OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW GUIDELINE

Fish Embryo Toxicity (FET) Test

INTRODUCTION

1. This Test Guideline describes a Fish Embryo Toxicity (FET) test mainly developed for use with the zebrafish (*Danio rerio*) but the test method can also be adapted to fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*) and other relevant species of interest (1). This Guideline intends to define lethal effects of chemicals on embryonic stages of fish and constitute an alternative test method to the acute toxicity tests with juvenile and adult fish, *i.e.*, the OECD Test Guideline 203 (2), thus providing a reduction in fish usage. The FET-test is mainly developed from studies and validation activities performed on zebrafish (1)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18), but also from studies on fathead minnow (1)(19)(20)(21)(22) and Japanese medaka (1)(23)(24)(25)(26)(27)(28)(29).

2. The Test Guideline is based on chemical exposure of newly fertilized zebrafish eggs for up to 48 hrs and is expected to reflect acute toxicity in fish in general. After 24 and 48 hrs, four apical endpoints are recorded as indicators of acute lethality in fish: (i) coagulation of fertilized eggs, (ii) lack of somite formation, (iii) lack of detachment of the tail-bud from the yolk sac, and (iv) lack of heart-beat.

3. See Annex 1 for definitions.

PRINCIPLE OF THE TEST

4. Zebrafish embryos are individually exposed in 24-well microtiter plates to a range of concentrations of the test substance. The test is initiated immediately after fertilization and is continued for 48 hours. Lethal effects, as described by four apical endpoints, are determined by comparison with controls to identify the LC_{50}, NOEC and LOEC-values. The test method is based on using a minimum of five test concentrations as well as appropriate controls, with ten individual embryos per exposure concentration. Each substance should be tested in parallel in two to three independent replicates.

INITIAL CONSIDERATIONS

5. Under normal conditions, the test can be terminated after 48 hrs. However, if indications or evidence of delayed toxicity exist (30), the test duration may be extended to up to a total of 6 days[1], *i.e.*, to up to 2 days post-hatch. The exposure should be stopped before the beginning of external feeding of the embryos.

[1] In zebrafish, the eleutheroembryo stage is restricted to maximum 2 days; in the medaka, this period extends to up to 3 days; since in the fathead minnow the eleutheroembryo stage is very short, extension of the exposure period seems inappropriate.

6. Water solubility and vapor pressure of the test substance should be 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 should be available. The following information should normally be available prior to undertaking this bioassay: structural formula, purity of the substance, stability in water and light, pK_a and K_{ow}. Regarding chemical stability, it may be useful to have available results from a ready biodegradability test (OECD TG 301)(31). Solubility and vapor pressure can be used to calculate Henry's constant, which will indicate if losses of the test substance may occur. If, for one or more of the reasons mentioned above, there is evidence that the concentrations of the test substance in solution cannot be satisfactorily maintained, a semi-static test should be performed. A ^{14}C tracer technique or a non-radiochemical analysis could be performed to ensure that the exposure chemical has penetrated the chorion and reached the target site.

7. A reference substance may be tested for estimation of an EC_{50}-value as a means of assuring that the test conditions are reliable. Toxicants previously used in ring-tests are recommended for this purpose (1)(18). Recurrent tests of a reference substance *e.g.*, 3,4-dichloroaniline should be performed preferably every three months to confirm the performance of the test.

LIMITATIONS OF THE TEST METHOD

8. Test substances which are characterized by a high lipophilicity and/or volatility and/or a lack of stability may not be adequately assessed by the test and another Test Guideline (*e.g.*, OECD TG 203) should be used. Size and charge of the test molecule may also be relevant, since the chorion and biological membranes have to be passed by the molecule to reach the target site(s) in the embryo.

DESCRIPTION OF THE METHOD

9. The following description of the test procedure mainly refers to the method for the zebrafish; however, minor modifications allow an adaptation of the method to, *e.g.*, Japanese medaka and fathead minnow (1). An overview of relevant maintenance and test conditions specific for the three species zebrafish, fathead minnow and Japanese medaka is available (1).

Test chambers

10. Glass or polystyrene exposure plates with a 2.5-5 ml filling capacity should be used (*e.g.*, 24-well multiplates). In case adsorption to polystyrene is suspected, inert materials (glass) should be used. Plates with round bottom are preferable for inspection with the stereo microscope, plates with flat bottom for inspection with the inverted microscope. It is desirable that test chambers be randomly positioned in the test area.

