INTERNATIONAL STANDARD

ISO 20227

First edition
2017-06

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*

Reference number
ISO 20227:2017(E)

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO 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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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)

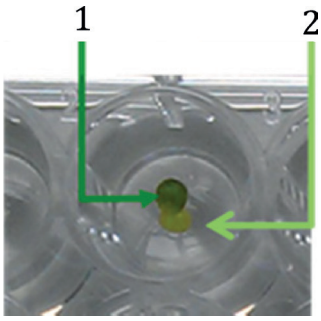
3.11
turion
small vegetative bud which develops from a colony of the duckweed under specific environmental conditions

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.

Add 2 ml of each of the stock solutions 1, 2 and 3, and 100 µl of each of the stock solutions 4, 5, 6, 7 and 8 to the calibrated flask.

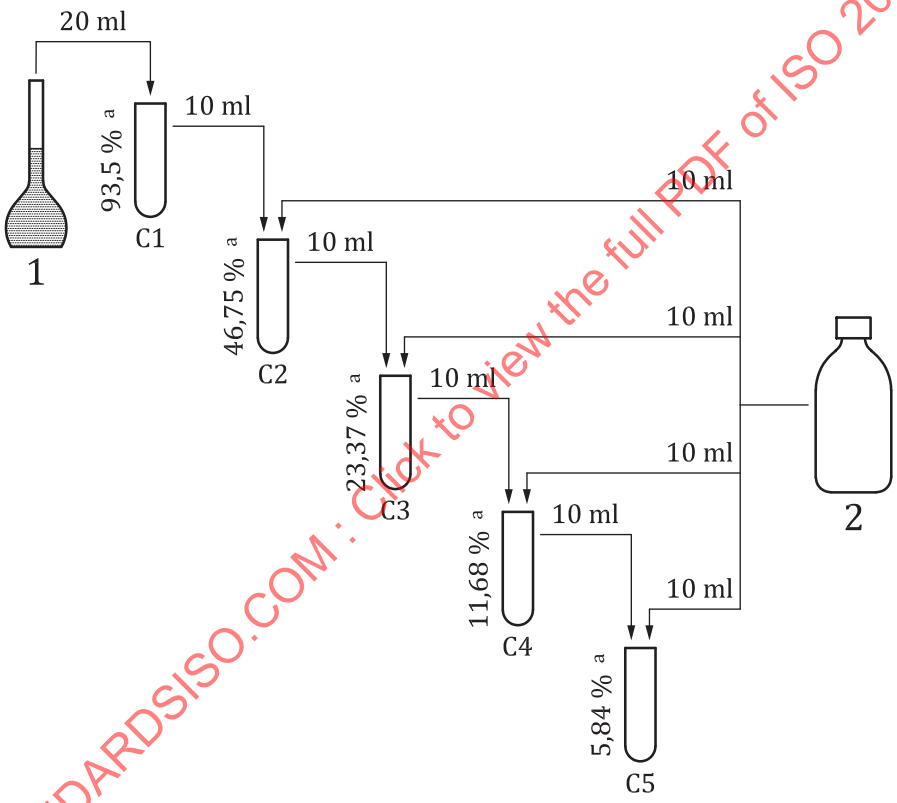
Fill the flask to the 100 ml mark with the effluent, stopper the flask and shake thoroughly to homogenize the contents.

NOTE The addition of 6,5 ml growth medium (3.4) to 93,5 ml effluent, dilutes the effluent sample by about 6 %. This means that the highest effluent concentration which will be tested is about 94 % of the original effluent sample.

9.2.2 Preparation of the effluent dilutions

A common procedure for the preparation of the dilution series is described in the following text. Depending on the purpose of the test and the statistical requirements concerning the test results, other dilution designs with concentrations in a geometric or a logarithmic series may be appropriate as well.

A 1:1 dilution series is prepared from the 94 % effluent (see Figure 2).



