

PRELIMINARY DRAFT OECD GUIDELINE FOR THE TESTING OF CHEMICALS

The Fish Screening Assay for Endocrine Active Substances

INTRODUCTION

1. The need to develop and validate a fish assay capable of detecting endocrine active substances originates from the concerns that environmental levels of chemicals may cause adverse effects in both humans and wildlife due to the interaction of these chemicals with the endocrine system. In 1998, the OECD initiated a high-priority activity to revise existing guidelines and to develop new guidelines for the screening and testing of potential endocrine disrupters (1). One element of the activity was to develop a test guideline for the screening of substances active on the endocrine system of fish species. The Fish Screening Assay then underwent an extensive validation programme which contains the intra- and inter-laboratory studies demonstrating the relevance and reliability of the assay. This test guideline is the outcome of the experience gained during the validation studies for the detection of estrogenic, aromatase inhibitors and androgenic substances.

2. This test guideline is based on an *in vivo* screening assay for identifying certain endocrine active chemicals in sexually dimorphic fish which are exposed to chemicals during a limited part of their life-cycle (normally 21 days). This Test Guideline is mainly intended to detect chemicals that affect estrogenic, aromatase inhibition or androgenic activities. This assay measures two core biomarker endpoints as indicators of endocrine activity, which are namely vitellogenin and secondary sexual characteristics.

3. This bioassay serves as an *in vivo* screening assay and its application should be seen in the context of the “OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals” (ANNEX 1). In this Conceptual Framework the Fish Screening Assay is contained in Level 4 as an *in vivo* assay providing data about multiple endocrine mechanism.

INITIAL CONSIDERATIONS AND LIMITATIONS

4. Vitellogenin is normally produced by the liver of female oviparous vertebrates in response to circulating endogenous oestrogen. 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. Vitellogenin is normally undetectable in the plasma of immature and male oviparous animals because they lack circulating oestrogen; however, their liver is capable of synthesizing and secreting vitellogenin into the blood in response to exogenous oestrogen stimulation.

5. Thus, the detection of estrogenic chemicals is possible via the measurement of vitellogenin level in male fish, and it has been abundantly documented in the scientific peer-reviewed literature (Sumpter and Jobling 1996). The biological relevance of the response evaluated is established. Furthermore, a reduction in the circulating level of oestrogen in females, for instance through the inhibition of the aromatase, causes a decrease in the vitellogenin level, which thus usefully serves for the detection of aromatase inhibiting substances (Ankley et al., 2002; Zerulla et al., 1998). The biological relevance of the response evaluated is

established. Finally, the decrease of vitellogenin production in females was also noted following exposure to non-aromatisable androgen (correlative relationship); however, the underlying mechanism is not well understood or documented yet and therefore the biological relevance is not established.

6. Several measurement methods have been successfully developed and standardised for routine use. This is the case of species-specific ELISA methods, using immunochemistry for the quantification of vitellogenin produced in small blood or liver samples collected from individual fish (Parks et al, 1999; Holbech et al, 2001; Nozaka et al, 2004). Blood of fathead minnow and zebrafish and liver of medaka are sampled for VTG measurement. In medaka, there is a good correlation between VTG measured from blood and from liver (Tatarazako et al, 2004).

7. Secondary sex characteristics in male fish of certain species are externally visible, quantifiable and responsive to circulating levels of endogenous androgens; this is the case for the fathead minnow and the medaka in particular. Similarly, females maintain the capacity to develop male secondary sex characteristics, when they are exposed to androgenic substances in water. Several studies are available in the scientific literature to document this type of response in fathead minnow (Ankley et al, 2003; Panter et al, 2004) and medaka (Seki et al, 2004) in particular, but also in other species.

8. In the fathead minnow, the main indicator of exogenous androgenic exposure used is the number of nuptial tubercles located on the snout of the female fish. In the medaka, the number of papillary processes constitutes the main marker of exogenous exposure to androgenic compounds in female fish.

