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Water quality — Marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*

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Qualité de l'eau — Essai d'inhibition de la croissance des algues marines avec Skeletonema costatum *et* Phaeodactylum tricornutum

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Foreword

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Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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International Standard ISO 10253 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

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Annex A of this International Standard is for information only.

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Water quality — Marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*

1 Scope

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

The method can be used for testing substances which are readily soluble in water and are not significantly degraded or eliminated from the test.

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NOTE 1 With minor changes, the method can also be used to determine the inhibitory effects of effluents. See however the note to table 2.

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2 Principle

Monospecific algal cells 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, seawater, stock solutions of the test substance, 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 intervals of 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

For the purposes of this International Standard, the following definitions 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.

See 8.2.2.

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

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

3.6 effective concentration, EC10 or EC50: The concentration of test substance which results in respectively a 10 % or 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 organisms

Use either of the following marine algae.

a) *Skeletonema costatum* (Greville) Cleve (CCAP 1077/1C, NIVA BAC 1, ISTPM P4 — Bouin).

or

b) *Phaeodactylum tricornutum* Bohlin (CCAP 1052/1A — Oban, 1090/1A Göttingen, NIVA BAC 2, ISTPM P1).

These algae are important and widely distributed planktonic phytoplankton species (phylum *Bacillariophyta*) in estuarine and coastal areas.

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

NIVA: Norwegian Institute for Water Research
P.O. Box 173 Kjelsås
N-0411 Oslo
Norway

ISTPM P1
ISTPM P4 —
Bouin: INERIS
9, rue de Rocroy
75010 Paris
France

CCAP: Dunstaffnage Marine Laboratory
P.O Box 3 Oban
Argyll PA34 4AD
United Kingdom

Göttingen: Collection of Algal Cultures
Institute of Plant Physiology
University of Göttingen
Nikolausberger Weg 18
D-3400 Göttingen
Germany

NOTE 2 Stock cultures may be maintained in the medium (see 4.3 and 6.1). Regular subculturing is necessary. Weekly intervals may be necessary for *Skeletonema*; every two or three weeks may be sufficient for *Phaeodactylum*.

4.2 Water

All water used in the preparation of the synthetic seawater, nutrient medium and test substance solutions shall be deionized or of equivalent purity. Take special care to avoid contamination of the water by inorganic or organic substances during preparation and storage. Equipment made of copper shall not be used.

4.3 Seawater

For culturing and testing *Phaeodactylum*, the medium (6.1) is made up by adding nutrients to either natural or synthetic seawater. For *Skeletonema*, the use of natural seawater is necessary for the long-term maintenance of cultures, and may also be necessary for the test medium because a synthetic seawater medium may not always support sufficient growth to meet the test quality criteria. If natural seawater [of salinity 30 ‰ $(m/m) \pm 5$ ‰ (m/m)] is used, care shall be taken to ensure that it is not polluted.

Prepare synthetic seawater with the composition given in table 1.

All the chemicals used shall be of analytical grade.

Table 1 — Synthetic seawater

Salt	Concentration of salt in synthetic seawater g/l
NaCl	22
$MgCl_2 \cdot 6H_2O$	9,7
Na_2SO_4 (anhydrous)	3,7
$CaCl_2$ (anhydrous)	1,0
KCl	0,65
$NaHCO_3$	0,20
Salts of H_3BO_3	0,023

Sterilize the seawater by membrane filtration (5.4).

4.4 Nutrients

Prepare three nutrient stock solutions in water, with the compositions given in table 2.

