C.3. ALGAL INHIBITION TEST

1. METHOD

1.1. INTRODUCTION

The purpose of this test is to determine the effects of a substance on the growth of a unicellular green algal species. Relatively brief (72 hours) tests can assess effects over several generations. This method can be adapted for use with several unicellular algal species, in which case a description of the method used must be provided with the test report.

This method is most easily applied to water-soluble substances which, under the conditions of the test, are likely to remain in the water.

The method can be used for substances that do not interfere directly with the measurement of algal growth.

It is desirable to have, as far as possible, information on the water solubility, vapour pressure, chemical stability, dissociation constants and biodegradability of the substance before starting the test.

Additional information (for instance structural formula, degree of purity, nature and percentage of significant impurities, presence and amount of additives, and n-octanol/water partition coefficient) should be taken into consideration in both the planning of the test and interpretation of the results.

1.2. DEFINITIONS AND UNITS

Cell density: the number of cells per rnillilitre;

Growth: the increase in cell density over the test period;

Growth rate: the increase in cell density per unit time;

EC_{50}: in this method, that concentration of test substance which results in a 50 % reduction in either growth (E_bC_{50}) or growth rate (E_rC_{50}) relative to the control;

NOEC (no observed effect concentration): in this method, the highest tested concentration at which no significant inhibition of growth is observed relative to the control.

All concentrations of the test substance are given in weight by volume (milligrams per litre). They may also be expressed as weight by weight ($mg.kg^{-1}$).

1.3. REFERENCE SUBSTANCES

A reference substance may be tested as a means of demonstrating that under the laboratory test conditions the sensitivity of the test species has not changed significantly.

If a reference substance is used, the results should be given in the test report. Potassium dichromate can be used as a reference substance, but its colour may affect the light quality and intensity available to the cells and also the spectrophotometric determinations if used. Potassium dichromate has been used in an international inter-laboratory test (see ref. (3) and Appendix 2).

1.4. PRINCIPLE OF THE TEST METHOD

A limit test may be performed at 100 mg per litre in order to demonstrate that the EC_{50} is greater than this concentration.

Exponentially-growing cultures of selected green algae are exposed to various concentrations of the test substance over several generations under defined conditions.

The test solutions are incubated for a period of 72 hours, during which the cell density in each solution is measured at least every 24 hours. The inhibition of growth in relation to a control culture is determined.

1.5. QUALITY CRITERIA

The quality criteria shall apply to the limit test as well as the full test method.

The cell density in the control cultures should have increased by a factor of at least 16 within three days.

The concentrations of the test substance shall be maintained to within 80 % of the initial concentrations/ throughout a time corresponding to the duration of the test.

For substances which dissolve easily in the test medium, yielding stable solutions i.e. those which will not to any significant extent volatilize, degrade, hydrolyze or adsorb, the initial concentration can be taken as being equivalent to the nominal concentration. Evidence shall be presented that the concentrations have been maintained throughout the test and that the quality criteria have been satisfied.

For substances that are:

(i) poorly soluble in the test medium, or
(ii) capable of forming stable emulsions or dispersions, or
(ii) not stable in aqueous solutions,

the initial concentration shall be taken as the concentration measured at the start of the test. The concentration shall be determined after a period of equilibration.

In any of these cases, further measurements must be made during the test to confirm the actual exposure concentrations or that the quality criteria have been met.

It is recognized that significant amounts of the test substance may be incorporated into the algal biomass during the period of the test. Therefore, for the purpose of demonstrating compliance with the above quality criteria, both the amount of the substance incorporated into the algal biomass and the substance in solution (or, if not technically possible measured in the water column) should be taken into account. However, as determination of the substance concentration in the algal biomass may pose significant technical problems, compliance with the quality criteria may be demonstrated by running a test vessel at the highest substance concentration but without algae and measuring concentrations in solution (or, if not technically possible in the water column) at the beginning and at the end of the test period.

1.6. DESCRIPTION OF THE TEST PROCEDURE

1.6.1. Reagents

1.6.1.1. Solutions of test substances

Stock solutions of the required strength are prepared by dissolving the substance in deionized water or water according to 1.6.1.2.

The chosen test concentrations are prepared by adding suitable aliquots to algal pre-cultures (see Appendix 1). Substances should normally only be tested up to the limit of solubility. For some substances (e.g. substances having low solubility in water, or high P_{ow} or those forming stable dispersion rather than true solution in water), it is acceptable to run a test concentration above the solubility limit of the substance to ensure that the maximum soluble stable concentration has been obtained. It is important, however, that this concentration will not otherwise disturb the test system (e.g. film of the substance on the water surface preventing the oxygenation of the water, etc.).

