains estrogenic activity data points collected for more than 8000 chemicals tested in binding, reporter gene, cell proliferation, and in vivo assays in 11 different species. The chemicals cover a broad chemical structure space and the data span a wide range of activities. A set of tools allow users to access EADB and evaluate potential endocrine activity of chemicals (Shen et al. 2013).

[35] http://echa.europa.eu/de/addressing-chemicals-of-concern/substances-of-potential-concern/endocrine-disruptor-expert-group

[36] http://www.fda.gov/ScienceResearch/BioinformaticsTools/EstrogenicActivityDatabaseEADB/default.htm

7. Excecutive summary of the VCI overview

The VCI overview on “endocrine active substances” examines the current state of scientific knowledge by discussing and addressing the following issues:

- Definition of “endocrine active substances” and “endocrine disrupters”,
- Basis of the evaluation,
- Background to the substances under discussion and to the question which hormone systems are under discussion in association with endocrine activity as well as to the possible accumulation of endocrine active substances in organisms,
- Examples of the potencies of estrogenic substances are described.
- Overview of regulatory significance and developments.

In separate sections, the human and environmental aspects are examined in more detail, e.g. the possible hazard to humans or the environment is assessed. In this process, test methods, test strategies, and the ongoing criteria-discussions are explained.

More general matters are also discussed, such as what is known about combination effects and the question of adverse effects in the low-dose range, as is the subject of the validity and quality of scientific studies.

In the last section, regulatory aspects are covered, e.g. the development of criteria for ED that can be used in the different regulations that address ED.

The VCI overview is intended to make a contribution to the scientific and objective discussion of endocrine active substances. To this end, regular updating of the document to reflect the current state of the discussion is envisaged.

For the following aspects additional VCI Position-papers are available:

- Endokrine Disruptors (general)
  https://www.vci.de/vci/downloads-vci/top-thema/argumente-positionen-endokrine-effekte-und-endokrine-schaedigungen-de.pdf
- Low-Dose Effects:
  www.vci.de/Downloads/PDF/Low%20Dose%20Effects_Englisch.pdf
- Thresholds and Limit Values
  www.vci.de/Downloads/PDF/VCI%20Position%20Threshold.pdf

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http://www.lanuv.nrw.de/

- River Basin Community Elbe:
http://www.fgg-elbe.de/start-en.html

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http://globalraptors.org/grin/SpeciesResults.asp?specID=8248

- International Commission for the Protection of the Rhine:
http://www.iksr.org/index.php?id=58&L=3&cHash=455fdab52ce6eafbf6f72632159564bf

- University of Cologne, Faculty of Mathematics and Natural Sciences:
http://mathnat.uni-koeln.de/10317.html

Appendices

Appendix 1: Toxicity test strategy (from Klotz, 2003)

General remarks:

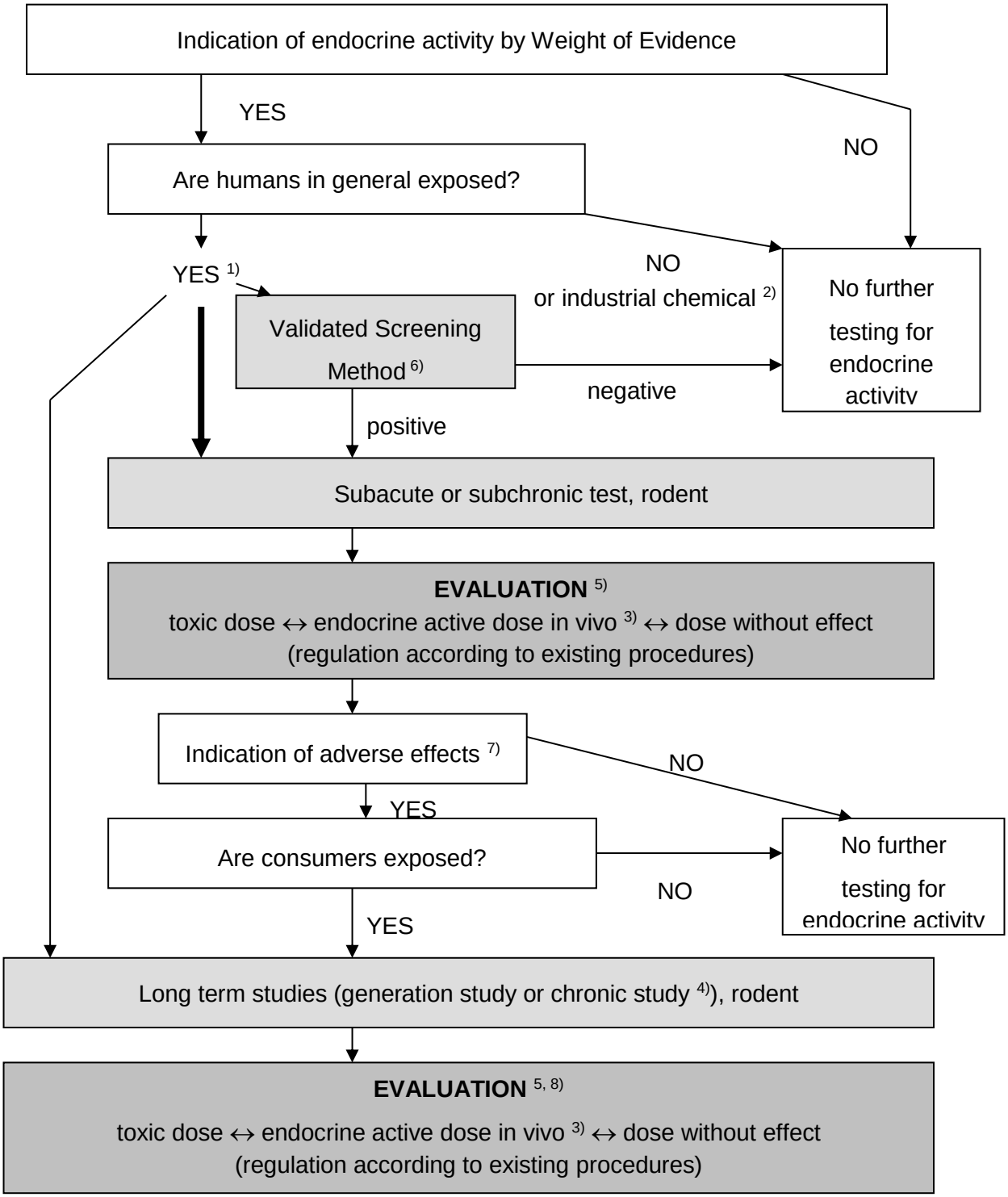
According to the so called “Weybridge Definition”, defined at the European Workshop on the Impact of Endocrine Disrupters on Human Health & Wildlife in 1996, “an endocrine disrupter is an exogenous substance that causes adverse health effects in the intact organism or its progeny, secondary/consequent to changes in endocrine function”.

Based on that definition, in the discussion of substances with scientific indications of endocrine activity the main question is, whether or not there is evidence of an adverse effect.

This decision tree is intended to give guidance for further testing of substances for which there is scientific reasonable indication of endocrine activity, e.g. in screening or non-validated tests. For clarification of an endocrine potential and to answer the question whether or not there is an adverse effect, in vivo guideline studies normally will be the preferred methods. In case that comprehensive repeated dose or reproductive toxicity data already exist, further testing may not be required.

The focus of the decision tree is on estrogenic/anti-estrogenic/androgenic/anti-androgenic activity. For other endocrine activities different test methods, which are not included in the decision tree, may be required.

The decision tree could help to guide the discussion of substances placed on e.g. priority lists for “endocrine disrupters”.


Indication of endocrine activity by Weight of Evidence
YES
NO
Are humans in general exposed?
YES 1)
NO
or industrial chemical 2)
No further testing for endocrine activity
Validated Screening Method 6)
negative
positive
Subacute or subchronic test, rodent
EVALUATION 5)
toxic dose ↔ endocrine active dose in vivo 3) ↔ dose without effect
(regulation according to existing procedures)
Indication of adverse effects 7)
NO
YES
Are consumers exposed?
No further testing for endocrine activity
NO
YES
Long term studies (generation study or chronic study 4)), rodent
EVALUATION 5, 8)
toxic dose ↔ endocrine active dose in vivo 3) ↔ dose without effect
(regulation according to existing procedures)

Legend:

1) A subacute toxicity test in rodents will be preferred in most cases (e.g. enhancement of OECD Guideline 407 including endocrine endpoints).

