# The medaka (*Oryzias latipes*) Full Life-Cycle Test Guideline

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# Fish Full Life-Cycle Test Guideline

## OBJECTIVE

This test guideline describes a fish full life-cycle test that can be used to evaluate the potential chronic effects of chemicals on fish populations. The method gives primary emphasis to potential population relevant effects (namely, adverse impacts on survival, development, growth and reproduction) for the calculation of the No-Observed Effect Concentration ($NOEC_{adverse}$). These effect observations should be augmented by secondary mechanistic biomarker responses (namely, vitellogenin, gonad somatic index [GSI], and gonad histology). The method is applicable to a variety of chemicals, including endocrine disrupters and general toxicants. The medaka (*Oryzias latipes*) is a suitable species for use in this test guideline, however, other species such as fathead minnow (*Pimephales promales*), sheepshead minnow (*Cyprinodon variegatus*), three spined stickleback (*Gasterosteus aculatus*) and zebrafish (*Danio rerio*) are also suitable.

## GLOSSARY OF TERMS

dph = days post-hatch; GSI = gonad somatic index; VTG = vitellogenin

## INTRODUCTION

This test guideline describes a confirmatory test based on full life-cycle of fish over two generations to give data relevant to ecological risk assessment of endocrine disrupting chemicals (EDCs). Fish full life-cycle test for ecotoxicological studies has already been established by the U.S. Environmental Protection Agency (U.S. EPA) in fathead minnows (*Pimephales promelas*) and sheepshead minnows (*Cyprinodon variegatus*) (U.S. EPA, 1986). Present protocol in this guideline is derived from work on medaka (*Oryzias latipes*) in Chemicals Evaluation and Research Institute, Japan (CERI) based on the original protocol by the U.S. EPA. This test described in this guideline has been evaluated by testing with OECD reference compounds (4-*tert*-pentylphenol; Seki et al., 2002 [submitted], ethinylestradiol and methyltestosterone; preparing the manuscripts for publication in a journal) and conducted with the known EDCs (4-nonylphenol; Yokota et al., 2001a, 4-*tert*-octylphenol and 17β-estradiol; preparing the manuscripts for publication in a journal) for their hazard assessments. In principal, the approach is also applicable to other OECD fish species, notably fathead minnow (Länge et al., 2001), sheepshead minnow (*Cyprinodon variegatus*) (Zillioux et al, 2001), three-spined stickleback (*Gasterosteus aculatus*) Katsiadaki et al., (2002) and zebrafish (*Danio rerio*).

2. This test guideline measures several biological endpoints. Primary emphasis is given to potential adverse effects on population relevant parameters including survival, gross development, growth and reproduction. Secondarily, in order to provide secondary mechanistic information and provide linkage between results from other kinds of field and laboratory studies, where there is *a priori* evidence for a chemical having potential endocrine disrupter activity (eg androgenic or oestrogenic activity) then other useful information can be obtained by measuring vitellogenin (VTG) levels, gonad somatic index [GSI] and evaluating gonad histology.

## PRINCIPAL OF THE TEST

3. Overviews of the relevant test conditions in medaka are provided in Annex 1. For medaka,

exposure of parental fish ($F_0$) is normally initiated with healthy embryos within several hours post-fertilization. This test is conducted at a minimum of five chemical concentrations, as well as appropriate controls with a minimum of 60 embryos for each treatment separated into four groups. Chemical delivery can be via an aqueous route (with or without carrier solvents). Monitoring continues for up to 100 dph and includes embryological development, hatching (hatchability and time to hatch), posthatch survival, growth (total length and body weight), sexual differentiation (secondary sex differentiation and gonadal histology), reproduction (fecundity and fertility), hepatic VTG levels, and gonadal development (GSI). Eggs spawned from the $F_0$ fish at least 3 d of the $F_0$ exposure are also exposed to the test substance and examined for hatching, and the progeny generation ($F_1$) is examined for survival after hatching, as well as growth, sexual differentiation, and hepatic VTG levels, until 60 dph.

