DRAFT PROPOSAL

Fish sexual Development Test

INTRODUCTION

1. This Guideline is based on a decision from 1998 to develop new or revise existing OECD Guidelines for the screening and testing of potential endocrine disrupters. The Fish Sexual Development Test (FSDT) was identified as a promising test method covering a sensitive fish life stage responsive to both estrogen and androgen-like chemicals. The test method went through an inter-laboratory validation exercise from 2006 to 2010 where Japanese medaka (Oryzias latipes) and zebrafish (Danio rerio) were fully validated; .. The protocol is in principle an enhancement of OECD Guideline No. 210 ‘Fish, Early Life Stage Toxicity Test’ [OECD TG 210 1992], where the exposure is continued until the fish are sexually differentiated, i.e. about 60 days post-hatch. The FSDT assesses early life-stage effects and potential adverse consequences of putative endocrine disrupting chemicals (e.g., estrogens, androgens and steroidogenesis inhibitors) on sexual development. The combination of the two core endocrine endpoints, vitellogenin concentration and the population-relevant sex ratio enable the test to be used for hazard and risk assessment when the chemical mode of action is known.

2. The protocol is based on exposure via water to chemicals during the sex labile period in which the fish is expected to be most sensitive toward the effects of endocrine disrupting chemicals. Two core endpoints are measured as indicators of endocrine-associated developmental aberrations: the vitellogenin (VTG) concentrations and sex ratios (proportions of sex) determined via gonad histology. Gonadal histopathology (evaluation and staging of oocytes and spermatogenetic cells) is optional. Additionally the genetic sex is determined whenever possible (e.g. in Japanese medaka). Other apical endpoints measured include hatching rate, survival, length and body weight. The test method might also be adaptable to other species than those mentioned above, provided fish are sexually differentiated at the end of the test, vitellogenin levels are sufficiently high to detect significant chemical-related variations, and sensitivity of the test system is established using chemicals already tested during the validation studies (OECD, 2011).

INITIAL CONSIDERATIONS AND LIMITATIONS

3. Vitellogenin (VTG) is normally produced by the liver of female oviparous vertebrates in response to circulating endogenous estrogen [Jobling et al. 1996]. It is a precursor of egg yolk proteins and, once produced in the liver, travels in the bloodstream to the ovary, where it is taken up and modified by developing eggs. The VTG synthesis is very limited, though detectable, in immature fish and adult male fish because they lack sufficient circulating estrogen; however, the liver is capable of synthesizing and secreting VTG in response to exogenous estrogen stimulation [Holbech et al. 2001; Tyler et al. 1999].

4. The measurement of vitellogenin serves for the detection of chemicals with various modes of action. The detection of estrogenic chemicals is possible via the measurement of VTG induction in male fish, and it has been abundantly documented in the scientific peer-reviewed literature. Vitellogenin induction has also been demonstrated following exposure to aromatizable androgens [Orn et al. 2003; Andersen et al. 2003]. A reduction in the circulating level of estrogen in females, for instance through...
the inhibition of the aromatase converting the endogenous androgen to the natural estrogen 17β-estradiol, causes a decrease in the VTG level, which is used to detect chemicals having aromatase inhibiting properties or steroidogenesis inhibitors more broadly [Kinnberg et al. 2007]. The biological relevance of the VTG response following estrogenic/aromatase inhibition is established and has been broadly documented. However, it is possible that production of VTG in females can also be affected by general toxicity and non-endocrine toxic modes of action, e.g. hepatotoxicity.

5. Several measurement methods have been successfully developed and standardised for routine use. This is the case for species included in this Guideline: species-specific Enzyme-Linked Immunosorbent Assay (ELISA) methods exists using immunochemistry for the quantification of VTG from blood, liver or head/tail homogenate samples collected from individual [Hahlbeck et al. 2004; Holbech et al. 2001; Nishi et al. 2002; Parks et al. 1999]. In both the Japanese medaka and the zebrafish, there is a good correlation between VTG measured from blood, liver and homogenate samples. Annex 5 provides the recommended procedures for sample collection for VTG analysis. Species-specific and validated ELISA-kits for the measurement of vitellogenin are widely available.

6. Change in the sex ratio (proportions of sex) is an endpoint reflecting phenotypic sex reversal. In principle, estrogens, anti-estrogens, androgens, anti-androgens and steroidogenesis inhibiting chemicals can affect the sex ratio of developing fish [Scholz and Kluver, 2009]. It has been shown that this sex reversal is partly reversible in zebrafish following estrogenic-related chemical exposure [Fenske et al. 2005], whereas sex reversal following androgenic-related chemical exposure is permanent [Morthorst et al. 2010]. The sex is defined as either female, male, intersex or undifferentiated; this is determined in individual fish via histological examination of the gonads.

7. Genetic sex is examined via genetic markers when they exist in a given fish species. In Japanese medaka the female xx or male xy genes can be detected by PCR, alternatively the Y-linked DM domain gene (DMY) can be analysed (DMY negative or positive) as described by [Kobayashi et al. 2004; Shinomiya et al. 2004]. Where the genetic sex can be individually linked to the phenotypic sex, the power of the test is improved and therefore genetic sex should be determined in species with documented genetic sex markers.

8. The two core endocrine endpoints VTG and sex ratio can in combination demonstrate the endocrine mode of action of the chemical (Table 1). The sex ratio is a population relevant biomarker [Kidd et al. 2007; Palace et al. 2009] and for some well defined modes of action, the FSDT results may be used for hazard and risk assessment purposes. These modes of action are at present androgens and steroidogenesis inhibitors. For estrogenic-like chemicals, reversibility of effects on the sexual differentiation may limit the use of the FSDT to a screening-type of assessment.

