INTERNATIONAL STANDARD

ISO 8692

Third edition
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Water quality — Fresh water algal growth inhibition test with unicellular green algae

Qualité de l'eau — Essai d'inhibition de la croissance des algues d'eau douce avec des algues vertes unicellulaires

Reference number
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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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

ISO 8692 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

This third edition cancels and replaces the second edition (ISO 8692:2004), which has been technically revised.

Water quality — Fresh water algal growth inhibition test with unicellular green algae

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

IMPORTANT — It is absolutely essential that tests conducted in accordance with this International Standard be carried out by suitably qualified staff.

1 Scope

This International Standard specifies a method for the determination of the growth inhibition of unicellular green algae by substances and mixtures contained in water or by waste water. This method is applicable for substances that are easily soluble in water.

With modifications to this method, as specified in ISO 14442 and ISO 5667-16, the inhibitory effects of poorly soluble organic and inorganic materials, volatile compounds, heavy metals and waste water can be tested.

A rapid algal growth inhibition screening test for waste water is described in Annex A.

An alternative test procedure with algae from algal beads, with direct measurement of algal growth in spectrophotometric cells, is described in Annex B.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. 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/TR 11044, *Water quality — Scientific and technical aspects of batch algae growth inhibition tests*

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

ISO/TS 20281, *Water quality — Guidance on statistical interpretation of ecotoxicity data*

3 Terms and definitions

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

3.1
cell density
n
number of cells per volume of medium

NOTE Cell density is expressed in cells per millilitre.

3.2
effective concentration
concentration of the test sample (EC_x) at which an effect of x % is measured, if compared to the control

NOTE To unambiguously denote an EC value deriving from growth rate, it is proposed to use the symbol "E_rC".

3.3
lowest ineffective dilution
LID
dilution level at which no inhibition, or only effects not exceeding the test-specific variability, are observed

NOTE Adapted from ISO 15088:2007[13], 3.5.

3.4
specific growth rate
μ
proportional rate of increase in cell density per time:

$$\mu = \frac{1}{n}\frac{\mathrm{d}n}{\mathrm{d}t}$$

where

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

t is the time, expressed in days.

NOTE Specific growth rate is expressed in reciprocal days (day^{-1}).

[ISO/TR 11044:2008, 3.2]

4 Principle

Monospecies algal strains are cultured for several generations in a defined medium containing a range of concentrations of the test sample, prepared by mixing appropriate quantities of growth medium, test sample, and an inoculum of exponentially growing algal cells. The test batches are incubated for a period of (72 ± 2) h during which the cell density in each test solution is measured at least every 24 h.

Inhibition is measured as a reduction in specific growth rate relative to control cultures grown under identical conditions.

5 Reagents and media

5.1 Test organism, using either of the following planktonic fresh water algae species:

a) *Desmodesmus subspicatus* (R. Chodat) E. Hegewald et A. Schmidt[1)] (86.81 SAG[2)]);

b) *Pseudokirchneriella subcapitata* (Korshikov) Hindak[3)] (ATCC® 22662™,[2)] CCAP 278/4[2)] or 61.81 SAG[2)]).

NOTE 1 The two species do not show identical responses to toxic agents.

NOTE 2 Both algae species are planktonic green algae belonging to the order of Sphaeropleales (Chlorophyta, Chlorophyceae) and are usually unicellular in culture.

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1) This species is formerly known as *Scenedesmus subspicatus* Chodat.

2) Trade names of strains are examples of suitable strains available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

3) This species is formerly known as *Selenastrum capricornutum* Prinz. The new name is currently cited by culture collections.

The strains recommended are available in unialgal, non-axenic cultures from the following collections:

— SAG — Sammlung von Algenkulturen Göttingen [Göttingen Algal Culture Collection], Germany

www.epsag.uni-goettingen.de (viewed 2012-01-30);

— ATCC — American Type Culture Collection, USA

www.atcc.org (viewed 2012-01-30);

— CCAP — Culture Collection of Algae and Protozoa, UK

www.ccap.ac.uk (viewed 2012-01-30);

— ALCP — Algothèque du Laboratoire de Cryptogamie, France

www.mnhn.fr (viewed 2012-01-30).

Stock cultures can be maintained in the medium specified in 5.3. and 7.1. However, frequent subculturing is necessary (once a week) to prevent failure of growth. The stock culture can be maintained for extended periods on richer algal media such as those recommended by the culture collection.

