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

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

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. 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.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

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

This second edition cancels and replaces the first edition (ISO 10253:1995), which has been technically revised.

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

WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This International Standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests conducted according to this International Standard be carried out by suitably trained staff.

1 Scope

This International Standard specifies a method for the determination of the inhibition of growth of the unicellular marine algae *Skeletonema costatum* and *Phaeodactylum tricornutum* by substances and mixtures contained in sea water.

The method can be used for testing substances that are readily soluble in water and are not significantly degraded or eliminated in any other way from the test medium.

NOTE With modifications, as described in ISO 14442 and ISO 5667-16, the inhibitory effects of poorly soluble organic and inorganic materials, volatile compounds, metal compounds, effluents, marine water samples and elutriates of sediments can be tested.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 5667-16, *Water quality — Sampling — Part 16: Guidance on biotesting of samples*

ISO 14442, *Water quality — Guidelines for algal growth inhibition tests with poorly soluble materials, volatile compounds, metals and waste water*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
cell density
number of cells per unit volume of medium (x cells/ml)

3.2
specific growth rate
μ
proportional rate of increase in cell density per unit of time:

$$\mu = \frac{1}{x} \times \frac{\mathrm{d}x}{\mathrm{d}t} \text{ (1/day)}$$

3.3
growth medium
mixture of sea water and nutrients which is used for pre-cultures and controls

3.4
test medium
mixture of sea water, nutrients (growth medium 3.3) and test material in which algal cells are incubated

3.5
test batch
mixture of sea water, nutrients and test material (test medium 3.4) inoculated with algae

3.6
control
mixture of sea water, nutrients (growth medium 3.3) without test material, inoculated with algae

3.7
effective concentration
EC$(r)_x$
concentration of test substance which results in an x % reduction in specific growth rate relative to the controls

4 Principle

Mono-specific 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, sea water, stock solutions of the test substance, and an inoculum of exponentially growing algal cells. The test solutions are incubated for a period of 72 h $\pm$ 2 h, during which the cell density in each is measured at intervals of at least every 24 h $\pm$ 2 h. Inhibition is measured as a reduction in specific growth rate, relative to control cultures grown under identical conditions.

5 Materials

5.1 Test organisms

Use either of the following marine algae:

a) *Skeletonema costatum* (Greville) Cleve (CCAP 1077/1C, NIVA BAC 1); or

b) *Phaeodactylum tricornutum* Bohlin (CCAP 1052/1A, SAG 1090-1a, NIVA BAC 2).

These algae are important and widely distributed 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

CCAP Dunstaffnage Marine Laboratory
P O Box 3 Oban
Argyll PA37 1QA
United Kingdom

SAG Collection of Algal Cultures
University of Göttingen
Albrecht-von-Haller Institute for Plant Science
Untere Karspüle 2
37073 Göttingen
Germany

Stock cultures may be maintained in the medium described in 7.1. Regular subculturing is necessary. Weekly intervals may be necessary for *Skeletonema*; every two or three weeks may be sufficient for *Phaeodactylum.* The stock cultures may also be maintained for extended periods on richer algal media such as those recommended by the culture collection. It is recommended to keep the stock culture in the medium described in 7.1 and in an exponential growth phase immediately before preparing the pre-culture for testing as described in 7.2.

NOTE Like many freshwater algae, the diatom *Phaeodactylum tricornutum* can also be stored for several months in alginate beads, without losing its viability [1]. The algae can be liberated from the algal beads when needed to perform the toxicity tests[1)].

5.2 Water

All water used in the preparation of the synthetic sea water, growth 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.

5.3 Sea water

For culturing and testing *Phaeodactylum,* the growth medium (7.1) is made up by adding nutrients to either natural [salinity = (30 ± 5) g/kg] or synthetic sea water (approximate salinity = 33 g/kg). For *Skeletonema,* the use of natural sea water may be necessary for the long-term maintenance of cultures and may also be necessary for the test medium, because a synthetic sea water medium may not always support sufficient growth to meet the test quality criteria. If natural sea water is used, care shall be taken to ensure that it is not polluted.

Prepare synthetic sea water with the composition given in Table 1 (approximate salinity = 33 g/kg). All the chemicals used shall be of analytical grade.

1) The algae beads supplied by MICROBIOTESTS Inc., Venecoweg 19, 9810 Nazareth, Belgium, Tel (32) 9 380 8545, Fax (32) 9 380 8546, Email microbiotests@skynet.be, are an example of a suitable commercially available product. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

Table 1 — Synthetic sea water

Salt	Concentration of salt in synthetic sea water 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
H_3BO_3	0,023

Filter the sea water through a 0,45 µm membrane filter in order to remove particulate material and algae.

5.4 Nutrients

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

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	3,0 mg/l (B)
Na_2EDTA	1 000 mg/l	15,0 mg/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; 0,438 mg/l P
$NaNO_3$	50,0 g/l	50,0 mg/l; 8,24 mg/l N
$Na_2SiO_3 \cdot 5H_2O$	14,9 g/l	14,9 mg/l; 1,97 mg/l Si

NOTE These stock solutions are eventually diluted (see 7.1 and Annex A) to obtain the final nutrient concentrations in the test solutions.