In vivo screening tests are mechanistic studies and should only be performed in special cases (e.g. priority setting) and when a validated method is available.

Long term studies may be the preferred testing if subacute/subchronic data already exists or if, e.g. due to consumer exposure, long term testing seems to be more appropriate.

2) Industrial chemicals: non-isolated chemicals, use in closed systems, accidental exposure only

3) Relevant route of exposure, endocrine active dose leading to an adverse effect

4) e.g. One- or two-generation study; 2-year study in rodents. Specific method to be decided case-by-case based on the database (toxicity and exposure) of the respective chemical.

5) For the evaluation a comparison of the NOAEL based on endocrine mechanism with the overall NOAEL for adverse effects is important. The lowest NOAEL will be normally most relevant for regulation according to existing procedures. Evaluation should lead to the following consequences:

A) Is an adverse effect predictable and assessable,

B) Further clarification of effect required?

6) Validated Screening Methods allowing judgment on likelihood of in vivo manifestations of adverse effect

7) Caused by endocrine mechanism in relevant exposure concentrations

8) In the evaluation process, it needs to be clarified if the long term study accounts for possible special vulnerability of sensitive subpopulations, e.g. children and by in utero exposure. If not, additional safety factors might have to be applied in the risk assessment process in case of exposure of that specific subpopulation.

Appendix 2: OECD

Conceptual Framework for Testing and Assessment of Endocrine Disrupters (as revised in 2012a)

as included in the Guidance Document 150 (Annex 1.4) published in the OECD Series on Testing and Assessment in August 2012. Very few editorial changes were necessary to be able to post it on the public website as a standalone document.

Mammalian and non mammalian Toxicology	
Level 1 Existing Data and Non-Test Information	• Physical & chemical properties, e.g., MW reactivity, volatility, biodegradability • All available (eco)toxicological data from standardized or non-standardized tests. • Read across, chemical categories, QSARs and other in silico predictions, and ADME model predictions

Level 2 In vitro assays providing data about selected endocrine mechanism(s) / pathways(s) (Mammalian and non mammalian methods)	• Estrogen or androgen receptor binding affinity • Estrogen receptor transactivation (OECD TG 455 – OECD TG 457) • Androgen or thyroid transactivation (If/when TGs are available) • Steroidogenesis in vitro (OECD TG 456) • MCF-7 cell proliferation assays (ER ant/agonist) • Other assays as appropriate

	Mammalian Toxicology	Non-Mammalian Toxicology
Level 3 In vivo assays providing data about selected endocrine mechanism(s) / pathway(s)[1]	• Uterotrophic assay (OECD TG 440) • Hershberger assay (OECD TG 441)	• Xenopus embryo thyroid signalling assay (When/if TG is available) • Amphibian metamorphosis assay (OECD TG 231) • Fish Reproductive Screening Assay (OECD TG 229) • Fish Screening Assay (OECD TG 230) • Androgenized female stickleback screen (GD 140)

Level 4 In vivo assays providing data on adverse effects on endocrine relevant endpoints[2]	• Repeated dose 28-day study (OECD TG 407) • Repeated dose 90-day study (OECD TG 408) • 1-generation reproduction toxicity study (OECD TG 415) • Male pubertal assay (see GD 150, Chapter C4.3)3 • Female pubertal assay (see GD 150, Chapter C4.4)3	• Fish sexual development test (OECD TG 234) • Fish Reproduction Partial Lifecycle Test (when/If TG is Available) • Larval Amphibian Growth & Development Assay (when TG is available) • Avian Reproduction Assay (OECD TG 206)

	• Intact adult male endocrine screening assay (see GD 150, Chapter Annex 2.5) • Prenatal developmental toxicity study (OECD TG 414) • Chronic toxicity and carcinogenicity studies (OECD TG 451-3) • Reproductive screening test (OECD TG 421 if enhanced) • Combined 28-day/reproductive screening assay (OECD TG 422 if enhanced) • Developmental neurotoxicity (OECD TG 426)	• Mollusc Partial Lifecycle Assays (when TG is available) [4] • Chironomid Toxicity Test (TG 218-219) [4] • Daphnia Reproduction Test (with male induction) (OECD TG 211) [4] • Earthworm Reproduction Test (OECD TG 222) [4] • Enchytraeid Reproduction Test (OECD TG 220) [4] • Sediment Water Lumbriculus Toxicity Test Using Spiked Sediment (OECD TG 225) [4] □ Predatory mite reproduction test in soil (OECD TG 226) [4] □ Collembolan Reproduction Test in Soil (TG OECD 232) [4]

