Integrated Environmental Assessment and Management — Volume 6, Number 4—pp. 653–662

Environmental Effect Assessment for Sexual Endocrine-Disrupting Chemicals: Fish Testing Strategy

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(Submitted 6 December 2009; Returned for Revision 2 April 2010; Accepted 15 April 2010)

Original Research

ABSTRACT

Current standard testing and assessment tools are not designed to identify specific and biologically highly sensitive modes of action of chemicals, such as endocrine disruption. This information, however, can be important to define the relevant endpoints for an assessment and to characterize thresholds of their sublethal, population-relevant effects. Starting a decade ago, compound-specific risk assessment procedures were amended by specifically addressing endocrine-disrupting properties of substances. In 2002, the Conceptual Framework, agreed upon by OECD's Task Force on Endocrine Disrupters Testing and Assessment, did not propose specific testing strategies, and appropriate testing methods had not yet been developed and approved. In the meantime, the OECD Test Guidelines Programme has undertaken important steps to revise established and to develop new test methods, which can be used to identify and quantify effects of endocrine-disrupting chemicals on mammals, birds, amphibians, fish, and invertebrates. For fish testing of endocrine-disrupting chemicals, the first Test Guidelines have recently been adopted by the OECD and validation of further test systems is under progress. Based on these test systems and the experience gained during their validation procedures, we propose a 3-step fish testing strategy: 1) Weight-of-evidence approach for identifying potential sexual endocrine-disrupting chemicals; even after advanced specification of systematic criteria, this step of establishing initial suspicion will still require expert judgment; 2) in vivo evaluation of sexual endocrine-disrupting activity in fish by applying in vivo fish screening assays; sufficient data are available to diagnose the aromatase-inhibition and estrogen-receptor agonist mechanisms of action by indicative endpoints (biomarkers), whereas the ability of the respective biomarkers in the screening assay to identify the estrogen-receptor antagonists and androgen-receptor agonists and antagonists requires further validation; 3) characterization of sexual endocrine-mediated adverse effects including threshold concentrations; in cases when the most sensitive population-relevant endpoints and the most sensitive time window for exposure are known for the mechanisms of action, the fish full life-cycle or 2-generation test, which are the normal definitive tests, might be abbreviated to, e.g., the fish sexual development test. In the European Union, the measurement of indicative endpoints in the definitive test might be crucial for the authorization procedure under REACH and plant-protection products. The results of the definitive tests can be used in existing schemes of compound-specific environmental risk assessments. Integr Environ Assess Manag 2010;6:653–662. © 2010 SETAC

Keywords: Fish testing Testing strategy Endocrine-disrupting chemicals Environmental risk assessment

INTRODUCTION

Before entering into the subject of this article, and by analogy to the definitions provided by Escher and Hermens (2002), we would like to specify the use of the terms ''mode of action'' and ''mechanism of action.'' Mode of action in this paper is a set of physiological and behavioral signs characterizing the biological response caused by sexual endocrine-disrupting chemicals (SEDCs); mechanism of action refers to the biochemical process or xenobiotic–biological interaction (e.g., estrogen-receptor agonism or aromatase inhibition) underlying a given mode of action. SEDCs are substances that interfere with the sexual development and reproduction of fish as outlined in more detailed in the following sections of this paper.

The issue of endocrine disruption (ED) was brought onto the agenda of regulatory authorities by environmentalists and researchers dedicated to studying the impact of chemicals on aquatic organisms under field conditions (Smith 1981; Woodward et al. 1993; Purdom et al. 1994; Sumpter and Johnson 2005). Data from these and other authors raised concern, because it became obvious that current testing and assessment tools are not appropriate to identify specifically designed and biologically highly active chemicals, which may result in sublethal population-relevant effects insufficiently addressed by available standard test methods. To improve the basis for regulatory and responsible care-driven decisions in chemicals safety management, activities were initiated for compound-specific environmental risk assessments (ERAs) to

All Supplemental Data may be found in the online version of this article.

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Published online 23 April 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/ieam.92

amend testing tools and assessment concepts specifically addressing endocrine-disrupting properties. The need for such amendments is common to all major types of substances, i.e., industrial chemicals, active ingredients of plant-protection and biocidal products, and environmentally relevant human and veterinary pharmaceuticals. The starting point for an ED-specific testing strategy is the identification of a substance as potentially endocrine disruptive, which depends on varying information levels according to the legal requirements of compound-specific ERAs.

The main objective of this paper is to propose a strategy for how to use the new and revised OECD testing methods with fish, for a comprehensive and efficient ERA for SEDCs. Before doing that, we outline the current state of risk assessment schemes with regard to EDCs, and we summarize the latest status of the OECD Conceptual Framework for testing of EDCs with emphasis on fish tests, which are designed to identify subtle, population-relevant effects caused by SEDCs.

STATUS OF RISK ASSESSMENT SCHEMES WITH REGARD TO ED ISSUES

ERA schemes address endocrine-disrupting properties of compounds as an indication for possibly increased risks to the environment (see, e.g., EC 2002a, 2002b, 2003). In most cases, however, the tiered standard risk assessment starts with data from short-term ecotoxicological studies, which cannot provide safe effect thresholds for endocrine disruptors.

