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Water quality — Determination of the toxic effect of water constituents and waste water on duckweed (*Lemna minor*) — Duckweed growth inhibition test

*Qualité de l'eau — Détermination de l'effet toxique des constituants de l'eau et des eaux résiduaires vis-à-vis des lentilles d'eau (*Lemna minor*) — Essai d'inhibition de la croissance des lentilles d'eau*

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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 20079 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Introduction

The duckweed species *Lemna minor* is used as model organism for higher water plants. Duckweeds are monocotyledonous, free-floating angiosperms and belong to the *Arales* within the subclass of *Aridae*. Duckweeds are fast growing higher plants, spreading from the tropic to the arctic zone. As primary producers they are a food source for waterfowl, fish and small animals and serve as physical support for a variety of small invertebrates.

Duckweed can be damaged by water constituents and effluents (see Annex B). The subsequent inhibition of growth is calculated from the observation parameters (frond number, frond area, chlorophyll, dry weight) by a number of defined calculation methods.

EC values are determined to allow for an assessment of toxic effects of water constituents (e.g. chemicals, plant protection products). The evaluation for at least two observation parameters is based on the average specific growth-rates.

The test is designed for measurement of response of substances dissolved in water. This includes the definition of a fixed dilution step, or a concentration of the test sample at which a parameter of observation (endpoint) is inhibited relative to a control for a defined percentage.

Water quality — Determination of the toxic effect of water constituents and waste water on duckweed (*Lemna minor*) — Duckweed growth inhibition test

WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This 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 according to this International Standard be carried out by suitably trained staff.

1 Scope

This International Standard specifies a method for the determination of the growth-inhibiting response of duckweed (*Lemna minor*) to substances and mixtures contained in water, treated municipal wastewater and industrial effluents.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 5667-16, *Water quality — Sampling — Part 16: Guidance on biotesting of samples*

ISO 10260, *Water quality — Measurement of biochemical parameters — Spectrometric determination of the chlorophyll-a concentration*

3 Terms and definitions

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

3.1
axenic cultures
monocultures of organisms from a single species, free from fungi, algae and other macrophyte species

3.2
calculation parameters
parameters for the estimation of toxicity derived from any parameters of observation by different methods of calculation

EXAMPLE Growth-rates derived from frond number, frond area, chlorophyll and dry weight are calculation parameters in this International Standard.

3.3
chlorosis
loss of pigment (yellowing of frond tissue)

3.4
colony
aggregate of mother and daughter fronds, attached to each other, sometimes referred to as a plant

3.5
control batch
control medium, including organisms used for testing

3.6
control medium
combination of dilution water and/or nutrient medium used in the test

3.7
dilution water
water added to the test sample to prepare a series of defined dilutions

3.8
doubling time
quotient of natural logarithm of 2 (ln 2) divided by average specific growth-rate

3.9
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 "EC(r)", followed by the observation parameter used, e.g. EC(r) (frond number).

3.10
frond
individual leaf-like structure on a duckweed colony; the smallest unit (i.e. individual), capable of reproducing

3.11
frond area
total area of all fronds visible from vertically above

3.12
frond number
all fronds protruding from a mother frond which are directly visible from above without magnification

3.13
growth
increase in biomass over time as the result of proliferation of new tissues

NOTE In this test it refers to any parameter of observation.

3.14
growth-rate
calculation parameter defined as quotient of the difference of the natural logarithms of a parameter of observation and the respective time period

NOTE If the time period comprises the total duration of the test, the term is referred to as average specific growth-rate. If the period between two measurements within the test period is used, the term is named segmented growth-rate (see 12.1.2).

3.15
inoculum
number of fronds (colonies) added to the test batch at the beginning of the test

3.16
necrosis
localised dead frond tissue (i.e. brown or white)

3.17
nutrient medium
solution of nutrients and micronutrients in water which are essential for the growth of duckweed

3.18
observation parameters
observed or measured biomass parameters like frond number, frond area, chlorophyll, dry weight, which are measured or counted once or repeatedly by observation or measurement

NOTE These parameters are relevant for the assessment of growth and vitality of the test organisms (e.g. frond number, frond area or chlorophyll content, dry weight).

3.19
pre-culture
culture of duckweed used for acclimatisation of test plants to the test conditions and for the growing of the plants to be used in the inoculum

3.20
root
that part of the *Lemna* plant that assumes a root-like structure

3.21
stock culture
culture of a single species of duckweed to conserve the original defined *Lemna* species in the laboratory and to provide inoculum for the pre-culture

NOTE It is necessary to use defined and verified strains, because of possible insecurities in species taxonomy. An address list of suppliers is given in Annex C.

3.22
test batch
test medium including organisms used for testing

3.23
test medium
combination of test sample, dilution water and/or nutrient medium used in the test

3.24
test sample
discrete portion of a sample (taken from i.e. receiving water, waste water, dissolved chemical substances or mixtures, products and compounds) pretreated according to the needs of this test (e.g. dissolution, filtering, neutralisation)

4 Principle

Plants of the species *Lemna minor* are allowed to grow as monocultures in different concentrations of the test sample over a period of seven days. The objective of the test is to quantify substance-related effects on vegetative growth over this period based on assessments of frond number, and also on assessments on biomass (total frond area, dry weight or chlorophyll). To quantify substance-related effects, the growth-rate in the test solutions is compared with that of the controls and the concentration resulting in a specified x % inhibition of growth-rate is determined and expressed as the $EC(r)_x$.

