**OECD Guidelines for the Testing of Chemicals 210 Fish, Early-life Stage Toxicity Test 1992-2013改定版比較表（仮）**

| **1992** | **2013** |
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| **INTRODUCTION** | **INTRODUCTION** |
| 1. Tests with the early-life stages of fish are intended to define the lethal and sub-lethal effects of chemicals on the stages and species tested. They yield information of value for the estimation of the chronic lethal and sub-lethal effects of the substance on other fish species. | 1. Tests with the early-life stages of fish are intended to define the lethal and sub-lethal effects of chemicals on the stages and species tested. They yield information of value for the estimation of the chronic lethal and sub-lethal effects of the chemical on other fish species. |
| 2. This guideline is based on a proposal from the United Kingdom which was discussed at a meeting of OECD experts convened at Medmenham (United Kingdom) in November 1988. | 2. This Test Guideline 210 is based on a proposal from the United Kingdom which was discussed at a meeting of OECD experts convened at Medmenham (United Kingdom) in November 1988 and further updated in 2013 to reflect experience in using the test and recommendations from an OECD workshop on fish toxicity testing, held in September 2010 (1). |
| **PRINCIPLE OF THE TEST** | **PRINCIPLE OF THE TEST** |
| 3. The early-life stages of fish are exposed to a range of concentrations of the test substance dissolved in water, preferably under flow-through conditions, or where appropriate, semi-static conditions. The test is begun by placing fertilised eggs in the test chambers and is continued at least until all the control fish are free-feeding. 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 (see Annex 1 for definitions). | 3. The early-life stages of fish are exposed to a range of concentrations of the test chemical dissolved in water. Flow-through conditions are preferred; however, if it is not possible semi-static conditions are acceptable. For details the OECD Guidance Document No. 23 on aquatic toxicity testing of difficult substances and mixtures should be consulted (2). The test is initiated by placing fertilised eggs in test chambers and is continued for a species-specific time period that is necessary for the control fish to reach a juvenile life-stage. Lethal and sub-lethal effects are assessed and compared with control values to determine the lowest observed effect concentration (LOEC) in order to determine the (i) no observed effect concentration (NOEC) and/or (ii) ECx (e.g. EC10, EC20) by using a regression model to estimate the concentration that would cause a x % change in the effect measured. Reporting of relevant effect concentrations and parameters may depend upon the regulatory framework. The test concentrations should bracket the ECx so that the ECx comes from interpolation rather than extrapolation (see Annex 1 for definitions). |
| **INFORMATION ON THE TEST SUBSTANCE** | **INFORMATION ON THE TEST CHEMICAL** |
| 4. Results of an acute toxicity test (see Guideline 203), preferably performed with the species chosen for this test, should be available. This implies that the water solubility and the vapour pressure of the test substance are known and a reliable analytical method for the quantification of the substance in the test solutions with known and reported accuracy and limit of detection is available. | 4. Test chemical refers to what is being tested. The water solubility (see Guideline 105) and the vapour pressure (see Guideline 104) of the test chemical should be known and a reliable analytical method for the quantification of the chemical in the test solutions with known and reported accuracy and limit of quantification should be available. Although not necessary to conduct the test, results from an acute toxicity test (see Guideline 203 or Guideline 236), preferably v performed with the species chosen for this test, may provide useful information. |
|  | 5. If the Test Guideline is used for the testing of a mixture, its composition should as far as possible be characterised, e.g., by the chemical identity of its constituents, their quantitative occurrence and their substance-specific properties (like those mentioned above). Before use of the Test Guideline for regulatory testing of a mixture, it should be considered whether it will provide acceptable results for the intended regulatory purpose. |
| 5. Useful information includes the structural formula, purity of the substance, stability in water and light, pKa, Pow and results of a test for ready biodegradability (see Guideline 301). | 6. Useful information includes the structural formula, purity of the substance, water solubility, stability in water and light, pKa, Pow and results of a test for ready biodegradability (e.g., Guideline 301 or Guideline 310). |
| **VALIDITY OF THE TEST** | **VALIDITY OF THE TEST** |
| 6. For a test to be valid the following conditions apply: | 7. For a test to be valid the following conditions apply: |
| - the dissolved oxygen concentration must be between 60 and 100 per cent of the air saturation value throughout the test; | ・ the dissolved oxygen concentration should be >60% of the air saturation value throughout the test; |
| - the water temperature must not differ by more than + 1.5oC 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 3 and 6); | ・ the water temperature should not differ by more than + 1.5oC 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 (Annex 2); |
| ~~- evidence must be available to demonstrate that the concentrations of the test substance in solution have been satisfactorily maintained within + 20% of the mean measured values;~~ |  |
|  | ・the analytical measure of the test concentrations is compulsory. |