Key

C1 to C5	test tubes with C1 to C5 test concentrations
1	effluent with growth medium
2	growth medium
a	Effluent

Figure 2 — Preparation of the 1:1 effluent dilution series

9.2.3 Procedure

Take five test tubes of 20 ml contents and label them, e.g. C1, C2, C3, C4 and C5.

Add 20 ml effluent (containing growth medium) to test tube C1.

Add 10 ml growth medium (as dilution medium) to the tubes C2, C3, C4 and C5.

Transfer 10 ml effluent from tube C1 to tube C2, and cap and shake the test tube.

Transfer 10 ml test dilution from tube C2 to tube C3, and cap and shake the test tube.

Repeat this procedure for the next dilutions.

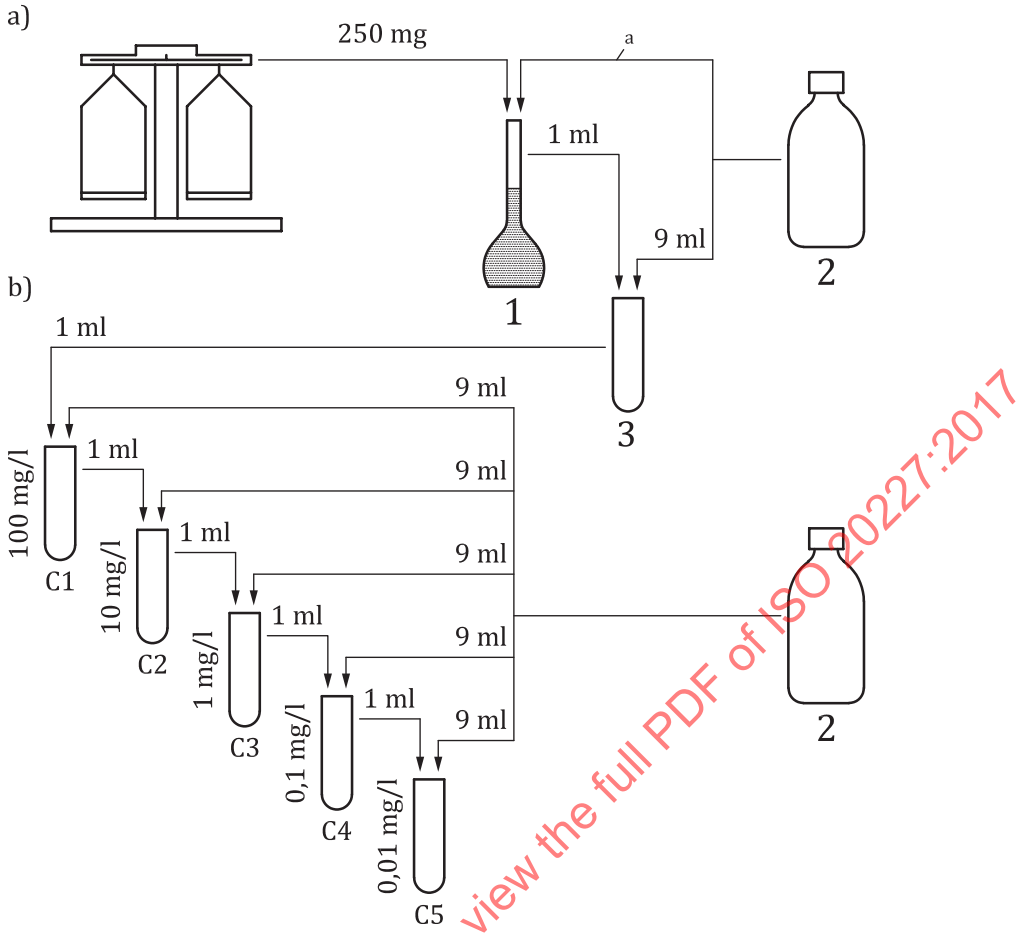
9.3 Tests on chemical compounds

If the approximate toxicity (= the order of magnitude) of the chemical compound to be tested is not known, a range finding test should be performed first to determine the 0 % to 100 % tolerance range of the duckweeds to the toxicant.