This conceptual shortcoming can be illustrated by, e.g., the human drug 17α-ethinylestradiol. Regulatory acceptable environmental risks would be indicated if the risk characterization were based exclusively on short-term aquatic toxicity data; unacceptable risks become obvious only when using data from long-term effect studies with fish (Länge et al. 2001; Wenzel et al. 2001). In the guideline on ERA of medicinal products for human use, the European Medicines Agency (EMEA) recommends long-term instead of short-term ecotoxicity tests as the starting point for effects assessment (EMEA/CHMP 2006). This modification provides an improved level of information (e.g., information on reproduction) for revealing ED-related effects for human drugs. The rationale provided by EMEA for proposing long-term tests, however, is based not on the interest in revealing ED activities of a compound but on the fact that human pharmaceuticals are continuously released to the aquatic environment via effluents from sewage treatment plants, so long-term exposure of organisms is a given, which should be compared with results from long-term effect studies. Moreover, pharmaceuticals are designed to exert biological highly specific sublethal activities, which are not adequately covered by standard testing methods for acute toxicity.

In cases when there is evidence that a compound shows endocrine-disrupting properties, adaptations of the ERA schemes are proposed in several other regulatory frameworks and corresponding guidance documents. For example, for plant-protection products, a nonstandard avian reproduction test and a fish life-cycle test should be considered, if there is evidence for endocrine-disrupting effects (EC 2002a, 2002b). Recently, the upcoming EU regulation on the placing of plant-protection products on the market passed the European Parliament, which calls for a nonauthorization of plant-protection products that have endocrine-disrupting properties (EU 2009).

Currently, the new European Chemicals Regulation REACH (EC 2006) is being implemented by comprehensively amending the existing Technical Guidance Documents for risk assessment (EC 2003). Highly concerning substance properties, for example, persistence, bioaccumulation, and endocrine disruption, are of particular significance as a trigger for entering the authorization procedure in REACH (Article 57 of EC 2006). Thus, it will become a key aspect for ERAs of industrial chemicals to identify, test, and assess substances that show endocrine activities. So far, more detailed technical guidance in regulatory frameworks is limited (see, e.g., ECHA 2008).

On the other hand, authorities of major economic regions have set up strategies to promote screening, assessment, and management of EDCs across regulatory schemes, such as the US Endocrine Disruptor Screening Program (EDSP; see http://epa.gov/endo/index.htm) or the EU strategy for EDs (see http://ec.europa.eu/environment/endocrine/strategy/index_en.htm, DG Environment; and http://ec.europa.eu/research/endocrine/activities_stategy_en.html, DG Research). Implementing these overarching strategies in substance-specific regulations, e.g., REACH or pesticide-approval regulations, requires operationalizing testing and assessment for individual substances in specific regulatory procedures.

Despite the progress made by implementing new regulation and the fact that a plethora of research papers have recently been published on endocrine effects (Sumpter and Johnson 2008), none of the guidance documents cited above provides technical details or consistent concepts on ED-specific testing and assessment.

THE OECD CONCEPTUAL FRAMEWORK FOR THE TESTING OF EDCs

The OECD Task Force on Endocrine Disrupters Testing and Assessment (EDTA) has agreed on a Conceptual Framework that ''attempts to identify tests of different levels suitable for assessing potential endocrine disrupters in situations of increasing biological complexity in both toxicological and ecotoxicological areas'' (Huet 2000; http://www.oecd.org/dataoecd/17/33/23652447.doc). Also casually called *toolbox*, the Framework is based on input from OECD member states, in particular the United States, Japan, and the European Union. With regard to the OECD Test Guidelines Programme, the principle of mutual acceptance of data requires that the selection of instruments for the toolbox is based on international consensus. The Framework consists of 5 levels:

1. Sorting and prioritization based on existing information
2. In vitro assays providing mechanistic data
3. In vivo assays providing data on single endocrine mechanisms and effects
4. In vivo assays providing data on multiple endocrine mechanisms and effects
5. In vivo assays providing data on effects from endocrine and other mechanisms

The hierarchical structure of the proposed levels implies a stepwise approach from simple to more complex and toxicologically or ecologically more relevant testing (OECD 2002), although this is not compulsory. Entering into and exiting from the Framework at any level are explicitly foreseen, depending on case-specific considerations of existing as well as legally required information and testing data. This

flexible approach is justified when considering the diverse complexities of specific regulatory cases and the need for efficient use of resources within ERA strategies (Fava et al. 1987; OECD 1995). The selection of tools for identifying and assessing EDCs might vary depending on compound-specific legislations in the OECD member countries.

Initiated by the Conceptual Framework, the OECD Test Guidelines Programme has undertaken important steps to amend established methods and develop new test methods that can be used to identify, characterize, and quantify effects of EDCs on mammals, birds, amphibians, fish, and invertebrates (OECD 1999, 2002; Gourmelon and Ahtiainen 2007). To integrate these ED-related testing methods into ERA schemes, 2 basic considerations are important.