9. The FSDT does not cover the reproductive life stage of the fish and therefore chemicals that are suspected to affect reproduction at lower concentrations than sexual development should be examined in a test that covers reproduction.

Table 1

<table>
<thead>
<tr>
<th>MOA</th>
<th>Vtg ♂</th>
<th>Vtg ♀</th>
<th>Sex ratio</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weak estrogen agonist</td>
<td>↑</td>
<td>↑</td>
<td>↑♀</td>
<td>[Panter et al. 2006]</td>
</tr>
<tr>
<td>Strong estrogen agonist</td>
<td>↑</td>
<td>↑</td>
<td>↑♀, No ♂</td>
<td>[Holbech et al. 2006]</td>
</tr>
<tr>
<td>Estrogen antagonist</td>
<td>?</td>
<td>↓</td>
<td>↓♀, ↑Undiff.</td>
<td>[Andersen et al. 2004]</td>
</tr>
<tr>
<td>Weak androgen agonist</td>
<td>?</td>
<td>?</td>
<td>?♂</td>
<td></td>
</tr>
<tr>
<td>Strong androgen agonist</td>
<td>↓ or -</td>
<td>↓ or -</td>
<td>↑♂, No ♀</td>
<td>[Holbech et al. 2006; Morthorst et al. 2010]</td>
</tr>
</tbody>
</table>
Androgen antagonist

<table>
<thead>
<tr>
<th>Androgen antagonist</th>
<th>↑</th>
<th>↑♀</th>
<th>↑intersex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aromatase inhibitor</td>
<td>↓</td>
<td>↓</td>
<td>↓♀</td>
</tr>
</tbody>
</table>

[Panter et al. 2004; Kiparissis et al. 2003]

[Kinnberg et al. 2007]

10. Definitions used in this Test Guideline are given in Annex 1.

11. The in vivo FSDT is intended to detect chemicals with androgenic or estrogenic properties as well as anti-androgenic, anti-estrogenic and aromatase inhibiting properties.

**PRINCIPLE OF THE TEST**

12. In the test, fish are exposed from newly fertilized eggs until sexual differentiation has finished to at least three concentrations of the test chemical dissolved in water, preferably under flow-through conditions. The test starts with the placing of 160 newly fertilized eggs per treatment divided between at least 4 replicates. For the validated fish species Japanese medaka and zebrafish, the test is terminated at 60 days post hatch (dph). At test termination, all fish are euthanized humanely. A biological sample (liver or head/tail homogenate) is collected for VTG analysis from each fish and the remaining part is fixed for histological evaluation of the gonads to determine the phenotypic sex; optionally histopathology can be performed. A biological sample for the determination of the genetic sex is also taken in species possessing appropriate markers. The combination of VTG measurements and sex ratio (analysed as proportion of sex) adds weight to the assessment of effects and likely mode of action (as seen in Table 1) of the chemical in many cases.

13. An overview of relevant test conditions specific for two validated species: Japanese medaka and zebrafish is provided in Annex 2.

**INFORMATION ON THE TEST SUBSTANCE**

14. Results from an acute toxicity test or other short-term toxicity assay preferably performed with the species chosen for this test, should be available. This implies that the water solubility and the vapor pressure of the test substance are known and a reliable analytical method for the quantification of the substance in the test solutions with known and reported accuracy and limit of detection is available.

15. Useful information includes the structural formula, purity of the substance, stability in water and light, pKₐ, Pₗw and results of a test for ready biodegradability (OECD TG 301).

**TEST ACCEPTANCE CRITERIA**

16. For the test results to be acceptable the following conditions apply:

- the dissolved oxygen concentration should be at least 60 per cent of the air saturation value throughout the test;

- the water temperature should not differ by more than ± 1.5 °C between test vessels at any one time during the exposure period and be maintained within a range of 2°C within the temperature ranges specified for the test species (Annex 2);

- evidence should be available to demonstrate that the concentrations of the test substance in solution have been satisfactorily maintained within ±20% of the mean measured values;

- overall survival of fertilized eggs in the controls and, where relevant, in the solvent controls should be greater than or equal to the limits defined in Annex 2;
- acceptance criteria related to growth and proportions of sex (control group/solvent control group mean values, based on data pooled from replicates at termination of the test):

<table>
<thead>
<tr>
<th></th>
<th>Japanese medaka</th>
<th>Zebrfish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth Weight (wet weight*)</td>
<td>&gt;150 mg</td>
<td>&gt;75 mg</td>
</tr>
<tr>
<td>Length (standard length)</td>
<td>&gt;20 mm</td>
<td>&gt;14 mm</td>
</tr>
<tr>
<td>Sex ratio (% males or females)</td>
<td>30-70 %</td>
<td>30-70 %</td>
</tr>
</tbody>
</table>

(*weight = Fish wet weight (blotted dry))

- When a solvent is used it should have no significant effect on survival and should not produce any other adverse effects on the early-life stages as revealed by a solvent control.

**DESCRIPTION OF THE METHOD**

**Test chambers**

17. Any glass, stainless steel or other chemically inert vessels can be used. The dimensions of the vessels should be large enough to allow compliance with loading rate criteria given below. It is desirable that test chambers be randomly positioned in the test area. A randomized block design with each treatment being present in each block is preferable to a completely randomized design. The test chambers should be shielded from unwanted disturbance.

**Selection of species**

18. The test has been validated using the Japanese medaka (*Oryzias latipes*) and the zebrafish (*Danio rerio*). The test might be adaptable to other species provided i) fish are sexually differentiated at the end of the test, ii) VTG levels are sufficiently high to detect significant chemical-related variations, and iii) sensitivity of the test system is established using chemicals that have been used in the validation studies (OECD, 2011). The rationale for the selection of the species and the experimental method should be reported in this case.