9. The assay is not designed to identify specific mechanisms of hormonal disruption (such as hormone receptor agonistic or antagonistic effects, or disturbance of hormone synthesis or metabolism). The concept for this protocol is derived from work on the fathead minnow, the Japanese medaka and the zebrafish. There is limited evidence that the protocol could also be used for the three spined stickleback (*Gasterosteus aculatus*)

10. The zebrafish should not be used in this assay for the detection of androgenic substances, due to the absence of quantifiable secondary sex characteristics responsive to androgenic induction. The interpretation of responses on secondary sex characteristics should be limited to their induction in females fish following exposure, as the decrease of such sexual characteristics in male fish may, taken on its own, be misinterpreted as a false positive outcome.

11. The 21-day fish assay does not include the evaluation of the gonado-somatic indices, nor the evaluation of fecundity, nor the evaluation of gonadal histopathology. Although these endpoints had been investigated earlier on in the validation studies using this assay, they did not demonstrate sufficient inter-laboratory reproducibility to be considered valid in the scope of this assay.

12. Definitions used in this Test Guideline are given in ANNEX 2.

PRINCIPLE OF THE TEST

13. In the assay, male and female fish in a reproductive status are exposed together in test vessels. Their adult and reproductive status enables a clear differentiation of each sex, and thus a sex-related analysis of each endpoint. An overview of the relevant bioassay conditions are provided in ANNEX 3. The assay is normally initiated with fish sampled from populations that are in spawning condition, senescent animals should not be used. Guidance on the age of fish is provided in the section on selection of test organisms. The assay is conducted using three chemical exposure concentrations as well as a water control. Two vessels (replicate) per treatment are used (each vessel containing 5 males and 5 females) in medaka and zebrafish, whereas four vessels (replicates) per treatment are used (each vessel containing 2 males and

4 females) in fathead minnow. The exposure is conducted for 21-days and sampling of fish is performed at day-21 of exposure.

14. On sampling at day 21, 20 fish (10 males and 10 females) per treatment level are killed humanely; secondary sex characteristics are measured and blood samples are collected for determination of vitellogenin (note - as an alternative to plasma collection, liver will be sampled for VTG analysis in medaka).

TEST ACCEPTANCE CRITERIA

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

- the mortality in the control(s) does not exceed 10 per cent at the end of the exposure period; as well, signs of disease are visible in less than 10 per cent of control animals during the course of the test.
- the dissolved oxygen concentration has been at least 60 per cent of the air saturation value (ASV) throughout the exposure period.
- the water temperature did not differ by more than ± 1 °C between test vessels at any one time during the exposure period and was maintained within a range of 2°C within the temperature ranges specified for the test species (ANNEX 1).

DESCRIPTION OF THE METHOD

Apparatus

16. Normal laboratory equipment and especially the following:

- (a) oxygen and pH meters;
- (b) equipment for determination of water hardness and alkalinity;
- (c) adequate apparatus for temperature control and preferably continuous monitoring;
- (d) tanks made of chemically inert material and of a suitable capacity in relation to the recommended loading and stocking density (see Appendix 1);
- (e) spawning substrate for fathead minnow and zebrafish, ANNEX 4 gives the necessary details.
- (f) suitably accurate balance (i.e. accurate to $\pm 0.5\%$).

Water

17. Any water in which the test species shows suitable long-term survival and growth may be used as test water. It should be of constant quality during the period of the test. The pH of the water should be within the range 6.5 to 8.5, but during a given test it should be within a range of ± 0.5 pH units. In order to ensure that the dilution water will not unduly influence the test result (for example by complexation of test substance), 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 (e.g. total organophosphorus and total organochlorine pesticides), total organic carbon and suspended solids should be made, for example, every three months where a dilution water is known to be relatively constant in quality. If water quality has been demonstrated to be constant over at least one year, determinations can be less frequent and intervals extended (e.g. every six months). Some chemical characteristics of acceptable dilution water are listed in ANNEX 5.

