DRAFT GUIDELINE 221
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OECD GUIDELINES FOR THE TESTING OF CHEMICALS

REVISED PROPOSAL FOR A NEW GUIDELINE 221

Lemna sp. Growth Inhibition Test

INTRODUCTION

1. This test Guideline is designed to assess the toxicity of substances to freshwater aquatic plants of the genus *Lemna* (duckweed). It is based on existing guidelines (1)(2)(3)(4)(5)(6) but includes modifications of those methods to reflect recent research and consultation on a number of key issues. The proposed method has been validated by international ring-test (7).

2. This Guideline describes toxicity testing using *Lemna gibba* and *Lemna minor*, both of which have been extensively studied and are the subject of the standards referred to above. The taxonomy of *Lemna* spp. is difficult, being complicated by the existence of a wide range of phenotypes. Although genetic variability in the response to toxins can occur with *Lemna*, there are currently insufficient data on this source of variability to recommend a specific clone for use with this Guideline. Short descriptions of duckweed species that have been used for toxicity testing are given in Annex 1. It should be noted that the test is not conducted axenically but steps are taken at stages during the test procedure to keep contamination by other organisms to a minimum.

3. Details of testing with renewal (semi-static and flow-through) and without renewal (static) of the test solution are described. Depending on the objectives of the test and the regulatory requirements, it is recommended to consider the application of semi-static and flow through methods, e.g. for substances that are rapidly lost from solution as a result of volatilisation, photodegradation, precipitation or biodegradation. Further guidance is given in the Guidance Document on Aquatic Toxicity Testing of Difficult Substance and Mixtures (8).

4. Reference substance(s) may be tested as means of checking the test procedure. Toxicants used in the international ring-test are recommended for this purpose (7). 3,5-dichlorophenol showed the best result in the ring-test, and therefore is recommended. It is advisable to test a reference substance at least twice a year or, where testing is carried out at a lower frequency, in parallel to the determination of the toxicity of a test substance.

5. Definitions used are given in Annex 2.

PRINCIPLE OF THE TEST

6. Plants of the genus *Lemna* are allowed to grow as monocultures in different concentrations of the test substance over a period of seven days. The objective of the test is to quantify substance-related effects on vegetative growth over this period based on assessments of frond number, and also on assessments of

biomass (total frond area, dry weight or fresh weight). To quantify substance-related effects, growth in the test solutions is compared with that of the controls and the concentration bringing about a specified x % inhibition of growth (e.g. 50 %) is determined and expressed as the EC_x (e.g. EC_{50}). In addition, the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) may be statistically determined.

INFORMATION ON THE TEST SUBSTANCE

7. An analytical method, with adequate sensitivity for quantification of the substance in the test medium, should be available.

8. Information on the test substance which may be useful in establishing the test conditions includes the structural formula, purity, water solubility, stability in water and light, pK_a , K_{ow}, vapour pressure and biodegradability. Water solubility and vapour pressure can be used to calculate Henry´s Law constant, which will indicate if significant losses of the test substance during the test period are likely. This will help indicate whether particular steps to control such losses should be taken. Where information on the solubility and stability of the test substance is uncertain, it is recommended that these be assessed under the conditions of the test, i.e. growth medium, temperature, lighting regime to be used in the test.

9. When pH control of the test medium is particularly important, e.g. when testing metals or substances which are hydrolytically unstable, the addition of a buffer to the growth medium is recommended (see paragraph 18). Further guidance for testing substances with physical-chemical properties that make them difficult to test is provided in (8).

VALIDITY OF THE TEST

10. For the test to be valid, the doubling time of frond number in the control must be less than 2.5 days (60 h), corresponding to approximately an seven-fold increase in seven days. Using the media and test conditions described in this Guideline, this criterion can be attained using a static test regime (5). It is also anticipated that this criterion will be achievable under semi-static and flow-through test conditions. Calculation of the doubling time is shown in paragraph 46.

DESCRIPTION OF THE METHOD

Apparatus

11. All equipment in contact with the test media should be made of glass or other chemically inert material. Glassware used for culturing and testing purposes should be cleaned of chemical contaminants that might leach into the test medium and sterile. The test vessels should be wide enough for the fronds of different colonies in the control vessels to grow without overlapping at the end of the test. It does not matter if the roots touch the bottoms of the test vessels, but a minimum depth of 20 mm and minimum volume of 100 ml in each test vessel is advised. The choice of test vessels is not critical as long as these requirements are met. Erlenmeyer flasks, crystallising dishes or glass petri dishes of appropriate dimensions have all proved suitable. Test vessels must be covered to minimise evaporation and accidental contamination. Suitable test vessels, and particularly covers, must avoid shadowing or changes in the spectral characteristics of light.

12. The cultures and test vessels should not be kept together. This is best achieved using separate environmental growth chambers, incubators, or rooms. Illumination and temperature must be controllable and maintained at a constant level (see paragraphs 33-34).