All the chemicals used shall be of reagent grade quality.

Sterilize stock solutions by filtration through a 0,2 µm membrane filter. Stock solutions 1 and 3 may also be sterilized by autoclaving at 120 °C for at least 15 min.

Store the stock solutions in the dark at 4 °C.

6 Apparatus

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

Use normal laboratory apparatus and in addition the following.

6.1 Temperature-controlled cabinet or **room**, with a white fluorescent light providing continuous even illumination, suitable for the lighting requirements specified for the test in 7.6.

6.2 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 for instance a fluorimeter (e.g. *in vitro* fluorescence [2]), 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 the inoculum cell density and to distinguish between algal growth and disturbing effects, for example, the presence of particulate matter and colour of the sample. Spectrophotometers may be sufficiently sensitive to measure 10^4 cells/ml providing a sufficient path length (up to 10 cm) can be used. However, this technique is particularly sensitive to interferences from suspended material and coloured substances at low cell densities.

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

6.4 Apparatus for membrane filtration, filters of mean pore diameter 0,2 µm and 0,45 µm.

6.5 Autoclave.

6.6 pH-meter.

7 Procedure

7.1 Preparation of growth 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 sea water (5.3) and then make up to 1 l with the same sea water.

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

NOTE 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. For guidance, see ISO 14442.

7.2 Preparation of pre-culture and inoculum

A pre-culture shall be started two to four days before the beginning of the test (see Note in 5.1).

Add sufficient cells from the algal stock culture to the growth medium (7.1) to obtain a sufficiently low cell density of, e.g. 2×10^3 cells/ml to 10^4 cells/ml for three days pre-culturing, in order to maintain exponential growth until the start of the test. The pre-culture shall be incubated under the same conditions as those in the test. Measure the cell density in the pre-culture immediately before use, in order to calculate the required inoculum volume.

7.3 Choice of test concentrations

Algae should be exposed to concentrations of the test substance in a geometric series with a ratio not exceeding 3,2 (e.g. 1,0 mg/l, 1,8 mg/l, 3,2 mg/l, 5,6 mg/l and 10 mg/l).

The concentrations should be chosen to obtain at least one inhibition below and one inhibition above the intended $EC(r)_x$ parameter. Additionally, at least two levels of inhibition between 10 % and 90 % should be included in order to provide data for regression analysis.

NOTE 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 not a requirement in the preliminary test.

7.4 Preparation of test substance stock solutions

Prepare stock solutions by dissolving the test substance in growth medium (7.1). Modifications are necessary when the test substance does not readily dissolve in the test medium, as described in ISO 14442 and ISO 5667-16.

When testing water samples (effluent, elutriates, etc.), spike them with the nutrient stock solutions (5.4) and, if appropriate, to avoid growth inhibition due to a too low salinity, with sea water salts (5.3) to bring the salinity of the sample up to the salinity of the growth medium. An example of a dilution scheme for sea water samples is found in Annex A.

Normally, carry out the test without adjusting the pH after addition of the test substance. 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 $8{,}0 \pm 0{,}2$, using either hydrochloric acid or sodium hydroxide solution. The concentration of acid or base should be such as the volume change is as small as possible.

7.5 Preparation of test and control batches

Prepare the test batches by mixing the appropriate volumes of test substance stock solutions (7.4), growth medium (7.1) and inoculum (7.2) in the test vessels. The total volume, concentration of added growth medium nutrients and cell density shall be the same in all test batches.

The initial cell density shall be sufficiently low to allow exponential growth in the control culture throughout the test duration, or for at least the time required to achieve a factor 64 increase of cell density, without a pH drift of more than 1,0 pH units (see Clause 8). Therefore, the initial cell densities shall not exceed 10^4 cells/ml.

A lower initial cell density (three to five-fold lower) is recommended for *Skeletonema* due to its higher cell volume and growth rate. Take into account the chain-formation of *Skeletonema* when determining the initial cell density.

Prepare at least three replicates for each test substance concentration. To a further 6 vessels, add only growth medium and inoculum with no test substance. These vessels serve as controls.

If appropriate (e.g. environmental, coloured or turbid samples), prepare a concentration series, single vessels only, of the test substance without algae to serve as a background for the cell density determinations.

The test design may be altered, based on statistical consideration, 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.

7.6 Incubation

The test vessels shall be sufficiently covered to avoid airborne contamination and to reduce water evaporation, but they shall not be airtight in order to allow CO_2 to enter the vessels. Incubate the 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 photon fluence rate at the average level of the test solutions shall be uniform and in the range 60 µmol/m^2·s to 120 µmol/m^2·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 (collector), affects 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 give higher readings for a multi-point light source of the type described in Note 1.

NOTE 1 The light intensity specified above could be obtained using between four to seven fluorescent lamps (power rating 30 W) 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 60081 [3] at a distance of approximately 0,35 m from the algal culture medium.

