SLOVENSKI STANDARD
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Kakovost vode - Preskus zaviranja rasti morskih alg s Skeletonema sp. in Phaeodactylum tricornutum (ISO/DIS 10253:2023)

Water quality - Marine algal growth inhibition test with Skeletonema sp. and Phaeodactylum tricornutum (ISO/DIS 10253:2023)

Wasserbeschaffenheit - Wachstumshemmtest mit marinen Algen Skeletonema sp. und Phaeodactylum tricornutum (ISO/DIS 10253:2023)

Qualité de l'eau - Essai d'inhibition de la croissance des algues marines avec Skeletonema sp. et Phaeodactylum tricornutum (ISO/DIS 10253:2023)

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Water quality — Marine algal growth inhibition test with Skeletonema sp. and Phaeodactylum tricornutum

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

ICS: 13.060.70

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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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

The committee responsible for this document is ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

This fourth edition cancels and replaces the second edition (ISO 10253:2016), which has been technically revised.

The main changes compared to the previous edition are as follows:

— in 5.2 Table 2, substitution of K_3PO_4 with K_2HPO_4 in stock solution 3;

— Annex D has been added to describe the marine algal growth inhibition test with *Phaeodactylum tricornutum* applied in 24-well-microwell plates.

Water quality — Marine algal growth inhibition test with Skeletonema sp. and Phaeodactylum tricornutum

WARNING — Persons using this document should be familiar with normal laboratory practice. This document 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 document be carried out by suitably trained staff.

1 Scope

This document specifies a method for the determination of the inhibition of growth of the unicellular marine algae *Skeletonema* sp. and *Phaeodactylum tricornutum* by substances and mixtures contained in sea water or by environmental water samples (effluents, elutriates, etc.).

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 documents are referred to in the text in such a way that some or all of their content constitutes requirements 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.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at https://www.electropedia.org/

— ISO Online browsing platform: available at https://www.iso.org/obp

3.1
cell density
number of cells per unit volume of medium

Note 1 to entry: The cell density is expressed as *x* cells/ml.

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

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

where

x is the cell density, expressed in cells per millilitre;

t is the time, expressed in days.

Note 1 to entry: Specific growth rate is expressed in inverse days (day^{-1}).

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

Note 1 to entry: The EC value is determined on the basis of the specific growth rate (r).

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 ± 2) h, during which the cell density in each is measured at intervals of at least every (24 ± 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* sp.[1)] (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 recommended algae are available in unialgal, non-axenic cultures from the following sources.

NIVA

Norwegian Institute for Water Research

Gaustadaléen 21

N 0349 Oslo

Norway

CCAP

Dunstaffnage Marine Laboratory

P O Box 3 Oban

Argyll PA37 1QA

United Kingdom

Experimental Phycology and Culture Collection of Algae at the University of Goettingen (EPSAG)

Nikolausberger Weg 18

37073 Goettingen

Germany

Stock cultures may be maintained in the medium described in 7.1. Regular subculturing is necessary. Weekly intervals may be necessary for *Skeletonema* sp., every two or three weeks may be sufficient for *Phaeodactylum tricornutum.* The stock cultures may also be maintained for extended periods on richer algal media such as those recommended by the culture collection or in (ninefold) concentrated growth medium (Annex D). It is recommended to keep the stock culture in the medium described in 7.1 and in

1) The previous editions of this document suggested the use of two strains of *Skeletonema costatum*. Following a taxonomic review of the *Skeletonema* genus, several strains originally identified as *S. costatum* may in fact be other species. In light of this and to enable continuity in the use of previously accepted strains, the present revision of this document has changed the reference from *Skeletonema costatum* to *Skeletonema* sp. to avoid non-compliance for labs that may be using different strains.

an exponential growth phase immediately before preparing the pre-culture for testing as described in 7.2.

NOTE Concentrated cultures of the diatom *Phaeodactylum tricornutum* can also be stored for several months without losing their viability. Stock cultures for the toxicity tests can easily be prepared from the stored concentrated cultures[2)].

5.2 Reagents

5.2.1 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.

5.2.2 Synthetic Sea water

For culturing and testing *Phaeodactylum tricornutum,* 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* sp., 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.

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 (synthetic as well as natural one) through a 0,45 µm membrane filter in order to remove particulate material and algae. Salinity of the (synthetic) sea water can be measured with e.g. a refractometer.

5.2.3 Nutrients

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

2) Concentrated *Phaeodactylum tricornutum* cultures can be supplied by MicroBioTests Inc. Mariakerke-Gent, Belgium. 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 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_2HPO_4	2,46 g/l	2,46 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

These stock solutions have to be 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 for a maximum of two months.

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 (e.g. Neubauer improved 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 (Reference [4])), 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.

Annex D describes a procedure to perform the test in 24-well-microwell plates with *in vivo* chlorophyll fluorimetric determination of algal growth of the species *Phaeodactylum tricornutum* in a microplate reader.

Spectrophotometers may be sufficiently sensitive to measure 10^4 algal 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.

Annex B describes a procedure to perform the spectrophotometric measurements of the algal cell density.

6.3 Culture flasks, e.g. 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.

6.7 Apparatus for salinity, e.g. a refractometer.

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 (5.2.2) 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 can 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 algal cells/ml to 10^4 algal 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. ratio of 1,8: 1,0 mg/l, 1,8 mg/l, 3,2 mg/l, 5,8 mg/l and 10,4 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.