Water

11. Dilution water in accordance with ISO standards (32)(33) or charcoal filtered drinking water may be used for fish maintenance. It should be of constant quality before and during the period of the test. The pH of the maintenance water should be within the range 6.8 to 8.4, but during a given test pH values in the controls should be kept between 6.8 and 8.0. Oxygen saturation in the maintenance tanks should be kept above 80%. Temperature should be adjusted to 26±1°C, during the whole test. In order to ensure that the dilution water will not unduly influence the test result (for example by complexion of test substance), samples should be taken at regular 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 (*e.g.*, total

organophosphorus and total organochlorine pesticides), total organic carbon and suspended solids should be made, for example, every three months in the cases for which 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 (1).

12. For the embryo toxicity tests, dilution water should be prepared from reconstituted water in accordance with ISO standards (32)(33). The resulting degree of hardness should be equivalent to 150-175 mg/L $CaCO_3$. The dilution water may be adapted to maintenance water of low hardness by dilution with deionized water up to a ratio of 1:5 resulting in a hardness of around 30-35 mg/L $CaCO_3$. The water is aerated to oxygen saturation prior to addition of the test substance. At the beginning of the test, oxygen saturation should be above 90 % (ca. 7.4 mg/L); during the test, oxygen saturation should be ≥ 50 % (ca. 4.1 mg/L; measurement is possible in the combined volumes of the test wells at the end of the experiment). Temperature should be kept at 26.0±1.0°C throughout the test.

Maintenance of brood fish

13. A breeding stock of unexposed, certified mature zebrafish with an age between 4 and 12 months is used for egg production. Fish should be free of macroscopically discernable symptoms of infection and disease. Spawners are maintained in aquaria with a loading capacity of a minimum of 1 L water per fish and a fixed 12-16 hour photoperiod (1)(4)(5)(34)(35). Females and males are continuously held together preferably in a ratio of 1:2. Optimal filtering rates should be adjusted; excess filtering rates causing heavy perturbation of the water should be avoided. Dry flake food is fed 3 to 5 times per day at a maximum of 3% fish weight per day. Live food (*e.g.*, *Artemia* nauplii, *Daphnia*) should be fed once daily *ad libitum*. Due to surplus feeding, water quality and cleanness of the aquaria should be monitored regularly and be reset to the initial state, if necessary.

Egg production

14. Mating and spawning take place within 30 minutes after turning on the lights (1)(4). To prevent adult zebrafish from egg predation, the egg traps are covered with stainless steel mesh with a grid size of 2 mm. Plant imitations of plastic or glass serving as spawning substrate are fastened to the mesh (1)(3)(4)(36). About 20-30 minutes after the onset of light, the egg traps are removed and the eggs collected. The traps should be replaced into the spawning tanks at the latest possible time or on the next day before the light is turned on. The collection of the fish eggs may take place in the maintenance tanks or in separated spawning tanks. A single mature female spawns about 50 - 80 eggs per day. The fertilization rate should be ≥50%. In case of fish spawning for the first time, fertilization rates may be lower in the first few spawns.

Egg differentiation

15. At 26°C, fertilized eggs undergo the first cleavage after about 15 min and the consecutive synchronous cleavages form 4, 8, 16 and 32 cell blastomers, respectively. At these stages, fertilized eggs can be clearly identified by the development of a blastula. Unfertilized eggs not undergoing cleavage or eggs showing obvious irregularities during cleavage (*e.g.*, asymmetry, vesicle formation) or injuries of the chorion are discarded.

Test solutions

16. Test solutions of the selected concentrations are prepared by dilution of a stock solution. The stock solutions should preferably be prepared by simply mixing or agitating the test substance in the dilution water by mechanical means (*e.g.*, stirring or ultrasonification). If the test substance is difficult to

dissolve in water, procedures described in the OECD Guidance Document No. 23 for handling difficult substances should be followed (30). The use of solvents or dispersants (solubilizing agents) 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 (30). Where a solubilizing agent is used to assist in stock solution preparation, its final concentration should not exceed 100 µl/L and should preferably be the same in all test vessels. Whereas dimethylsulfoxide (DMSO) proved to be a useful solubilizing agent, acetone should be avoided, since it has been shown to produce side effects (30).