Alternatively, algae can be stored for several months on agar plates or in alginate beads[4)] without losing their viability[1]. The algae can be easily recovered from the agar or liberated from the algal beads (see Annex C) when needed to perform the toxicity tests.

The appearance of the cells and the identity of the test organisms should be confirmed by microscopy.

5.2 Water, deionized or of equivalent purity (conductivity <10 µS/cm), for use in the preparation of the growth medium and test substance solutions.

Take special care to avoid contamination of the water by inorganic or organic substances during preparation and storage. Do not use equipment made of copper.

5.3 Nutrients

Prepare four nutrient stock solutions in water, according to the compositions given in Table 1.

These solutions are eventually diluted (see 7.1 and 7.4) to achieve the final nutrient concentrations in the test solutions. However, the macronutrients may instead be added directly to the water.

All chemicals used shall be of reagent-grade quality.

Sterilize the stock solutions by membrane filtration (mean pore diameter 0,2 µm) or by autoclaving [(120 ± 2) °C, 15 min]. Store the solutions in the dark at 4 °C.

Do not autoclave stock solution 4 in order to avoid evaporative loss of $NaHCO_3$, but sterilize it by membrane filtration.

4) The algal beads supplied by MicroBioTests Inc., Mariakerke-Gent, Belgium are an example of a suitable product available commercially. 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 the validity criteria specified in this document are fulfilled.

Table 1 — Mass concentrations of nutrients in the test solution

Stock solution	Nutrient	Mass concentration in stock solution	Final mass concentration in test solution
1: Macronutrients	NH_4Cl	1,5 g/l	15 mg/l (N: 3,9 mg/l)
	$MgCl_2{\cdot}6H_2O$	1,2 g/l	12 mg/l (Mg: 2,9 mg/l)
	$CaCl_2{\cdot}2H_2O$	1,8 g/l	18 mg/l (Ca: 4,9 mg/l
	$MgSO_4{\cdot}7H_2O$	1,5 g/l	15 mg/l (S: 1,95 mg/l)
	KH_2PO_4	0,16 g/l	1,6 mg/l (P: 0,36 mg/l)
2: Fe-EDTA	$FeCl_3{\cdot}6H_2O$	64 mg/l	64 µg/l (Fe: 13 µg/l)
	$Na_2EDTA{\cdot}2H_2O$	100 mg/l	100 µg/l
3: Trace elements	H_3BO_3[a]	185 mg/l	185 µg/l (B: 32 µg/l)
	$MnCl_2{\cdot}4H_2O$	415 mg/l	415 µg/l (Mn: 115 µg/l)
	$ZnCl_2$	3 mg/l	3 µg/l (Zn: 1,4 µg/l)
	$CoCl_2{\cdot}6H_2O$	1,5 mg/l	1,5 µg/l (Co: 0,37 µg/l)
	$CuCl_2{\cdot}2H_2O$	0,01 mg/l	0,01 µg/l (Cu: 3,7 ng/l)
	$Na_2MoO_4{\cdot}2H_2O$	7 mg/l	7 µg/l (Mo: 2,8 µg/l)
4: $NaHCO_3$	$NaHCO_3$	50 g/l	50 mg/l (C: 7,14 mg/l)

[a] H_3BO_3 can be dissolved by the addition of 0,1 mol/l NaOH.

6 Apparatus

All equipment that comes in contact with the test medium shall be made of glass or other chemically inert material.

Usual laboratory apparatus and, in particular, the following.

6.1 Temperature-controlled cabinet or room, with white fluorescent light, providing continuous, uniform 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 capable of counting particles in the size range 2,5 µm to 25 µm (spherical diameters), or a microscope and a counting chamber.

Alternatively, the algal densities may be determined by an indirect procedure using for instance a fluorimeter (e.g. *in vitro* fluorescence[2] or DCMU[5]-enhanced *in vivo* fluorescence[3]), when sufficiently sensitive and if shown to be sufficiently well correlated with cell density. The apparatus used shall be capable of measuring cell densities as low as 10^4 cells/ml and of distinguishing between algal growth and disturbing effects, e.g. the presence of particulate matter and the colour of the sample. Spectrophotometers may be sufficiently sensitive to measure 10^4 cells/ml, providing a sufficient pathlength (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 (see ISO/TR 11044).

