DRAFT REVISED GUIDELINE 201
July 2002

OECD GUIDELINES FOR THE TESTING OF CHEMICALS

PROPOSAL FOR UPDATING GUIDELINE 201

Freshwater Alga and Cyanobacteria, Growth Inhibition Test

INTRODUCTION

1. OECD Guidelines for Testing of Chemicals are periodically reviewed in the light of scientific progress. With respect to Guideline 201, Alga, Growth Inhibition Test (adopted June 1984), the need to extend the Guideline to include additional species and update it to meet the requirements for hazard assessment and classification of chemicals has been identified. The revision has been performed on the basis of extensive practical experience and scientific progress in the field of algal toxicity studies.

2. Definitions used are given in Annex 1.

PRINCIPLE OF THE TEST

3. The purpose of this test is to determine the effects of a substance on the growth of fresh water microalgae (algae and cyanobacteria). Exponentially growing test algae are exposed to the test substance in batch cultures over a period of normally 72 hours. In spite of the relatively brief test duration, effects over several generations can be assessed.

4. The system response is the reduction of growth in a series of algal cultures (test units) exposed to various concentrations of a test substance. The response is evaluated as a function of the exposure concentration by comparison with the average growth of replicate, unexposed control cultures. For full expression of the system response to toxic effects (optimal sensitivity), the cultures are allowed unrestricted exponential growth under nutrient sufficient conditions and continuous light for a sufficient period of time to measure reduction of the specific growth rate. Growth and growth inhibition are quantified from measurements of the algal biomass density as a function of time.

5. The test endpoint is inhibition of growth, expressed as logarithmic algal biomass increase (average growth rate) during the exposure period. From the average growth rates recorded in a series of test solutions 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. Optionally the log-biomass integral (area under the growth curve) may be used as an additional endpoint. This option may be attractive in situations with highly irregular growth in inhibited cultures.

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

INFORMATION ON THE TEST SUBSTANCE

8. Information on the test substance which may be useful in establishing the test conditions includes the structural formula, purity, stability in light, stability under the conditions of the test, light absorption properties, pKa, and results of a degradation test.

9. The water solubility, octanol water partition coefficient (P_{ow}) and the vapour pressure of the test substance should be known and a validated method for the quantification of the substance in the test solutions with reported recovery efficiency and limit of detection should be available.

VALIDITY OF THE TEST

10. For the test to be valid, the following performance criteria should be met:

- The biomass concentration in the control cultures should have increased by a factor of at least 16 within the test period. (This criterion applies to the test algae *Pseudokirchneriella subcapitata* and *Scenedesmus subspicatus* only. Experience with the other recommended species is inadequate for suggesting a definite criterion at this time for these species)

- The coefficient of variation daily growth rates in the control cultures during the course of the test (days 0-1, 1,2 and 2-3) must not exceed 35%

- Coefficient of variation of average growth in replicate control cultures must not exceed 15%

- pH in the control cultures shall not increase more than 1.5 unit. (For test compounds that partly ionise at a pH around the test pH, it may be necessary to limit the pH drift to obtain reproducible and well defined results. A drift of no more than 0.3 pH units is technically feasible and can be achieved by ensuring an adequate CO_2 mass transfer rate from the surrounding air to the test solution, e.g. by increasing the shaking rate. Another possibility is to reduce the demand for CO_2 by reducing the initial biomass density or the test duration)

APPLICABILITY OF THE TEST

11. This Guideline is most easily applied to water-soluble substances which, under the conditions of the test, are likely to remain in the water. For testing of substances that are volatile, strongly adsorbing, coloured, having a low solubility in water or substances that may affect the availability of nutrient or minerals in the test medium, certain modifications of the described procedure may be required (e.g. closed system, conditioning of the test vessels). Guidance on appropriate modifications is given in (1), (2) and (3).

DESCRIPTION OF THE METHOD

Apparatus

12. Test vessels and other apparatus, which will come into contact with the test solutions should be made entirely of glass or other chemically inert material. If other materials are used it has to be demonstrated that the availability of the test substance is not affected. The items should be thoroughly washed to ensure that no organic or inorganic contaminants may interfere with the algal growth or composition of the test solutions.

13. The test vessels will normally be glass flasks of sufficient volume to obtain a surface volume ratio of at least 0.15 cm^2/ml (e.g. 250 ml conical flasks are suitable when the volume of the test solution is 100 ml).

14. In addition some or all of the following equipment will be required:

- Culturing apparatus: a cabinet or chamber is recommended, in which a temperature in the range of 21-24°C can be maintained at ± 2°C.

- Light measurement instruments: It is important to note that the method of measurement of light intensity, and in particular the type of receptor (collector), will affect the measured value. Measurements should preferably be made using a spherical (4 π) receptor (which responds to direct and reflected light from all angles above and below the plane of measurement). If a cosine (2 π) receptor is used (which responds to light from all angles above the measurement plane), measurements should be made of the direct and reflected light and the two values added up.

