INTERNATIONAL STANDARD

ISO 8692

First edition
1989-11-15

Water quality — Fresh water algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum*

Qualité de l'eau — Essai d'inhibition de la croissance des algues d'eau douce avec Scenedesmus subspicatus *et* Selenastrum capricornutum

Reference number
ISO 8692 : 1989 (E)

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

Draft International Standards adopted by the technical committees are circulated to the member bodies for approval before their acceptance as International Standards by the ISO Council. They are approved in accordance with ISO procedures requiring at least 75 % approval by the member bodies voting.

International Standard ISO 8692 was prepared by Technical Committee ISO/TC 147, *Water quality.*

Annex A of this International Standard is for information only.

International Organization for Standardization
Case postale 56 • CH-1211 Genève 20 • Switzerland

Printed in Switzerland

Water quality — Fresh water algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum*

1 Scope

This International Standard specifies a method for the determination of the toxic effects of chemical compounds on the growth of planktonic freshwater algae.

The test can be used for readily water-soluble substances which are not significantly degraded or eliminated from the test system.

2 Principle

Monospecific algal strains are cultured for several generations in a defined medium containing a range of concentrations of the test substance prepared by mixing appropriate quantities of nutrient concentrate, water, test substance stock solutions, and an inoculum of exponentially growing algal cells. The test solutions are incubated for a minimum period of 72 h, during which the cell density in each is measured at least every 24 h.

Inhibition is measured as a reduction in growth or growth rate relative to control cultures grown under identical conditions.

3 Definitions and abbreviations

For the purposes of this International Standard, the following definitions and abbreviations apply.

3.1 cell density : Number of cells per unit volume.

3.2 growth : Increase in cell density.

3.3 growth rate : Expression of rate of increase in cell density with respect to time as given in 8.2.2.

3.4 test solution : Mixture of water, nutrients and test substance in which algal cells are incubated.

3.5 control : Mixture of water, nutrients and algal cells without test substance.

3.6 median effective concentration (EC_{50}) : The concentration of test substance which results in a 50 % reduction in either growth or growth rate relative to the controls.

3.7 no observed effect concentration (NOEC) : The highest concentration tested at which there is no statistically significant reduction of growth or growth rate relative to the controls.

4 Materials

4.1 Test organism

Use either of the following planktonic freshwater algae :

a) *Scenedesmus subspicatus* Chodat (86.81 SAG)

or

b) *Selenastrum capricornutum* Printz (ATCC 22662 or CCAP 278/4).[1)]

NOTE — Both species are planktonic green algae belonging to the order of *Chlorococcales (Chlorophyta, Chlorophyceae),* and are usually unicellular in culture.

The strains recommended are available in unialgal, non-axenic cultures from the following collections :

86.81 SAG : Collection of Algal Cultures
Inst. Plant Physiology
University of Göttingen
Nikolausberger Weg 18
D-3400 Göttingen
Germany, F.R.

ATCC 22662 : American Type Culture Collection
12301 Parklane Drive
Rockville
Maryland 20852
USA

1) This species is now systematically named *Raphidocelis subcapitata* Korsikov nov. comb. [1].

CCAP 278/4 : Culture Centre of Algae and Protozoa
Freshwater Biological Association
The Ferry House
Ambleside
Cumbria LA22 OLP, UK

Algothèque du laboratoire de Cryptogamie
Muséum d'histoire naturelle
12, rue Buffon
F-75005 Paris, France

4.2 Water

All water used in the preparation of the nutrient medium and test substance solutions shall be deionized or of equivalent quality. Take special care to avoid contamination of the water by inorganic or organic substances during preparation and storage. No copper equipment shall be used.

4.3 Nutrients

Prepare four stock solutions in water, according to the compositions given in table 1.

NOTE — These solutions will eventually be diluted (see 6.1 and 6.4) to achieve the final nutrient concentrations in the test solutions.

Table 1

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

All the chemicals used shall be of reagent grade quality.

Sterilize the stock solutions by membrane filtration (mean pore diameter 0,2 µm) or by autoclaving (120 °C, 15 min). Store the solutions in the dark at 4 °C.

Do not autoclave stock solution 4 ($NaHCO_3$) but sterilize it only by membrane filtration.

5 Apparatus

All equipment in contact with the test medium shall be made of glass or chemically inert material.

Ordinary laboratory apparatus and

5.1 Temperature-controlled cabinet or room with continuous even illumination by white fluorescent light suitable to meet requirements with respect to lighting conditions during the test as specified in 6.6.

