Adopted :
23 March 2006

OECD GUIDELINES FOR THE TESTING OF CHEMICALS

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 an 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 toxicants can occur with *Lemna*, there are currently insufficient data on this source of variability to recommend a specific clone for use with this Guideline. 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 (8).

4. Definitions used are given in Annex 1.

PRINCIPLE OF THE TEST

5. Exponentially growing plant cultures 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 selected measurement variables. Frond number is the primary measurement variable. At least one other measurement variable (total frond area, dry weight or fresh weight) is also measured, since some substances may affect other measurement variables much more than frond numbers. 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})

6. The test endpoint is inhibition of growth, expressed as logarithmic increase in measurement variable (average specific growth rate) during the exposure period. From the average specific growth rates recorded in a series of test solutions, the concentration bringing about a specified x % inhibition of growth rate (e.g. 50%) is determined and expressed as the E_rC_x (e.g. E_rC_{50}).

7. An additional response variable used in this Guideline is yield, which may be needed to fulfil specific regulatory requirements in some countries. It is defined as measurement variables at the end of the

exposure period minus the measurement variables at the start of the exposure period. From the yield recorded in a series of test solutions, the concentration bringing about a specified x % inhibition of yield (e.g., 50%) is calculated and expressed as the E_yC_x (e.g. E_yC_{50}).

8. In addition, the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) may be statistically determined.

INFORMATION ON THE TEST SUBSTANCE

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

10. 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.

11. 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 21). Further guidance for testing substances with physical-chemical properties that make them difficult to test is provided in (8).

VALIDITY OF THE TEST

12. 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 a seven-fold increase in seven days and an average specific growth rate of 0.275 d^{-1}. 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 49.

REFERENCE SUBSTANCE

13. Reference substance(s), such as 3,5-dichlorophenol used in the international ring test (7), may be tested as a means of checking the test procedure. 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.

DESCRIPTION OF THE METHOD

Apparatus

14. 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 should be 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. Glass beakers, crystallising dishes or glass petri dishes of appropriate

dimensions have all proved suitable. Test vessels must be covered to minimise evaporation and accidental contamination, while allowing necessary air exchange. Suitable test vessels, and particularly covers, must avoid shadowing or changes in the spectral characteristics of light.

15. 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 35-36).

Test organism

16. The organism used for this test is either *Lemna gibba* or *Lemna minor*. Short descriptions of duckweed species that have been used for toxicity testing are given in Annex 2. 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.

17. 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.

18. 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

19. 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 may 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.

20. 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.

Test medium

21. 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.

22. A modification of the Swedish standard (SIS) *Lemna* growth medium is recommended for culturing and testing with *L. minor*. The composition of this medium is given in Annex 4.

23. The growth medium, 20X - AAP, as described in Annex 4, is recommended for culturing and testing with *L. gibba*.

24. Steinberg medium, as described in Annex 4, is also suitable for *L. minor*, but may also be used for *L. gibba* as long as the validity criteria are met.

Test solutions

25. Test solutions are usually prepared by dilution of a stock solution. Stock solutions of the test substance are normally prepared by dissolving the substance in growth medium.

26. 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 µL/L include acetone and dimethylformamide. If a solvent or dispersant is used, its final concentration should be reported and kept to a minimum ($\leq$ 100 µL/L), and all treatments and controls should contain the same concentration of solvent or dispersant. Further guidance on the use of dispersants is given in (8).

Test and control groups

27. 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. In the definitive toxicity test, there should normally be at least five test concentrations arranged in a geometric series. Preferably the separation factor between test concentrations should not exceed 3.2, but a larger value may be used where the concentration-response curve is flat. Justification should be provided if fewer than five concentrations are used. At least three replicates should be used at each test concentration.

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

- To determine 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 mg/L).

29. Every test should include controls consisting of the same nutrient medium, number of fronds and colonies, 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 (and solvent vessels, if applicable) should be at least equal to, and ideally twice, the number of vessels used for each test concentration.

