C.15. FISH, SHORT-TERM TOXICITY TEST ON *EMBRYO* AND SAC-FRY STAGES

1. METHOD

This short-term toxicity test method is a replicate of the OECD TG 212 (1998).

1.1 INTRODUCTION

This short-term toxicity test on Fish Embryo and Sac-Fry stages is a short-term test in which the life stages from the newly fertilized egg to the end of the sac-fry stage are exposed. No feeding is provided in the embryo and sac-fry test, and the test should thus be terminated while the sac-fry are still nourished from the yolk-sac.

The test is intended to define lethal, and to a limited extent, sublethal effects of chemicals on the specific stages and species tested. This test would provide useful information in that is could (a) form a bridge between lethal and sublethal tests, (b) be used as a screening test for either a Full Early Life Stage test or for chronic toxicity tests and (c) be used for testing species where husbandry techniques are not sufficiently advanced to cover the period of change from endogenous to exogenous feeding.

It should be borne in mind that only tests incorporating all stages of the life-cycle of fish are generally liable to give an accurate estimate of the chronic toxicity of chemicals to fish, and that any reduced exposure with respect to life stages may reduce the sensitivity and thus underestimate the chronic toxicity. It is therefore expected that the embryo and sac-fry test would be less sensitive than a Full Early Life Stage test, particularly with respect to chemicals with high lipophilicity (log P_{ow} > 4) and chemicals with a specific mode of toxic action. However smaller differences in sensitivity between the two tests would be expected for chemicals with a non-specific, narcotic mode of action (1).

Prior to the publication of this test, most experience with this embryo and sac-fry test has been with the freshwater fish *Danio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae – common name zebrafish). More detailed guidance on test performance for this species is therefore given in Annex 1. This does not preclude the use of other species for which experience is also available (Table 1).

1.2 DEFINITIONS

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 $p < 0.05$) when compared with the control. However, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC.

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

1.3 PRINCIPLE OF THE TEST

The embryo and sac-fry stages of fish are exposed to a range of concentrations of the test substance dissolved in water. Within the protocol a choice is possible between a semi-static and a flow-through procedure. The choice depends on the nature of the test substance. The test is begun by placing fertilised eggs in the test chambers and is terminated just before the yolk-sac of any larvae in any of the test chambers has been completely absorbed or before mortalities by starvation start in controls. Lethal and sub-lethal effects are assessed and compared with control values to determine the lowest observed effect concentration and hence the no observed effect concentration. Alternatively, they may be analysed using a regression model in order to estimate the concentration that would cause a given percentage effect (i.e. LC/ECx, where x is a defined % effect).

1.4 INFORMATION ON THE T EST SUBSTANCE

Results of an acute toxicity test (see Method C. 1) preferably performed with the species chosen for this test, should be available. The results may be useful in selecting an appropiate range of test concentrations in the early life stages test. Water solubility (including solubility in the test water) and the vapour pressure of the test substance should be known. 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.

Information on the test substance which is useful in establishing the test conditions includes the structural formula, purity of the substance, stability in light, stability under the conditions of the test, pKa, P_{ow} and results of a test for ready biodegradability (see Method C. 4).

1.5 VALIDITY OF THE TEST

For a test to be valid, the following conditions apply:

— overall survival of fertilised eggs in the controls and where relevant, in the solvent-only vessels must be greater than or equal to the limits defined in Annexes 2 and 3;

— the dissolved oxygen concentration must be between 60 and 100 % of the air saturation value (ASV) throughout the test;

— the water temperature must not differ by more than ± 1.5 °C between test chambers or between successive days at any time during the test and should be within the temperature ranges specified for the test species (Annexes 2 and 3).

1.6 DESCRIPTION OF THE TEST METHOD

1.6.1 **Test chambers**

Any glass or other chemically inert vessels can be used. The dimensions of the vessels should be large enough to allow compliance with the loading rate (see section 1.7.1.2). It is recommended that test chambers be randomly positioned in the test area. A randomised block design with each treatment being present in each block is preferable to a completely randomised design when there are systematic effects in the laboratory that can be controlled using blocking. Blocking, if used, should be taken account of in the subsequent data analysis. The test chambers should be shielded from unwanted disturbance.