Test solutions

18. Test solutions of the chosen concentrations are prepared by dilution of a stock solution. The stock solution should preferably be prepared by simply mixing or agitating the test substance in the dilution water by using mechanical means (e.g. stirring or ultrasonication). Saturation columns (solubility columns) can be used for achieving a suitable concentrated stock solution. The use of a solvent carrier is not recommended. However, in case a solvent would be used, a solvent control should be added, using the same solvent concentration as in the chemical treatments.

19. A flow-through test system will be used. Such a system continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) in order to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water should be checked at intervals, preferably daily, during the test and should not vary by more than 10% throughout the test. Care should be taken to avoid the use of low-grade plastic tubing or other materials that may contain biologically active substances.

Selection of fish species

20. The exposure phase is started with sexually dimorphic adult fish from a laboratory supply of reproductively mature animals, in spawning conditions. It is recommended that fish used should be reproductively mature (namely, with clear secondary sexual characteristics visible) and actively spawning. For general guidance only (and not to be considered in isolation from observing the actual reproductive status of a given batch of fish), fathead minnows should be approximately 20 (± 2) weeks of age, assuming they have been cultured at $25\pm 2^\circ\text{C}$ throughout their lifespan. Japanese medaka should be approximately 16 (± 2) weeks of age, assuming they have been cultured at $25\pm 2^\circ\text{C}$ throughout their lifespan. Zebrafish should be approximately 15 (± 2) weeks of age, assuming they have been cultured at $25\pm 2^\circ\text{C}$ throughout their lifespan.

Holding of fish

21. Test fish shall be selected from a laboratory population, preferably from a single stock, which has been acclimated for at least two weeks prior to the test under conditions of water quality and illumination similar to those used in the test (note, this acclimation period is not an *in situ* pre-exposure period). Fish should be fed *ad libitum* throughout the holding period and during the exposure phase. Note- fish should not be fed within 12 hours of necropsy.

22. Following a 48-hour settling-in period, mortalities are recorded and the following criteria applied:

- mortalities of greater than 10% of population in seven days: reject the entire batch;
- mortalities of between 5% and 10% of population: acclimation for seven additional days; if more than 5% mortality during second seven days, reject the entire batch;
- mortalities of less than 5% of population in seven days: accept the batch

23. Fish should not receive treatment for disease in the two week acclimation period preceding the test, or during the exposure period.

TEST DESIGN

24. Three concentrations of the test substance, one control (water) and one concentration of the positive control are used per experiment. The data may be analyzed in order to define the Lowest

Observed Effect Concentration or the No-Observed Effect Concentration based on endocrine sensitive endpoints (LOEC and NOEC respectively). Calculation of these statistical parameters will be useful in order to establish whether any further longer term testing for adverse effects (namely, survival, development, growth and reproduction) is required for the chemical.

25. A positive control substance should be run regularly to ensure that the test system is performing correctly. One concentration (100µg/l) of 17β-estradiol (CAS. 50-28-2) should be used as a positive control.

26. For zebrafish and medaka, on day-21 of the experiment, 10 males and 10 females from each treatment level (5 males and 5 females for each of the two replicates) and from the control are sampled for the measurement of the two endpoints. For fathead minnow, on day 21 of exposure, 8 males and 16 females (2 males and 4 females for each of the four replicates) and from the control are sampled for the measurement of the two endpoints.

PROCEDURE

Regulatory compliance and laboratory verification

27. The assay should be conducted in conformity with the OECD Good Laboratory Practice and quality Assurance Procedures (**ref**).

Selection and weighing of test fish

28. It is important to minimise variation in weight of the fish at the beginning of the assay. Suitable size ranges for the different species recommended for use in this test are given in ANNEX 3. For the whole batch of fish used in the test, the range in individual weights at the start of the test should be kept, if possible, within ± 20% of the arithmetic mean weight. It is recommended to weigh a subsample of fish before the test in order to estimate the mean weight.