**Holding of the brood fish**

19. Details on holding the brood stock under satisfactory conditions may be found in OECD Guideline No. 210 (OECD, 1992). Parental fish should be fed once or twice a day with appropriate food. To avoid genetic bias, eggs are collected from a minimum of three breeding pairs or groups, mixed and randomly selected to initiate the test.

**Handling of embryos and larvae**

20. Initially, embryos and larvae may be exposed within the main vessel in smaller glass or stainless steel vessels, fitted with mesh sides or ends to permit a flow of test solution through the vessel. Non-turbulent flow through small vessels may be induced by suspending them from an arm arranged to move the vessel up and down but always keeping the organisms submerged.

21. Where egg containers, grids or meshes have been used to hold eggs within the main test vessel, these restraints should be removed after the larvae hatch, except that meshes should be retained to prevent the escape of the fish. If there is a need to transfer the larvae, they should not be exposed to the air, and
nets should not be used to release fish from egg containers. The timing of this transfer varies with the species and transfer may not always be necessary.

**Water**

22. Any water in which the test species shows control survival at least as good as that described in Annex 3 is suitable as test water. It should be of constant quality during the period of the test. In order to ensure that the dilution water will not unduly influence the test result (for example by complexation of test substance) or adversely affect the performance of the brood stock, samples should be taken at intervals for analysis. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl, S0₄), pesticides, total organic carbon, conductivity, pH and suspended solids should be made, for example every three months where a dilution water is known to be relatively constant in quality.

**Test solutions**

23. Flow-through system is recommended. For flow-through tests, a system which continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, and saturator system) is required to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water should be checked at intervals during the test and should not vary by more than 10% throughout the test. A flow rate equivalent to at least five test chamber volumes per 24 hours has been found suitable [OECD, 1992]. Care should be taken to avoid the use of plastic tubing or other materials, some of which may contain biologically active substances or may adsorb the test substance.

24. The stock solution should preferably be prepared without the use of solvents by simply mixing or agitating the test substance in the dilution water by using mechanical means (e.g. stirring or ultrasonication). If the test substance is difficult to dissolve in water, procedures described in the OECD Guidance for handling difficult substances should be followed ([OECD, 2000]). The use of solvents should be avoided but may be required in some cases in order to produce a suitably concentrated stock solution. Examples of suitable solvents are given in the Guidance Document on difficult to test substances (OECD, 2000).

25. Semi-static test conditions should be avoided unless there are compelling reasons associated with the test chemical (e.g., stability, limited availability, high cost or hazard). For the semi-static technique, two different renewal procedures may be followed. Either new test solutions are prepared in clean vessels and surviving eggs and larvae gently transferred into the new vessels, or the test organisms are retained in the test vessels whilst a proportion (at least two thirds) of the test water is changed daily.

**PROCEDURE**

**Conditions of Exposure**

**Duration**

26. The test should start as soon as possible after the eggs have been fertilized, the embryos preferably being immersed in the test solutions before cleavage of the blastodisc commences, or as close as possible after this stage. The test should continue until sexual differentiation in the control group is completed (60 days post hatch for Japanese medaka and zebrafish).
Loading

27. The number of fertilized eggs at the start of the test should be at least 160 per treatment divided between a minimum of 4 replicates. The eggs should be randomly distributed among treatments. The loading rate (fish biomass per volume of test solution) should be low enough in order that a dissolved oxygen concentration of at least 60% of the air saturation value (ASV) can be maintained without directly aeration of the aquaria units. For flow-through tests, a loading rate not exceeding 0.5 g fish/l per 24 hours and not exceeding 5 g fish/l of solution at any time has been recommended.

Light and temperature

28. The photoperiod and water temperature should be appropriate for the test species (see Annex 2).

Feeding

29. Food and feeding are critical, and it is essential that the correct food for each stage should be supplied from an appropriate time and at a level sufficient to support normal growth. Feeding should be ad libitum whilst minimizing the surplus. To obtain a sufficient growth rate it is recommended that fish are fed at least twice daily (accepting once daily in weekends), separated by at least three hours between each feeding. Surplus food and faeces should be removed as necessary to avoid accumulation of waste. Detailed feeding regimes are given in OECD Guideline 210 ([OECD, 1992]) but, as experience is gained, food and feeding regimes are continually being refined to improve survival and optimize growth. Effort should therefore be made to confirm the proposed regime with acknowledged experts. Feeding should be withheld 24 hours before ending the test.

Test concentrations

30. Test substances should be spaced as described in Annex 4. A minimum of three test concentrations in at least four replicates should be used. The curve relating LC₅₀ to period of exposure in the acute studies available should be considered when selecting the range of test concentrations. Five test concentrations are recommended if the data are to be used for risk assessment.

31. Concentrations of the substance higher than 10% of the acute adult LC₅₀ or 10 mg/l, whichever is the lower, need not be tested. The maximum test concentration should be 10% of the LC₅₀ on the larval/juvenile life-stage.

Controls

32. One set of dilution-water control replicates and also, if relevant, one control containing the solvent should be run in addition to the test series. Only solvents that has been investigated not to have any significant influence on the test endpoints should be used in the test.

33. Where a solvent is used, its final concentration should not be greater than 0.1 ml/L and should be the same in all test vessels. However, every effort should be made to avoid the use of such materials or keep concentrations to a minimum.

Frequency of Analytical Determinations and Measurements

34. Chemical analysis of the test substance water concentration should be performed before initiation of the test to check compliance with the validity criteria. All replicates should be analyzed at the beginning and termination of the test. One replicate per test concentration should be analyzed at least once per week during the test, changing systematically between replicates. If samples are stored to be analyzed at a later
point, the storage method of the samples should be previously validated. Samples should be filtered (e.g. using a 0.45 µm pore size) or centrifuged to ensure that the determinations are made on the substance in true solution. Results shall be based on measured concentrations.