## DESCRIPTION OF THE METHOD

### Test animals and exposure system

#### Test animals

4. This test should be started with embryos within several hours post-fertilization from a ready supply of known-quality animals. Candidate species for this assay include medaka (*Oryzias latipes*), fathead minnow (*Pimephales promelas*), sheepshead minnow (*Cyprinodon variegatus*) and zebrafish (*Danio rerio*). Relevant modification is necessary for test fish species other than medaka. Field-collected fish generally should not be used to initiate cultures for the testing.

5. Parental fish for the spawning eggs subjected to exposure should be selected from a population of a single stock. The fish should be paired and acclimatized for at least two weeks prior to the test under conditions of water quality and illumination similar to those used in the test (Annex 1). Fish should be fed exclusively with *Artemia nauplii* (<24 h after hatching) twice a day.

#### Water

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. 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, $SO_4$), pesticides, total organic carbon and suspended solids should be made, for example, every six months where a dilution water is known to be relatively constant in quality. Some chemical characteristics of an acceptable dilution water are listed in Annex 2.

#### Exposure system

7. The design and materials used for the exposure system are optional. Glass, stainless steel, or other chemically inert material should be used for construction of the test system. For medaka, an exposure system is well suited as follows (Yokota et al., 2000, 2001a; Kang et al., 2002; Seki et al., 2002): The exposure system consists of a continuous-flow mini-diluter system (Benoit et al., 1982). Stock solution of the test chemical should be injected in this exposure system by an appropriate pump. The flow rate of the stock solution should be calibrated in accordance with analytical confirmation of the test solutions before the initiation of exposure, and checked volumetrically

periodically during the test. The test solution in each chamber is renewed adequately depending on the test chemical stability and water quality.

## Experimental Design

8. It is recommended to use a minimum of five chemical concentrations. Prior knowledge of the test substance (e.g. results of the tests at tier 1 and 2 in OECD testing scheme, in vitro receptor binding assay, subchronic toxicity, and range-finding test) should help in selection of appropriate test concentrations.

9. For the fish full life-cycle test in weak estrogens, the highest concentration used in the definitive test should be selected to cause significant subchronic toxicity, because weak estrogens may exert subchronic toxicity and abnormal sex differentiation at similar concentration ranges (Seki et al., 2002 [submitted]). If possible, the range-finding test may be conducted under conditions (water quality, test system, animal loading) similar to those used for the definitive test (Annex 1). For the test in strong estrogen or androgen, histological measurements are much more responsive than subchronic effects, therefore, the highest concentration used in the definitive test should be selected to cause considerable sex reversal. The lowest concentration should be a factor of 10- to 100-times lower than the highest concentration. The use of five concentrations in this test enables not only dose-response relationships, but also providing the lowest-observed-effect concentration and no-observed-effect concentration which are necessary for risk assessment. At present, a minimum of four replicate test chambers (each containing 15 fish) except the reproductive trial (a minimum of six replicate test chambers) is recommended per treatment in medaka (Yokota et al., 2001a). It has been demonstrated that this sample size enables detection of statistically-significant differences for the majority of the endpoints in control fish versus animals treated with "model" EDCs, including 4-nonylphenol (Yokota et al., 2001a), 4-*tert*-pentylphenol (Seki et al., 2002 [submitted]), ethinylestradiol, methyltestosterone, 4-*tert*-octylphenol and 17β-estradiol (preparing the manuscripts for publication in a journal). Based on this test, a minimum of 360 fish (15 fish in each of four replicates for five treatments, plus one control) is required per assay. This would, of course, increase when additional control treatments are required.

## Administration of test substance via water and analytical determination

10. It is recognized that the environmentally relevant routes of exposure are via the water or the diet. For water soluble chemicals, theoretically there are several options for delivery of test chemical to the fish via the water.

11. 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 can be used for achieving a suitable concentrated stock solution. It is recommended that, whenever possible, solvents not be used to generate stock solutions for conducting chemical exposures via the water.

12. In some cases it may be necessary to use solvents to generate stock solutions for aqueous testing; this could occur when a chemical is very insoluble, unstable in a saturator system, or so expensive/limited in availability that the use of saturators is not practical. Generally, examples of suitable solvents in fish chronic toxicity test are acetone, dimethylformamide, dimethylsulfoxide, ethanol, methanol and triethylene glycol. However, there are relatively few studies on the possible effects of these solvents on the fish endocrine system. Hence, it is essential that any test utilizing a carrier solvent include both solvent-exposed and non-exposed controls.