First, endocrine-disrupting properties of a compound have to be evaluated in a transparent weight-of-evidence approach (see, e.g., ECHA 2008). This will be a key issue for regulatory testing and assessment procedures and requires particular consideration, which is, however, not included in this paper. The crucial point is to agree upon what existing or newly generated screening information could provide the basis for initial concern of a given substance to act as an endocrine disruptor. For fish, such concern would be confirmed or falsified by testing and assessment procedures proposed below.

Second, in regulatory risk assessment, the risk characterization is a result of comparing exposure (e.g., predicted environmental concentrations; PEC) with effect concentrations (e.g., predicted no effect concentrations; PNEC), and risk is defined as PEC $\geq$ PNEC (Vermeire and Van Der Zandt 1996). This regulatory principle also applies to EDCs, given that all endocrine-mediated, population-relevant adverse effects are appropriately covered in PNEC derivation.

ED-RELATED FISH TEST METHODS

Two OECD expert consultation meetings (OECD 1999, 2000) developed a concept for in vivo screening and definitive testing of EDCs, which would affect predominantly the endocrine regulation of sexual development and reproduction. Specifically, the concept aims at investigating the impact of substances acting as estrogens, androgens, or aromatase inhibitors. EDTA recommended that the test methods should be applicable to the fish species fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*); in addition, the three-spined stickleback (*Gasterosteus aculeatus*) has been recommended as a test fish (Hahlbeck, Katsiadaki, et al. 2004; Hahlbeck, Griffiths, et al. 2004; Katsiadaki et al. 2006). The OECD Validation Management Group for the Screening and Testing of Endocrine Disrupters for Ecotoxicological Effects (VMG-Eco) coordinates revision, development, and validation of several methods focusing on the sexual hormone system of fish.

The progress made so far by VMG-Eco is based mainly on research with compounds used as active ingredients in medicinal products and pesticides. Hence, the mechanisms of action described for these compounds, i.e., estrogenic or antiestrogenic, androgenic or antiandrogenic, and aromatase inhibiting, are known from drug development or toxicological studies with mammals and are related mainly to interactions with the reproductive system of vertebrates. Consequently, when proposing to use the OECD toolbox for fish testing with EDCs for regulatory purposes, it is important to keep in mind that impacts of chemicals on other mechanisms of action under endocrine control, which are related to physiological processes such as stress, maintenance, and regulation of energy, have been almost neglected. The only exception is the amphibian metamorphosis assay, which serves as a model for studying thyroid axis functions in vertebrates (OECD 2004a, 2004b). More recently, Gourmelon and Ahtianinen (2007) in the journal *Ecotoxicology*, as part of a special issue on endocrine disruption in invertebrates, described the status of and need for aquatic invertebrate test methods for the assessment of chemicals including potential endocrine-active substances.

Table 1 shows important features of the in vivo fish screening assays and the definitive fish tests, and Figure 1 presents a scheme of the OECD concept for in vivo fish screening assays and definitive tests of EDCs. For reasons of acceptability in all OECD member countries, rather than fulfilling a scientific need, 2 in vivo fish screening assays have been adopted by the OECD (OECD 2009a, 2009b), which are both conducted with reproductively active fish and correspond to level 4 of the Conceptual Framework.

According to the guideline OECD 229 Fish Short Term Reproduction Assay (STRA; OECD 2009a), the biomarker endpoints vitellogenin and secondary sexual characteristics are determined. Additionally, fecundity is quantitatively monitored, and gonads are preserved for histopathological evaluation to assess the reproductive fitness of the test animals. Neither fecundity nor histopathology is intended to identify unequivocally specific cellular mechanisms of endocrine action. The STRA provides data on multiple endocrine mechanisms. Fathead minnow is the preferred test species, because only for this species have all endpoints been validated.

The guideline 230 21-day Fish Assay: A Short Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition (21d-FA; OECD 2009b) serves as an indicator for the 3 specific endocrine mechanisms of action by determining the same biomarker endpoints as in OECD 229. For fathead minnow, medaka, and zebrafish, the validation study confirmed the mechanisms of action for potent (17β-estradiol) and weak (4-*tert*-pentylphenol) estrogenic substances, aromatase inhibitors (fadrozole, prochloraz), and potent androgenic substances (17β-trenbolone, androstendione). Estrogenic substances induced dose-dependent vitellogenin (VTG) synthesis in males. Androgenic activity could be determined by measuring secondary sex characteristics in females of fathead minnow and medaka, which are sexually dimorphic species, in contrast to zebrafish. Aromatase inhibitors caused a dose-dependent decrease of VTG levels in females of all 3 fish species, together with cessation of spawning. For the antiandrogen flutamide, especially in zebrafish, histological investigation of the gonads or measurements of 11-ketotestosterone were needed for detecting this mechanism of action, as secondary sex characteristics and VTG measurements did not show treatment-related responses (OECD 2006a, 2006b, 2007, 2008; Owens 2007; USEPA 2007).