30. If determination of NOEC is not required, the test design may be altered to increase the number of concentrations and reduce the number of replicates per concentration. However, the number of control replicates must be at least three.

Exposure

31. 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 fronds and colonies 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 4 to 7% of inhibition calculated by growth rate (10 to 15% calculated by yield) between treatments (7).

32. 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.

33. 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 may be needed to maintain near-constant concentrations of highly unstable or volatile substances. In some circumstances, a flow-through procedure may be required (8)(10).

34. The exposure scenario through a foliar application (spray) is not covered in this Guideline; instead, see (11).

Incubation conditions

35. Continuous warm or cool white fluorescent lighting should be used to provide a light intensity selected from the range of 85-135 $\mu E \cdot m^{-2} s^{-1}$ when measured in a photosynthetically active radiation (400-700 nm) at points the same distance from the light source as the *Lemna* fronds (equivalent to 6500-10000 lux). Any differences from the selected light intensity over the test area should not exceed the range of ± 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.

36. 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

invalidate the test when it can be shown that validity criteria are met. Additional care is needed on pH drift in special cases such as when testing unstable substances or metals. See (8) for further guidance.

Duration

37. The test is terminated 7 days after the plants are transferred into the test vessels.

Measurements and analytical determinations

38. At the start of the test, frond number in the test vessels is counted and recorded, taking care to ensure that protruding, distinctly visible fronds are accounted for. Frond numbers appearing normal or abnormal, need to be determined at the beginning of the test, at least once every 3 days during the exposure period (i.e. on at least 2 occasions during the 7 day period), and at test termination. Changes in plant development, e.g. in frond size, appearance, indication of necrosis, chlorosis or gibbosity, colony break-up or loss of buoyancy, and in root length and appearance, 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.

39. In addition to determinations of frond number during the test, effects of the test substance on one (or more) of the following measurement variables are also assessed:

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

40. Total frond area has an advantage, in that it can be determined for each test and control vessel at the start, during, and at the end of the test. Dry or fresh weight should be determined at the start of the test from a sample of the inoculum culture representative of what is used to begin the test, and at the end of the test with the plant material from each test and control vessel. If frond area is not measured, dry weight is preferred over fresh weight.

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

(i) Total frond area: The total frond area of all 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: All 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: All 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 measurements and analytical determinations

42. If a static test design is used, the pH of each treatment should be measured at the beginning and at the 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.

43. 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.

44. During the test, the concentrations of the test substance are determined at appropriate intervals. In static tests, the minimum requirement is to determine the concentrations at the beginning and at the end of the test.

45. 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 33). 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).

46. 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.

47. 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 not within ± 20 %, analysis of the results should be based on the geometric mean concentration during exposure or models describing the decline of the concentration of the test substance (8).

Limit test

48. Under some circumstances, e.g. when a preliminary test indicates that the test substance has no toxic effects at concentrations up to 100 mg/L 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 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

Doubling time

49. To determine the doubling time *(T_d)* of frond number and adherence to this validity criterion by the study (paragraph 12), the following formula is used with data obtained from the control vessels:

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

where μ is the average specific growth rate determined as described in paragraphs 54-55.

Response variables

50. The purpose of the test is to determine the effects of the test substance on the vegetative growth of *Lemna*. This Guideline describes two response variables, as member countries have different preferences and regulatory needs. In order for the test results to be acceptable in all member countries, the effects should be evaluated using both response variables (a) and (b) described below.

(a) Average specific growth rate: this response variable is calculated on the basis of changes in the logarithms of frond numbers, and in addition, on the basis of changes in the logarithms of another measurement parameter (total frond area, dry weight or fresh weight) over time (expressed per day) in the controls and each treatment group. It is sometimes referred to as relative growth rate (12).