- Apparatus to determine algal biomass are e.g. electronic particle counter, microscope with counting chamber, fluorimeter, spectrophotometer and colorimeter. A conversion factor between the unit measured and biomass concentration (mg/l) should be known. In order to provide useful measurements at low cell concentrations when using a spectrophotometer, it may be necessary to use cuvettes with a light path of at least 4 cm.

Test organisms

15. Several species of non-attached microalgae and cyanobacteria may be used. The strains listed in Annex 2 have been shown to be suitable using the test procedure specified in this Guideline:

16. If other species are used, the strain should be reported. It has to be confirmed that exponential growth of the selected test alga can be maintained throughout the test period under the prevailing conditions.

Test medium

17. Two alternative test media, the OECD and the AAP medium are recommended. The composition of these media is shown in Annex 3. Note that the initial pH value and the buffering capacity (regulating pH increase) of the two media are different. Therefore the result of the test may be different with the two media, particularly with ionising substances.

18. Modification of the test media may be necessary for certain purposes, e.g. testing of metals and chelating agents or testing at different pH values. Use of modified media must be described in detail and justified.

TEST DESIGN

Initial cell concentration

19. The initial cell concentration in the test cultures must be sufficiently low to allow exponential growth in the control culture throughout the incubation period without risk of nutrient depletion, and in any case not exceeding a biomass density of 0.5 mg/l as dry weight. The following initial cell concentrations are recommended:

Pseudokirchneriella subcapitata:	$5x10^3$ - 10^4	cells/ml
Scenedesmus subspicatus	$2\text{-}5x10^3$	cells/ml
Navicula pelliculosa	10^4	cells/ml
Anabaena flos aquae	10^4	cells/ml

Synechococcus leopoldensis $5x10^4$ - 10^5 cells/ml

Concentrations of test substance

20. The concentration range in which effects are likely to occur may be determined on the basis of results from range-finding tests. For the final test, at least five concentrations arranged in a geometric series should be selected. The concentration series should preferably cover the range causing 0-90% inhibition of algal growth.

Replicates and controls

21. The test design should include three replicates at each test concentration and ideally twice that number of controls. If justified, and 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.

22. A separate set of test solutions may be prepared for analytical determinations of test substance concentrations (See paragraph 32).

23. When a solvent is used to solubilise the test substance, additional controls containing the solvent at the same concentration as used in the test cultures should be included in the test design.

PROCEDURE

Preparation of inoculum culture

24. In order to adapt the test alga to the test conditions, an inoculum culture in the test medium is prepared 2-3 days before start of the test. The algal biomass should be adjusted in order to allow exponential growth to prevail in the inoculum culture until test start. The inoculum culture shall be incubated under the same conditions as the test cultures. Measure the increase in biomass in the inoculum culture to ensure that growth is within the normal range for the test strain under the culturing conditions. An example of procedure for the culturing is described in Annex 4.

Preparation of test solutions

25. Test solutions of the chosen concentrations are usually prepared by mixing a stock solution of the test substance with test medium and inoculum culture. Stock solutions are normally prepared by dissolving the substance in test medium.

26. Solvents, e.g. acetone, t-butyl alcohol and dimethylsulfoxide, may be used as carriers to add substances of low water solubility to the test medium (1), (2). The concentration of solvent should not exceed 100 µl/l, and the same concentration of solvent should be added to all cultures in the test series. Separate controls with the same concentration of solvent have to be included in the test design.

Incubation

27. Cap the culture flasks with air-permeable stoppers The flasks are shaken and placed in the culturing apparatus. During the test it is necessary to keep the algae in suspension and to facilitate transfer of CO_2. To this end constant shaking or stirring should be used. The cultures should be maintained at a temperature in the range of 21 to 24°C, controlled at ± 2°C. It is recommended to place the flasks randomly in the incubator.

28. The surface where the cultures are incubated shall receive continuous, uniform fluorescent illumination of « cool-white » or « daylight » type. The light intensity at the level of the test solutions shall be in the range of 60-120 $\mu E/m^2/s$ when measured in the photosynthetically effective wavelength range of 400-700 nm using an appropriate receptor. (Note that strains of algae and cyanobacteria vary in their light requirements. The light intensity should be selected, within the recommended range, to suit the test organism used.) For light-measuring instruments calibrated in lux, an equivalent range of 6000-10000 lux is acceptable. Make sure that the illumination is uniform over the incubation area.

Measurements and analytical determinations

29. The algal biomass density in each flask is determined at least daily during the test period, which is normally 72 ± 2 hours for the recommended species. For slow-growing algae, extension of the exposure duration may be accepted as long as the validity criteria (see paragraph 10) are fulfilled. If measurements are made on small volumes removed from the test solution by pipette, these should not be replaced.

30. Measurement of biomass by manual cell counting by microscope or an electronic particle counter (cell counts and/or biovolume, the biovolume correlates directly with biomass) are preferred. Alternative techniques, eg. *in vitro* chlorophyll fluorescence (4), or optical density can be used providing a satisfactory correlation with biomass (dry weight mg/l) can be demonstrated over the range occurring in the test.

31. The pH of the solutions shall be measured at the beginning and at the end of the test.

32. Providing an analytical procedure for determination of the test substance in the concentration range used is available, the test solutions should be analysed to verify the initial concentrations and maintenance of the exposure concentrations during the test.