| - overall survival of fertilised eggs in the controls and, where relevant, in the solvent~~-only~~ controls must be greater than or equal to the limits defined in Annexes 3 and 6; | ・ overall survival of fertilised eggs and post-hatch success in the controls and, where relevant, in the solvent controls should be greater than or equal to the limits defined in Annex 2. |
| ~~- when a solubilising agent is used it must have no significant effect on survival nor produce any other adverse effects on the early-life stages as revealed by a solvent-only control.~~ |  |
|  | 8. If a minor deviation from the validity criteria is observed, the consequences should be considered in relation to the reliability of the test data and these considerations should be included in the report. Effects on survival, hatch or growth occurring in the solvent control, when compared to the negative control, should be reported and discussed in the context of the reliability of the test data. |
| **DESCRIPTION OF THE METHOD** | **DESCRIPTION OF THE METHOD** |
| **Test chambers** | **Test chambers** |
| 7. Any glass, stainless steel or other chemically inert vessels can be used. The dimensions of the vessels should be large enough to allow compliance with loading rate criteria given below. It is desirable that test chambers be randomly positioned in the test area. A randomised block design with each treatment being present in each block is preferable to a completely randomised design. The test chambers should be shielded from unwanted disturbance. | 9. Any glass, stainless steel or other chemically inert vessels can be used. As silicone is known to have a strong capacity to absorb lipophilic substances, the use of silicone tubing in flow-through studies and use of silicone seals in contact with water should be minimised by the use of e.g. monoblock glass aquaria. The dimensions of the vessels should be large enough to allow proper growth in the control, maintenance of dissolved oxygen concentration (e.g. for small fish species, a 7 L tank volume will achieve this) and compliance with the loading rate criteria given in paragraph 19. It is desirable 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. The test chambers should be shielded from unwanted disturbance. The test system should preferably be conditioned with concentrations of the test chemical for a sufficient duration to demonstrate stable exposure concentrations prior to the introduction of test organisms. |
| **Selection of species** | **Selection of species** |
| 8. Recommended fish species are given in Table 1~~a~~. This does not preclude the use of other species ~~(and 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. | 10. Recommended fish species are given in Table 1. This does not preclude the use of other species, 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. |
| **Holding of the brood fish** | **Holding of the brood fish** |
| 9. Details on holding the brood stock under satisfactory conditions may be found in Annex 2 and the references cited (1)(2)(3). | 11. Details on holding the brood stock under satisfactory conditions may be found in Annex 3 and the references cited (3)(4)(5). |
| **Handling of embryos and larvae** | **Handling of fertilised eggs, embryos and larvae** |
| 10. Initially, embryos and larvae may be exposed within the main vessel in smaller glass or stainless steel vessels, fitted with mesh sides or ends to permit a flow of test solution through the vessel. Non-turbulent flow through these small vessels may be induced by suspending them from an arm arranged to move the vessel up and down but always keeping the organisms submerged. Fertilised eggs　of salmonid fishes can be supported on racks or meshes with apertures sufficiently large to allow larvae to drop through after hatching. | 12. Initially, fertilised eggs, embryos and larvae may be exposed within the main vessel in smaller glass or stainless steel vessels, fitted with mesh sides or ends to permit a flow of test solution through the vessel. Non-turbulent flow-through in 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. Fertilised eggs of salmonid fishes can be supported on racks or meshes with apertures sufficiently large to allow larvae to drop through after hatching. |
| 11. 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, according to the advice in Annex 2, except that meshes should be retained to prevent the escape of the fish. If there is a need to transfer the larvae, they should not be exposed to the air and nets should not be used to release fish from egg containers. The timing of this transfer varies with the species and transfer may not always be necessary. | 13. 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, according to the guidance in Annex 3, except that meshes should be retained to prevent the escape of the larvae. 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 larvae from egg containers. The timing of this transfer varies with the species and should be documented in the report. However, a transfer may not always be necessary. |
| **Water** | **Water** |
| 12. Any water in which the test species shows control survival at least as good as that described in Annexes 3 and 6 is suitable as a 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, S04), 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. Some chemical characteristics of an acceptable dilution water are listed in Annex 4. | 14. Any water in which the test species shows suitable long-term survival and growth may be used as test water (see Annex 4). 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 chemical), or adversely affect the performance of the brood stock, samples should be taken at intervals for analysis. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl, SO4), ammonia, total residual chlorine pesticides, total organic carbon and suspended solids should be made, for example, on a bi-annual basis where a dilution water is known to be relatively constant in quality. If the water is known to be of variable quality the measurements have to be conducted more often; the frequency is dependent of how variable the quality is. Some chemical characteristics of an acceptable dilution water are listed in Annex 4. |