5 Interferences

Non-soluble, poorly soluble, volatile, bio- or photodegradable substances or substances reacting with the dilution water or the nutrient medium or changing their state during the test, may falsify or reduce the reproducibility of the results (see ISO 5667-16). Special consideration is necessary in the case of substances accumulated at the water surface as this may increase the effects on duckweed.

6 Apparatus

The test design determines the requirements for the apparatus.

6.1 Cylindrical vessels, (glass beakers, crystallising dishes, Petri dishes).

Minimum volume of 150 ml (for 2/3 of total volume, i.e. 100 ml of test solution).

6.2 Uniform glass coverings.

Covers may be provided to minimize evaporation and accidental contamination.

6.3 Facilities with constant temperature and illumination, temperature controlled room or water bath, incubator or environmental chamber.

6.4 Spectrometer to monitor chlorophyll, 665 nm and 750 nm.

6.5 Lumino-meter, to be used to measure light intensity.

6.6 pH-meter.

6.7 Tweezers.

6.8 Glassware, for the preparation of different concentration series and nutrient medium (volumetric flasks, graduated cylinders, pipettes, Petri dishes).

6.9 Image analysis system, to measure frond number and frond area.

6.10 Autoclave.

6.11 Filtration device, for sterile filtration.

7 Reagents

Use only reagents of recognised analytical grade.

7.1 Dilution water, distilled or deionised water or water of equivalent purity, conductivity $\leqslant$ 10 µS/cm.

7.2 Hydrochloric acid, for example c(HCl) = 0,1 mol/l.

7.3 Sodium hydroxide solution, for example c(NaOH) = 0,1 mol/l.

7.4 Glucose, $C_6H_{12}O_6$.

7.5 Agar medium.

See Annex A.

7.6 Nutrient media, *modified STEINBERG* medium (see Table 1).

Generally the *modified STEINBERG* medium shall be used for all applications within the scope of the guideline, i.e. water constituents and wastewaters. In some cases, use of the media described in Annex A may also be suitable as long as all validity criteria are fulfilled.

Table 1 — pH-stabilised *STEINBERG* medium (modified by Altenburger)

Substance		Nutrient medium	
Macroelements	**Molecular mass**	mg/l	mmol/l
KNO_3	101,12	350,00	3,46
$Ca(NO_3)_2 \cdot 4H_2O$	236,15	295,00	1,25
KH_2PO_4	136,09	90,00	0,66
K_2HPO_4	174,18	12,60	0,072
$MgSO_4 \cdot 7H_2O$	246,37	100,00	0,41
Microelements	**Molecular mass**	µg/l	µmol/l
H_3BO_3	61,83	120,00	1,94
$ZnSO_4 \cdot 7H_2O$	287,43	180,00	0,63
$Na_2MoO_4 \cdot 2H_2O$	241,92	44,00	0,18
$MnCl_2 \cdot 4H_2O$	197,84	180,00	0,91
$FeCl_3 \cdot 6H_2O$	270,21	760,00	2,81
EDTA Disodium-dihydrate	372,24	1 500,00	4,03

7.6.1 Concentrations and stock solutions (see Tables 2 and 3).

Prepare the nutrient medium from single solutions. The required concentrations of pre-culture and test medium are obtained by dilution if 10-fold concentrated medium is prepared.

Table 2 — Stock solutions (macroelements)

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 3 — Stock solutions (microelements)

Microelements (1 000-fold concentrated)	mg/l
Stock solution 4:	
H_3BO_3	120,0
Stock solution 5:	
$ZnSO_4 \cdot 7H_2O$	180,0
Stock solution 6:	
$Na_2MoO_4 \cdot 2H_2O$	44,0
Stock solution 7:	
$MnCl_2 \cdot 4H_2O$	180,0
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).

For longer shelf life, treat stock solutions in an autoclave at 121 °C for 20 min or alternatively carry out a sterile filtration (0,2 µm). For stock solution 8, sterile filtration (0,2 µm) is strongly recommended.

7.6.2 Preparation of the final concentration of *modified STEINBERG* medium

Add 20 ml each of stock solutions 1, 2 and 3 (see Table 2) to about 900 ml water (7.1).

Then add 1,0 ml each of stock solutions 4, 5, 6, 7 and 8 (see Table 3) to avoid precipitation.

The pH should be 5,5 ± 0,2 [adjust by addition of a minimised volume of NaOH solution (7.3) or HCl (7.2)].

Adjust with water (7.1) to 1 000 ml.

If stock solutions are sterilized and appropriate water is used, no further sterilisation is necessary. If sterilisation is done with the final medium, stock solution 8 should be added after autoclaving (at 121 °C for 20 min).

7.6.3 Preparation of 10-fold-concentrated *modified STEINBERG* medium

Add 20 ml each of stock solutions 1, 2 and 3 (see Table 2) to about 30 ml water (7.1).

Then add 1,0 ml each of stock solutions 4, 5, 6, 7 and 8 (see Table 3) to avoid precipitation. Adjust with water (7.1) to 100 ml.

If stock solutions are sterilized and appropriate water is used, no further sterilisation is necessary. If sterilisation is done with the final medium, stock solution 8 should be added after autoclaving (at 121 °C for 20 min).

The pH of the medium (final concentration) should be 5,5 ± 0,2.

For the assessment of mining effluents, metal substances added to water or other samples which may contain pre-dominantly metals, it may be appropriate to use a modified APHA test medium. Using modified APHA (i.e. without EDTA, see Annex A) would make it necessary to change medium from *modified STEINBERG* medium to modified APHA between pre-culture and an acclimatization phase before the test [12]. This change does not conform with 9.2, but this is to be accepted in this case. All details on handling and use of APHA are included in Annex A.