Proposed definitive fish tests corresponding to level 5 of the Conceptual Framework are the Fish Full Life Cycle/2-Generation Test (FLCT/2-genT) and the Fish Sexual Development Test (FSDT). The FSDT can be seen as an extension of the existing OECD Test Guideline 210 (OECD 1992) by continued exposure until the fish reach sexual maturity. The enhancement allows—in addition to the

Table 1. Comparison of important features determining the design of in vivo fish screening assays and definitive fish tests

	In vivo fish screening assays		Definitive fish tests		
	STRA (fish short-term reproduction assay)	**21d-FA (21-d fish assay)**	**FSDT (fish sexual development test)**	**FLCT (fish full-life cycle test)**	**2-GenT (fish 2-generation test)**
Duration of exposure	21 d	21 d	60 d	4–5 Months	5–6 Months
Nr of treatments	3	3	5	5	5
Nr of replicates	2–4	2–4	4	4	4
Nr of fish per replicate	10	10	40 (introduced as fertilized eggs)	P generation:	P generation:
				Start with 50 fertilized eggs, reduction to 20 (adults)	20 adults
				F1 generation:	F1 generation:
				Start with 50 fertilized eggs	Start with 50 fertilized eggs, reduction to 20 (adults)
					F2 generation:
					Start with 50 fertilized eggs
Important endpoints	Vitellogenin, secondary sex characteristics, fecundity, fertility, histology	Vitellogenin, secondary sex characteristics	Vitellogenin, hatch, survival of early life stages, sex ratio, histology	Hatch, survival of early life stages, juvenile growth, fecundity, fertility, sex ratio, histology	Hatch, survival of early life stages, juvenile growth, fecundity, fertility, sex ratio, histology
OECD Guideline	229	230	Validation ongoing	Draft guideline (not yet validated)	Draft guideline (not yet validated)

endpoints mortality; malformations; and growth of embryos, larvae, and juveniles—recording of the sexual development, i.e., sex ratio as determined via histological examination of the gonads, and VTG production. The test aims at studying the impact of sexual endocrine disruptors in fish at a very sensitive stage of their life (Holbech et al. 2006).

Both the FLCT and the 2-genT allow assessing the effects on developmental and reproductive endpoints. Additionally, the 2-genT allows determining transgenerational transfer of effects. Depending on the species used, the total test duration may vary considerably. Measurement endpoints in both definitive tests include developmental and reproductive endpoints (hatching, sex ratio, survival, growth, time to first spawn, fecundity, fertility, and behavior), as well as biochemical, histological, and morphological markers that are indicative of specific mechanism of endocrine disruption.

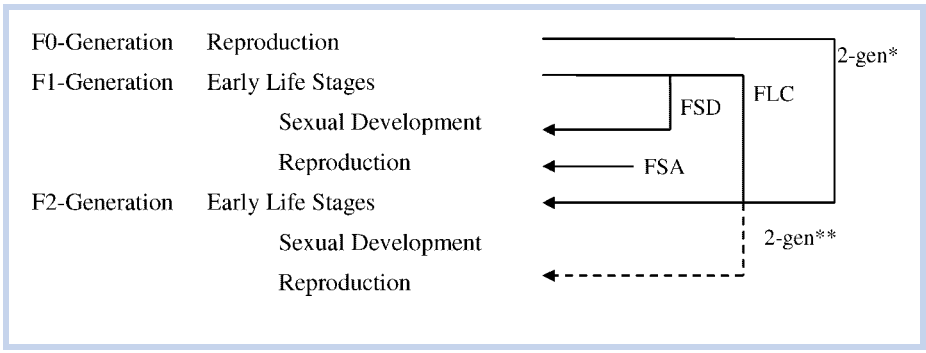

Figure 1. Schematic presentation of OECD's Test Guidelines Programme to amend established and to develop new test methods, which can be used to identify and quantify effects of endocrine-disrupting chemicals on fish (solid lines). F0 = parental; F = filial; 2-gen* = 2-generation test according to US-EPA (from F0, reproduction, to F2, early life stages); 2-gen** = 2-generation test proposed in this paper (from F1, early life stages, to F2, reproduction; dotted lines indicate the extension of the FLC to 2-gen test); FLC = fish full life cycle test; FSD = fish sexual development test; FSA = fish screening assays (OECD Guidelines 229 and 230).

Recently, 2-generation studies with zebrafish exposed to flutamide, trenbolone, and tamoxifen have been completed (Braunbeck et al. 2009; see also Supplemental Data S1–S3). Apart from sexual reproduction, the stages of sexual differentiation are considered to be most sensitive for SEDCs.

