Adopted:
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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Daphnia magna Reproduction Test

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 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 second version (1998) of the Test Guideline are:

(a) the recommended species to be used is *Daphnia magna*;
(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);
(d) more specific recommendations have been made with regard to test medium and feeding conditions.

The main differences between the second version (1998) and this version are:

(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 EC_x (e.g. EC_{10} or EC_{50}) and for NOEC/LOEC approach.

(h) A limit test is introduced.

3. Definitions used are given in Annex 1.

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 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 offspring 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 EC_x 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. EC_{10}) 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 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

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, P_{ow} and results of a test for ready biodegradability (see Test Guidelines 301 and 310).

VALIDITY OF THE TEST

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 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

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.

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);
- adequate apparatus for temperature control;
- pH-meter;
- 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);
- adequate apparatus for the control of the lighting regime and measurement of light intensity.

Test Organism

11. The species to be used in the test is *Daphnia magna* Straus [1].

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 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-

(1) Other 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.

test acclimation period of normally about 3 weeks (i.e. one generation) to avoid stressing the parent animals.

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), 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 (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 (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 $CaCO_3$) is recommended. Tests at this level and above have demonstrated reproductive performance in compliance with the validity criteria (11) (12).

Test solutions

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. 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

Conditions of Exposure

Duration

20. The test duration is 21 days.

Loading

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.

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.

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.

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). 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, *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) 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 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 re-suspension in *Daphnia* culture medium.

Light

30. 16 hours light at an intensity not exceeding 15-20 $\mu E \bullet m^{-2} \bullet s^{-1}$ 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

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

32. The test vessels should not be aerated during the test.

Test design

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

35. Normally there should be at least five test concentrations, bracketing effective concentration (e.g. EC_x), 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:

(i) When EC_x for effects on reproduction is estimated, it is advisable that sufficient concentrations are used to define the EC_x with an appropriate level of confidence. Test concentrations used should preferably bracket the estimated EC_x such that EC_x 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.

(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.

(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.

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

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.

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.

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

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 paragraph 44), additional observations may be required.

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 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

43. Mortality among the parent animals should be recorded preferably daily, or at least as frequently as offspring are counted.

Other parameters

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

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.

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.

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 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

Treatment of results

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 accidentally[2] or inadvertently[3] 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

[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

assigned as an effect of the test substance and the replicates should not be excluded from the analysis of the test result.

52. In summary, when LOEC and NOEC or EC_x 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 EC_x 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.

EC_x

55. EC_x-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 EC_{10}, EC_{50} or any other EC_x, 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 $p \leq 0.05$.

57. Normal distribution and variance homogeneity can be tested using an appropriate statistical test, e.g. the Shapiro-Wilk test and Levene test, respectively ($p \leq 0.05$). 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 ($p \leq 0.05$) 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 test such 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

60. The test report includes the following:

Test substance:

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

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.

Test conditions:

- test procedure used (e.g. semi-static or flow-through, volume, loading in number of *Daphnia* per litre);
- photoperiod and light intensity;
- test design (e.g. number of replicates, number of parents per replicate);
- 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;
- 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 should be given, when used).

Results:

- 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;
- 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 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 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 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
- 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 EC_x 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;
- an explanation for any deviation from the Test Guideline.

LITERATURE

(1) OECD Test Guidelines Programme. Report of the Workshop on the *Daphnia magna* Pilot Ring Test, Sheffield University, U.K., 20-21 March 1993.

(2) OECD (1997). Report of the Final Ring Test of the *Daphnia magna* Reproduction Test. Environmental Health and Safety Publications, Series on Testing and Assessment No.6. OECD, Paris.

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ANNEX 1

DEFINITIONS

For the purposes of this Guideline the following definitions are used:

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.

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 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.

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 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 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 should be reported together with its reference).