(b) Yield: this response variable is calculated on the basis of changes in frond number, and in addition, on the basis of changes in another measurement parameter (total frond area, dry weight or fresh weight) in the controls and in each treatment group until the end of the test.

51. It should be noted that toxicity values calculated by using these two response variables are not comparable and this difference must be recognised when using the results of the test. EC_x values based upon average specific growth rate (E_rC_x) will generally be higher than results based upon yield (E_yC_x) if the test conditions of this Guideline are adhered to, due to the mathematical basis of the respective approaches. This should not be interpreted as a difference in sensitivity between the two response variables, simply that the values are different mathematically. The concept of average specific growth rate is based on the general exponential growth pattern of duckweed in non-limited cultures, where toxicity is estimated on the basis of the effects on the growth rate, without being dependent on the absolute level of the specific growth rate of the control, slope of the concentration-response curve or on test duration. In contrast, results based upon the yield response variable are dependent upon all these other variables. E_yC_x is dependent on the specific growth rate of the duckweed species used in each test and on the maximum specific growth rate that can vary between species and even different clones. This response variable should not be used for comparing the sensitivity to toxicants among duckweed species or even different clones. While the use of average specific growth rate for estimating toxicity is scientifically preferred, toxicity estimates based on yield are also included in this Guideline to satisfy current regulatory requirements in some countries.

52. Toxicity estimates should be based on frond number and one additional measurement variable (total frond area, dry weight or fresh weight), because some substances may affect other measurement variables much more than the frond number. This effect would not be detected by calculating frond number only.

53. The number of fronds as well as any other recorded measurement variable, i.e. total 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

54. The average specific growth rate for a specific period is calculated as the logarithmic increase in the growth variables -frond numbers and one other measurement variable (total frond area, dry weight or fresh weight) - using the formula below for each replicate of control and treatments:

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

where:

- $\mu_{i\text{-}j}$: average specific growth rate from time i to j
- N_i : measurement variable in the test or control vessel at time i
- N_j : measurement variable in the test or control vessel at time j
- t : time period from i to j

For each treatment group and control group, calculate a mean value for growth rate along with variance estimates.

55. The average specific growth rate should be calculated for the entire test period (time "i" in the above formula is the beginning of the test and time "j" is the end of the test). For each test concentration and control, calculate a mean value for average specific growth rate along with the variance estimates. In addition, the section-by-section growth rate should be assessed in order to evaluate effects of the test substance occurring during the exposure period (e.g. by inspecting log-transformed growth curves). Substantial differences between the section-by-section growth rate and the average growth rate indicate deviation from constant exponential growth and that close examination of the growth curves is warranted. In this case, a conservative approach would be to compare specific growth rates from treated cultures during the time period of maximum inhibition to those for controls during the same time period.

56. Percent inhibition of growth rate (I_r) may then be calculated for each test concentration (treatment group) 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

Yield

57. Effects on yield are determined on the basis of two measurement variables, frond number and one other measurement variable (total frond area, dry weight or fresh weight) present in each test vessel at the start and at the end of the test. For dry weight or fresh weight, 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 20). For each test concentration and control, calculate a mean value for yield along with variance estimates. The mean percent inhibition in yield (%I_y) may be calculated for each treatment group as follows:

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

where:

- $\% \ I_y$: percent reduction in yield
- b_C : final biomass minus starting biomass for the control group
- b_T : final biomass minus starting biomass in the treatment group

Plotting concentration-response curves

58. Concentration-response curves relating mean percentage inhibition of the response variable (I_r, or I_y calculated as shown in paragraph 56 or 57) and the log concentration of the test substance should be plotted.

EC_x estimation

59. Estimates of the EC_x (e.g., EC_{50}) should be based upon both average specific growth rate (E_rC_x) and yield (E_yC_x), each of which should in turn be based upon frond number and one additional measurement variable (total frond area, dry weight, or fresh weight). This is because there are test substances that impact frond number and other measurement variables differently. The desired toxicity parameters are therefore four EC_x values for each inhibition level x calculated: E_rC_x (frond number); E_rC_x (total frond area, dry weight, or fresh weight); E_yC_x (frond number); and E_yC_x (total frond area, dry weight, or fresh weight).