Level 5 In vivo assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism[2]	• Extended one-generation reproductive toxicity study (OECD TG 443)[5] • 2-Generation reproduction toxicity study (OECD TG 416 most recent update)	• FLCTT (Fish LifeCycle Toxicity Test) (when TG is available) • Medaka Multigeneration Test (MMGT) (when TG is available) • Avian 2 generation reproductive toxicity assay (when TG is available) • Mysid Life Cycle Toxicity Test (when TG is available)[4] • Copepod Reproduction and Development Test (when TG is available)[4] • Sediment Water Chironomid Life Cycle Toxicity Test (OECD TG 233) [4] • Mollusc Full Lifecycle Assays (when TG is available) [4] • Daphnia Multigeneration Assay (if TG is available) [4]

1 Some assays may also provide some evidence of adverse effects.

2 Effects can be sensitive to more than one mechanism and may be due to non-ED mechanisms.

3 Depending on the guideline/protocol used, the fact that a substance may interact with a hormone system in these assays does not necessarily mean that when the substance is used it will cause adverse effects in humans or ecological systems.

4 At present, the available invertebrate assays solely involve apical endpoints which are able to respond to some endocrine disrupters and some non-EDs. Those in Level 4 are partial lifecycle tests, while those in Level 5 are full- or multiple lifecycle tests.

5 The Extended one-generation reproductive Toxicity Study (OECD TG 443) is preferable for detecting endocrine disruption because it provides an evaluation of a number of endocrine endpoints in the juvenile and adult F1, which are not included in the 2-generation study (OECD TG 416) adopted in 2001

Notes to the OECD Revised Conceptual Framework

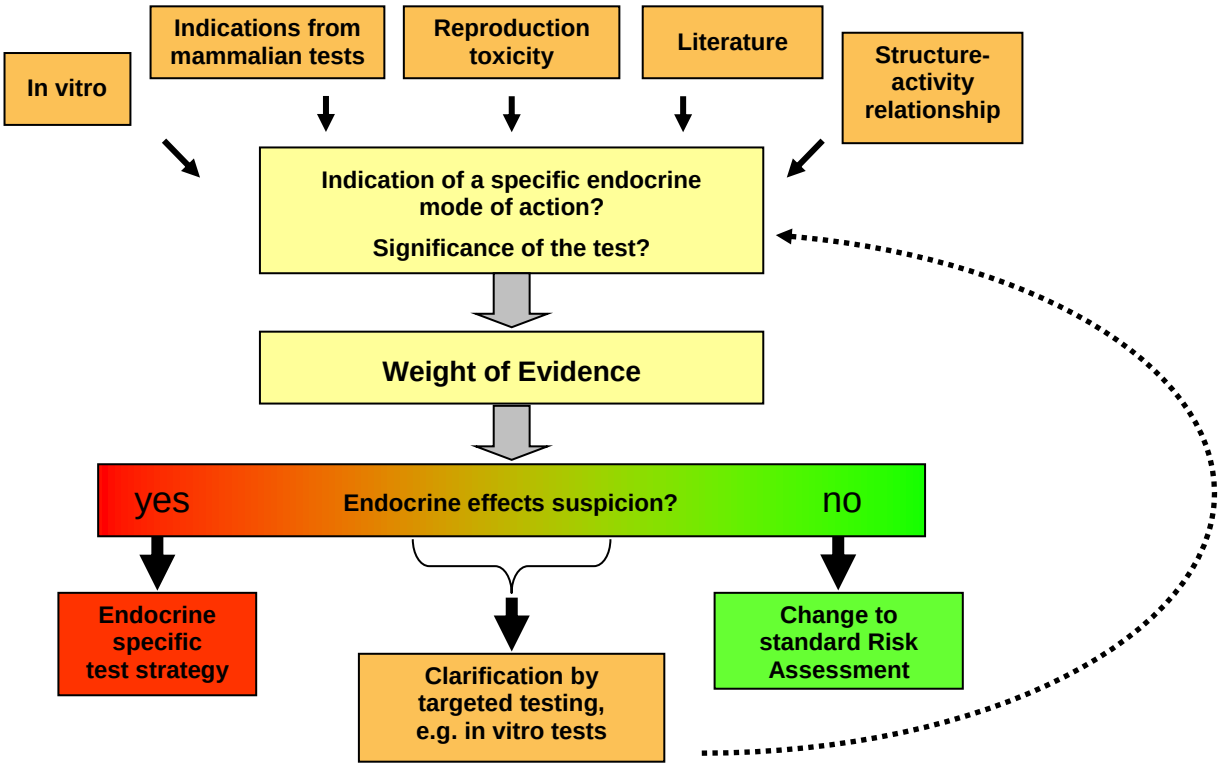
Note 1: Entering at all levels and exiting at all levels is possible and depends upon the nature of existing information and needs for testing and assessment.