9.3.1 Range finding test

A dilution series 1:10 is prepared in test tubes of 10 ml, in growth medium (as dilution medium).

Figure 3 shows an example for preparation of a concentration range of a chemical from 100 mg/l down to 0,01 mg/l.



Key

C1 to C5	test tubes
a)	stock solution
b)	test concentrations
1	25 ml volumetric flask
2	growth medium
3	test tube
a	Fill to the mark

Figure 3 — Preparation of a 100 mg/l to 0,01 mg/l dilution series for a range finding test on a pure chemical

9.3.2 Definitive test

The dilution series to be prepared spans the range of the lowest concentration producing 100 % effect, to the highest one producing less than 10 % effect in the range finding test.

A dilution series is prepared, starting with a test concentration of the toxicant which was the lowest one giving 100 % growth inhibition in the range finding test.

A logarithmic dilution series (e.g. 10 mg/l, 5,6 mg/l, 3,2 mg/l, 1,8 mg/l, 1 mg/l + the negative control) is then prepared in 10 ml test tubes, with the volumes of growth medium and toxicant indicated in Figure 4.

The actual concentrations of the toxicant in the tubes (which will be needed for the EC_{50} or EC_x determination) are calculated as follows:

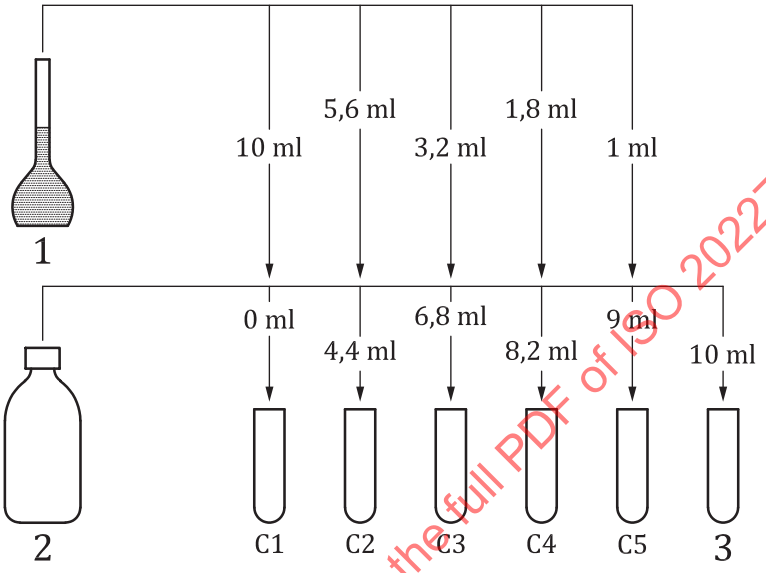
C1 = ... mg/l

C2 = 0,56 × C1 = ... mg/l

C3 = 0,32 × C1 = ... mg/l

C4 = 0,18 × C1 = ... mg/l

C5 = 0,10 × C1 = ... mg/l



Key

C1 to C5	test tubes
1	25 ml toxicant solution (in growth medium) = C1
2	growth medium
3	control

Figure 4 — Preparation of a logarithmic dilution series for a definitive test on a pure chemical

9.4 Filling of the test plate with the toxicant dilutions

9.4.1 General

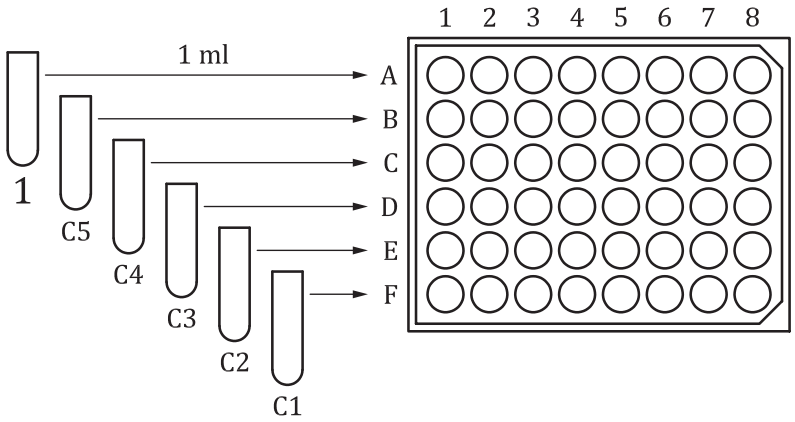
The test is performed in a 6 × 8 multiwell cup (7.7), with eight replicates per test concentration and the negative control.