Statistical procedures

60. The aim is to obtain a quantitative concentration-response relationship by regression analysis. It is possible to use a weighted linear regression after having performed a linearising transformation of the response data, for instance into probit or logit or Weibull units (13), but non-linear regression procedures are preferred techniques that better handle unavoidable data irregularities and deviations from smooth distributions. Approaching either zero or total inhibition such irregularities may be magnified by the transformation, interfering with the analysis (13). It should be noted that standard methods of analysis using probit, logit, or Weibull transforms are intended for use on quantal (e.g. mortality or survival) data, and must be modified to accommodate growth rate or yield data. Specific procedures for determination of EC_x values from continuous data can be found in (14), (15), and (16).

61. For each response variable to be analysed, use the concentration-response relationship to calculate point estimates of EC_x values. When possible, the 95% confidence limits for each estimate should be determined. Goodness of fit of the response data to the regression model should be assessed either graphically or statistically. Regression analysis should be performed using individual replicate responses, not treatment group means.

62. EC_{50} estimates and confidence limits may also be obtained using linear interpolation with bootstrapping (17), if available regression models/methods are unsuitable for the data.

63. For estimation of the LOEC and hence the NOEC, it is necessary to compare treatment means using analysis of variance (ANOVA) techniques. The mean for each concentration must then be compared with the control mean using an appropriate multiple comparison or trend test method. Dunnett's or Williams'test may be useful (18)(19)(20)(21). It is necessary to assess whether the ANOVA assumption of homogeneity of variance holds. This assessment may be performed graphically or by a formal test (22). Suitable tests are Levene's or Bartlett's. Failure to meet the assumption of homogeneity of variances can

sometimes be corrected by logarithmic transformation of the data. If heterogeneity of variance is extreme and cannot be corrected by transformation, analysis by methods such as step-down Jonkheere trend tests should be considered. Additional guidance on determining the NOEC can be found in (16).

64. Recent scientific developments have led to a recommendation of abandoning the concept of NOEC and replacing it with regression based point estimates EC_x. An appropriate value for x has not been established for this *Lemna* test. However, a range of 10 to 20 % appears to be appropriate (depending on the response variable chosen), and preferably both the EC_{10} and EC_{20} should be reported.

Reporting

65. The test report must include the following:

Test substance:

- physical nature and physical-chemical properties, including water solubility limit;
- chemical identification data (e.g., CAS Number), including purity (impurities).

Test species:

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

Test conditions:

- test procedure used (static, semi-static or flow-through);
- date of start of the test and its 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, light intensity and homogeneity;
- 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 measurement variables, e.g. dry weight, fresh weight or frond area;
- all deviations from this Guideline.

Results:

- raw data: number of fronds and other measurement variables in each test and control vessel at each observation and occasion of analysis;
- means and standard deviations for each measurement variable;

- growth curves for each concentration (recommended with log transformed measurement variable, see paragraph 55);
- doubling time/growth rate in the control based on the frond number;
- calculated response variables for each treatment replicate, with mean values and coefficient of variation for replicates;
- graphical representation of the concentration/effect relationship;
- estimates of toxic endpoints for response variables e.g. EC_{50}, EC_{10}, EC_{20}, and associated confidence intervals. If calculated, LOEC and/or NOEC and the statistical methods used for their determination;
- if ANOVA has been used, the size of the effect which can be detected (e.g. the least significant difference);
- 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

(1) ASTM International. (2003). Standard Guide for Conducting Static Toxicity Test With *Lemna gibba* G3. E 1415-91 (Reapproved 1998). pp. 733-742. In, Annual Book of ASTM Standards, Vol. 11.05 Biological Effects and Environmental Fate; Biotechnology; Pesticides, ASTM, West Conshohocken, PA.