| **Test solutions** | **Test solutions** |
| 13. For flow-through tests, a system which continually dispenses and dilutes a stock solution of the test substance (eg 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 during the test and should not vary by more than 10% throughout the test. A flow rate equivalent to at least five test chamber volumes per 24 hours has been found suitable (1). | 15. For flow-through tests, a system which continually dispenses and dilutes a stock solution of the test chemical (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 during the test and should not vary by more than 10% throughout the test. A flow rate equivalent to at least five test chamber volumes per 24 hours has been found suitable (3). However, if the loading rate specified in paragraph 18 is respected, a lower flow rate of e.g. 2-3 test chamber volumes is possible to prevent quick removal of food. |
| ~~14. The use of solvents or dispersants (solubilising agents) may be required in some cases in order to produce a suitably concentrated stock solution.~~ |  |
|  | 16. 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 chemical in dilution water by using mechanical means (e.g. stirring and/or ultrasonication). Saturation columns (solubility columns) or passive dosing methods (6) can be used for achieving a suitable concentrated stock solution. The use of a solvent carrier is not recommended. However, in case a solvent is necessary, a solvent control should be run in parallel, at the same solvent concentration as the chemical treatments; i.e. the solvent level should preferably be equal across all concentrations as well as the solvent control. For some diluter systems this might be technically difficult; here the solvent concentration in the solvent control should be equal to the highest solvent concentration in the treatment group. For difficult to test substances, the OECD Guidance Document No. 23 on aquatic toxicity testing of difficult substances and mixtures should be consulted (2). If a solvent is used, the choice of solvent will be determined by the chemical properties of the substance. The OECD Guidance Document No. 23 recommends a maximum concentration of 100 μl/L. To avoid potential effect of the solvent on endpoints measured (7), it is recommended to keep solvent concentration as low as possible. |
| 15. For the semi-static technique, two different renewal procedures may be followed. Either new test solutions are prepared in clean vessels and survivings eggs and larvae gently transferred into the new vessels, or the test organisms are retained in the test vessels whilst a proportion (at least two thirds) of the test water is changed. | 17. For a semi-static test, two different renewal procedures may be followed. Either new test solutions are prepared in clean vessels and surviving eggs and larvae gently transferred into the new vessels, or the test organisms are retained in the test vessels whilst a proportion (at least two thirds) of the test solution / control volume is changed. |
| **PROCEDURE** | **PROCEDURE** |
| ~~16. Useful information on the performance of fish early-life stage tests is available in the literature, some examples of which are included in the literature section of this text (1)(4)(5)(6)(7)(8).~~ |  |
| **Conditions of Exposure** | **Conditions of Exposure** |
| **Duration** | **Duration** |
| 17. The test should start as soon as possible after the eggs have been fertilised,~~the embryos~~ preferably being immersed in the test solutions before cleavage of the blastodisc commences, or as close as possible after this stage. ~~The test should continue at least until all the control fish have been free-feeding.~~ Test duration will depend upon the species used. Some recommended durations are given in Annexes 3 and 6. | 18. The test should start as soon as possible after the eggs have been fertilised and preferably being immersed in the test solutions before cleavage of the blastodisc commences, or as close as possible after this stage. The test duration will depend upon the species used. Some recommended durations are given in Annex 2. |
| **Loading** | **Loading** |
| 18. 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 60 eggs, divided equally between at least two 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% of the air saturation value ~~(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 been recommended (1). | 19. 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 80 eggs, divided equally between at least four 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% of the air saturation value can be maintained without aeration during the egg and larval stage. For flow-through tests, a loading rate not exceeding 0.5 g/L wet weight per 24 hours and not exceeding 5 g/L of solution at any time has been recommended (3). |
| **Light and temperature** | **Light and temperature** |
| 19. The photoperiod and water temperature should be appropriate for the test species (see Annex 3). | 20. The photoperiod and water temperature should be appropriate for the test species (see Annex 2). |
| **Feeding** | **Feeding** |
