**OECD Guidelines for the Testing of Chemicals 211 *Daphnia magna* Reproduction Test 2008-2012改定版比較表（仮）**

| **2008** | **2012** |
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| **INTRODUCTION** | **INTRODUCTION** |
| 1. OECD Test Guidelines for Testing of Chemicals are periodically reviewed in the light of scientific progress. With respect to Guideline 202, Part II, *Daphnia* sp Reproduction Test (adopted April 1984), it had generally been acknowledged that data from tests performed according to this Guideline could be variable. This led, ~~in recent years~~, to considerable effort being devoted to the identification of the reasons for this variability with the aim of producing a better test method. This updated Guideline is based on the outcome of these research activities and ring-tests performed in 1992 (1) and 1994 (2).  | 1. OECD Test Guidelines for Testing of Chemicals are periodically reviewed in the light of scientific progress. With respect to Guideline 202, Part II, *Daphnia* sp. Reproduction Test (adopted April 1984), it had generally been acknowledged that data from tests performed according to this Guideline could be variable. This led, to considerable effort being devoted to the identification of the reasons for this variability with the aim of producing a better test method. This Test Guideline (TG) is based on the outcome of these research activities, ring-tests and validation studies performed in 1992 (1), 1994 (2) and 2008 (3). |
| 2. The main differences between the initial version (1984) and the second version (1998) of the Guideline are:  | 2. The main differences between the initial version (1984), and second version (1998) and this version of the Guideline are: |
| (a) the species to be used is *Daphnia magna;*  | (a) the recommended species to be used is *Daphnia magna;* |
| (b) the test duration is 21 days;  | (b) the test duration is 21 days; |
| (c) for semi-static tests, the number of animals to be used at each test concentration has been reduced from at least 40, preferably divided into four groups of 10 animals, to at least 10 animals held individually (although different designs can be used for flow-through tests);  | (c) for semi-static tests, the number of animals to be used at each test concentration has been reduced from at least 40, preferably divided into four groups of 10 animals, to at least 10 animals held individually (although different designs can be used for flow-through tests); |
| (d) more specific recommendations have been made with regard to test medium and feeding conditions.  | (d) more specific recommendations have been made with regard to test medium and feeding conditions. |
| The main difference between the second version (1998) and this version is:  | The main differences between the second version (1998) and this version are: |
| (e) Annex 7 has been added to describe procedures for the identification of neonate sex if required. In line with previous versions of this guideline sex ratio is an optional endpoint.  | (e) In 2008, Annex 7 has been added to describe procedures for the identification of neonate sex if required. In line with previous versions of this TG sex ratio is an optional endpoint; |
|  | (f) In 2012, the response variable number of living offspring produced per surviving parental animal has been supplemented with an additional response variable for Daphnia reproduction ,i.e. the total number living offspring produced at the end of the test per parent daphnia at the start of the test excluding from the analysis parental accidental and/or inadvertent mortality. The purpose of the added response variable is to align this response variable with other OECD reproduction Test Guidelines on invertebrates. Furthermore, in relation to this response variable, it is possible, in this TG, to remove a source of error, namely the effect of inadvertent and/or accidental parental mortality, should that occur during the exposure period. |
|  | (g) Additional statistical guidance for test design and for treatment of results has been included both for ECx (e.g. EC10 or EC50) and for NOEC/LOEC approach. |
|  | (h) A limit test is introduced. |
| 3. Definitions used are given in Annex 1. | 3. Definitions used are given in Annex 1. |
| **PRINCIPLE OF THE TEST** | **PRINCIPLE OF THE TEST** |
| 4. The primary objective of the test is to assess the effect of chemicals on the reproductive output of *Daphnia magna*. To this end, young female *Daphnia* (the parent animals), aged less than 24 hours at the start of the test, are exposed to the test substance added to water at a range of concentrations. The test duration is 21 days. At the end of the test, the total number of living offspring produced ~~per parent animal alive~~ at the end of the test is assessed. ~~This means that juveniles produced by adults that die during the test are excluded from the calculations.~~ Reproductive output of the parent animals can be expressed in otherways (e.g. number of living offspring produced per animal per day from the first day offspring were observed) but these should be reported in addition to the total number of juveniles produced ~~per parent alive~~ at the end of the test. ~~The reproductive output of the animals exposed to the test substance is compared to that of the control(s) in order to determine the lowest observed effect concentration (LOEC) and hence the no observed effect concentration (NOEC).~~ In addition, and as far as possible, the data are analysed using a regression model in order to estimate the concentration that would cause ~~a~~ x % reduction in reproductive output, i.e. ECx (e.g. EC50, EC20 or EC10). | 4. The primary objective of the test is to assess the effect of chemicals on the reproductive output of *Daphnia magna.* To this end, young female *Daphnia* (the parent animals), aged less than 24 hours at the start of the test, are exposed to the test substance added to water at a range of concentrations. The test duration is 21 days. At the end of the test, the total number of living offspring produced is assessed. Reproductive output of the parent animals can be expressed in other ways (e.g. number of living offspring produced per animal per day from the first day offsprings were observed) but these should be reported in addition to the total number of living offspring produced at the end of the test. Because of the particular design of the semi-static test compared to other OECD invertebrate reproduction Test Guidelines, it is also possible to count the number of living offspring produced by each individual parent animal. This enables that, contrary to other OECD invertebrate reproduction tests, if the parent animal dies accidentally and/or inadvertently during the test period, its offspring production can be excluded from data assessment. Hence, if parental mortality occurs in exposed replicates, it should be considered whether or not the mortality follows a concentration-response pattern, e.g. if there is a significant regression of the response versus concentration of the test substance with a positive slope (a statistical test like the Cochran-Armitage trend test may be used for this). If the mortality does not follow a concentration-response pattern, then those replicates with parental mortality should be excluded from the analysis of the test result. If the mortality follows a concentration-response pattern, the parental mortality should be assigned as an effect of the test substance and the replicates should not be excluded from the analysis. If the parent animal dies during the test i.e. accidentally from mishandling or accident, or inadvertently due to unexplained incident not related to the effect of the test substance or turns out to be male, then the replicate is excluded from the analysis (see more in paragraph 51). The toxic effect of the test substance on reproductive output is expressed as ECx by fitting the data to an appropriate model by non-linear regression to estimate the concentration that would cause x % reduction in reproductive output, respectively, or alternatively as the NOEC/LOEC value (4). The test concentrations should preferably bracket the lowest of the used effect concentrations (e.g. EC10) which means that this value is calculated by interpolation and not extrapolation. |
| 5. The survival of the parent animals and time to production of first brood must also be reported. Other substance-related effects on parameters such as growth (e.g. length), and possibly intrinsic rate of increase, may also be examined.  | 5. The survival of the parent animals and time to production of first brood should also be reported. Other substance-related effects on parameters such as growth (e.g. length), and possibly intrinsic rate of population increase, can also be examined (see paragraph 44). |
| **INFORMATION ON THE TEST SUBSTANCE** | **INFORMATION ON THE TEST SUBSTANCE** |