(2) US EPA - United States Environmental Protection Agency. (1996). OPPTS 850.4400 Aquatic Plant Toxicity Test Using *Lemna* spp., "Public draft". EPA 712-C-96-156. 8pp.

(3) AFNOR - Association Française de Normalisation. (1996). XP T 90-337: Détermination de l´inhibition de la croissance de *Lemna minor*. 10pp.

(4) SSI - Swedish Standards Institute. (1995). Water quality - Determination of growth inhibition (7-d) *Lemna minor*, duckweed. SS 02 82 13. 15pp. (in Swedish).

(5) Environment Canada. (1999). Biological Test Method: Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte, *Lemna minor*. EPS 1/RM/37 - 120 pp.

(6) Environment Canada. (1993) Proposed Guidelines for Registration of Chemical Pesticides: Non-Target Plant Testing and Evaluation. Canadian Wildlife Service, Technical Report Series No. 145.

(7) Sims I., Whitehouse P. and Lacey R. (1999) The OECD *Lemna* Growth Inhibition Test. Development and Ring-testing of draft OECD Test Guideline. R&D Technical Report EMA 003. WRc plc - Environment Agency.

(8) OECD (2000). Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. OECD Environmental Health and Safety Publications, Series on Testing and Assessment No.23. Organisation for Economic Co-operation and Development, Paris.

(9) International Organisation for Standardisation. ISO DIS 20079. Water Quality - Determination of the Toxic Effect of Water Constituents and Waste Water to Duckweed (*Lemna minor*) - Duckweed Growth Inhibition Test.

(10) Walbridge C. T. (1977). A flow-through testing procedure with duckweed (*Lemna minor* L.). Environmental Research Laboratory - Duluth, Minnesota 55804. US EPA Report No. EPA-600/3-77 108. September 1977.

(11) Lockhart W. L., Billeck B. N. and Baron C. L. (1989). Bioassays with a floating plant (*Lemna minor*) for effects of sprayed and dissolved glyphosate. *Hydrobiologia*, 118/119, 353 - 359.

(12) Huebert, D.B. and Shay J.M. (1993) Considerations in the assessment of toxicity using duckweeds. *Environmental Toxicology and Chemistry*, 12, 481-483.

(13) Christensen, E.R.,, Nyholm, N. (1984): Ecotoxicological Assays with Algae: Weibull Dose-Response Curves. *Env. Sci. Technol*. 19, 713-718.

(14) Nyholm, N. Sørensen, P.S., Kusk, K.O. and Christensen, E.R. (1992): Statistical treatment of data from microbial toxicity tests. *Environ. Toxicol. Chem*. 11, 157-167.

(15) Bruce R.D. and Versteeg D.J. (1992) A statistical procedure for modelling continuous toxicity data. *Environmental Toxicology and Chemistry*, 11, 1485-1494.

(16) OECD. (2005). Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application. Organisation for Economic Co-operation and Development, Paris.

(17) Norberg-King T.J. (1988) An interpolation estimate for chronic toxicity: The ICp approach. National Effluent Toxicity Assessment Center Technical Report 05-88. US EPA, Duluth, MN.

(18) Dunnett, C.W. (1955) A multiple comparisons procedure for comparing several treatments with a control. J. Amer. Statist. Assoc., 50, 1096-1121.

(19) Dunnett, C.W. (1964) New tables for multiple comparisons with a control. Biometrics, 20, 482-491.

(20) Williams, D.A. (1971) A test for differences between treatment means when several dose levels are compared with a zero dose control. Biometrics, 27: 103-117.

(21) Williams, D.A. (1972) The comparison of several dose levels with a zero dose control. Biometrics, 28: 519-531.

(22) Brain P. and Cousens R. (1989). An equation to describe dose-responses where there is stimulation of growth at low doses. *Weed Research*, 29, 93-96.