- A new section on dietary exposure would be useful

### Analytical determination and measurements

13. During the test, the concentrations of the test substance are determined at appropriate intervals (e.g. every other week in each of the test treatments).

14. During the test, the flow rates of diluent and stock solution should be checked at intervals accordingly (e.g. five times a week). It is recommended that the test substance concentrations fall within ± 20 % of nominal values (i.e. within the range 80-120 %). If the concentrations of the test substance are not maintained within ± 20 % of nominal values, it is preferable within ± 20 % of mean measured values. However, in case of some chemicals which can markedly accumulate in fish body, the concentrations of the test substance may be decreased depending on fish growth. In that case, it is recommended that renewal rate of the test solution in each chamber be raised.

15. It is recommended that results be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance has been satisfactory maintained within ± 20 % of the nominal, the results can be based on nominal or measured values.

16. During the test, dissolved oxygen, pH, and temperature should be measured in one test vessel of all treatment groups and the controls. As a minimum, these measurements should be measured once a week through the exposure periods.

## PERFORMANCE OF THE TEST

### Embryological phase

17. Exposure should be initiated shortly after fertilization (at the latest, < several hours post-fertilization). The 60 embryos employed for each treatment are randomly separated into four groups of 15 for testing in quadruplicate. Each group of embryos may be placed directly in the test chamber or in a cylindrical glass egg cup (e.g. diameter, 5 cm; depth, 10 cm), which is covered with stainless steel mesh (e.g. No. 32) on the bottom. If the egg cups are used, these may be swung vertically at a slow pace (e.g. 20-25 times/min.) in the test chamber. The developing embryos should be observed daily under a stereoscopic microscope. Any dead embryo should be discarded; live ones should be returned to the chamber. This procedure should be repeated until all the living embryos have hatched. Generally, hatchability and time to hatch of medaka in the controls at 24 °C are ≥ 90 % and 9-10 d, respectively (Yokota et al, 2000; Yokota et al, 2001a).

### Larval-juvenile phase

18. After hatching, the larvae should be fed an adequate amount of *Artemia* nauplii (<24 h after hatching) twice a day; some nauplii remain for at least 1 h after feeding. Daily observation should be made to examine mortality, abnormal behavior and appearance until 60-d posthatch, and dead fish should be removed as soon as possible.

19. At 60-d posthatch, 20 individuals from each treatment group (e.g. five individuals from each of the four test chambers) should be randomly removed and euthanized with an adequate anesthetic (e.g. FA-100, Tanabe Seiyaku, Osaka, Japan). The fish should be observed for external secondary sex characteristics based on the shape of the dorsal and anal fins under a stereoscopic microscope.

Notably, some chemicals cause skewed occurrence of secondary sex characteristics in medaka; for example, when exposed to androgen receptor agonist, methyltestosterone, the sex ratio estimated from secondary sex characteristics at 60-d posthatch can skew toward male (preparing the manuscripts for publication in a journal). Then the fish are drained on filter paper; body weight and total length should be measured.

**Gonadal histology**

20. After observation of secondary sex characteristics and measuring growth, the fish should be devoted for gonadal histology. The fish should be placed in an appropriate fixative (e.g. Bouin's solution, 4% formaldehyde or 1% glutaraldehyde) and embedded in paraffin or plastic after dehydrated. Fish should be sectioned longitudinally at 3-5 μm in thickness. Stepwise sectioning of the gonad ensures respective sections of the ensure gonad but does not guarantee detection of testis-ova (Gray et al, 1999a). It is not clear that how many sections should be prepared to satisfy both detecting abnormal tissues and be cost-effective. The section can be stained with hematoxylin and eosin, mounted with adequate agent (e.g. malinol, Muto pure chemicals, Tokyo, Japan) and then examined under a light microscope.