33. Analysis at the start and end of the test of a low and high test concentration and a concentration around the expected EC_{50} may be sufficient where it is likely that exposure concentrations will vary less than 20% from nominal values during the test. Analysis of all test concentrations at the start and end of the test is recommended where concentrations are unlikely to remain within 80-120 % of nominal. For volatile or strongly adsorbing test substances additional sampling for analysis at 24 hours intervals during the exposure period is recommended in order to better define the loss function. In all cases, determination of test substance concentrations need only be performed on one replicate vessel at each test concentration (or the contents of the vessels pooled by replicate).

34. Test media prepared specifically for analysis of exposure concentrations should be treated identical to those used for testing, i.e. they should be inoculated with algae and incubated under identical conditions. If analysis of the dissolved test substance concentration is required, it may be necessary to separate algae from the medium. Separation should preferably be made by centrifugation at a low g-force, sufficient to settle the algae.

35. 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. If the deviation from the nominal or measured initial concentration is greater than ± 20 %, analysis of the results should be based on average concentration during exposure.

36. The alga growth inhibition test is a more dynamic test system than most other short-term aquatic toxicity tests. As a consequence, the actual exposure concentrations may be difficult to define, especially for adsorbing substances tested at low concentrations. In such cases, disappearance of the substance from solution by adsorption to the increasing algal biomass does not mean that it is loosed from the test system.

When the result of the test is analysed, it should be checked whether a decrease in concentration of the test substance in the course of the test is accompanied by a decrease in growth inhibition. If not, it may be appropriate to base the analysis of the results on the initial (nominal or measured) concentrations. Expert judgement should be applied to determine the relevant exposure concentrations in the test, based on the physical and chemical properties of the test substance, results of analytical determinations and the growth response of the algae.

Other observations

37. Microscopic observation should be performed to verify a normal and healthy appearance of the inoculum culture and to observe any abnormal appearance of the algae (as may be caused by the exposure to the test substance) at the end of the test.

Limit test

38. 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 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

Plotting growth curves

39. Calculate preferably the equivalent biomass (dry weight) concentration from the indirect parameter measured. Alternatively, calculate cell numbers and use the cell number as a surrogate biomass ignoring variability in cell size.

40. Tabulate the estimated biomass concentrations in test cultures and controls together with the concentrations of test material and the times of measurement, recorded with a resolution of at least whole hours, to produce plots of growth curves. Both logarithmic scales and linear scales can be useful at this first stage, but logarithmic scales are mandatory and generally allow a better and more balanced (more symmetrical) presentation.

41. Using the plots, examine whether control cultures grow exponentially at the expected rate throughout the test. Examine all data points and the appearance of the graphs critically and check raw data and procedures for plausibility. Check in particular any data point that seems to deviate by a systematic error. If it is obvious that procedural mistakes can be identified and/or considered highly likely, the specific data point is marked as an outlier and not included in subsequent statistical analysis. (A zero algal concentration in 1 out of 2 or 3 replicate flasks is such an example of evident mistake which for example could be accidentally missing inoculation or improper cleaning of the one specific flask). Reasons for rejection of a datapoint as an outlier must be clearly stated in the test report. Accepted reasons are only (rare) procedural mistakes and not just bad precision. Statistical procedures for outlier identification are of limited use for this type of problem.and cannot replace expert judgement. Outliers (marked as such) should preferably be retained among the data points shown in any subsequent graphical or tabular data presentation.

Calculation of growth rates

42. The average specific growth rate for a specific period is calculated as the logarithmic increase in biomass from the equation:

$$\mu_{i-j} = \frac{\ln B_j - \ln B_i}{t_j - t_i} \ d^{-1}$$

where:

μ_{i-j} is the average specific growth rate from moment time i to j;
t_i is the moment time for the start of the period;
t_j is the moment time for the end of the period;
B_i is the biomass concentration at time *i*;
B_j is the biomass concentration at time *j*

43. Calculate average specific growth rate over the test duration (normally days 0-3), using the nominally inoculated biomass concentration as the starting value rather than a measured starting concentration, because in this way greater precision is normally obtained. Calculate also the average daily growth rates for each day during the course of the test (days 0-1, 1-2 and 2-3) and examine whether the growth rate remains constant (See validity criteria, paragraph 10). A lower growth rate on day one than the total average growth rate reveals a lag phase. While a lag phase can be minimized and practically eliminated in control cultures by proper propagation of the pre-culture, a lag phase in exposed cultures may indicate recovery after initial toxic stress or reduced exposure due to loss of test substance (including sorption onto the algal biomass) after initial exposure.

44. Calculate the percent inhibition of growth rate for each treatment replicate from the equation:

$$\%I = \frac{\mu_C - \mu_T}{\mu_C} \times 100$$

where:

%I: percent inhibition in average specific growth rate;
μ_0 mean value for μ in the control;
μ_T value for growth rate in the treatment.