Test organism

13. The organism used for this test is either *Lemna gibba* or *Lemna minor*. Plant material may be obtained from a culture collection, another laboratory or from the field. If collected from the field, plants should be maintained in culture in the same medium as used for testing for a minimum of eight weeks prior to use. Field sites used for collecting starting cultures must be free of obvious sources of contamination. If obtained from another laboratory or a culture collection they should be similarly maintained for a minimum of three weeks. The source of plant material and the species and clone (if known) used for testing should always be reported.

14. Monocultures, that are visibly free from contamination by other organisms such as algae and protozoa, should be used. Healthy plants of *L. minor* will consist of colonies comprising between two and five fronds whilst healthy colonies of *L. gibba* may contain up to seven fronds. Throughout this Guideline the number of fronds is used to denote the quantity of plant material.

15. The quality and uniformity of the plants used for the test will have a significant influence on the outcome of the test and should therefore be selected with care. Young, rapidly growing plants without visible lesions or discoloration (chlorosis) should be used. Good quality cultures are indicated by a high incidence of colonies comprising at least two fronds. A large number of single fronds is indicative of environmental stress, e.g. nutrient limitation, and plant material from such cultures should not be used for testing.

Cultivation

16. To reduce the frequency of culture maintenance (e.g. when no *Lemna* tests are planned for a period), cultures can be held under reduced illumination and temperature (4 - 10°C). Details of culturing are given in Annex 3. Obvious signs of contamination by algae or other organisms will require surface sterilisation of a sub-sample of *Lemna* fronds, followed by transfer to fresh medium (see Annex 3). In this eventuality the remaining contaminated culture should be discarded.

17. At least seven days before testing, sufficient colonies are transferred aseptically into fresh sterile medium and cultured for 7 - 10 days under the conditions of the test.

Water

18. Different media are recommended for *Lemna minor* and *Lemna gibba*, as described below. Careful consideration should be given to the inclusion of a pH buffer in the test medium (MOPS (4-morpholinepropane sulphonic acid, CAS No: 1132-61-2) in *L. minor* medium and $NaHCO_3$ in *L. gibba* medium) when it is suspected that it might react with the test substance and influence the expression of its toxicity. Steinberg Medium (9) is also acceptable as long as the validity criteria are met.

Growth medium for *Lemna minor*

19. A modification of the Swedish standard (SIS) *Lemna* growth medium is recommended for culturing and testing with *L. minor*. The composition of the nutrient stock solutions used to prepare this medium is given in Annex 4.

20. To prepare one litre of SIS medium, the following are added to 900 ml of deionised water:

- 10 ml of stock solution I
- 5 ml of stock solution II
- 5 ml of stock solution III
- 5 ml of stock solution IV
- 1 ml of stock solution V
- 5 ml of stock solution VI
- 1 ml of stock solution VII (optional)

Note: *A further stock solution VII (MOPS buffer) may be needed for certain test substances (see paragraph 9).*

21. The pH is adjusted to 6.5 ± 0.2 with either 0.1 or 1 M HCl or NaOH, and the volume adjusted to one litre with deionised water.

Growth medium for *Lemna gibba*

22. The growth medium, 20X - AAP, is recommended for culturing and testing with *L. gibba*. Five nutrient stock solutions (A1, A2, A3, B and C) are prepared for 20X - AAP medium, as indicated in Annex 4, using reagent-grade chemicals. 20 ml of each nutrient stock solution is added to approximately 850 ml deionised water to produce the culture medium. The pH is adjusted to 7.5 ± 0.1 with either 0.1 or 1 M HCl or NaOH, and the volume adjusted to one litre with deionised water. The medium is then filtered through a 0.2 μm (approximate) membrane filter into a sterile container.

23. Growth medium intended for testing should be prepared 1-2 days before use to allow the pH to stabilise. The pH of the culture medium should be checked prior to use and readjusted if necessary by the addition of 0.1 or 1 M NaOH or HCl as described above.

Test solutions

24. Test solutions are usually prepared by dilution of a stock solution. Stock solutions of the test substance are prepared by dissolving the substance in culture medium.

25. The highest tested concentration of the test substance should not normally exceed the water solubility of the substance under the test conditions. It should be noted however that *Lemna* spp. float on the surface and may be exposed to substances that collect at the water-air interface (e.g poorly water-soluble or hydrophobic substances or surface-active substances). Under such circumstances exposure will result from material other than in solution and test concentrations may, depending on the characteristics of the test substance, exceed water solubility. For test substances of low water solubility it may be necessary to prepare a concentrated stock solution or dispersion of the substance using an organic solvent or dispersant in order to facilitate the addition of accurate quantities of the test substance to the test medium and aid in its dispersion and dissolution. Every effort should be made to avoid the use of such materials. There should be no phytotoxicity resulting from the use of auxiliary solvents or dispersants. For example, commonly used solvents which do not cause phytotoxicity at concentrations up to 100 mgl^{-1} include acetone and dimethylformamide. If a solvent or dispersant is used, its final concentration should be reported and kept to a minimum ($\leq$ 0.01%, i.e. $\leq$ 100 mgl^{-1}), and all treatments should contain the same concentration of solvent or dispersant. Further guidance on the use of dispersants is given in (8).