17. Whenever possible, the test should be carried out without adjustment of pH. If the pH of the test solutions does not remain within a range between pH 6.5 and 8.5, a second test should be carried out, adjusting the pH of the stock solution to that of the dilution water before addition of the test substance. This pH adjustment should be made in such a way that the stock solution concentration is not significantly changed and that no chemical reaction or precipitation of the test substance is caused. Use of HCl and NaOH is recommended.

PROCEDURE

18. The embryos may be statically exposed to the test substance, unless there is evidence that the concentrations of the test substance in solution cannot be satisfactorily maintained. In this case, a semi-static technique should be used. For renewal, new test solutions are prepared in clean vessels, and surviving eggs are gently transferred into the new chambers. In any case, care should be taken to minimize any stress to the embryos.

19. The following instructions refer to performing the test in 24-well multi-plates with five concentrations of the test substance and a control group, which is the minimum number of concentrations required to meet the statistical requirements. Two-three replicates should be individually performed. If different test chambers (*e.g.*, small Petri dishes) are used or more treatments are tested, instructions have to be adjusted accordingly.

Start of exposure and duration of test

20. The test is initiated as soon as possible after fertilization of the eggs, the embryos preferably being immersed in the test solutions before cleavage of the blastodisc commences, or as close as possible after this stage. Not later than 60 minutes post fertilization (onset of light), viable fertilized eggs should be separated from unfertilized eggs and transferred into the test solutions. At latest 120 minutes post fertilization (past the onset of light), the fertilized eggs should be transferred into the test chambers.

21. To start exposure with minimum delay, about 40 eggs per exposure group are transferred into dilution water (*e.g.*, in 100 ml crystallization dishes) at latest 60 minutes post-fertilization. Under a stereo microscope with a minimum of 30-fold magnification, fertilized eggs are separated from non-fertilized eggs and transferred into freshly prepared test solutions (*e.g.*, in 100 ml crystallization dishes) by means of pipettes or small sieves. From these, eggs should be transferred to 24-wells plates with freshly prepared test solutions. Direct transfer from dilution water to the test wells should be avoided in order to minimize dilution errors. In 20 wells, fertilized eggs are placed individually in 2 ml of the respective test solutions. The remaining 4 wells of each plate are filled with 2 ml dilution water and one egg per well as an internal control, amounting to at least a total of 20 controls (4).

Test concentrations

22. Normally, five concentrations of the test substance spaced by a constant factor not exceeding 2.2 are required to meet statistical requirements. Justification should be provided if fewer than five concentrations are used. The highest concentration tested should preferably result in 100% mortality, and the lowest concentration tested should preferably give no observable effect. A range-finding test properly conducted before the definitive test enables the choice of the appropriate concentration range.

23. There should be evidence that the concentration of the substance being tested has been satisfactorily maintained, and preferably it should be at least 80% of the nominal concentration throughout the test. If the deviation from the nominal concentration is higher than 20%, results should be based on the measured concentration.

24. In case a solubilizing agent is used, its concentration should be the same in all test chambers. However, every effort should be made to avoid the use of such materials.

Controls

25. On each 24-well plate, four cavities are filled with dilution water only as an internal control.

26. In case a solubilizing agent is used, an additional group of the same size as the internal control group (20 embryos) is exposed to the solubilizing control on a separate 24-well plate, thus serving as a second control (30). The solvent should be demonstrated to have no significant effects on survival, nor produce any other adverse effects on the embryos.

Observations

27. Detailed description of the normal development of zebrafish embryos is available (37).

28. Overall survival of fertilized eggs in the controls and, where relevant in the solvent control, should be at least 90%. Coagulation of embryos, irregularities in somite formation, non-detachment of the tail as well as lack of heart-beat are recorded as apical endpoints according to table 1. Usually, observation of endpoints after 24 and 48 h is sufficient. Zebrafish are considered dead, if one of the apical endpoints is recorded as positive.

Table 1. Apical endpoints of acute toxicity in zebrafish embryos. The 24/48 hrs are the minimum observation times.

	Exposure time						
	4 h	**8 h**	**12**	**16 h**	**24 h**	**36 h**	**48 h**
Number of coagulated eggs	+	+	+	+	+	+	+
Formation of somites				+	+	+	+
Tail detachment					+	+	+
Presence of heart-beat						+	+

31. After 24 and 48 hours the number of coagulated eggs is determined. Coagulated eggs are milky white and appear dark under the microscope.

