

To be send out for commenting by 1 of July!!

Phase 2 report: 15 August + all per-reviewed articles. Husk tables from John Green

Peer-reviev: August/September

1st commenting round on draft TG: mid October – end November

2nd commenting round on draft TG: mid December – to mid February

WNT; Mid April 2011

DRAFT PROPOSAL

Fish sexual Development Test

INTRODUCTION

1. This ~~G~~guideline is based on a decision ~~from 1998 in OECD in 1998,~~ to develop new ~~or revise existing~~ OECD Gguidelines for the screening and testing of potential endocrine disrupters. ~~The~~In 2005 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 included in the OECD working program and it went through an inter-laboratory two international validation exercisephases from 2006 to 2010 where Japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) were fully validated; and fathead minnow (*pimephales promelas*) and three spined stickleback (*Gasterosteus aculeatus*) were partially validated. The protocol describes a Fish Sexual Development Test (FSDT) for evaluation of effects of endocrine disrupting chemicals in fish. 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. is population relevant and the FSDT can in certain cases be included in risk and hazard assessment of endocrine disrupters. The concept for this protocol is derived from previous studies on the effects of endocrine disrupters on fish

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 ~~an optional~~. Additionally the genetic sex is determined whenever possible (e.g. in Japanese medaka). Other apical endpoints ~~that are~~ 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 proficiency chemicals.

INITIAL CONSIDERATIONS AND LIMITATIONS (MODIFIED FROM TG 229 AND TG 230)

3. Vitellogenin (VTG) is normally produced by the liver of female oviparous vertebrates in response to circulating endogenous estrogen. 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.

5. 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. 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 17 β -estradiol, causes a decrease in the VTG level, which is used to detect chemicals having aromatase inhibiting properties or steroidogenesis inhibitors more broadly. 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.

6. Several measurement methods have been successfully developed and standardised for routine use. This is the case of species-specific Enzyme-Linked Immunosorbent Assay (ELISA) methods using immunochemistry for the quantification of VTG from blood, liver, whole body- or head/tail homogenate samples collected from individual. In both Japanese medaka and zebrafish, there is a good correlation between VTG measured from blood, liver and homogenate samples. Annex 4 provides the recommended procedures for sample collection for vitellogenin analysis. Species-specific and validated ELISA-kits for the measurement of vitellogenin are widely available.

7. Change in the sex ratio (proportions of sex) is an endpoint ~~indirectly showing~~ reflecting phenotypic sex reversal. In principle, eEstrogens, anti-estrogens, androgens, anti-androgens and steroidogenesis inhibiting chemicals can affect the sex ratio of developing fish. It has been shown that this sex reversal is partly reversible ~~for estrogens~~ in zebrafish (schäfers et al ?) following estrogenic-related chemical exposure, whereas ~~androgenic~~ sex reversal following androgenic-related chemical exposure is permanent ~~in the same specie~~. (Morthorst et al 2010). The sex is defined as either female, male, intersex or undifferentiated determined in individual fish via histological examination of the gonads.

8. Genetic sex is ~~examined~~ measured via genetic markers when they exist in a given ~~in the~~ fish species where this is possible. In Japanese medaka the female xx or male xy genes can be detected by PCR ~~ore~~ g1 HH2 the Y-linked DM domain gene (DMY) can be analysed (DMY negative or positive) as described by (Shinomiya et al 2004). Where the genetic sex can be individually linked to the phenotypic sex, the power of the test is improved s.

9. The two core endocrine endpoints; VTG and sex ratio can in combination demonstrate the endocrine mode of action of the ~~exposure~~ chemical. The sex ratio is a population relevant biomarker and for some well defined modes of action, the FSDT results may ~~can~~ be used for ~~the hazard and risk~~ assessment purposes of risk and hazard of the exposure chemical. These modes of action are at present androgens and ~~steroidogenesis inhibitors~~ aromatase 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.

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

11. The *in vivo* FSDT is intended to detect chemicals with androgenic or estrogenic properties as well as anti-androgenic g3 HH4, anti-estrogenic and aromatase inhibiting properties.