PROCEDURE

Test and control groups

26. Prior knowledge of the toxicity of the test substance to *Lemna*, e.g. from a range-finding test, will help in selecting suitable test concentrations. There should normally be at least five test concentrations arranged in a geometric series, with a separation factor preferably not exceeding 3.2. Justification should be provided if fewer than five concentrations are used. At least three replicates should be used at each test concentration and for the controls.

27. In setting the range of test concentrations (for range-finding and/or for the definitive toxicity test), the following should be considered:

- If determining an EC_x, test concentrations should bracket the EC_x value to ensure an appropriate level of confidence. For example, if estimating the EC_{50}, the highest test concentration should be greater than the EC_{50} value. If the EC_{50} value lies outside of the range of test concentrations, associated confidence intervals will be large and a proper assessment of the statistical fit of the model may not be possible.

- If the aim is to estimate the LOEC/NOEC, the lowest test concentration should be low enough so that growth is not significantly less than that of the control. In addition, the highest test concentration should be high enough so that growth is significantly lower than that in the control. If this is not the case, the test will have to be repeated using a different concentration range (unless the highest concentration is at the limit of solubility or the maximum required limit concentration, e.g. 100 mgl^{-1}).

28. Every test should include controls consisting of the same nutrient medium, number of fronds, environmental conditions and procedures as the test vessels but without the test substance. If an auxiliary solvent or dispersant is used, an additional control treatment with the solvent/dispersant present at the same concentration as that in the vessels with the test substance should be included. The number of replicate control vessels should be the same as that used for the test concentrations.

Exposure

29. Colonies consisting of 2 to 4 visible fronds are transferred from the inoculum culture and randomly assigned to the test vessels under aseptic conditions. Each test vessel should contain a total of 9 to 12 fronds. The number of colonies and fronds should be the same in each test vessel. Experience gained with this method and ring-test data have indicated that using three replicates per treatment, with each replicate containing 9 to 12 fronds initially, is sufficient to detect differences in growth of approximately 10 to 15% (4 to 7% of inhibition calculated by growth rate) between treatments (7).

30. A randomised design for location of the test vessels in the incubator is required to minimise the influence of spatial differences in light intensity or temperature. A blocked design or random repositioning of the vessels when observations are made (or repositioning more frequently) is also required.

31. If a preliminary stability test shows that the test substance concentration cannot be maintained (i.e. the measured concentration falls below 80 % of the measured initial concentration) over the test duration (7 days), a semi-static test regime is recommended. In this case, the colonies should be exposed to freshly prepared test and control solutions on at least two occasions during the test (e.g. days 3 and 5). The frequency of exposure to fresh medium will depend on the stability of the test substance; a higher frequency will be required to maintain concentrations of highly unstable or volatile substances. In some circumstances, a flow-through procedure may be required (10).

32. For some substances, e.g. pesticides, a foliar application (spray) of the test substance directly onto the fronds may be applicable if this is considered to be the most likely exposure scenario and/or if it is required by regulation (7)(11).

Incubation conditions

33. Continuous warm or cool white fluorescent lighting should be used to provide a light intensity range of 6500-10000 lux and a photosynthetically-active radiation (400-700 nm) of 85-125 $\mu E^{-2}s^{-1}$, as measured at points the same distance from the light source as the *Lemna* fronds. Any differences in light intensity over the test area should not exceed ± 15 %. The method of light detection and measurement, in particular the type of sensor, will affect the measured value. Spherical sensors (which respond to light from all angles above and below the plane of measurement) and "cosine" sensors (which respond to light from all angles above the plane of measurement) are preferred to unidirectional sensors, and will give higher readings for a multi-point light source of the type described here.

34. The temperature in the test vessels should be 24 ± 2 °C. The pH of the control medium should not increase by more than 1.5 units during the test. However, deviation of more than 1.5 units would not unvalidate the test when it can be shown that validity criteria are met.

Duration

35. The test is terminated 7 days after the plants were transferred into the test vessels.

Observations

36. At the start of the test, frond and colony numbers in the test vessels are counted and recorded, taking care to ensure that overlapping but distinctly visible fronds are accounted for. Frond and colony numbers (normal and abnormal) and their appearance need only be determined at the beginning and end of the test when effects are to be assessed in terms of the average specific growth rate over the full duration of the test (see paragraph 47). Counts of frond and colony numbers after intermediate exposure periods will be required if average specific growth rate is to be determined at intervals during the period of the test. In this case frond numbers will need to be determined at least once every 3 days, i.e. on at least 2 occasions during the 7 day exposure period, and at test termination. Changes in plant development (e.g. frond size, appearance, necrosis, chlorosis or gibbosity, colony break-up or loss of buoyancy, root length, morphology or breakdown, should be noted. Significant features of the test medium (e.g. presence of undissolved material, growth of algae in the test vessel) should also be noted.