Calculation of log-biomass integral

45. Optionally the log- biomass integral (area under the growth curve) may be used as an additional endpoint. This option may be attractive in situations with highly irregular growth in inhibited cultures and a focus on the high inhibition part of the concentration response curve i.e. EC_x.with high x value such as for determination of algicidal concentration levels (algal "kill" levels).

46. By utilising the log biomass integral, the effective test time (the same for all treatments) need not coincide with the last measurement, but can be any time shorter than that: The corresponding biomass estimate is found by interpolation between two adjacent data points if constant exponential growth prevails - or it is found by extrapolation from the last data point in the exponential growth region discarding the next datapoint.

47. Log biomass integral is calculated for each test culture from the equation:

$$A = \frac{\ln B_1 - \ln B_0}{2} \times t_1 + \frac{\ln B_1 + \ln B_2 - 2\ln B_0}{2} \times (t_2 - t_1) + ... + \frac{\ln B_{n-1} - 1 + \ln B_n - 2\ln B_0}{2} \times (t_n - t_{n-1})$$

where:

A = area;
B_0 = nominal biomass concentration at time t_0;
B_1 = measured biomass concentration at t_1;
B_n = measured biomass concentration at time t_n;
t_1 = time of first measurement after beginning of test;
t_n = time of n^{th} measurement after beginning of test

48. It is noted that for idealized conditions with constant exponential growth at all dose levels the two endpoints, average growth rate and log-biomass integral produce identical results. Generally only minor differences between the two growth inhibition endpoints can be expected and the data can be analyzed using the same statistical methods.

49. Calculate the percent inhibition of the log-biomass integral for each treatment replicate from the equation:

$$\%I = \frac{A_C - A_T}{A_C} \times 100$$

where:

%I: percent reduction in log-biomass integral;
A_C: mean value for log-biomass integral in the control;
A_T: value for log-biomass integral in the treatment.

Plotting concentration response curve

50. Plot, for each individual test flask, the percentage of inhibition against the logarithm of the test substance concentration and examine the plot closely, disregarding any such data point that was singled out as an outlier in the first phase. Fit a smooth line through the data points by eye or by computerized interpolation to get a first impression of the concentration response relationship, and then proceed with a more detailed method, preferably a computerized statistical method. Depending on the intended usage of data; the quality (precision) and amount of data as well as the availability of data analysis tools, it may be decided (and sometimes well justified)to stop the data analysis at this stage and simply read the key figures EC_{50} and EC_{10} (and/or EC_{20}) from the eye fitted curve (see also section below on stimulatory effects). Valid reasons for not using a statistical method may include:

- Data are too few and/or uncertain for computerized methods to produce any more reliable results than can be obtained by expert judgement - in such situations some computer programs may even fail to produce a reliable solution (iterations may not converge and etc.)
- Stimulatory growth responses cannot be handled adequately using available computer programs (see below).

Statistical procedures

51. 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 linearizing transformation of the response data - for instance into probit or logit or Weibul units (6), but non-linear regression directly on the data is a preferred technique that better handles unavoidable data irregularities and deviations from smooth distributions. Approaching either zero or total inhibition such irregularities may be magnified by the transformation, disturbing the analysis (6). Non-linear regression analysis is detailed in Annex 5.

52. Calculate by inverse estimation characteristic point estimates EC_x´s, and report as a minimum the EC_{50} and lower estimates (e.g. EC_{10} and EC_{20}).

53. For estimation of the LOEC, and hence the NOEC, for effects of the test substance on average growth rate, it is necessary to calculate the mean growth rate across replicates for each concentration and the pooled residual standard deviation, and this can be done using analysis of variance (ANOVA). The mean for each concentration must then be compared with the control mean using an appropriate multiple comparison method. Dunnett's or Williams' tests may be useful, (7), (8), (9), (10). It is necessary to check whether the ANOVA assumption of homogeneity of variance holds. It is recommended that this is done graphically rather than via a formal significance test (11); a suitable alternative is to run a Bartlett's test. If this assumption does not hold, then consideration should be given to transforming the data to homogenise variances prior to performing the ANOVA, or carrying out a weighted ANOVA.

54. Recent developments have led to a recommendation from the scientific community 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 algal test 10 to 20 % appears to be the appropriate range, and preferably both EC_{10} and EC_{20} should be reported.

Initial stimulation

55. Initial growth stimulation (negative inhibition) is sometimes observed. This can result from either hormesis ("toxic stimulation") or from addition of stimulating growth factors with the test material to the minimal medium used. Note that the addition of inorganic nutrients should not have any direct effect because the test medium should maintain a surplus of nutrients throughout the test. Ways of handling initial stimulation in data analysis are described in Annex 5.

Non toxic growth inhibition

56. Light absorbing test materials may give rise to a growth rate reduction because shading reduces the amount of available light. Such physical types of effects should be separated from toxic effects by modifying the test conditions and be reported separately. Guidance may be found in (1) and (2).

Test report

57. The test report must include the following:

Test substance:
- physical nature and relevant physiochemical properties;
- chemical identification data, including purity.

Test species:
- the strain, supplier or source and the culture conditions used.