1.6.2 **Selection of fish species**

Recommended fish species are given in Table 1A. This does not preclude the use of other species (examples are given in Table 1B), but the test procedure may have to be adapted to provide suitable test conditions. The rationale for the selection of the species and the experimental method should be reported in this case.

1.6.3 **Holding of the brood fish**

Details on holding the brood stock under satisfactory conditions may be found in OECD TG 210 [1] and in references (2)(3)(4)(5)(6).

1.6.4 **Handling of embryos and larvae**

Embryos and larvae may be exposed, within the main vessel, in smaller 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; a siphon-flush system can also be used. Fertilised eggs of salmonid fishes can be supported on racks or meshes with apertures sufficiently large to allow larvae to drop through after hatching. The use of pasteur pipettes is appropriate to remove the embryos and larvaes in the semi-static tests with complete daily renewal (see paragraph 1.6.6)

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 [1], 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 (such a caution may not be necessary for some less fragile species, e.g. the carp). The timing of this transfer varies with the species and transfer may not always be necessary. For the semi-static technique, beakers or shallow containers may be used, and, if necessary, equipped with a mesh screen slightly elevated above the bottom of the beaker. If the volume of these containers is sufficient to comply with loading requirements, (see 1.7.1.2) no transfer of embryo or larvae may be necessary.

1.6.5 **Water**

Any water which conforms to the chemical characteristics of an acceptable dilution water as listed in Annex 4 and in which the test species shows control survival at least as good as that described in Annexes 2 and 3 is suitable as a test water. It should be of constant quality during the period of the test. The pH should remain within a range of $\pm$ 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), 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 and Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl and 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, 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).

[1] OECD, Paris, 1992, Test Guideline 210, " Fish, Early-life Stage Toxicity Test".

1.6.6 **Test Solutions**

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 and ultrasonication). Saturation colums (solubility columns) can be used for achieving a suitable concentrated stock solution. As far as possible, the use of solvents or dispersants (solubilising agents) should be avoided; however, such coupounds may be required in some cases in order to produced a suitably concentrated stock solution. Examples of suitable solvents are acetone, ethanol, methanol, dimethylformamide and triethyleneglycol. Examples of suitable dispersants are Cremophor RH40, Tween 80, methylcellulose 0.01 % and HCO-40. Care should be taken when using readily biodegradable agents (e.g. acetone) and/or highly volatile as these can cause problems with bacterial built-up in flow-through tests. When a solubilising agent is used it must have no significant effect on survival nor visible adverse effect on the early-life stages as revealed by a solvent-only control. However, every effort should be made to avoid the use of such materials.

For the semi-static technique, two different renewal procedures may be followed; either (i) new test solutions are prepared in clean vessels and surviving eggs and larvae gently transferred into the new vessels in a small volume of old solution, avoiding exposure to air, or (ii) the test organisms are retained in the vessels whilst a proportion (at least three-quarters) of the test water is changed. The frequency of medium renewal will depend on the stability of the test substance, but a daily water renewal is recommended. If, from preliminary stability tests (see section 1.4), the test substance concentration is not stable (i.e.outside the range 80- 120 % of nominal or falling below 80 % of the measured initial concentration) over the renewal period, consideration should be given to the use of a flow-through test. In any case, care should be taken to avoid stressing the larvae during the water renewal operation.

For flow-through tests, a system which continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, 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, preferably daily, 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 (2).

1.7 PROCEDURE

Useful information on the performance of fish embryo and sac-fry toxicity tests is available in the literature, some examples of which are included in the literature section of this text (7)(8)(9).

1.7.1 **Conditions of exposure**

1.7.1.1 *Duration*

The test should start preferably within 30 minutes after the eggs have been fertilised. The embryos are immersed in the test solution before, or as soon as possible after, commencement of the blastodisc cleavage stage and in any case before the onset of the gastrula stage. For eggs obtained from commercial supplier, it may not be possible to start the test immediately after fertilisation. As the sensitivity of the test may be seriously influenced by delaying the start of the test, the test should be initiated within 8 hours after fertilisation. As larvae are not fed during the exposure period, the test should be terminated just before the yolk sac of any larvae in any of the test chambers has been completely absorbed or before mortalities by starvation start in controls. The duration will depend upon the species used. Some recommended durations are given in Annexes 2 and 3.

