



# FINAL DRAFT International Standard

## ISO/FDIS 10253

### 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**

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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 document 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](http://www.iso.org/directives)).

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

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

The main changes are as follows:

- in [Table 2](#),  $K_3PO_4$  has been substituted 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.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at [www.iso.org/members.html](http://www.iso.org/members.html).

# 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 terminology databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <https://www.electropedia.org/>

### 3.1

#### cell density

number of cells per unit volume of medium

Note 1 to entry: The cell density is expressed as  $x$  cells  $\text{ml}^{-1}$ .

### 3.2 specific growth rate

$\mu$   
proportional rate of increase in *cell density* (3.1) per unit of time:

$$\mu = \frac{1}{x} \times \frac{dx}{dt}$$

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<sup>-1</sup>).

### 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 and 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* (3.2) relative to the controls

Note 1 to entry: The EC value is determined based on 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  $(72 \pm 2)$  h, during which the cell density in each is measured at intervals of at least every  $(24 \pm 2)$  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* sp. (CCAP 1077/1C, NIVA BAC 1); or
- b) *Phaeodactylum tricornutum* Bohlin (CCAP 1052/1A, SAG 1090-1a, NIVA BAC 2).

NOTE 1 Editions of this document before the year 2016 suggested the use of two strains of *Skeletonema costatum*. Following a taxonomic review of the *Skeletonema* genus, several strains originally identified as *S. costatum* can in fact be other species. In light of this and to enable continuity in the use of previously accepted strains, since the 2016 edition of this document, the reference from *Skeletonema costatum* to *Skeletonema* sp. has changed to avoid nonconformity for laboratories that possibly use different strains.

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

Algae in unialgal, non-axenic cultures are available from the following sources<sup>1)</sup>.

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

SAG

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 can be necessary for *Skeletonema* sp., every two or three weeks can 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 an exponential growth phase immediately before preparing the pre-culture for testing as described in 7.2.

NOTE 2 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<sup>2)</sup>.

1) This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these suppliers.

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.

## 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 Hydrochloric acid or sodium hydroxide solution

If pH-adjustment is necessary ([7.1](#), [7.4](#)) use hydrochloric acid or sodium hydroxide solution:

- hydrochloric acid: HCl, e.g.  $c(\text{HCl}) = 1 \text{ mol/l}$ ; or
- sodium hydroxide: NaOH,  $c(\text{NaOH}) = 1 \text{ mol/l}$ .

### 5.2.3 Synthetic sea water

For culturing and testing *Phaeodactylum tricornutum*, the growth medium ([7.1](#)) is made up by adding nutrients to either natural [salinity of  $(30 \pm 5) \text{ g/kg}$ ] or synthetic (approximate salinity of  $33 \text{ g/kg}$ ) sea water. For *Skeletonema* sp., the use of natural sea water can be necessary for the long-term maintenance of cultures and can also be necessary for the test medium, because a synthetic sea water medium doesn't 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 =  $33 \text{ g/kg}$ ). 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	22,0
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	9,7
$\text{Na}_2\text{SO}_4$ (anhydrous)	3,7
$\text{CaCl}_2$ (anhydrous)	1,0
KCl	0,65
$\text{NaHCO}_3$	0,20
$\text{H}_3\text{BO}_3$	0,023

Filter the sea water (synthetic as well as natural one) through a  $0,45 \mu\text{m}$  membrane filter to remove particulate material and algae. Salinity of the (synthetic) sea water can be measured with e.g. a refractometer.