| 20. Food and feeding are critical, and it is essential that the correct food for each stage ~~should~~ be supplied from an appropriate time and at a level sufficient to support normal growth. Feeding should be ad libitum whilst minimising the surplus. Surplus food and faeces should be removed as necessary to avoid accumulation of waste. Detailed feeding regimes are given in Annex 2 but, as experience is gained, food and feeding regimes are continually being refined to improve survival and optimise growth. ~~Effort should therefore be made to confirm the proposed regime with acknowledged experts.~~ | 21. Food and feeding are critical, and it is essential that the correct food for each life-stage is supplied from an appropriate time and at a level sufficient to support normal growth. Feeding should be approximately equal across replicates unless adjusted to account for mortality. Surplus food and faeces should be removed as necessary, to avoid accumulation of waste. Detailed feeding regimes are given in Annex 3 but, as experience is gained, food and feeding regimes are continually being refined to improve survival and optimise growth. Live food provides a source of environmental enrichment and therefore should be used in place of or in addition to dry or frozen food whenever appropriate to the species and life stage. |
| **Test concentrations** | **Test concentrations** |
| 21. Normally five concentrations of the test substance spaced by a constant factor not exceeding 3.2 are required. The curve relating LC50 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 LC50 or 10 mg/l, whichever is the lower, need not be tested. | 22. Normally five concentrations of the test chemical, with a mimimum of four replicates per concentration, spaced by a constant factor not exceeding 3.2 are required. If available, information on the acute testing, preferable with the same species and/or a range finding test should be considered (1) when selecting the range of test concentrations. However, all sources of information should be considered when selecting the range of test concentrations, including sources like e.g., read across, fish embryo acute toxicity test data. A limit test, or an extended limit test, with fewer than five concentrations as the definitive test may be acceptable where empirical NOECs only are to be established. Justification should be provided if fewer than five concentrations are used. Concentrations of the test chemical higher than the 96 hour LC50 or 10 mg/L, whichever is the lower, need not be tested. |
| ~~22. Where a solubilising agent is used its concentration should not be greater than 0.1 ml/l and should be the same in all test vessels. However, every effort should be made to avoid the use of such materials.~~ |  |
| **Controls** | **Controls** |
| 23. One dilution-water control and ~~also~~, if relevant, one control containing the solubilising agent should be run in addition to the test series. | 23. A dilution-water control and, if needed, a solvent control containing the solvent carrier only should be run in addition to the test chemical concentration series (see paragraph 16). |
| **Frequency of Analytical Determinations and Measurements** | **Frequency of Analytical Determinations and Measurements** |
| 24. During the test, the concentrations of the test substance are determined at regular intervals to check compliance with the validity criteria. A minimum of five determinations is necessary. In studies lasting more than one month determinations should be made at least once a week. Samples may need to be filtered (e.g. using a 0.45 m pore size) or centrifuged to ensure that the determinations are made on the substance in true solution. | 24. Prior to initiation of the exposure period, proper function of the chemical delivery system across all replicates should be ensured (for example, by measuring test concentrations). Analytical methods required should be established, including an appropriate limit of quantification (LOQ) and sufficient knowledge on the substance stability in the test system. During the test, the concentrations of the test chemical are determined at regular intervals to characterise exposure. A minimum of five determinations is necessary. In flow-through systems, analytical measurements of the test chemical in one replicate per concentration should be made at least once a week changing systematically amongst replicates. Additional analytical determinations will often improve the quality of the test outcome. Samples may need to be filtered to remove any particulate matter (e.g. using a 0.45 μm pore size) or centrifuged to ensure that the determinations are made on the chemical in true solution. In order to reduce adsorption of the test chemical, the filters should be saturated before the use. When the measured concentrations do not remain within 80-120% of the nominal concentration, the effect concentrations should be determined and expressed relative to the arithmetic mean concentration for flow-through tests (see Annex 6 of the Test Guideline 211 for the calculation of the arithmetic mean (8)), and expressed relative to the geometric mean of the measured concentrations for semi-static tests (see Chapter 5 in the OECD Guidance Document No. 23 on aquatic toxicity testing of difficult substances and mixtures (2)). |
| 25. During the test, dissolved oxygen, pH, ~~total hardness and salinity (if relevant)~~ and temperature should be measured in all test vessels. ~~As a minimum, dissolved oxygen, salinity (if relevant) and temperature should be measured weekly, and pH and hardness at the beginning and end of the test.~~ Temperature should preferably be monitored continuously in at least one test vessel. | 25. During the test, dissolved oxygen, pH, and temperature should be measured in all test vessels, at least weekly, and salinity and hardness, if warranted, at the beginning and end of the test. Temperature should preferably be monitored continuously in at least one test vessel. |