1.7.1.2 *Loading*

The number of fertilised eggs at the start of the test should be sufficient to meet statistical requirements. They should be randomly distributed among treatments, and at least 30 fertilised eggs, divided equally (or as equally as possible since it can be difficult to obtain equal batches when using some species) between at least three replicate test chambers, should be used per concentration. The loading rate (biomass per volume of test solution) should be low enough in order that a dissolved oxygen concentration of at least 60 % ASV can be maintained without aeration. 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 bean recommended (2).

1.7.1.3 *Light and temperature*

The photoperiod and test water temperature should be appropriate for the test species (Annex 2 and 3). For the purpose of temperature monitoring, it may be appropriate to use an additional test vessel.

1.7.2 **Test concentrations**

Normally, five concentrations of the test substance spaced by a constant factor not exceeding 3.2 are required. The curve relating LC_{50} to period of exposure in the acute study should be considered when selecting the range of test concentrations. The use of fewer than five concentrations, for example in limit tests, and a narrower concentration interval may be appropriate in some circumstances. Justification should be provided if fewer than five concentrations are used. Concentrations of the substance higher than the 96 hour LC_{50} or 100 mg/l, whichever is the lower, need not be tested. Substances should not be tested above their solubility limit in in the test water.

When a solubilising agent is used to aid preparation of test solutions (see section 1.6.6), its final concentration in the test vessels should not be greater than 0.1 ml/l and should be the same in all test vessels.

1.7.3 **Controls**

One dilution-water control (replicated as appropriate) and also, if relevant, one control containing the solubilising-agent (replicated as appropriate) should be run in addition to the test series.

1.7.4 **Frequency of analytical determinations and measurements**

During the test, the concentrations of the test substance are determined at regular intervals.

In semi-static tests where the concentration of the test substance is expected to remain within ± 20 % of the nominal (i.e. within the range 80 - 120 %; see section 1.4 and 1.6.6), it is recommended that, as a minimum, the highest and lowest test concentrations be analysed when freshly prepared and immediately prior to renewal on at least three occasions spaced evenly over the test (i.e. analyses should be made on a sample from the same solution - when freshly prepared and at renewal).

For tests where the concentration of the test substance is not expected to remain within ± 20 % of nominal (on the basis of stability data of the substance), it is necessary to analyse all test concentrations ,when freshly prepared and at renewal, but following the same regime (i.e. on at least three occasions spaced evenly over the test). Determination of test substance concentrations prior to renewal need only be performed on one replicate vessel at each test concentration. Determinations should be made no more than seven days apart. It is recommended that results be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance in solution has been satisfactorily maintained within ± 20 % of the nominal or measured initial concentration throughout the test, then results can be based on nominal or measured initial values.

For flow-through tests, a similar sampling regime to that described for semi-static tests is appropriate (but measurement of 'old' solutions is not applicable in this case). However, if the test duration is more than seven days, it may be advisable to increase the number of sampling occasions during the first week (e.g. three sets of measurements) to ensure that the test concentrations are remaining stable.

Samples may need to be centrifuged or filtered (e.g. using a 0.45 µm pore size). However, since neither centrifuging nor filtration appears always to separate the non-bioavailable fraction of the test substance from that which is bioavailable, samples may not be subjected to those treatments.

During the test, dissolved oxygen, pH and temperature should be measured in all test vessels. Total hardness and salinity (if relevant) should be measured in the controls and one vessel at the highest concentration. As a minimum, dissolved oxygen and salinity (if relevant) should be measured three times (at the beginning, middle and end of the test). In semi-static tests, it is recommended that dissolved oxygen be measured more frequently, preferably before and after each water renewal or at least once at week. pH should be measured at the beginning and end of each water renewal in semi-static test and at least weekly in flow-through tests. Hardness should be measured once each test. Temperature should be measured daily and it should preferably be monitored continuously in at least one test vessel.

1.7.5 **Observations**

1.7.5.1 *Stage of embryonic development*

The embryonic stage (i.e. gastrula stage) at the beginning of exposure to the test substance should be verified as precisely as possible. This can be done using a representative sample of eggs suitably preserved and cleared. The literature may also be consulted for the description and illustration of embryonic stages (2)(5)(10)(11).

