BS EN ISO 10253:2016

BSI Standards Publication

Water quality — Marine algal growth inhibition test with Skeletonema sp. and Phaeodactylum tricornutum (ISO 10253:2016)

bsi.

National foreword

This British Standard is the UK implementation of EN ISO 10253:2016. It supersedes BS EN ISO 10253:2006 which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee EH/3/5, Biological Methods.

A list of organizations represented on this committee can be obtained on request to its secretary.

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Published by BSI Standards Limited 2016

ISBN 978 0 580 87012 5

ICS 13.060.70

Compliance with a British Standard cannot confer immunity from legal obligations.

This British Standard was published under the authority of the Standards Policy and Strategy Committee on 31 December 2016.

Amendments/corrigenda issued since publication

Date	Text affected

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN ISO 10253

November 2016

ICS 13.060.70

Supersedes EN ISO 10253:2006

English Version

Water quality - Marine algal growth inhibition test with Skeletonema sp. and Phaeodactylum tricornutum (ISO 10253:2016)

Qualité de l'eau - Essai d'inhibition de la croissance des algues marines avec Skeletonema sp. et Phaeodactylum tricornutum (ISO 10253:2016)

Wasserbeschaffenheit - Wachstumshemmtest mit marinen Algen Skeletonema sp. und Phaeodactylum tricornutum (ISO 10253:2016)

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Ref. No. EN ISO 10253:2016 E

European foreword

This document (EN ISO 10253:2016) has been prepared by Technical Committee ISO/TC 147 "Water quality" in collaboration with Technical Committee CEN/TC 230 "Water analysis" the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by May 2017, and conflicting national standards shall be withdrawn at the latest by May 2017.

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

This document supersedes EN ISO 10253:2006.

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Endorsement notice

The text of ISO 10253:2016 has been approved by CEN as EN ISO 10253:2016 without any modification.

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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).

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

This third edition cancels and replaces the second edition (ISO 10253:2006), which has been technically revised.

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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 http://www.electropedia.org/

— ISO Online browsing platform: available at http://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.

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

$$\mu = \frac{1}{x} \times \frac{dx}{dt} (1 / \text{day})$$

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

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.

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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

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* 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. It is recommended to keep the

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.

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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 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 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.

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.

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Table 2 — Nutrient stock solutions

Nutrient	Concentration in stock solution	Final concentration in test solution
Stock solution 1		
$FeCl_3 \cdot 6H_2O$	48 mg/l	149 µg/l (Fe)
$MnCl_2 \cdot 4H_2O$	144 mg/l	605 µg/l (Mn)
$ZnSO_4 \cdot 7H_2O$	45 mg/l	150 µg/l (Zn)
$CuSO_4 \cdot 5H_2O$	0,157 mg/l	0,6 µg/l (Cu)
$CoCl_2 \cdot 6H_2O$	0,404 mg/l	1,5 µg/l (Co)
H_3BO_3	1 140 mg/l	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

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.

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. 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.

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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.

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. 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 (5.2.3) and, if appropriate, to avoid growth inhibition due to a too low salinity, with sea water salts (5.2.2) 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 given 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

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in series) to 8,0 ± 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 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.

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 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 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 ± 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.

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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 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.

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 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).

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Calculate mean values of μ for the control. Calculate the percentage inhibition for each individual test flask from Formula (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 nonlinear model to the experimental data points by regression analysis (for example, see References [5], [6] and [2]) 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), 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 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 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 10253:2016;

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;

— 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.

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Annex A
(informative)

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

A.1 General

When testing mixtures in sea water (waste water or elutriates) in dilution series, the natural or synthetic sea water (see 5.2.2) 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 stock solution 1, 4,5 ml stock solution 2 and 9 ml stock solution 3 to approximately 700 ml 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.

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 5.2.2) 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 (5.2.2) 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 5	5	10	20	60	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	3,125	76,875	10	100
Control batch		10	—	80	10	100

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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, wastewaters 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 and which bypasses 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 ISO 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.

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).

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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 Bürker 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 and transfer the contents into the preculturing long cell to ensure the total transfer of the algal inoculum.

---

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 International Standard 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.

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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.

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.

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, Bürker chamber).

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. 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 A_1. With N_2 equal to 1×10^6 algal cells/ml, calculate from the N_1/N_2 ratio the dilution factor which needs to be applied to reach an OD equal to A_2, corresponding to an algal density of 1×10^6 algal cells/ml (see Figure B.1).

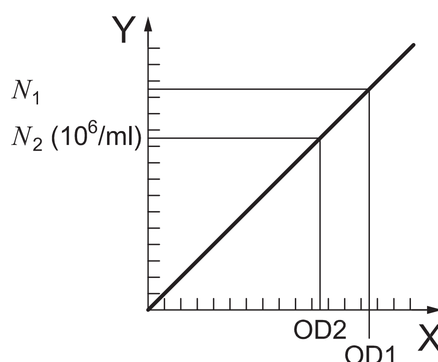

Figure B.1 — Diagram for calculation of the dilution factor to make up a concentrated 10^6 algal cells/ml suspension

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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 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, and with addition of 1 ml of concentrated algal inoculum (1×10^6 algal cells/ml) to each flask, to obtain a start concentration of 10^4 algal cells/ml.

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 up the cover at one end to keep them open for gas exchange.

NOTE A plastic strip of a few centimetres width 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 in order to take into account possible changes in colour which could 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.

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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 the same as 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 years 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 years period clearly points to the 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 interlaboratory test carried out in 1989/1990 with 10 laboratories (see Table C.1).

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In 2014, 12 laboratories, from six different countries, participated in an International Interlaboratory Comparison, using the reference toxicant potassium dichromate and this procedure. The outcome and the details of this ringtest are given in a detailed report (Reference [7]).

One laboratory, however, 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.

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

Table B.2 — Mean EC(r)$_{50}$ values, s_r intralab repeatability, s_R interlab reproducibility, CV % variation coefficient in %, 95 % upper (UCL) and lower confidence limits (LCL)

n lab	*n* replicates	Mean	s_r	CV %	s_R	CV %	UCL	LCL
11	3	17,87	1,11	6,22	4,38	24,53	26,46	9,28

The mean EC(r)$_{50}$ and the inter-laboratory variability of this ringtest 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 CV % 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 and the requirements given in Clause 12.

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Annex C
(informative)

Performance data

An interlaboratory test 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 — 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

In 2014, 12 laboratories, from six different countries, participated in an International Interlaboratory Comparison, using the reference toxicant potassium dichromate and the direct measurement of algal growth in 10 cm spectrophotometric cells, as described in Annex B. The outcome and the details of this ringtest 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.

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