21. When exposed to estrogens in medaka, induction of testis-ova composed of both testicular germ cells and oocytes in the gonad has been well described in many in vivo studies (Egami, 1955; Yamamoto, 1965; Wester and Canton, 1986; Gary et al, 1997; Gronen et al, 1999; Gray et al, 1999a; Gray et al, 1999b; Metcalfe et al, 2001; Kang et al, 2002; Seki et al, 2002). This condition can be observed when exposed to estrogens from embryo to 60-d posthatch in medaka (Yokota et al, 2000; Yokota et al, 2001a; Seki et al, 2002 [submitted]). The rate of testis-ova induction can be increased dose-dependently, and the extent of oocyte-containing parenchyma in each testis-ova specimen can increase with increasing estrogen concentrations (Yokota et al, 2001a; Seki et al, 2002 [submitted]). Since small testis-ova appear similar in structure to enlarged spermatogonia, it is recommended that oocyte surrounded by an obvious band of follicular epithelium in the testis be testis-ova (Gray et al, 1999a). On occasion, abnormal connective tissues can develop in medaka when exposed to estrogens (Egami, 1955; Gray et al, 1999a; Kang et al, 2002; Seki et al, 2002). This condition can be observed when exposed to estrogens from fertilized eggs to 60-d posthatch in medaka (Yokota et al, 2001a; Seki et al, 2002 [submitted]). The ovary is evaluated based on relative number of previtellogenic, vitellogenic, and postvitellogenic oocytes. Strong estrogen, ethinylestradiol can inhibit ovarian development or cause many previtellogenic oocytes in female medaka (Scholz et al, 2000; Seki et al, 2002). We observed testis-ova condition when exposed to androgen, methyltestosterone from fertilized eggs to 60-d posthatch in medaka (preparing the manuscripts for publication in a journal). However, it was not clear whether this condition had been developed in genetically female fish by androgenic activity of methyltestosterone or in genetically male fish by estrogenic effect of aromatized chemical. We recommend nonaromatizable androgen may be suitable for androgenic reference compound. When exposed to anti-androgen, vinclozolin in medaka, induction of testis-ova has been reported (Kogar et al, 1999). Further research to characterize the effect of sex steroid antagonists (anti-androgen and anti-estrogen) on sexual differentiation and to verify the applicability of the present protocol to these chemicals is needed.

**Reproductive trial**

22. At 70-d posthatch, the sex of the surviving fish should be distinguished by their external secondary sex characteristics, and six mating pairs from each treatment and the controls should be selected to examine fecundity and fertility. If sex ratio is skewed toward male or female, no pairs can be selected. Each pair should be assigned to a test chamber and exposed until 100-d posthatch. The water temperature may be increased from 24 °C to 28 °C to stimulate spawning at 70-d

posthatch. The eggs spawned from each female should be counted, and assessed for viability microscopically for 30 consecutive days (71-100 d posthatch). Using weak estrogen, this test protocol has been verified to detect significant reproductive impairment (Seki et al, 2002 [submitted]).

23. On the day after the end of the reproductive examination (101-d posthatch), all pairs examined should be removed from their chambers and euthanized. If possible, the number of androgen-dependent papillary processes on the anal fin in males may be counted under a stereoscopic microscope. After body weights are measured, the gonads and livers should be removed and weighed for GSI calculation (100 x gonad wt/body wt) and measurement of hepatic VTG concentrations. For example, typical GSI values for reproductively active medaka at about 100-d posthatch range from 7 to 10 % for females, and 0.9 to 1.3 % for males (Yokota et al, 2001a; Seki et al, 2002 [submitted]). Nonylphenol that reduce fertility also can inhibit the maturation of the testis (Yokota et al, 2001a).

**Vitellogenin (VTG)**

24. The livers removed should be stored at –70 °C until the VTG measurements. For the assay, they should be individually homogenized with enzyme-linked immunosorbent assay (ELISA) buffer and centrifuged, and the collected supernatants are used for measuring VTG in ELISA (Yokota et al, 2001b).