ANNEX 1

DEFINITIONS

The following definitions and abbreviations are used for the purposes of this Guideline:

Biomass is the dry weight of living matter present in a population. In this test, surrogates for biomass, such as frond counts or frond area are typically measured and the use of the term "biomass" thus refers to these surrogate measures as well.

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 (to be mentioned explicitly if deviating from full or normal test duration) . To unambiguously denote an EC value deriving from growth rate or yield the symbol "E_rC" is used for growth rate and "E_yC" is used for yield, followed by the measurement variable used, e.g. E_rC (frond number).

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 measurement variable, e.g. frond number, dry weight, wet weight or frond area, over the test period.

Growth rate (average specific growth rate) is the logarithmic increase in biomass during the exposure 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.

Measurement variables are any type of variables which are measured to express the test endpoint using one ore more different response variables. In this guideline frond number, frond area, fresh weight and dry weight are measurement variables.

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.

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

Response variable are variables for the estimation of toxicity derived from any measured variables describing biomass by different methods of calculation. For this guideline growth rates and yield are response variables derived from measurement variables like frond number, frond area, fresh weight or dry weight.

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 endpoint describes the general factor that will be changed relative to control by the test chemical as aim of the test. In this guideline the test endpoint is inhibition of growth which may be expressed by different response variables which are based on one or more measurement variables.

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

Yield is value of a measurement variable to express biomass at the end of the exposure period minus the measurement variable at the start of the exposure period.

ANNEX 2

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

Lemna aequinoctialis: Eklund, B. (1996). The use of the red alga *Ceramium strictum* and the duckweed *Lemna aequinoctialis* in aquatic ecotoxicological bioassays. Licentiate in Philosophy Thesis 1996:2. Dep. of Systems Ecology, Stockholm University.

Lemna major: Clark, N. A. (1925). The rate of reproduction of *Lemna major* as a function of intensity and duration of light. J. phys. Chem., 29: 935-941.

Lemna minor: United States Environmental Protection Agency (US EPA). (1996). OPPTS 850.4400 Aquatic Plant Toxicity Test Using *Lemna* spp., "Public draft". EPA 712-C-96-156. 8pp.
Association Française de Normalisation (AFNOR). (1996). XP T 90-337: Détermination de l´inhibition de la croissance de *Lemna minor*. 10pp.
Swedish Standards Institute (SIS). (1995). Water quality - Determination of growth inhibition (7-d) *Lemna minor*, duckweed. SS 02 82 13. 15pp. (in Swedish).

Lemna gibba: ASTM International. (2003). Standard Guide for Conducting Static Toxicity Test With *Lemna gibba* G3. E 1415-91 (Reapproved 1998). pp. 733-742.
United States Environmental Protection Agency (US EPA). (1996). OPPTS 850.4400 Aquatic Plant Toxicity Test Using *Lemna* spp., "Public draft". EPA 712-C-96-156. 8pp.

Lemna paucicostata: Nasu, Y., Kugimoto, M. (1981). *Lemna* (duckweed) as an indicator of water pollution. I. The sensitivity of *Lemna paucicostata* to heavy metals. Arch. Environ. Contam. Toxicol., 10:1959-1969.

Lemna perpusilla: Clark, J. R. et al. (1981). Accumulation and depuration of metals by duckweed (*Lemna perpusilla*). Ecotoxicol. Environ. Saf., 5:87-96.

Lemna trisulca: Huebert, D. B., Shay, J. M. (1993). Considerations in the assessment of toxicity using duckweeds. Environ. Toxicol. and Chem., 12:481- 483.

Lemna valdiviana: Hutchinson, T.C., Czyrska, H. (1975). Heavy metal toxicity and synergism to floating aquatic weeds. Verh.-Int. Ver. Limnol., 19:2102-2111.