5.2 Apparatus for measuring algal cell density, preferably a particle counter, or microscope with counting chamber. Alternatively determine the state of growth of the algal cultures by an indirect procedure using a spectrophotometer, turbidimeter or fluorimeter when sufficiently sensitive and if shown to be sufficiently well correlated with cell density. The apparatus used shall be capable of measuring accurately cell densities as low as 10^4 cells per millilitre.

5.3 Culture flasks, e.g. 250 ml conical flasks with air-permeable stoppers.

5.4 Apparatus for membrane filtration, using filters of mean pore diameter 0,2 µm.

5.5 Autoclave.

5.6 pH meter.

6 Procedure

6.1 Preparation of nutrient concentrate

Prepare a nutrient concentrate as follows (for 1 000 ml) :

Add to 100 ml of stock solution 1 (4.3) :

10 ml of stock solution 2 (4.3)
10 ml of stock solution 3 (4.3)
and 10 ml of stock solution 4 (4.3).

Make up to 1 000 ml with water.

Prepare the nutrient concentrate freshly before each test. Before use equilibrate it by leaving overnight in contact with air, or by bubbling filtered air through it for 30 min. After equilibration, adjust the pH if necessary to 8,3 ± 0,2, using either 1 mol/l hydrochloric acid or 1 mol/l sodium hydroxide solution.

6.2 Preparation of inoculum

The algal inoculum for the test shall be taken from an exponentially growing pre-culture. Set up the pre-culture 3 days before the start of the test as described below.

Mix one part by volume of nutrient concentrate (6.1) with eight parts of water. Add sufficient cells from the algal stock culture so that, when made up to 10 parts with water, the cell density is of the order of 10^4 cells per millilitre.

Maintain the pre-culture under the same conditions as used in the test (see 6.6) for 3 days, after which time it should be in exponential growth and of sufficient cell density to be used as an inoculum for the test.

Measure the cell density in the preculture immediately before use (see 6.7), in order to calculate the required inoculum volume.

6.3 Choice of test concentrations

The concentrations of test substance to be tested shall normally follow a geometric progression, for example 10; 3,2; 1,0; 0,32; . . .; 0,01 $mg \cdot l^{-1}$.

If possible the concentrations shall be chosen to obtain several (4 to 5) levels of effect ranging from $\leqslant$ 10 % to $\geqslant$ 90 % inhibition of growth.

NOTE — A suitable concentration range is best determined by carrying out a preliminary range-finding test covering several orders of magnitude of difference in test concentration. Replication of test concentrations is unnecessary in the preliminary test.

6.4 Preparation of test substance stock solutions

Prepare a stock solution of the test substance in water in which the concentration of the test substance is at least twice that of the highest concentration to be tested. Dilute this stock solution as required to produce a series of stock solutions corresponding to the range of test concentrations.

Normally the test shall be carried out without adjustment of pH. However, some substances may exert a toxic effect through extreme acidity or alkalinity. In order to investigate the toxicity of a substance other than that due to pH, adjust the pH of the first stock solution (before the serial dilution) to 7,0 using either 1 mol/l hydrochloric acid or 1 mol/l sodium hydroxide solution.

NOTE — The pH adjustment should not cause a chemical reaction with the substance to be tested (e.g. precipitation, complexation) and should not change the concentration of the test substance solution significantly.

6.5 Preparation of test solutions

Prepare the test solutions by mixing the appropriate volumes of test substance stock solutions, water, nutrient concentrate (7.1) and inoculum (7.2) in the test vessels.

The total volume shall be the same in all vessels.

The amount of nutrient concentrate added to all the vessels shall be one part in ten of the total volume.

The amount of inoculum added to all the vessels shall be sufficient to give an initial cell density in the test solutions of 10^4 cells per millilitre.

To some vessels add only water, nutrient concentrate and inoculum, with no test substance. These vessels serve as controls.

Prepare three replicates of each test substance concentration, and six identical controls.

Measure the pH of a sample of the test solutions at each concentration and control.

6.6 Incubation

Incubate the stoppered test vessels at 23 °C ± 2 °C, under continuous white light. The light intensity at the average level of the test solutions shall be in the range 60 $\mu E/m^2/s$ to 120 $\mu E/m^2/s$ (35×10^{18} photons/m^2/s to 70×10^{18} photons/m^2/s) when measured in the photosynthetically effective wavelength range of 400 nm to 700 nm using an appropriate receptor.