25. VTG is an estrogen-dependent glycoprotein which is usually only synthesized in the liver of mature females (Denslow et al, 1999). Therefore, when detected in the serum of male fish, VTG can be used as a biomaker of exposure to estrogenic chemicals in a variety of fish species, including medaka (Gronen et al, 1999; Metcalfe et al, 2000; Yokota et al, 2001b; Kang et al, 2002; Seki et al, 2002), fathead minnow (Ankley et al, 2001; Harries et al, 2000; Panter et al, 1998; Kramer et al, 1998; Parks et al, 1999; Tyler et al, 1999), and rainbow trout (Jobling et al, 1996; Thorpe et al, 2000). Different methods are available to assess VTG production in fish; a measurement technique that is both relatively sensitive and specific is determination of protein concentrations in plasma or liver via ELISA or radioimmunoassay (RIA). For ELISA, monoclonal medaka VTG antibody and purified VTG protein from medaka are utilized (Yokota et al, 2001b). In fathead minnow, polyclonal fathead minnow VTG antibody and VTG protein have also been purified (Korte et al, 2000; Parks et al, 1999). Until now, the VTG induction assay using plasma have been developed and validated in fathead minnow (Tyler et al, 1999; Ankley et al, 2001) and rainbow trout (Thorpe et al, 2000). In small fish species such as medaka, collecting the plasma is technically difficult. Therefore hepatic VTG induction assay have been developed and validated in medaka (Yokota et al, 2001b).

**$F_1$ embryo phase**

26. Eggs spawned by females in the last 3 d of the reproductive trial (98 to 100-d posthatch) should be subjected to the exposure study of the progeny generation ($F_1$). To evaluate their hatchability and time to hatch, the fertilized eggs from each spawning in treatment groups and in the controls should be collected and exposed until hatching, under the same flow-through conditions as those used for the $F_0$ generation.

**$F_1$ larval–juvenile phase**

27. The newly hatched larvae in the treatments and the controls should be randomly transferred to four test chambers in each treatment and kept in the chambers until the last hatching. Then 15

larvae in each chamber should randomly be selected (60 larvae in each treatment group). Then they may be treated until 60-d posthatch in the same flow-through system for the $F_0$ generation. The fish should be checked daily until 60-d posthatch for mortality, abnormal behavior, and appearance. Dead fish should be removed as soon as possible. At 60-d posthatch, the external secondary sex characteristics of all the surviving $F_1$ fish should be observed, and their weights and lengths should be measured after overdosing with anesthetic. They should be fixed and prepared for the gonadal histology. Some estrogenic chemicals can have adverse effects at lower concentrations in the progeny than in their parent generations (Yokota et al, 2001a; Seki et al, 2002 [submitted]). Further work to elucidate the enhanced response in the progeny by transgenerational exposure need to be investigated.

## VALIDITY OF THE TEST

28. This test has not been performed extensively, but based on our study with medaka to date, we suggest the following criteria for the test acceptability:

- the dissolved oxygen concentration must be between 60 and 100 % of the air saturation value throughout the test;
- the water temperature must not differ by more than ± 2.0 °C between test chamber or between successive days at any time during the test;
- there should be more than 80 % survival of control animals after hatching through 60-d posthatch in each $F_0$ and $F_1$ generation.

29. As experience is gained with this test, additional biological performance criteria can be incorporated.

## DATA REPORTING

### Statistical analysis

30. The primary goal of the data analyses are to calculate the No-Observed Effect Concentration and the Lowest Observed Effect Concentration based on potentially adverse population relevant effects ($NOEC_{adverse}$ and $LOEC_{adverse}$, respectively). These calculations should address the effects on survival, gross development, growth and reproduction. Secondarily, the data for VTG, GSI and gonad histology may be used to calculate the No-Observed Effect Concentration and the Lowest Observed Effect Concentration based on mechanistic biomarker reponses ($NOEC_{biomarker}$ and $LOEC_{biomarker}$, respectively).

New paragraph. Different options are available for data analysis, we suggest the following flow acceptability; If carrier solvent is used in the test, appropriate analytical method may be used prior to data analysis to determine whether there are differences between the solvent control and control groups. If no differences are found, these groups are pooled for subsequent analysis. If differences are found, the control group without solvent is excluded from the subsequent analyses because of few studies on the possible effects of the solvents on the fish endocrine system. To identify potential endocrine activity by a chemical, biological responses except for sex ratios may be compared between in treatments versus controls groups using analysis of variance (ANOVA) followed by multiple comparison test (e.g. Dunnett's multiple comparison test). If the required assumptions for parametric methods are not met, non-parametric test may be conducted. The data on sex ratios may be assessed by chi-squared analysis.