35. During the test, dissolved oxygen, pH, total hardness, conductivity and salinity (if relevant) and temperature should be measured in all test vessels. As a minimum dissolved oxygen, salinity (if relevant) and temperature should be measured weekly, and pH, conductivity and hardness at the beginning and end of the test. Temperature should preferably be monitored continuously in at least one test vessel.

36. Results should always be based on measured concentrations and included in reporting.

Observations

37. **Stage of embryonic development**: The exposure should begin less than 24 h post fertilisation to ensure exposure during early embryonic development.

38. **Hatching and survival**: Observations on hatching and survival should be made at least once daily and numbers recorded. Dead embryos, larvae and juvenile fish should be removed as soon as observed since they can decompose rapidly and may be broken up by the actions of the other fish. Extreme care should be taken when removing dead individuals not to knock or physically damage adjacent eggs/larvae, these being extremely delicate and sensitive. Criteria for death vary according to life stage:

- for eggs: particularly in the early stages, a marked loss of translucency and change in coloration, caused by coagulation and/or precipitation of protein, leading to a white opaque appearance;
- for larvae and juvenile fish: immobility and/or absence of respiratory movement and/or absence of heart-beat and/or white opaque coloration of central nervous system and/or lack of reaction to mechanical stimulus.

39. **Abnormal appearance**: The number of larvae or fish showing abnormality of body form should be recorded at adequate intervals and the nature of the abnormality described. It should be noted that abnormal embryos and larvae occur naturally and can be of the order of several per cent in the control(s) in some species. Abnormal animals should only be removed from the test vessels on death. However, this may be in conflict with some local regulations, therefore if considerable suffering (very severe and death can be reliably predicted) is observed, animals should be anaesthetized and euthanized according to the description in paragraph 44 and treated as mortality for data analysis.

40. **Abnormal behaviour**: Abnormalities, e.g. hyperventilation, uncoordinated swimming, atypical quiescence and atypical feeding behaviour should be recorded at adequate intervals depending on the duration of the test.

41. **Weight**: At the end of the test all surviving fish should be blotted dry and individually weighed.

42. **Length**: At the end of the test, measurement of individual lengths (standard length) is recommended.

43. These observations will result in some or all of the following data being available for statistical analysis:

- cumulative mortality;
- numbers of healthy fish at end of test;
- time to start of hatching and end of hatching;
- length and weight of surviving animals;
Version of 2 November 2010

numbers of deformed larvae;
numbers of fish exhibiting abnormal behaviour.

**Sampling of fish**

44. Fish sampling is performed at termination of the test. Sampled fish should be anaesthetized and euthanized with e.g. MS-222 (100-500 mg per L buffered with 200 mg NaHCO3 per L) or FA-100 (4-allyl-2-methoxyphenol: eugenol) and individually measured and weighed as wet weight (blotted dry).

**Sampling for VTG analysis and sex determination via histological evaluation**

45. All fish should be sampled and prepared for analysis VTG and sex determination via gonadal histology. For the VTG measurements, a sub-sampling from at least 10 fish from each replicate is accepted. More fish should be analyzed for VTG if the results of the sub-sampling turn out to be unclear.

46. The sampling procedure for VTG and sex determination is dependant on the VTG analysis method:

*Head/tail homogenate method for VTG analysis*

47. Head and tail of each fish are separated from the body of the fish by cuts made: right behind the pectoral fins, and right behind the dorsal fin, using a scalpel. See Figure 1. The head and tail part from each fish are pooled, weighed and individually numbered, frozen in liquid nitrogen and stored at -70º or less for VTG analysis. The body part of the fish is numbered and fixed in an appropriate fixative for histological evaluation. By use of this method, VTG and histopathology are evaluated on each individual and a possible change in the VTG level can thus be related to the sex of the fish. **Annex 6 of OECD Guideline 230 (OECD, 2009)** provides further guidance on homogeneisation of head and tail and further quantification of VTG.

*Liver homogenate method*

48. The liver is dissected out and stored at -70º or less. Guidance on procedures for liver excision and pre-treatment is available from **Annex 6 of OECD Guideline 230 (OECD, 2009)**. Livers are then individually homogenized and the supernatant collected is then used for measuring VTG with a homologous ELISA technique (see **Annex 5 for quantification in zebrafish**). Following this approach, it is also possible to have individual fish data on both VTG and gonad histology.

*Plasma method*

49. Blood is collected from the anaesthetized fish and centrifuged for plasma collection. The plasma is stored at -70º or less until use. The whole fish is euthanized and fixed for histology. Both plasma samples and fish are numbered individually to relate VTG levels to the sex of the fish.

![Figure 1: How to cut a fish for measurement of VTG in head/tail homogenate and histological evaluation of the mid section](image-url)
Genetic sex determination

50. A biological sample for the determination of the genetic sex is taken from individual fish in species possessing appropriate markers. For Japanese medaka a description is given in Annex 8 including tissue sampling and sex determination by a PCR-method.

Vitellogenin (VTG) measurement

51. The measurement of VTG should be based upon a quantitative and validated method. Information should be available upon the intra-assay and inter-assay variability of the method used in a given laboratory. Different methods are available to assess VTG production in the fish species considered in this assay. A measurement technique that is both relatively sensitive and specific is determination of protein concentrations via enzyme-linked immunosorbant assay (ELISA). It is recommended to use homologous antibodies (raised against VTG of the same species) and most important homologous standards. Validation of a quantitative method is essential to obtain meaningful results. Ideally, an internal standard, and VTG standards should be widely available.