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Table 2 — Nutrient stock solutions

Nutrient	Concentration in stock solution	Final concentration in test solution
Stock solution 1		
$FeCl_3 \cdot 6H_2O$	48 mg/l	149 µg/l (Fe)
$MnCl_2 \cdot 4H_2O$	144 mg/l	605 µg/l (Mn)
$ZnSO_4 \cdot 7H_2O$	45 mg/l	150 µg/l (Zn)
$CuSO_4 \cdot 5H_2O$	0,157 mg/l	0,6 µg/l (Cu)
$CoCl_2 \cdot 6H_2O$	0,404 mg/l	1,5 µg/l (Co)
H_3BO_3	1 140 mg/l	17,1 µg/l
Na_2EDTA [1]	1 000 mg/l	15,0 µg/l
Stock solution 2		
Thiamin hydrochloride	50 mg/l	25 µg/l
Biotin	0,01 mg/l	0,005 µg/l
Vitamin B_{12} (cyanocobalamin)	0,10 mg/l	0,05 µg/l
Stock solution 3		
K_3PO_4	3,0 g/l	3,0 mg/l
$NaNO_3$	50,0 g/l	50,0 mg/l
$Na_2SiO_3 \cdot 5H_2O$	14,9 g/l	14,9 mg/l

1) Complexing of heavy metals by the relatively high concentration of EDTA present in the nutrient medium may preclude the testing of effluents containing heavy metals.

NOTE 3 These stock solutions will eventually be diluted (see 6.1) to obtain the final nutrient concentrations in the test solutions.

All the chemicals used shall be of analytical grade.

Sterilize stock solutions 1 and 3 by autoclaving at 120 °C for at least 15 min, and stock solution 2 by membrane filtration (5.4).

Store the solutions in the dark at 4 °C.

5 Apparatus

All equipment which will come into contact with the test medium shall be made of glass or a chemically inert material.

Normal laboratory apparatus and

5.1 Temperature-controlled cabinet or room, with a white fluorescent light providing continuous even illumination, suitable for the lighting requirements specified for the test in 6.6.

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

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5.3 Culture flasks, for example conical flasks of capacity 250 ml, with air-permeable stoppers.

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

5.5 Autoclave.

5.6 pH-meter.

6 Procedure

6.1 Preparation of culture medium

Add 15 ml of nutrient stock solution 1, 0,5 ml of nutrient stock solution 2 and 1 ml of nutrient stock solution 3 (see table 2) to approximately 900 ml of natural or synthetic seawater (4.3) and then make up to 1 litre with the same seawater.

Adjust the pH to $8,0 \pm 0,2$ by adding dilute hydrochloric acid or sodium hydroxide solution.

6.2 Preparation of inoculum

The algal inoculum for the test shall be taken from an exponentially growing pre-culture. The pre-culture shall be set up 3 d ± 1 d before the start of the test, as follows.

Add sufficient cells from the algal stock culture to the culture medium (6.1) to obtain an initial cell density of approximately 2×10^3 to 10^4 cells per millilitre. Maintain the pre-culture under the same conditions as those in the test (see 6.6) for 3 d ± 1 d. After this, the pre-culture 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 pre-culture immediately before use (see 6.7), in order to calculate the required inoculum volume.

6.3 Choice of test concentrations

The concentrations of substance to be tested shall normally follow a geometric progression, for example 10 mg/l; 3,2 mg/l; 1,0 mg/l; 0,32 mg/l; ...; 0,01 mg/l.

If possible, the concentrations shall be chosen to obtain several (i.e. 4 or 5) levels of inhibition of growth ranging from less than 10 % to greater than 90 %.

NOTE 4 A suitable concentration range is best determined by carrying out a preliminary range-finding test covering several orders of magnitude of difference between test concentrations. Replication of test concentrations is unnecessary during this preliminary test.

6.4 Preparation of test substance stock solution

Prepare stock solutions of the test substance, where necessary, in the algal growth medium by dilution. The concentration of test substance in the stock solutions shall be such that, when added to the test vessels containing growth medium inoculated with the algae, the intended range of test concentrations is obtained.

Normally, the test shall be carried out without adjusting the pH. However, some substances may exert a toxic effect due to extreme acidity or alkalinity. In order to determine the toxicity of a substance independent of pH, adjust the pH of the master stock solution (before the dilution in series) to that of the culture medium, using either 1 mol/l hydrochloric acid or 1 mol/l sodium hydroxide solution.

6.5 Preparation of test solutions

Prepare the test solutions by mixing the appropriate volumes of test substance stock solutions (6.4), cul-

ture medium (6.1) and inoculum (6.2) in the test vessels. The total volume shall be the same in all the vessels.