1.7.5.2 *Hatching and survival*

Observations on hatching and survival should be made at least once daily and numbers recorded. It may be desirable to make more frequent observations at the beginning of the test (e.g. each 30 minutes during the first three hours), since in some cases, survival times can be more relevant than only the number of deaths (e.g. when there are acute toxic effects). Dead embryos and larvae should be removed as soon as observed since they can decompose rapidly. 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 embryos**: absence of body movement and/or absence of heart beat and/or opaque discoloration in species whose embryos are normally translucent;

— **for larvae**: immobility and/or absence of respiratory movement and/or absence of heart-beat and/or white opaque colouration of central nervous system and/or lack of reaction mechanical stimulus.

1.7.5.3 *Abnormal appearance*

The number of larvae showing abnormality of body form and/or pigmentation, and the stage of yolk-sac absorption, should be recorded at adequate intervals depending on the duration of the test 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.

1.7.5.4 *Abnormal behaviour*

Abnormalities, e.g. hyperventilation, uncoordinated swimming, and atypical quiescence should be recorded at adequate intervals depending on the duration of the test. These effects, although difficult to quantify, can, when observed, aid in the interpretation of mortality data i.e. provide information on the mode of toxic action of the substance.

1.7.5.5 *Length*

At the end of the test, measurement of individual lengths is recommended; standard, fork or total length may be used. If however, caudal fin rot or fin erosion occurs, standard lengths should be used. Generally, in a well-run test, the coefficient of variation for length among replicates in the controls should be ≤ 20 %.

1.7.5.6 *Weight*

At the end of the test, individual weights can be measured; dry weights (24 hours at 60 °C) are preferable to wet weights (blotted dry). Generally, in a well-run test, the coefficient of variation for weight among replicates in the controls should be ≤ 20 %.

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

— cumulative mortality;

— numbers of healthy larvae at end of test;

— time to start of hatching and end of hat ching (i.e. 90 % hatching in each replicate);

— numbers of larvae hatching each day;

— length (and weight) of surviving animals at end of the test;

— numbers of larvae that are deformed or of abnormal appearance;

— numbers of larvae exhibiting abnormal behaviour.

2. DATA AND REPORTING

2.1 TREATMENT OF RESULTS

It is recommended that a statistician be involved in both the design and analysis of the test since the method allows for considerable variation in experimental design as, for example, in the number of test chambers, number of test concentrations, starting number of fertilised eggs and in the parameters measured. In view of the options available in test design, specific guidance on statistical procedures is not given here.

If LOEC/NOECs are to be estimated, it will be necessary for variations to be analysed within each set of replicates using analysis of variance (ANOVA) or contingency table procedures. In order to make a multiple comparison between the results at the individual concentrations and those for the controls, Dunnett's method may be found useful (12)(13). Other useful examples are also available (14)(15). The size of the effect detectable using ANOVA or other procedures (i.e. the power of the test) should be calculated and reported. It should be noted that not all the observations listed in section 1.7.5.6 are suitable for statistical analysis using ANOVA. For example, cumulative mortality and numbers of healthy larvae at the end of the test could be analysed using probit methods.

If LC/EC_xs are to be estimated, (a) suitable curve(s), such as the logistic curve, should be fitted to the data of interest using a statistical method such as least squares or non-linear least squares. The curve(s) should be parameterised so that the LC/EC_x of interest and its standard error can be estimated directly. This will greatly ease the calculation of the confidence limits around the LC/EC_x. Unless there are good reasons to prefer different confidence levels, two-sided 95 % confidence should be quoted. The fitting procedure should preferably provide a means for assessing the significance of the lack of fit. Graphical methods for fitting curves can be used. Regression analysis is suitable for all observations listed in section 1.7.5.6.

2.2 INTERPRETATION OF RESULTS

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. The interpretation of results for concentrations above the water solubility of the substance should also be made with care.

2.3 THE TEST REPORT

The test report must include the following information:

2.3.1 **Test substance:**

— physical nature and relevant physical-chemical properties;

— chemical identification data, including purity and analytical method for quantification of the tests substance where appropriate.

2.3.2 **Test species:**

— scientific name, strain, numbers of parental fish (i.e. how many females were used for providing the required numbers of eggs in the test), source and method of collection of the fertilised eggs and subsequent handling.