In Table 2, effects of ED substances representing different mechanisms of action induced in the 21d-FA on indicative endpoints (biomarkers) are compared with effects measured for population-relevant endpoints in definitive tests (FLCT/2-genT) with the same zebrafish strain, mostly conducted in the same laboratory; this means that data with the same test substances, but different fish species or zebrafish strains, are not taken into consideration for this comparison. Also, not all studies shown in Table 2 might be in compliance with standards (e.g., number of analytical measurements during the exposure period of fish; statistical evaluation: EC50 vs. NOEC) applied to studies used for regulatory purposes. Nevertheless, the data show that, for all mechanisms of action except androgen-receptor agonists, the level of sensitivity of measured biomarkers and population-relevant endpoints is rather similar, the EC50 diverging by a factor of less than 3. In consequence, for zebrafish, the combination of VTG and 11-ketotestosterone measurements seems to be appropriate to indicate all mechanisms of sexual endocrine action except for one, namely, androgen-receptor agonists. For the latter, as long as a suitable biomarker remains to be found, the use of fish species with secondary sex characteristics should be recommended, or gonad histology should be included.

PROPOSAL FOR A TESTING STRATEGY: REGULATORY USE OF OECD FISH-TESTING TOOLS FOR SEDCs IN ERA SCHEMES

The proposed testing strategy for SEDCs in fish is based on data compiled in Table 2 and the outcome of a workshop held at Berlin, Germany, on 10 and 11 December, 2007 (Schäfers et al. 2008). The conceptual background as provided by Huet (2000) and more general considerations on screening assays and definitive tests as published by Hutchinson et al. (2006) are essentials of the proposed testing strategy. It consists of 3 distinctive steps (Schäfers and Teigeler 2005): 1) weight-of-evidence approach for identifying potential SEDCs, 2) in vivo confirmation of sexual endocrine-disrupting activity associated with specific mechanisms of action, and 3) characterization of sexual endocrine-mediated adverse effects, including their thresholds. Each of the 3 steps will be outlined briefly in the following sections and is schematically presented in Figure 2.

Table 2. Effect concentrations (EC50) determined for zebrafish when exposed to different sexual endocrine-disrupting substances

		21-FA (biomarker)		FLCT/2-genT (population-relevant endpoint)	
SED-MoA	**Substance**	**EC50**	**Endpoint**	**EC50**	**Endpoint**
ER agonist					
Strong	Ethinylestradiol	3.1 ng/L	VTG+	1.1 ng/L[d]	Fertility
Weak	Bisphenol A	640 µg/L	VTG+	1410 µg/L[e]	Fertility
ER antagonist	Tamoxifen-citrate[a]	3.0 µg/L	VTG−	1.5 µg/L	Sex ratio
AR agonist	Trenbolone[b]	220 ng/L	VTG−	2.2 ng/L[f]	Sex ratio
AR antagonist	Flutamide[c]	560 µg/L	11kT+	760 µg/L	Fecundity
Aromatase inhibitor	Azole fungicide	62 µg/L	VTG−	90 µg/L[g]	Sex ratio
Inhibitor of basic steroid synthesis	3,4-DCA	150 µg/L	VTG−	140 µg/L[h]	Fecundity
Inhibitor of basic steroid synthesis	Atrazin	730 µg/L	VTG−	30–4300 µg/L[i]	Fecundity

Data (based on measured concentrations) obtained from Teigeler et al. (2007) and Braunbeck et al. (2009). Further data sources are explained in footnotes below. SED-MoA = sexual endocrine-disrupting mechanisms of action; ER = estrogen receptor; AR = androgen receptor; DCA = dichloroaniline. Biomarker: VTG+ = increase of vitellogenin in both sexes; VTG− = decrease of vitellogenin in females; 11kT+ = increase of 11-ketotestosterone in males. Population-relevant endpoint: fertility (decreased percentage of fertilized eggs), fecundity (decreased number of eggs due to decreased mating), sex ratio (increased number of males).

[a]Supplemental Data S1: 2-generation test with zebrafish (*Danio rerio*); Teigeler and Schäfers. EC50 values were determined by applying probit analysis (maximum likelihood regression) according to Finney (1971).

[b]Supplemental Data S2: 2-generation test with zebrafish (*D. rerio*); Böttcher et al. EC50 values were determined by applying probit analysis (maximum likelihood regression) according to Finney (1971).

[c]Supplemental Data S3: 2-generation test with zebrafish (*D. rerio*); Teigeler. EC50 values were determined by applying probit analysis (maximum likelihood regression) according to Finney (1971).

[d]Wenzel et al. (2001)

[e]Raw data from IDEA (Segner et al. 2003). Semistatic exposure; 3 renewals per week; data based on mean measured initial concentrations.

[f]Values are nominal concentrations. During the exposure period of 6 months, four analytical verifications of all treatment levels showed mean recoveries ± SD between 35 ± 12% and 50 ± 14%.

[g]Raw data from a confidential GLP study; for the SED-MoA confirmed by Schäfers (2007).

[h]Raw data from Ensenbach (1991), only fecundity (ELS more sensitive, different MoA).

[i]Nagel et al. (2004).

