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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

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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.

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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.

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

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

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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.

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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:

$$\mu = \frac{1}{n}\frac{dn}{dt}$$

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]

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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.

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.

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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.

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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.

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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.

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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.

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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).

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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 ± 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.

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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).

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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.

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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.

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

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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.

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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.

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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 ± 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.

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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.

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