6.3 Culture vessels (glass), e.g. 250 ml conical flasks with air-permeable stoppers.

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

6.5 Autoclave.

6.6 pH meter.

5) DCMU is 3-(3,4-dichlorophenyl)-1,1-dimethylurea (CAS No. 330-54-1).

7 Procedure

7.1 Preparation of growth medium

Prepare a growth medium by adding an appropriate volume of the nutrient stock solutions (5.3) to water (5.2).

Add to approximately 500 ml of water (5.2):

— 10 ml of stock solution 1 (5.3);

— 1 ml of stock solution 2 (5.3);

— 1 ml of stock solution 3 (5.3);

— 1 ml of stock solution 4 (5.3).

Make up to 1 000 ml with water.

Before use, equilibrate the medium by leaving overnight in contact with air, or by bubbling filtered air through it for 30 min. After equilibration, adjust the pH if necessary to 8,1 $\pm$ 0,2, using either 1 mol/l hydrochloric acid or 1 mol/l sodium hydroxide solution.

This growth medium is buffered by hydrogencarbonate and atmospheric CO_2. Different pH values may be obtained by modifying the concentration of HCO_3^- and/or the atmospheric CO_2 concentration (requires closed vessels) as specified in ISO 14442. Should such modifications be required in order to perform a test at a different, specific pH value, these should be clearly motivated and reported.

7.2 Preparation of pre-culture and inoculum

A pre-culture shall be started 2 d to 4 d before the beginning of the test. Growth medium (7.1) is inoculated at a sufficiently low cell density (e.g. 5×10^3 cells/ml to 10^4 cells/ml for 3 d pre-culturing) in order to maintain exponential growth until test start. The pre-culture shall be incubated under the same conditions as those in the test (7.6).

This exponentially growing pre-culture is used as an inoculum for 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 sample concentrations

Algae should be exposed to concentrations of the test sample 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 E_rC_x parameter. Additionally, at least two levels of inhibition between 10 % and 90 % should be included in order to provide data for regression analysis.

A limit test with only one concentration can be conducted to demonstrate absence of toxicity. The number of replicates for this one concentration should be at least six.

In case the “lowest ineffective dilution” (LID) of a waste water is to be determined, the following dilution series shall be used: 1:1,25, 1:2, 1:3, 1:4, 1:6, 1:8, 1:12.

NOTE A suitable concentration range is best determined by carrying out a preliminary range-finding test covering several orders of magnitude of difference in test concentration. Replication of test concentrations is not a requirement in the preliminary test.

7.4 Preparation of test sample and stock solutions

Test sample may be aqueous (e.g. waste water) or non-aqueous (e.g. chemical substance or mixture of chemicals) for which the inhibitory effects on the growth of algae shall be determined.

If the test sample is aqueous (e.g. waste water), pre-treatment (e.g. filtration, neutralization) should be considered depending on the nature of the sample and the purpose of the test. Add nutrient stock solutions (5.3), prepared in accordance with 7.1, to the sample.

For non-aqueous test samples, preparation of stock solutions is generally necessary. The method for preparation of the stock solutions should be carefully chosen based on the properties of the sample. Stock solutions are usually prepared by dissolving the test sample in growth medium. Modifications are necessary when the test sample does not readily dissolve in the growth medium as specified in ISO 14442 and ISO 5667-16.

Usually, the test shall be carried out without adjustment of the pH of the medium after addition of the test sample. However, some substances may exert a toxic effect due to extreme acidity or alkalinity. In order to determine the toxicity of a sample independent of pH, adjust the pH of the aqueous sample or stock solution (before the dilution in series) to that of the culture medium using either 1 mol/l hydrochloric acid or 1 mol/l sodium hydroxide solution (see ISO 5667-16).

7.5 Preparation of test and control batches

Prepare the test batches by mixing the appropriate volumes of test sample or test sample stock solutions (7.4), growth medium (7.1) and inoculum (7.2) in the test vessels. The total volume, concentrations of added growth medium nutrients and cell density shall be the same in all vessels. Prepare at least three replicate batches for each test sample concentration.

The initial cell density shall be sufficiently low to allow exponential growth in the control culture throughout the test duration without a pH drift of more than 1,5 pH units (see Clause 8). Therefore, the initial cell densities shall not exceed 10^4 cells/ml.