Conditions of exposure

Duration

29. The test duration is 21 days.

Loading rates and stocking densities

30. It is important that the loading rate and stocking density (for definitions, see ANNEX 2) is appropriate for the test species used (see ANNEX 3). If the stocking density is too high, then overcrowding stress will occur leading to reduced growth rates and possibly to disease. If it is too low, territorial behavior may be induced which could also affect growth. In any case, the loading rate should be low enough in order that a dissolved oxygen concentration of at least 60% ASV can be maintained without aeration.

Feeding

31. The fish should be fed *ad libitum* with an appropriate food (ANNEX 3) at a sufficient rate to maintain body condition (2). Care should be taken to avoid microbial growth and water turbidity. The daily ration may be divided into two or three equal portions for multiple feeds per day, separated by at least

three hours between each feed. Food should be withheld from the fish for 12 hours prior to the day of sampling.

32. Fish food, other than the one recommended in ANNEX 3, should be evaluated for the presence of contaminants such as organochlorine pesticides, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and any estrogenic activity.

33. Uneaten food and fecal material should be removed from the test vessels at least twice weekly, e.g. by carefully cleaning the bottom of each tank using suction.

Light and temperature

34. The photoperiod and water temperature should be appropriate for the test species (see ANNEX 3).

Frequency of analytical determinations and measurements

35. Prior to initiation of the exposure period, proper function of the chemicals delivery system should be ensured. All analytical methods needed should be established, including sufficient knowledge on the substances stability in the test system. During the test, the concentrations of the test substance are determined at regular intervals, as follows: the flow rates of diluent and toxicant stock solution should be checked at intervals, at least twice per week, and should not vary by more than 15% throughout the test. It is recommended that the actual test chemical concentrations be measured in all vessels at the start of the test and at weekly intervals thereafter.

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

37. Samples may need to be filtered (e.g. using a 0.45 µm pore size) or centrifuged. If needed, then centrifugation is the recommended procedure. However, if the test material does not adsorb to filters, filtration may also be acceptable.

38. During the test, dissolved oxygen, temperature, and pH should be measured in all test vessels at least once per week. Total hardness and alkalinity should be measured in the controls and one vessel at the highest concentration at least once per week. Temperature should preferably be monitored continuously in at least one test vessel.

Observations

39. A number of general (e.g. survival) and core biological responses (e.g. vitellogenin levels) are assessed over the course of the assay. Collection of these endpoints and their utility are described below:

Survival

40. Fish should be examined daily during the test period and any external abnormalities (such as hemorrhage, discoloration) noted. Any mortality should be recorded and the dead fish removed as soon as possible. Dead fish should not be replaced in either the control or treatment vessels.

Behaviour

41. Any abnormal behavior (relative to controls) should be noted; this might include signs of general toxicity including hyperventilation, uncoordinated swimming, loss of equilibrium, and atypical quiescence or feeding. Such behavioral observations may provide useful qualitative information to inform potential

future fish testing requirements (for example, territorial aggressiveness in normal males or masculinized females has been observed in fathead minnows under androgenic exposure).

Spawning status

42. Daily qualitative observations of spawning in the control vessel will be recorded, as a check that fish are in spawning condition. Eggs will be removed daily from the test chambers. A spawning substrate must be placed in the test chamber for the fathead minnow and zebrafish to enable fish to spawn in normal conditions. ANNEX 4 gives further details of recommended spawning substrates for zebrafish (4A) and fathead minnow (4B). It is not considered useful to provide spawning substrate for medaka.