2.3.3 **Test conditions:**

— test procedure used (e.g. semi-static or flow-through, time period from fertilisation to start the test, loading, etc);

— photoperiod(s);

— test design (e.g. number of test chambers and replicates, number of embryos per replicate);

— 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 measured values, their means and their standard deviations in the test vessels and the method by which these were attained and, if the test substance is soluble in water at concentrations below those tested, evidence should be provided that the measurements refer to the concentrations of the test substance in solution;

— dilution water characteristics: pH, hardness, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), total organic carbon, suspended solids, salinity of the test medium (if measured) and any other measurements made;

— water quality within test vessels: pH, hardness, temperature and dissolved oxygen concentration.

2.3.4 **Results:**

— results from any preliminary studies on the stability of the test substance;

— evidence that controls met the overall survival acceptability standard of the test species (Annexes 2 and 3);

— data on mortality/survival at embryo and larval stages and overall mortality/survival;

— days to hatch and numbers hatched;

— data for length (and weight);

— incidence and description of morphological abnormalities, if any;

— incidence and description of behavioural effects, if any;

— statistical analysis and treatment of data;

— for tests analysed using ANOVA, the lowest observed effect concentration (LOEC) at p=0.05 and the no observed effect concentration (NOEC) for each response assessed, including a description of the statistical procedures used and an indication of what size of effect could be detected;

— for tests analysed using regression techniques, the LC/EC_x and confidence intervals and a graph of the fitted model used for its calculation;

— explanation for any deviation from this testing method.

3. REFERENCES

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TABLE 1A: FISH SPECIES RECOMMENDED FOR TESTING

FRESHWATER
Oncorhynchus mykiss Rainbow trout (9)(16)
Danio rerio Zebrafish (7)(17)(18)
Cyprinus caprio Common carp (8)(19)
Oryzias latipes Japanese ricefish/Medaka (20)(21)
Pimephales promelas Fathead minnow (8)(22)

TABLE 1B: EXAMPLES OF OTHER WELL-DOCUMENTED SPECIES WHICH HAVE ALSO BEEN USED

FRESHWATER	**SALTWATER**
Carassius auratus Goldfish (8) *Lepomis macrochirus* Bluegill (8)	*Menidia peninsulae* Tidewater silverside (23)(24)(25) *Clupea harengus* Herring (24)(25)
	Gadus morhua Cod (24)(25)
	Cyprinodon variegatus Sheepshead minnow (23)(24)(25)

ANNEX 1

GUIDANCE ON PERFORMANCE OF A TOXICITY TEST ON EMBRYOS AND SAC-FRY OF ZEBRAFISH *(Brachydanio rerio)*

INTRODUCTION

The zebrafish originates from the Coromandel coast of India where it inhabits fast-flowing streams. It is a common aquarium fish of the carp family, and information about procedures for its care and culture can be found in standard reference books on tropical fish. Its biology and use in fishery research have been reviewed by Laale (1).

The fish rarely exceeds 45 mm in length. The body is cylindrical with 7-9 dark-blue horizontal silvery stripes. These stripes run into the caudal and anal fins. The back is olive-green. Males are slimmer than females. Females are more silvery and the abdomen is distended, particularly prior to spawning.

Adult fishes are able to tolerate large fluctuations in temperature, pH and hardness. However, in order to get healthy fish which produce eggs of good quality, optimal conditions should be provided.

During spawning the male pursues and butts the female, and as the eggs are expelled they are fertiliZed. The eggs, which are transparent and non-adhesive, fall to the bottom where they may be eaten by the parents. Spawning is influenced by light. If the morning light is adequate, the fish usually spawns in the early hours following daybreak.

A female can produce batches of several hundreds of eggs at weekly intervals.

CONDITIONS OF PARENTAL FISH, REPRODUCTION AND EARLY-LIFE STAGES

Select a suitable number of healthy fish and keep these in a suitable water (e.g. Annex 4) for at least 2 weeks prior to the intended spawning. The group of fish should be allowed to breed at least once before producing the batch of eggs used in the test. The density of fish during this period should not exceed 1 gramme of fish per litre. Regular changes of water or the use of purification systems will enable the density to be higher. The temperature in the holding tanks should be maintained at 25 ± 2 °C. The fish should be provided with a varied diet, which may consist of, for example, appropriate commercial dry food, live newly hatched Arthemia, chironomids, Daphnia, white worms (Enchytraeids).