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ANNEX 2

PREPARATION OF FULLY DEFINED ELENDT M7 AND M4 MEDIA

Acclimation to Elendt M7 and M4 media

Some laboratories have experienced difficulty in directly transferring *Daphnia* to M4 (1) and M7 media. However, some success has been achieved with gradual acclimation, i.e. moving from own medium to 30% Elendt, then to 60% Elendt and then to 100% Elendt. The acclimation periods may need to be as long as one month.

Preparation

Trace elements

Separate stock solutions (I) of individual trace elements are first prepared in water of suitable purity, e.g. deionised, distilled or reverse osmosis. From these different stock solutions (I) a second single stock solution (II) is prepared, which contains all trace elements (combined solution), i.e:

Stock solution(s) I (single substance)	Amount added to water mg/l	Concentration (related to medium M4)	To prepare the combined stock-solution II add the following amount of stock solution I to water mL/L	
			M 4	M 7
H_3BO_3	57 190	20 000-fold	1.0	0.25
$MnCl_2$•4 H_2O	7 210	20 000-fold	1.0	0.25
LiCl	6 120	20 000-fold	1.0	0.25
RbCl	1 420	20 000-fold	1.0	0.25
$SrCl_2$•6 H_2O	3 040	20 000-fold	1.0	0.25
NaBr	320	20 000-fold	1.0	0.25
Mo Na_2O_4•2 H_2O	1 260	20 000-fold	1.0	0.25
$CuCl_2$•2 H_2O	335	20 000-fold	1.0	0.25
$ZnCl_2$	260	20 000-fold	1.0	1.0
$CoCl_2$•6 H_2O	200	20 000-fold	1.0	1.0
KI	65	20 000-fold	1.0	1.0
Na_2SeO_3	43.8	20 000-fold	1.0	1.0
NH_4VO_3	11.5	20 000-fold	1.0	1.0
Na_2EDTA•2 H_2O	5 000	2 000-fold	-	-
$FeSO_4$•7 H_2O	1 991	2 000-fold	-	-
Both Na_2EDTA and $FeSO_4$ solutions are prepared singly, poured together and autoclaved immediately. This gives:				
Fe-EDTA solution		1 000-fold	20.0	5.0

M4 and M7 media

M4 and M7 media are prepared using stock solution II, the macro-nutrients and vitamins as follows:

	Amount added to water mg/l	Concentration (related to medium M4)	Amount of stock solution added to prepare medium mL/L	
			M 4	M 7
Stock solution II (combined trace elements)		20-fold	50	50
Macro nutrient stock solutions (single substance)				
$CaCl_2 \cdot 2 H_2O$	293 800	1 000-fold	1.0	1.0
$MgSO_4 \cdot 7 H_2O$	246 600	2 000-fold	0.5	0.5
KCl	58 000	10 000-fold	0.1	0.1
$NaHCO^3$	64 800	1 000-fold	1.0	1.0
$Na_2SiO_3 \cdot 9 H_2O$	50 000	5 000-fold	0.2	0.2
$NaNO_3$	2 740	10 000-fold	0.1	0.1
KH_2PO_4	1 430	10 000-fold	0.1	0.1
K_2HPO_4	1 840	10 000-fold	0.1	0.1
Combined Vitamin stock	-	10 000-fold	0.1	0.1
The combined vitamin stock solution is prepared by adding the 3 vitamins to 1 litre water, as shown below:				
	mg/l			
Thiamine hydrochloride	750	10 000-fold		
Cyanocobalamine (B_{12})	10	10 000-fold		
Biotine	7.5	10 000-fold		

The combined vitamin stock is stored frozen in small aliquots. Vitamins are added to the media shortly before use.

N.B: To avoid precipitation of salts when preparing the complete media, add the aliquots of stock solutions to about 500 - 800 mL deionized water and then fill it up to 1 litre.