Appearance and observation of secondary sex characteristics

43. Secondary sexual characteristics are under endocrine control; therefore observations of physical appearance of the fish should be made over the course of the test, and at conclusion of the study. Experience to date with fathead minnows suggests that some endocrine active chemicals may initially induce changes in the following external characteristics: body colour (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and specialized secondary sex characteristics (size of dorsal nape pad, number of nuptial tubercles in male fathead minnow, ovipositor size in females, papillary processes in male medaka). Notably, chemicals with certain modes of action may cause abnormal occurrence of secondary sex characteristic in animals of the opposite sex; for example, androgen receptor agonists, such as methyltestosterone and dihydrotestosterone, can cause female fathead minnows to develop pronounced nuptial tubercles (3)(12) (13). It also has been reported that estrogen receptor agonists can decrease nuptial tubercle numbers and size of the dorsal nape pad in adult males (14) (15). Such gross morphological observations may provide useful qualitative and quantitative information to inform potential future fish testing requirements.

44. Because some aspects of appearance (primarily color) can change quickly with handling, it is important that qualitative observations be made prior to removal of animals from the test system. Other endpoints, such as the number and size of nuptial tubercles in fathead minnow and papillary processes in medaka, can be quantified directly or in preserved specimens (3). Standard operating procedures for the evaluation of secondary sex characters in fathead minnow and medaka are available as ANNEX 6.

Humane killing of fish

45. At day 21, i.e. at conclusion of the exposure, the fish should be anaesthetized with appropriate amounts of MS-222 (100 ... 500 mg per L buffered with 200 mg NaHCO₃ per L) or with FA-100 and ice-cold water, individually weighed as wet weights (blotted dry) and blood collected from the caudal artery/vein with a heparinised microhematocrit capillary tubule. Depending upon the size of the fish, collectable blood volumes generally range from 20 to 80 µl per individual for fathead minnows (1) (16) and 5-15 µl per individual for zebrafish (17). Plasma is separated from the blood via centrifugation (3 min; 15,000 g; room temperature), and stored with protease inhibitors at -80°C, until analyzed for vitellogenin. Alternatively, in medaka the liver will be used as a tissue-source for vitellogenin determination.

Vitellogenin (VTG)

46. VTG is a phospholipoglycoprotein precursor to egg yolk protein that normally occurs in sexually-active females of all oviparous species; the production of VTG is controlled by interaction of estrogens with the estrogen receptor. Significantly, males maintain the capacity to produce VTG in response to stimulation with estrogen receptor agonists; as such, induction of VTG in males and immature

females has been successfully exploited as a biomarker specific for estrogenic compounds in a variety of OECD fish species (1) (2) (3) (28) (29) (30) (31) (32) (33).

47. The measurement of VTG should be based upon a validated homologous ELISA method, using homologous VTG standard and homologous antibodies. It is recommended to use method capable to detect VTG levels as low as few ng/ml plasma (or liver for medaka), which is the background level in unexposed male fish.

DATA AND REPORTING

Evaluation of Biomarker Responses by Analysis of Variance (ANOVA) or by Regression Analysis

48. To identify potential endocrine activity by a chemical, responses are compared between treatments versus controls groups using analysis of variance (ANOVA). All biological response data should be analyzed and reported separately by sex (either females or males). If the required assumptions for parametric methods are not met - non-normal distribution (e.g. Shapiro-Wilk's test) or heterogeneous variance (Bartlett's test or Levene's test), consideration should be given to transforming the data to homogenize variances prior to performing the ANOVA, or to carrying out a weighted ANOVA. Dunnett's test (parametric) on multiple pair-wise comparisons or a Mann-Whitney with Bonferroni adjustment (non-parametric) may be used. Other statistical tests may be used (e.g. Jonckheere-Terpstra test) provided the number of replicates is sufficient. Additional information can be obtained from the OECD Draft Guidance Document on Statistical Analysis of Ecotoxicity Data, available on the OECD public website (**ref**).

49. To identify potential endocrine activity following chemical exposure, responses across the different treatment concentrations could also be examined based on regression analysis (based on three or more exposure concentrations per chemical). All biological response data should be analyzed and reported separately by sex (either females or males). Direct observation of concentration-related trends in biological responses can also usefully inform the outcome of such a study for the intrinsic endocrine activity of a test chemical.

Interpretation of results

50. 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.