37. In addition to determinations of frond number during the test, effects of the test substance on final biomass are also assessed, based on one (or more) of the following parameters:

- (i) total frond area
- (ii) dry weight, or
- (iii) fresh weight, or

Total frond area is preferred followed by dry weight and then fresh weight. Dry or fresh weight should be determined at the start of the test from a sample of the inoculum culture, and at the end of the test with the plant material from each test and control vessel. Total frond area can be determined for each test and control vessel at the start, in the course, and at the end of the test.

38. Total frond area, dry weight and fresh weight may be determined as follows:

(i) Total frond area: The total frond area of colonies may be determined by image analysis. A silhouette of the test vessel and plants can be captured using a video camera (i.e. by placing the vessel on a light box) and the resulting image digitised. By calibration with flat shapes of known area, the total frond area in a test vessel may then be determined. Care should be taken to exclude interference caused by the rim of the test vessel. An alternative but more laborious approach is to photocopy test vessels and plants, cut out the resulting silhouette of colonies and determine their area using a leaf area analyser or graph paper. Other techniques (e.g. paper weight ratio between silhouette area of colonies and unit area) may also be appropriate.

(ii) Dry weight: Colonies are collected from each of the test vessels and rinsed with distilled or deionised water. They are blotted to remove excess water and then dried at 60 °C to a constant weight. Any root fragments should be included. The dry weight should be expressed to an accuracy of at least 0.1 mg.

(iii) Fresh weight: Colonies are transferred, to pre-weighed polystyrene (or other inert material) tubes with small (1 mm) holes in the rounded bottoms. The tubes are then centrifuged at 3000 rpm for 10 minutes at room temperature. Tubes, containing the now dried colonies, are re-weighed and the fresh weight is calculated by subtracting the weight of the empty tube.

Frequency of analytical determinations and measurements

39. If a static test design is used, the pH of each treatment should be measured at the beginning and end of the test. If a semi-static test design is used, the pH should be measured in each batch of 'fresh' test solution prior to each renewal and also in the corresponding 'spent' solutions.

40. Light intensity should be measured in the growth chamber, incubator or room at points the same distance from the light source as the *Lemna* fronds. Measurements should be made at least once during the test. The temperature of the medium in a surrogate vessel held under the same conditions in the growth chamber, incubator or room should be recorded at least daily.

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

42. In semi-static tests where the concentration of the test substance is not expected to remain within ± 20 % of the nominal concentration, it is necessary to analyse all freshly prepared test solutions and the same solutions at each renewal (see paragraph 31). However, for those tests where the measured initial concentration of the test substance is not within ± 20% 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 % of the initial concentration), chemical determinations may be carried out on only 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 (or the contents of the vessels pooled by replicate).

43. If a flow-through test is used, a similar sampling regime to that described for semi-static tests, including analysis at the start, mid-way through and at the end of the test, is appropriate, but measurement of 'spent' solutions is not appropriate in this case. In this type of test, the flow-rate of diluent and test substance or test substance stock solution should be checked daily.

44. If there is evidence that the concentration of the substance being tested has been satisfactorily maintained within ± 20 % of the nominal or measured initial concentration throughout the test, analysis of the results can be based on nominal or measured initial values. If the deviation from the nominal or measured initial concentration is greater than ± 20 %, analysis of the results should be based on the time-weighted mean (see Annex 5).

LIMIT TEST

45. Under some circumstances, e.g. when a preliminary test indicates that the test substance is non-toxic at concentrations up to 100 mg l^{-1} or up to its limit of solubility in the test medium (whichever is the lower), a limit test involving a comparison of responses in a control group and one treatment group (100 mg·l^{-1} or a concentration equal to the limit of solubility), may be undertaken. It is strongly recommended that this be supported by analysis of the exposure concentration. All previously described test conditions and validity criteria apply to a limit test, with the exception that the number of treatment replicates should be doubled. Growth in the control and treatment group may be analysed using a statistical test to compare means, e.g. a Student's t-test.

DATA AND REPORTING

Treatment of results

46. To determine the doubling time (T_d) of frond number and adherence to this validity criterion for the study (paragraph 10), the following formula is used with data from the control vessels:

$$T_d = \ln 2 / \mu$$

where μ is the average specific growth determined as described in paragraphs 51-52.