31. Any endpoints that are significantly impacted by the test chemical should be reported.

## Test report

32. The test report must include the following:

Test substance:

- physical nature and, where relevant, physicochemical properties;
- chemical identification data.

Test species:

- scientific name, strain, source and method of collection of the fertilized eggs and subsequent handling.

Test conditions:

- photoperiod(s);
- test design (e.g. chamber size, number of test chambers and replicates, number of embryos per replicates);
- method of preparation of stock solutions and frequency of renewal (the solubilising agent and its concentration must be given, when used);
- the nominal test concentrations, the means of the measured values and their standard deviations;
- dilution water characteristics: pH, hardness, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), total organic carbon, suspended solids and other measurements made;
- water quality within test vessels, pH, temperature and dissolved oxygen concentration;
- detailed information on feeding (e.g. type of foods, source, amount given and frequency).

Results:

- Data for the control (plus solvent control when used) and the treatment groups as follows, embryological development, hatching (hatchability and time to hatch), posthatch survival, growth (total length and body weight), sexual differentiation (secondary sex characteristics and gonadal histology), reproduction (fecundity and fertility), gonadosomatic index (GSI) and hepatic vitellogenin (VTG);
- Statistical analysis used and results of this.

## LITERATURE

Ankley GT, Jensen KJ, Kahl MD, Korte JJ, Makynen EA. 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem* 20:1276–1290.

Benoit DA, Mattson VR, Olson DL. 1982. A continuous-flow mini-diluter system for toxicity testing. *Water Res* 16:457–464.

Denslow ND. 1999. Vitellogenin as a biomaker of exposure for estrogen or estrogen mimics. *Ecotoxicology* 8:385-398.

Egami N. 1955. Production of testis-ova in adult males of *Oryzias latipes* I. Testis-ova in the fish receiving estrogens. *Jpn J Zool* 11:353–365.

Gary MA, Metcalfe CD. 1997. Induction of testis-ova in Japanese medaka (*Oryzias latipes*) exposed to *p*-nonylphenol. *Environ Toxicol Chem* 16:1082–1086.

Gray MA, Niimi AJ, Metcalfe CD. 1999a. Factors affecting the development of testis-ova in medaka, *Oryzias latipes*, exposed to octylphenol. *Environ Toxicol Chem* 18:1835–1842.

Gray MA, Teather KL, Metcalfe CD. 1999b. Reproductive success and behavior of Japanese medaka (*Oryzias latipes*) exposed to 4-tert-octylphenol. *Environ Toxicol Chem* 18:2587–2594.

Gronen S, Denslow N, Manning S, Barnes S, Barnes D, Brouwer M. 1999. Serum vitellogenin levels and reproductive impairment of male Japanese medaka (*Oryzias latipes*) exposed to 4-*tert*-octylphenol. *Environ Health Perspect* 107: 385-390.

Harries JE, Runnalls T, Hill E, Harris C, Maddix S, Sumpter JP, Tyler CR. 2000. Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*). *Environ Sci Technol* 34:3003-3011.

Jobling S, Sheahan D, Osborne JA, Matthiessen P, Sumpter JP. 1996. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. *Environ Toxicol Chem* 15:194–202.

Kang IJ, Yokota H, Oshima Y, Tsuruda Y, Yamaguchi T, Maeda M, Imada N, Tadokoro H, Honjo T. 2002. Effects of 17β-estradiol on the reproduction of Japanese medaka (*Oryzias latipes*). *Chemosphere* 47:71–80.

Katsiadaki I, Scott AP, Hurst M, Matthiessen PM, Mayer I. 2002. Detection of environmental andrgens: a novel method based on ELISA of spigin, the stickleback glue protein. *Environ Toxicol Chem* 21: 1946-1954

Koger CS, Teh SJ, Hinton DE. 1999. *p*-Tert-octylphenol and/or vinclozolin induced-intersex in developing medaka (*Oryzias latipes*). Society of Toxicology 1999 Annual Meeting, New Orleans, LA, p.268.

