International Standard

ISO 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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Contents

Page

Foreword

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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).

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This document was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 230, *Water analysis*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

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;

— in Table A.1, dilution step 5 was changed to dilution step 6.

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.

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.

IMPORTANT — It is absolutely essential that tests conducted according to this document be carried out by suitably qualified 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 ml^{-1}.

3.2
specific growth rate
μ
proportional rate of increase in *cell density* (3.1) per unit of time:

$$\mu = \frac{1}{x} \times \frac{\mathrm{d}x}{\mathrm{d}t}$$

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 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 ± 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. (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[1].

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

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1)   This information is given for the convenience of users of this document and does not constitute an endorsement by ISO. The stock cultures are not commercial products, but they are provided by the given sources, and they are tested and validated for the method described in this document. Equivalent products may be used if they can be shown to lead to the same results.

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[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   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. $c(HCl) = 1$ mol/l; or

— sodium hydroxide: NaOH, $c(NaOH) = 1$ 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 ± 5) g/kg] or synthetic (approximate salinity of 33 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 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
$MgCl_2 \cdot 6H_2O$	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 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.

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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
NOTE During the preparation of stock solution 3, difficulties can occur in the solubilisation of the silicates with K_2HPO_4. Therefore, a 14,9 g/l $Na_2SiO_3 \cdot 5H_2O$ 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 (5 ± 3) °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 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 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 (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 ± 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. 2×10^3 algal cells ml^{-1} to 10^4 algal cells 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 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 ± 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 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 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.

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 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 ± 2) h. These measurements are usually made on small volumes which are removed from the test solution and not replaced. Before measurement, the test batches should be mixed thoroughly.

The test shall last for (72 ± 2) h. At the end of the test, measure the pH of each test batch (7.5) and of the controls (7.5). 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   The growth rate of the algae under the specified conditions can vary among different strains of the species. Results from validation interlaboratory trials indicate that growth rates above 1,0 d^{-1} are usually 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.

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 should always be kept as low as possible, for example, by 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 sample 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 Formula (1):

$$\mu = \frac{\ln N_L - \ln N_0}{t_L - t_0} \tag{1}$$

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 using Formula (2):

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

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 nonlinear model to the experimental data points by regression analysis (for example, see References [2], [5] and [6]) 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 may 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, clearly indicate 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, usually in milligrams per litre (mg/l), millilitres per litre (ml/l), or %.

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 account 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 Test report

This test report shall contain at least the following information:

a) the test method used, together with a reference to this document, i.e. ISO 10253:2024;

b) all data required for identification of the test sample, e.g. 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 in temperature-controlled cabinet or incubator;

— 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.

Annex A
(informative)

Preparation of dilution series of mixtures in sea water(effluents or elutriates)

A.1 General

When testing mixtures in sea water (wastewater or elutriates) in dilution series, the natural or synthetic sea water (see 5.2.3) should be used as dilution water and added nutrient concentrations should be the same in all dilutions and equal to the final test concentration stated in 5.2.3. For that reason, the use of a concentrated growth medium is recommended.

A.2 Preparation of concentrated growth medium

Add 135 ml of stock solution 1, 4,5 ml of stock solution 2 and 9 ml of stock solution 3 to approximately 700 ml natural or synthetic sea water (5.2.3) and then make up to 1 l with the same sea water.

Adjust the pH to 8,0 ± 0,2 by adding diluted hydrochloric acid or sodium hydroxide solution (5.2.2).

A.3 Preparation of the dilution series of test media

Prepare the dilution series of test media by mixing the volumes of concentrated growth medium (A.2), inoculum (7.2), sample (effluent or elutriates) and dilution water (natural or synthetic sea water 5.2.3) in the test vessels following the scheme of Table A.1. The total volume should be the same in all the vessels.

For further instruction concerning initial cell density and test design, follow 7.5.