Note 2: The assessment of each chemical should be made on a case by case basis, taking into account all available information.

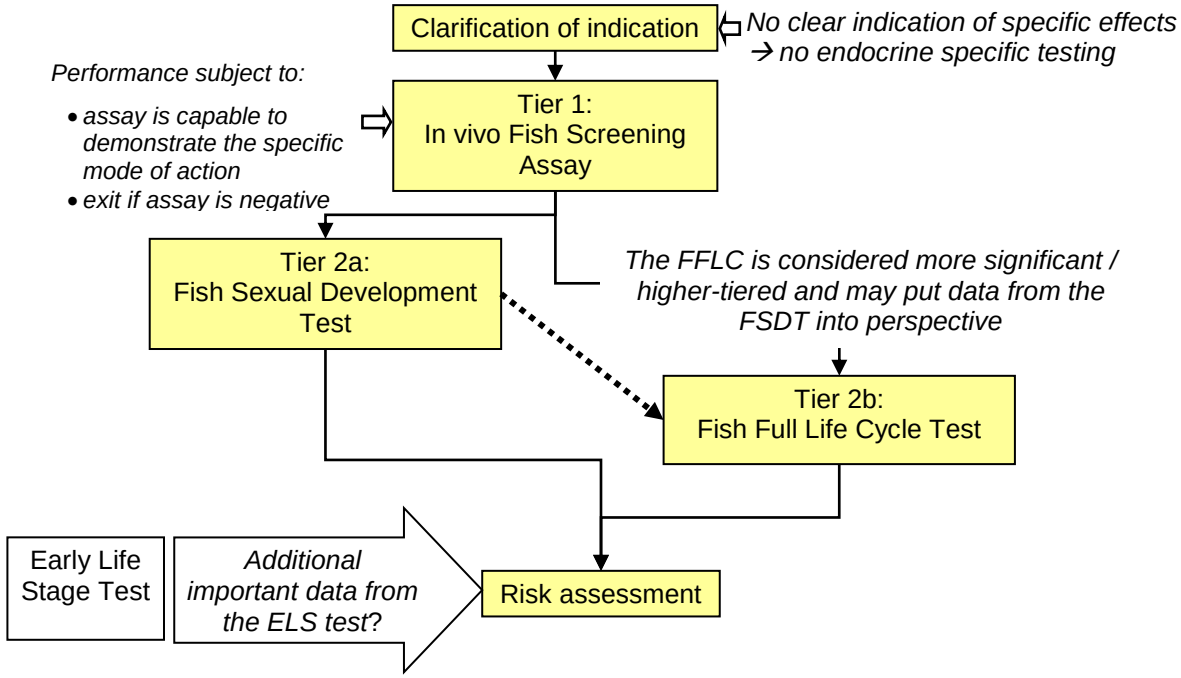
Note 3: The framework should not be considered as all inclusive at the present time. At levels 2, 3, 4 and 5 it includes assays that are either available or for which validation is under way. With respect to the latter, these are provisionally included.

Appendix 3: Ecotoxicity test strategy

Initial considerations: Criteria for specific tests on endocrine effects



Fish test strategy:



IMPORTANT: Intelligent test strategy, no fixed hierarchy

Appendix 4:

VCI position paper on the subject “is a separate hazard category required?”