Korte JJ, Kahl MD, Jensen KM, Pasha MS, Parks LG, LeBlanc GA, Ankley GT. 2000. Fathead minnow vitellogenin: complementary DNA sequence and messenger RNA and protein expression after 17β-estradiol treatment. *Environ Toxicol Chem* 19:972–981.

Kramer VJ, Miles-Richardson S, Pierens SL, Giesy JP. 1998. Reproductive impairment and induction of alkaline-labile phosphate, a biomarker of estrogen exposure, in fathead minnows (*Pimephales promelas*) exposed to warterborne 17β-estradiol. *Aquat Toxicol* 40:335–360.

Länge R, Hutchinson TH, Croudace CP, Siegmund F, Schweinfurth H, Hampe P, Panter GH, Sumpter JP. 2001. Effects of the synthetic estrogen 17α-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem* 20:1216–1227.

Metcalfe TL, Metcalfe CD, Kiparissis Y, Niimi AJ, Foran CM, Benson WH. 2000. Gonadal

development and endocrine responses in Japanese medaka (*Oryzias latipes*) exposed to *o*,*p*'-DDT in water or through maternal transfer. *Environ Toxicol Chem* 19:1893–1900.

Metcalfe CD, Metcalfe TL, Kiparissis Y, Koenig BG, Khan C, Hughes RJ, Croley TR, March RE, Potter T. 2001. Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by in vivo assays with Japanese medaka (*Oryzias latipes*). *Environ Toxicol Chem* 20:297–308.

Panter GH, Thompson RS, Sumpter JP. 1998. Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone. *Aquat Toxicol* 42:243-253.

Parks LG, Cheek AO, Denslow ND, Heppell SA, McLachlan JA, LeBlanc GA, Sullivan CV. 1999. Fathead minnow (*Pimephales promelas*) vitellogenin: Purification, characterization, and quantitative immunoassay for the detection of estrogenic compounds. *Comp Biochem Physiol* 132C:113-125.

Seki M, Yokota H, Matsubara H, Tsuruda Y, Maeda M, Tadokoro H, Kobayashi K. 2002. Effect of ethinylestradiol on the reproduction and induction of vitellogenin and testis-ova in medaka (*Oryzias latipes*). *Environ Toxicol Chem* 21: (in press).

Seki M, Yokota H, Matsubara H, Maeda M, Tadokoro H, Kobayashi K. 2002. Fish full life-cycle testing of the weak estrogen 4-*tert*-pentylphenol in medaka (*Oryzias latipes*) *Environ Toxicol Chem* : (submitted).

U.S. Environmental Protection Agency. 1986. Fish life-cycle toxicity tests; Hazard Evaluation Division Standard Procedure EPA 540/9–86–137; Office of Pesticide Programs: Washington, USA.

Scholz S, Gutzeit HO. 2000. 17-α-ethinylestradiol affects reproduction, sexual differentiation and aromatase gene expression of the medaka (*Oryzias latipes*). *Aquat Toxicol* 50:363–373.

Thorpe KL, Hutchinson TH, Hetheridge MJ, Sumpter JP, Tyler CR. 2000. Development of an in vivo screening assay for estrogenic chemicals using juvenile rainbow trout (*Oncorhynchus mykiss*). *Environ Toxicol Chem* 19:2812–2820.

Tyler CR, Aerle RV, Hutchinson TH, Maddix S, Trip H. 1999. An in vivo testing system for endocrine disruptors in fish early life stages using induction of vitellogenin. *Environ Toxicol Chem* 18:337–347.

Wester PW, Canton JH. 1986. Histopathological study of *Oryzias latipes* (medaka) after long-term β-hexachlorocyclohexane exposure. *Aquat Toxicol* 9:21-45.

Yamamoto T. 1965. Estriol-induced XY females of the medaka (*Oryzias latipes*) and their progenies. *Gen Comp Endocrinol* 5:527–533.

Yokota H, Tsuruda Y, Maeda M, Oshima Y, Tadokoro H, Nakazono A, Honjo T, Kobayashi K. 2000. Effect of bisphenol A on the early life stage in Japanese medaka (*Oryzias latipes*). *Environ Toxicol Chem* 19:1925–1930.