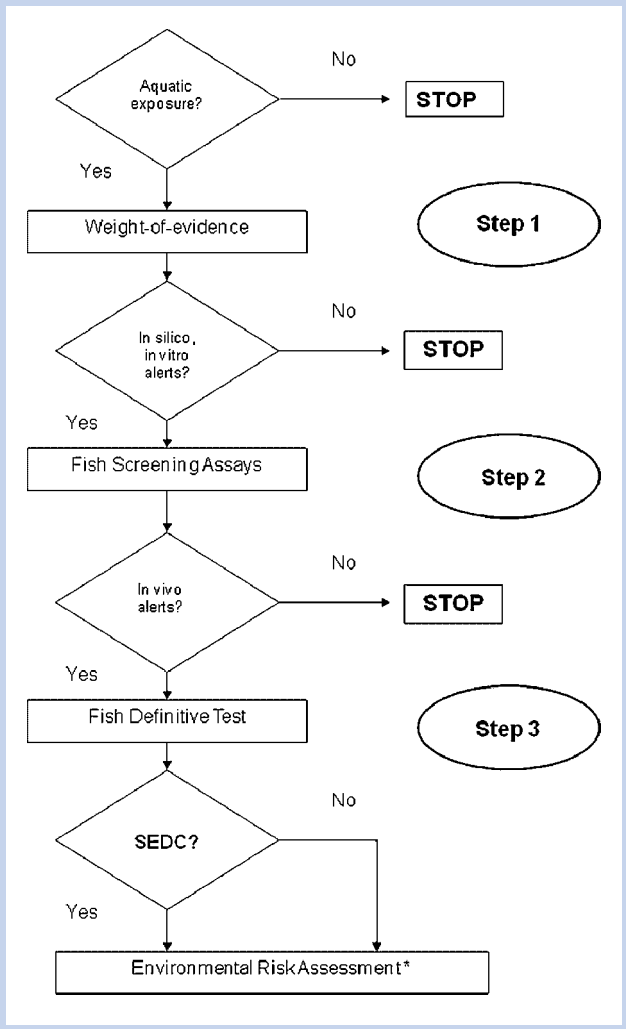

Figure 2. Schematic presentation of the proposed testing strategy with fish for sexual endocrine-disrupting chemicals. *In some legislation, the identification of a substance as an SEDC may initiate an authorization procedure or may lead to a nonregistration of the substance.

First step: Weight-of-evidence approach for identifying potential SEDCs

There is, so far, no standardized and cost-efficient screening method to identify all potential ED properties of chemical substances comprehensively without risking false-negative responses. Hence, for a substance released into the aquatic environment, all available information has to be assessed to evaluate the possibility of whether the substance might cause endocrine effects in fish (weight-of-evidence approach). When exposure of the aquatic compartment to the substance in question is demonstrated not to be relevant, the evaluation of data and specific testing to assess endocrine effects can be waived. Based on legal requirements, the competent authorities request a specific set of data according to the use of a chemical. This results in varying information levels for substances with different use patterns when evaluating the possibility of a substance of being a potential SEDC. Important information to justify the assumption of endocrine-disrupting properties of a chemical is in principle available from the following.

- Large acute to chronic ratio (ACR) with regard to the toxicity in fish (threshold value for ACR > 20 recommended by participants of the workshop Characterisation of Endocrine Mediated Effects on Fish held in Berlin, Germany, on 10 and 11 December, 2007)
- Evaluation of studies with mammals and birds with regard to endocrine-specific effects
- Reliable and relevant (preferably fully validated) in vitro assays
- (Q)SARs, read-across, chemical categories
- Knowledge of the mechanism of action (normally applicable only to active ingredients)

The ECHA (2008) gives advice on what data can be useful to evaluate the endocrine-disrupting potential of a substance. The guidance emphasizes that (Q)SARs, read-across, and chemical categories provide nontesting data, whereas estrogen- and androgen-receptor binding assays, reporter gene assays, and VTG and steroid genesis assays provide in vitro screening data. However, both the nontesting and in vitro screening methods are still under development. For compounds other than industrial chemicals, in particular, for pesticides and pharmaceuticals, extensive ecotoxicological data are usually available and can be reviewed. The complete set of compiled information requires a comprehensive, transparent evaluation to decide whether there is sufficient evidence to move on to the second step of the testing strategy.

Second step: In vivo confirmation of sexual endocrine-disrupting activity associated with specific mechanisms of action

When the weight-of-evidence approach reveals potential endocrine effects of the substance on fish, this finding has to be assessed by testing the substance in in vivo screening assays. The biomarkers measured according to the OECD guidelines for the screening assays (OECD 2009a, 2009b) allow the identification of 4 mechanisms of action related to sexual endocrine disruption. The measurement of 11-ketotestosterone, the androgen-receptor antagonist, which indicates the fifth mechanism of action, is not yet included as a validated endpoint in the screening assays. The following list denominates the mechanisms of action and their biomarkers, which, when affected by the exogenous substance, would indicate that the compound is an SEDC.

1. Estrogen-receptor agonist: Substance increases the VTG level in both sexes of fish, most clearly indicated in male fish.
3. Estrogen-receptor antagonist: Substance reduces the VTG level in fish; however, particularly in male fish, the reduction is difficult to measure, because VTG levels are already rather low under normal physiological conditions.
4. Androgen-receptor agonist: Substance is indicated by secondary sex characteristics for fathead minnow and medaka, whereas for zebrafish histopathological analyses are required.
5. Androgen-receptor antagonist: Substance causes compensatory increase of 11-ketotestosterone levels in fish.