Two procedures are outlined below, which in practice have led to a sufficient batch of healthy, fertilized eggs for a test to be run:

i. Eight females and 16 males are placed in a tank containing 50 litres of dilution water, shielded from direct light and left as undisturbed as possible for at least 48 hours. A spawning tray is placed at the bottom of the aquarium in the afternoon the day before start of the test. The spawning tray consists of a frame (plexi-glass or other suitable material), 5-7 cm high with a 2-5 mm coarse net attached at the top and a 10-30 µm fine net at the bottom. A number of 'spawning-trees', consisting of untwisted nylon rope, are attached to the coarse net of the frame. After the fish have been left in dark for 12 hours, a faint light is turned on which will initiate the spawning. Two to four hours after spawning, the spawning tray is removed and the eggs collected. The spawning tray will prevent the fish from eating the eggs and at the same time permit an easy collection of the eggs. The group of fish should have spawned at least once before the spawning from which eggs are used for testing.

ii. Five to 10 male and female fish are housed individually at least 2 weeks prior to the intended spawning. After 5-10 days, the abdomens of the females will be distended and their genital papillae visible. Male fish lack papillae. Spawning is performed in spawning tanks equipped with a false mesh bottom (as above). The tank is filled with dilution water, so that the depth of water above the mesh is 5-10 cm. One female and two males are placed in the tank the day before the intended spawning. The water temperature is gradually increased one degree higher than the acclimatisation temperature. The light is turned off and the tank is left as undisturbed as possible. In the morning a faint light is turned on which will initiate spawning. After 2-4 hours, the fish are removed and the eggs collected. If larger batches of eggs are needed than can be obtained from one female, a sufficient number of spawning tanks may be set-up in parallel. By recording the reproduction success of the individual females prior to the test (size of batch and quality), those females with highest reproduction success may be selected for breeding.

The eggs should be transferred to the test vessels by means of glass tubes (inner diameter not less than 4 mm) provided with a flexible suction bulb. The amount of water accompanying the eggs on their transfer should be as small as possible. The eggs are heavier than water and sink out of the tube. Care should be taken to prevent eggs (and larvae) coming into contact with the air. Microscopic examination of sample(s) of the batch(es) should be carried out to ensure that there are no irregularities in the first developmental stages. Disinfection of the eggs is not allowed.

The mortality rate of the eggs is highest within the first 24 hours after fertilisation. A mortality of 5-40 percent is often seen during this period. Eggs degenerate as a result of unsuccessful fertilization or development failures. The quality of the batch of eggs seems to depend on the female fish, as some females consistently produce good quality eggs, others never will. Also the development rate and the rate of hatching vary from one batch to another. The successfully fertilized eggs and the yolk sac larvae survive well, normally above 90 percent. At 25 °C the eggs will hatch 3-5 days after fertilization and the yolk sac will be absorbed approximately 13 days after fertilization.

The embryonic development has been well defined by Hisaoka and Battle (2). Due to the transparency of the eggs and post-hatch larvae, the development of the fish may be followed and the presence of malformations may be observed. Approximately 4 hours after spawning, the non-fertilized eggs may be distinguished from the fertilized (3). For this examination, eggs and larvae are placed in test vessels of small volume and studied under a microscope.

The test conditions, which apply to the early life stages, are listed in Annex 2. Optimal values for pH values and hardness of the dilution water are 7.8 and 250 mg $CaCO_3$/l respectively.

CALCULATIONS AND STATISTICS

A two-stage approach is proposed. First, the data on mortality, abnormal development and hatching-time are analysed statistically. Then, for those concentrations at which no adverse effects on any of these parameters have been detected, the body length is statistically evaluated. This approach is advisable since the toxicant may selectively kill smaller fish, delay hatching-time and induce gross malformations, thus leading to biased length measurements. Furthermore, there will be roughly the same number of fish to be measured per treatment, ensuring the validity of the test statistics.

LC_{50} AND EC_{50} DETERMINATIONS

The percentage of surviving eggs and larvae is calculated and corrected for mortality in the controls in accordance with Abbott's formula (4):

$$P = 100 - \left(\frac{C - P}{C} x 100\right)$$

where,

P = corrected % survival
P′ = % survival observed in the test concentration
C = % survival in the control

If possible, the LC_{50} is determined by a suitable method at the end of the test.