| 6. Results of an acute toxicity test (see Guideline 202: *Daphnia* sp. Acute Immobilisation Test) performed with *Daphnia magna* ~~should be available. The result~~ may be useful in selecting an appropriate range of test concentrations in the reproduction tests. The water solubility and the vapour pressure of the test substance should be known and a reliable analytical method for the quantification of the substance in the test solutions with reported recovery efficiency and limit of determination should be available. | 6. Results of an acute toxicity test (see Guideline 202: *Daphnia* sp. Acute Immobilisation Test) performed with *Daphnia magna* may be useful in selecting an appropriate range of test concentrations in the reproduction tests. The water solubility and the vapour pressure of the test substance should be known and a reliable analytical method for the quantification of the substance in the test solutions with reported recovery efficiency and limit of determination should be available. |
| 7. Information on the test substance which may be 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, Pow and results of a test for ready biodegradability (see Guideline 301). | 7. Information on the test substance which may be 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, Pow and results of a test for ready biodegradability (see Test Guidelines 301 and 310). |
| **VALIDITY OF THE TEST** | **VALIDITY OF THE TEST** |
| 8. For a test to be valid, the following performance criteria should be met in the control(s): | 8. For a test to be valid, the following performance criteria should be met in the control(s): |
| - the mortality of the parent animals (female *Daphnia*) does not exceed 20% at the end of the test; | - the mortality of the parent animals (female *Daphnia*) does not exceed 20% at the end of the test; |
| - the mean number of live offspring produced per parent animal surviving at the end of the test is ≥60. | - the mean number of living offspring produced per parent animal surviving at the end of the test is ≥60. |
|  | Note: The same validity criterion (20%) can be used for accidental and inadvertent parental mortality for the controls as well as for each of the test concentrations. |
| **DESCRIPTION OF THE METHOD** | **DESCRIPTION OF THE METHOD** |
| **Apparatus** | **Apparatus** |
| 9. Test vessels and other apparatus which will come into contact with the test solutions should be made entirely of glass or other chemically inert material. The test vessels will normally be glass beakers. | 9. Test vessels and other apparatus, which will come into contact with the test solutions should be made entirely of glass or other chemically inert material. The test vessels will normally be glass beakers. |
| 10. In addition some or all of the following equipment will be required: | 10. In addition some or all of the following equipment will be required: |
| - oxygen meter (with microelectrode or other suitable equipment for measuring dissolved oxygen in low volume samples); | - oxygen meter (with microelectrode or other suitable equipment for measuring dissolved oxygen in low volume samples); |
| - adequate apparatus for temperature control; | - adequate apparatus for temperature control; |
| - pH-meter; | - pH-meter; |
| - equipment for the determination of the hardness of water; | - equipment for the determination of the hardness of water; |
| - equipment for the determination of the total organic carbon concentration (TOC) of water or equipment for the determination of the chemical oxygen demand (COD); | - equipment for the determination of the total organic carbon concentration (TOC) of water or equipment for the determination of the chemical oxygen demand (COD); |
| - adequate apparatus for the control of the lighting regime and measurement of light intensity. | - adequate apparatus for the control of the lighting regime and measurement of light intensity. |
| **Test Organism** | **Test Organism** |
| 11. The species to be used in the test is *Daphnia magna* Straus 1. | 11. The species to be used in the test is *Daphnia magna* Straus 1. |
| 1Other *Daphnia* species may be used provided they meet the validity criteria as appropriate (the validity criterion relating to the reproductive output in the controls should be relevant for the *Daphnia* species). If other ~~species of~~ *Daphnia* are used they must be clearly identified and their use justified. | 1Other daphnids may be used provided they meet the validity criteria as appropriate (the validity criterion relating to the reproductive output in the controls should be relevant for *all* species). If other *daphnid* are used they should be clearly identified and their use justified. |
| 12. Preferably, the clone should have been identified by genotyping. Research (1) has shown that the reproductive performance of Clone A (which originated from IRCHA in France) (3) consistently meets the validity criterion of a mean of ≥ 60 offspring per parent animal surviving when cultured under the conditions described in this Guideline. However, other clones are acceptable provided that the *Daphnia* culture is shown to meet the validity criteria for a test.  | 12. Preferably, the clone should have been identified by genotyping. Research (1) has shown that the reproductive performance of Clone A (which originated from IRCHA in France) (5) consistently meets the validity criterion of a mean of ≥ 60 living offspring per parent animal surviving when cultured under the conditions described in this Guideline. However, other clones are acceptable provided that the *Daphnia* culture is shown to meet the validity criteria for the test. |
| 13. At the start of the test, the animals should be less than 24 hours old and must not be first brood progeny. They should be derived from a healthy stock (i.e. showing no signs of stress such as high mortality, presence of males and ephippia, delay in the production of the first brood, discoloured animals, etc). The stock animals must be maintained in culture conditions (light, temperature, medium, feeding and animals per unit volume) similar to those to be used in the test. If the *Daphnia* culture medium to be used in the test is different from that used for routine *Daphnia* culture, it is good practice to include a pre-test acclimation period of normally about 3 weeks (i.e. one generation) to avoid stressing the parent animals. | 13. At the start of the test, the animals should be less than 24 hours old and should not be first brood progeny. They should be derived from a healthy stock (i.e. showing no signs of stress such as high mortality, presence of males and ephippia, delay in the production of the first brood, discoloured animals, etc). The stock animals should be maintained in culture conditions (light, temperature, medium, feeding and animals per unit volume) similar to those to be used in the test. If the *Daphnia* culture medium to be used in the test is different from that used for routine *Daphnia* culture, it is good practice to include a pre-test acclimation period of normally about 3 weeks (i.e. one generation) to avoid stressing the parent animals. |
| **Test medium** | **Test medium** |
| 14. It is recommended that a fully defined medium be used in this test. This can avoid the use of additives (e.g. seaweed, soil extract ~~etc~~), which are difficult to characterise, and therefore improves the opportunities for standardisation between laboratories. Elendt M4 (4) and M7 media (see Annex 2) have been found to be suitable for this purpose. However, other media (e.g. (5) (6)) are acceptable provided the performance of the *Daphnia* culture is shown to meet the validity criteria for the test.  | 14. It is recommended that a fully defined medium be used in this test. This can avoid the use of additives (e.g. seaweed, soil extract), which are difficult to characterise, and therefore improves the opportunities for standardisation between laboratories. Elendt M4 (6) and M7 media (see Annex 2) have been found to be suitable for this purpose. However, other media (e.g. (7) (8)) are acceptable provided the performance of the *Daphnia* culture is shown to meet the validity criteria for the test. |
| 15. If media are used which include undefined additives, these additives should be specified clearly and information should be provided in the test report on composition, particularly with regard to carbon content as this may contribute to the diet provided. It is recommended that the total organic carbon (TOC) and/or chemical oxygen demand (COD) of the stock preparation of the organic additive be determined and an estimate of the resulting contribution to the TOC/COD in the test medium made. It is further recommended that TOC levels in the medium (i.e. before addition of the algae) be below 2 mg/l (7).  | 15. If media are used which include undefined additives, these additives should be specified clearly and information should be provided in the test report on composition, particularly with regard to carbon content as this may contribute to the diet provided. It is recommended that the total organic carbon (TOC) and/or chemical oxygen demand (COD) of the stock preparation of the organic additive be determined and an estimate of the resulting contribution to the TOC/COD in the test medium made. It is further recommended that TOC levels in the medium (i.e. before addition of the algae) be below 2 mg/l (9). |