47. The purpose of the test is to determine the effects of the test substance on the vegetative growth of *Lemna*. This Guideline describes three approaches for determining the effects of a test substance as follows:

(a) Average specific growth rate: this is calculated on the basis of changes in frond number or frond area determined during the course of the 7 day exposure period in controls and in each treatment group. It is sometimes referred to as 'relative growth rate' (12).

(b) Final biomass: this is calculated on the basis of changes in logarithmic value for total frond area, dry weight or fresh weight in the controls and in each treatment group at the end of the test.

(c) Area under the growth curve: this is also calculated on the basis of frond number determined during the test in controls and each treatment group but integrates the logarithmic value for frond number over the exposure period.

48. It is recommended that toxicity estimates be based on average specific growth rate for frond number, but it is preferable to use the measurement of biomass (total frond area, dry weight or fresh weight) in addition, because some substances may affect the frond size without affecting the frond number. Of the three parameters for biomass, total frond area is often the most sensitive and therefore preferred.

Another advantage of frond area is that it can be measured during the test period. If frond area is not measured, dry weight is preferred over fresh weight.

49. If the doubling time criterion is met (see paragraph 10) but there is evidence that growth in the controls is not exponential, or if significant periods of lag or stagnancy are observed, or if the course of the growth curve is not monotonous, then it is preferable to base estimates of toxicity on area under the growth curve rather than average specific growth rate.

50. The number of fronds as well as any other recorded parameter of growth, i.e. frond area, dry weight or fresh weight, are tabulated together with the concentrations of the test substance for each measurement occasion. Subsequent data analysis e.g. to estimate a LOEC, NOEC or EC_x should be based on the values for the individual replicates and not calculated means for each treatment group.

Average specific growth rate

51. To determine the average specific growth rate for each test concentration and for the controls, frond numbers for each replicate in the controls and each treatment at each observation time are plotted against time as a semi-logarithmic graph to produce growth curves. The average specific growth rate for a specific period is calculated as the slope of the logarithmic growth curve from the equation:

$$\mu_{i-j} = \frac{\ln(N_j) - \ln(N_i)}{t_j - t_i}$$

where:

- μ_{i-j} : average specific growth rate from moment time i to j
- N_i : number of fronds observed in the test or control vessel at time i
- N_j : number of fronds observed in the test or control vessel at time j
- t_i : moment time for the start of the period
- t_j : moment time for the end of the period

52. If there is exponential growth in the controls (or growth is close to an exponential pattern) and if no significant periods of lag or stagnancy are observed and if the course of the growth curve is monotonous, the average specific growth rate can be derived from the slope of the regression line in a plot of ln N *versus* time.

53. Percent inhibition of growth rate (I_r) may then be calculated for each test concentration according to the following formula:

$$\% \ I_r = \frac{(\mu_C - \mu_T)}{\mu_C} \times 100$$

where:

- $\% \ I_r$: percent inhibition in average specific growth rate
- μ_C : mean value for μ in the control
- μ_T : mean value for μ in the treatment group

Final biomass

54. Effects on final biomass may be determined on the basis of either frond area, dry weight or fresh weight present in each test vessel at the start and end of the test [NB. the starting biomass is determined on the basis of a sample of fronds taken from the same batch used to inoculate the test vessels (see paragraph

17)]. The mean percent inhibition in final biomass (%I_b) may be calculated for controls and each treatment group as follows:

$$\% \, I_b = \frac{(b_c - b_T)}{b_c} \times 100$$

where:

- % I_b: percent reduction in biomass
- b_C : ln (final biomass) minus ln(starting biomass) for the control group
- b_T : ln(final biomass) minus ln(starting biomass) in the treatment group

Area under the curve

55. The area under the growth curves can be calculated for each control and treatment replicate according to the following equation:

$$A = \frac{\ln N_1 - \ln N_0}{2} t_1 + \frac{\ln N_1 + \ln N_2 - 2 \ln N_0}{2} (t_2 - t_1) + ... + \frac{\ln N_{n-1} + \ln N_n - 2 \ln N_0}{2} (t_n - t_{n-1})$$

where:

- A : area under the growth curve
- N_0 : number of fronds observed in the test or control vessel at the start of the test (t_0)
- N_1 : number of fronds observed in the test or control vessel at time t_1
- N_n : number of fronds observed in the test or control vessel at time t_n
- t_1 : time of first measurement after beginning of test
- t_n : time of the nth measurement after beginning of test

The area should be calculated for the entire test period, or a rationale for selecting only a portion of the growth curve provided. A mean area is calculated for each test concentration and control with variance estimates.

56. Percent inhibition of area under the curve, I_a, may be calculated for each test concentration according to the following formula:

$$\% \, I_a = \frac{(A_C - A_T)}{A_C} \times 100$$

where:

- A_C : mean value for area under the curve in the control group
- A_T : mean value for area under the curve in the treatment group

Concentration-growth curves

57. Concentration-growth curves relating mean percentage inhibition of the growth parameters (I_r, I_b, or I_a, calculated as shown in paragraph 53, 54 or 56) and the log concentration of the test substance should be plotted.