If the inclusion of morphological abnormalities in the EC_{50} statistic is desired, guidance can be found in Stephan (5).

ESTIMATION OF LOEC AND NOEC

An objective of the egg and sac-fry test is to compare the non-zero concentrations with the control, i.e. to determine the LOEC. Therefore multiple comparison procedures should be utilised (6)(7)(8)(9)(10).

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ANNEX 2

TEST CONDITIONS, DURATION AND SURVIVAL CRITERIA FOR RECOMMENDED SPECIES

SPECIES	TEMP (^{0}C)	SALINITY (0/00)	PHOTO - PERIOD (hrs)	DURATION OF STAGES (days)		TYPICAL DURATION OF TEST	SURVIVAL OF CONTROL, (MINIMUM %)	
				Embryo	Sac-fry		Hatching success	Post-hatch
FRESHWATER								
Brachydanio rerio Zebrafish	25 ± 1	–	12 – 16	3 – 5	8 – 10	As soon as possible after fertilisation (early gastrula stage) to 5 days post-hatch (8-10 days)	80	90
Oncorhynchus mykiss Rainbow trout	10 ± 1[1] 12 ± 1[2]	–	0 [a]	30 – 35	25 – 30	As soon as possible after fertilisation (early gastrula stage) to 20 days post-hatch (50-55 days)	66	70
Cyprinus carpio Common carp	21 – 25	–	12 – 16	5	> 4	As soon as possible after fertilisation (early gastrula stage) to 4 days post-hatch (8 - 9 days)	80	75
Oryzias latipes Japanese ricefish/Medaka	24 ± 1[1] 23 ± 1[2]	–	12 – 16	8 – 11	4 – 8	As soon as possible after fertilisation (early gastrula stage) to 5 days post-hatch (13 - 16 days)	80	80
Pimephales promelas Fathead minnow	25 ± 2	–	16	4 – 5	5	As soon as possible after fertilisation (early gastrula stage) to 4 days post-hatch (8 - 9 days)	60	70

[1]For embryos [2]For larvae [a]Darkness for embryo and larvae until one week after hatching except when they are being inspected. Then subdued lighting throughout the test

ANNEX 3

TEST CONDITIONS, DURATION AND SURVIVAL CRITERIA FOR OTHER WELL DOCUMENTED SPECIES

SPECIES	TEMP (°C)	SALINITY (0/00)	PHOTO -PERIOD (hrs)	DURATION OF STAGES (days)		TYPICAL DURATION OF EMBRYO AND SAC -FRY TEST	SURVIVAL OF CONTROL (MINIMUM %)	
				EMBRYO	SAC -FRY TEST		Hatching success	Post-hatch
FRESHWATER								
Carassius auratus Goldfish	24 ± 1	–	–	3 – 4	> 4	As soon as possible after fertilisation (early gastrula stage) to 4 days post-hatch (7 days)	–	80
Leopomis macrochirus Blugill sunfish	21 ± 1	–	16	3	> 4	As soon as possible after fertilisation (early gastrula stage) to 4 days post-hatch (7 days)	–	75
SALTWATER								
Menidia peninsulae Tidewater silverside	22 - 25	15 – 22	12	1.5	10	As soon as possible after fertilisation (early gastrula stage) to 5 days post-hatch (6-7 days)	80	60
Clupea harengus Herring	10 ± 1	8 – 15	12	20 – 25	3 – 5	As soon as possible after fert ilisation (early gastrula stage) to 3 days post-hatch (23-27 days)	60	80
Gadus morhua Cod	5 ± 1	5 – 30	12	14 – 16	3 – 5	As soon as possible after fertilisation (early gastrula stage) to 3 days post-hatch (18 days)	60	80
Cyprinodon variegatus Sheepshead m innow	25 ± 1	15 – 30	12	–	–	As soon as possible after fertilisation (early gastrula stage) to 4/7 days post-hatch (28 days)	> 75	80

ANNEX 4

SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

SUBSTANCE	CONCENTRATIONS
Particulate matter	< 20 mg/l
Total organic carbon	< 2 mg/l
Unionised 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