Sex determination

52. Dependent on the VTG sampling procedure, whole fish or the remaining mid-section of each fish is placed in a pre-labeled processing cassette and fixed in an appropriate fixative for histological determination of sex and evaluation of gonadal staging. Guidance on fixation and embedding is provided in Section 2 Post-mortem and histotechnical procedures of the OECD Guidance Document No.123 on the Diagnosis of Endocrine-Related Histopathology of Fish Gonads [OECD, 2010]. After processing, 6-8 individuals are embedded in each paraffin block. The individuals should be placed longitudinally in the paraffin block. At least six longitudinal sections (3-5 µm in thickness) in a frontal plane including gonadal tissue from both gonads are taken from each individual. The interval between these sections should be approximately 50 µm for males and 250 µm for females. However, since each block will often contain both males and females, the interval between sections from these blocks should be approximately 50 µm until at least six sections of the gonads from each male are obtained. Thereafter, the interval between sections can be increased to approximately 250 µm for the females. Sections are stained with haematoxylin and eosin and examined by light-microscopy with focus on sex (male, female, intersex or undifferentiated) and staging of ovaries and testis by determination of presence of vitellogenic oocytes (yes/no) and spermatozoa (yes/no), respectively. It should be noted that some fish species naturally lack a fully developed pair of gonads and only one gonad may be present (e.g. Japanese medaka). All such observations should be recorded.

53. Genetic sex determination in individual Japanese medaka is based on the presence or absence of the medaka male-sex determining gene, DMY, which is located on the Y chromosome. The genotypic sex of medaka can be identified by sequencing the DMY gene from DNA extracted from for instance a piece of anal fin. The presence of DMY indicates a XY (male) individual regardless of phenotype, while the absence of DMY indicates a XX (female) individual regardless of phenotype [Kobayashi et al. 2004]. Guidance for tissue preparation and PCR method is given in Annex 7.

54. The occurrence of testicular oocytes should be diagnosed. Concerning interpretation of intersex determination, it should be noted that a baseline level of 5% individuals of a population with mild intersex is regarded as normal in zebrafish. In Japanese medaka, a baseline level of intersex of 3% is regarded as normal. In case these rates were found at a higher incidence or severity per individual (i.e. change>> mild), this should be weighed against historical control data and subjected to investigator’s judgment. However, in the interpretation, other factors must be weighed, such as dose-responsiveness, biological plausibility, additional estrogenic or anti-androgenic effects etc. A grading system, such as recommended in the OECD Guidance Document No.123 [OECD, 2010] could be helpful.
Secondary sexual characteristics

55. Secondary sexual characters are under endocrine control in species like the Japanese medaka; therefore observations of physical appearance of the fish should if possible be made at the end of the exposure. In the Japanese medaka, the papillary formation on the posterior part of the anal fin in females is androgen sensitive. OECD Guidelines 230 provides relevant photographs of male secondary sex characteristics and androgenised females.

DATA AND REPORTING

Treatment of results

56. A decision flow-chart is available in Annex 6 to help with the most appropriate statistical test to use based on the characteristic of the data obtained from the test.

Proportions of sex

57. The proportions of sex should be analysed for significant effect (NOEC/LOEC approach) of exposure by Jonckheere-Terpstra (Trend test) if a monotone dose-response exists. If non-monotonicity is found then a pair wise test should be applied: Use Dunnett’s test if normality and homogenous variance can be obtained. Use Tamhane-Dunnett if heterogeneous variance is present. Otherwise use exact Mann-Whitney test with Bonferroni-Holm adjustment.

Vitellogenin (VTG) concentrations

58. VTG concentrations should be analysed for significant effect (NOEC/LOEC approach) of exposure by a parametric one way ANOVA followed by Multiple Comparisons versus Control Group (Bonferroni t-test): If normality and homogenous variance can not be obtained, then a Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Multiple Comparisons versus Control Group (Dunn's Method) should be used.

Interpretation of results

59. The results should be interpreted with caution where measured toxicant concentrations in test solutions occur at levels near the detection limit of the analytical method.

Test report

60. The test report should include the following information:

Test substance: Relevant physical-chemical properties; chemical identification data including purity and analytical method for quantification of the test substance where appropriate.

Test conditions: Test procedure used (e.g. semi-static/renewal, flow-through); test design including test concentrations, method of preparation of stock solutions, frequency of renewal (the solubilising agent and its concentration should be given, when used); the nominal test concentrations, the means of the measured values and their standard deviations in the test vessels and the method by which these were attained and evidence that the measurements refer to the concentrations of the test substance in true solution; dilution water characteristics (including pH, hardness, alkalinity, temperature, dissolved oxygen concentration, residual chlorine levels, total organic carbon, suspended solids and any other measurements made); water quality within test vessels: pH, hardness, temperature and dissolved oxygen concentration;
detailed information on feeding (e.g. type of food(s), source, amount given and
frequency and analyses for relevant contaminants (e.g. PCBs, PAHs and
organochlorine pesticides).

Results: Evidence that controls met the validity criteria, data on mortalities occurring in any
of the exposed groups; statistical analytical techniques used, treatment of data and
justification of techniques used. Clear indication of the results obtained on the
different endpoints observed: embryo survival and hatching success; external
abnormalities; vitellogenin measurements (ng/g fish, ng/ml blood or ng/mg liver);
gonadal histology and sex ratio. Incidence of any unusual reactions by the fish and
any visible effects produced by the test substance.

The results should be presented as mean values ± standard deviation (SD) or standard error (SE).
Statistics should be reported as a minimum as NOEC/LOEC and confidence intervals. The statistical flow
chart (Annex 8) should be followed.