LOEC/NOEC determination

58. To estimate the LOEC, and hence the NOEC, ANOVA is used to calculate the mean average specific growth rate, area under the curve or final biomass and pooled residual standard deviation across replicates for each test concentration. The resulting mean for each test concentration is then compared

with the control mean (all controls pooled, including negative and solvent controls if used [1]) using an appropriate multiple comparison method e.g. Dunnett's (13)(14) or Williams' tests (15)(16). Other multiple comparison techniques may be preferred if deemed appropriate.

59. A test for normality of the data is advised e.g. by calculating the Shapiro-Wilk's statistic (17) and, if the replicate data reveal a normally distributed error structure, a test for homogeneity of variances across treatment groups is recommended e.g. using Bartlett's or Levene's test (18)(19). If the variances are not homogeneous, it may be necessary to carry out a transformation of the data prior to carrying out ANOVA. The log transformation is recommended for average specific growth rate and area under the curve, and the square root transformation for final biomass. Non-parametric analysis, e.g. Wilcoxon Rank Sum Test, may be used when the assumptions of normality and homogeneity of variances are not satisfied (20).

60. If a one-tailed test to compare means is used, rejection of the null hypothesis implies that the mean of the treatment group is less than the mean of the control group. If a two-tailed test is used, rejection of the null hypothesis implies that the mean of the treatment group could be either less (inhibition) or more (stimulation) than the mean of the control group. The type of means comparison test should therefore be described and also whether a one-tailed or two-tailed procedure was employed.

61. In addition, the size of the effect which can be detected using ANOVA (the least significant difference) must be reported.

ECx estimation

62. Estimates of EC_x (e.g. EC_{50}) should be based on frond number and at least one other growth parameter i.e. final dry weight, fresh weight or total frond area. An EC_x can be obtained by non-linear regression analysis of the concentration-response curve using an appropriate mathematical function. Suitable functions, which are fitted to the replicate level % inhibition data, are:

(i) the logistic curve
(ii) the cumulative normal model (21)
(iii) linear interpolation with bootstrapping (22)

The formulae for these models can be solved for any percentage x, e.g. 50 %, 20 %, 10 %. The linear interpolation model is most suitable when the responses to the test substance are not monotonic. All three models assume that 0 % inhibition corresponds to a replicate value equal to the control mean and that 100 % inhibition corresponds to a replicate value of zero. Consequently, including treatment levels that have negative values for any of the endpoints (i.e. hormesis) may distort the results for the models. This in turn can result in an underestimate of EC_x values, particularly with the cumulative normal model. If hormesis is substantial, resulting in a poor fit with the~~se~~ models, or a substantial portion of the data set must be left out in order to achieve a reasonable fit, then a hormesis model may need to be considered (23).

63. Variance within treatments may not be constant across concentrations, in which case consideration may need to be given to a weighted analysis in which greater weight is accorded to treatments having less variability. Where possible, two-sided 95 % confidence limits around the estimated EC_x should be quoted. Therefore, the fitted curve should be parameterised so that the EC_x of interest and its standard error can be estimated directly. Goodness of fit should also be assessed, either graphically or

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[1] Before pooling data for the negative and solvent controls (if used), they should be statistically tested to see that they are not significantly different, using two-tailed t-test.

by dividing the residual sum of squares into 'lack of fit' and 'pure error components' and performing a significance test for lack of fit.

64. It should be noted that the statistical methods indicated are provided as examples and that the best statistics should be used to match the study design used.

Reporting

65. The test report must include the following:

Test substance:

- physical nature and physicochemical properties, including water solubility limit and percent purity;
- identification data, e.g. CAS Number.

Test species:

- scientific name, clone (if known) and source.

Test conditions:

- test procedure used (static, semi-static or flow-through);
- test duration;
- test medium;
- description of the experimental design: test vessels and covers, solution volumes, number of colonies and fronds per test vessel at the beginning of the test;
- test concentrations (nominal and measured as appropriate) and number of replicates per concentration;
- methods of preparation of stock and test solutions including the use of any solvents or dispersants;
- temperature during the test;
- light source and light intensity;
- pH values of the test and control media;
- test substance concentrations and the method of analysis with appropriate quality assessment data (validation studies, standard deviations or confidence limits of analyses);
- methods for determination of frond number and other parameters, e.g. dry weight, fresh weight or frond area;
- all deviations from this guideline.

Results:

- raw data: number of fronds and other parameters in each test and control vessel at each observation and occasion of analysis;
- means and standard deviations for each measured growth parameter;
- growth curves for each concentration;
- doubling time in the control based on the frond number;
- estimates of toxic endpoints e.g. EC_{50}, LOEC and NOEC, and the statistical methods used for their determination;
- any stimulation of growth found in any treatment;
- any visual signs of phytotoxicity as well as observations of test solutions;

- discussion of the results, including any influence on the outcome of the test resulting from deviations from this Guideline.