51. Study data should include:

Testing facility:

- Responsible personnel and their study responsibilities

Test Substance:

- Characterization of test substances
- Physical nature and were relevant physicochemical properties
- Method and frequency of preparation of dilutions
- Information on stability and biodegradability
- Weakly measured concentrations of the test solutions and analytical method used

Solvent:

- Characterization of solvent (nature, concentration used)
- Justification of choice of solvent (if other than water)

Test animals:

- Species and strain
- Supplier and specific supplier facility
- Age of the fish at the start of the test
- Details of animal acclimatation procedure
- Body weight of the fish at the start of the exposure

Test Conditions:

- Test procedure used (test-type, loading rate, stocking density, etc.)
- Method of preparation of stock solutions and flow-rate
- The nominal test concentrations, the means of the measured values and 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 if necessary (e.g. PCBs, PAHs and organochlorine pesticides)

Results

- Evidence that controls met the validity criterion for survival
- Data on mortalities occurring in any of the test concentrations and control
- Statistical analytical techniques used, statistics based on fish, treatment of data and justification of techniques used
- Data on biological observations of gross morphology, including secondary sex characteristics and vitellogenin
- Results of the statistical analysis preferably in tabular and graphical form
- Incidence of any unusual reactions by the fish and any visible effects produced by the test substance

ANNEX 1

Note: Document prepared by the Secretariat of the Test Guidelines Programme based on the agreement reached at the 6th Meeting of the EDTA Task Force

OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals

<p>Level 1 Sorting & prioritization based upon existing information</p>	<ul style="list-style-type: none"> - physical & chemical properties, e.g., MW, reactivity, volatility, biodegradability, - human & environmental exposure, e.g., production volume, release, use patterns - hazard, e.g., available toxicological data
<p>Level 2 <i>In vitro</i> assays providing mechanistic data</p>	<ul style="list-style-type: none"> - ER, AR, TR receptor binding affinity - Transcriptional activation - Aromatase and steroidogenesis <i>in vitro</i> - Aryl hydrocarbon receptor recognition/binding - QSARs
<p>Level 3 <i>In vivo</i> assays providing data about single endocrine Mechanisms and effects</p>	<ul style="list-style-type: none"> - High Through Put Prescreens - Thyroid function - Fish hepatocyte VTG assay - Others (as appropriate)
<p>Level 4 <i>In vivo</i> assays providing data about multiple endocrine Mechanisms and effects</p>	<ul style="list-style-type: none"> - Uterotrophic assay (estrogenic related) - Hershberger assay (androgenic related) - Non-receptor mediated hormone function - Others (e.g. thyroid)
<p>Level 5 <i>In vivo</i> assays providing data on effects from endocrine & other mechanisms</p>	<ul style="list-style-type: none"> - enhanced OECD 407 (endpoints based on endocrine mechanisms) - male and female pubertal assays - adult intact male assay
<ul style="list-style-type: none"> - 1-generation assay (TG415 enhanced)¹ - 2-generation assay (TG416 enhanced)¹ - reproductive screening test (TG421 enhanced)¹ - combined 28 day/reproduction screening test (TG 422 enhanced)¹ <p><small>1. Potential enhancements will be considered by VMG mam</small></p>	<ul style="list-style-type: none"> - Fish gonadal histopathology assay - Frog metamorphosis assay
	<ul style="list-style-type: none"> - Fish VTG (vitellogenin) assay (estrogenic related)
	<ul style="list-style-type: none"> - Partial and full life cycle assays in fish, birds, amphibians & in vertebrates (developmental and reproduction)

ANNEX 2

ABBREVIATIONS & DEFINITIONS

CV – coefficient of variation

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.