Ultrasonic dispersion, organic solvents, emulsifiers or dispersants may be used as an aid to prepare stock solutions of substances with low aqueous solubility or to help to disperse these substances in the test medium. When such auxiliary substances are used, all test concentrations should contain the same amount of auxiliary substances, and additional controls should be exposed to the same concentration of the auxiliary substance as that used in the test series. The concentration of such auxiliaries should be minimized, but in no case should exceed 100 mg per litre in the test medium.

The test should be carried out without adjustment of the pH. If there is evidence of marked change in the pH, it is advised that the test should be repeated with pH adjustment and the results reported. In that case, the pH value of the stock solution should be adjusted to the pH value of the solution water unless there are specific reasons not to do so. HCl and NaOH are preferred for this purpose. This pH adjustment should be made in such a way that the concentration of test substance in the stock solution is not changed to any significant extent. Should any chemical reaction or physical precipitation of the test compound be caused by the adjustment, this should be reported.

1.6.1.2. Test medium

The water should be good-quality distilled water, or deionized water with a conductivity less than 5 $\mu S.cm^{-1}$. The apparatus for distillation of water must not contain any part made of copper.

The following medium is recommended.

Four stock solutions are prepared, according to the following table. The stock solutions are sterilised by membrane filtration or by autoclaving, and stored in the dark at 4 °C. Stock solution no.4 should be sterilised only by membrane filtration. These stock solutions are diluted to achieve the final nutrient concentrations in the test solutions.

Nutrient	Concentration in the stock solution	Final concentration in the test solution
Stock solution 1: macro-nutrients		
NH_4Cl	1,5 g/l	15 mg/l
$MgCl_2.6H_2O$	1,2 g/l	12mg/l
$CaCl_2.2H_2O$	1,8 g/l	18 mg/l
$MgSO_4.7H_2O$	1,5 g/l	15 mg/l
KH_2PO_4	0,16 g/l	1,6 mg/l
Stock solution 2: Fe-EDTA		
$FeCl_3.6H_2O$	80 mg/l	0,08 mg/l
$Na_2EDTA.2H_2O$	100 mg/l	0,1 mg/l
Stock solution 3: trace elements		
H_3BO_3	185 mg/l	0,185 mg/l
$MnCl_2.4H_2O$	415 mg/l	0,415 mg/l
$ZnCl_2$	3 mg/l	3×10^{-3} mg/l
$CoCl_2.6H_2O$	1,5 mg/l	$1,5 \times 10^{-3}$ mg/l
$CuCl_2.2H_2O$	0,01 mg/l	10^{-5} mg/l
$Na_2MoO_4.2H_2O$	7 mg/l	7×10^{-3}mg/l
Stock solution 4: $NaHCO_3$		
$NaHCO_3$	50 g/l	50 mg/l

The pH of the medium after equilibration with air is approximately 8.

1.6.2. Apparatus

-Normal laboratory equipment,

-Test flasks of suitable volume (e.g. 250 ml conical flasks are suitable when the volume of the test solution is 100 ml). All test flasks should be identical as regards to material and dimensions.

-Culturing apparatus: cabinet or chamber in which a temperature in the range 21 °C to 25 °C can be maintained at ± 2 °C, and continuous uniform illumination provided in the spectral range 400 to 700 nm. If algae in control cultures have achieved the recommended growth rates, it can be assumed that the conditions for growth, including light intensity, have been adequate.

It is recommended to use, at the average level of the test solutions, a light intensity in the range 60 to 120 $\mu E.m^{-2}.s^{-1}$ (35 to 70 x 10^{18} $photons.m^{-2}.s^{-1}$) when measured in the range 400 to 700 nm using an appropriate receptor. For light measuring instruments calibrated in lux, an equivalent range of 6000 to 10000 lx is acceptable.

The light intensity could be obtained using four to seven 30 W fluorescent lamps of the universal white type (colour temperature of approximately 4300 K), at a distance of 0,35 m from the algal culture.

-Cell density measurements should be made using a direct counting method of living cells, e.g. a microscope with counting chambers. However, other procedures (photometry, turbidimetry,...) may be used if sufficiently sensitive and if shown to be sufficiently well correlated with cell density .

1..6.3. Test organisms

It is suggested that the species of green algae used be a fast-growing species that is convenient for culturing and testing. The following species are preferred:

-*Selenastrum capricornutum* , e.g. ATCC 22662 or CCAP 278/4,

-*Scenedesmus subspicatus*, e.g. 86.81 SAG,

Note:

ATCC = American Type Culture Collection (U.S.A.)