| 16. When testing substances containing metals, it is important to recognise that the properties of the test medium (e.g. hardness, chelating capacity) may have a bearing on the toxicity of the test substance. For this reason, a fully defined medium is desirable. However, at present, the only fully defined media which are known to be suitable for long-term culture of *Daphnia magna* are Elendt M4 and M7. Both media contain the chelating agent EDTA. Work has shown (2) that the 'apparent toxicity' of cadmium is generally lower when the reproduction test is performed in M4 and M7 media than in media containing no EDTA. M4 and M7 are not, therefore, recommended for testing substances containing metals, and other media containing known chelating agents should also be avoided. For metal-containing substances it may be advisable to use an alternative medium such as, for example, ASTM reconstituted hard fresh water (7), which contains no EDTA, ~~with added seaweed extract (8)~~. This combination of ASTM reconstituted hard fresh water and seaweed extract is ~~also~~ suitable for long-term culture ~~and testing~~ of *Daphnia magna* (2)~~, although it still exerts a mild chelating action due to the organic component in the added seaweed extract.~~ | 16. When testing substances containing metals, it is important to recognise that the properties of the test medium (e.g. hardness, chelating capacity) may have a bearing on the toxicity of the test substance. For this reason, a fully defined medium is desirable. However, at present, the only fully defined media which are known to be suitable for long-term culture of Daphnia magna are Elendt M4 and M7. Both media contain the chelating agent EDTA. Work has shown (2) that the 'apparent toxicity' of cadmium is generally lower when the reproduction test is performed in M4 and M7 media than in media containing no EDTA. M4 and M7 are not, therefore, recommended for testing substances containing metals, and other media containing known chelating agents should also be avoided. For metal-containing substances it may be advisable to use an alternative medium such as, for example, ASTM reconstituted hard fresh water (9), which contains no EDTA . This combination of ASTM reconstituted hard fresh water and seaweed extract (10) is suitable for long-term culturing of *Daphnia magna* (2). |
| 17. The dissolved oxygen concentration should be above 3 mg/l at the beginning and during the test. The pH should be within the range 6 - 9, and normally it should not vary by more than 1.5 units in any one test. Hardness above 140 mg/l (as CaCO3) is recommended. Tests at this level and above have demonstrated reproductive performance in compliance with the validity criteria (9) (10). | 17. The dissolved oxygen concentration should be above 3 mg/l at the beginning and during the test. The pH should be within the range 6 - 9, and normally it should not vary by more than 1.5 units in any one test. Hardness above 140 mg/l (as CaCO3) is recommended. Tests at this level and above have demonstrated reproductive performance in compliance with the validity criteria (11) (12). |
| **Test solutions** | **Test solutions** |
| 18. Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. Stock solutions should preferably be prepared by dissolving the substance in test medium. | 18. Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. Stock solutions should preferably be prepared, without using any solvents or dispersants if possible, by mixing or agitating the test substance in test medium　using mechanical means such as agitating, stirring or ultrasonication, or other appropriate methods. It is preferable to expose test systems to concentrations of the test substance to be used in the study for as long as is required to demonstrate the maintenance of stable exposure concentrations prior to the introduction of test organisms. If the test substance is difficult to dissolve in water, procedures described in the OECD Guidance for handling difficult substances should be followed (13). The use of solvents or dispersants should be avoided, but may be necessary in some cases in order to produce a suitably concentrated stock solution for dosing. |
| ~~19. The use of organic solvents or dispersants may be required in some cases in order to produce a suitably concentrated stock solution, but every effort should be made to avoid the use of such materials. Examples of suitable solvents are acetone, ethanol, methanol, dimethylformamide and triethylene glycol. Examples of suitable dispersants are Cremophor RH40, methylcellulose 0.01% and HCO-40. In any case, the test substance in the test solutions should not exceed the limit of solubility in the test medium.~~ |  |
| ~~Solvents are used to produce a stock solution which can be dosed accurately into water. At the recommended solvent concentration in the final test medium (i.e. ≤ 0.1 ml/l), the solvents listed above will not be toxic and will not increase the water solubility of a substance.~~ |  |
| ~~Dispersants may assist in accurate dosing and dispersion. At the recommended concentration in the final test medium (≤ 0.1 ml/l), the dispersants listed above will not be toxic and will not increase the water solubility of a substance.~~ |  |
|  | 19. A dilution water control with adequate replicates and, if unavoidable, a solvent control with adequate replicates should be run in addition to the test concentrations. Only solvents or dispersants that have been investigated to have no significant or only minimal effects on the response variable should be used in the test. Examples of suitable solvents (e.g. acetone, ethanol, methanol, dimethylformamide and triethylene glycol) and dispersants (e.g. Cremophor RH40, methylcellulose 0.01% and HCO-40) are given in (13). Where a solvent or dispersant is used, its final concentration should not be greater than 0.1 mL/L (13) and it should be the same concentration in all test vessels, except the dilution water control. However, every effort should be made to keep the solvent concentration to a minimum. |
| **PROCEDURE** | **PROCEDURE** |
| **Conditions of Exposure** | **Conditions of Exposure** |
| **Duration** | **Duration** |
| 20. The test duration is 21 days. | 20. The test duration is 21 days. |
| **Loading** | **Loading** |
| 21. Parent animals are maintained individually, one per test vessel, with 50 - 100 ml of medium in each vessel. | 21. Parent animals are maintained individually, one per test vessel, usually with 50 - 100 mL (for *Daphnia magna*, smaller volumes may be possible especially for smaller daphnids e.g. *Ceriodaphnia dubia*) of medium in each vessel, unless a flow-through test design is necessary for testing. |
| 22. Larger volumes may sometimes be necessary to meet requirements of the analytical procedure used for determination of the test substance concentration, although pooling of replicates for chemical analysis is also allowable. If volumes greater than 100 ml are used, the ration given to the *Daphnia* may need to be increased to ensure adequate food availability and compliance with the validity criteria. ~~For flow-through tests, alternative designs may, for technical reasons, be considered (e.g. four groups of 10 animals in a larger test volume), but any changes to the test design should be reported.~~ | 22. Larger volumes may sometimes be necessary to meet requirements of the analytical procedure used for determination of the test substance concentration, although pooling of replicates for chemical analysis is also allowable. If volumes greater than 100 mL are used, the ration given to the *Daphnia* may need to be increased to ensure adequate food availability and compliance with the validity criteria. |
| **Test animals** | **Test animals** |
| 23. For semi-static tests, at least 10 animals individually held at each test concentration and at least 10 animals individually held in the control series. | 23. For semi-static tests, at least 10 animals individually held at each test concentration and at least 10 animals individually held in the control series. |
| 24. For flow-through tests, 40 animals divided into four groups of 10 animals at each test concentration has been shown to be suitable (1). A smaller number of test organisms may be used and a minimum of 20 animals per concentration divided into two or more replicates with an equal number of animals (e.g. four replicates each with five daphnids) is recommended. Note that for tests where animals are held in groups, it will not be possible to express the reproductive output as the total number of living offspring produced per parent animal alive at the end of the test, if parent animals die. In these cases reproductive output should be expressed as 'total number of living offspring produced per parent present at the beginning of the test'. | 24. For flow-through tests, 40 animals divided into four groups of 10 animals at each test concentration has been shown to be suitable (1). A smaller number of test organisms may be used and a minimum of 20 animals per concentration divided into two or more replicates with an equal number of animals (e.g. four replicates each with five daphnids) is recommended. Note that for tests where animals are held in groups, it will not be possible to exclude any offspring from the statistical analysis if inadvertent/ accidental parental mortality occurs when the reproduction has begun, and hence in these cases the reproductive output should be expressed as 'total number of living offspring produced per parent present at the beginning of the test'. |