LITERATURE

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ANNEX 1

ABBREVIATIONS & DEFINITIONS

FSDT: Fish Sexual Development Test
TG: Test Guideline
SOP: Standard Operational Procedure
GLP: Good Laboratory Practice
ELISA: Enzyme-Linked Immunosorbent Assay
Loading rate - the wet weight of fish per volume of water.
Stocking density - is the number of fish per volume of water.
VTG - vitellogenin
HPG axis: hypothalamic-pituitary-gonadal axis
## ANNEX 2

### EXPERIMENTAL CONDITIONS FOR THE FISH SEXUAL DEVELOPMENT TEST

1. **Recommended species**
   - Japanese medaka (*Oryzias latipes*)
   - Zebrafish (*Danio rerio*)

2. **Test type**
   - Flow-through or semi-static
   - Flow-through or semi-static

3. **Water temperature**
   - 25 ± 2 °C
   - 27 ± 2 °C

4. **Illumination quality**
   - Fluorescent bulbs (wide spectrum)
   - Fluorescent bulbs (wide spectrum)

5. **Light intensity**
   - 10-20 µE/M²/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)
   - 10-20 µE/M²/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)

6. **Photoperiod**
   - 16 h light, 8 h dark
   - 16 h light, 8 h dark

7. **Loading rate**
   - <5 g per L at any time and not exceeding 0.5 g/L/24h
   - <5 g per L at any time and not exceeding 0.5 g/L/24h

8. **Volume exchanges of test solutions**
   - Minimum of 5 daily
   - Minimum of 5 daily

9. **Age of test organisms at start of exposure**
   - Newly fertilised eggs
   - Newly fertilised eggs

10. **No. of eggs per treatment**
    - Minimum 160
    - Minimum 160

11. **No. of treatments**
    - Minimum 3 (plus appropriate controls)
    - Minimum 3 (plus appropriate controls)

12. **No. replicates per treatment**
    - 4 (minimum)
    - 4 (minimum)

13. **Feeding regime**
    - Live *Artemia*, frozen adult brine shrimp, flake food, etc. It is recommended to feed twice daily
    - Live *Artemia*, frozen adult brine shrimp, flake food, etc. It is recommended to feed twice daily

14. **Aeration**
    - None unless DO concentration falls below 70% saturation
    - None unless DO concentration falls below 70% saturation

15. **Dilution water**
    - Clean surface, well or reconstituted water
    - Clean surface, well or reconstituted water

16. **Chemical exposure duration**
    - 60-dph
    - 60-dph

17. **Biological endpoints**
    - Hatching success, Survival, Gross- morphology, VTG, gonadal histology, Genetic sex, Sex ratio
    - Hatching success, Survival, Gross- morphology, VTG, gonadal histology, Sex ratio

18. **Test acceptability**
    - Hatching success > 80%
    - Hatching success > 80%
    - Post hatch survival ≥ 70% of fish in the controls
    - Post hatch survival ≥ 70% of fish in the controls
ANNEX 3

SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

<table>
<thead>
<tr>
<th>SUBSTANCE</th>
<th>CONCENTRATIONS</th>
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<tbody>
<tr>
<td>Particular matter</td>
<td>&lt; 20 mg/l</td>
</tr>
<tr>
<td>Total organic carbon</td>
<td>&lt; 2 mg/l</td>
</tr>
<tr>
<td>Unionised ammonia</td>
<td>&lt; 1 ug/l</td>
</tr>
<tr>
<td>Residual chlorine</td>
<td>&lt; 10 ug/l</td>
</tr>
<tr>
<td>Total organophosphorus pesticides</td>
<td>&lt; 50 ng/l</td>
</tr>
<tr>
<td>Total organochlorine pesticides plus polychlorinated biphenyls</td>
<td>&lt; 50 ng/l</td>
</tr>
<tr>
<td>Total organic chlorine</td>
<td>&lt; 25 ng/l</td>
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</table>
ANNEX 4

(Might go out)

FROM TG 215/GUIDANCE ON TEST CONCENTRATIONS

<table>
<thead>
<tr>
<th>Column (Number of concentrations 100 and 10, or between 10 and 1)*</th>
<th>1</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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</tbody>
</table>

* A series of three (or more) successive concentrations may be chosen from a column. Mid-points between concentrations in column (x) are found in column (2x + 1). The values listed can represent concentrations expressed as percentage per volume or weight (mg/l or μg/l). Values can be multiplied or divided by any power of 10 as appropriate. Column 1 might be used if there was considerable uncertainty on the toxicity level.

ANNEX 5

GUIDANCE: QUANTIFICATION OF HEAD & TAIL HOMOGENATE VITELLOGENIN IN ZEBRAFISH (DANIO RERIO) (MODIFIED FROM HOLBECH ET AL., 2001)

1. Microtiterplates (certified Maxisorp F96, Nunc, Roskilde Denmark) previously coated with 5 µg/ml anti-zebrafish lipovitellin-IgG are thawed and washed 3 times with washing buffer*.

2. Battelle zebrafish Standard AP4.6.04 (1.18 mg/ml (AAA)) is serially diluted to 0.2, 0.5, 1, 2, 5, 10 and 20 ng/ml in dilution buffer** and samples are diluted at least 200 times (to prevent matrix effect) in dilution buffer and applied to the plates. An assay control is applied in duplicate. 150 µl are applied to each well. Standards are applied in duplicate and samples in triplicate. Incubate overnight at 4°C on a shaker.

3. The plates are washed 5 times with washing buffer*

4. AMDEX (HRP coupled to a dextran chain (AMDEX A/S, Denmark)) conjugated antibodies are diluted in washing buffer; Actual dilution differs by batch and age. 150 µl are applied to each well and the plates are incubated for 1 hour at room temperature on a shaker.

