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Water quality — Aquatic toxicity test based on root re-growth in *Lemna minor*

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Foreword

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

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Lemna gibba and *L. minor* have been most extensively used in phytotoxicity testing and there are several standard methods which have been adopted by major international standardization agencies e.g. ISO 20079:2005, U.S. Environmental Protection Agency,[4] Organization for Economic Cooperation and Development.[5] Tests with duckweed have typically favoured measurements of frond (e.g. their number, biomass, area, carbon uptake, chlorophyll content[9]) require standard exposure durations of at least 7 d to detect toxicity.

On the other hand, tests based on root elongation are some of the most widely used phytotoxicity methodologies for terrestrial angiosperms because of their simplicity and rapidity. Despite reports that roots of *L. minor* are highly sensitive to environmental stressors and that they play important ecological roles by providing stability[6],[7],[8] little attention has been paid to the roots in *Lemna* since it was generally considered that root fragility made their handling for measurements difficult and that it was impractical to obtain sufficient numbers of individual plants with identical root lengths to initiate tests. However, the ecotoxicological significance of the root endpoint has been re-evaluated and root length was shown to be a sensitive, precise and ecologically significant endpoint in comparison with more traditional frond growth or biomass endpoints.[9]

The proposed root re-growth bioassay differs in several key aspects from three internationally standardized methods (ISO, OECD and US EPA):

a) the test can be completed within 72 h;

b) the test vessel is a 24-well cell plate;

c) the required volume of test water samples is 3 ml;

d) roots were excised prior to exposure with subsequent measurements on newly developed roots. The technique of excising roots prior to exposure means that there is no requirement to pre-select roots of uniform length, which reduces the handling of these fragile roots.

Artificial severance of roots may never happen in natural settings since root abscission in *Lemna* has not been reported previously. However, according to recent studies the tiny globally distributed water ferns of the genus *Azolla* lost their roots under stress conditions[10] a phenomenon known as rapid root abscission. Such shedding sets its fronds free from root-entangled mats and facilitates their dispersion to a potentially better environment. Therefore, rapid root abscission is considered an important survival strategy of *Azolla*.[10] This may indicate that the endpoint root re-growth has its ecological relevance.

It is also well known that *Lemna* will thrive without any roots. Thus, *Lemna* roots appear nonessential organs, but are nonetheless important for plant anchorage, nutrient absorption and cytokinin biosynthesis. Therefore, the manipulation of roots by simple severance may not be a serious issue and does not justify the conclusion that removal of roots prior to ecotoxicological testing is inappropriate.

The 3 d root re-growth test is useful for the rapid screening of either wastewater effluents or hazardous contaminants in natural waters[11] as it is easy to perform, quick to run, and cost-effective to operate for wastewater toxicity screening and may have an operational benefit of testing time since management decision should be made in a timely manner in the case of unexpected pollution events.

The root re-growth endpoint from this 72-hour protocol is not a direct substitute for the 7-day growth rate/biomass endpoints.

The present protocol provides detailed information on how to set up and conduct the root re-growth test with *Lemna minor* as well as how to analyse toxicity data. This protocol is intended for use with *Lemna minor*, but it can also be applied to other *Lemna* species and *Spirodela* species with some modifications.

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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.

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 root re-growth in duckweeds (*Lemna minor*) by substances and mixtures contained in water or waste water. This method applies to environmental water samples including treated municipal wastewater and industrial effluents.

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 20079, *Water quality — Determination of the toxic effect of water constituents and waste water on duckweed (Lemna minor) — Duckweed growth inhibition test*

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:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

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

[SOURCE: ISO 20079:2005, 3.1]

3.2
coefficient of variation
CV
relative standard deviation, expressed as a percentage

3.3
colony
aggregate of mother and daughter fronds, attached to each other, sometimes referred to as a plant

[SOURCE: ISO 20079:2005, 3.4]

3.4
effective concentration
EC_x
concentration of test sample (EC_x) at which an effect of x % is measured, if compared to the control

[SOURCE: ISO 20079:2005, 3.9, modified — Note 1 to entry deleted]

3.5
frond
individual leaf-like structure on a duckweed colony; the smallest unit (i.e. individual), capable to reproduce

[SOURCE: ISO 20079:2005, 3.10]

3.6
growth
increase in biomass over time as the result of proliferation of new tissues

Note 1 to entry: In this test it refers to any parameter of observation.

[SOURCE: ISO 20079:2005, 3.13]

3.7
growth medium
pure water to which reagent-grade chemicals (micronutrients) have been added

3.8
non-axenic culture
monoculture of organisms from a single species (i.e. free from other macrophyte species), which has not been treated with antimycotic or antibiotic solutions to remove naturally associated bacteria and fungi

3.9
pre-culture
culture of duckweed used for acclimation of test plants to the test conditions and for the growing of the plants to be used in the inoculum

[SOURCE: ISO 20079:2005, 3.19]

3.10
pure water
deionized or distilled water

[SOURCE: ISO 19827:2016, 3.4]

3.11
root
the part of the *Lemna* plant that assumes a root-like structure

[SOURCE: ISO 20079:2005, 3.20]

3.12
stock culture
culture of a single species of duckweed to conserve the original *Lemna* species in the laboratory and to provide inoculum for the pre-culture

Note 1 to entry: It is necessary to use defined and verified strains, because of possible insecurities in species taxonomy.