NOTE 2 For light-measuring instruments calibrated in lx, an equivalent range of 6 000 lx to 10 000 lx is acceptable for the test.

Continuously and gently shake the cultures in order to keep the cells in free suspension and to facilitate CO_2 mass transfer from air to water, and in turn, reduce pH shift.

7.7 Measurements

Measure the cell density in each test vessel, including the controls, at least every 24 h ± 2 h. These measurements are usually made on small volumes which are removed from the test solution and not replaced.

The test shall last for 72 h ± 2 h. At the end of the test, measure the pH of samples of each concentration of the test substance and of the controls. Confirm the appearance of the cells and the identity of the test organism by microscopy.

8 Validity criteria

Consider the test valid if the following conditions are met.

a) The control cell density shall have increased by a factor of more than 16 in 72 h. This increase corresponds to a specific growth rate (9.2) of 0,9 d^{-1}.

NOTE 1 The growth rate of the algae under the specified conditions may vary among different strains of the species. Results from interlaboratory tests indicate that growth rates above 1,0 d^{-1} are normally obtained with both species.

b) The variation coefficient of the control specific growth rates should not exceed 7 %.

c) The control pH shall not have increased by more than 1,0 during the test.

NOTE 2 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.

9 Interpretation of data

9.1 Plotting growth curves

Tabulate the cell density measurements, or other parameters correlated with cell density in the test media, 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. A linear growth curve indicates exponential growth, whereas a levelling off indicates that cultures have entered the stationary phase.

If the control cultures show declining growth rate towards the end of the exposure period, inhibited cultures may tend to catch up with the controls, falsely indicating a decreased growth inhibiting effect. In this case, perform the calculations of growth rate and growth inhibition based on the last measurement within the exponential growth period in the control cultures.

9.2 Calculation of percentage inhibition

Calculate first the average specific growth rate, μ, for each test culture, using Equation (1):

$$\mu = \frac{\ln N_L - \ln N_o}{t_L - t_o} \quad (1)$$

where

t_0 is the time of test start;

t_L is the time of test termination or the time of the last measurement within the exponential growth period in the control (9.1.);

N_0 is the nominal initial cell density;

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 (9.1).

Calculate mean values of μ for the control. Calculate the percentage inhibition for each individual test flask from Equation (2):

$$I_{\mu i} = \frac{\bar{\mu}_c - \mu_i}{\bar{\mu}_c} \times 100 \quad (2)$$

where

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

μ_i is the growth rate for test flask i;

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

9.3 Determination of EC$(r)_x$

Tabulate and plot for each individual flask the percentage inhibition ($I_{\mu i}$) against the test concentration on a logarithmic scale. If the scatter of data points is large, plot means of replicates with corresponding standard deviations.

Fit a suited non-linear model to the experimental data points by regression analysis (for example, see References [4], [5] and [6]) in order to determine EC$(r)_x$ values, preferably with their confidence intervals.

If data are too few or uncertain for regression analysis, or if inhibitions appear not to follow a regular concentration response relation (e.g. stimulation occurs), then a graphical method might be applied. In this case, draw a smooth eye-fitted curve of the concentration response relationship and read EC$(r)_x$ values from this graph.

10 Expression of results

Denote EC_{10} and EC_{50} values based on growth rate as EC$(r)_{10}$ and EC$(r)_{50}$. Also indicate clearly the time span used for the determination, for example, EC$(r)_{50}$ (0 h to 72 h). Quote EC$(r)_{10}$ and EC$(r)_{50}$ values, normally in milligrams per litre (mg/l) or millilitres per litre (ml/l).

11 Interpretation of results

EC_{10} and EC_{50} 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 EC_{10} and EC_{50} 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.

12 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.

Table 3 — Interlaboratory test results

Test organism and test substance	Participants	Outliers	Mean value EC$(r)_{50}$ mg/l	Standard deviation mg/l	Coefficient of variation %
Skeletonema costatum					
Potassium dichromate	9	2	2,5 ($n = 7$)	1,1	44
3,5-dichlorophenol	7	2	1,6 ($n = 5$)	0,3	18
Phaeodactylum tricornutum					
Potassium dichromate	10	3	20,1 ($n = 7$)	5,3	26
3,5-dichlorophenol	10	3	2,7 ($n = 7$)	0,2	8,6

Reference substance may be tested for checking the test procedure and sensitivity. It is advisable to test the reference substances regularly and to use control charts for measuring within laboratory precision and monitoring culture health.

13 Test report

The test report shall include the following information:

a) a reference to this International Standard (ISO 10253:2006);

b) test substance chemical identification data;

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

d) test details:

- start date and duration,
- method of preparations,
- nominal and measured concentrations tested,
- composition of medium,
- source and salinity of sea water,
- 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, and, if appropriate, method to correct for background values;

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,
- $EC(r)_{10}$ value and method of determination,
- $EC(r)_{50}$ value and method of determination,
- other observed effects,
- if appropriate, results of positive controls, control chart.