### 5.2.4 Nutrient stock solution

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
<b>Stock solution 1</b>		
FeCl <sub>3</sub> ·6H <sub>2</sub> O	48 mg/l	149 µg/l (Fe)
MnCl <sub>2</sub> ·4H <sub>2</sub> O	144 mg/l	605 µg/l (Mn)
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	45 mg/l	150 µg/l (Zn)
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0,157 mg/l	0,6 µg/l (Cu)
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0,404 mg/l	1,5 µg/l (Co)
H <sub>3</sub> BO <sub>3</sub>	1 140 mg/l	3,0 mg/l (B)
Na <sub>2</sub> EDTA	1 000 mg/l	15,0 mg/l
<b>Stock solution 2</b>		
Thiamin hydrochloride	50 mg/l	25 µg/l
Biotin	0,01 mg/l	0,005 µg/l
Vitamin B <sub>12</sub> (cyanocobalamin)	0,10 mg/l	0,05 µg/l
<b>Stock solution 3</b>		
K <sub>2</sub> HPO <sub>4</sub>	2,46 g/l	2,46 mg/l; 0,438 mg/l P
NaNO <sub>3</sub>	50,0 g/l	50,0 mg/l; 8,24 mg/l N
Na <sub>2</sub> SiO <sub>3</sub> ·5H <sub>2</sub> O	14,9 g/l	14,9 mg/l; 1,97 mg/l Si
NOTE During the preparation of stock solution 3, difficulties can occur in the solubilisation of the silicates with K <sub>2</sub> HPO <sub>4</sub> . Therefore, a 14,9 g/l Na <sub>2</sub> SiO <sub>3</sub> ·5H <sub>2</sub> O solution can be prepared separately as “stock solution 4”. In this case, add 1 ml of this solution when preparing the growth medium (7.1).		

These stock solutions shall 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 (2 ± 8) °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 usual laboratory equipment 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 can be sufficiently sensitive to measure  $10^4$  algal cells  $\text{ml}^{-1}$  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  $\mu\text{m}$  and 0,45  $\mu\text{m}$ .

**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 ([Table 2](#)) to approximately 900 ml of natural or synthetic sea water ([5.2.3](#)) and then make up to 1 l with the same sea water ([5.2.3](#)).

Adjust the pH to  $8,0 \pm 0,2$  by adding diluted hydrochloric acid or sodium hydroxide solution ([5.2.2](#)).

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 2 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.  $2 \times 10^3$  algal cells  $\text{ml}^{-1}$  to  $10^4$  algal cells  $\text{ml}^{-1}$  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, 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  $\text{EC}(r)_x$  parameter. Additionally, at least two levels of inhibition between 10 % and 90 % should be included 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 required when the test substance does not readily dissolve in the test medium, as described in ISO 14442 and ISO 5667-16.

Use a concentrated growth medium as specified in [Annex A](#) and, if necessary, when testing water samples (effluents, elutriates, etc.), to avoid growth inhibition due to low salinity. Use seawater salts ([5.2.3](#)) to adjust the salinity of the sample to the salinity of the growth medium. An example of a dilution scheme for sea water samples is given in [Annex A](#).

Carry out the test without adjusting the pH after addition of the test substance. However, some substances can exert a toxic effect due to extreme acidity or alkalinity. To determine the toxicity of a substance independent of pH, adjust the pH of the stock solution (before the dilution in series) to  $8,0 \pm 0,2$ , using either hydrochloric acid or sodium hydroxide solution ([5.2.2](#)). The concentration of acid or base should be such that 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, the concentration of added growth medium nutrients and the cell density shall be the same in all test batches.

The initial cell density shall be sufficiently low to allow for exponential growth in the control culture throughout the test duration, or for at least the time required to achieve a factor 16 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  $10^4$  algal cells  $\text{ml}^{-1}$ .

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

Prepare at least three replicates for each test substance concentration. To a further six 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 to allow  $\text{CO}_2$  to enter the vessels. Incubate the test vessels at a nominal temperature of  $20\text{ }^\circ\text{C}$ , under continuous white light. The temperature shall not vary by more than  $2\text{ }^\circ\text{C}$  during the test. The photon fluence rate at the average level of the test solutions shall be uniform and in the range  $60\text{ }\mu\text{mol}/\text{m}^2\text{ s}$  to  $120\text{ }\mu\text{mol}/\text{m}^2\text{ s}$ , when measured in the photosynthetically effective wavelength range of 400 nm to 700 nm using an appropriate receptor.

**NOTE 1** 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 2.

**NOTE 2** The light intensity specified above can 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 at a distance of approximately 0,35 m from the algal culture medium.

**NOTE 3** For light-measuring instruments calibrated in lx, an equivalent range of 3 000 lx to 6 000 lx is acceptable for the test.

Continuously and gently shake the cultures to keep the cells in free suspension and to facilitate  $\text{CO}_2$  mass transfer from air to water, and in turn, reduce pH shift.