N.N.B. The first publication of the M4 medium can be found in Elendt, B.P. (1990). Selenium deficiency in crustacea; an ultrastructural approach to antennal damage in *Daphnia magna* Straus. Protoplasma, 154, 25-33.

ANNEX 3

TOTAL ORGANIC CARBON (TOC) ANALYSIS AND PRODUCTION OF A NOMOGRAPH FOR TOC CONTENT OF ALGAL FEED

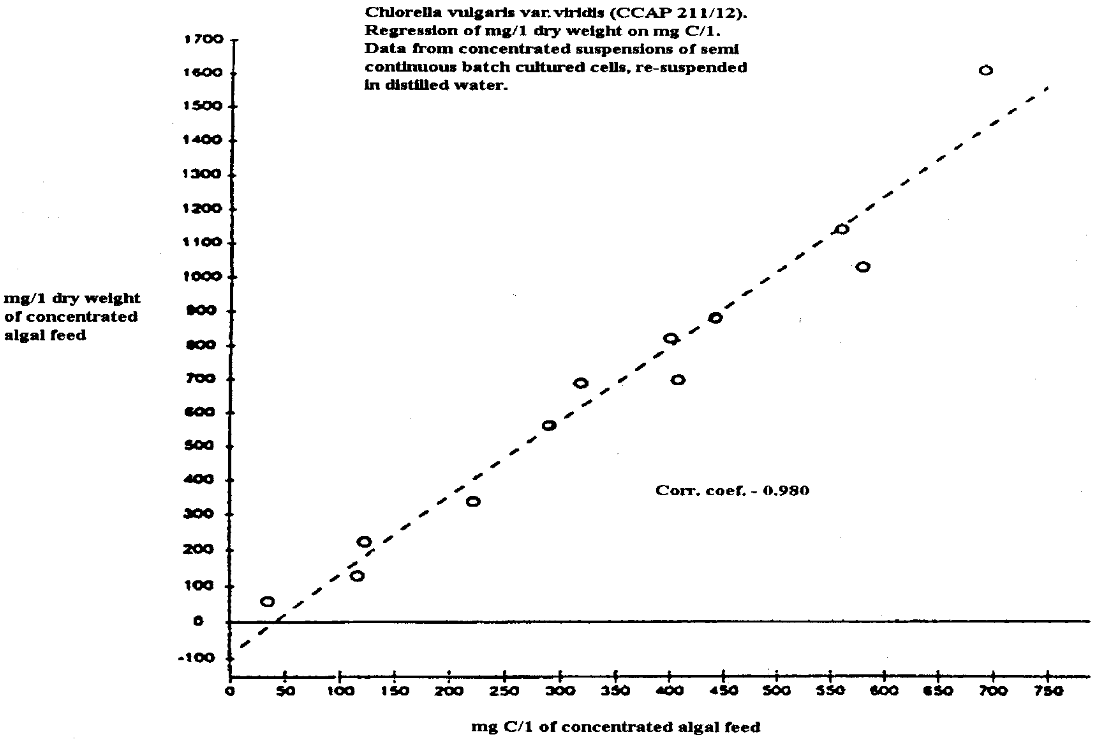
It is recognised that the carbon content of the algal feed will not normally be measured directly but from correlations (i.e. nomographs) with surrogate measures such as algal cell number or light absorbance).