LITERATURE

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ANNEX 1: DESCRIPTION OF *LEMNA* SPP.

The aquatic plant commonly referred to as duckweed, *Lemna* spp., belongs to the family *Lemnaceae* which has a number of world-wide species in four genera. Their different appearance and taxonomy have been exhaustively described (1)(2). *Lemna gibba* and *L. minor* are species representative of temperate areas and are commonly used for toxicity tests. Both species have a floating or submerged discoid stem (frond) and a very thin root emanates from the centre of the lower surface of each frond. *Lemna* spp. rarely produce flowers and the plants reproduce by vegetatively producing new fronds (3). In comparison with older plants the younger ones tend to be paler, have shorter roots and consist of two to three fronds of different sizes. The small size of *Lemna*, its simple structure, asexual reproduction and short generation time makes plants of this genus very suitable for laboratory testing (4)(5).

Because of probable interspecies variation in sensitivity, only comparisons of sensitivity within a species are valid.

Examples of *Lemna* species which have been used for testing: Species Reference

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ANNEX 2: DEFINITIONS

The following definitions and abbreviations are used in the **context of this** guideline:

Chlorosis is yellowing of frond tissue.

Clone is an organism or cell arisen from a single individual by asexual reproduction. Individuals from the same clone are, therefore, genetically identical.

Colony means an aggregate of mother and daughter fronds (usually 2 to 4) attached to each other. Sometimes referred to as a plant.

EC_x is the concentration of the test substance dissolved in test medium that results in a x % (e.g. 50%) reduction in growth of Lemna within a stated exposure period.

Flow-through is a test in which the test solutions are replaced continuously.

Frond is an individual/single "leaf-like" structure of a duckweed plant. It is the smallest unit, i.e. individual, capable of reproduction.

Gibbosity means fronds exhibiting a humped or swollen appearance.

Growth is an increase in the recorded parameter, e.g. frond number, dry weight, wet weight or frond area, over the test period.

Lowest Observed Effect Concentration (LOEC) is the lowest tested concentration at which the substance is observed to have a statistically significant reducing effect on growth (at $p < 0.05$) when compared with the control, within a given exposure time. 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.

Monoculture is a culture with one plant species.

Necrosis is dead (i.e. white or water-soaked) frond tissue.

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 given exposure time.

Phenotype is the observable characteristics of an organism determined by the interaction of its genes with its environment.

Semi-static (renewal) test is a test in which the test solution is periodically replaced at specific intervals during the test.

Static test is a test method without renewal of the test solution during the test.

Test medium is the complete synthetic culture medium on which test plants grow when exposed to the test substance. The test substance will normally be dissolved in the test medium.

ANNEX 3: MAINTENANCE OF STOCK CULTURE

Stock cultures can be maintained under lower temperatures (4-10°C) for longer times without needing to be re-established. The *Lemna* culture medium may be the same as that used for testing but other nutrient rich media can be used for stock cultures.

Under normal conditions of temperature (24 ^{o}C) and illumination (6500 - 10000 lux), monthly sub-culturing of stock cultures is advised. A number of young, light-green plants are removed to new culture vessels containing fresh medium using an aseptic technique. Under the cooler conditions suggested here, sub-culturing may be conducted less frequently. Intervals of up to three months have been found to be acceptable.

Chemically clean (acid-washed) and sterile glass culture vessels should be used and aseptic handling techniques employed. In the event of contamination of the stock culture e.g. by algae, steps are necessary to eliminate the contaminating organisms. In the case of algae and most other contaminating organisms, this can be achieved by surface sterilisation. A sample of the contaminated plant material is taken and the roots cut off. The material is then shaken vigorously in clean water, followed by immersion in a 0.5% (v/v) sodium hypochlorite solution for between 30 seconds and 5 minutes. The plant material is then rinsed with sterile water and transferred, as a number of batches, into culture vessels containing fresh culture medium. Many fronds will die as a result of this treatment, especially if longer exposure periods are used, but some of those surviving will usually be free of contamination. These can then be used to re-inoculate new cultures.

ANNEX 4: MEDIA

Different growth media are recommended for *L. minor* and *L. gibba*. For *L. minor*, a modified Swedish Standard (SIS) medium is recommended whilst for *L. gibba*, 20X AAP medium is recommended. Compositions of both media are given below. When preparing these media, reagent or analytical-grade chemicals should be used and deionised water.

Swedish Standard (SIS) *Lemna* growth medium

- Stock solutions I - V are sterilised by autoclaving (120 °C, 15 minutes) or by membrane filtration (approximately 0.2 µm pore size).
- Stock VI (and optional VII) are sterilised by membrane filtration only; these should not be autoclaved.
- Sterile stock solutions should be stored under cool and dark conditions. Stocks I - V should be discarded after six months whilst stocks VI (and optional VII) have a shelf life of one month.