Sources of *Lemna* species

University of Toronto Culture Collection of Algae and Cyanobacteria
Department of Botany, University of Toronto
Toronto, Ontario, Canada, M5S 3 B2
Tel: +1-416-978-3641
Fax:+1-416-978-5878
e-mail: jacreman@botany.utoronto.ca
http://www.botany.utoronto.ca/utcc

North Carolina State University
Forestry Dept
Duckweed Culture Collection
Campus Box 8002
Raleigh, NC 27695-8002
United States
phone 001 (919) 515-7572
astomp@unity.ncsu.edu

Institute of Applied Environmental Research (ITM) Stockholm University
SE-106 91
STOCKHOLM
SWEDEN
Tel: +46 8 674 7240
Fax +46 8 674 7636

Federal Environmental Agency (UBA)
FG III 3.4
Schichauweg 58
12307 Berlin
Germany
e-mail: lemna@uba.de
http://www.umweltbundesamt.de/contact.htm

Literature

(1) Hillman, W.S. (1961). The Lemnaceae or duckweeds: A review of the descriptive and experimental literature. The Botanical Review, 27:221-287.

(2) Landolt, E. (1986). Biosystematic investigations in the family of duckweed (*Lemnaceae*). Vol. 2. Geobotanischen Inst. ETH, Stiftung Rubel, Zürich, Switzerland.

(3) Björndahl, G. (1982). Growth performance, nutrient uptake and human utilization of duckweeds (*Lemnaceae* family). ISBN 82-991150-0-0. The Agricultural Research Council of Norway, University of Oslo.

(4) Wang, W. (1986). Toxicity tests of aquatic pollutants by using common duckweed. Environmental Pollution, Ser B, 11:1-14.

(5) Wang, W. (1990). Literature review on duckweed toxicity testing. Environmental Research, 52:7-22.

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* growth medium may be the same as that used for testing but other nutrient rich media can be used for stock cultures.

Periodically, 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 at intervals of up to three months.

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 or funghi, 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 growth 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 ^{o}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/L)	Concentration in prepared medium (mg/•L)	Prepared medium	
				Element	Concentration (mg/•L)
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	4.0	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 $2H_2O$	0.28	1.4	-	-
VII	MOPS (buffer)	490	490	-	-

- 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 11).

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

20X AAP growth medium

Stock solutions are prepared in sterile distilled or deionised water.

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

Five nutrient stock solutions (A1, A2, A3, B and C) are prepared for 20X - AAP medium, using reagent-grade chemicals. The 20 mL of each nutrient stock solution is added to approximately 850 mL deionised water to produce the growth medium. The pH is adjusted to 7.5 ± 0.1 with either 0.1 or 1 mol 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.

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

Stock solution No.	Substance	Concentration in stock solution (g/•L)*	Concentration in prepared medium (mg/•L)*	Prepared medium	
				Element	Concentration (mg/•L)*
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	66 µg/L	Zn	31 µg/L
	$CoCl_2.6H_2O$	1.4 mg/L	29 µg/L	Co	7.1 µg/L
	$Na_2MoO_4.2H_2O$	7.3 mg/L	145 µg/L	Mo	58 µg/L
	$CuCl_2.2H_2O$	0.012 mg/L	0.24 µg/L	Cu	0.080 µg/L
C	$NaHCO_3$	15	300	Na;C	220; 43

*Unless noted

footnote:
The theoretically appropriate final bicarbonate concentration (which will avoid appreciable pH adjustment) is 15 mg/L, not 300 mg/L. However, the historical use of 20X-AAP medium, including the ring test for this guideline, is based upon 300 mg/L. (I. Sims, P. Whitehouse and R. Lacey. (1999) The OECD *Lemna* Growth Inhibition Test. Development and Ring-testing of draft OECD Test Guideline. R&D Technical Report EMA 003. WRc plc - Environment Agency.)