CCAP = Culture Centre of Algae and Protozoa (U.K.)

SAG = Collection of algal culture (Gottingen, F.R.G.)

If other species are used, the strain should be reported.

1.6.4. Test procedure

The concentration range in which effects are likely to occur is determined on the basis of results from range-finding tests.

The two measures of growth (biomass and growth rate) may result in widely disparate measures of growth inhibition; both should be used in the range finding test to ensure that the geometric progression of concentrations will allow estimation of both the E_bC_{50} and the E_rC_{50}.

Initial cell density

It is recommended that the initial cell density in the test cultures be approximately 10^4 cells/ml for *Selenastrum capricornutum* and *Scenedesmus subspicatus*. When other species are used the biomass should be comparable.

Concentrations of test substance

For the test, at least five concentrations are made up in a geometric series at a concentration ratio not exceeding 2,2. The lowest concentration tested should have no observed effect on the growth of the algae. The highest concentration tested should inhibit growth by at least 50 % relative to the control and, preferably, stop growth completely.

Replicates and controls

The test design should include three replicates at each test concentration. Three controls without test substance are run and, if relevant, three controls containing the auxiliary substance are also run. If justified, the test design may be altered to increase the number of concentrations and reduce the number of replicates per concentration.

Performance of the test

Test cultures containing the desired concentrations of test substance and the desired quantity of algal inoculum are prepared by adding aliquots of stock solutions of the test substance to suitable amounts of algal pre-cultures (see Appendix 1).

The culture flasks are shaken and placed in the culturing apparatus. The algal cells are kept in suspension by shaking, stirring or bubbling with air, in order to improve gas exchange and reduce pH variation in the test solutions. The cultures should be maintained at a temperature in the range of 21 to 25 °C, controlled at ± 2 °C.

The cell density in each flask is determined at least at 24, 48 and 72 hours after the start of the test. Filtered algal medium containing the appropriate concentration of the test chemical is used to determine the background when using cell density measurements other than direct counting methods.

The pH is measured at the beginning of the test and at 72 hours.

The pH of the controls should not normally deviate by more than 1,5 units during the test.

Testing volatile substances

There is to date no generally accepted way to test volatile substances. When a substance is known to have a tendency to vaporize, closed test flasks with increased head-space may be used. The possibility of shortage of CO_2 should be considered when calculating the head-space of the closed flasks. Variations to this method have been proposed (see reference (4)).

Attempts should be made to determine the amount of the substance which remains in solution, and extreme caution is advised when interpreting results of tests with volatile chemicals using closed systems.

Limit test

Using the procedures described in this method, a limit test may be performed at 100 mg per litre in order to demonstrate that the EC_{50} is greater than this concentration.

If the nature of the substance is such that a concentration of 100 mg per litre in the test water cannot be attained, the limit test should be performed at a concentration equal to the solubility of the substance (or the maximum concentration forming a stable dispersion) in the medium used (see also point 1.6.1.1).

The limit test should be performed at least in triplicate, with the same number of controls. The two measures of growth (biomass and growth rate) should be used for the limit test.

If, in a limit test, a mean decrease of 25 % or more is found in either biomass or growth rate between the limit test and the control, a full test should be carried out.

2. **DATA AND EVALUATION**

The measured cell density in the test cultures and controls are tabulated together with the concentrations of the test substance and the times of measurements. The mean value of the cell density for each test substance concentration and for the controls is plotted against time (0-72 h) to produce growth curves.

To determine the concentration/ effect relationship, the two following approaches should be used. Some substances can stimulate the growth at low concentrations. Only data points indicating inhibition between 0 and 100 % should be considered.

2.1. COMPARISON OF AREAS UNDER THE GROWTH CURVES

The area between the growth curves and the horizontal line $N = N_0$ may be calculated according to the formula:

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

where

A = area,

N_o = number of cells/ml at time t_0 (beginning of the test), $_o$,

N_1 = measured number of cells/ml at t_1,

N_n = measured number of cells/ml at time,

t_l = time of first measurement after beginning of test,

t_n = time of n_{th} measurement after beginning of test.

n = number of measurements taken after the beginning of the test.

The percentage inhibition of the cell growth at each test substance concentration (I_A) is calculated according to the formula:

$$I_A = (A_c - A_t) / A_c \times 100$$

where

A_c = area between the control growth curve and the horizontal line $N = N_o$.