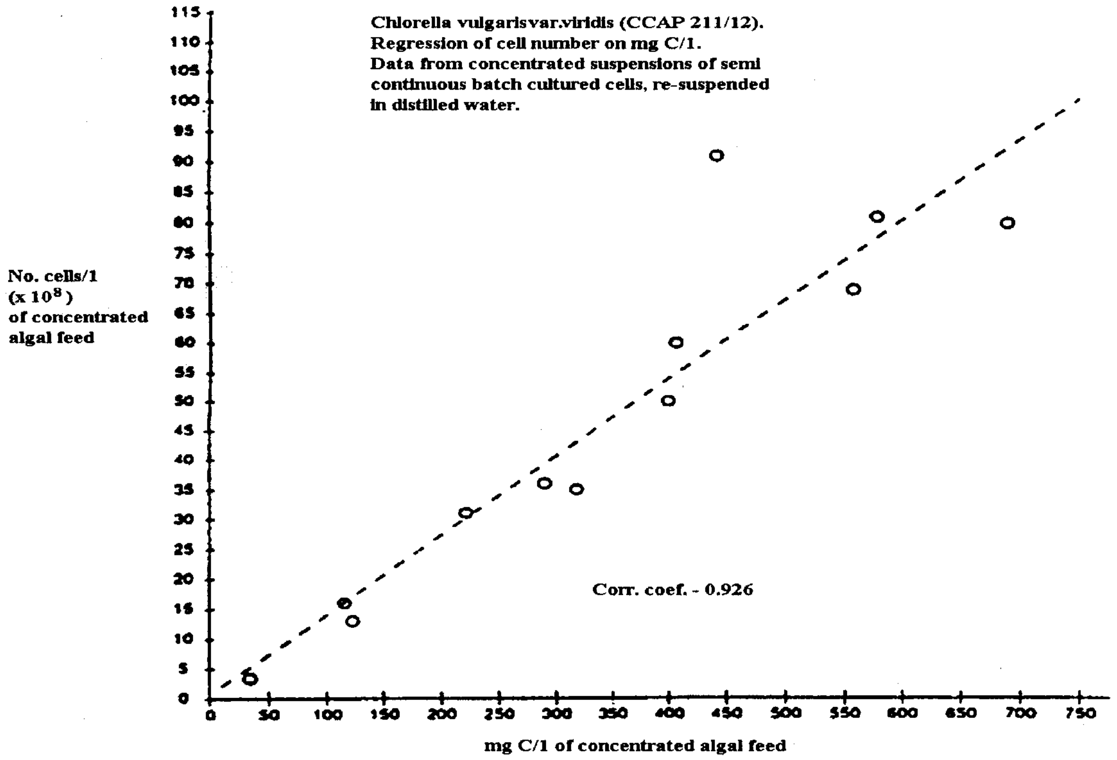
TOC should be measured by high temperature oxidation rather than by UV or persulphate methods. (For advice see: The Instrumental Determination of Total Organic Carbon, Total Oxygen Demand and Related Determinands 1979, HMSO 1980; 49 High Holborn, London WC1V 6HB).