Hutchinson et al. (2006) proposed a stepwise procedure for measuring the various biomarkers in the screening assays, starting with the biomarker that requires the least resources. Whenever a specific impact on a biomarker is indicated, the

analysis of the remaining biomarkers can be terminated, and the third step of the testing strategy, the definitive testing, can be initiated.

The 21d-FA, if subjected to further amendment by integrating the measurement of 11-ketotestosterone, would identify all SEDCs with regard to their intrinsic properties and thus contribute to the definition of the European Commission that ''an endocrine disrupter is an exogenous substance or mixture that alters functions of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations'' (EC 1999, 2007). However, the identification of a compound as an SEDC in either the extended 21d-FA or the STRA does not allow conducting a quantitative ERA, because population-relevant endpoints are not adequately addressed by the in vivo screening assays (Hutchinson et al. 2006). For substances without indication for an endocrine activity via the mechanisms covered by the extended 21d-FA, no further endocrine-specific fish testing is considered necessary. Eventually, this approach requires that the extended 21d-FA can confirm all of the 5 sexual endocrine mechanisms of action, which, for the time being, has been sufficiently demonstrated only for aromatase-inhibiting substances and estrogen-receptor agonists. Hence, when step 1 would not provide sufficient evidence to specify a probable mechanism of endocrine action, or as long as the assumed mechanism is not covered by available step 2 test systems, step 2 might be skipped, with direct progression to a life-cycle study according to step 3.

Third step: Characterization of sexual endocrine-mediated adverse effects, including their thresholds for regulatory use

A positive response of one of the in vivo screening assays triggers the performance of a definitive test to determine regulatory useful threshold concentrations, e.g., no observed effect concentration (NOEC), for population-relevant endpoints, i.e., survival, growth, and reproduction, including sexual development, which are required for ERAs. Publicly available experimental data from fish life-cycle studies (Teigeler et al. 2007; Braunbeck et al. 2009) showed that, for the already-mentioned sexual endocrine mechanisms of action, the following population-relevant endpoints were most sensitive.

- Aromatase inhibition: Sex ratio shift toward males, delays in growth and sexual development, impaired fecundity (number of eggs); the variability between the NOECs of these endpoints was in the range of a factor of 3.
- Estrogen-receptor agonist: Fertilization rate, growth, time to first spawn, and sex ratio shift toward females (medaka, fathead minnow) or delay of male sex development (zebrafish); the sensitivity of these endpoints was rather similar; however, depending on the fish species tested, the sequence from the most to the least sensitive endpoint can vary.
- Estrogen-receptor antagonist: Sex ratio shift toward males.
- Androgen-receptor agonist: Sex ratio shift toward males.
- Androgen-receptor antagonist: Spawning and fecundity (number of eggs) are affected.

The number of tested aromatase-inhibiting substances and estrogen-receptor agonists is large enough to justify acceptable confidence in the identified most sensitive population-relevant endpoints (Teigeler et al., 2007). For both mechanisms of action, tests exposing different developmental time windows demonstrate the sexual development phase to be the most critical life stage with respect to sensitivity and maintenance of effects.

The OECD Test Guidelines Programme has not yet finalized the protocols for definitive fish tests. However, definitive studies to be used in ERA should cover the following population-relevant endpoints.

- Mortality (e.g., survival of embryos, larvae, juveniles and adults)
- Growth and development (e.g., time to hatch, success rate of hatch, body weight and length, malformations, time to first spawn, secondary sex characteristics, sex ratio)
- Reproduction (e.g., fecundity; i.e., number of eggs laid; fertility, i.e., rate of fertilized eggs; sexual behavior, i.e., courtship)

Apart from sex ratio, time to first spawn, secondary sex characteristics, and behavior, all endpoints are also covered by USEPA's fish life-cycle test method (USEPA 1996).

From a general point of view, the measurement of indicative endpoints (biomarkers) such as VTG, secondary sexual characteristics, histological findings, and 11-ketotestosterone is not required in definitive tests. If, however, observed population-relevant effects in a definitive test can be associated with other than sexual endocrine-disrupting mechanisms of action, this might be important, e.g., for the authorization procedure according to article 57(f) under REACH (EC 2006) and according to the upcoming EU regulation on the placing of plant-protection products on the market (EU 2009). Indicative endpoint measurements can exclude or substantiate that adverse effects observed in a definitive test are caused by endocrine mechanisms (cf. Weybridge definition of EDs). Additionally, the integration of indicative endpoints into definitive tests can improve the differentiation between effects caused by an endocrine mode of action and effects caused by systemic toxicity. Crane et al. (2009) describe an approach using multicriteria decision analyses (MCDA) for selecting test endpoints in fish life-cycle studies.

