Adopted:
3 October 2008

OECD GUIDELINES 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, 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).

2. The main differences between the initial version (1984) and the second version (1998) of the Guideline are:

(a) the 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 difference between the second version (1998) and this version is:

(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.

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 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 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 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. EC_x (e.g. EC_{50}, EC_{20} or EC_{10}).

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.

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.

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 Guideline 301).

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 live offspring produced per parent animal surviving at the end of the test is $\geq$ 60.

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) (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.

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.

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.

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).

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.

(1) Other *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.

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 (9) (10).

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.

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.

PROCEDURE

Conditions of Exposure

Duration

20. The test duration is 21 days.

Loading

21. Parent animals are maintained individually, one per test vessel, with 50 - 100 ml of medium in each vessel.

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.

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 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'.

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). 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.

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).

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.

Light

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

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). It may be appropriate to use an additional test vessel for the purposes of temperature monitoring.

Aeration

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

Test concentrations

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.

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. 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) 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) If 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**.** If the EC_{50} for effects on reproduction is estimated, it is advisable that the highest test concentration is greater than this EC_{50}. Otherwise, although it will still be possible to estimate the EC_{50}, the confidence interval for the EC_{50} will be very wide and it may not be possible to satisfactorily assess the adequacy of the fitted model.

(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.

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 paragraphs 5 and 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 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.

Mortality

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

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).

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 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).

DATA AND REPORTING

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.

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 EC_{50}), 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 EC_{50} and its standard error can be estimated directly. This would greatly ease the calculation of the confidence limits about the

EC_{50}. 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:

$$Y = \frac{c}{1 + \left(\frac{x}{x_0}\right)^b}$$

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
x_0 = the EC_{50} 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 EC_{10} or EC_{20} can also be estimated, although it may be preferable to use a different parameterisation of the model from that used to estimate the EC_{50}.

Test report

57. The test report must include 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 must 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 living offspring 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 fecundity (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;
- 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 EC_x 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;
- 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

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ANNEX 1

DEFINITIONS

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

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.

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.

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.

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.

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 must 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.a.:

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
Na_2MoO_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:				
21 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)				
$CaCl_2$•2 H_2O	293 800	1 000-fold	1.0	1.0
$MgSO_4$•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•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

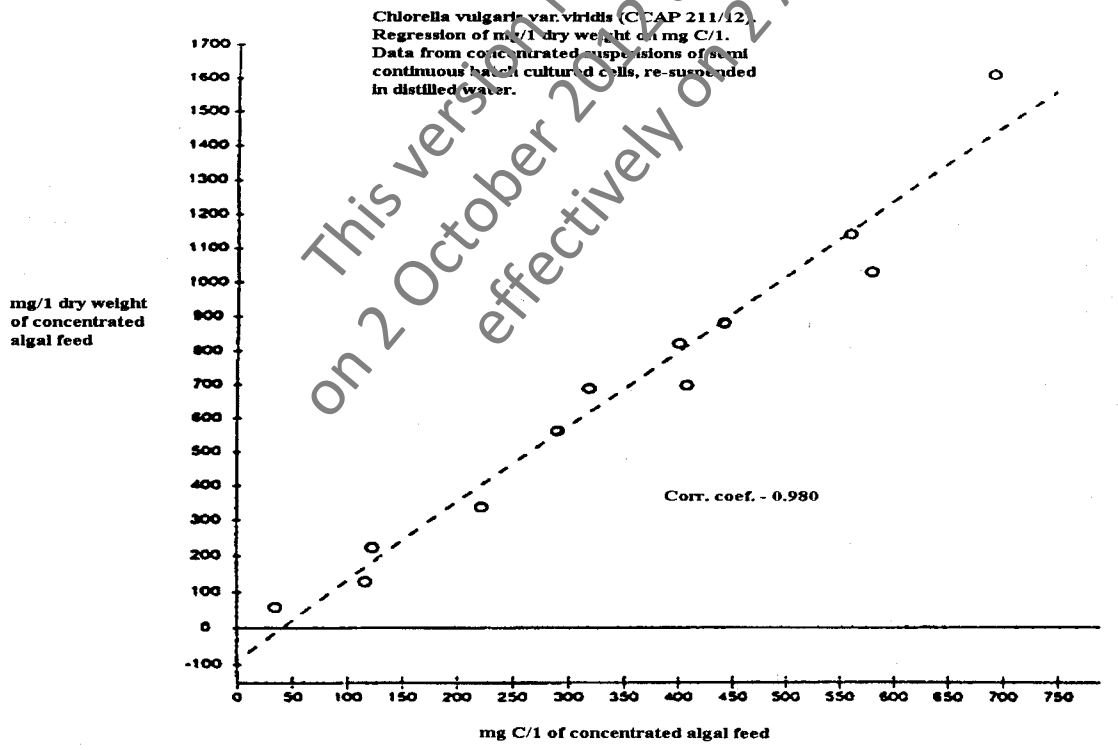
It is recognised that the carbon content of the algal feed will not normally be measured directly but from correlations (i.e. nomographs) with surrogate measures such as algal cell number or light absorbance).