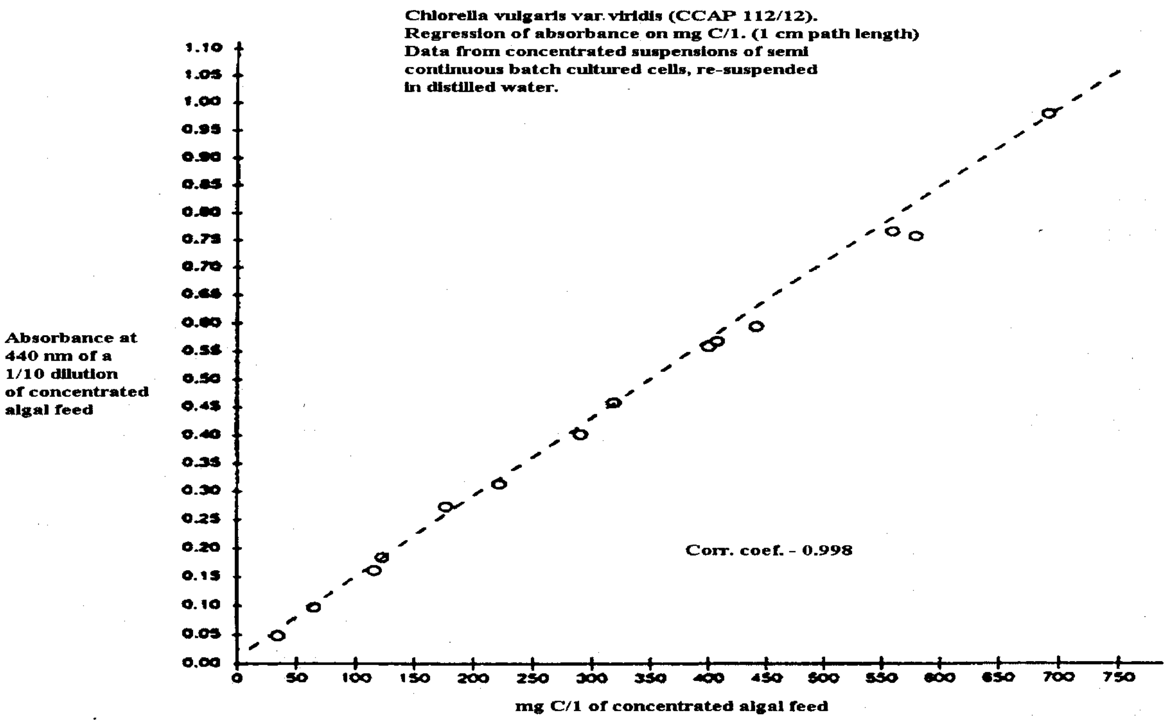
For nomograph production, algae should be separated from the growth medium by centrifugation followed by resuspension in distilled water. Measure the surrogate parameter and TOC concentration in each sample in triplicate. Distilled water blanks should be analysed and the TOC concentration deducted from that of the algal sample TOC concentration.

Nomographs should be linear over the required range of carbon concentrations. Examples are shown below.

N.B. THESE SHOULD NOT BE USED FOR CONVERSIONS; IT IS ESSENTIAL THAT LABORATORIES PREPARE THEIR OWN NOMOGRAPHS.




Chlorella vulgarisvar.viridis (CCAP 211/12).
Regression of cell number on mg C/1.
Data from concentrated suspensions of semi
continuous batch cultured cells, re-suspended
in distilled water.
No. cells/1
(x 10⁸)
of concentrated
algal feed
Corr. coef. - 0.926
mg C/1 of concentrated algal feed
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115
0 50 100 150 200 250 300 350 400 450 500 550 600 650 700 750


Chlorella vulgaris var. viridis (CCAP 112/12).
Regression of absorbance on mg C/1. (1 cm path length)
Data from concentrated suspensions of semi
continuous batch cultured cells, re-suspended
in distilled water.
Absorbance at
440 nm of a
1/10 dilution
of concentrated
algal feed
Corr. coef. - 0.998
mg C/1 of concentrated algal feed
0.00 0.05 0.10 0.15 0.20 0.25 0.30 0.35 0.40 0.45 0.50 0.55 0.60 0.65 0.70 0.75 0.80 0.85 0.90 0.95 1.00 1.05 1.10
0 50 100 150 200 250 300 350 400 450 500 550 600 650 700 750

ANNEX 4
EXAMPLE DATA SHEET FOR RECORDING MEDIUM RENEWAL, PHYSICAL/CHEMICAL MONITORING DATA, FEEDING, DAPHNIA REPRODUCTION AND PARENT MORTALITY

Experiment No: Date started: Clone: Medium: Type of food: Test Substance: Nominal conc:

Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
Medium renewal (tick)																								
pH*																							new	
																							old	
O_2 (mg/l)*																							new	
																							old	
Temp (°C)*																							new	
																							old	
Food provided (tick)																								
No. live offspring**																								Total
Vessel 1																								
2																								
3																								
4																								
5																								
6																								
7																								
8																								
9																								
10																								
Cumulative parent mortality***																							Total	