9.4.2 Procedure

Put 1 ml growth medium (3.4) in the eight cups of control row A (see Figure 5).

Put 1 ml of each toxicant concentration in the rows B to F.

The distribution of the test solutions shall always be carried out starting with the control row on top of the multiwell (row A), followed in sequence by the rows receiving increasing toxicant concentrations (rows B to F).



Key

C5 to C1 test tubes with C5-C1 test concentrations

1 control medium

Figure 5 — Filling of the multiwell test plate with the toxicant dilutions (C1 to C5)

9.5 Transfer of the germinated turions in the test cups

Take the Petri dish (7.5) with the turions out of the incubator and transfer, with the aid of a spatula, one germinated turion into each cup of the multiwell test plate.

NOTE Germinated turions can easily be distinguished from turions which have not germinated, by the presence of a small first frond on one side of the turion, and by small roots underneath the frond.

The transfer of the germinated turions in the test cups shall be started with the control row (row A on top of the multiwell) and continued in the next rows, in sequence of the increasing test concentrations.

The transfers shall be carried out randomly, i.e. one has to avoid picking up and transferring the turions with the largest fronds first.

9.6 Photo of the multiwell at the start of the toxicity test

On completion of the transfers of the germinated turions (with their small first fronds), take a digital photo (at t = 0 h) and transfer the picture to a computer file.

The digital camera (7.9) should not be held too close to the multiwell since this will lead to a distortion of the view of the cups in the columns on the right and on the left side. The latter cups should also have a round (and not an oval) look.

To increase the contrast between the turions and the first small fronds in the photo, it is advised to place the multiwell on a light table, or on a white background.

9.7 Incubation of the multiwell

Put the cover on the multiwell and put the test plate in the incubator (7.1).

Incubate the multiwell at (25 ± 1) °C for 3 d (72 ± 1) h with a continuous illumination of 6 000 lx (measured at the top of the test plate).

Similarly, as mentioned above for the germination of the turions, the prescribed temperature and illumination should be respected as closely as possible.

9.8 Photo of the multiwell at the end of the toxicity test

At the end of the 3 d incubation (at t = 72 h), take the multiwell out of the incubator, remove the lid, and take again a digital photo (preferably on a light table or a white background), with subsequent transfer of the picture to a computer file.

9.9 Measurement of the area of the first fronds

Area measurements of the first fronds have to be made in all the test cups, on the (computer stored) photos of the multiwell test plate taken at t = 0 h and at t = 72 h.

The area measurements are made with the aid of an appropriate image analysis (7.10) program (e.g. image J, which is an open access image analysis program).

In some cups, after 3 d incubation, a second frond will have developed from the germinated turion but the area measurements shall be restricted to the surface of the largest frond (= the first frond).

The data of all the area measurements shall be scored on the data report templates for the t = 0 h and the t = 72 h measurements (see Table 3 and Table 4).

Table 3 — Data report template for the area measurements at the start of the toxicity test (t = 0 h values)

Replicate	Control	C5	C4	C3	C2	C1
1						
2						
3						
4						
5						
6						
7						
8						

Table 4 — Data report template for the area measurements at the end of the toxicity test (t = 72 h values)

Replicate	Control	C5	C4	C3	C2	C1
1						
2						
3						
4						
5						
6						
7						
8						

10 Data treatment — Calculation of the growth inhibition

The effect parameter in this document on the *Spirodela polyrhiza* microbiotest is the growth inhibition of the duckweeds.