Yokota H, Seki M, Maeda M, Oshima Y, Tadokoro H, Honjo T, Kobayashi K. 2001a. Life-cycle toxicity of 4-nonylphenol to medaka (*Oryzias latipes*). *Environ Toxicol Chem* 20:2552–2560.

Yokota H, Morita H, Nakano N, Kang IJ, Tadokoro H, Oshima Y, Honjo T, Kobayashi K. 2001b. Development of an ELISA for determination of the Hepatic vitellogenin in Medaka (*Oryzias latipes*). *Jpn J Environ Toxicol* 4:87-98.

Zillioux EJ, Johnson IC, Kiparissis Y, Metcalfe CD, Wheat JV, Ward SG, Liu H. 2001. The sheepshead minnow as an in vivo model for endocrine disruption in marine teleosts: a partial life-cycle test with 17α-ethinylestradiol. *Environ Toxicol Chem* 20:1968–1978.

## ANNEX 1

### Test Conditions For The Fish Full Life-cycle Test Guideline

| | |
|---|---|
| 1. Recommended species | Medaka (*Oryzias latipes*)* |
| 2. Test type | Flow-through |
| 3. Water temperature | 24 ± 2 °C (28 ± 2 °C at reproductive phase) |
| 4. Illumination quality | Fluorescent bulbs (wide spectrum) |
| 5. Light intensity | 10-20 μE/$M^2$/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels) |
| 6. Photoperiod | 16 h light, 8h dark |
| 7. Loading rate | <5g per L |
| 8. Test chamber size | 2.5 L |
| 9. Test solution volume | 1.8 L |
| 10. egg cup | (If possible,) glass cylinder (e.g. diameter, 5 cm; depth, 10 cm), with a base of stainless steel mesh (e.g. No. 32) |
| 11. Volume exchanges of test solutions | Minimum of 6 daily |
| 12. Age of test organisms | Fertilized eggs (< several hours post-fertilization) |
| 13. No. of fish per test vessel | 15 (from embryo to 69-d posthatch)<br>1F + 1M (reproductive trial from 70 to 100-d posthatch) |
| 14. No. of treatments | ≥5 (plus appropriate controls) |

**ANNEX 1 continued**

| | |
|---|---|
| 15. No. of vessels per treatment | 4 (6 at reproductive trial) |
| 16. No. of fish per test concentration | 60 (6F + 6M at reproductive trial) |
| 17. Feeding regime | Live *Artemia* nauplii (< 24 h after hatching) 2x daily |
| 18. Aeration | None unless DO reaches <5.0 mg/L (<4.7 mg/L at reproductive trial) |

| | |
|---|---|
| 19. Dilution water | Clean surface, well, reconstituted water, or dechlorinated tap water |
| 20. Dilution factor | ≤ 3.2 |
| 21. Chemical exposure duration | 180-days |
| 22. Primary endpoints | $F_0$ and $F_1$: embryological development, hatching (hatchability and time to hatch), posthatch survival, growth (total length and body weight), sexual differentiation (secondary sex characteristics and gonadal histology) and hepatic vitellogenin (VTG)<br>$F_0$: reproduction (fecundity and fertility) and gonadosomatic index (GSI) |
| 23. Test acceptability | Dissolved oxygen ≥ 60 % of saturation; mean temperature of 24 ± 2 °C (28 ± 2 °C at reproductive phase); ≥ 80 % post-hatch survival of fish at 60-d posthatch in the controls |

* Other recommended species such as fathead minnow, sheepshead minnow and zebrafish is not mentioned here.

## ANNEX 2

## SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

| SUBSTANCE | CONCENTRATION |
|---|---|
| Particular matter | < 20 mg/L |
| Total organic carbon | <2 mg/L |
| Unionized ammonia | <1 µg/L |
| Residual chlorine | <10 µg/L |
| Total organophosphorus pesticides | <50 ng/L |
| Total organochlorine pesticides plus polychlorinated biphenyls | <50 ng/L |
| Total organic chlorine | <25 ng/L |