Prepare six replicate control batches by adding the appropriate volume of inoculum to growth medium.

Measure the pH of a replicate batch at each test concentration and in one control replicate.

If appropriate, prepare a single concentration series of the test sample without algae to serve as background for the cell density determinations.

The number of replicates per concentration can be reduced based on statistical considerations (see ISO/TS 20281), if increasing the number of concentrations and reducing the concentration spacing.

If chemicals are tested for registration purposes, the exposure concentration at the start, during, and at the end of exposure shall be verified by specific chemical analysis. This can require preparation of additional batches for analysis. Further information can be found in OECD 201[4].

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 (a small hole is sufficient). Incubate the test vessels at (23 ± 2) °C, under continuous white light. The light intensity at the average level of the test media shall be homogeneous within ±10 % 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, in particular the type of receptor (collector), affects the measured value. Spherical receptors (which respond to 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. They give higher readings for a multipoint light source of the type described in the Note.

NOTE The light intensity specified in the first paragraph of this subclause can be obtained using four to six fluorescent lamps of the universal white (natural) type, i.e. a rated colour of standard colour 2 (colour temperature of 4 300 K). The optimum distance of the lamps is approximately 0,35 m from the algal culture medium.

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

Testing of coloured test solutions requires specific modifications as specified in ISO 14442.

Continuously shake, stir or aerate 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 drift.

7.7 Measurements

Measure the cell density in each test batch (including the controls) at least every 24 h. Mix the test batches thoroughly before measurement. Aliquots removed from the test vessels for measurements should preferably not be replaced.

The nominal cell density can be used as the initial cell density and no initial cell density measurement is then required.

The test shall last for (72 $\pm$ 2) h.

At the end of the test, measure the pH of samples of at least one replicate batch at each test sample concentration and one control replicate.

8 Validity criteria

Consider the test valid if the following conditions are met.

a) The average growth rate in the control replicates shall be at least 1,4 d^{-1}. This growth rate corresponds to an increase in cell density by a factor 67 in 72 h.

b) The variation coefficient of the growth rate in the control replicates shall not exceed 5 %.

c) The pH in the control shall not have increased during the test by more than 1,5 relative to the pH of the growth medium.

An increase in pH during the test can have significant influence on the results and therefore a limit of 1,5 units is set. These variations, however, should always be kept as low as achievable, e.g. by performing continuous shaking during the test.

If these criteria are not met, examine experimental techniques and use inocula from other sources, if necessary.

9 Calculation

9.1 Plotting of growth curves

Tabulate the cell density measurements for each test batch according to the concentration of the test sample and the duration 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

First calculate the specific growth rates, μ, for each test and control batch replicate, using Equation (1).

$$\mu = \frac{\ln n_L - \ln n_0}{t_L - t_0} \quad (1)$$

where

n_0 is the initial cell density;

n_L is the measured cell density at time t_L;

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 batches (9.1)].

Alternatively, determine the specific growth rate from the slope of the regression line in a plot of the natural logarithm of the mean cell density against time (9.1).

Calculate the mean value of μ for the replicate control batches. Then, calculate the percentage inhibition (growth rate) for each test batch replicate i, $I_{\mu i}$, using Equation (2).

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

where

μ_i is the growth rate for test batch i;

μ_c is the mean growth rate for the control batches.

9.3 Determination of E_rC_x (e.g. E_rC_{10} and E_rC_{50})

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

Fit a suitable non-linear model to the experimental data points by regression analysis (e.g. see ISO/TS 20281, References [5] to [8] in order to determine E_rC_x values, 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), then a graphical method can be applied. In this case, draw a smooth eye-fitted curve of the concentration–response relationship and read E_rC_x values from this graph. If extreme stimulation at intermediate concentrations of the test substance is observed, use of a hormesis model should be considered[8].

If chemicals are tested for registration purposes, the E_rC_x should be based on time-weighted average concentrations calculated from measured concentrations at the start, during, and at the end of the test.

10 Expression of results

Denote EC_{10} and EC_{50} values based on growth rate as E_rC_{10} and E_rC_{50}. Also indicate clearly the time span used for the determination, e.g. E_rC_{50} (0 h to 72 h). Report E_rC_{10} and E_rC_{50} values in milligrams per litre or as percentages with the corresponding confidence intervals.