STEINBERG medium (After ISO 20079)

Concentrations and stock solutions

- The modified Steinberg medium is used in ISO 20079 for *Lemna* minor alone (as only *Lemna minor* is allowed there) but tests showed good results could be reached with *Lemna gibba* too.
- When preparing the medium, reagent- or analytical grade chemicals and deionised water should be used.
- Prepare the nutrient medium from stock solutions or the 10 fold concentrated medium which allows maximum concentration of the medium without precipitation.

Table 1 — pH-stabilised STEINBERG medium (modified acc. to Altenburger)

Substance		Nutrient medium	
Macroelements	mol weight	mg/L	mmol/L
KNO_3	101.12	350.00	3.46
$Ca(NO_3)_2 \cdot 4H_2O$	236.15	295.00	1.25
KH_2PO_4	136.09	90.00	0.66
K_2HPO_4	174.18	12.60	0.072
$MgSO_4 \cdot 7H_2O$	246.37	100.00	0.41
Microelements	mol weight	µg/L	µmol/L
H_3BO_3	61.83	120.00	1.94
$ZnSO_4 \cdot 7H_2O$	287.43	180.00	0.63
$Na_2MoO_4 \cdot 2H_2O$	241.92	44.00	0.18
$MnCl_2 \cdot 4H_2O$	197.84	180.00	0.91
$FeCl_3 \cdot 6H_2O$	270.21	760.00	2.81
EDTA Disodium-dihydrate	372.24	1 500.00	4.03

Table 2 — Stock solutions (Macroelements)

1. Macroelements (50-fold concentrated)	g/L
Stock solution 1:	
KNO_3	17.50
KH_2PO_4	4.5
K_2HPO_4	0.63
Stock solution 2:	
$MgSO_4 \cdot 7H_2O$	5.00
Stock solution 3:	
$Ca(NO_3)_2 \cdot 4H_2O$	14.75

Table 3 — Stock solutions (Microelements)

2. Microelements (1 000-fold concentrated)	mg/L
Stock solution 4: H_3BO_3	120.0
Stock solution 5: $ZnSO_4 \cdot 7H_2O$	180.0
Stock solution 6: $Na_2MoO_4 \cdot 2H_2O$	44.0
Stock solution 7: $MnCl_2 \cdot 4H_2O$	180.0
Stock solution 8: $FeCl_3 \cdot 6H_2O$ EDTA Disodium-dihydrate	760.00 1 500.00

- Stock solutions 2 and 3 and separately 4 to 7 may be pooled (taking into account the required concentrations).
- For longer shelf life treat stock solutions in an autoclave at 121 °C for 20 min or alternatively carry out a sterile filtration (0.2 µm). For stock solution 8 sterile filtration (0.2 µm) is strongly recommended.

Preparation of the final concentration of STEINBERG medium (modified)

- Add 20 mL of stock solutions 1, 2 and 3 (see table 2) to about 900 mL deionised water to avoid precipitation.
- Add 1.0 mL of stock solutions 4, 5, 6, 7 and 8 (see table 3).
- The pH should be to 5.5 +/- 0.2 (adjust by addition of a minimised volume of NaOH solution or HCl).
- Adjust with water to 1000 mL.
- If stock solutions are sterilised and appropriate water is used no further sterilisation is necessary. If sterilisation is done with the final medium stock solution 8 should be added after autoclaving (at 121 °C for 20 min).

Preparation of 10-fold-concentrated STEINBERG medium (modified) for intermediate storage

- Add to 20 mL of stock solutions 1, 2 and 3 (see table 2) to about 30 mL water to avoid precipitation.
- Add 1.0 mL of stock solutions 4, 5, 6, 7 and 8 (see table 3). Adjust with water to 100 mL.
- If stock solutions are sterilized and appropriate water is used no further sterilisation is necessary. If sterilisation is done with the final medium stock solution 8 should be added after autoclaving (at 121 °C for 20 min).
- The pH of the medium (final concentration) should be 5.5+/- 0.2.