The growth inhibition is calculated as the difference (= the decrease) of the size of the first fronds in the cups of the rows with toxicant concentrations versus their size in the cups in the control row after 3 d exposure.

With the data scored in the data report templates (Tables 4 and 5) calculate for the eight replicates in the control and in the five test concentrations, the mean area of the initial first frond (size at t = 0 h) and of the final first frond (size at t = 72 h).

Score these values on the data treatment sheet (see Table 5).

Calculate for the control and for the five test concentrations, the mean growth of the first frond (i.e. the difference between the mean t = 72 h area values and the mean t = 0 h area values).

Calculate the percentage growth inhibition using Formula (1):

$$\frac{A-B}{A}\times 100 \quad (1)$$

where

A is the mean growth of the first frond in the control;

B is the mean growth of the first frond in the five test concentrations.

The 72 h EC_{50} (and other EC_x values, if needed) can be calculated from the percentages growth inhibition with the aid of an appropriate statistical program.

It is recommended to use a nonlinear regression of the concentration-response curve with a suitable model (Reference [3]).

Table 5 — Data treatment sheet

	Control	C5	C4	C3	C2	C1
Mean area of initial first frond at t = 0 h (= I)						
Mean area of final first frond at t = 72 h (= F)						
Mean growth of the first frond (= F – I)						
Percentage growth inhibition						

11 Validity criterion

The mean growth of the first fronds in the cups of the control row after 3 d incubation should be ≥ 10 mm^2.

12 Test sensitivity

The sensitivity of the *Spirodela polyrhiza* microbiotest has first been compared with the sensitivity of the duckweed tests with *Lemna* species for the two reference chemicals indicated in ISO 20079[2] and subsequently for 22 inorganic and organic chemicals.

The results of these comparisons are detailed and commented in Annex B.

13 Test with reference chemicals

A reference substance should be tested regularly to check the test procedure, the sensitivity of the test organisms and the conformity to the test procedure.

3,5-dichlorophenol and potassium chloride are the two preferred reference chemicals for quality control tests on the *Spirodela polyrhiza* microbiotest.

The 72 h EC_{50} should be between 2,2 mg/l and 3,8 mg/l for 3,5-dichlorophenol and between 5,5 g/l and 10,0 g/l for potassium chloride.

NOTE The ranges for 3,5-dichlorophenol and KCl are based on the data of Annex B.

Annex C reports the performance data obtained in an international interlaboratory comparison with the reference chemical KCl.

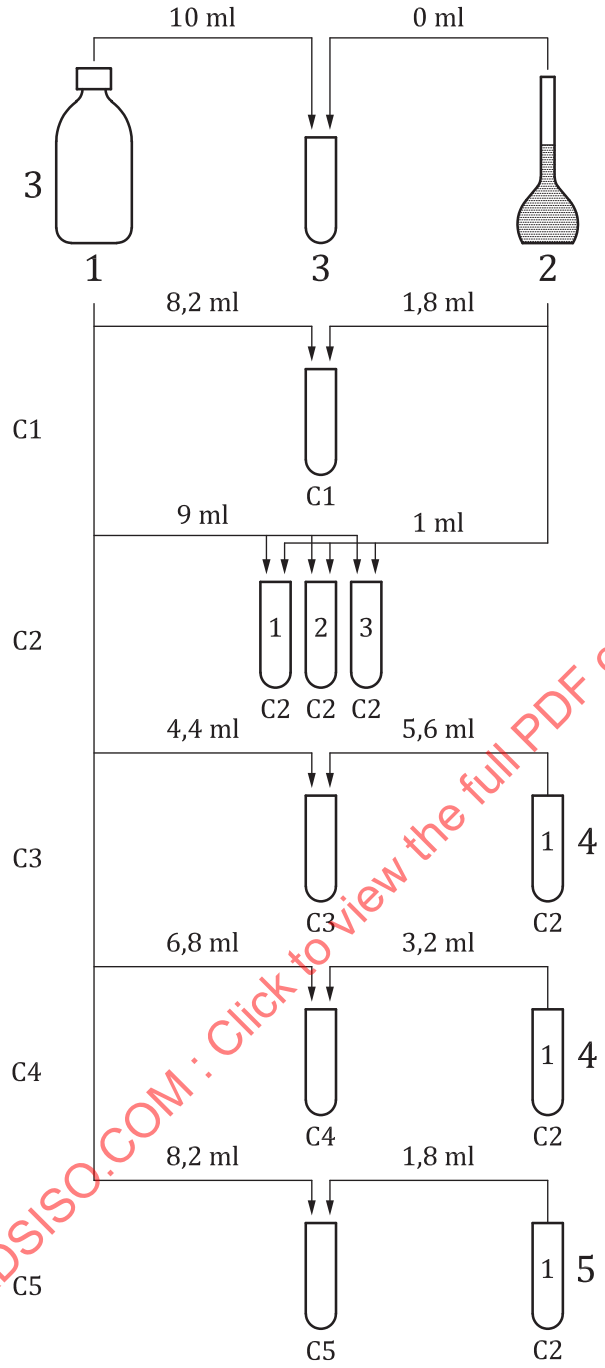
Figure 6 shows the details for the preparation of a reference test with potassium chloride, in a dilution series 18 000 mg/l – 10 000 mg/l – 5 600 mg/l – 3 200 mg/l – 1 800 mg/l.