| 25. Treatments should be allocated to the test vessels and all subsequent handling of the test vessels should be done in a random fashion. Failure to do this may result in bias that could be construed as being a concentration effect. In particular, if experimental units are handled in treatment or concentration order, then some time-related effect, such as operator fatigue or other error, could lead to greater effects at the higher concentrations. Furthermore, if the test results are likely to be affected by an initial or environmental condition of the test, such as position in the laboratory, then consideration should be given to blocking the test. | 25. Treatments should be allocated to the test vessels and all subsequent handling of the test vessels should be done in a random fashion. Failure to do this may result in bias that could be construed as being a concentration effect. In particular, if experimental units are handled in treatment or concentration order, then some time-related effect, such as operator fatigue or other error, could lead to greater effects at the higher concentrations. Furthermore, if the test results are likely to be affected by an initial or environmental condition of the test, such as position in the laboratory, then consideration should be given to blocking the test. |
| **Feeding** | **Feeding** |
| 26. For semi-static tests, feeding should preferably be done daily, but at least three times per week (i.e. corresponding to media changes). Deviations from this (e.g. for flow-through tests) should be reported. | 26. For semi-static tests, feeding should preferably be done daily, but at least three times per week (i.e. corresponding to media changes). The possible dilution of the exposure concentrations by food addition should be taken into account and avoided as much as possible with well concentrated algae suspensions. Deviations from this (e.g. for flow-through tests) should be reported. |
| 27. During the test the diet of the parent animals should preferably be living algal cells of one or more of the following: Chlorella sp, *Selenastrum capricornutum* [now *Pseudokirchneriella subcapitata*, (11)] and *Scenedesmus subspicatus*. The supplied diet should be based on the amount of organic carbon (C) provided to each parent animal. Research (12) has shown that, for *Daphnia magna*, ration levels of between 0.1 and 0.2 mg C/*Daphnia*/day are sufficient for achieving the required number of offspring to meet the test validity criteria. The ration can be supplied either at a constant rate throughout the period of the test, or, if desired, a lower rate can be used at the beginning and then increased during the test to take account of growth of the parent animals. In this case, the ration should still remain within the recommended range of 0.1 - 0.2 mg C/*Daphnia*/day at all times. | 27. During the test, the diet of the parent animals should preferably be living algal cells of one or more of the following: *Chlorella* sp, (formerly *Selenastrum capricornutum*) *Pseudokirchneriella subcapitata*, (11b) and *Desmodesmus subspicatus* (formerly *Scenedesmus subspicatus*). The supplied diet should be based on the amount of organic carbon (C) provided to each parent animal. Research (14) has shown that, for *Daphnia magna*, ration levels of between 0.1 and 0.2 mg C/*Daphnia*/day are sufficient for achieving the required number of living offspring to meet the test validity criteria. The ration can be supplied either at a constant rate throughout the period of the test, or, if desired, a lower rate can be used at the beginning and then increased during the test to take account of growth of the parent animals. In this case, the ration should still remain within the recommended range of 0.1 - 0.2 mg C/*Daphnia*/day at all times. |
| 28. If surrogate measures, such as algal cell number or light absorbance, are to be used to feed the required ration level (i.e. for convenience since measurement of carbon content is time consuming), each laboratory must produce its own nomograph relating the surrogate measure to carbon content of the algal culture (see Annex 3 for advice on nomograph production). Nomographs should be checked at least annually and more frequently if algal culture conditions have changed. Light absorbance has been found to be a better surrogate for carbon content than cell number (13). | 28. If surrogate measures, such as algal cell number or light absorbance, are to be used to feed the required ration level (i.e. for convenience since measurement of carbon content is time consuming), each laboratory should produce its own nomograph relating the surrogate measure to carbon content of the algal culture (see Annex 3 for advice on nomograph production). Nomographs should be checked at least annually and more frequently if algal culture conditions have changed. Light absorbance has been found to be a better surrogate for carbon content than cell number (15). |
| 29. A concentrated algal suspension should be fed to the *Daphnia* to minimise the volume of algal culture medium transferred to the test vessels. Concentration of the algae can be achieved by centrifugation followed by resuspension in ~~distilled water, deionised water or~~ *Daphnia* culture medium. | 29. A concentrated algal suspension should be fed to the *Daphnia* to minimise the volume of algal culture medium transferred to the test vessels. Concentration of the algae can be achieved by centrifugation followed by re-suspension in *Daphnia* culture medium. |
| **Light** | **Light** |
| 30. 16 hours light at an intensity not exceeding 15-20 μE•m-2•s-1. | 30. 16 hours light at an intensity not exceeding 15-20 μE•m-2•s-1 measured at the water surface of the vessel. For light-measuring instruments calibrated in lux, an equivalent range of 1000 \_ 1500 lux for cool white light corresponds close to the recommended light intensity 15-20 μE・m-2・s-1. |
| **Temperature** | **Temperature** |
| 31. The temperature of the test media should be within the range 18-22°C. However, for any one test, the temperature should not, if possible, vary by more than 2°C within these limits (e.g. 18-20, 19-21 or 20-22°C). It may be appropriate to use an additional test vessel for the purposes of temperature monitoring. | 31. The temperature of the test media should be within the range 18-22°C. However, for any one test, the temperature should not, if possible, vary by more than 2°C within these limits (e.g. 18-20, 19-21 or 20-22°C) as daily range. It may be appropriate to use an additional test vessel for the purposes of temperature monitoring. |
| **Aeration** | **Aeration** |
| 32. The test vessels must not be aerated during the test. | 32. The test vessels should not be aerated during the test. |
| **Test concentrations** | **Test design** |
| ~~33. Prior knowledge of the toxicity of the test substance (e.g. from an acute test and/or from range-finding studies) should help in selecting appropriate test concentrations.~~ |  |
|  | **Range finding test** |
|  | 33. When necessary, a range-finding test is conducted with, for example five test substance concentrations and two replicates for each treatment and control. Additional information, from tests with similar compounds or from literature, on acute toxicity to *Daphnia* and/or other aquatic organisms may also be useful in deciding on the range of concentrations to be used in the range-finding test. |
|  | 34. The duration of the range-finding test is 21 days or of a sufficient duration to reliably predict effect levels. At the end of the test, reproduction of the *Daphnia* is assessed. The number of parents and the occurrence of offspring should be recorded. |
|  | **Definitive test** |
| 34. Normally there should be at least five test concentrations arranged in a geometric series with a separation factor preferably not exceeding 3.2, and the appropriate number of replicates for each test concentration should be used (see paragraphs 23-24). Justification should be provided if fewer than five concentrations are used. Substances should not be tested above their solubility limit in test medium. | 35. Normally there should be at least five test concentrations, bracketing effective concentration (e.g. ECx), and arranged in a geometric series with a separation factor preferably not exceeding 3.2 An appropriate number of replicates for each test concentration should be used (see paragraphs 24-25). Justification should be provided if fewer than five concentrations are used. Substances should not be tested above their solubility limit in test medium. Before conducting the experiment it is advisable to consider the statistical power of the tests design and using appropriate statistical methods (4). In setting the range of concentrations, the following should be borne in mind: |