| **Observations** | **Observations** |
| 26. Stage of embryonic development: the embryonic 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. | 26. Stage of embryonic development: the embryonic stage at the beginning of exposure to the test chemical should be verified as precisely as possible. This can be done using a representative sample of eggs suitably preserved and cleaned. |
| 27. Hatching and survival: observations on hatching and survival should be made at least once daily and numbers recorded. Dead embryos, larvae and juvenile fish should be removed as soon as observed since they can decompose rapidly and may be broken up by the actions of the other fish. Extreme care should be taken when removing dead individuals not to ~~knock or~~ physically damage adjacent eggs/larvae, ~~these being extremely delicate and sensitive. Criteria for death vary according to life stage:~~ | 27. Hatching and survival: observations on hatching and survival should be made at least once daily and numbers recorded. If fungus on eggs is observed early in embryonic development (e.g., at day one or two of test), those eggs should be counted and removed. Dead embryos, larvae and juvenile fish should be removed as soon as observed since they can decompose rapidly and may be broken up by the actions of the other fish. Extreme care should be taken when removing dead individuals not to physically damage adjacent eggs/larvae. Signs of death vary according to species and life stage. For example: |
| - 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 fertilised 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; | ・for embryos, larvae and juvenile fish: immobility and/or absence of respiratory movement and/or absence of heartbeat and/or lack of reaction to mechanical stimulus. |
| - for larvae and juvenile fish: 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 to mechanical stimulus. |
| 28. Abnormal appearance: the number of larvae or fish showing abnormality of body form 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 percent in the control(s) in some species. ~~Abnormal animals should only be removed from the test vessels on death.~~ | 28. Abnormal appearance: the number of larvae or juvenile fish showing abnormality of body form 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 larvae and juvenile fish occur naturally and can be of the order of several percent in the control(s) in some species. Where deformities and associated abnormal behaviour are considered so severe that there is considerable suffering to the organism, and it has reached a point beyond which it will not recover, it may be removed from the test. Such animals should be euthanised and treated as mortalities for subsequent data analysis. Normal embryonic development has been documented for most species recommended in this Guideline (9) (10) (11) (12). |
| 29. Abnormal behaviour: abnormalities, e.g. hyperventilation, unco-ordinated swimming, atypical quiescence and atypical feeding behaviour 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 ~~and influence a decision to extend the exposure period beyond the recommended duration.~~ | 29. Abnormal behaviour: abnormalities, e.g. hyperventilation, uncoordinated swimming, atypical quiescence and atypical feeding behaviour should be recorded at adequate intervals depending on the duration of the test (e.g. once daily for warm water species). These effects, although difficult to quantify, can, when observed, aid in the interpretation of mortality data. |
| 30. Weight: at the end of the test all surviving fish must be weighed. ~~Individual weights are preferred but, if the fish are especially small, they may be weighed in groups by test vessel. Dry weights (24 hours at 60℃) are preferable to wet weights (blotted dry).~~ | 30. Weight: at the end of the test, all surviving fish are weighed at least on a replicate basis (reporting the number of animals in the replicate and the mean weight per animal): wet weight ? (blotted dry) is preferred, however, dry weight data may also be reported (13). |
| 31. 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. | 31. Length: at the end of the test, individual lengths are measured. Total length is recommended, if however, caudal fin rot or fin erosion occurs, standard lengths can be used. The same method should be used for all fish in a given test. Individual length can be measured either by e.g. callipers, digital camera, or calibrated ocular micrometer. Typical minimum lengths are defined in Annex 2. |
| ~~32. These observations will result in some or all of the following data being available for statistical analysis:~~ |  |
| ~~- cumulative mortality;~~  ~~- numbers of healthy fish at end of test;~~  ~~- time to start of hatching and end of hatching;~~  ~~- numbers of larvae hatching each day;~~  ~~- length and weight of surviving animals;~~  ~~- numbers of deformed larvae;~~  ~~- numbers of fish exhibiting abnormal behaviour.~~ |  |
| **DATA AND REPORTING** | **DATA AND REPORTING** |
| **Treatment of results** | **Treatment of results** |