PRINCIPLE OF THE TEST

12. In the test, fish are exposed from newly fertilized egg 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 humanly. A biological sample for VTG analysis (blood, 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; and optionally histopathology can be performed. A biological sample for the determination of the genetic sex is also taken in species possessing appropriate markers whenever relevant. The combination of the endpoints VTG measurements and sex ratio (analysed as proportion of sex) in individual fish adds weight to the assessment of effects and likely gives the opportunity to determine the mode of action of the exposure 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 (e.g., OECD TG 203, TG 204, TG 212, TG 215) 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, P_{Ka} , P_{ow} 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 temperatureranges 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 $\pm 20\%$ 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):

		Japanese medaka	Zebrafish
Growth	Weight (wet weight*)	>150 mg	>75 mg
	Length (standard length)	<u>>20mm</u> ^[5]	>14 mm

Sex ratio (% males or females) 30-70 % 30-70 %

(*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. Recommended fish species are given in ANNEX 24. [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\) vitellogenin levels are sufficiently high to detect significant chemical-related variations, and iii\) sensitivity of the test system is established using proficiency chemicals.](#) 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. 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 these 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 1 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, SO₄), 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. Some chemical characteristics of acceptable dilution water are listed in ANNEX 2.

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 TG 210 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 GD 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 [OECD GD 2000]. Where a solvent is used to assist in stock solution preparation, its final concentration should not be greater than 100 µl/l and should be the same in all test vessels. Every effort should be made to keep solvent concentrations to a minimum.

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.

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 (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/l per 24 hours and not exceeding 5 g/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 1).

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 TG 210 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 43. 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 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

33. 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. Samples should be filtered (e.g. using a 0.45 m pore size) or centrifuged to ensure that the determinations are made on the substance in true solution. Results shall be based on measured concentrations.

34. 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.

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

Observations

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

37. **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 colouration, 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.

38. **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 33 and treated as mortality for data analysis.

39. **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.

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

41. **Length:** At the end of the test, measurement of individual lengths (standard length) is recommended. ~~standard length, If however, caudal fin rot or fin erosion occurs standard lengths should be used.~~

42. 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;
- numbers of deformed larvae;
- numbers of fish exhibiting abnormal behaviour.

Sampling of fish

43. **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 ~~histological sex ratio~~ determination via histological evaluation

44. ~~All surviving fish should be sampled for VTG and sex ratio determination.~~ All fish should be sampled and prepared for analysis of sex and VTG. All fish should be analyzed histologically to determine sex. For the VTG measurements, a sub-sampling~~A sub-analysis of VTG~~ from at least 10 fish from each replicate, is accepted. More fish should be analyzed for VTG if the results of the sub-sampling turns out to be unclear.

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

Head/tail homogenate method for VTG analysis

46. 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 -70° 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 (for further information see the SOP for homogenization (ANNEX 54) and the SOP for VTG quantification (ANNEX 65).

Liver homogenate method

47. The liver is dissected out and stored at -70° or less. Standard operating procedures for liver excision and pre-treatment are available from the protocol on the OECD Guidelines TG229 or TG230, and will be added as an annex to this protocol if there is sufficient interest. Livers are then individually homogenized as described in the SOP. The supernatant collected is then used for measuring VTG with a homologous ELISA technique (see ANNEX 65 for quantification in zebrafish). Following this approach, it is also possible to have individual fish data on both VTG and gonad histology.

Plasma method

48. Blood is collected from the anaesthetized fish and centrifuged for plasma collection. The plasma is stored at -70° 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.

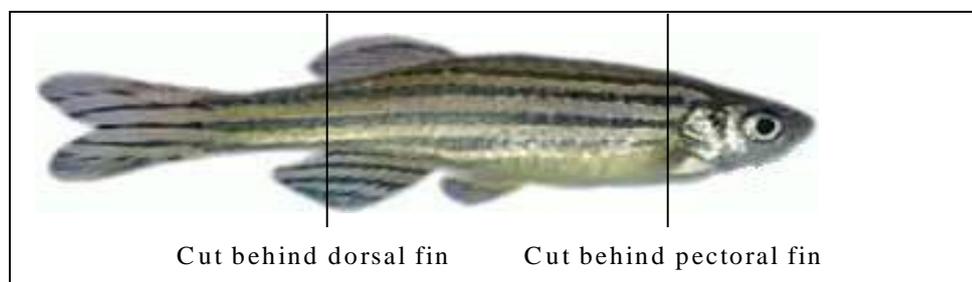


Figure 1: How to cut a fish for measurement of VTG in head/tail homogenate and histological evaluation of the mid section