When testing waste water by means of a graduated dilution, D, the test medium with the highest concentration at which an inhibition <5 % is observed is termed the lowest ineffective dilution (LID). This dilution is expressed as the reciprocal of the volume fraction of waste water in the test medium [e.g. if the waste water content is one part in four (25 % volume fraction), the dilution factor is $D = 4$]; see ISO 5667-16:1998, Annex A.

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 hazard, but cannot be used directly to predict effects in the natural environment.

When interpreting EC_{10} and EC_{50}, take into consideration the shape of the growth curves. Certain features of these curves (e.g. delayed onset growth, good initial growth but not sustained) may help to indicate the mode of action of the toxic substance concerned.

12 Precision

Interlaboratory tests based on the test specified in this International Standard were carried out in 1980 and 1981. The results obtained with the reference substances $K_2Cr_2O_7$ and 3,5-dichlorophenol are shown in Table 2. At the time of publication, review of the reference tests indicates that the sensitivity of the strains has not changed significantly.

Table 2 — Interlaboratory test results for E_rC_{50}

Test organism and test substance	Number of laboratories	Outliers	Mean value mg/l	Standard deviation mg/l	Coefficient of variation C_V %
D. subspicatus					
Potassium dichromate	20	4	0,84	0,12	14
3,5-Dichlorophenol	18	2	6,42	2,38	37
P. subcapitata					
Potassium dichromate	9[a]	4	1,19	0,27	23
3,5-Dichlorophenol	9[a]	4	3,38	1,30	38

[a] The high number of outliers in the tests with *P. subcapitata* is due to the use of different growth media (with different pH values). Results from tests using media whose pH deviates from the growth medium specified in this International Standard have been excluded.

To prove the validity of the test system, it is recommended to test at least one reference substance (e.g. when using a strain or after changing test conditions). Results should be compared to those given in Table 2.

NOTE The mean control growth rates determined in the interlaboratory test were 1,74 d^{-1} (coefficient of variation, C_V = 27 %) for *D. subspicatus* and 1,91 d^{-1} (C_V = 23 %) for *P. subcapitata*. These growth rates suggest an increase in cell density by at least 150.

13 Test report

The test report shall contain at least the following information:

a) a reference to this International Standard (ISO 8692:2012);

b) all data required for complete identification of the test sample;

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

d) test details:

   1) start date and duration,

   2) method of preparation of sample and test batches,

   3) concentrations tested,

4) composition of medium,

5) culturing apparatus and incubation procedure,

6) light intensity and quality,

7) temperature,

8) pH of test solutions including the controls at start and end of test,

9) method for measuring cell density and, if appropriate, method to correct for background values;

e) results:

1) cell density in each batch at each measuring point,

2) mean cell density for each test concentration (and control) at each measuring point,

3) growth curves (logarithm of mean cell density against time) for each test concentration and control,

4) relationship between the concentration and effect (percentage inhibition values against concentration) in tabular or graphical representation, e.g. percentage of inhibition on probit-scaled ordinate against concentration in logarithmic-scaled abscissa,

5) E_rC_x values such as E_rC_{10} and E_rC_{50} with their confidence intervals, including the method of determination or, alternatively, if waste water is tested by means of a graduated dilution, the LID;

6) other observed effects such as bleaching of algal cells.

Annex A
(informative)

Rapid screening of waste water algal growth inhibition

A.1 General

This method can be applied to the testing of effluents, waste waters and other environmental aqueous samples. The following modifications address mainly requirements for carrying out screening tests in various types of test containers, e.g. microplates.

A.2 Sampling and storage

Samples need to be tested as soon as possible after collection or occasionally frozen and thawed, filtered or centrifuged. The recommendations of ISO 14442 and ISO 5667-16 should be considered before planning waste water testing.

A.3 Culture vessels

Use flasks, vials or microplates with appropriate culture volumes. Choose the material and geometry of the culture vessels to avoid:

a) release of potentially toxic substances;

b) adsorption of components from the test media;

c) evaporative losses of important waste water constituents;

d) light inhomogeneities among replicates and treatments.

A.4 Choice of test concentrations

Prepare a dilution series of the water sample as specified in A.5. The dilution series should follow a geometric progression covering the desired range of response. If microplates or automated systems are used, an increased number of tested dilutions is recommended in order to ensure compliance with this requirement. A range-finding test may be carried out to define the dilution series.