| 33. It is recommended that ~~a statistician be involved in both the design and analysis of the test since this Test Guideline 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.~~ | 32. It is recommended that the design of the experiment and selection of statistical test permit adequate power (80% or higher) to detect changes of biological importance in endpoints where a NOEC is to be reported. Reporting of relevant effect concentrations and parameters may depend upon the regulatory framework. If an ECx is to be reported, the design of the experiment and selection of regression model should permit estimation of ECx so that (i) the 95% confidence interval reported for ECx does not contain zero and is not overly wide, (ii) the 95% confidence interval for the predicted mean at ECx does not contain the control mean (iii) there is no significant lack-of-fit of regression model to the data. Either approach requires the identification of the percent change in each endpoint that is important to detect or estimate. The experimental design should be tailored to allow that. When the above conditions for determining the ECx are not satisfied, the NOEC approach should be used. It is not likely that the same percent change applies to all endpoints, nor is it likely that a feasible experiment can be designed that will meet these criteria for all endpoints, so it is important to focus on the endpoints, which are important for the respective experiment in designing the experiment appropriately. Statistical flow diagrams and guidance for each approach are available in Annexes 5 and 6 to guide in the treatment of data and in the choice of the most appropriate statistical test or model to use. Other statistical approaches may be used, provided they are scientifically justified. |
| 34. ~~In view of the options available in test design, specific guidance on statistical procedures is not given here. However~~ it will be necessary for variations to be analysed within each set of replicates using analysis of variance 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 (9)(10). However,~~ care must be taken where applying such a method to ensure that chamber to chamber variability is estimated and ~~is acceptably low. Other useful examples are also available (1)(6)(11).~~ | 33. It will be necessary for variations to be analysed within each set of replicates using analysis of variance or contingency table procedures and appropriate statistical analysis methods be used based on this analysis. In order to make a multiple comparison between the results at the individual concentrations and those for the controls, the step-down Jonckheere-Terpstra or Williams’ test is recommended for continuous responses and a step-down Cochran-Armitage test for quantal responses that are consistent with a monotone concentration-response and with no evidence of extra-binomial variance (14). When there is evidence of extra-binomial variance, the Rao-Scott modification of the Cochran-Armitage test is recommended (15) (16) or Williams or Dunnett’s (after an arcsin-square-root transform) or Jonckheere-Terpstra test applied to replicate proportions. Where the data are not consistent with a monotone concentration-response, Dunnett's or Dunn’s or the Mann-Whitney method may be found useful for continuous responses and Fisher’s Exact test for quantal responses (14) (17) (18). Care should be taken where applying any statistical method or model to ensure that the requirements of the method or model are satisfied (e.g. chamber to chamber variability is estimated and accounted for in the experimental design and test or model used). Data are to be evaluated for normality and Annex 5 indicates what should be done on the residuals from an ANOVA. Annex 6 discusses additional considerations for the regression approach. Transformations to meet the requirements of a statistical test should be considered. However, transformations to enable the fitting of a regression model require great care, as, for example, a 25% change in the untransformed response does not correspond to a 25% change in a transformed response. In all analyses, the test chamber, not the individual fish, is the unit of analysis and the experimental unit and both hypothesis tests and regression should reflect that (3) (14) (19) (20). |
| **~~Interpretation of results~~** |  |
| ~~35. The results should be interpreted with caution where measured toxicant concentrations in test solutions occur at levels near the detection limit of the analytical method.~~ |  |
| **Test report** | **Test report** |
| 36. The test report must include the following information: | 34. The test report should include the following information: |
| **Test substance:** | **Test chemical:** |
|  | **Mono-constituent substance** |
| - physical nature and, where relevant, physicochemical properties; | - physical appearance, water solubility, and additional relevant physicochemical properties; |
| - chemical identification data. | - chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate. |
|  | **Multi-constituent substance, UVBCs and mixtures:** |
|  | - characterised as far as possible, e.g., by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents |
| **Test species:** | **Test species:** |
| - scientific name, strain, source and method of collection of the fertilised eggs and subsequent handling. | - scientific name, strain, source and method of collection of the fertilised eggs and subsequent handling. |
| **Test conditions:** | **Test conditions:** |
| - test procedure used (e.g. semi-static or flow-through, loading); | - test procedure used (e.g. semi-static or flow-through, loading); |
| - photoperiod(s); | - photoperiod(s); |
| - test design (e.g. number of test chambers and replicates, number of embryos per replicate); | - test design (e.g. number of test chambers and replicates, number of eggs per replicate, material and size of the test chamber (height, width, volume), water volume per test chamber); |
| - method of preparation of stock solutions and frequency of renewal (the solubilizing agent and its concentration must be given, when used); | - method of preparation of stock solutions and frequency of renewal (the solubilising agent and its concentration should be given, when used); |
|  | - method of dosing the test chemical (e.g. pumps, diluting systems) |