Test conditions:

- date of start of the test and its duration;
- description of test design: test vessels, culture volumes, biomass density at the beginning of the test);
- composition of the medium;
- test design (e.g. number of replicates, number of test concentrations and geometric progression used);
- description of the preparation of test solutions, including use of solvents etc.
- culturing apparatus;
- light intensity and quality (source);
- temperature;
- concentrations tested: the nominal test concentrations and any results of analyses to determine the concentration of the test substance in the test vessels. The recovery efficiency of the method and the limit of quantification should be reported.;
- all deviations from this Guideline;
- method for determination of biomass density and evidence of correlation between the measured parameter and dry weight;

Results:
- pH values at the start and end of the test at all treatments;
- biomass concentration for each flask at each measuring point and method for measuring biomass concentration;
- growth curves (plot of biomass concentration versus time);
- calculated test endpoint(s); average growth rate (and log-biomass integral) for each treatment replicate, with mean values and variation coefficient for replicates;
- graphical presentation of the concentration/effect relationship;
- estimates of toxic endpoints e.g. EC_{50} , EC_{10} , EC_{20} LOEC and NOEC, and the statistical methods used for their determination;
- any other observed effects, e.g. morphological changes of the algae;
- discussion of the results, including any influence on the outcome of the test resulting from deviations from this Guideline.

LITERATURE

(1) ISO 1998: Water quality – Guidance for algal growth inhibition tests with poorly soluble materials, volatile compounds, metals and waster water. ISO/DIS 14442

(2) OECD 2000: Draft Guidance Document on Aquatic Toxicity Testing of Difficult Substances and mixtures. Environmental Health and Safety Publications. Series on Testing and Assessment, no. 23.

(3) ISO #: Water quality – Sampling – Part 16: General Guidance for Biotesting. ISO 5667-16.

(4) Mayer, P., Cuhel, R. and Nyholm, N. (1997). A simple in vitro fluorescence method for biomass measurements in algal growth inhibition tests. *Water Research* 31: 2525-2531.

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

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

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

(9) 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.

(10) Williams, D.A. (1972) The comparison of several dose levels with a zero dose control. Biometrics 28: 510-531.

(11) Draper, N.R. and Smith, H. (1981). Applied Regression Analysis, second edition. Wiley, New York.

(12) ISO 1993: Water quality – Algal growth inhibition test. ISO 8692

(13) EPA 1971: Algal Assay Procedure: Bottle Test. U.S. Environmental Protection Agency, Corvallis, OR.

(14) ASTM 1998: Standard Guide for Conducting Static 96-h Toxicity Tests with Microalgae. ASTM E 1218-97a.

ANNEX 1: DEFINITIONS

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

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 the text organism within a stated 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.

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.

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 2: STRAINS SHOWN TO BE SUITABLE FOR THE TEST

Green algae

- *Pseudokirchneriella subcapitata,* (formerly known as *Selenastrum capricornutum*) , ATCC 22662, CCAP 278/4, 61.81 SAG
- *Scenedesmus subspicatus,* 86.81 SAG

Diatoms

- *Navicula pelliculosa,* UTEX 664

Cyanobacteria

- *Anabaena flos-aquae,* UTEX 1444, ATCC 29413, CCAP 1403/13A
- *Synechococcus leopoldensis,* UTEX 625, CCAP 1405/1

Sources of Strains

The strains recommended are available in unialgal cultures from the following collections:

SAG: Collection of Algal Cultures
Inst. Plant Physiology
University of Göttingen
Nicholausberger Weg 18
D-3400 GÖTTINGEN
Germany

ATTC: American Type Culture Collection
10801 University Boulevard
Manassas
Virginia 20110-2209
USA

UTEX Culture Collection of Algae
Section of Molecular, Cellular and Developmental Biology
School of Biological Sciences
the University of Texas at Austin
AUSTIN TX 78712
USA.

CCAP, Culture Collection of Algae and Protozoa
Institute of Freshwater Ecology,
Windermere Laboratory
Far Sawrey, Amblerside
Cumbria LA22 0LP
UK

Specific Recommendations on Culturing and Handling of Recommended Test Species

Pseudokirchneriella subcapitata and *Scenedesmus subspicatus*

These green algae are generally easy to maintain in various culture media. Information on suitable media is available from the culture collections. The cells are normally solitary, and biomass measurements can easily be performed using an electronic particle counter or microscope.

Anabaena flos aquae

Various growth media may be used for keeping a stock culture. It is particularly important to avoid allowing the batch culture to go past log phase growth when renewing, recovery is difficult at this point.

Anabaena flos-aque develops aggregates of nested chains of cells. The size of these aggregates may vary with culturing conditions. It may be neccesary to break up these aggregates when microscope counting or an electronic particle counter is used for determination of biomass.

Sonication of sub-samples may be used to break up chains to reduce count variability and eye strain. Normally you should only have to sonicate for 15 to 30 seconds to break the chains into shorter lengths. Longer sonication may destroy the cells. Sonication intensity and duration must be identical for each treatment. Note that *Anabaena* used for testing and culturing should never be sonicated. Only cells which will be counted and discarded should be subjected to sonication.