Use at least three replicates per treatment (including a control) and five concentrations unless there is sufficient technical justification for a different test design. The number of replicates per concentration can be reduced based on statistical considerations, by increasing the number of concentrations and reducing the concentration spacing.

A.5 Preparation of test batches

Prepare the series of test batches in a manner that ensures that all batches receive the same concentrations of spiked nutrients and algal inoculum. This can, for example, be achieved by spiking the test water sample with nutrient stock solutions (5.3) as specfied in 7.1 and mixing the spiked water sample with appropriate volumes of growth medium and inoculum culture. This protocol allows testing concentrations up to approximately 98 %.

A.6 Background control

Dilutions of waste water without algae are required in order to determine possible interferences for algal density measurements (A.9), in order to allow background corrections or to select a suited measurement method.

There shall be no algal growth in the background control at the end of the test.

A.7 Reference substance

Testing with a reference substance (see Clause 12) at regular intervals is recommended for quality assurance and to check the sensitivity of the algae. If the growth inhibition test is carried out with only two measurements (at the start and at the end of the test), or, if the nominal cell density is used, only one measurement at the end of the test, the reference test may be conducted with this simplified procedure.

A.8 Test organisms

Use *D. subspicatus* or *P. subcapitata* (see 5.1).

A.9 Measurement of algal cell density

Microscopic or electronic counting are the reference methods. However, indirect methods such as turbidimetry, photometry or fluorimetry may be used when sufficiently sensitive and if shown to be sufficiently well correlated with cell density. Fluorimetry is recommended if the test sample is turbid in order to keep the interference due to the turbidity as low as possible[2].

A.10 Test duration

The test duration shall be at least (48 $\pm$ 2) h, but can be extended to (72 $\pm$ 2) h.

A.11 Frequency of measurement

Daily measurement of algal cell density is generally recommended. With waste water samples, the cell density can only be measured at the beginning and at the end of the test. Alternatively, the nominal cell density may be used as initial cell density. In that case, only measurement at the end of the test is required.

A.12 pH measurement

The pH may be measured in one control vessel at the end of the test. In a microplate test, pooling of control replicates can be necessary for the pH measurement.

A.13 Calculation of percentage inhibition

The inhibition of growth rate (see 9.2) is the recommended endpoint for this screening test, as it is rather independent of the biomass levels, the growth conditions and the test duration.

A.14 Determination of E_rC_x and LID values

ErC*x* values can be determined as specified in 9.3. The E_rC_x can also be determined by straight-line graphical interpolation. The E_rC_x values can be reported in, for instance, millilitres per litre or percentage of waste water in test media (volume fraction).

The results may also be expressed in LID (lowest ineffective dilution, see ISO 5667-16:1998, Annex A), which is the lowest dilution with an effect lower than 5 %.

A.15 Validity criteria

The validity criteria listed in Clause 8 are also applicable to this annex. The following additional criterion (see A.6) shall be fulfilled.

There shall be no interfering algal growth in the background control at the end of the test.

A.16 Test report

The test report shall include at least the following information:

a) a statement that the test has been performed in accordance with ISO 8692:2012, Annex A;

b) in addition to the data required in Clause 13, the nature of the sample tested (waste water, effluent, leachate, etc.), origin, labelling, sampling method, date of collection, conservation, duration of exposure, appearance, pre-treatment (settling, filtration, centrifugation, adjustment of pH);

c) the endpoint;

d) the calculation method.

Annex B
(informative)

Test procedure with algae from algal beads, 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, waste waters and other environmental aqueous samples.

This test procedure is based on optical density (OD) measurements of the algal growth in spectrophotometric cells of pathlength 10 cm, which serve as test vessels for the assays. The algae are obtained from alginate algal beads 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 high density are obtained by separating the algae from the inert alginate matrix in which the algae are immobilized and stored.

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 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 h in a temperature-controlled cabinet or incubator under continuous illumination, 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.

B.3 Materials

B.3.1 Test organisms

Use *P. subcapitata* or *D. subspicatus* for the assays (see 5.1).

B.3.2 Algal beads

Algal beads in a calcium or sodium alginate matrix are prepared from exponentially growing cultures of either *P. subcapitata* or *D. subspicatus* according to the technique described in Annex C and several scientific papers (see References [9] to [12]).

The algal beads can be stored for up to 6 months in a refrigerator at (4 ± 2) °C in darkness, in test tubes containing a 10 times concentrated ISO growth medium (7.1) and 10 beads to 15 beads per tube.