Table A.1 — Preparation of dilution series — Concentration of test and control batch

Dilution	Dilution step D	Inoculum (7.2) ml	Sample (7.4) ml	Sea water (5.2.3) ml	Concentrated growth medium (A.2) ml	End volume ml
1 in 1,25	1	10	80	–	10	100
1 in 2	2	10	50	30	10	100
1 in 3	3	10	33,33	46,67	10	100
1 in 4	4	10	25	55	10	100
1 in 6	6	10	16,67	63,33	10	100
1 in 8	8	10	12,5	67,5	10	100
1 in 12	12	10	8,33	71,67	10	100
1 in 16	16	10	6,25	73,75	10	100
1 in 24	24	10	4,17	75,83	10	100
1 in 32	32	10	3,13	76,88	10	100
Control batch		10	–	80	10	100

Annex B
(informative)

Test procedure starting from stored algal inocula, and with direct measurement of algal growth in spectrophotometric cells

B.1 General

This method can be applied to testing of pure chemicals as well as to effluents, wastewater, and other environmental aqueous samples.

This test procedure is based on optical density (OD) measurements of the algal growth in spectrophotometric cells of 10 cm path length, which serve as test vessels for the assays. The algae are obtained from algal inocula in test tubes which can be stored for several months, bypassing the need for continuous culturing of algal stocks.

B.2 Principle

Monospecies algal suspensions in the exponential growth phase are obtained by 3 d preculturing of algal inocula.

A dilution series of the chemical or the water sample is prepared with the algal growth medium (7.1) and inoculated with a specific volume of the concentrated algal suspension to obtain a start density of 10^4 algal cells ml^{-1}.

The algal suspensions are transferred into 10 cm spectrophotometric cells (hereafter called "long cells") covered in part with a lid to preclude evaporation, but still allowing for gas exchange with the environment.

The long cells are incubated for (72 ± 2) h in a temperature-controlled cabinet or incubator at (20 ± 2) °C under continuous white illumination (7.6), with daily measurement of the algal growth in each long cell by determination of the OD of the algal suspension in a spectrophotometer at 670 nm.

The test procedure, materials and equipment are similar to those described in ISO 8692:2012, Annex B.

B.3 Materials

B.3.1 Test organisms

The algal species used as stored algal inocula is *Phaeodactylum tricornutum* (see 5.1).

B.3.2 Nutrients

For the algal growth medium used for this test, see Table 1.

The three stock solutions (see Table 2) are preferably dispensed in penicillin vials. When stored in the refrigerator at (4 ± 2) °C in darkness, the vials with the nutrient solutions have a shelf life of up to one year.

B.3.3 Algal inoculum

Prepare a stock culture of the algae in the exponential growth phase according to (7.2).

Transfer 2,5 ml of the algal suspension in test tubes and add 7,5 ml algal growth medium (7.1) to each tube.

Put and keep the test tubes in the refrigerator in darkness, prior to proceeding to a 3 d preculturing for subsequent performance of the toxicity test.

NOTE   Algal inocula in test tubes can be stored for several months without losing their viability.

Test tubes with *Phaeodactylum tricornutum* inocula can also be obtained from a commercial source.[3]

B.4 Apparatus

B.4.1   Temperature-controlled cabinet or room, or incubator with white, fluorescent light, providing continuous uniform illumination suitable for the lighting requirements of algal growth inhibition tests, as specified in 7.6.

B.4.2   Spectrophotometer, equipped with a holder for 10 cm cells.

B.4.3   pH-meter.

B.4.4   Cell counter.

B.5 Laboratory materials

B.5.1   Spectrophotometric cells, path length 10 cm.

Spectrophotometric cells made of glass and provided with a lid may be used. Disposable 10 cm spectrophotometric cells in inert materials (e.g. polystyrene) may also be used and are commercially available.[4]

B.5.2   Laboratory glassware.

Conventional laboratory flasks, pipettes, and test tubes.

B.5.3   Holding tray in transparent plastic, for housing the long cells during the incubation period.