Classification and labelling of endocrine active substances

in respect of toxicological endpoints: integration in the existing and future classification and labelling system

(July 2002 based on EU 67/548/EWG; update March 2010 based on EU 1272/2008)

In the course of the discussion of the effects of chemicals on the endocrine system, there are occasional calls for a separate category for endocrine active substances. However, representatives of the scientific community and the authorities, e.g. the SCTEE( ) are of the unanimous opinion that endocrine activity is not a separate or new type of toxic property, nor is it a hazard that has not previously been detected. Rather, it involves specific mechanisms that could, but would not necessarily, result in adverse effects, particularly after long-term exposure. Such hazards due to endocrine mechanisms and corresponding adverse effects are already detected by existing toxicity test methods, particularly by studies of reproductive toxicity, repeated administration or chronic toxicity/carcinogenicity. As described below, national and international regulations concerning hazardous substances and products already cover these effects and will continue to do so in future:

- Possible adverse effects of endocrine active substances on the reproductive system, such as impairment of fertility or embryotoxic effects, are covered by a number of risk phrases (R60, R61, R62 and R63 or H360 or H361 respectively). Postnatal effects which may result from transmission in the mother’s milk are covered by R64 (or H362) in the current classification system, effects on development until the end of puberty are covered by R39*, R68*, or R48*, (H370, H371, H372, H373) depending on the duration of exposure and the severity of the finding.
- Irrespective of the underlying mechanism, all functional or morphological changes - as a function of the duration of exposure and the severity of the finding - can be described by risk phrases R39*, 68*, or 48* (H370, H371, H372, H373). This includes neurotoxic, immunotoxic, or other adverse effects due to endocrine effects.
- If a carcinogenic effect is due to the endocrine activity of a substance, suitable risk phrases are also available (R45, R40 or H350, H351 respectively).

In summary, it can be stated that adverse toxic effects - that could be induced by an endocrine mechanism - are covered by existing classification systems. Accordingly, the introduction of a separate hazard category for endocrine active substances is not necessary or justified, according to the criteria of the European Union or the planned Globally Harmonized System.

Appendix: Relevant risk phrases (July 2002 based on EU 67/548/EWG; update March 2010 based on EU 1272/2008):

EU 67/548/EWG

R60 – May impair fertility

R62 – Possible risk of impaired fertility

R61 – May cause harm to the unborn child

R63 – Possible risk of harm to the unborn child

R64 – May cause harm to breast-fed babies

R39 – Danger of very serious irreversible effects

R68 – Possible risk of irreversible effects

R48 – Danger of serious damage to health by prolonged exposure

R45 – May cause cancer

R40 – Limited evidence of a carcinogenic effect

EU 1272/2008

H360 – May damage fertility or the unborn child

H361 – Suspected of damaging fertility or the unborn child

H362 – May cause harm to breast-fed children

H370 – Cause damage to organs

H371 – May cause damage to organs

H372 – Causes damage to organs through prolonged or repeated exposure

H373 – May cause damage to organs through prolonged or repeated exposure

H350 – May cause cancer*

H351 – Suspected of causing cancer*

* In combination with the route of exposure

Appendix 5: Proposal for criteria for identification of endocrine disruptors‡

Human Health	Environment
Data from intact animals*: Strong WoE**: • Adverse effect[4] via relevant route and at relevant doses, and • Causal relationship between primary ED MoA and the adverse effect[5], and • Relevance to humans cannot be excluded	**Data from intact animals*:** Strong WoE**: • Adverse population-relevant effect[4] via relevant route, and • Causal relationship between primary ED MoA and the adverse effect[5]

High concern in terms of:
- Potency[6]
- Severity of effects[7]
- Lead toxic effect/decisive for the overall (eco)toxicological profile[7]

UNLESS (FOR ENVIRONMENTAL HEALTH)

- *Unless* it is scientifically demonstrated that under relevant field conditions there is no unacceptable effect[8]

*** When there are data from humans or animal species living in the environment they should be taken into account**

Strong WoE**:
- Adverse effect (population relevant for environmental health), and
- Demonstrated primary ED Mode of Action[1], and
- Relevant (eco)epidemiological data[2] providing evidence of causality for a specific substance