Algal beads from *P. subcapitata* can to date also be obtained from commercial sources[6)].

6) The algal beads supplied by MicroBioTests Inc., Mariakerke-Gent, Belgium are an example of a suitable product available commercially. 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 the validity criteria specified in this document are fulfilled.

B.3.3 Nutrients

The algal growth medium used for this test is the same as that described in Table 1.

The four stock solutions are preferably dispensed in penicilline vials. When stored in a refrigerator at (4 ± 2) °C in darkness, the vials with the nutrient solutions have a shelf-life of up to 1 year.

B.3.4 De-immobilization medium

The de-immobilization medium for the algal beads is a 20 g/l solution of sodium metaphosphate. When stored in a refrigerator at (4 ± 2) °C, the de-immobilization medium has a shelf life of 1 year.

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 Laboratory centrifuge.

B.4.4 pH meter.

B.4.5 Bürker cell counter.

B.5 Laboratory materials

B.5.1 Spectrophotometric cells, pathlength 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 available commercially[7)].

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 Preparation of algal growth medium

The algal growth medium shall be prepared according to the procedure specified in 7.1.

B.6.2 De-immobilization of the algae from the algal beads

Take a tube with algal beads, decant the liquid and add 5 ml de-immobilization medium (B.3.4). Cap the tube and shake vigorously by hand at regular intervals to dissolve the matrix. A vortex shaker can be used to speed up the process.

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7) The long cells supplied by MicroBioTests Inc., Mariakerke-Gent, Belgium are an example of a suitable product available commercially. 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 the validity criteria specified in this document are fulfilled.

When the matrix is totally dissolved (after 5 min to 10 min), centrifuge the tube for 10 min at 3 000 r/min. Decant the supernatant, add 10 ml deionized water, cap the tube and shake to resuspend the algal clot. Centrifuge again for 10 min at 3 000 r/min and decant the supernatant. Add 10 ml algal growth medium, cap the tube and shake to resuspend the algae.

B.6.3 Determination of the optical density to number of algae relationship for spectrophotometric measurements in long cells

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

Add the algal suspension obtained from one tube with algal beads (according to B.6.2.) to a 25 ml calibrated flask and add algal growth medium to the mark. Cap the flask and shake to produce a homogeneous algal suspension again. Take two long cells and mark them as “Calibration cell” and “Algal stock cell” respectively.

Fill the calibration cell with 25 ml algal culture medium, close with the lid, and insert the cell into the spectrophotometer. Zero-calibrate the instrument at 670 nm.

Transfer the algal suspension into the algal stock cell, close the cell tightly and shake to distribute the algal suspension evenly.

Put the algal stock cell in the spectrophotometer and read the OD at 670 nm.

Take a small sample of algal suspension from the algal stock cell with a pipette and count the number of algae under the microscope with a counting chamber (hemacytometer, Bürker chamber).

Dilute the algal suspension in the algal stock cell by 20 % by removing 5 ml of suspension and replacing it with 5 ml algal culture medium. Close the cell tightly and shake to obtain again a homogenous algal suspension.

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

Remove another 5 ml algal suspension from the algal stock cell and replace it with 5 ml culture medium, to obtain an algal suspension which is again 20 % less concentrated than the previous one, and determine the OD of this algal suspension in the stock cell.

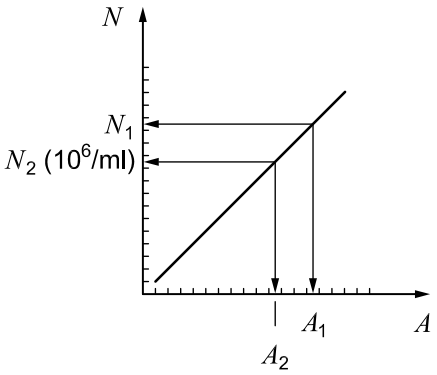
Repeat the dilution steps and the measurements until the measured OD value has decreased to approximately 0,05.

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.6.4 Preparation of the concentrated algal inoculum

Take a tube with algal beads and proceed as specified in B.6.2 and in the five first paragraphs of B.6.3.

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



Key

N number

A optical density

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

Transfer the algal suspension from the algal stock cell into a 100 ml flask and add the volume of algal culture medium needed to make up a 10^6 cells/ml suspension in the flask. Stopper and shake the flask to distribute the algae evenly.