* Indicate which vessel was used for the experiment

** Record aborted broods as 'AB' in relevant box

*** Record mortality of any parentalanimals as 'M' in relevant box

ANNEX 5

EXAMPLE DATA SHEET FOR RECORDING RESULTS OF CHEMICAL ANALYSIS

(a) Measured concentrations

Nominal conc.	Week 1 sample		Week 2 sample		Week 3 sample	
	Fresh	Old	Fresh	Old	Fresh	Old

(b) Measured concentrations as a percentage of nominal

Nominal conc.	Week 1 sample		Week 2 sample		Week 3 sample	
	Fresh	Old	Fresh	Old	Fresh	Old

ANNEX 6

CALCULATION OF A TIME-WEIGHTED MEAN

Time-weighted mean

Given that the concentration of the test substance can decline over the period between medium renewals, it is necessary to consider what concentration should be chosen as representative of the range of concentrations experienced by the parent *Daphnia*. The selection should be based on biological considerations as well as statistical ones. For example, if reproduction is thought to be affected mostly by the peak concentration experienced, then the maximum concentration should be used. However, if the accumulated or longer term effect of the toxic substance is considered to be more important, then an average concentration is more relevant. In this case, an appropriate average to use is the time-weighted mean concentration, since this takes account of the variation in instantaneous concentration over time.

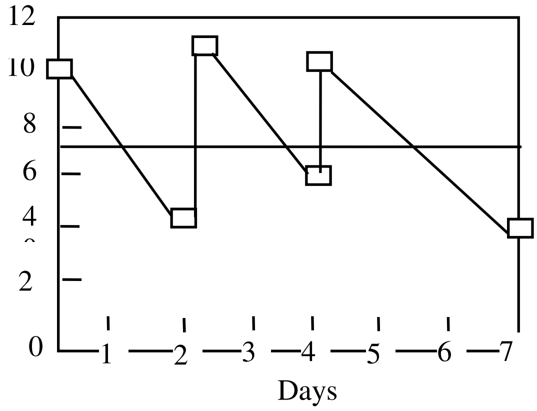


Figure 1: Example of time-weighted mean

Figure 1 shows an example of a (simplified) test lasting seven days with medium renewal at Days 0, 2 and 4.

- The thin zig-zag line represents the concentration at any point in time. The fall in concentration is assumed to follow an exponential decay process.
- The 6 plotted points represent the observed concentrations measured at the start and end of each renewal period.
- The thick solid line indicates the position of the time-weighted mean.

The time-weighted mean is calculated so that the area under the time-weighted mean is equal to the area under the concentration curve. The calculation for the above example is illustrated in Table 1.

Table 1: Calculation of Time-weighted mean

Renewal No.	Days	Conc 0	Conc 1	Ln(Conc 0)	Ln(Conc 1)	Area
1	2	10.000	4.493	2.303	1.503	13.767
2	2	11.000	6.037	2.398	1.798	16.544
3	3	10.000	4.066	2.303	1.403	19.781
Total Days:	7				Total Area:	50.092
					TW Mean:	7.156

Days is the number of days in the renewal period
Conc 0 is the measured concentration at the start of each renewal period
Conc 1 is the measured concentration at the end of each renewal period
Ln(Conc 0) is the natural logarithm of Conc 0
Ln(Conc 1) is the natural logarithm of Conc 1

Area is the area under the exponential curve for each renewal period. It is calculated by:

$$Area = \frac{Conc\ 0 - Conc\ 1}{Ln(Conc\ 0) - Ln(Conc\ 1)} \times Days$$

The time-weighted mean *(TW Mean)* is the *Total Area* divided by the *Total Days.*

Of course, for the *Daphnia* reproduction test the table should be extended to cover 21 days.

It is clear that when observations are taken only at the start and end of each renewal period, it is not possible to confirm that the decay process in, in fact, exponential. A different curve would result in a different calculation for *Area*. However, an exponential decay process is not implausible and is probably the best curve to use in the absence of other information.