Vitellogenin (VTG) measurement

49. 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

50. After separation of head and tail for VTG measurement 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 (ANNEX 6). After processing, 6-8 specimens are embedded in each paraffin block. The specimens should be placed longitudinally in the paraffin block. Three sections 3-5 µm in thickness including gonadal tissue are taken from each individual. The interval between these sections should be approximately 50 µm. Sections are stained with haematoxylin and eosin and examined by light-microscopy with focus on sex (male, female, intersex) 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. [Clear definition on a female; male and intersex! How many oocytes is needed for deeming intersex!](#)^[g6]

[Medaka can be sexed by the genetic marker](#)^{[g7][HH8]}

Secondary sexual characteristics

51. 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 E.g. in 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.](#) ~~The shape of dorsal and anal fins is also sensitive to endocrine active substances.~~

DATA AND REPORTING

Treatment of results

52. [A decision flow-chart is available in ANNEX 8 to help with the most appropriate statistical test to use based on the characteristic of the data obtained from the test.](#)

Proportions of sex

53. [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

54. [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^[g9][HH10]

55. 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

56. 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.

57. The results should be presented as mean values \pm [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 [87](#)) should be followed.

[58.](#) The following issues should be addressed:

- [Matrix VTG/Sex ratio](#)
- [Sex ratio in relation to VTG](#)
- [How to use the results in relation to Risk Assessment](#)
- [Consideration of all the endpoint; Matrix; Intersex; Risk Assessment](#)

^[g11][HH12]

References

1. OECD GD, 2009. Guidance document on aquatic toxicity testing of difficult substances and mixtures. OECD Environmental Health and Safety Publications. OECD Environmental Health and Safety Publications.
2. OECD TG 210, 1992. OECD Guideline No. 210. (1992) Fish, Early Life Stage Toxicity Test. OECD Guidelines.

ANNEX ~~1XXX~~

ABBREVIATIONS & DEFINITIONS

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 is a phospholipoglycoprotein precursor to egg yolk protein that normally occurs in sexually active females of all oviparous species.

HPG axis: hypothalamic-pituitary-gonadal axis

ANNEX 21**EXPERIMENTAL CONDITIONS FOR THE FISH SEXUAL DEVELOPMENT TEST**

1. Recommended species	Japanese medaka (<i>Oryzias latipes</i>)	Zebrafish (<i>Danio rerio</i>)
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/M ² /s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)	10-20 µE/M ² /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 <i>Artemia</i> , frozen adult brine shrimp, flake food, etc. It is recommended to feed twice daily	Live <i>Artemia</i> , 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% - Post hatch survival ≥ 70% of fish in the controls	- Hatching success > 80% - Post hatch survival ≥ 70% of fish in the controls

ANNEX 32

SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

SUBSTANCE	CONCENTRATIONS
Particular matter	< 20 mg/l
Total organic carbon	< 2 mg/l
Unionised ammonia	< 1 ug/l
Residual chlorine	< 10 ug/l
Total organophosphorus pesticides	< 50 ng/l
Total organochlorine pesticides plus polychlorinated biphenyls	< 50 ng/l
Total organic chlorine	< 25 ng/l

ANNEX 43 (Might go out)**FROM TG 215 /GUIDANCE ON TEST CONCENTRATIONS**

Column (Number of concentrations 100 and 10, or between 10 and 1)*						
1	2	3	4	5	6	7
100	100	100	100	100	100	100
32	46	56	63	68	72	75
10	22	32	40	46	52	56
3.2	10	18	25	32	37	42
1.0	4.6	10	16	22	27	32
	2.2	5.6	10	15	19	24
	1.0	3.2	6.3	10	14	18
		1.8	4.0	6.8	10	13
		1.0	2.5	4.6	7.2	10
			1.6	3.2	5.2	7.5
			1.0	2.2	3.7	5.6
				1.5	2.7	4.2
				1.0	1.9	3.2
					1.4	2.4
					1.0	1.8
						1.3
						1.0

* 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.

(1) Environment Canada. (1992). Biological test method: toxicity tests using early life stages of salmonid fish (rainbow trout, coho salmon, or Atlantic salmon). Conservation and Protection, Ontario, Report EPS 1/RM/28, 81 p.

ANNEX 54

GUIDANCE: HOMOGENISATION OF HEAD & TAIL FROM JUVENILE ZEBRAFISH, FATHEAD MINNOW, THREE SPINED STICKLEBACK AND JAPANESE MEDAKA

The purpose of this section is to describe the procedures that occur prior to the quantification of the vitellogenin concentration. Other procedures that result in comparable vitellogenin quantification can be used.