The limited data available do not reveal conclusively whether the 2-genT is more sensitive than the FLCT (Schäfers et al. 2007). However, the 2-genT is required to identify potential transgenerational effects, i.e., effects that become obvious in the second generation after life-time exposure of the parental generation. Substances with high bioaccumulation potential, which might result in in ovo exposure (i.e., intrinsic exposure to a contaminant deposited in the egg following maternal exposure), and SEDCs, which affect the quality of eggs or sperm during early developmental stages, might be candidates for causing transgenerational effects. The proposed 2-genT design starts with the exposure of reproducing adults (P) and includes exposure of the first filial generation (F1) and the early life stages of the second filial generation (F2).

Alternatively, as with the FLCT, the 2-genT should start with the exposure of fertilized eggs and end with the sex determination of the following generation. This design has several advantages: 1) the number of test fish would be reduced by one-third, 2) the number of reproductive phases, which are very labor-intensive as well as causing high variability in the test results, would be reduced from 2 to 1

and, 3) the number of sexual maturation phases as the most important time windows of exposure would be enhanced from 1 to 2. In comparison with starting the 2-genT with reproductive adults, this would improve the statistical homogeneity of the first life-time-exposed generation. Starting with fertilized pooled eggs from several clutches improves the genetic homogeneity of eggs exposed to the different treatment levels. Properly planned and prepared, each FLCT can be transformed to a 2-genT when extended to the reproductive phase of the filial generation (Figure 1).

Instead of the FLCT or 2-genT, shorter tests might be acceptable as definitive test as long as the most sensitive time window of exposure and the most sensitive endpoints related to the specific sexual endocrine-disrupting mechanism of action of the tested substance are covered. For example, for aromatase-inhibiting substances, it has been shown that the NOEC values derived from FLCT and the NOEC values derived from FSDT are almost identical, because the most sensitive endpoints linked to sexual development are measured in both tests (Teigeler et al. 2007). Hence, if the introductory information and the in vivo screening assays reveal the mechanism of action as being aromatase inhibition for a chemical, the definitive test can be the FSDT. Possibly, for estrogen-receptor agonists, the FSDT can also replace the FLCT when the slightly lower sensitivity observed in the FSDT (fertilization rate not covered; Teigeler et al. 2007) is compensated for by a higher assessment factor. For the remaining sexual endocrine-disrupting mechanisms of action, the available data to compare results obtained from the FLCT with results from partial life cycle tests are still scarce.

CONCLUSIONS

The proposed testing strategy is a comprehensive concept that uses components of OECD's Conceptual Framework in such a way that sexual endocrine-disrupting chemicals can be identified and their ecotoxicological effects on fish measured and integrated into established compound-specific ERA schemes. With regard to fish testing, a similar, less specific approach has been proposed by ECETOC, which focuses on hazard rather than risk assessment and which, in addition to fish, includes other groups of organisms (ECETOC 2009).

In particular, the first step (weight-of-evidence approach) of the testing strategy, which describes the rules, the nontesting and in vitro screening methods for identifying potential sexual endocrine-disrupting chemicals, still requires more specific guidance, criteria, and supportive data. Another example for future validation work is the measurement of 11-ketotestosterone as an additional endpoint, which would allow the indication of androgen-receptor antagonistic action and could be used in all 3 recommended fish test species, i.e., fathead minnow, medaka, and zebrafish, in the in vivo fish screening assays or definitive fish tests. Moreover, for some mechanisms of endocrine action, i.e., the estrogen-receptor antagonist and androgen-receptor agonist and antagonist, more data generated with the endocrine-specific fish tests will be needed to confirm the assumed capacity of biomarkers to predict specific mechanisms. Several elements of the available guidance for FLCT and 2-genT should be harmonized, validated, and eventually transferred to an internationally accepted OECD Test Guideline or Guidance Document. The proposed testing strategy has to prove its practicability in real cases from different regulatory frameworks. Based on accumulating experience, the strategy should be subjected to further amendments and specification when necessary.

Testing strategies and ERA procedures related to substances that are not SEDCs should be based on endpoints such as mortality, growth, reproduction, and behavior, which integrate across all potential impacts of endocrine-disrupting chemicals at the organizational level of organisms (see, e.g., Ankley and Johnson 2004). In other words, because currently available screening assays cover only mechanisms of endocrine action related to SEDCs, for substances other than SEDCs there is no possibility to apply a tiered testing strategy. Such cases directly require the performance of life-cycle and/or multigenerational tests to eliminate reasonable suspicion of an endocrine mode of action.

SUPPLEMENTAL DATA

Supplemental Data S1. Tamoxifen Citrate SEDC fish testing stategy. (611 KB DOC)

Supplemental Data S2. Trenbolone SEDC fish testing stategy. (1619 KB DOC)

Supplemental Data S3. Flutamide SEDC fish testing stategy. (694 KB DOC)

Acknowledgment—The preparation of this paper was part of the project Studies To Support the Validation of a Definitive Fish Test (Full Life-Cycle/Two-Generation), sponsored by the German Environmental Protection Agency (UBA), Dessau, R + D project FKZ 204 67 454/02. The conclusions and recommendations made in this paper reflect the views of the authors as individual scientists and do not represent a position of the organizations with which the authors are affiliated. Furthermore, we thank 3 anonymous reviewers who initiated with their useful comments a significant improvement of the revised manuscript.

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