However, a word of caution is required if the chemical analysis fails to find any substance at the end of the renewal period. Unless it is possible to estimate how quickly the substance disappeared from the solution, it is impossible to obtain a realistic area under the curve, and hence it is impossible to obtain a reasonable time-weighted mean.

ANNEX 7

GUIDANCE FOR THE IDENTIFICATION OF NEONATE SEX

Production of male neonates can occur under changing environmental conditions, such as shortening photoperiod, temperature, decreasing food concentration, and increasing population density (Hobaek and Larson, 1990; Kleiven et al., 1992). Male production is also a known response to certain insect growth regulators (Oda et al., 2005). Under conditions where chemical stressors are inducing a decrease in reproductive offspring from the parthenogenic females, an increased number of males would be expected (OECD, 2008). On the basis of available information, it is not possible to predict which of the sex ratio or of the reproduction endpoint will be more sensitive; however, there are indications (reference "validation report", part 1) this increase in the number of males might be less sensitive than the decrease in offspring. Since the primary purpose of the Test Guideline is to assess the number of offspring produced, the appearance of males is an optional observation. If this optional endpoint is evaluated in a study, then an additional test validity criterion of no more than 5% males in the controls should be employed.

The most practical and easy way to differentiate sex of Daphnia is to use their phenotypic characteristics, as males and females are genetically identical and their sex is environmentally determined. Males and females are different in the length and morphology of the first antennae, which are longer in males than females (Fig. 1). This difference is recognizable right after birth, although other secondary sex characteristics develop as they grow up (e.g., see Fig. 2 in Olmstead and LeBlanc, 2000).

To observe the morphological sex, neonates produced by each test animal should be transferred by pipet and placed into a petri dish with test medium. The medium is kept to a minimum to restrain movement of the animals. Observation of the first antennae can be conducted under a stereomicroscope (×10-60).

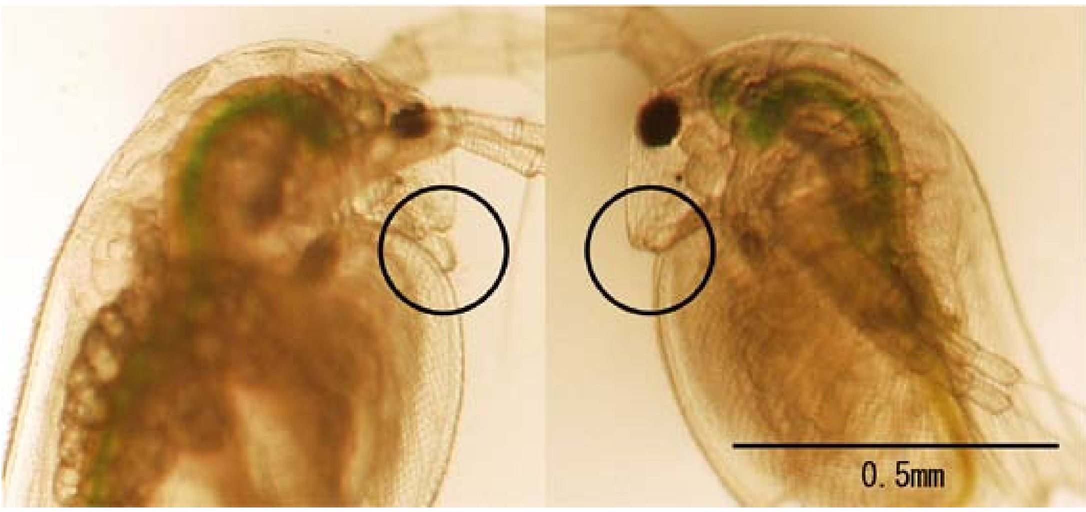


Fig. 1 24-hour-old male (left) and female (right) of *D. magna*. Males can be distinguished from females by the length and morphology of the first antennae as shown in the circles (Tatarazako et al., 2004).

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