| - the nominal test concentrations, the means of the measured values and their standard deviations in the test vessels and the method by which these were attained and evidence that the measurements refer to the concentrations of the test substance in true solution; | - the recovery efficiency of the method and the nominal test concentrations, the limit of quantification, the means of the measured values and their standard deviations in the test vessels and the method by which these were attained and evidence that the measurements refer to the concentrations of the test chemical in true 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; | - dilution water characteristics: pH, hardness, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), total organic carbon (if measured), suspended solids (if measured), salinity of the test medium (if measured) and any other measurements made; |
| - water quality within test vessels, pH, hardness, temperature and dissolved oxygen concentration; | - water quality within test vessels, pH, hardness, temperature and dissolved oxygen concentration; |
| - detailed information on feeding (e.g. type of food(s), source, amount given and frequency). | - detailed information on feeding (e.g. type of food(s), source, amount given and frequency). |
| **Results:** | **Results reported individually (or on a replicate basis) and as mean and coefficient of variation, as appropriate, for the following endpoints:** |
| - evidence that controls met the overall survival acceptability standard of the test species (Annexes 3 and 6); | - evidence that controls met the overall survival acceptability standard of the test species (Annex 2); |
| - data on mortality~~/survival~~ at embryo, larval and juvenile stages and overall mortality~~/survival~~; | - data on mortality at each stage (embryo, larval and juvenile) and cumulative mortality; |
| - days to hatch and numbers hatched; | - days to hatch, numbers of larvae hatched each day, and end of hatching; |
|  | - number of healthy fish at end of test; |
| - data for length and weight; | - data for length (specify either standard or total) and weight of surviving animals; |
| - incidence and description of morphological abnormalities, if any; | - incidence, description and number of morphological abnormalities, if any; |
| - incidence and description of behavioural effects, if any; | - incidence, description and number of behavioural effects, if any; |
| - statistical analysis and treatment of data; | - approach for the statistical analysis (regression analysis or analysis of the variance) and treatment of data (statistical test or model used); |
| - no observed effect concentration for each response assessed (NOEC); | - no observed effect concentration for each response assessed (NOEC); |
| - lowest observed effect concentration (at p = 0.05) for each response assessed (LOEC); | - lowest observed effect concentration (at p = 0.05) for each response assessed (LOEC); |
| ~~- any concentration-response data and curves available.~~ |  |
|  | - ECx for each response assessed, if applicable, and confidence intervals (e.g. 90% or 95%) and a graph of the fitted model used for its calculation, the slope of the concentration-response curve, the formula of the regression model, the estimated model parameters and their standard errors. |
|  | Any deviation from the Test Guideline. |
| Discussion of the results. | Discussion of the results, including any influence of deviations from the Guideline on the outcome of the test. |

**TABLE 1~~A~~: FISH SPECIES RECOMMENDED FOR TESTING**

| **1992** | **2013** | **1992** | **2013** |
| --- | --- | --- | --- |
| **FRESHWATER** | **FRESHWATER** | **SALTWATER** | **ESTUARINE and MARINE** |
| *Oncorhynchus mykiss*  (Rainbow trout)  *Pimephales promelas*  (Fathead minnow)  *Brachydanio rerio*  (Zebra fish)  *Oryzias latipes*  (Ricefish) | *Oncorhynchus mykiss*  (Rainbow trout)  *Pimephales promelas*  (Fathead minnow)  *Danio rerio*  (Zebrafish)  *Oryzias latipes*  (Japanese ricefish or Medaka) | *Cyprinodon variegatus*  (Sheepshead minnow) | *Cyprinodon variegatus*  (Sheepshead minnow)  *Menidia* sp.  (Silverside) |

**ANNEX 2(1992)　3(2013) FEEDING AND HANDLING REQUIREMENTS OF BROOD AND TEST ANIMALS OF RECOMMENDED SPECIES**

| **SPECIES** | **adapted** | **FOOD\*** | | | | | **POST-HATCH**  **TRANSFER TIME ~~(ifapplicable)~~** | **TIME TO FIRST**  **FEEDING** |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Brood fish** | **Newly-hatched larvae** | **Juveniles** | | |
| **Type** | **~~Amount~~** | **Frequency** |
| **Freshwater:** |  |  |  |  |  |  |  |  |
| *Oncorhynchus mykiss*  (Rainbow trout) | 1992 | trout food | none(a) | trout starter | ~~4% body wt per day~~ | 2-4 feeds per day | 14-16 days post-hatch or at swim-up (not essential) | 19 days post-hatch or at swim-up |
| 2013 | trout food | None (a) | trout starter BSN |  | 2-4 feeds per day | 14-16 days post-hatch or at swim-up (not essential) | 19 days post hatch or at swim-up |
| *Pimephales promelas*  (Fathead minnow) | 1992 | FBS | BSN | BSN48 |  | ad lib. | once hatching is 90% | within 2 days of hatching |
| 2013 | BSN, flake food FBS | BSN | BSN48, flake food |  | 2-3 times a day | once hatching is 90% | 2 day post hatch |
| *Brachydanio rerio*(1992)  *Danio rerio*(2013)  (Zebra fish) | 1992 | BSN48, flake food | protozoa(b), protein(c) | BSN48 |  |  | not necessary | 6-7 days after spawning |
| 2013 | BSN, flake food | Commercial larvae food, protozoa(b), protein(c) | BSN48, flake food, |  | BSN once daily; flake food twice daily | once hatching is 90% | 2 days post hatch |
| *Oryzias latipes*  (Ricefish(1992)  Japanese ricefish or Medaka(2013)) | 1992 | flake food | BSN, flake food (or protozoa or rotifers) | BSN48, flake food (or rotifers) |  | BSN once daily; flake food twice daily or flake food and rotifers once daily | from hatch to swim-up | within 24h of hatch/swim-up |