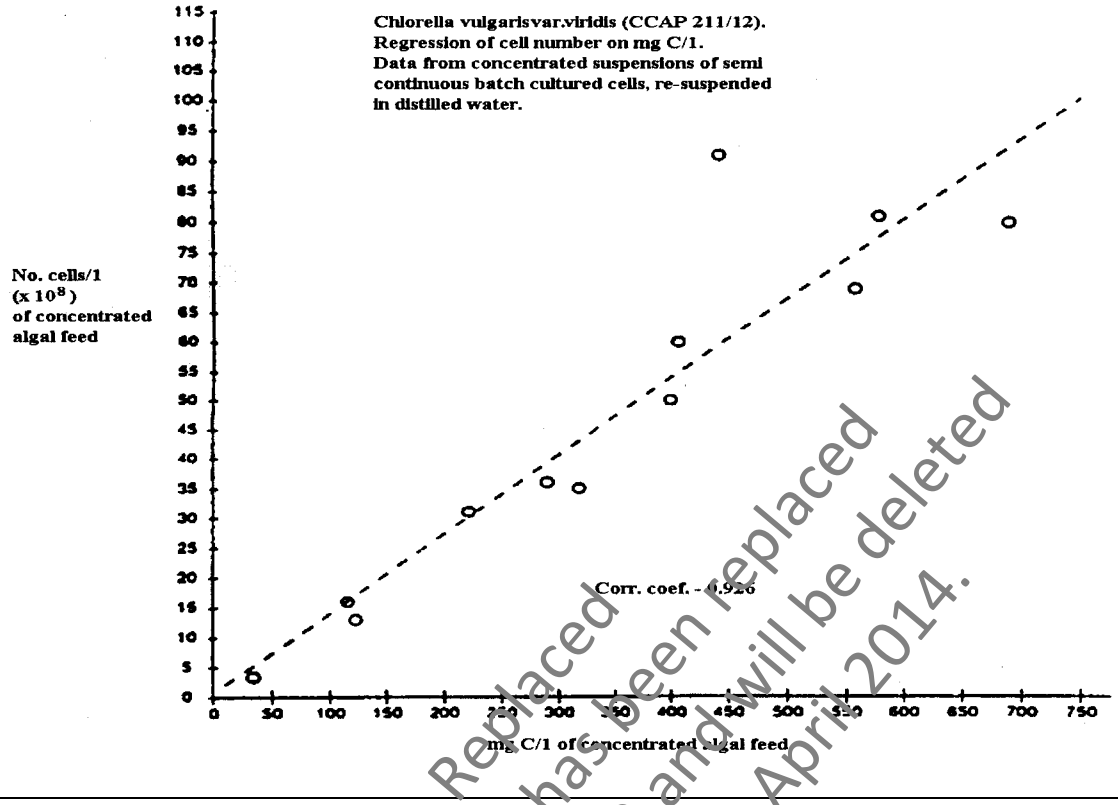
TOC should be measured by high temperature oxidation rather than by UV or persulphate methods. (For advice see: The Instrumental Determination of Total Organic Carbon, Total Oxygen Demand and Related Determinands 1979, HMSO 1980; 49 High Holborn, London WC1V 6HB).

