

**"Alga, Growth Inhibition Test"****1. INTRODUCTORY INFORMATION****• Prerequisites**

- Water solubility
- Vapour pressure

**• Guidance information**

- Structural formula
- Purity of the substance
- Chemical stability in water and light
- Methods of analysis for quantification of the substance in water
- pK<sub>a</sub> value
- n-Octanol/water partition coefficient
- Results of a ready biodegradability test (see Test Guideline 301)

**• Qualifying statements**

- This guideline is suitable for a number of fresh-water green algae.
- 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 substances with limited solubility in the test medium, it may not be possible to quantitatively determine the EC 50 (see Definitions, below).
- This guideline can be used for substances that do not interfere directly with the measurement of algal growth.

**• Standard documents**

See Section 4, Literature.

**2. M E T H O D****A. INTRODUCTION, PURPOSE, SCOPE, RELEVANCE, APPLICATION AND LIMITS OF TEST**

The purpose of this test is to determine the effects of a substance on the growth of a unicellular green algal species. Relatively brief tests can assess effects over several generations. This guideline 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.

- D e f i n i t i o n s

Cell concentration is the number of cells per ml.

Growth is the increase in cell concentration over the test period.

Growth rate is the increase in cell concentration per unit of time.

EC 50 in this guideline is that concentration of test substance which results in a 50 per cent reduction in either growth or growth rate relative to the control.

NOEC (no observed effect concentration) in this guideline is the highest concentration tested at which the measured parameter(s) show(s) no significant inhibition of growth relative to control values.

- R e f e r e n c e   s u b s t a n c e s

A reference substance may be tested as a means of detecting unsatisfactory test conditions. If a reference substance is used, the results should be given in the test report. Potassium dichromate can be used as a reference substance.

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- Principle of the test method

Exponentially-growing cultures of selected green algae are exposed to various concentrations of the test substance over several generations under defined conditions. The inhibition of growth in relation to a control culture is determined over a fixed period.

- Conditions for the validity of the test

- The cell concentration in the control cultures should have increased by a factor of at least 16 within three days.
- Disappearance of the test substance from the water into the biomass does not necessarily invalidate the test.

### B. DESCRIPTION OF THE TEST PROCEDURE

- Preparations

*Equipment*

- 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).
- Culturing apparatus: a cabinet or chamber is recommended in which a temperature in the range 21 to 25°C can be maintained at  $\pm 2^\circ\text{C}$  and continuous uniform illumination provided with a quantum flux of  $0.72 \times 10^{20}$  photons/m<sup>2</sup>s  $\pm 20$  per cent\* in the spectral range 400-700 nm.

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\* A quantum flux of  $0.72 \times 10^{20}$  photons/m<sup>2</sup>s equals 120  $\mu\text{E/m}^2\text{s}$ . This quantum flux can be obtained with universal white-type fluorescent lamps [light-temperature of approximately 4200K] yielding approximately 8000 Lux measured with a spherical collector.

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- Apparatus to determine cell concentrations, e.g. electronic particle counter, microscope with counting chamber, fluorimeter, spectrophotometer, colorimeter [Note: 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].

***Algal medium***

The following medium is recommended:

NH <sub>4</sub> Cl	15	mg/l
MgCl <sub>2</sub> .6H <sub>2</sub> O	12	mg/l
CaCl <sub>2</sub> .2H <sub>2</sub> O	18	mg/l
MgSO <sub>4</sub> .7H <sub>2</sub> O	15	mg/l
KH <sub>2</sub> PO <sub>4</sub>	1.6	mg/l
FeCl <sub>3</sub> .6H <sub>2</sub> O	0.08	mg/l
Na <sub>2</sub> EDTA.2H <sub>2</sub> O	0.1	mg/l
H <sub>3</sub> BO <sub>3</sub>	0.185	mg/l
MnCl <sub>2</sub> .4H <sub>2</sub> O	0.415	mg/l
ZnCl <sub>2</sub>	3x10 <sup>-3</sup>	mg/l
CoCl <sub>2</sub> .6H <sub>2</sub> O	1.5x10 <sup>-3</sup>	mg/l
CuCl <sub>2</sub> .2H <sub>2</sub> O	10 <sup>-5</sup>	mg/l
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	7x10 <sup>-3</sup>	mg/l
NaHCO <sub>3</sub>	50	mg/l

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

The use of other media is not precluded by the above recommendation, provided, however, that following limits of essential constituents are respected:

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P	≤ 0.7	mg/l
N	≤ 10	mg/l
chelators	≤ 10 <sup>-3</sup>	mmol/l
hardness (Ca + Mg)	≤ 0.6	mmol/l

The recommended medium and the medium given in reference (6) meet this requirement.