It is an option to determine the vitellogenin concentration in blood or liver instead of head/tail homogenate

Procedure

1. The fish are anaesthetised and euthanised in accordance with the test description.
2. The head and tail are cut of the fish in accordance with the test description. **Important:** *All dissection instruments, and the cutting board should be rinsed and cleaned properly (e.g. with 96% ethanol) between handling of each single fish to prevent "vitellogenin pollution" from females or induced males to un-induced males.*
3. The weight of the pooled head and tail from each fish is measured to the nearest mg.
4. After being weighed, the parts are placed in appropriate tubes (e.g. 1.5 ml eppendorf) and frozen at -80°C until homogenisation or directly homogenised on ice with two plastic pistils. (Other methods can be used if they are performed on ice and the result is a homogenous mass). **Important:** *The tubes should be numbered properly so that the head and tail from the fish can be related to their respective body-section used for gonad histology.*
5. When a homogenous mass is achieved an amount of 4-10 x the tissue weight of ice-cold **homogenisation buffer*** is added (note the dilution). Keep working with the pistils until the mixture is homogeneous. **Important note:** *New pistils are used for each fish.*
6. The samples are placed on ice until centrifugation at 4°C at 50000 x g for 30 min.
7. Use a pipette to dispense portions of 20 to 50 μl (note the amount) supernatant into **at least two** tubes by dipping the tip of the pipette below the fat layer on the surface and carefully sucking up the supernatant without fat- or pellet fractions.
8. The tubes are stored at -80°C until use.

***Homogenisation buffer:**

(50 mM Tris-HCl pH 7,4; 1% Protease inhibitor cocktail (Sigma)): 12 ml Tris-HCl pH 7,4 + 120 μl Protease inhibitor cocktail.

TRIS: TRIS-ULTRA PURE (ICN) e.g. from Bie & Berntsen, Denmark.

Protease inhibitor cocktail: From Sigma (for mammalian tissue) Product number **P 8340**.

NOTE: *The homogenisation buffer has to be used the same day as manufactured. Place on ice during use*

ANNEX 65

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 over night 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 76

GUIDANCE: PREPARATION OF TISSUE SECTIONS FOR SEX DETERMINATION AND STAGING OF GONADS

The purpose of this section is to describe the procedures that occur prior to the evaluation of histological sections. Other procedures that result in similar sex determination and gonadal staging can be used.

With a few exceptions these procedures are similar for fathead minnow (FHM), Japanese medaka (JMD), zebrafish (ZBF),).

EUTHANASIA, NECROPSY, AND TISSUE FIXATION

Objectives:

1. Provide for the humane sacrifice of fish.
2. Obtain necessary body weights and measurements.
3. Evaluate secondary sex characteristics.
4. Dissect tissues for vitellogenin analysis.
5. Fixation of the gonads.

Procedures:

1. Fish should be sacrificed immediately prior to necropsy. Therefore, unless multiple processors are available, multiple fish should not be sacrificed simultaneously.
2. Using the small dip net, a fish is removed from the experimental chamber and transported to the necropsy area in the transport container.
3. The fish is placed in the euthanasia solution. The fish is removed from the solution when there is cessation of respiration and the fish is unresponsive to external stimuli.
4. The fish is wet weighed.
5. For preparation of tissues for vitellogenin analysis the fish can be placed on a corkboard on the stage of a dissecting microscope.
 - a. For FHM and ZF the head is cut right behind the pectoral fin and tail is cut right behind the dorsal fin.
 - b. For JM the abdomen is opened via a carefully made incision that extends along the ventral midline from the pectoral girdle to a point just cranial to the anus. Using the small forceps and small scissors, the liver is carefully removed.
6. Specimen for vitellogenin analysis are placed in eppendorf tubes and immediately frozen in liquid nitrogen.
7. The carcass including the gonads is placed into a pre-labelled plastic tissue cassette, which is transferred into Davidson's or Bouin's fixative. The volume of fixative should be at least 10 times the approximated volume of the tissues. The fixative container is gently agitated for five seconds to dislodge air bubbles from the cassette.

8. a. All tissues remain in Davidson's fixative overnight, followed by transfer to individual containers of 10 % neutral buffered formalin the next day. Containers with cassettes are gently agitated for 5 seconds to ensure adequate penetration of formalin into cassettes.
- b. Tissues remain in Bouins fixative for 24 h, followed by transfer to 70 % ethanol.