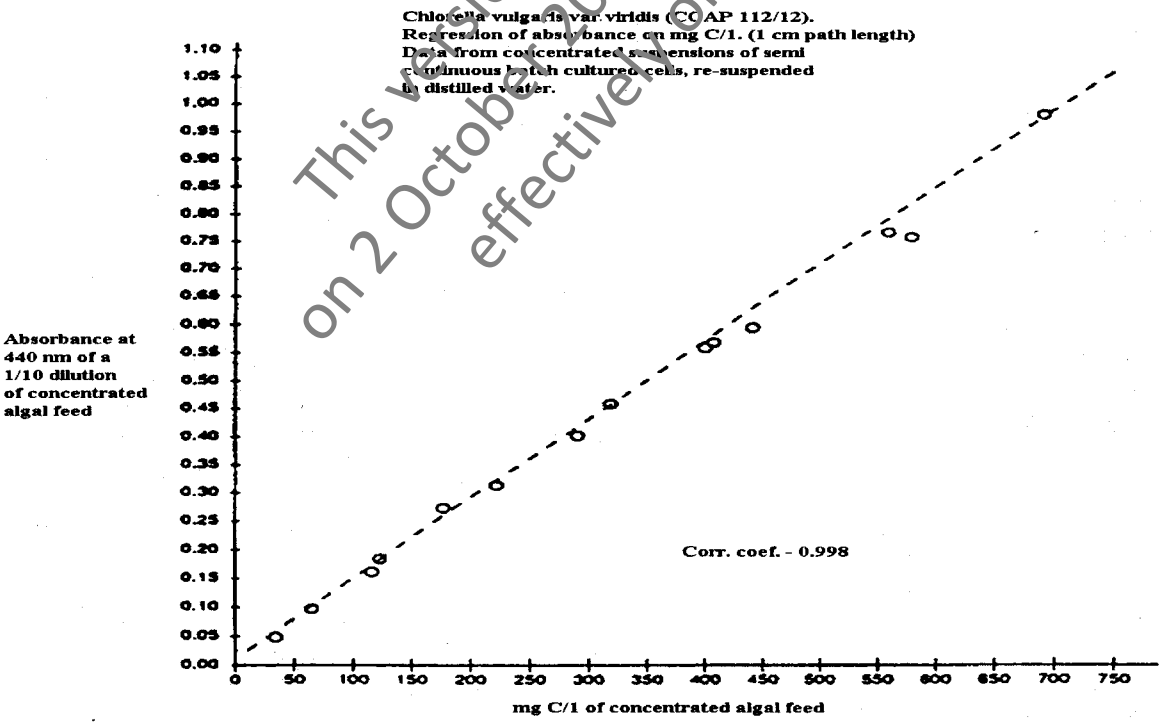
For nomograph production, algae should be separated from the growth medium by centrifugation followed by resuspension in distilled water. Measure the surrogate parameter and TOC concentration in each sample in triplicate. Distilled water blanks should be analysed and the TOC concentration deducted from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







ANNEX 4: EXAMPLE DATA SHEET FOR RECORDING MEDIUM RENEWAL, PHYSICAL/CHEMICAL MONITORING DATA, FEEDING, DAPHNIA REPRODUCTION AND ADULT 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																								
																							Total	
Cumulative adult mortality***																								

* Indicate which vessel was used for the experiment ** Record aborted broods as 'AB' in relevant box *** Record mortality of any adult animals 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.

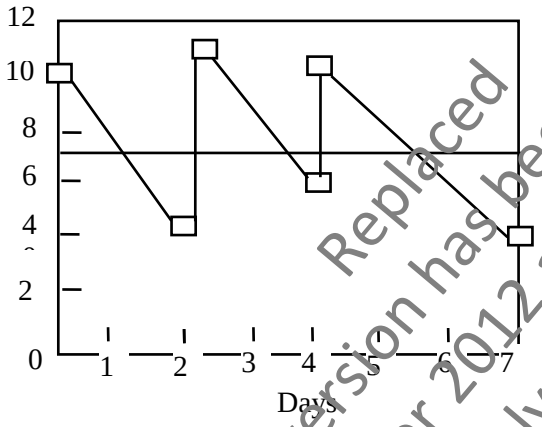


Figure 1: Example of time-weighted mean

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 would have to 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 is, 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).

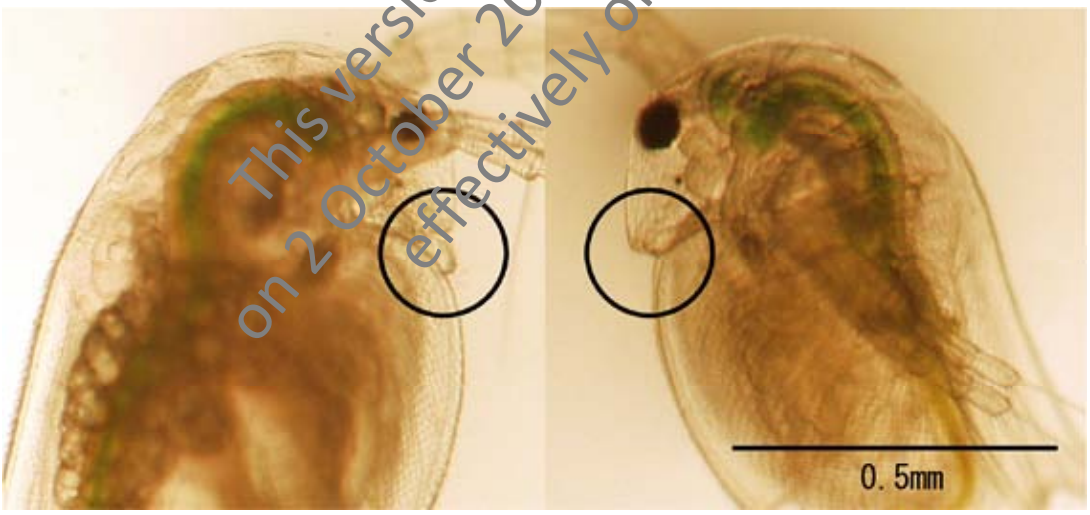


Fig. 1 24-hour-old male (left) and female (right) of *D. magna*. Males can be distinguished from females by the length and morphology of the first antennae as shown in the circles (Tatarazako et al., 2004).

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