Count enough fields on the hemocytometer (at least 100 cells), to help compensate for variability. This will improve reliability of microscopic density determinations.

An electronic particle counter can be used for determination of total cell volume of *Anabaena* after sonification.

Use a vortex mixer or similar appropriate method to make sure the algae used to inoculate test vessels is well mixed and homogeneous.

Test vessels should be placed on an orbital or reciprocate shaker table at about 150 revolutions per minute. The test vessels should also be removed daily and agitated to disintegrate algal clumps.

Synechococcus leopoldensis.

Various growth media may be used for keeping a stock culture. Information on suitable media is available from the culture collections.

Synechococcus leopoldensis grows as solitary rod-shaped cells. The cells are very small, which complicates the use of microscope counting or electronic particle counters for biomass measurements. *In vitro* fluorometric measurements are recommended.

Navicula pelliculosa

Various growth media may be used for keeping a stock culture. Information on suitable media is available from the culture collections. Note that silicate is required in the medium.

Navicula pelliculosa may form aggregates under certain growth conditions. Due to production of lipids the algal cells sometimes tend to accumulate in the surface film. Under those circumstances special measures have to be taken when sub-samples are taken for biomass determination in order to obtain representative samples. Vigorous shaking, e.g. using a vortex mixer may be required.

ANNEX 3: TEST MEDIA

One of the following two test media may be used:

OECD medium : Original medium of OECD TG 201, also according to ISO 8692 (12)
US. EPA medium AAP (13) also according to ASTM (14).

When preparing these media, reagent or analytical-grade chemicals should be used and deionised water.

Composition of The AAP-medium (US. EPA) and the OECD TG 201 medium.

Component	EPA		OECD	
	mg/l	mM	mg/l	mM
$NaHCO_3$	15,000	0,178550	50,00000	0,59516724
$NaNO_3$	25,500	0,300000		
NH_4Cl			15,00000	0,28037383
$MgCl_2 \cdot 6(H_2O)$	12,160	0,059804	12,00000	0,05901736
$CaCl_2 \cdot 2(H_2O)$	4,410	0,029984	18,00000	0,12238248
$MgSO_4 \cdot 7(H_2O)$	14,600	0,059232	15,00000	0,06085440
K_2HPO_4	1,044	0,005994	1,60000	0,00918590
$FeCl_3 \cdot 6(H_2O)$	0,160	0,000591	0,08000	0,00029595
$Na_2EDTA \cdot 2(H_2O)$	0,300	0,000806	0,10000	0,00026864
H_3BO_3	0,185500	0,003000	0,18500	0,00299159
$MnCl_2 \cdot 4(H_2O)$	0,415400	0,002099	0,41500	0,00209691
$ZnCl_2$	0,003270	0,000024	0,00300	0,00002201
$CoCl_2 \cdot 6(H_2O)$	0,001428	0,000006	0,00150	0,00000630
$Na_2MoO_4 \cdot 2(H_2O)$	0,007260	0,000030	0,00700	0,00002893
$CuCl_2.2(H_2O)$	0,000012	0,00000007	0,00001	0,00000006
pH	7,5		8,3	

In test with the diatom *Navicula pelliculosa* both media must be supplemented with $Na_2SiO_3 \cdot 9H_20$ to obtain a concentration of 1.4 mg Si/l.

The pH-values are adjusted to the specified values using solutions of hydrochloric acid or sodium hydroxide.

Element composition of test media

Element	EPA	OECD
	mg/l	mg/l
C	2,144	7,148
N	4,202	3,927
P	0,186	0,285
K	0,469	0,718
Na	11,044	13,704
Ca	1,202	4,905
Mg	2,909	2,913
Fe	0,033	0,017
Mn	0,115	0,115

ANNEX 4: EXAMPLE OF A PROCEDURE FOR THE CULTURING OF ALGAE

General observations

The purpose of culturing on the basis of the following procedure is to obtain algal cultures for toxicity tests.

Suitable methods must be used to ensure that the algal cultures are not infected with bacteria. Axenic cultures may be desirable but unialgal cultures must be established and used.

All operations must be carried out under sterile conditions in order to avoid contamination with bacteria and other algae.

Equipment and materials

See under Test Guideline: Apparatus.

Procedures for obtaining algal cultures

Preparation of nutrient solutions (media):

All nutrient salts of the medium are prepared as concentrated stock solutions and stored dark and cold. These solutions are sterilised by filtration or by autoclaving.

The medium is prepared by adding the correct amount of stock solution to sterile distilled water, taking care that no infections occur. For solid medium 0.8 per cent of agar is added.

Stock culture:

The stock cultures are small algal cultures that are regularly transferred to fresh medium to act as initial test material. If the cultures are not used regularly they are streaked out on sloped agar tubes. These are transferred to fresh medium at least once every two months.

The stock cultures are grown in conical flasks containing the appropriate medium (volume about 100 ml). When the algae are incubated at 20°C with continuous illumination, a weekly transfer is required.

During transfer an amount of "old" culture is transferred with sterile pipettes into a flask of fresh medium, so that with the fast-growing species the initial concentration is about 100 times smaller than in the old culture.