- Experimental organisms

### *Selection of species*

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 considered suitable:

- *Selenastrum capricornutum* ATCC 22662
- *Scenedesmus subspicatus* 86.81 SAG
- *Chlorella vulgaris* CCAP 211/11b

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

- Test design

### *Initial cell concentration*

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

### *Concentrations of test substance*

The concentration range in which effects are likely to occur is determined on the basis of results from range-finding tests. For the test, at least five concentrations arranged in a geometric series, should be selected. The lowest concentration tested should have no observed effect on the growth of the algae.

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The highest concentration tested should inhibit growth by at least 50 per cent relative to the control and, preferably, stop growth completely.

### *Replicates and controls*

The test design should include preferably three replicates at each test concentration and ideally twice that number of controls. If justified the test design may be altered to increase the number of concentrations and reduce the number of replicates per concentration.

When a vehicle is used to solubilise the test substance additional controls containing the vehicle at the highest concentration used in the test cultures should be included in the test design.

- P e r f o r m a n c e o f t h e t e s t

This section contains guidance for the testing of readily soluble and poorly soluble substances and of volatile substances.

(1) Testing readily water-soluble substances

Test cultures containing the desired concentrations of test substance and the desired quantity of algal inoculum are prepared by diluting with filtered algal medium aliquots of stock solutions of the test substance and of algal suspension.

The culture 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<sub>2</sub>. To this end shaking, stirring or aeration may be used. The cultures should be maintained at a temperature in the range of 21 to 25°C, controlled at ±2°C.

The cell concentration in each flask is determined at least at 24, 48 and 72 hours after the start of the test. Filtered algal medium is used to determine the background when using particle counters or as a blank when using spectrophotometers.

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The pH is measured at the beginning of the test and at 72 hours. The pH of the solutions should not normally deviate by more than one unit during the test.

### (2) Testing substances with limited water solubility

When the solubility of the test substance is of the order of the highest concentration used in the test, only slight deviations from the above procedure are necessary to make up the test solutions. A saturated solution may serve as the stock solution of the test substance. Another approach can be to dissolve the test substance at the desired concentration in the algal medium prior to the introduction of algal suspension.

Stock solutions of substances of low water-solubility may be prepared by mechanical dispersion or by the use of vehicles of low toxicity to algae, such as organic solvents, emulsifiers or dispersants. When a vehicle is used the concentration should not exceed 100 mg/l, and additional controls, in which the vehicle is incorporated at the highest concentration present in the test solutions, must be included in the test design.

### (3) 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 vaporise, closed test flasks with increased head-space may be used. Variations to this method have been proposed (see reference 11). 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.

## **3. DATA AND REPORTING**

### **Treatment of results**

The measured cell concentrations in the test cultures and controls are tabulated together with the concentrations of the test substance and the times of measurements.

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The mean value of the cell concentration for each test substance concentration and for the controls is plotted against time to produce growth curves.

To determine the concentration effect relationship one of the following recommended approaches can be used.

- (1) Comparison of areas under the growth curves

The area below the growth curves may be calculated according to the formula:

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

where

A = area

$N_0$  = nominal number of cells/ml at time  $t_0$

$N_1$  = measured number of cells/ml at  $t_1$

$N_n$  = measured number of cells/ml at time  $t_n$

$t_1$  = time of first measurement after beginning of test

$t_n$  = time of  $n^{\text{th}}$  measurement after beginning of test



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The percentage inhibition of the cell growth at each test substance concentration ( $I_A$ ) is calculated as the difference between the area under the control growth curve ( $A_c$ ) and the area under the growth curve at each test substance concentration ( $A_t$ ) as:

$$I_A = \frac{A_c - A_t}{A_c} \times 100$$

$I_A$  values are plotted on semilogarithmic paper or on semilogarithmic probit paper against the corresponding concentrations. The points if plotted on probit paper are fitted by a straight line by eye, or, when a log-normal distribution of values can be assumed, a computed regression line may be drawn.