| 35. In setting the range of concentrations, the following should be borne in mind: |
| (i) If the aim is to obtain the LOEC/NOEC, the lowest test concentration must be low enough so that the fecundity at that concentration is not significantly lower than that in the control. If this is not the case, the test will have to be repeated with a reduced lowest concentration. | (ii) When estimating the LOEC and/or NOEC, the lowest test concentration should be low enough so that the reproductive output at that concentration is not significantly lower than that in the control. If this is not the case, the test should be repeated with a reduced lowest concentration. |
| (ii) If the aim is to obtain the LOEC/NOEC, the highest test concentration must be high enough so that the fecundity at that concentration is significantly lower than that in the control. If this is not the case, the test will have to be repeated with an increased highest concentration. | (iii) When estimating the LOEC and/or NOEC, the highest test concentration should be high enough so that the reproductive output at that concentration is significantly lower than that in the control. If this is not the case, the test should be repeated with an increased highest concentration unless the maximum required test concentration for chronic effects testing (i.e., 10 mg/L) was used as the highest test concentration in the initial test. |
| (iii) If ECx for effects on reproduction is estimated, it is advisable that sufficient concentrations are used to define the ECx with an appropriate level of confidence. If the EC50 for effects on reproduction is estimated, it is advisable that the highest test concentration is greater than this EC50. Otherwise, although it will still be possible to estimate the EC50, the confidence interval for the EC50 will be very wide and it may not be possible to satisfactorily assess the adequacy of the fitted model. | (i) When ECx for effects on reproduction is estimated, it is advisable that sufficient concentrations are used to define the ECx with an appropriate level of confidence. Test concentrations used should preferably bracket the estimated ECx such that ECx is found by interpolation rather than extrapolation. It is an advantage for the following statistical analysis to have more test concentrations (e.g. 10) and fewer replicates of each concentration (e.g. 5 thus holding the total number of vessels constant) and with 10 controls. |
| ~~(iv) The range of test concentrations should preferably not include any concentrations that have a statistically significant effect on adult survival since this would change the nature of the test from simply a reproduction test to a combined reproduction and mortality test requiring much more complex statistical analysis.~~ |  |
| ~~36. Where a solvent or dispersant is used to aid preparation of test solutions (see paragraph 19), 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.~~ |  |
|  | 36. If no effects are observed at the highest concentration in the range-finding test (e.g. at 10 mg/l), or when the test substance is highly likely to be of low/ no toxicity based on lack of toxicity to other organisms and/or low/no uptake, the reproduction test may be performed as a limit test, using a test concentration of e.g.10 mg/l and the control. Ten replicates should be used for both the treatment and the control groups. When a limit test might need to be done in a flow-through system less replicates would be adequate. A limit test will provide the opportunity to demonstrate that there is no statistically significant effect at the limit concentration, but if effects are recorded a full test will normally be required. |
| **Controls** | **Controls** |
| 37. One test-medium control series and also, if relevant, one control series containing the solvent or dispersant should be run in addition to the test series. When used, the solvent or dispersant concentration should be the same as that used in the vessels containing the test substance. The appropriate number of replicates should be used (see paragraphs 23-24). | 37. One test-medium control series and also, if relevant, one control series containing the solvent or dispersant should be run in addition to the test series. When used, the solvent or dispersant concentration should be the same as that used in the vessels containing the test substance. The appropriate number of replicates should be used (see paragraphs 23-24). |
| 38. Generally in a well-run test, the coefficient of variation around the mean number of living offspring produced per parent animal in the control(s) should be ≤ 25%, and this should be reported for test designs using individually held animals. | 38. Generally in a well-run test, the coefficient of variation around the mean number of living offspring produced per parent animal in the control(s) should be ≤ 25%, and this should be reported for test designs using individually held animals. |
| **Test medium renewal** | **Test medium renewal** |
| 39. The frequency of medium renewal will depend on the stability of the test substance, but should be at least three times per week. If, from preliminary stability tests (see paragraph 7), 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 maximum renewal period (i.e. 3 days), consideration should be given to more frequent medium renewal, or to the use of a flow-through test. | 39. The frequency of medium renewal will depend on the stability of the test substance, but should be at least three times per week. If, from preliminary stability tests (see paragraph 7), 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 maximum renewal period (i.e. 3 days), consideration should be given to more frequent medium renewal, or to the use of a flow-through test. |
| 40. When the medium is renewed in semi-static tests, a second series of test vessels are prepared and the parent animals transferred to them by, for example, a glass pipette of suitable diameter. The volume of medium transferred with the *Daphnia* should be minimised. | 40. When the medium is renewed in semi-static tests, a second series of test vessels are prepared and the parent animals transferred to them by, for example, a glass pipette of suitable diameter. The volume of medium transferred with the *Daphnia* should be minimised. |
| **Observations** | **Observations** |
| 41. The results of the observations made during the test should be recorded on data sheets (see examples in Annexes 4 and 5). If other measurements are required (see paragraphs ~~5 and~~ 44), additional observations may be required. | 41. The results of the observations made during the test should be recorded on data sheets (see examples in Annexes 4 and 5). If other measurements are required (see paragraph44), additional observations may be required. |
| **Offspring** | **Offspring** |
| 42. The offspring produced by each parent animal should preferably be removed and counted daily from the appearance of the first brood to prevent them consuming food intended for the adult. For the purpose of this guideline it is only the number of living offspring that needs to be counted, but the presence of aborted eggs or dead offspring should be recorded. | 42. The offspring produced by each parent animal should preferably be removed and counted daily from the appearance of the first brood to prevent them consuming food intended for the parent. For the purpose of this guideline it is only the number of living offspring that needs to be counted, but the presence of aborted eggs or dead offspring should be recorded. |
| **Mortality** | **Mortality** |
| 43. Mortality among the parent animals should be recorded preferably daily, at least at the same times as offspring are counted. | 43. Mortality among the parent animals should be recorded preferably daily, or at least as frequently as offspring are counted. |
| **Other parameters** | **Other parameters** |