NOTE — It is important to note that the method of measurement, in particular the type of receptor (collecter) will affect the measured value. Spherical receptors (which respond to light from all angles above and below the plane of measurement) and "cosine" receptors (which respond to light from all angles above the measurement plane) are preferred to unindirectional receptors and will give higher readings for a multi-point light source of the type described below.

The intensity specified above could be obtained using 4 W to 7,3 W fluorescent lamps of the universal white (natural) type [i.e. a rated colour of standard colour 2 (a colour temperature of 4 300 K) according to IEC 81] at a distance of approximately 0,35 m from the algal culture medium.

For light-measuring instruments calibrated in lux, an equivalent range of 6 000 lux to 10 000 lux is acceptable for the test.

Keep the algal cells in suspension by shaking, stirring or aerating in order to improve gas exchange and reduce pH variation in the test solutions.

6.7 Measurements

Measure the cell density in each test vessel (including the controls) at least every 24 h. These measurements shall be made on small volumes (e.g. 5 ml) removed from the test solution by pipette, and not replaced.

The test shall last for a minimum period of 72 h.

Measure the pH of a sample of the test solutions at each concentration (and control) at the end of the test.

7 Validity criteria

Consider the test invalid if the following conditions are not met :

a) the control cell density shall have increased by a factor of more than 16 in 72 h. This increase corresponds to a growth rate (8.2) of 0,9 d^{-1}. Under normal experimental conditions growth rates of 1,5 to 1,9 d^{-1} can be achieved;

b) the control pH shall not have varied by more than 1,5 units during the test.

NOTE — Variations in pH during the test can have significant influence on results and therefore a limit of 1,5 units is set. Variations in pH, however, should always be as low as achievable. e.g. by performing continuous shaking during the test.

8 Expression of results

8.1 Plotting growth curves

Tabulate the cell density measurements or other parameters correlated with cell density in the test cultures according to the concentration of test substance and the time of measurement.

Plot a growth curve for each test concentration and control, as a graph of the logarithm of the mean cell density against time.

8.2 Calculation of percentage inhibition

Base the assessment of the inhibition of growth in the test on the two following parameters.

8.2.1 Area under the growth curve (biomass integral)

Calculate the area, A, under the double linear growth curve for each test culture, from the equation

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

where

t_1 is the time of the first measurement after the beginning of the test;

t_n is the time of the nth measurement after the beginning of the test;

N_0 is the nominal initial cell density;

N_1 is the measured cell density at time t_1;

N_n is the measured cell density at time t_n.

Calculate mean values of A for each test concentration and control. From these calculate the percentage inhibition for each test concentration, from the equation

$$I_{Ai} = \frac{A_c - A_i}{A_c} \times 100$$

where

I_{Ai} is the percentage inhibition (area) for test concentration i;

A_i is the mean area for test concentration i;

A_c is the mean area for the control.

8.2.2 Growth rate

Calculate the growth rate, μ, for each test culture, from the equation

$$\mu = \frac{\ln N_n - \ln N_0}{t_n}$$

where

t_n is the time of the final measurement after the beginning of the test;

N_0 is the nominal initial cell density;

N_n is the measured final cell density.

Alternatively, determine the growth rate from the slope of the regression line in a plot of the logarithm of the cell density against time.

Calculate mean values of μ for each test concentration and control. From these calculate the percentage inhibition for each test concentration, from the equation

$$I_{\mu i} = \frac{\mu_c - \mu_i}{\mu_c} \times 100$$

where

$I_{\mu i}$ is the percentage inhibition (growth rate) for test concentration i;

μ_i is the mean growth rate for test concentration i;

μ_c is the mean growth rate for the control.

8.3 Determination of EC_{50}

Tabulate values of I_{Ai} or $I_{\mu i}$ against the corresponding test concentrations, and plot these data on semilogarithmic or logarithmic-probit paper (test concentration on the logarithmic scale) as appropriate. Fit a line to the data by eye, and read the EC_{50} (the test concentration corresponding to 50 % inhibition) from this graph.

Alternatively, calculate the EC_{50} value by a regression analysis technique, e.g. probit analysis.

8.4 Determination of NOEC

The NOEC is the highest tested concentration at which no significant inhibition of growth is observed relative to the control. Determine this by a suitable statistical procedure for multisample comparison (e.g. analysis of variance and Dunnett's test), using the individual replicate values of A or μ.