B.6 Test procedure

B.6.1 Preculturing of the algae

Take a test tube with algal inoculum, handshake it vigorously and pour the contents into a long cell (called preculturing long cell).

Rinse the tube twice with 7,5 ml algal growth medium (7.1) and transfer the contents into the preculturing long cell to ensure the total transfer of the algal inoculum.

Close the preculturing long cell with the lid and incubate this long cell for 3 d at (20 ± 2) °C with an appropriate illumination and in the conditions described in 7.6.

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3)   *Phaeodactylum tricornutum* inocula supplied by MicroBioTests Inc., Mariakerke-Gent, Belgium 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.

4)   The long cells supplied by MicroBioTests Inc., Mariakerke-Gent, Belgium 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.

B.6.2 Determination of the relationship "optical density (OD) to number of algae (*N*)" for spectrophotometric measurements in long cells

The relationship between optical density (OD) and number of algae *N* is specific for each batch of algae inocula and for each type of spectrophotometer.

Take one long cell, mark it as "Calibration cell" and fill it with 25 ml algal growth medium (7.1).

Close the long cell with the lid, insert it in the spectrophotometer and zero-calibrate the instrument at 670 nm.

Take the preculturing long cell, close it tightly with the lid and shake to distribute the algal suspension evenly.

Put the preculturing long cell in the spectrophotometer and read the optical density at 670 nm.

Take a small sample of algal suspension from the preculturing long cell with a pipette and count the number of algae under the microscope with a counting chamber (hemocytometer).

Dilute the algal suspension in the preculturing long cell by 20 % by removing 5 ml suspension and replacing it by 5 ml algal growth medium (7.1). Close the cell tightly and shake to obtain again a homogenous algal suspension.

Measure the OD in the preculturing cell containing the diluted algal suspension.

Remove again 5 ml algal suspension from the preculturing long cell and replace it by 5 ml growth medium, to obtain an algal suspension which is again 20 % less concentrated than the previous one. Determine again the OD of this algal suspension in the preculturing long cell.

Repeat the dilution steps and the measurements until the measured OD value has decreased to approximately 0,05.

For each dilution, calculate the corresponding number of algae (which is 20 % lower than in the previous dilution) and make a plot of values of OD against *N* to calculate the OD to *N* regression.

B.6.3 Preparation of the concentrated algal inoculum

Take the OD to *N* plot and look up the number of algae N_1 corresponding to the measured OD value OD_1. With N_2 equal to 1×10^6 algal cells ml^{-1}, calculate from the N_1/N_2 ratio the dilution factor which needs to be applied to reach an OD equal to OD_2, corresponding to an algal density of 1×10^6 algal cells ml^{-1} (see Figure B.1).
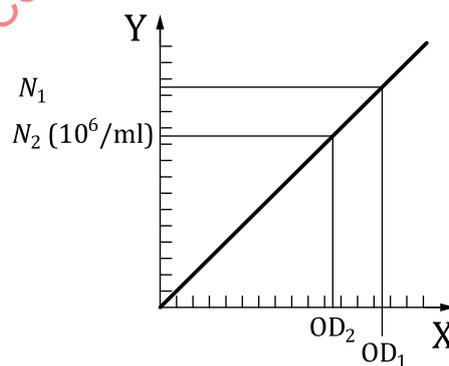
Key

X	optical density
Y	concentration of algae
OD_1, OD_2	optical density 1 and optical density 2
N_1, N_2	number of algae

Figure B.1 — Diagram for calculation of the dilution factor to make up a concentrated 10^6 algal cells ml^{-1} suspension

Transfer the algal suspension from the preculturing cell into a 100 ml flask and add the volume of algal growth medium needed to make up a 1×10^6 algal cells ml^{-1} suspension in the flask. Stopper and shake the flask to distribute the algae evenly.

B.6.4 Preparation of toxicant dilution series

Toxicant dilutions shall be prepared according to the procedure described in 7.4.