| 44. Although this guideline is designed principally to assess effects on reproduction, it is possible that other effects may also be sufficiently quantified to allow statistical analysis. Growth measurements are highly desirable since they provide information on possible sublethal effects which may be more useful than reproduction measures alone; the measurement of the length of the parent animals (i.e. body length excluding the anal spine) at the end of the test is recommended. Other parameters that can be measured or calculated include time to production of first brood (and subsequent broods), number and size of broods per animal, number of aborted broods, presence of male neonates (OECD, 2008) or ephippia and possibly the intrinsic rate of population increase (see Annex 1 for definition and Annex 7 for the identification of the sex of neonates). | 44. Although this guideline is designed principally to assess effects on reproductive output, it is possible that other effects may also be sufficiently quantified to allow statistical analysis. Reproductive output per surviving parent animal, i.e. number of living offspring produced during the test per surviving parent, may be recorded. This may be compared with the main response variable (reproductive output per parent animal in the start of the test which did not inadvertently or accidentally die during the test). If parental mortality occurs in exposed replicates it should be considered whether or not the mortality follows a concentration-response pattern, e.g. if there is a significant regression of the response versus concentration of the test substance with a positive slope (a statistical test like the Cochran-Armitage trend test may be used for this). If the mortality does not follow a concentration-response pattern, then those replicates with parental mortality should be excluded from the analysis of the test result. If the mortality follows a concentration-response pattern, the parental mortality should be assigned as an effect of the test substance and the replicates should not be excluded from the analysis of the test result.Growth measurements are highly desirable since they provide information on possible sublethal effects which may be useful in addition to reproduction measures alone; the measurement of the length of the parent animals (i.e. body length excluding the anal spine) at the end of the test is recommended. Other parameters that can be measured or calculated include time to production of first brood (and subsequent broods), number and size of broods per animal, number of aborted broods, presence of male neonates (OECD, 2008) or ephippia and possibly the intrinsic rate of population increase (see Annex 1 for definition and Annex 7 for the identification of the sex of neonates). |
| **Frequency of analytical determinations and measurements** | **Frequency of analytical determinations and measurements** |
| 45. Oxygen concentration, temperature, hardness and pH values should be measured at least once a week, in fresh and old media, in the control(s) and in the highest test substance concentration. | 45. Oxygen concentration, temperature, hardness and pH values should be measured at least once a week, in fresh and old media, in the control(s) and in the highest test substance concentration. |
| 46. During the test, the concentrations of test substance are determined at regular intervals. | 46. During the test, the concentrations of test substance are determined at regular intervals. |
| 47. In semi-static tests where the concentration of the test substance is expected to remain within ± 20 per cent of the nominal (i.e. within the range 80 - 120 per cent- see paragraphs 6, 7 and 39), it is recommended that, as a minimum, the highest and lowest test concentrations be analysed when freshly prepared and at the time of renewal on one occasion during the first week of the test (i.e. analyses should be made on a sample from the same solution - when freshly prepared and at renewal). These determinations should be repeated at least at weekly intervals thereafter. | 47. In semi-static tests where the concentration of the test substance is expected to remain within ± 20 per cent of the nominal (i.e. within the range 80 - 120 per cent- see paragraphs 6, 7 and 39), it is recommended that, as a minimum, the highest and lowest test concentrations be analysed when freshly prepared and at the time of renewal on one occasion during the first week of the test (i.e. analyses should be made on a sample from the same solution - when freshly prepared and at renewal). These determinations should be repeated at least at weekly intervals thereafter. |
| 48. For tests where the concentration of the test substance is not expected to remain within ± 20 per cent of the nominal, it is necessary to analyse all test concentrations, when freshly prepared and at renewal. However, for those tests where the measured initial concentration of the test substance is not within ± 20 per cent of nominal but where sufficient evidence can be provided to show that the initial concentrations are repeatable and stable (i.e. within the range 80 - 120 per cent of initial concentrations), chemical determinations could be reduced in weeks 2 and 3 of the test to the highest and lowest test concentrations. In all cases, determination of test substance concentrations prior to renewal need only be performed on one replicate vessel at each test concentration. | 48. For tests where the concentration of the test substance is not expected to remain within ± 20 per cent of the nominal, it is necessary to analyse all test concentrations, when freshly prepared and at renewal. However, for those tests where the measured initial concentration of the test substance is not within ± 20 per cent of nominal but where sufficient evidence can be provided to show that the initial concentrations are repeatable and stable (i.e. within the range 80 - 120 per cent of initial concentrations), chemical　determinations could be reduced in weeks 2 and 3 of the test to the highest and lowest test concentrations. In all cases, determination of test substance concentrations prior to renewal need only be performed on one replicate vessel at each test concentration. |
| 49. If a flow-through test is used, 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, 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. In these types of test, the flow-rate of diluent and test substance should be checked daily. | 49. If a flow-through test is used, 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, 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. In these types of test, the flow-rate of diluent and test substance should be checked daily. |
| 50. If there is evidence that the concentration of the substance being tested has been satisfactorily maintained within ± 20 percent of the nominal or measured initial concentration throughout the test, then results can be based on nominal or measured initial values. If the deviation from the nominal or measured initial concentration is greater than ± 20 per cent, results should be expressed in terms of the time-weighted mean (see guidance for calculation in Annex 6). | 50. If there is evidence that the concentration of the substance being tested has been satisfactorily maintained within ± 20 per cent of the nominal or measured initial concentration throughout the test, then results can be based on nominal or measured initial values. If the deviation from the nominal or measured initial concentration is greater than ± 20 per cent, results should be expressed in terms of the time-weighted mean (see guidance for calculation in Annex 6). |
| **DATA AND REPORTING** | **DATA AND REPORTING** |
| **Treatment of results** | **Treatment of results** |
| 51. The purpose of this test is to determine the effect of the test substance on the total number of living offspring produced per parent animal alive at the end of the test. The total number of offspring per parent animal should be calculated for each test vessel (i.e. replicate). ~~If, in any replicate the parent animal dies during the test or turns out to be male, then the replicate is excluded from the analysis. The analysis will then be based on a reduced number of replicates.~~ | 51. The purpose of this test is to determine the effect of the test substance on the reproductive output. The total number of living offspring per parent animal should be calculated for each test vessel (i.e. replicate). In addition, the reproduction can be calculated based on the production of living offspring by the surviving parent organism. However, the ecologically most relevant response variable is the total number of living offspring produced per parent animal which does not die accidentally2 or inadvertently3　during the test. If the parent animal dies accidentally or inadvertently during the test, or turns out to be male, then the replicate is excluded from the analysis. The analysis will then be based on a reduced number of replicates. If parental mortality occurs in exposed replicates it should be considered whether or not the mortality follows a concentration-response pattern, e.g. if there is a significant regression of the response versus concentration of the test substance with a positive slope (a statistical test like the Cochran-Armitage trend test may be used for this). If the mortality does not follow a concentration-response pattern, then those replicates with parental mortality should be excluded from the analysis of the test result. If the mortality follows a concentration-response pattern, the parental mortality should be　assigned as an effect of the test substance and the replicates should not be excluded from the analysis of the test result. |
|  | 2 Accidental mortality: non substance related mortality caused by an accidental incidence (i.e. known cause) |
|  | 3 Inadvertent mortality: non substance related mortality with no known cause |