32. The formation of somites is examined after 24 and 48 hours. At 26±1°C, about 20 somites have formed after 24 hours; however, it is not possible to determine the exact number at this time. A normally

developed embryo shows vigorous side-to-side contractions at this stage which may result in rotating movements within the chorion. The formation of somites or not is therefore a sufficient criterion.

33. After 24 and 48 hours, detachment of the tail from the yolk following posterior elongation of the embryonic body is recorded.

34. After 48 hours only, the presence of active heart-beat is recorded.

DATA AND REPORTING

Treatment of results

35. For the zebrafish FET-test, the cumulative percentage mortality for each exposure period (24, 48 h) is plotted against concentration on logarithmic probability paper. For calculation of the LC_{50}-value and the confidence limits (95%) appropriate statistical methods should be applied (38)(39)(40)(41)(42)(43) and the OECD Guidance Document No. 54 should be consulted (44). In addition to the LC_{50}, the LOEC and NOEC values should be determined.

36. Where the data obtained are inadequate for the use of standard methods of calculating the LC_{50}, the highest concentration causing no mortality (NOEC) and the lowest concentration producing 100 % mortality should be used as an approximation for the LC_{50} (this being considered the geometric mean of these two concentrations).

Test report

31. The test report should include the following information:

Test substance:

- physical nature, purity and where relevant, physicochemical properties (including isomerisation);
- identification data and Chemical abstract Services Registry Number, if known.

Test organisms:

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

Test conditions:

- test procedure used (*e.g.*, standard or extended test; static or semi-static);
- photoperiod;
- test design (*e.g.*, number of test chambers and replicates);
- water quality characteristics in fish stock maintenance (pH, hardness, temperature);
- test solution characteristics at the beginning of the test: pH, temperature, dissolved oxygen saturation and any other measurements made;
- dissolved oxygen concentration, pH and temperature of the test solutions after 48 h (in semi-static systems the pH should be measured prior to and after water renewal);
- method of preparation of stock solutions and test solutions as well as frequency of renewal;
- justification for use of solvent and justification for choice of solvent, if other than water;

- history or concurrent data demonstrating that the solvent does not interfere with the outcome of the study;
- nominal test concentrations;
- information on concentrations of the test substance in the test solutions at the beginning and upon termination of exposure (actual concentrations);
- evidence that controls met the overall survival acceptability standard;

Results:

- cumulative mortality at each concentration at the recommended observation times;
- LC_{50} values, with 95% confidence limits, at each of the recommended observation times, if possible;
- maximum concentration causing no mortality within the period of the test (NOEC);
- minimum concentration causing 100% mortality within the period of the test;
- graph of the concentration-mortality curve at the end of the test;
- mortality in the controls;
- data on response for each parameter assessed;
- NOEC/LOEC for each response assessed;
- incidence and description of morphological and physiological abnormalities, if any;
- incidents in the course of the test which might have influenced the results;
- statistical analysis and treatment of data (*e.g.*, probit analysis, logistic regression model or geometric mean for LC_{50}; ANOVA, Dunnetts, William´s for NOEC/LOEC)
- LC_{50} for lethal response parameters
- slope and confidence limits of the regression of the (transformed) concentration-response curve.

Discussion and interpretation of results.

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ANNEX 1

DEFINITIONS

Blastula: The blastula is a cellular formation around the animal pole that covers a certain part of the yolk cell.

Median Effective Concentration (EC_{50}): Statistically derived concentration of a substance in an environmental medium expected to produce a certain effect in 50% of test organisms in a given population under a defined set of conditions.

Eleutheroembryo stage: The stage between hatching and start of intake of external feed (yolk-sac fry stage)

Lowest Observed Effect Concentration (LOEC) is the lowest tested concentration of a test substance at which the substance is observed to have a significant effect (at a p-value of <0.05) when compared with the control. However, all test concentrations above the LOEC should have a harmful effect equal to, or greater, than those observed at the LOEC.

Median Lethal Concentration (LC_{50}) is the concentration of a test substance that is estimated to be lethal to 50% of the test organisms.

No Observed Effect Concentration (NOEC) is the test concentration immediately below the LOEC.

Semi-static test is a test without flow of solution, but with occasional batchwise renewal of the test solution after prolonged periods (*e.g.*, 24 hours).

Static test is a test with aquatic organisms in which no flow of test solution occurs. Solutions remain unchanged throughout the duration of the test.