The growth rate of a species can be determined from the growth curve. If this is known, it is possible to estimate the density at which the culture should be transferred to new medium. This must be done before the culture reaches the death phase.

Pre-culture:

The pre-culture is intended to give an amount of algae suitable for the inoculation of test cultures. The pre-culture is incubated under the conditions of the test and used when still exponentially growing, normally after an incubation period of about 3 days. When the algal cultures contain deformed or abnormal cells, they must be discarded.

ANNEX 5: DATA ANALYSIS BY NONLINEAR REGRESSION

General considerations

The response is by nature a continuous or metric variable - a relative reduction of a process rate, here of biomass growth rate which is normalised by referencing to a non-exposed control. In the uncomplicated situation with a monotonous concentration response relationship, the controls grow at maximum rate μ_{max} and exposed cultures grow at rates less than or equal to μ_{max}. The relative response r then decreases monotonically from 1 to 0 while the corresponding inhibition increases from 0 to 100 per cent. This type of bioassay response is different from the more commonly known "categorical" type of response. With categorical data an EC_x typically relates to x per cent of a limited number of individuals in a population being visibly impaired, and the response of untreated controls is zero (or background) and therefore not influencing the relative response very much. Conversely, for metric variables the control response is non-zero, and the associated variability may significantly influence the relative response especially for small values of x) . High precision on the control response estimate is therefore important, and it is generally recommended to include more (rule of thumb twice as many) control replicates than test replicates, when planning the experiment. The variance distribution of the measured absolute responses for metric variables generally relates to the physical measurements (here measurements of algal biomass), and to variability between test units in handling and exposure conditions (e.g,, inoculation or light exposure), and curves can be fitted using simple minimum least squares as goodness of fit criterion. By contrast, for categorical types of responses, the dominant variance contribution may relate to tolerance or sensitivity differences between the individual organisms of the test population and thus be an intrinsic biological property of the batch of organisms. The data are often binomially distributed and curve-fits performed using maximum likelyhood estimation..

Regression analysis

The type of statistical method to be used for processing the data is a computerised regression analysis of the concentration - response relationship. The analysis aims at quantitatively describing the concentration response curve in the form of a mathematical regression function $Y = f(C)$. Used inversely $C = f^{-1}(Y)$, EC_x values, including the EC_{50}, EC_{10} and EC_{20}, and their 95% confidence limits can then be calculated. The advantage of a such analysis over a graphical estimation is in particular that it is objective, and allows the possibility of estimating confidence limits around the reported figures

Several simple mathematical functional forms have proved to successfully describe concentration - response relationships obtained in algal growth inhibition tests. Functions include for instance the logistic equation, the Weibul equation and the log normal distribution function, which are all sigmoid curves asymptotically approaching one for $C \rightarrow 0$ and zero for $C \rightarrow$ infinity. Note that the analysis can be a simple minimisation of sums of residual squares (assuming constant variance) or weighted squares if variance heterogeneity are compensated through weighting The functional relationship can be any suitable equation and be derived partly on a mechanistic basis with certain simplifying conditions. Now select an appropriate functional equation, $Y = f(C)$, and fit it to the data by non-linear regression. Use preferably the measurements from each individual flask rather than means of replicates, in order to extract as much information from the data as possible. If the variance is high, on the other hand, practical experience suggests that means of replicates may provide a more robust mathematical estimation less influenced by systematic errors in the data, than with each individual data point retained.

Plot the fitted curve and the measured data and examine whether the curve fit is appropriate. Analysis of residuals may be a particular helpful tool for this purpose. If the chosen functional relationship to fit the concentration response does not describe well the whole curve or some essential part of it, such as

the response at low concentrations, choose another curve fit option - e.g., a non-symmetrical curve like the Weibul function instead of a symmetrical one, as for example the logistic function. It may also be appropriate to make separate curve fits on parts of the curve such as the low inhibition part.

Calculate from the fitted equation (by "inverse estimation", $C = f^{-1}(Y)$, characteristic point estimates EC_x´s, and report as a minimum the EC_{50} and the EC_{10} and/or the EC_{20}. Experience from algal testing has shown that the precision of the test normally allows a reasonably accurate estimation at the 10 % inhibition level. The precision of an EC_{20} estimate is often considerably better than that of the EC_{10}, on the other hand, and sometimes the EC_{10} estimate is difficult to interpret because of growth stimulation. So while the EC_{10} is normally obtainable it may nevertheless be desired to report both figures EC_{10} and the $EC_{20.}$

The calculation of non-linear regression confidence intervals by inverse estimation is rather complex and not an available standard option in ordinary statistical computer program packages. Approximate confidence limits may be obtained with standard non-linear regression programs with reparameterisation , which involves rewriting the mathematical equation with the desired point estimates, e.g. the EC_{10} and the EC_{50} as the parameters to be estimated. (Let the function be I = f (α, β, Concentration) and utilize the definition relationships f (α, β, EC_{10}) = 0.1 and f (α, β, EC_{50}) = 0.5 to substitute f (α, β, concentration) with an equivalent function g(EC_{10}, EC_{50}, concentration).