| ~~52. For the estimation of the LOEC, and hence the NOEC, for effects of the chemical on reproductive output, it is necessary to calculate the mean reproductive output across replicates for each concentration and the pooled residual standard deviation, and this can be done using analysis of variance (ANOVA). The mean for each concentration must then be compared with the control mean using an appropriate multiple comparison method. Dunnett's or Williams' tests may be useful (14)(15)(16)(17). It is necessary to check whether the ANOVA assumption of homogeneity of variance holds. It is recommended that this be done graphically rather than via a formal significance test (18); a suitable alternative is to run a Bartlett’s test. If this assumption does not hold, then consideration should be given to transforming the data to homogenise variances prior to performing the ANOVA, or to carrying out a weighted ANOVA. The size of the effect detectable using ANOVA (i.e. the least significant difference) should be calculated and reported.~~ |  |
| ~~53. For the estimation of the concentration which would cause a 50% reduction in reproductive output (i.e. the EC50), a suitable curve, such as the logistic curve, should be fitted to the data using a statistical method such as least squares. The curve could be parameterised so that the EC50 and its standard error can be estimated directly. This would greatly ease the calculation of the confidence limits about the EC50. Unless there are good reasons to prefer different confidence levels, two-sided 95% confidence limits should be quoted. The fitting procedure should preferably provide a means for assessing the significance of the lack of fit. This can be done graphically or by dividing the residual sum of squares into 'lack of fit' and 'pure error components' and performing a significance test for lack of fit. Since treatments giving high fecundity are likely to have greater variance in the number of juveniles produced than treatments giving low fecundity, consideration to weighting the observed values to reflect the different variances in the different treatment groups should be given. Useful background information can be found in (18).~~ |  |
| ~~54. In the analysis of the data from the final ring test (2), a logistic curve was fitted using the following model, although other suitable models can be used:~~ |  |
| ~~Y=c/[1+(x/x0)^b]~~ |  |
| ~~where:~~ |  |
| ~~Y is the total number of juveniles per parent animal alive at the end of the test (calculated for each vessel) and x is the concentration.~~ |  |
| ~~c = the expected number of juveniles when x=0~~ |  |
| ~~x0 = the EC50 in the population~~ |  |
| ~~b = the slope parameter~~ |  |
| ~~55. This model is likely to be adequate in a large number of situations, but there will be tests for which it is not appropriate. A check should be made on the validity of the model as suggested in paragraph 54. In some cases, a hormesis model in which low concentrations give enhanced effects may be appropriate (19).~~ |  |
| ~~56. Other Effect Concentrations, such as the EC10 or EC20 can also be estimated, although it may be preferable to use a different parameterisation of the model from that used to estimate the EC50.~~ |  |
|  | 52. In summary, when LOEC and NOEC or ECx are being used to express the effects, it is recommended to calculate the effect on reproduction by the use of both response variables mentioned above i.e. |
|  | ● as the total number of living offspring produced per parent animal which does not die accidentally or inadvertently during the test and; |
|  | ● as the number of living offspring produced per surviving parental animal; |
|  | and then to use as the final result the lowest NOEC and LOEC or ECx value calculated by using either of these two response variables. |
|  | 53. Before employing the statistical analysis, e.g. ANOVA procedures, comparison of treatments to the control by Student t-test, Dunnett’s test, Williams’ test, or stepdown Jonckheere-Terpstra test, it is recommended to consider transformation of data if needed for meeting the requirements of the particular statistical test. As non-parametric alternatives one can consider Dunn’s or Mann-Whitney’s tests. 95% confidence intervals are calculated for individual treatment means. |
|  | 54. The number of surviving parents in the untreated controls is a validity criterion, and should be documented and reported. Also all other detrimental effects, e.g. abnormal behavior and toxicological significant findings, should be reported in the final report as well. |
|  | **ECx** |
|  | 55. ECx-values, including their associated lower and upper confidence limits, are calculated using appropriate statistical methods (e.g. logistic or Weibull function, trimmed Spearman-Karber method, or simple interpolation). To compute the EC10, EC50 or any other ECx, the complete data set should be subjected to regression analysis. |
|  | **NOEC/LOEC** |
|  | 56. If a statistical analysis is intended to determine the NOEC/LOEC appropriate statistical methods should be used according to OECD Document 54 on the Current Approaches in the Statistical Analysis of Ecotoxicity Data: a Guidance to Application (4). In general, adverse effects of the test substance compared to the control are investigated using one-tailed hypothesis testing at p ≤ 0.05. |
|  | 57. Normal distribution and variance homogeneity can be tested using an appropriate statistical test, e.g. the Shapiro-Wilk test and Levene test, respectively (p ≤ 0.05). One-way ANOVA and subsequent multi-comparison tests can be performed. Multiple comparisons (e.g. Dunnett's test) or step-down trend tests (e.g. Williams' test, or stepdown Jonckheere-Terpstra test) can be used to calculate whether there are significant differences (p ≤ 0.05) between the controls and the various test substance concentrations (selection of the recommended test according to OECD Guidance Document 54 (4)). Otherwise, non-parametric methods (e.g. Bonferroni-U-test according to Holm or Jonckheere-Terpstra trend test) could be used to determine the NOEC and the LOEC. |
|  | **Limit test** |
|  | 58. If a limit test (comparison of control and one treatment only) has been performed and the prerequisites of parametric test procedures (normality, homogeneity) are fulfilled, metric responses can be evaluated by the Student test (t-test). An unequal-variance t-test (such as Welch test) or a non-parametric testsuch as the Mann-Whitney-U-test may be used, if these requirements are not fulfilled. |
|  | 59. To determine significant differences between the controls (control and solvent or dispersant control), the replicates of each control can be tested as described for the limit test. If these tests do not detect significant differences, all control and solvent control replicates may be pooled. Otherwise all treatments should be compared with the solvent control. |
| **Test report** | **Test report** |
| 57. The test report ~~must~~ include the following: | 60. The test report includes the following: |
| **Test substance:** | **Test substance:** |
| - physical nature and relevant physicochemical properties; | - physical nature and relevant physicochemical properties; |
| - chemical identification data, including purity. | - chemical identification data, including purity. |
| **Test species:** | **Test species:** |
| - the clone (whether it has been genetically typed), supplier or source (if known) and the culture conditions used. If a different species to *Daphnia magna* is used, this should be reported and justified. | - the clone (whether it has been genetically typed), supplier or source (if known) and the culture conditions used. If a different species to *Daphnia magna* is used, this should be reported and justified. |
| **Test conditions:** | **Test conditions:** |
| - test procedure used (e.g. semi-static or flow-through, volume, loading in number of *Daphnia*　per litre); | - test procedure used (e.g. semi-static or flow-through, volume, loading in number of *Daphnia* per litre); |
| - photoperiod and light intensity; | - photoperiod and light intensity; |
| - test design (e.g. number of replicates, number of parents per replicate); | - test design (e.g. number of replicates, number of parents per replicate); |
| - details of culture medium used; | - details of culture medium used; |
| - if used, additions of organic material including the composition, source, method of preparation, TOC/COD of stock preparations, estimation of resulting TOC/COD in test medium; | - if used, additions of organic material including the composition, source, method of preparation, TOC/COD of stock preparations, estimation of resulting TOC/COD in test medium; |
| - detailed information on feeding, including amount (in mg C/*daphnia*/day) and schedule (e.g. type of food(s), including, for algae the specific name (species) and, if known, the strain, the culture conditions); | - detailed information on feeding, including amount (in mg C/*daphnia*/day) and schedule (e.g. type of food(s), including, for algae the specific name (species) and, if known, the strain, the culture conditions); |