| 2013 | flake food | BSN, flake food (or protozoa or rotifers) | BSN48, flake food (or rotifers) |  | BSN once daily; flake food twice daily or flake food and rotifers once daily | not applicable | 6-7 days post spawn |
| **SALTWATER:** |  |  |  |  |  |  |  |  |
| *Cyprinodon variegatus*  (Sheepshead minnow) | 1992 | FBS or flake food | BSN | BSN48 |  | 2-3 feeds per day | not applicable | ~~within~~ 1 day frist hatch |
| 2013 | BSN, flake food, FBS | BSN | BSN48 |  | 2-3 feeds per day | not applicable | 1 day post hatch/swim-up |
| *Menidia* sp.  (Silverside) | 2013 | BSN48, flake food | BSN | BSN48 |  | 2-3 feeds per day | not applicable | 1 day post hatch/swim-up |

**key**

| **1992** | **2013** |
| --- | --- |
|  | \*Food should be given to satiation. Surplus food and faeces should be removed, as necessary to avoid accumulation of waste |
| FBS frozen brine shrimps; adults Artemia sp | FBS frozen brine shrimps; adults Artemia sp |
| BSN brine shrimp nauplii; newly hatched | BSN brine shrimp nauplii; newly hatched |
| BSN48 brine shrimp nauplii; 48 hours old | BSN48 brine shrimp nauplii; 48 hours old |
| (a) yolk-sac larvae require no food | (a) yolk-sac larvae require no food |
| (b) filtered from mixed culture | (b) filtered from mixed culture |
| (c) granules from fermentation process | (c) granules from fermentation process |

**ANNEX 3(1992)　2(2013)　TEST CONDITIONS, DURATION AND SURVIVAL CRITERIA FOR RECOMMENDED SPECIES**

| **SPECIES** | **adapted** | **TEST CONDITIONS** | | | **RECOMMENDED DURATION OF TEST** | **Typical minimum mean total length of control fish at the end of the study (mm)(1)** | **SURVIVAL OF CONTROLS**  **(minimum)** | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Temperature (℃)** | **Salinity (‰)** | **Photoperiod (hrs)** | **Hatching**  **success** | **Post-hatch success** |
| **Freshwater:** |  |  |  |  |  |  |  |  |
| *Oncorhynchus mykiss*  (Rainbow trout) | 1992 | 10 ± 2 (a)  12 ± 2 (b)(1) |  | (c) | 2 weeks after controls are free-feeding (or 60 days post-hatch) |  | >66% | 70% |
| 2013 | 10 ± 1.5(2) |  | 12 - 16(3) | 2 weeks after controls are free-feeding (or 60 days post-hatch) | 40 | 75% | 75% |
| *Pimephales promelas*  (Fathead minnow) | 1992 | 25 ± 2 |  | 16 | 32 days from start of test (or 28 days posthatch) |  | >66% | 70% |
| 2013 | 25 ± 1.5 |  | 16 | 32 days from start of test (or 28 days post-hatch) | 18 | 70% | 75% |
| *Brachydanio rerio*(1992)  *Danio rerio*(2013)  (Zebra fish) | 1992 | 25 ± 2 |  | 12 - 16(4) | 30 days post-hatch |  |  | 70 |
| 2013 | 26 ± 1.5 |  | 12 - 16(4) | 30 days post-hatch | 11 | 70% | 75 |
| *Oryzias latipes*  (Ricefish(1992)  Japanese ricefish or Medaka(2013)) | 1992 | 24 ± 1 (a)  23 ± 2(b)(2) |  | 12 - 16(4) | 30 days post-hatch |  |  | 80% |
| 2013 | 25 ± 2 |  | 12 - 16(4) | 30 days post-hatch | 17 | 80% | 80% |
| **SALTWATER:** |  |  |  |  |  |  |  |  |
| *Cyprinodon variegatus*  (Sheepshead minnow) | 1992 | 25 ± 2 | 15-30(3) | 12 - 16(4) | 32 days from start of test (or 28 days posthatch) |  | ~~>~~75% | 80% |
| 2013 | 25 ± 1.5 | 15-35(5) | 12 - 16(4) | 32 days from start of test (or 28 days post-hatch) | 17 | 75% | 80% |
| *Menidia* sp.  (Silverside) | 2013 | 22 - 25 | 15-35(5) | 13 | 28 days | 20 | 80% | 60% |

**key**

| **1992** | **2013** |
| --- | --- |
| ~~(a) for embryos.~~ |  |
| ~~(b) for larvae and juvenile fish.~~ |  |
| (c) darkness for larvae until one week after hatching except when they are being inspected, then subdued lighting throughout test (12-16 hour photoperiod (4)). | (3) Darkness for larvae until one week after hatching except when they are being inspected, then subdued lighting throughout test (12-16 hour photoperiod)(4). |
| (1) the particular strain of rainbow trout tested may necessitate the use of other temperatures. Brood stock must be held at the same temperature as that to be used for the eggs. | (2) The particular strain of rainbow trout tested may necessitate the use of other temperatures. Brood stock must be held at the same temperature as that to be used for the eggs. After receipt of eggs from a commercial breeder, a short adaptation (*e.g.* 1-2 h) to test temperature after arrival is necessary. |
| ~~(2) this supersedes the requirement for temperature control given earlier on in the test.~~ |  |
| (3) for any given test this shall be performed to ±2‰. | (5) For any given test this shall be performed to ±2‰. |
| (4) for any given test conditions, light regime should be constant. | (4) For any given test conditions, light regime should be constant. |
|  | (1) Typical minimum mean total length is not a validity criterion but deviations below the figure indicated should be carefully examined in relation to the sensitivity of the test. The minimum mean total length is derived from a selection of data available at the current time. |

**ANNEX 4 SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER**

| **SUBSTANCE** | **adapted** | **CONCENTRATIONS** |
| --- | --- | --- |
| **Limit concentration** |
| Particular matter | 1992 | <20 mg/l |
| 2013 | 5mg/L |
| Total organic carbon | 1992 | ~~<~~2 mg/l |
| 2013 | 2mg/L |
| Unionised ammonia | 1992 | ~~<~~1 ug/l |
| 2013 | 1μg/L |
| Residual chlorine | 1992 | ~~<~~10 ug/l |
| 2013 | 10 μg/L |
| Total organophosphorus pesticides | 1992 | ~~<~~50 ng/l |
| 2013 | 50 ng/L |
| Total organochlorine pesticides plus polychlorinated biphenyls | 1992 | ~~<~~50 ng/l |
| 2013 | 50 ng/L |
| Total organic chlorine | 1992 | ~~<~~25 ng/l |
| 2013 | 25 ng/L |
| Aluminium | 2013 | 1 μg/L |
| Arsenic | 2013 | 1 μg/L |
| Chromium | 2013 | 1 μg/L |
| Cobalt | 2013 | 1 μg/L |
| Copper | 2013 | 1 μg/L |
| Iron | 2013 | 1 μg/L |
| Lead | 2013 | 1 μg/L |
| Nickel | 2013 | 1 μg/L |
| Zinc | 2013 | 1 μg/L |
| Cadmium | 2013 | 100 ng/L |
| Mercury | 2013 | 100 ng/L |
| Silver | 2013 | 100 ng/L |

※他のAnnexは省略