The present test procedure is based on five test concentrations and a negative control with three replicates per test concentration and the control.

Prepare for each toxicant dilution (and the control) 100 ml solution in calibrated flasks, in algal growth medium (7.1), and with addition of 1 ml of concentrated algal inoculum (1×10^6 algal cells ml^{-1}) to each flask, to obtain a start concentration of 10^4 algal cells ml^{-1}.

B.6.5 Transfer of the algae-toxicant suspensions into test long cells

Take 18 long cells and label them in sets of three replicates for each test concentration and the control.

After thorough shaking, transfer 25 ml algae-toxicant dilution from each flask into the corresponding three replicate long cells and close the cells with their lid.

B.6.6 Incubation of the test vials

Put the inoculated 18 long cells in a transparent holding tray, in a random way. Open the long cells slightly by lifting the cover at one end to keep them open for gas exchange.

NOTE   A plastic strip, a few centimetres wide, can be slid under the lids along the total length of the holding tray to keep the long cells slightly open.

Incubate the cells in the holding tray for (72 ± 2) h at (20 ± 2) °C with an appropriate illumination and in the conditions described in 7.6.

B.6.7 Measurements

Measure the OD in each long cell after (24 ± 2) h, (48 ± 2) h and (72 ± 2) h incubation and record the data on a results sheet.

Prior to each measurement the algae shall be resuspended by thoroughly shaking the contents of the closed long cells.

B.6.8 Tests on coloured natural samples

Interferences by colour can be taken into account by filling five additional long cells with 25 ml of each toxicant concentration prior to the addition of the concentrated algal suspension.

Zero-calibrate the spectrophotometer at 670 nm with the long cells containing toxicant solution without algae, prior to the OD measurement of the long cell containing the corresponding algae/toxicant solution.

The long cells containing the coloured toxicant dilutions without algae shall be incubated alongside the other long cells to take into account possible changes in colour which can occur during the exposure time.

B.6.9 pH measurement

At the end of the test, the contents of the three control long cells shall be pooled and the pH measured.

B.7 Calculation of the percentage inhibition

Transform the ODs scored on the results sheet into cell numbers, N, with the aid of the OD to N regression.

Subsequently calculate the inhibition of the growth rate using Formulae (1) and (2) as specified in 9.2 and the EC$(r)_x$ as indicated in 9.3.

B.8 Validity criteria

The validity criteria of the test procedure in long cells are those indicated in Clause 8.

B.9 Precision

The repeatability of the method with determination of the optical density of the algal growth in long cells, and departing from stored algal inocula, has been determined on a substantial number of batches of stored *Phaeodactylum tricornutum* inocula, over a 5-year-period (2008 to 2013).

Fourteen "reference" toxicity tests have been performed with the chemical potassium dichromate in a laboratory in Belgium, with calculation of the 72 h EC$(r)_{50}$ values for each assay. Table B.1 shows the individual EC$(r)_{50}$ data and their mean value (16,21 mg/l).

Table B.1 — Repeatability test results

Phaeodactylum tricornutum batch	72 h EC$(r)_{50}$
PH031008	16,66
PH130109	15,41
PH070509	17,41
PH030909	16,10
PH201109	14,82
PH110310	17,00
PH190511	15,28
PH240811	18,98
PH081211	14,37
PH020412	16,02
PH110712	15,18
PH151112	14,93
PH200313	14,54
PH050713	20,21
Mean	16,21
Standard deviation	1,72
Coefficient of variation %	10,59

The low variation coefficient (10,59 %) for the multiple assays carried out over a 5-year-period clearly indicates a high repeatability of the algal assays departing from stored algal inocula and performed in 10 cm spectrophotometric cells.

The mean EC$(r)_{50}$ value is furthermore quite close to the mean EC$(r)_{50}$ (20,1 mg/l) obtained with potassium dichromate on *Phaeodactylum tricornutum* in the validation interlaboratory trial carried out in 1989/1990 with 10 laboratories (see Table C.1).