ANNEX 3

EXPERIMENTAL CONDITIONS FOR THE NON-SPAWNING FISH ENDOCRINE SCREENING PROTOCOL

1. Recommended species	Fathead minnow (<i>Pimephales promelas</i>)	Medaka (<i>Oryzias latipes</i>)	Zebrafish (<i>Danio rerio</i>)
2. Test type	Flow-through	Flow-through	Flow-through
3. Water temperature	25 ± 2°C	24± 2°C	26 ± 2°C
4. Illumination quality	Fluorescent bulbs (wide spectrum)	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)	10-20 µE/M ² /s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)
6. Photoperiod (dawn / dusk transitions are optional, however not considered necessary)	16 h light, 8 h dark	16 h light, 8 h dark	16 h light, 8 h dark
7. Loading rate	<5 g per L	<5 g per L	<5 g per L
8. Test chamber size	10 L (minimum)	2 L (minimum)	4 L (minimum)
9. Test solution volume	8 L (minimum)	1.5 L (minimum)	4 L (minimum)
10. Volume exchanges of test solutions	Minimum of 6 daily	Minimum of 5 daily	Minimum of 5 daily
11. Age of test organisms	Reproductively mature fish (not senescent)	Reproductively mature fish (not senescent)	Reproductively mature fish (not senescent)
12. Wet weight of adult fish (g)	Females: 1.5 ± 20% Males: 2.5 ± 20%	Females: 0.35 ± 20% Males: 0.35 ± 20%	Females: 0.65 ± 20% Males: 0.5 ± 20%
13. No. of fish per test vessel	10	10	10
14. No. of treatments	= 3 (plus appropriate controls)	= 3 (plus appropriate controls)	= 3 (plus appropriate controls)

ANNEX 3 (continued)

15. No. vessels per treatment	2 minimum	2 minimum	2 minimum
16. No. of fish per test concentration	10 adult females and 10 males (5 females and 5 males in each replicate vessel)	10 adult females and 10 males (5 females and 5 males in each replicate vessel)	10 adult females and 10 males (5 females and 5 males in each replicate vessel)
17. Feeding regime	Frozen adult brine shrimp twice daily (<i>ad libitum</i>)	Brine shrimp nauplii twice daily (<i>ad libitum</i>)	Frozen adult brine shrimp twice daily (<i>ad libitum</i>)
18. Aeration	None unless DO concentration falls below 4.9 mg per L	None unless DO concentration falls below 4.9 mg per L	None unless DO concentration falls below 4.9 mg per L
19. Dilution water	Clean surface, well or reconstituted water	Clean surface, well or reconstituted water	Clean surface, well or reconstituted water
20. Dilution factor	max 10	max 10	max 10
21. Pre-exposure period	none	None	None
22. Chemical exposure duration	21-d	21-d	21-d
23. Biological endpoints	Non-specific: - survival - behavior Endocrine biomarkers: - gross morphology (incl. 2y sex characteristics) - VTG - gonadal histology	Non-specific: - survival - behavior Endocrine biomarkers: - gross morphology (incl. 2y sex characteristics), - VTG - gonadal histology	Non-specific: - survival - behavior Endocrine biomarkers: - gross morphology, - VTG - gonadal histology
24. Test acceptability	Dissolved oxygen $\geq 60\%$ of saturation; mean temperature of $25 \pm 2^\circ\text{C}$; 90% survival of fish in the controls	Dissolved oxygen $\geq 60\%$ of saturation; mean temperature of $24 \pm 2^\circ\text{C}$; 90% survival of fish in the controls	Dissolved oxygen $\geq 60\%$ of saturation; mean temperature of $26 \pm 2^\circ\text{C}$; 90% survival of fish in the controls

ANNEX 4

SPAWNING SUBSTRATE

ANNEX 5

CHARACTERISTICS OF ACCEPTABLE WATER

ANNEX 6

PROCEDURES FOR SECONDARY SEX CHARACTERISTICS MEASUREMENT

ANNEX 6

This will be attached later.(SPAWNING SUBSTRATE)

LITERATURE

- (1) OECD (1998). Report of the First Meeting of the OECD Endocrine Disrupter Testing and Assessment (EDTA) Task Force, 10th – 11th March 1998, ENV/MC/CHEM/RA(98)5.

Other literatures will be added later.