An EC 50 value results from the intercept of the (regression) line with the parallel drawn to the abscissa at  $I_A = 50\%$ . To denote this value unambiguously in relation to this method of calculation it is proposed to use the symbol  $E_bC 50$ . In relation to this guideline which specifies measurements at 24, 48 and 72 hours, the symbol becomes  $E_bC 50 (0-72h)$ .

Other EC values, like  $E_bC 10$ , can also be derived from the plot of  $I_A$  versus log concentration.

### (2) Comparison of growth rates

The average specific growth rate ( $\mu$ ) for exponentially growing cultures can be calculated as

$$\mu = \frac{\ln N_n - \ln N_1}{t_n - t_1}$$

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 reduction in average growth rate at each test substance concentration compared to the control value is plotted against the logarithm of the concentration. The EC 50

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may be read from the resulting graph. To denote unambiguously the EC 50 derived by this method it is proposed to use the symbol  $E_tC_{50}$ . The times of measurement must be indicated, e.g. if the value relates to observation times 24 and 48 hours the symbol becomes  $E_tC_{50}$  (24-48h).

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

- Test report

The test report should 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 one unit are observed]
- vehicle and method used for solubilising the test substance and concentration of the vehicle in the test solutions
- light intensity and quality
- concentrations tested (measured or nominal)

Results:

- cell concentration for each flask at each measuring point and method for measuring cell concentration

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- mean values of cell concentrations
- growth curves
- graphical presentation of the concentration effect relationship
- EC values and method of calculation
- NOEC
- other observed effects

### 4. L I T E R A T U R E

#### • S t a n d a r d P r o c e d u r e s

1. Draft International Standard ISO/TC 147/SC5/W5/N67: Toxicity with Respect to Algae.
2. NEN 6506: Water-Determination of Toxicity with Algae, (Dutch standard) Nederlands Normalisatie Instituut, Rijswijk (1979).
3. DIN 38 412, Teil 1: Testverfahren mit Wasserorganismen (Gruppe L) Allgemeine Hinweise zur Planung, Durchführung und Auswertung biologischer Testverfahren (German standard) Deutsches Institut für Normung e.V, Berlin (June 1982).
4. DIN *Draft* 38 412, Teil 9: Testverfahren mit Wasserorganismen (Gruppe L). Bestimmung der Wirkung von Wasserinhaltsstoffen auf Grünalgen (Scenedesmus-Zellvermehrungshemmtest) (German standard) Deutsches Institut für Normung e.V., Berlin (April 1982).
5. U.S. EPA: Algal Assay Procedure: Bottle Test, National Environmental Research Centre, Corvallis, Oregon (1971).
6. U.S. EPA: The *Selenastrum capricornutum* Printz Algal Assay Bottle Test, EPA-600/9-78-018 (July 1978).
7. AFNOR T 90 304 (French standard).

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8. A.O. Hanstveit, in *Degradability, Ecotoxicity and Bioaccumulation: the Determination of the Possible Effects of Chemicals and Wastes on the Aquatic Environment*, Chapter 5, Government Publishing Office, the Hague (1980).
9. G. Bringmann and R. Kühn, *Water Research* 14, 231-241 (1980).
10. A.G. Payne and R.H. Hall, in *Aquatic Toxicology*, (edited by L.L. Marking and R.A. Kimble), ASTM STP 667, pp. 171-180 American Society for Testing and Materials, (1979).
11. S. Galassi and M. Vighi, *Chemosphere* 10, 1123-1126 (1981).
12. Standard Methods for Examination of Water and Waste Water, Part 800. 15th ed., American Public Health Association, Washington, D.C. (1980).

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**"Alga, Growth Inhibition Test"****5. A N N E X****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 may be carried out under sterile conditions in order to avoid contamination with bacteria and other algae.

***Equipment and materials***

See under 2B: Preparations and Experimental Organisms.

***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.

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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 preculture 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.