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Water quality — Determination of the growth inhibition effects of waste waters, natural waters and chemicals on the duckweed *Spirodela polyrhiza* — Method using a stock culture independent microbiotest

Qualité de l'eau — Détermination des effets d'inhibition sur la croissance de la lentille d'eau Spirodela polyrhiza *par les eaux usées, les eaux naturelles et les produits chimiques — Méthode utilisant un bioessai miniaturisé indépendant d'une culture mère*

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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).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

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

Introduction

Duckweeds are free-floating higher water plants commonly used in ecotoxicological research for the assessment of the toxicity of waste waters, natural waters and chemicals (see ISO 20079 and References [6] to [11] and in particular plant protection products, see Reference [12]).

Duckweeds are fast growing plants, many of which have a cosmopolitan distribution, and they are, hence, well suited as primary producers for hazard assessment of pollutants in freshwater environments.

Contrary to terrestrial plants, for which bioassays can be started from the "dormant" life stages (seeds), toxicity tests with duckweeds require continuous culturing and maintenance of live stocks, with the inherent biological, technical and financial costs.

A few duckweed species, however, produce dormant vegetative buds (turions) which can be stored for long periods of time, and which can be germinated on demand at the time of performance of the bioassay.

One of the duckweeds producing turions is *Spirodela polyrhiza*, and this species was eventually selected for a simple and practical microbiotest which is independent of the stock culturing and maintenance of live stocks.

Spirodela polyrhiza was found to be as sensitive to toxicants as the conventional bioassays with duckweeds.

The microbiotest procedure for this document involves a 3 d germination of the turions, followed by a 3 d toxicity test in a multiwell test plate, with determination of the growth inhibition of the first fronds via image analysis.

The *Spirodela polyrhiza* microbiotest is very simple and easy to perform:

a) the assay does not require culturing or maintenance of live stocks of the test species, and can be performed "anytime, anywhere" by the use of stored turions;

b) stored turions have a shelf life of several months with a high germination success;

c) the microbiotest requires minimal bench and incubation space, and minimal equipment;

d) the area measurements of the first fronds do not need to be made immediately and can be postponed to an appropriate timing;

e) the area measurements by image analysis are very rapid and precise, and take less than 1 h for a complete test.

Water quality — Determination of the growth inhibition effects of waste waters, natural waters and chemicals on the duckweed *Spirodela polyrhiza* — Method using a stock culture independent microbiotest

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 the growth of the first fronds of *Spirodela polyrhiza* germinated from turions, by substances and mixtures contained in water or waste water, including treated municipal waste water and industrial effluents.

The test is also applicable to pure chemicals and in particular, plant protection products and pesticides.

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*

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

3.2
frond
leaf-like structure which develops from a germinated turion

3.3
growth
increase in biomass over time as the result of proliferation of new tissues

Note 1 to entry: In this test, it refers to the increase in size of the first frond developing from a germinated turion.

3.4
growth medium
combination of dilution water and/or nutrient medium used in the test

Note 1 to entry: In this test, it refers to the nutrient medium used for the germination of the turions and the growth of the fronds.

3.5
inoculum
transfer of a germinated turion with its small frond in all the test wells at the start of the toxicity test

3.6
pure water
deionized or distilled water with a conductivity below 10 µS/cm

[SOURCE: ISO 19827:2016, 3.4]

3.7
root
part of the *Spirodela polyrhiza* plant that assumes a root-like structure and develops underneath a frond

3.8
stock culture
culture of a single species of duckweed for the production of the turions

3.9
test medium
combination of test sample, dilution water and/or nutrient medium used in the test

[SOURCE: ISO 20079:2005, 3.23]

3.10
test sample
discrete portion of a sample (taken from i.e. receiving water, waste water, dissolved chemical substances or mixtures, products and compounds) pre-treated according to the needs of this test (e.g. dissolution, filtering, neutralisation)

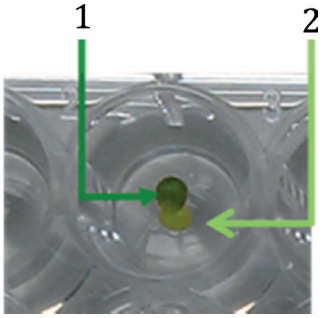
3.11
turion
small vegetative bud which develops from a colony of the duckweed under specific environmental conditions

4 Principle

Turions produced by culturing *Spirodela polyrhiza*, or taken from test tubes in which they are stored (see Annex A) are transferred to a Petri dish containing growth medium, and incubated for 3 d at 25 °C with continuous illumination of at least 6 000 lx (corresponding approximately to 85 µE m^{-2} s^{-1}).

During this time, the turions germinate and produce a small (first) frond (see Figure 1).

One germinated turion with its first frond is then taken from the Petri dish and inoculated into each cup of a 6 × 8 multiwell test plate which contains the toxicant dilutions and the negative control (each of which is prepared in growth medium).



Key

1 turion

2 first frond

Figure 1 — Enlargement of a germinated turion with its first frond, in a cup of the test plate

On completion of the inoculations, a photo of the multiwell is taken (at t = 0 h) with a digital camera and transferred to a computer file.

The multiwell is subsequently incubated for 3 d at (25 ± 1) °C with continuous illumination of minimum 6 000 lx, after which a photo is again taken (at t = 72 h) and transferred to a computer file.