Stock solution No.	Substance	Concentration in stock solution ($g \cdot l^{-1}$)	Concentration in prepared medium ($mg \cdot l^{-1}$)	Prepared medium	
				Element	Concentration ($mg \cdot l^{-1}$)
I	$NaNO_3$	8.50	85	Na ; N	32 ; 14
	KH_2PO_4	1.34	13.4	K ; P	6.0 ; 2.4
II	$MgSO_4 . 7H_2O$	15	75	Mg ; S	7.4 ; 9.8
III	$CaCl_2 . 2H_2O$	7.2	36	Ca ; Cl	9.8 ; 17.5
IV	Na_2CO_3	4.00	20	C	2.3
V	H_3BO_3	1.0	1.00	B	0.17
	$MnCl_2 . 4H_2O$	0.20	0.20	Mn	0.056
	$Na_2MoO_4 . 2H_2O$	0.010	0.010	Mo	0.0040
	$ZnSO_4 . 7H_2O$	0.050	0.050	Zn	0.011
	$CuSO_4 . 5H_2O$	0.0050	0.0050	Cu	0.0013
	$Co(NO_3)_2 . 6H_2O$	0.010	0.010	Co	0.0020
VI	$FeCl_3 . 6H_2O$	0.17	0.84	Fe	0.17
	Na_2-EDTA 2H₂O	0.28	1.40	-	-
VII	MOPS (buffer)	490	490	-	-

20X AAP growth medium

Stock solutions A1, A2, A3 and B are sterilised by autoclaving (120 °C, 15 minutes) or by membrane filtration (approximately 0.2 µm pore size).

Stock solution C is only to be sterilised by membrane filtration.

Sterile stock solutions should be stored under cool and dark conditions. Under these conditions the stock solutions will have a shelf life of 6 – 8 weeks.

Stock solution No.	Substance	Concentration in stock solution ($g \bullet l^{-1}$)*	Concentration in prepared medium ($mg \bullet l^{-1}$)*	Prepared medium	
				Element	Concentration ($mg \bullet l^{-1}$)*
A1	$NaNO_3$	26	510	Na;N	190;84
	$MgCl_2.6H_2O$	12	240	Mg	58.08
	$CaCl_2.2H_2O$	4.4	90	Ca	24.04
A2	$MgSO_4.7H_2O$	15	290	S	38.22
A3	$K_2HPO_4.3H_2.O$	1.4	30	K;P	9.4;3.7
B	H_3BO_3	0.19	3.7	B	0.65
	$MnCl_2.4H_2O$	0.42	8.3	Mn	2.3
	$FeCl_3.6H_2O$	0.16	3.2	Fe	0.66
	$Na_2EDTA.2H_2O$	0.30	6.0	-	-
	$ZnCl_2$	3.3 $mg.l^{-1}$	66 $\mu g.l^{-1}$	Zn	31 $\mu g.l^{-1}$
	$CoCl_2.6H_2O$	1.4 $mg.l^{-1}$	29 $\mu g.l^{-1}$	Co	7.1 $\mu g.l^{-1}$
	$Na_2MoO_4.2H_2O$	7.3 $mg.l^{-1}$	145 $\mu g.l^{-1}$	Mo	58 $\mu g.l^{-1}$
	$CuCl_2.2H_2O$	0.012 $mg.l^{-1}$	0.24 $\mu g.l^{-1}$	Cu	0.080 $\mu g.l^{-1}$
C	$NaHCO_3$	15	300	Na;C	220; 43

*Unless noted

ANNEX 5: ESTIMATION OF THE 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 *Lemna*. The selection should be based on biological considerations as well as statistical ones and should be justified in the test report. For example if growth 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 hereunder shows an example of a (simplified) test lasting seven days with medium renewal at days 0, 2 and 4.

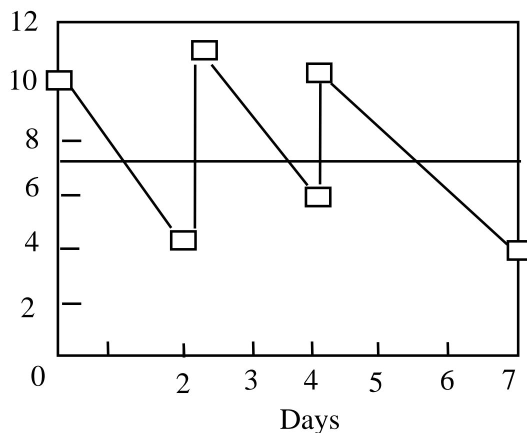

Figure 1: Example of time-weighted mean

- The thin zigzag 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	2.203	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*.

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.