In 2014, 12 laboratories, from six different countries, participated in a validation interlaboratory trial, using the reference toxicant potassium dichromate and this procedure. The result and the details of the validation interlaboratory trial are given in a detailed report (Reference [7]).

One laboratory did not perform the assay according to the prescribed test procedure, and had carried out the assay in 1 cm spectrophotometric cells instead of the 10 cm long cells. Therefore, the results of this laboratory were discarded.

The validation interlaboratory trial revealed that the accepted results from 11 participating laboratories were all consistent, without outliers or stragglers (Table B.2).

Table B.2 — Precision data for EC$(r)_{50}$

l	n	$\overline{\overline{x}}$	s_r	$C_{V,r}$ %	s_R	$C_{V,R}$ %	95 % UCL	95 % LCL
11	3	17,87	1,11	6,22	4,38	24,53	26,46	9,28

Key

l number of laboratories

n number of individual test results after outlier rejection

$\overline{\overline{x}}$ overall mean of results (without outliers) of EC$(r)_{50}$

s_r repeatability standard deviation

$C_{V,r}$ coefficient of variation of repeatability

s_R reproducibility standard deviation

$C_{V,R}$ coefficient of variation of reproducibility

UCL upper confidence limits

LCL lower confidence limits

The mean EC$(r)_{50}$ and the interlaboratory variability of this validation interlaboratory trial performed in long cells are very near to those obtained in the repeatability tests reported in Table B.1 and to those of the 1989/1990 ring test reported in Table C.1 for the assays in culture flasks.

The growth rate of the 11 participating laboratories varies from a minimum of 1,24 d^{-1} to a maximum of 1,62 d^{-1}, with a corresponding range of standard deviation (3 replicates) between 0,001 and 0,005, and a coefficient of variation between 0,035 % and 2,81 %. The mean growth rate of the controls was 1,45 d^{-1}, with an interlaboratory standard deviation of 0,13 and a coefficient of variation % of 9,27.

B.10 Test report

The test report shall include a statement that the test was performed in accordance with this document, Annex B. For the content and structure of the test report see Clause 12.

Annex C
(informative)

Performance data

A validation interlaboratory trial based on the test described in this document 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 C.1.

Table C.1 — Results interlaboratory trial

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

In 2014, 12 laboratories, from six different countries, participated in a validation interlaboratory trial, using the reference toxicant potassium dichromate and the direct measurement of algal growth in 10 cm spectrophotometric cells, as described in Annex B. The result and details of the validation interlaboratory trial are given in Annex B and in a detailed report (Reference [7]).

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.

Annex D
(informative)

Marine algal growth inhibition test with *Phaeodactylum tricornutum* applied in 24-well microwell plates

D.1 General

This annex specifies a procedure for the determination of the growth inhibition of chemicals, marine water samples and elutriates to the marine algae *Phaeodactylum tricornutum* applied in 24-well microwell plates as test vessels. The cell density is indirectly determined by in vivo chlorophyll fluorescence measurement with a microplate reader. The procedure is based on a calibration (linear regression line) of (net) fluorescence as a function of algal cell number of *Phaeodactylum tricornutum*. For highly volatile substances and substances with a high n-octanol-water partition coefficient (log k_{ow}) further modification (e.g. Reference [9]) and validation are necessary.

D.2 Principle

See Clause 4.

The test procedure is miniaturized in order to save resources, chemicals, water, test material, space and time. Therefore, more tests can run in parallel.

The stock culture (5.1) of *Phaeodactylum tricornutum* is cultured in concentrated growth medium (D.5.2). In order to achieve exponential growth in the test culture, it is recommended to establish two successive pre-cultures, each lasting 3 d.

A calibration (linear regression line) of (net) fluorescence as a function of algal cell number of *Phaeodactylum tricornutum* is prepared and should be regularly validated.