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.

A lower initial cell density (3 to 5-fold lower) is recommended for *Skeletonema* due to its higher cell volume. The chain-formation of *Skeletonema* shall be taken into account when determining the initial cell density.

Prepare three replicates for each test substance concentration. To a further six vessels, add only culture medium and inoculum, with no test substance. These vessels serve as controls.

Prepare a single concentration series of the test substance without algae to serve as a background for the cell density determinations.

If there is sufficient technical justification, the test design may be altered to increase the number of concentrations and reduce the number of replicates per concentration.

Measure the pH of samples of each concentration of the test solution and of the controls.

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6.6 Incubation

Incubate the stoppered test vessels at a nominal temperature of 20 °C, under continuous white light. The temperature shall not vary by more than 2 °C during the test. The light intensity at the average level of the test solutions shall be uniform and in the range 60 $\mu E/(m^2 \cdot s)$ to 120 $\mu E/(m^2 \cdot s)$ [35×10^{18} photons/$(m^2 \cdot s)$ to 70×10^{18} photons/$(m^2 \cdot s)$], when measured in the photosynthetically effective wavelength range of 400 nm to 700 nm using an appropriate receptor.

It is important to note that the method of measurement, and in particular the type of receptor (collecter), will affect the measured value. Spherical receptors (which respond to direct and reflected 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 unidirectional receptors and will give higher readings for a multi-point light source of the type described in note 5.

NOTES

5 The light intensity specified in this subclause could be obtained using 4 W to 7,30 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[1]] at a distance of approximately 0,35 m from the algal culture medium.

6 For light-measuring instruments calibrated in lux, an equivalent range of 6 000 lx to 10 000 lx 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 are usually made on small volumes (e.g. 5 ml) which are removed from the test solution and not replaced.

The test shall last for at least 72 h. At the end of the test, measure the pH of samples of each concentration of the test solution (6.5) and of the controls (6.5).

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 (8.2.2) of 0,04/h.

NOTE 7 The control growth rates determined in the interlaboratory test were as follows:

Skeletonema costatum: 0,10/h ± 0,02/h;

Phaeodactylum tricornutum: 0,072/h ± 0,007/h.

In the interlaboratory test, outlier results were consistently found in tests having a control growth rate of < 0,06/h.

b) The control pH shall not have varied by more than ± 1,0 unit during the test.

NOTE 8 Variations in pH during the test can have a significant influence on the results and therefore a limit of ± 1,0 unit is set. These variations, however, should always be kept as low as possible, for example 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 culture, 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 area under the growth curve (8.2.1) and the growth rate (8.2.2). Calculate for the period during which growth is considered to be exponential in the control cultures [indicated as the linear part of the logarithmic plot of the growth curve (8.1)].

8.2.1 Area under the growth curve (biomass integral)

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Calculate the area, A, under the double-linear growth curve (not the logarithmic growth curve) for each test culture separately, from the equation

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

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where

t_1 is the time, in hours, between the first measurement and the beginning of the test;

t_2 is the time, in hours, between the second measurement and the beginning of the test;

t_{n-1} is the time, in hours, between the $(n-1)$th measurement and the beginning of the test;

t_n is the time, in hours, between the nth measurement and the beginning of the test;

N_0 is the nominal initial cell density (see note 9);

N_1 is the measured cell density at time t_1;

N_2 is the measured cell density at time t_2;

N_{n-1} is the measured cell density at time t_{n-1};

N_n is the measured cell density at time t_n.

NOTE 9 Cell density is expressed as cells per millilitre or another appropriate unit dependent on the method used (5.2).

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

$$I_{Ai} = \frac{\bar{A}_c - \bar{A}_i}{\bar{A}_c} \times 100$$

where

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

$\bar{A}_i$ is the mean area for test concentration i;

$\bar{A}_c$ is the mean area for the control.

8.2.2 Growth rate

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

$$\mu = \frac{\ln N_L - \ln N_0}{t_L}$$

where

t_L is the time, in hours, between the last measurement of the exponential growth period (8.2) and the beginning of the test;

N_0 is the nominal initial cell density (see note 9);

N_L is the measured cell density at time t_L.