A stock solution of 100 000 mg/l KCl is prepared by weighing 10 g KCl on an analytical balance.

Transfer the 10 g KCl in a calibrated 100 ml flask, which is then filled to the 100 ml mark with growth medium.

When the salt is dissolved, a KCl dilution series is prepared in 10 ml test tubes according to the prescriptions shown in Table 5.

Three test tubes with the C2 test concentration are needed.



Key

C1 to C5	test tubes with C5-C1 test concentrations
1	growth medium
2	KCl stock solution (= 100 000 mg/l)
3	control
4	tube 1
5	tube 2

Figure 6 — Preparation of the toxicant dilutions for a reference test with potassium chloride

14 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 20227:2017;

b) name of the laboratory performing the test;

c) date of test performance;

d) test organism (species, origin);

e) designation of test material (type of the sample or name of the chemical tested);

f) sample pre-treatment (if any);

g) dilutions or concentrations tested;

h) image analysis program used for the area measurements;

i) result sheet with the mean initial and final frond areas, and the calculation of the growth inhibition;

j) statistical data treatment method used for the EC calculations;

k) comments on the test results, if necessary.

Annex A
(informative)

Spirodela polyrhiza stock culturing for turion production

Stocks of *Spirodela polyrhiza* can be cultured in facilities with controlled temperature (20 °C to 25 °C) and light (fluorescent lamps – 4 000 lx to 8 000 lx – photoperiod 14 L:10 D).

The medium for *Spirodela polyrhiza* stock culturing can be the same as that used for the toxicity test, but other nutrient rich media can also be used for stock cultures.

The specific environmental conditions triggering the production of dormant vegetative stages (turions) can be simulated in the laboratory.

The shift from the normal growth of the duckweed colonies to the formation of turions can be induced by several environmental variables such as, for example, nutrient limitation (N and/or P shortage), and lower illumination and temperature.

After about one month in the triggering conditions – which have to be determined by trial and error on an individual basis – *Spirodela polyrhiza* cultures start to produce turions (see Figure A.1).

Figure A.1 — Fronds of a *Spirodela polyrhiza* culture with (smaller and darker) turions

Mature turions eventually detach from the parent plants and sink to the bottom of the culturing container from which they can be collected.

Freshly harvested turions are in a dormant stage for at least one month. They will only germinate after termination of the dormancy, when brought into appropriate germination conditions (9.1).

Turions should be stored in the refrigerator (at 4 °C) in darkness, in pure water.

The germination success of the turions is batch dependent, and the shelf life ranges from a few months to more than half a year.

NOTE 48 (germinated) turions are needed for each toxicity test. Departing from, for example, a 70 % germination success, it is advised to store the turions in test tubes with at least 75 turions per tube.