The algal test culture (inoculum) has a density of 10^5 algal cells ml^{-1} (±10 %), thus by pipetting according to scheme (D.5.6) a start density of 10^4 (±10 %) algal cells ml^{-1} in the well is obtained. A concentration series of the test substance or a dilution series of the water or elutriate sample is prepared with synthetic sea water (5.2.). The test and control batches are prepared by pipetting the volumes of concentrated growth medium, test concentration or sample, synthetic sea water and inoculum according to scheme (D.5.6) in the wells. In addition, for each concentration and for the negative controls a blank without algae is prepared on the 24-well microwell plates. The total volume of 2 ml should be the same in all wells.

The 24-well microwell plates are closed with their regular cover, so that gas exchange in the incubator is possible. They are placed on a shaking device and are gently agitated (approximately 120 r/min to 150 r/min). The 24-well microwell plates are incubated for (72 ± 2) h in a temperature-controlled cabinet or incubator at (20 ± 2) °C under continuous white illumination (7.6). The algal growth is measured at the beginning of the test, every (24 ± 2) h and after (72 ± 2) h with a fluorescence microplate reader (excitation wavelength (430 ± 35) nm, emission wavelength (670 ± 25) nm). The blank for each dilution step is subtracted from the initial (gross) fluorescence value. The (net) fluorescence values are transferred to cell numbers by applying the equation for the linear regression of fluorescence as a function of algal cell number of *Phaeodactylum tricornutum*. The growth rate and inhibition are calculated according to Clause 9 and Clause 10.

D.3 Materials

D.3.1 Test organisms

Phaeodactylum tricornutum (5.1).

D.3.2 Reference substance

3,5-dichlorophenol (CAS Registry Number®:[5] 591-35-5) is recommended as reference substance (see Clause 12, Annex C, D.5.4, D.5.6, D.7 and D.9). All chemicals used shall be of analytical grade.

D.3.3 Laboratory materials

See 5.1 test organism *Phaeodactylum tricornutum* and 5.2 reagents. In addition: 24-well microwell plates (polystyrene, transparent, flat bottom, untreated and uncoated surface) are used as test vessels. A test volume of 2,0 ml per well is applied.

D.4 Apparatus

See 6.1 to 6.7 for the test culture *Phaeodactylum tricornutum* and test procedure.

In addition:

D.4.1 Fluorescence microplate reader

The chlorophyll fluorescence of *Phaeodactylum tricornutum* is optimally detected at a wavelength excitation of 435 nm and a wavelength emission of 685 nm. Thus, use appropriate filter with a wavelength excitation = (430 ± 35) nm, wavelength emission = (670 ± 25) nm to measure the chlorophyll fluorescence in the wells.

D.4.2 Orbital shaker for 24-well microwell plates

D.5 Procedure

D.5.1 Preparation of growth medium

See 7.1 for preparation of growth medium.

D.5.2 Preparation of concentrated growth medium

A ninefold concentrated growth medium is prepared as follows:

Add 135 ml of stock solution 1, 4,5 ml of stock solution 2 and 9 ml of stock solution 3 (Table 2) to approximately 700 ml synthetic sea water (5.2.3) 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 (5.2.2).

NOTE   In general, the test algae culture (D.5.3) is prepared with a high percentage of fresh growth medium. Therefore, 200 µl ninefold concentration growth medium and 200 µl test algae in fresh growth medium add up to approximately tenfold nutrient concentration and is sufficient for a volume of 2 ml in the test vessel.

D.5.3 Pre-culturing of the algae and inoculum

The stock culture (5.1) is cultured in concentrated growth medium (D.5.2). To achieve exponential growth in the test culture, it is recommended to establish two successive pre-cultures in growth medium (D.5.1, 7.1), each lasting 3 d.

Carry out a microscopic evaluation of the algae at the beginning from the pre-culture and from the negative control batch at the end of the test. The cells are examined for unusual cell shapes, colour differences, chloroplast differences, precipitates and clots.