5. The plates are washed 5 times with washing buffer* and the bottom of the plates is carefully cleaned with ethanol.

6. 150 µl TMB plus*** are applied to each well. Protect the plate against light with tinfoil, and watch the colour development on a shaker.

7. When the standard curve is fully developed the enzyme activity is stopped by adding 150 µl 0.2 M H₂SO₄ to each well.

8. The absorbance is measured at 450 nm (e.g. on a Molecular Devices Thermomax plate reader). Data are analysed on the associated software (e.g. Softmax).

* Washing buffer:

PBS-stock**** 500.0 ml
BSA 5.0 g
Tween 20 5.0 ml
Adjust pH to 7.3 and fill to 5 l with millipore H₂O. Store at 4°C.

** Dilution buffer

PBS-Stock*** 100.0 ml
BSA 3.0 g
Tween 20 1.0 ml
Adjust pH to 7.3 and fill to 1 l with millipore H₂O. Store at 4°C.

*** TMB plus is a “ready-to-use” substrate produced by KemEnTec (Denmark). It is sensitive to light.
Store at 4°C.

**** PBS stock

NaCl 160.0 g
KH₂PO₄ 4.0 g
Na₂HPO₄,2H₂O 26.6 g
KCl 4.0 g
Adjust pH to 6.8 and fill with millipore H₂O to 2 l. Store at room temperature.
ANNEX 6

Statistical Flow Chart for vitellogenin analysis

Both solvent control and non-solvent control are present.

Yes

Compare controls using Wilcoxon or T-test

Do controls differ?

No

Yes

Drop water control

Combine controls, retain subgroups

Determine whether Dose-Response is monotone

Monotone

Step-down trend test on replicate means

Rep means normal & homogeneous

Step-down Jonckheere or Williams test

Rep means not normal or not homogeneous

Not monotone

Rep means normally distributed

Variances equal

Dunn test

Variances unequal

Dunn or Mann-Whitney test

Rep means not normally distributed

Normalizing transform?

No

Yes

Nested ANOVA normal

Nested ANOVA not normal

<=>3 reps per conc

Dunn Test

Dunn or Mann-Whitney test

>=4 reps per conc

Tamhane-Dunnet test

Dunnett test on replicate means

Dunnett test on nested ANOVA

Variance stabilizing transform?
Statistics Flow-Diagram for Sex Ratio Response-NOEC

Is solvent used?

Yes

Compare controls using t-test. Do controls differ?

Yes

Drop water control†

No

Combine controls†

No

Are data consistent with monotone dose-response?

Yes

Apply step-down Jonckheere-Terpstra test * to determine NOEC

No

Are data normally distributed?*

Yes

Dunn or Mann-Whitney U-test w/ Bonferroni-Holm adjustment to determine NOEC

No

† Or other agreed control selection

* After arcsin square-root transform

+ With fewer than 5 experimental units per treatment, exact J-T or M-W tests should be used if available.
ANNEX 7
GUIDANCE ON TISSUE SAMPLING FOR GENETIC SEXDETERMINATION BY PCR METHOD IN THE JAPANESE MEDAKA

Tissue sampling

1. With fine scissors the anal or the dorsal fin will be cut off in each individual fish and placed into a tube filled with 100 µL of extraction-buffer 1 (details on buffer preparation see below). The scissors will be cleaned after each single fish in a beaker filled up with dest. H2O and dried with a paper tissue.
2. Now the fin-tissues will be homogenized by a micro tube teflon pistil for the lysis of cells. For each tube a new pistil will be used to prevent any contaminations. The pistils will be placed overnight in 0.5 M NaOH, rinse for 5 minutes in dest. H2O and stored in ethanol or sterile after autoclave until use.
3. It is also possible to store the fin tissue without any extraction-buffer 1 on dry-ice and then at -80°C refrigerator to prevent any degeneration of the DNA. But the extraction runs better, if you extract the DNA at the same time (handling see above; samples must be thawed on ice after storing at -80°C before the buffer will be filled in the tubes).
4. After homogenizing all tubes will be placed in a water bath and boiled for 15 minutes at 100°C.
5. Then 100 µL of the extraction buffer 2 (details on buffer preparation see below) will be pipetted into each tube. The samples will be stored at room temperature for 15 minutes and in the meantime they will be sometimes gently shaken by hand.
6. Afterwards all tubes will be placed in the water bath again and boiled for another 15 minutes at 100°C.
7. Until further analysis the tubes will be frozen at -20°C.
8. Important: Before further use of the sample, the thawed tubes have to be centrifuged in an Eppendorf centrifuge (for 30 sec at max. speed). For PCR use the clear supernatant. It has to be absolutely avoided that any traces of Chelex are transferred to the PCR reaction, because this will interfere with the Taq polymerase activity.

Buffer preparation:

1. PCR-buffer 1:
   a. 500 mg N-Lauroylsarcosine (Merck KGaA, Darmstadt, GE)
   b. 2 mL 5M NaCl (Carl Roth GmbH + Co. KG, Karlsruhe, GE)
   c. ad 100 mL dest. H2O → autoclave

2. PCR-buffer 2:
   a. 20 g Chelex (Biorad, Munich, GE)
   b. To swell in 100 mL dest. H2O
Preparation of samples for PCR analysis

The prepared and frozen tubes will be thawed on ice. After that they will be centrifuged using an Eppendorf centrifuge (30 sec at max. speed, at room temperature). For the PCR the clear supernatant separated from the precipitate will be used. It has absolutely to be avoided that any traces of Chelex (localized in the precipitate) are transferred to the PCR reaction, because this will interfere with the “Taq”-polymerase activity. The supernatant will be used directly or can be stored frozen (at -20 °C) and rethawed again in several cycles without negative impact on the DNA for later analyses.