B.6.5 Preparation of toxicant dilution series

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

This 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 (10^6 cells/ml) to each flask, to obtain a start concentration of 10^4 cells/ml.

B.6.6 Transfer of 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.6.7 Incubation of the test vials

Put the 18 inoculated 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 width a few centimetres 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 3 d at (23 ± 2) °C with an appropriate illumination and in the conditions described in 7.6.

B.6.8 Measurements

Measure the OD in each long cell after 24 h, 48 h and 72 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.6.9 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 and 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.6.10 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 recorded 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 and the E_rC_x using Equations (1) and (2) as specified in 9.2.

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 precision of the method using algal beads and in long cells was determined during an international interlaboratory exercise which took place in 2006, with the participation of 28 laboratories from 14 countries.

The intercalibration was made with the reference chemical potassium dichromate ($K_2Cr_2O_7$) and the results were compared with those obtained in tests carried out by various laboratories, with algae from laboratory stock cultures, and performed in glass vessels or in microplates.

The findings and the conclusions of this international ring test are reported in Reference [10].

In 2007, an ecotoxicological ring test was organized by the EU in which (among other assays) algal tests had to be performed on waste eluates. During this exercise several laboratories submitted results from algal tests departing from algal beads and performed in long cells, with measurements of optical densities (see References [11][12]).

The major conclusion of both these studies is that the overall precision (repeatability and reproducibility) of algal assays departing from algal beads and performed in 10 cm spectrophotometric cells is as good as that of assays performed with algae from stock cultures, and performed in glass vessels or microplates.

B.10 Test report

The test report shall include a statement that the test was performed in accordance with ISO 8692:2012, Annex B, and the requirements given in Clause 13.

Annex C
(informative)

Procedure for immobilization of algae in alginate beads

C.1 Preparation of alginate beads

Prepare a culture of algae in the normal growth medium (7.1).

When the culture has reached the exponential growth phase, concentrate the algae by centrifuging the suspension for 5 min at low speed (3 000 r/min with a small laboratory centrifuge at 1 200 g to 1 400 g) and decant the supernatant.

Add an equal volume of a 30 g l^{-1} sodium alginate solution to the algae suspension and stir gently until the algae are evenly distributed.

Draw the algae–alginate suspension into a syringe.

Place a beaker containing a 10 g l^{-1} calcium chloride solution under the syringe and allow the algae–alginate suspension to drip slowly from the syringe tip into the calcium chloride solution.

Leave the beads with immobilized algae to harden in the calcium chloride solution for 15 min to 30 min.

Separate the beads from the calcium chloride solution using a sieve with a smaller mesh than the diameter of the algal beads, and gently rinse the algal beads with distilled water.

Transfer the algal beads into a container with 10 times concentrated algal culture medium and store the beads in a refrigerator at (4 $\pm$ 2) °C until use.

C.2 Storage of alginate beads

Algal beads can be stored for several months in a refrigerator at (4 $\pm$ 2) °C prior to use, and their shelf-life and the capability of the algae to resume their growth rapidly after de-immobilization depends on various factors such as the “health state” of the inoculum at the time of immobilization of the algae in beads, the density of the algae in the beads, and the diameter of the algal beads.

Depending on these factors, it can occasionally happen that de-immobilized algae do not reach the requested average growth rate of 1,4 d^{-1} in the controls at the end of the test, in which case the test results are invalid. De-immobilized algae can, however, be “re-activated” to a good physiological condition after a few days.

C.3 De-immobilization of algae from alginate beads

To recover the algae from the beads, the “de-immobilization medium” specified in B.3.4, i.e. a 20 g/l solution of sodium metaphosphate, can be used.

As indicated in B.6.2, de-immobilization is performed by transferring algal beads in the sodium metaphosphate solution, in a tube or in a beaker, and in a ratio of 10 to 15 algal beads per 5 ml de-immobilization medium.

The suspension shall then be shaken till the matrix surrounding the algae is completely dissolved.

The algal suspension shall subsequently be centrifuged at low speed (1 200 g to 1 400 g) and the supernatant decanted to eliminate the de-immobilization medium.

The algal clot is then resuspended in deionized water and centrifuged again.

After decanting the “rinsing” water, algal growth medium is added and the algae shall be resuspended to obtain the algal stock culture.

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