Alternatively, determine the growth rate from the slope of the regression line in a plot of the logarithm of the mean cell density against time (8.1).

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

$$I_{\mu i} = \frac{\bar{\mu}_c - \bar{\mu}_i}{\bar{\mu}_c} \times 100$$

where

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

$\bar{\mu}_i$ is the mean growth rate for test concentration i;

$\bar{\mu}_c$ is the mean growth rate for the control.

8.3 Determination of EC10 and EC50

Tabulate values of I_{Ai} or $I_{\mu i}$ against the corresponding test concentrations, and plot these values on semi-logarithmic or logarithmic-probit paper (with test concentrations on the logarithmic scale) as appropriate. Fit a line to the data by eye and read the EC50 (the test concentration corresponding to 50 % inhibition) and EC10 (the test concentration corresponding to 10 % inhibition) from this graph.

Alternatively, calculate the EC10 and EC50 values by a regression analysis technique (for example see [2] and [3] in annex A).

8.4 Determination of NOEC

Determine the NOEC as the highest tested concentration at which no significant inhibition of growth is observed relative to the control.

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9 Denotation of results

Denote EC10 and EC50 values based on growth curve area (biomass integral) as $EC10_b$ and $EC50_b$ and those based on growth rate as $EC10_r$ and $EC50_r$. Denote NOEC values as $NOEC_b$ for values based on growth curve areas, or $NOEC_r$ for values based on growth rate. Also indicate clearly the time span used for the determination, for example $EC50_b$ (0-72 h). Quote EC10, EC50 and NOEC values to two significant digits, normally in milligrams per litre.

10 Interpretation of results

EC10, EC50 and NOEC values are toxicological data derived from a laboratory experiment carried out under defined standard conditions. They give an indication of potential hazards, but cannot be used directly to predict effects in the natural environment. When interpreting EC10, EC50 and NOEC values, take into consideration the shape of the growth curves. Certain features of these curves (for example, delayed onset of growth; good initial growth that is not sustained) can help to indicate the mode of action of the toxic substance concerned.

11 Reproducibility

An interlaboratory test based on the test described in this International Standard was carried out by 10 laboratories in 1989/1990. The results obtained with the reference substances potassium dichromate ($K_2Cr_2O_7$) and 3,5-dichlorophenol ($Cl_2C_6H_3OH$) and the strains ISTPM/BAC/CCAP (1077/1C and 1052/1B) are shown in table 3.

12 Test report

The test report shall include the following information:

a) a reference to this International Standard;

b) test substance: chemical identification data;

c) test organism: species, origin, strain number, method of cultivation;

d) test details:

— start date and duration,

— concentrations tested,

— composition of medium,

— culturing apparatus and incubation procedure,

— light intensity and quality,

— temperature,

— pH of test solutions at the start and end of the test,

— method for measuring cell density;

e) results:

— cell density in each test vessel at each measuring point,

— mean cell density for each test concentration (and control) at each measuring point,

— growth curves (logarithm of cell density against time),

— relationship between concentration and effect (percentage inhibition values against concentration) in table or graphical representation; for example, percentage inhibition on probit-scaled ordinate against concentration on logarithmic-scaled abscissa,

— EC10 values and method of determination,

— EC50 values and method of determination,

— NOEC values and method of determination,

— other observed effects.

Table 3 — Interlaboratory test results

Test organism and test substance	Participants	Outliers	Parameter	Mean value mg/l	Standard deviation mg/l	Coefficient of variation %
Skeletonema costatum						
Potassium dichromate	9 9	2 2	$EC50_r$ $EC50_b$	2,5 2,1	1,1 0,8	44 37
3,5-dichlorophenol	7	2	$EC50_r$ $EC50_b$	1,6 1,3	0,3 0,1	18 7
Phaeodactylum tricornutum						
Potassium dichromate	10	3	$EC50_r$ $EC50_b$	20,1 6,0	5,3 1,8	26 31
3,5-dichlorophenol	10	3	$EC50_r$ $EC50_b$	2,7 1,5	0,2 0,3	8,6 20

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