TISSUE PROCESSING

Objectives:

1. Dehydrate tissue for adequate penetration of paraffin.
2. Impregnate the tissue with paraffin to maintain tissue integrity and create a firm surface for microtomy.

Procedures:

1. Labelled tissue cassettes are removed from formalin/ethanol storage and the cassettes are placed in the processing basket(s). The processing basket is loaded in the tissue processor.
2. The processing schedule is selected.
3. After the tissue processor has completed the processing cycle, the basket(s) may be transferred to the embedded station.

EMBEDDING

Objective:

Properly orient the specimen in solidified paraffin for microtomy.

Procedures:

1. The basket(s) of cassettes is/are removed from the processor and immersed in the paraffin-filled front chamber of the embedding station thermal console or the cassettes are moved to a separate paraffin heater.
2. The first cassette to be embedded is removed from the front chamber of the thermal console or the paraffin heater. The cassette lid is removed and discarded, and the cassette label is checked against the animal records to resolve potential discrepancies prior to embedding.
3. An appropriately sized embedding mould is selected.
4. The mould is held under the spout of the dispensing console and filled with molten paraffin.
5. The specimen is removed from the cassette and placed in the molten paraffin in the mould. This is repeated with 4-8 specimens for each paraffin mould. The position of individual fish is marked by putting fish no 1 in 180 degrees to fish 2-4/8.
6. Additional paraffin is added to cover the specimen.
7. The mould with the cassette base is placed on the cooling plate of the cryo console.

8. After the paraffin has solidified, the block (i.e., the hardened paraffin containing the tissues and the cassette base) is removed from the mould.

MICROTOMY

Objective:

Cut and mount histological sections for staining.

Procedures:

1. The initial phase of microtomy termed “facing” is conducted as follows:
 - a. The paraffin block is placed in the chuck of the microtome.
 - b. The chuck is advanced by rotating the microtome wheel and thick sections are cut from the paraffin surface of the block until the knife reaches the embedded tissues.
 - c. The section thickness on the microtome is set between 4 – 10 microns. The chuck is advanced and multiple sections are cut from the block to remove any artefacts created on the cut surface of the tissue during rough trimming.
 - d. The block can be removed from the chuck and placed facedown on ice to soak the tissue.
2. The next phase of microtomy is final sectioning and mounting of tissue sections on slides. These procedures are conducted as follows:
 - a. If the block has been placed on ice, the block is removed from the ice and replaced in the chuck of the microtome.
 - b. With the section thickness on the microtome set to 4 – 5 microns, the chuck is advanced by rotating the microtome wheel. Sections are cut from the block until a “ribbon” containing at least one acceptable section including the gonads has been produced. (As necessary during sectioning, the block may be removed from the chuck, placed on ice to soak the tissue, and replaced in the chuck.)
 - c. The sections are floated flat on the surface of the water in the water bath. An attempt is made to obtain at least one section that contains no wrinkles and has no air bubbles trapped beneath it.
 - d. A microscope slide is immersed beneath the best section, which is lifted out of the water using the slide. This process is referred to as “mounting” the section on the slide.
 - e. Three sections are prepared for a set of fish. The second and third sections are taken at 50 micron intervals following the first section. If the fish are not embedded with their gonads in the same sectioning level, more sections are to be made to ensure that at least three sections including the gonads are obtained from each fish.
 - f. With a slide-marking pen, the block number from which the slide was produced is recorded on the slide.
 - g. The slide is placed in a staining rack.
 - h. The block is removed from the chuck and placed facedown for storage.

STAINING, COVER SLIPPING, AND SLIDE LABELLING

Objectives:

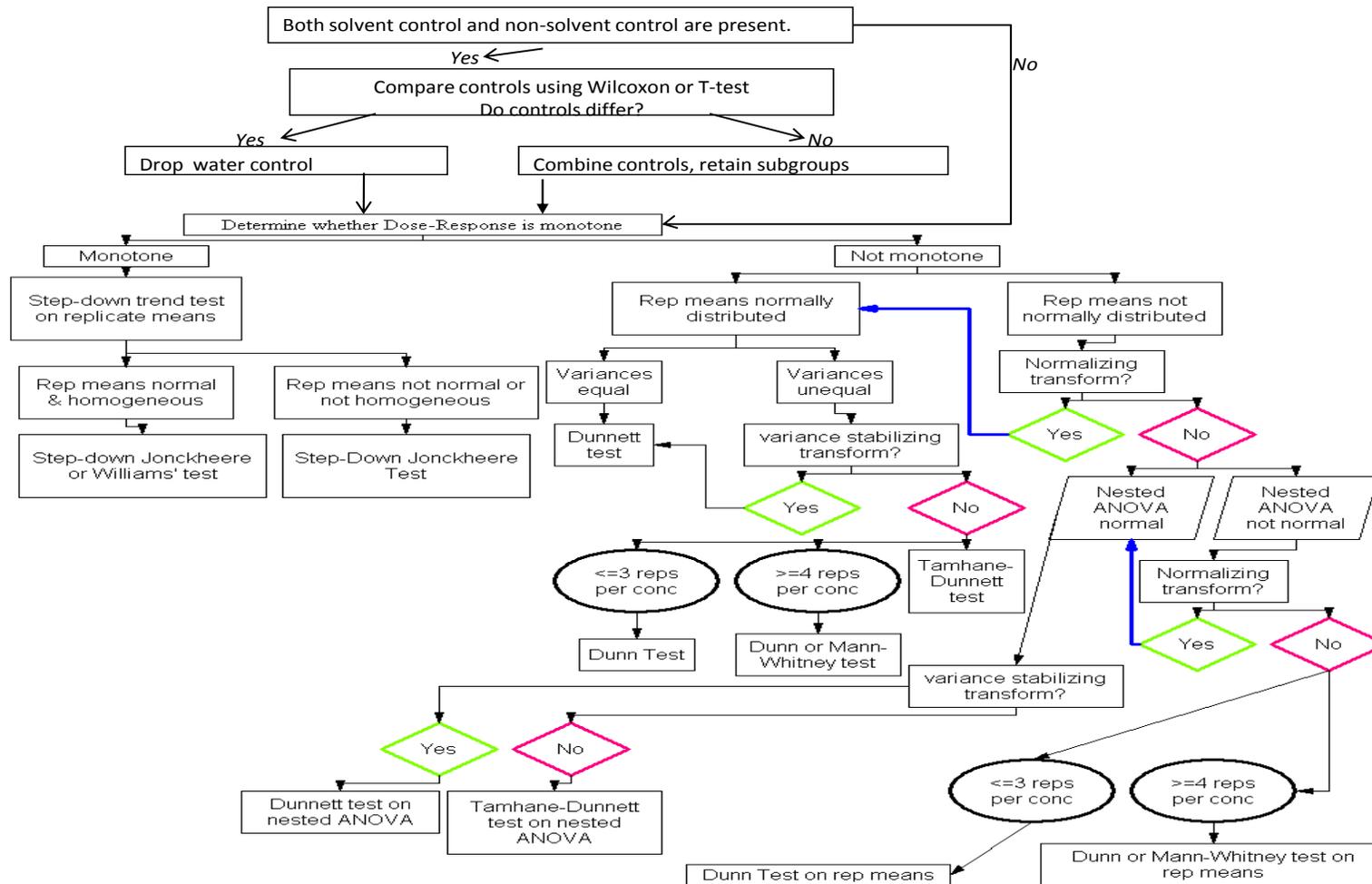
1. Stain the sections for histopathological examination
2. Permanently seal mounted and stained tissues.
3. Permanently identify stained sections in a manner that allows complete trace ability.

Procedures:

1. Staining
 - a. Slides are air-dried overnight before staining.
 - b. The sections are stained by Hematoxylin-Eosin.
2. Cover slipping
 - a. Cover slips can be applied manually or automatically.
 - b. A slide is dipped in xylene or TissueClear, and the excess xylene/TissueClear is gently knocked off the slide.
 - c. Approximately 0.1 ml of mounting medium is applied near the end of the slide opposite to the frosted end or on the cover slip.
 - d. The cover slip is tilted at a shallow angle as it is applied to the slide.
3. Labelling
 - a. Each slide label should contain the following information.
 - i. Laboratory name
 - ii. Species
 - iii. Specimen No. / Slide No.
 - iv. Chemical / Treatment group
 - v. Date

ANNEX 87

Statistical Flow Chart for vitellogenin analysis



Statistical Flow Chart for sex ratio analysis

Statistics Flow-Diagram for Sex Ratio Response-NOEC