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5) Chemical Abstract Service (CAS) Registry Number® is a trademark of the American Chemical Society (ACS). This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

Prepare a calibration (linear regression line) of (net) fluorescence as a function of algal cell number of *Phaeodactylum tricornutum* as described in D.5.8 and use the calibration line to prepare the proper algae cell density for the inoculum.

The pre-culture and growth medium (D.5.1, 7.1) are used to prepare the test algae culture (inoculum) containing 10^5 cells ml^{-1} resulting in a final cell density of 10^4 cells ml^{-1} (± 10 %) in the wells (dilution by a factor of 10 in the batch), thus allowing an exponential growth of the algae during the test duration. The precise cell density at the beginning of the test is important for the validity of the test.

NOTE   It is assumed that a high percentage of fresh growth medium (D.5.1, 7.1) is used to prepare the test algae culture.

## D.5.4   Preparation of test substance dilution series

Prepare dilution series of test substance or water sample or elutriate sample in extra vessels (outside of the 24-well microwell plates) according to the procedure described in 7.3 and 7.4, by using synthetic seawater (5.2.3, Table 1) instead of growth medium as diluent.

D.5.5   Test design – number of 24-well microwell plates and well position

See 7.5 for preparation of test and control batches.

Depending on the number of test substance or sample dilutions, it can be necessary to use more than one 24-well microwell plate. In this case, negative control and blank batches shall be placed on each 24-well microwell plate.

Figure D.1 shows the test design with two 24-well microwell plates with the position of test and control batches used in the validation interlaboratory trial (D.9) for informative purposes. It is recommended to (pseudo) randomize the position of the dilution series of test substance or sample and negative controls, although no significant side effects were detected on additional control well plates in the validation interlaboratory trial (Reference [8]).

a) Plate no. 1   b) Plate no. 2
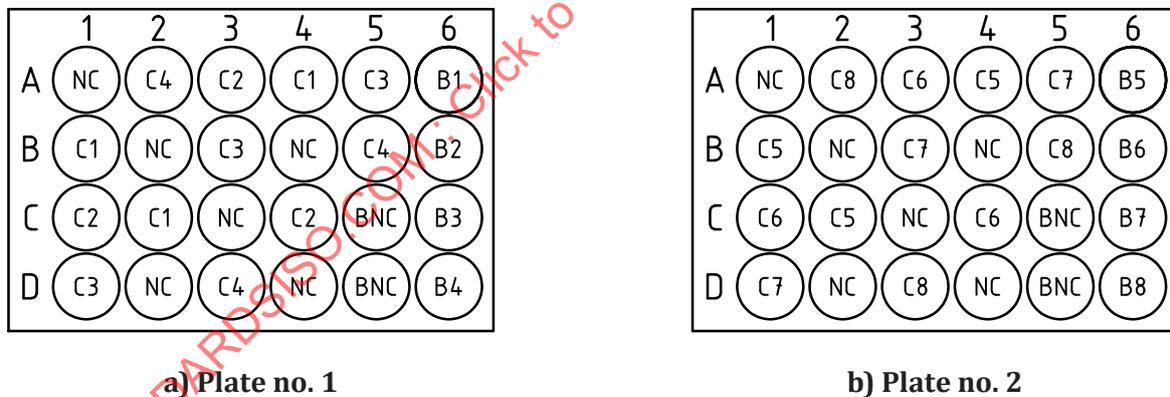
Key

C1…C8   concentration dilution step
NC   negative control
B1…B8   blanks of C1 to C8
BNC   blank negative control (see Tables D.1 and D.2)

Figure D.1 — Example of test design with two 24-well microwell plates and the well position of test and control batches

D.5.6   Preparation of test and control batches

Use the following pipetting scheme for the preparation of test and negative control batches in the wells (Table D.1, Table D.2). The total volume of 2 ml should be the same in all wells.