| - method of preparation of stock solutions and frequency of renewal (the solvent or dispersant and its concentration must be given, when used). | - method of preparation of stock solutions and frequency of renewal (the solvent or dispersant and its concentration should be given, when used). |
| **Results:** | **Results:** |
| - results from any preliminary studies on the stability of the test substance; | - results from any preliminary studies on the stability of the test substance; |
| - the nominal test concentrations and the results of all analyses to determine the concentration of the test substance in the test vessels (see example data sheets in Annex 5); the recovery efficiency of the method and the limit of determination should also be reported; | - the nominal test concentrations and the results of all analyses to determine the concentration of the test substance in the test vessels (see example data sheets in Annex 5); the recovery efficiency of the method and the limit of determination should also be reported; |
| - water quality within the test vessels (i.e. pH, temperature and dissolved oxygen concentration, and TOC and/or COD and hardness where applicable) (see example data sheet in Annex 4); | - water quality within the test vessels (i.e. pH, temperature and dissolved oxygen concentration, and TOC and/or COD and hardness where applicable) (see example data sheet in Annex 4); |
| - the full record of living offspring by each parent animal (see example data sheet in Annex 4); | - the full record of the production of living offspring during the test by each parent animal (see example data sheet in Annex 4); |
| - the number of deaths among the parent animals and the day on which they occurred (see example data sheet in Annex 4); | - the number of deaths among the parent animals and the day on which they occurred (see example data sheet in Annex 4); |
| - the coefficient of variation for control fecundity (based on total number of living offspring per parent animal alive at the end of the test); | - the coefficient of variation for control reproductive output (based on total number of living offspring per parent animal alive at the end of the test); |
| - plot of total number of living offspring per parent animal (for each replicate) alive at the end of the test vs concentration of the test substance; | - plot of total number of living offspring produced per parent animal in each replicate excluding any parent animal which may have accidentally or inadvertently died during the test vs. concentration of the test substance; |
|  | - as appropriate plot of total number of living offspring produced per surviving parent animal in each replicate vs. concentration of the test substance |
| - the Lowest Observed Effect Concentration (LOEC) for reproduction, including a description of the statistical procedures used and an indication of what size of effect could be detected and the No Observed Effect Concentration (NOEC) for reproduction; where appropriate, the LOEC/NOEC for mortality of the parent animals should also be reported; | - where appropriate the Lowest Observed Effect Concentration (LOEC) for reproduction, including a description of the statistical procedures used and an indication of what size of effect could be expected to be detected (a power analysis can be performed before the start of the experiment to provide this) and the No Observed Effect Concentration (NOEC) for reproduction; information on which response variable that has been used for calculating the LOEC and NOEC value (either as total living offspring per maternal organism which did not die accidentally or inadvertently during the test or as total number of living offspring per surviving maternal organism), where appropriate, the LOEC or NOEC for mortality of the parent animals should also be reported; |
| - where appropriate, the ECx for reproduction and confidence intervals and a graph of the fitted model used for its calculation, the slope of the dose-response curve and its standard error; | - where appropriate, the ECx for reproduction 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 and its standard error; |
| - other observed biological effects or measurements: report any other biological effects which were observed or measured (e.g. growth of parent animals) including any appropriate justification; | - other observed biological effects or measurements: report any other biological effects which were observed or measured (e.g. growth of parent animals) including any appropriate justification; |
| - an explanation for any deviation from the Test Guideline. | - an explanation for any deviation from the Test Guideline. |

**ANNEX1 DEFINIIONS**

| **2008** | **2012** |
| --- | --- |
| For the purposes of this Guideline the following definitions are used: | For the purposes of this Guideline the following definitions are used: |
|  | **Fecundity**: number of living offspring produced per mother animal within the test period |
|  | **Reproductive output:** number of living offspring produced by parental animals within the test period |
| **Parent Animals** are those female *Daphnia* present at the start of the test and of which the reproductive output is the object of study. | **Parent Animals** are those female *Daphnia* present at the start of the test and of which the reproductive output is the object of study. |
| **Offspring** are the young *Daphnia* produced by the parent animals in the course of the test. | **Offspring** are the young *Daphnia* produced by the parent animals in the course of the test. |
|  | **Accidental mortality:** non substance related mortality caused by an accidental incidence (i.e. known cause) |
|  | **Inadvertent mortality**: non substance related mortality with no known cause |
| **Lowest Observed Effect Concentration (LOEC)** is the lowest tested concentration at which the substance is observed to have a statistically significant effect on reproduction and parent mortality (at p < 0.05) when compared with the control, within a stated exposure period. However, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC. When these two conditions cannot be satisfied, a full explanation must be given for how the LOEC (and hence the NOEC) has been selected. | **Lowest Observed Effect Concentration (LOEC)** is the lowest tested concentration at which the substance is observed to have a statistically significant effect on reproduction and parent mortality (at p < 0.05) when compared with the control, within a stated exposure period. However, all test concentrations above the LOEC should have a harmful effect equal to or greater than those observed at the LOEC. When these two conditions cannot be satisfied, a full explanation should be given for how the LOEC (and hence the NOEC) has been selected. |
| **No Observed Effect Concentration (NOEC)** is the test concentration immediately below the LOEC, which when compared with the control, has no statistically significant effect (p < 0.05), within a stated exposure period. | **No Observed Effect Concentration (NOEC)** is the test concentration immediately below the LOEC, which when compared with the control, has no statistically significant effect (p < 0.05), within a stated exposure period. |
| **ECx** is the concentration of the test substance dissolved in water that results in a x per cent reduction in reproduction of *Daphnia ~~magna~~* within a stated exposure period. | **ECx** is the concentration of the test substance dissolved in water that results in a x per cent reduction in reproduction of *Daphnia* within a stated exposure period. |
| **Intrinsic rate of increase** is a measure of population growth which integrates reproductive output and age-specific mortality (1) (2) (3). In steady state populations it will be zero. For growing populations it will be positive and for shrinking populations it will be negative. Clearly the latter is not sustainable and ultimately will lead to extinction. | **Intrinsic rate of population increase** is a measure of population growth which integrates reproductive output and age-specific mortality (1) (2) (3). In steady state populations it will be zero. For growing populations it will be positive and for shrinking populations it will be negative. Clearly the latter is not sustainable and ultimately will lead to extinction. |
| **Limit of detection** is the lowest concentration that can be detected but not quantified. | **Limit of detection** is the lowest concentration that can be detected but not quantified. |
| **Limit of determination** is the lowest concentration that can be measured quantitatively. | **Limit of determination** is the lowest concentration that can be measured quantitatively. |
| **~~Mortality.~~** ~~An animal is recorded as dead when it is immobile, i.e. when it is not able to swim, or if there is no observed movement of appendages or postabdomen, within 15 seconds after gentle agitation of the test container. (If another definition is used, this must be reported together with its reference).~~ |  |

※ANNEX2以降は省略