The area of the first frond in each test cup is measured with the aid of an image analysis programme, on the two photos of the multiwell (i.e. taken at t = 0 h and at t = 72 h).

The growth of the first fronds in the controls and in the test concentrations or dilutions is calculated as the difference between the t = 72 h areas and the t = 0 h areas, after which the growth inhibition and the 72 h EC_{50} or EC_x values are determined.

5 Test organisms

The test species used in this document is the duckweed *Spirodela polyrhiza* (L.) Schleid.

The test organisms are obtained by germination of (stored) turions.

Turions can be produced in the laboratory according to the procedure described in Annex A.

They can also be purchased from a commercial source[1)].

6 Growth medium

The growth medium (3.4) used for the germination of the turions and the growth of the duckweeds during the toxicity test is the modified Steinberg medium which is described and used in ISO 20079[2] and the OECD guideline for testing chemicals (Reference [8]).

This medium is also used to prepare the toxicant dilutions.

The growth medium is composed of macroelements and microelements of which stock solutions are prepared according to Table 1 and Table 2 respectively.

1) The turions supplied by MicroBioTests Inc. 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.

6.1 Preparation of stock solutions

Prepare the eight stock solutions by adding the prescribed weight of the chemicals to 1 l of pure water (3.6).

Table 1 — Macroelements stock solutions

Macroelements (50-fold concentrated)		g/l
Stock solution 1	KNO_3	17,50
	KH_2PO_4	4,5
	K_2HPO_4	0,63
Stock solution 2	$MgSO_4 \cdot 7H_2O$	5,00
Stock solution 3	$Ca(NO_3)_2 \cdot 4H_2O$	14,75

Table 2 — Microelements stock solutions

Microelements (1 000-fold concentrated)		mg/l
Stock solution 4	H_3BO_3	120,00
Stock solution 5	$ZnSO_4 \cdot 7H_2O$	180,00
Stock solution 6	$Na_2MoO_4 \cdot 2H_2O$	44,0
Stock solution 7	$MnCl_2 \cdot 4H_2O$	180,00
Stock solution 8	$FeCl_3 \cdot 6H_2O$	760,00
	EDTA disodium-dihydrate	1 500,00

Stock solutions 2 and 3, and 4 to 7 may be pooled (taking into account the required concentrations).

6.2 Preparation of the final concentration of modified Steinberg medium

Add 20 ml each of stock solutions 1, 2 and 3 to about 900 ml pure water (3.6) in a 1 l volumetric flask.

Then add 1,0 ml each of stock solutions 4, 5, 6, 7 and 8.

Fill the volumetric flask to 1 000 ml with pure water.

The pH of the growth medium shall be 5,5 ± 0,2 and shall be adjusted with either HCl or NaOH.

Once prepared, the growth medium has a relatively short shelf life and shall be used within two weeks after preparation.

7 Apparatus

Usual laboratory equipment and in particular the following.

7.1 Temperature-controlled cabinet or room, or incubator, with white fluorescent light providing continuous uniform illumination of at least 6 000 lx at the surface of the turion germination Petri dish and the multiwell test plate.

7.2 Lux meter, for the measurement of the light intensity at the surface of the turion germination Petri dish and the multiwell test plate.

7.3 pH meter, for checking and/or adjustment of the pH of the growth medium.

7.4 Laboratory glassware, for the preparation of the test concentrations (volumetric flasks, graduated cylinders, pipettes, test tubes).

7.5 Petri dishes, diameter 9 cm, with lid, for the germination of the turions.

7.6 Microsieve, 100 µm mesh, for rinsing the stored turions.

7.7 Multiwells, 6 × 8 cups, as test plates.

7.8 Plastic spatula, for the transfer of the germinated turions in the multiwell cups.

7.9 Digital camera, to take a picture of the multiwell with the growing duckweeds.

7.10 Image analysis system, for the measurement of the area of the first fronds.

8 Reference chemicals

8.1 3,5-dichlorophenol, analytical grade > 99 % purity.

8.2 Potassium chloride, KCl, analytical grade > 99 % purity.

9 Procedure

9.1 Germination of the *Spirodela polyrhiza* turions

When using turions from a culture of *Spirodela polyrhiza*, place the turions in a Petri dish (7.5) and pour 30 ml growth medium (3.4) into it.

Starting from stored turions, take a tube with stored turions and shake it slightly to re-suspend the turions.

Pour the contents of the tube in the microsieve (7.6) and rinse with pure water (3.6) to remove the storage medium.

Put 10 ml growth medium (3.4) in the Petri dish (7.5).

Turn the microsieve upside down and flush all the turions in the Petri dish by pouring 10 ml growth medium over the surface of the microsieve.

Fill the Petri dish further by adding 10 ml growth medium.

Cover the Petri dish with the transparent lid and place it in the incubator or in the temperature conditioned room (7.1).

Incubate the Petri dish for 3 d (72 ± 1) h at (25 ± 1) °C, with continuous illumination (at least 6 000 lx at the surface of the Petri dish).

NOTE Both germination of the turions and the growth of the first fronds are very substantially dependent on temperature and illumination conditions. It is therefore important that the prescribed values of temperature and illumination be respected as closely as possible.

9.2 Tests on effluents (and waste waters)

Sampling and samples preparation shall be done according to ISO 5667-16.

9.2.1 Addition of concentrated growth medium to the effluent sample

Transfer about 80 ml effluent in a 100 ml calibrated flask.