An example with the Weibul equation is:

Weibul functional relationship: relative response, $r = \exp(-k \cdot C^{\eta})$
At 50 % inhibition, $r = 0.5 = \exp(-k \cdot EC_{50}^{\eta})$ and at 10% inhibition, $0.9 = \exp(- k \cdot EC_{10}^{\eta})$

Taking the natural logarithm twice:

$$\ln(\ln(2)) = \ln(k) + \eta \cdot \ln(EC_{50})$$
$$\ln(-\ln(0.9) = \ln k + \eta \cdot \ln(EC_{10})$$

Solving these for k and η

$$\eta = 1.8839/ \{\ln(EC_{50}/EC_{10})\}$$
$$k = 0.6931/ (EC_{50})^{\eta}$$

Replace now η and k in the Weibul equation with the corresponding right sides of the above equations and let EC_{10} and EC_{50} be the unknown parameters to be estimated using a standard non-linear regression routine. Most such routines include approximate confidence intervals for the parameters estimated.

A more direct calculation is performed by retaining the original equation and calculating the variance and co-variance using a Taylor expansion around the means of r_i and $r_{0.}$ (Subscript i refers to concentration level i and subscript 0 to the controls.)

$$\xi_i = \text{Relative response} = r_i/r_0 = 1 - \text{Inhibition} = f(C_i)$$

with a variance:

$$\text{Var}(\xi_i) = \text{Var}(r_i/r_0) \cong (\partial \xi_i / \partial r_i)^2 \cdot \text{Var}(r_i) + (\partial \xi_i / \partial r_0)^2 \cdot \text{Var}(r_0)$$

Since $(\partial \xi_i / \partial r_i) = 1/r_0$ and $(\partial \xi_i / \partial r_0) = r_i/r_0^2$
and with normally distributed data and m_i and m_0 replicates: $Var(r_i) = \sigma^2/m_i$
the total variance and covariance of the relative response ξ_i thus become:

$$Var(\xi_i) = \sigma^2/(r_0^2 \cdot m_i) + r_i^2 \cdot \sigma^2/r_0^4 \cdot m_0$$
$$Cov(r_i, r_j) = (r_i \cdot r_j \cdot \sigma^2) / (r_0^4 \cdot m_0)$$

EC values are;

$$EC \cong f^{-1}(\xi(C)) = g(\xi(C))$$

And with only 2 parameters in f (and g) α and β , the Variance of an EC estimate, EC_x will be approximately:

$$Var(EC_x) = (\partial g/\partial \alpha)^2 \cdot Var(\alpha) + (\partial g/\partial \beta)^2 \cdot Var(\beta) + 2 \cdot (\partial g \partial g/\partial \alpha \partial \beta) \cdot Cov(\alpha, \beta)$$

Still assuming that the estimated EC´s are normally distributed a 95% confidence interval for an ECx becomes

$$C.I. = [EC - 1.96 \cdot (Var(EC))^{1/2}; EC + 1.96 \cdot (Var(EC))^{1/2}]$$

Normalising by the control response thus gives rise to a rather complicated variance structure. Moreover, the underlying experimental variance need not be constant at all - a proportional variance is probably more typical than a constant absolute variance, and a weighted regression is therefore advantageously carried out. Weighting factors for such an analysis are normally assumed inversely proportional to the variance:

$$W_i = 1/Var(r_i)$$

Many regression programs allow the option of weighted regression analysis with weighting factors listed in a table. Conveniently weighting factors should be normalised by multiplying them by $n/\Sigma W_i$ (n is the number of datapoints) so their sum be one.

It pays making an effort obtaining a precise control response estimate. Note also that confidence limits calculated for this type of estimate can vary somewhat depending on the assumptions and approximations made. Confidence intervals are therefore intended used primarily as guidance information on the error of the reported point estimates A plot of exposure concentration versus percent inhibition and its confidence bands is a particularly useful illustration of the precision of the experiment. (Note that the axis of this error plot has been interchanged from the normal concentration- response type plot.)

Recently "boot strap methods" have become popular. Such methods use the measured data and a random number generator directed frequent resampling to estimate an empirical variance distribution.

Recent developments have led to a recommendation from the scientific community of abandoning the concept of the ANOVA derived NOEC and replacing it with regression based point estimates EC_x. For this algal test 10% seems an appropriate x., although 20 % also has supporters. The use of continuous threshold function models (e.g. Kooijman et al. 1996) is a recently proposed or alternative. Such models assume no effects at concentrations below a certain threshold that is estimated by extrapolation of the response concentration relationship to intercept the concentration axis. This

extrapolation is done using a simple continuous (but not differentiable) function with no tolerance distribution statistics or other probabilistic elements involved, and the axis intercept may be defined as an empirical EC0. It appears, however, that at present definitions and concepts are not sufficiently harmonised and clarified, for consensus interpretation of such EC0 estimates.

Reference

Kooijman, S.A.L.M.; Hanstveit, A.O.; Nyholm, N. (1996): No-effect concentrations in algal growth inhibition tests. *Water Research*, 30, 1625-1632.