1. Preparation of the “Reaction Mix” (25 µL per sample):

<table>
<thead>
<tr>
<th>Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5µl-2µl</td>
<td>Template DNA</td>
</tr>
<tr>
<td>2.5µl</td>
<td>10xPCR-buffer with MgCl2</td>
</tr>
<tr>
<td>4µl (5mM)</td>
<td>Nucleotides (each of dATP, dCTP, dGTP, dTTP)</td>
</tr>
<tr>
<td>0.5µl</td>
<td>Forward Primer (10µM)</td>
</tr>
<tr>
<td>0.5µl</td>
<td>Reverse Primer (10µM)</td>
</tr>
<tr>
<td>1.25µl</td>
<td>DMSO</td>
</tr>
<tr>
<td>up to 25µl</td>
<td>Water (PCR grade)</td>
</tr>
<tr>
<td>0.3µl</td>
<td>Taq E- Polymerase (Genaxxon)</td>
</tr>
</tbody>
</table>

10xPCR-buffer with MgCl2: 670mM Tris/HCl (pH8.8 at 25°C), 160mM (NH₄)₂SO₄, 25mM MgCl₂, 0.1%Tween 20

For each PCR (see below 3-5) the special primer as a new combination of “Reaction-Mix” and the adequate needed amount of template DNA for each sample (see above) is needed. The respective volumes will be transferred into new tubes using pipettes. After that all tubes will be closed, stirred (ca. 10 sec) and centrifuged (10 sec, at room temperature). Now the respective PCR-programs can be started. Additionally a positive control (exemplary DNA sample with known activity and clear results) and a negative control (1 µL dest. H₂O) will be used in each PCR-program.

2. Preparation of the agarose gel (1 %) – During running PCR-programs:
- Solve 3 g agarose in 300mL 1 x TAE-buffer (1% agarose gel)
- This solution has to be boiled using an microwave (ca. 2-3 min)
- Transfer the hot solution into a special casting box, which lies on ice
- After ca. 20 min the agarose gel is ready to use
- Storage the agarose gel in 1x TAE-buffer until the end of the PCR-programs
3. **Actin-PCR-program:**

This PCR-reaction is aimed to demonstrate that the DNA in the sample is not harmed.

- **Special primer:**
  - “M act 1(upper/forward)” \( \Rightarrow \) TTC AAC AGC CCT GCC ATG TA
  - “M act 2(lower/reverse)” \( \Rightarrow \) GCA GCT CAT AGC TCT TCT CCA GGG AG

- **Program:**
  - 5 min 95 °C
  - Cycle (35-times):
    - Denaturation \( \Rightarrow \) 45 sec at 95 °C
    - Annealing \( \Rightarrow \) 45 sec at 56 °C
    - Elongation \( \Rightarrow \) 1 min at 68 °C
  - 15 min 68 °C

4. **X- and Y-Gene-PCR-program:**

The samples with intact DNA will be used in this PCR-program to detect the X- and Y-Genes. Male DNA should show one double-band and female DNA should show one single band (after staining and gel-electrophoresis). For this program-run one positive control for males (XY-sample) and one for females (XX-sample) has to be included.

- **Special primer:**
  - “PG 17.5” (upper/forward) \( \Rightarrow \) CCG GGT GCC CAA GTG CTC CCG CTG
  - “PG 17.6” (lower/reverse) \( \Rightarrow \) GAT CGT CCC TCC ACA GAG AAG AGA

- **Program:**
  - 5 min 95 °C
  - Cycle (40-times):
    - Denaturation \( \Rightarrow \) 45 sec at 95 °C
    - Annealing \( \Rightarrow \) 45 sec at 55 °C
    - Elongation \( \Rightarrow \) 1 min 30 sec at 68 °C
  - 15 min 68 °C

5. **Y-Gene-PCR-program as “control” for X- and Y-Gene-PCR-program:**

This PCR-program verifies the results of the “X- and Y-Gene-PCR-program”. The “male-samples” should show one band and the “female-samples” shouldn’t show any band (after staining and gel-electrophoresis).

- **Special primer:**
  - “DMTYa (upper/forward)” \( \Rightarrow \) GGC CGG GTC CCC GGG TG
  - “DMTYd (lower/reverse)” \( \Rightarrow \) TTT GGG TGA ACT CAC ATG G
• Program:
  o 5 min 95 °C
  o Cycle (40-times):
    ▪ Denaturation ➞ 45 sec at 95 °C
    ▪ Annealing ➞ 45 sec at 56 °C
    ▪ Elongation ➞ 1 min at 68 °C
  o 15 min 68 °C

6. Staining of the PCR-samples:
• Staining solution:
  o 50 % Glycerin
  o 100 mM EDTA
  o 1 % SDS
  o 0.25 % Bromphenolblue
  o 0.25 % Xylenxyanol
• Pipette 1 µL of the staining solution into each single tube

7. Start of the Gel-Electrophoresis:
• The prepared 1 % agarose gel will be transferred into a gel-electrophoresis-chamber filled with 1 x TAE-Puffer
• 10 - 15 µL of each stained PCR-sample will be pipetted into an agarose gel slot
• Also 5 - 15 µL of the 1kb-“Ladder”(Invitrogen) will be pipetted into a separate slot
• Start the electrophoresis by 200 V
• Stop after 30-45 min

8. Determination of the bands:
• Clean the agarose gel in dest. H₂O
• Now transfer the agarose gel into Ethidiumbromid for 15 - 30 min
• After that a picture of the agarose gel has to be taken in an UV-light-box
• Finally the samples are analyzed in comparison to the positive control-band (or bands) and the ladder