A_t = area between the growth curve at the concentration t and the horizontal line $N = N_o$.

I_A values are plotted on semilogarithmic paper or on semilogarithmic probit paper against the corresponding concentrations. If plotted on probit paper, the points are fitted by a straight line, either by eye or by a computed regression.

The EC_{50} is estimated from the regression line by reading off the concentration that is equivalent to a 50 % inhibition (I_A = 50%). To denote this value unambiguously in relation to this method of calculation, it is proposed to use the symbol E_bC_{50}. It is essential that the E_bC_{50} is quoted with the appropriate exposure period, e.g. E_bC_{50} (0-72h).

2.2. COMPARISON OF GROWTH RATES

The average specific growth rate (μ) for exponentially growing cultures can be calculated as

$$\mu = (\ln N_n - \ln N_0) / (t_n - t_0)$$

where t_o is the time at the beginning of the test.

Alternatively, the average specific growth rate may be derived from the slope of the regression line in a plot of ln N *versus* time.

The percentage inhibition of specific growth rate at each test substance concentration ($I_\mu t$) is calculated according to the formula:

$$I_\mu t = (\mu_c - \mu_t) / \mu_c \times 100$$

where

μ_c = mean control specific growth rate

μ_t = mean specific growth rate for the test concentration t

The percentage reduction in average specific growth rate at each test substance concentration compared to the control value is plotted against the logarithm of the concentration. The EC_{50} may be read from the resulting graph. To denote unambiguously the EC_{50} derived by this method it is proposed to use the symbol E_rC_{50}. The times of measurement must be indicated, e.g. if the value relates to times 0 and 72 hours, the symbol becomes E_rC_{50} (0-72h).

Note: specific growth rate is a logarithmic term, and small changes in growth rate may lead to great changes in biomass. E_bC and E_rC values are therefore not numerically comparable.

2.3. CALCULATION OF THE NOEC

The No Observed Effect Concentration is determined by a suitable statistical procedure for multisample comparison (e.g. analysis of variance and Dunnett's test), using the individual replicates values of the areas under the growth curves A (see point 2.1) or the specific growth rates μ (see point 2.2).

3. **REPORTING**

The test report shall, if possible, include the following information:

-test substance: chemical identification data;

-test organisms: origin, laboratory culture, strain number, method of cultivation;

-test conditions:

- -date of the start and the end of the test and its duration,
- -temperature,
- -composition of medium,
- -culturing apparatus,
- -pH of solutions at the start and end of the test (an explanation should be provided if pH deviations of more than 1,5 unit are observed),
- -vehicle and method used for solubilizing the test substance and concentration of the vehicle in the test solutions,
- -light intensity and quality,
- -concentrations tested (measured or nominal).

-results:

- -cell density for each flask at each measuring point and method for measuring cell density,
- -mean values of cell density,
- -growth curves,
- -graphical presentation of the concentration effect relationship,
- -EC values and method of calculation,
- -NOEC,
- -other observed effects.

4. **REFERENCES**

(1) OECD, Paris, 1981, Test Guideline 201, Decision of the Council C(81) 30 Final.

(2) Umweltbundesamt, Berlin, 1984, Verfahrensvorschlag 'Hemmung der Zellvermehrung bei der Grünalge Scenedesmus subspicatus', in: Rudolph/Boje: bkotoxikologie, ecomed, Landsberg, 1986.

(2) ISO 8692- Water quality -Fresh water algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum.*

(4) S.Galassi and M.Vighi -Chemosphere, 1981, vol.10, 1123-1126.

Appendix 1

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 should be used to ensure that the algal cultures are not infected with bacteria (ISO 4833). Axenic cultures may be desirable but unialgal cultures are essential.

All operations should be carried out under sterile conditions in order to avoid contamination with bacteria and, other algae. Contaminated cultures should be rejected.

Procedures for obtaining algal cultures

Preparation of nutrient solutions (media):

The medium can be prepared by diluting concentrated stock solutions of nutrients. For solid medium, 0,8 % of agar is added. The medium used should be sterile. Sterilisation by autoclaving may lead to a loss of NH_3.

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 three days. When the algal cultures contain deformed or abnormal cells, they must be discarded.

Appendix 2

The ISO 8692 -Water quality -Fresh water algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum* reports the following results for an inter-laboratory test among 16 laboratories, testing potassium dichromate:

	Means (mg/l)	Range (mg/l)
E_rC_{50} (0 - 72h)	0,84	0,60 to 1,03
E_bC_{50} (0 - 72 h)	0,53	0,20 to 0,75