****WoE = Weight of evidence:** 'the process of considering the strengths and weaknesses of various pieces of information in reaching and supporting a conclusion concerning a property of the substance'[3]

[1] EFSA Scientific Opinion March 2013 (p. 17) http://www.efsa.europa.eu/en/efsajournal/pub/3132.htm,
[2] per STROBE Guidelines
[3] Scientific Committee on Emerging and Newly Identified Health Risks 2012 http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_s_001.pdf, ECHA Practical Guide 2, 2010 (p. 2) http://echa.europa.eu/documents/10162/13655/pg_report_weight_of_evidence_en.pdf
[4] WHO/IPCS 2002 http://who.int/ipcs/publications/en/ch1.pdf , OECD CF level IV & V http://www.oecd.org/env/ehs/testing/OECD%20Conceptual%20Framework%20for%20Testing%20and%20Assessment%20of%20Endocrine%20Disrupters%20for%20the%20public%20website.pdf
[5] Bradford Hill Criteria: Hill, Austin Bradford (1965). "The Environment and Disease: Association or Causation?". *Proceedings of the Royal Society of Medicine* 58 (5): 295-300 and OECD CF level IV & V
[6] Based on Specific Target Organ Toxicity Repeated Exposure (STOT-RE) as seen in DE/UK position paper 2011 (p. 6)
[7] EFSA Scientific Opinion March 2013 (p. 42)
[8] Biocides Regulation (EU) No. 528/2012, Annex VI, also relevant to industrial chemicals and pesticides

‡ *This proposal is for criteria for the purposes of hazard assessment; substances should be subject to comprehensive risk assessment, considering both hazard and exposure.*

Appendix 6: Abbreviations

BMU	Bundesministerium für Umwelt, Naturschutz und Reaktorsicherheit [Federal Ministry for the Environment, Nature Conservation and Nuclear Safety]
BfG	Bundesanstalt für Gewässerkunde [Federal Institute of Hydrology]
BIAC	Business and Industry Advisory Committee to the OECD
BPR	Biocides Product Regulation
BUA	Beratergremium für Altstoffe [Advisory Committee on Existing Chemicals]
CSTEE	Scientific Committee on Toxicity, Ecotoxicity and the Environment (until 2004, suceeeded by SCHER)
DDT	Dichlorodiphenyltrichloroethane
DG	Directorate General
DGPT	Deutsche Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie [German Society for Experimental and Clinical Pharmacology and Toxicology]
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECHA	European Chemicals Agency
ED	Endocrine disruption
EDTA	Task Force on Endocrine Disruptors Testing and Assessment
EFSA	European Food Safety Authority
EMEA	European Medicines Agency
FGG Elbe	Flussgebietsgemeinschaft Elbe [River Basin Community Elbe]
GSI	Gonadosomatic Index
HBMOS	Hygiene-based margin of safety
ICPR	International Commission for the Protection of the Rhine]
IPCS	International Program on Chemical Safety
IUPAC	International Union of Pure and Applied Chemistry
JRC	Joint Research Center
LANUV NRW	Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen [North-Rhine-Westphalia State Environment Agency]
NOAEL	No observed adverse effect level
NOEC	No observed effect concentration

NOEL	No observed effect level
NTP	National Toxicology Program
OBELIX	OBesogenic Endocrine disrupting chemicals: LInking prenatal eXposure to the development of obesity later in life
OECD	Organization for Economic Co-operation and Development
PCB	Polychlorinated biphenyls
PPPR	Plant protections products regulation
SCCS	Scientific Committee on Consumer Safety
SCCP	Scientific Committee on Consumer Products (until 2009, suceeded by SCCS)
SCTEE	Scientific Committee for Toxicity, Ecotoxicity and the Environment
SCHER	Scientific Committee on Health and Environmental Risks
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
STOT RE	Specific Target Organ Toxicity after repeated exposure
SVHC	Substance of very high concern
TBT	Tributyltin
UBA	Umweltbundesamt [German Federal Environmental Agency]
UNEP	United Nations Environment Programme
US EPA	United States Environmental Protection Agency
VMG	Validation Management Group
WHO	World Health Organization
WNT	Working Group of the National Coordinators of the (OECD) Test Guidelines Programme