[SOURCE: ISO 20079:2005, 3.21, modified — "An address list of suppliers is given in Annex C" deleted in the note]

3.13
stock solution
solution with accurately known analyte concentration (s), prepared from chemicals with an appropriate purity

[SOURCE: ISO 5667-16:2017, 3.21]

3.14
test sample
discrete portion of sample (taken from i.e. receiving water, waste water, dissolved chemical substances or mixtures, products and compounds) pre-treated according the to the needs of this test (e.g. dissolution, filtering, neutralization)

[SOURCE: ISO 20079:2005, 3.24]

3.15
test medium
aqueous solution that consists of a particular concentration of prepared test sample mixture of test water and the sample under test

[SOURCE: ISO 21427-1:2006, 3.4]

4 Principle

All roots (3.11) shall be removed from *Lemna minor* fronds (3.4), grown in axenic (3.7) or non-axenic cultures (3.8), prior to exposure to test sample and the growth of newly developed roots during the exposure period of 72 h will be measured.

To quantify substance-related effects, the root length in the test medium (3.15) is compared with that of the controls and the concentration resulting in a relative inhibition of root length to be determined and expressed as the EC(r)x.

5 Test organisms

The standard test organism of this test is duckweed, *Lemna minor*, which is a freshwater-floating plant.

6 Growth medium

6.1 Preparation of stock solution

Prepare stock solution (3.13) by adding the weighed chemicals according to Table 1 to the desired volume of pure water (3.10) for the growth medium and test compound solutions. Pure water should be used for the dilution of liquid media and test substances.

pH of liquid media shall be adjusted to 6,9 ± 0,1 after adding pure water to each stock solution. Nutrients should be added in the order of I-II-III-IV-V while preparing the solutions to prevent precipitation.

NOTE A pH of 7 is ideal for the re-growth of *Lemna* roots and pH 6,9 ± 0,1 is proposed to be the appropriate pH for the toxicity tests, as this is also the same pH range that is measured in the Steinberg medium.

Liquid media can be stored for up to one month at room temperature in the dark.

Table 1 — Main ingredients of STEINBERG medium

Stock solution	Chemicals	Stock concentration g L^{-1}	Final concentration ml L^{-1}
Macro-elements			

Table 1 *(continued)*

Stock solution	Chemicals	Stock concentration g L^{-1}	Final concentration ml L^{-1}
I	KNO_3	17,5	20
	K_2HPO_4	4,5	
	KH_2PO_4	0,63	
II	$MgSO_4 \cdot 7H_2O$	5	20
III	$Ca(NO_3)_2 \cdot 4H_2O$	14,75	20
Micro-elements			
IV	H_3BO_3	0,12	1
	$ZnSO_4 \cdot 7H_2O$	0,18	
	$Na_2MoO_4 \cdot 2H_2O$	0,044	
	$MnCl_2 \cdot 4H_2O$	0,18	
V	$FeCl_3 \cdot 6H_2O$	0,76	1
	Na_2-EDTA$\cdot 2H_2O$	1,5	

6.2 Storage and cultivation

Culture fronds of *Lemna minor* in growth medium (3.6) as shown in Table 1. The fronds (3.4) shall be cultured at (25 ± 1) °C, given 24 h continuous white light at an intensity of 30 µmol m^{-2} s^{-1} to 40 µmol m^{-2} s^{-1} (1,500 lx to 2,000 lx). The medium shall be replaced at an interval of 7 d and the storage culture can be kept continually unless uncontrolled contamination occurs. Cloudy medium in a *Lemna minor* stock culture (3.12) indicates bacterial contamination, whereas contamination with mould may not be clearly evident until large colonies appear in the medium or a slime layer develops on the vessel. Contaminated *Lemna minor* cultures shall be discarded.

7 Apparatus

The test requires usual laboratory equipment and the following.

7.1 Temperature-controlled cabinet or room, with a white fluorescent light, providing, continuous, uniform illumination in accordance with the requirements specified in Table 2.

Table 2 — Summary test conditions for the *Lemna* root re-growth test

Type of test:	Static; 72 h test
Lighting:	90 µmol m^{-2} s^{-1} to 100 µmol m^{-2} s^{-1} (4,500 lx to 5,000 lx; 400 nm to 700 nm)
Photoperiod:	Continuous cool white fluorescent light
Temperature:	(25 ± 1) °C
Salinity:	0 psu
pH:	6,9 ± 0,1
Test vessel:	24-well plate (85,4 mm × 127,6 mm; well dimension 15,6 mm diameter)
Growth medium:	Steinberg medium
Volume of test solution:	3,0 ml/well
Number of colonies per well:	One colony; an individual colony has two fronds to three fronds each with one root

Table 2 *(continued)*

Type of test:	Static; 72 h test
Number of replicates:	At least three (6 control replicates are recommended)
Endpoint:	Root length

NOTE As 25 °C is optimal for the re-growth of *Lemna minor* roots, the temperature range was determined, taking into account temperature changes due to electricity in the culture chamber.

The test shall be performed after adjusting the pH if the pH is outside the acceptance range.

7.2 Light-meter, to be used to measure in photon irradiance expressed in micromoles per square meter per second (µmol m^{-2} s^{-1}) or in lx.

Measure the light intensity once per test in at least five characteristic points of the test area in a realistic test environment. It shall not vary by more than ±15 % of the selected light intensity. The measurement of light with a spherical head quantifies all light that would reach the plants if the test solution is clear.

The use of a random design with changes at the observation times is recommended but does not compensate high deviations of light intensity and temperature between different places of the test area. Before a toxicity test is conducted with new test facilities, it is desirable to conduct a non-toxicant test, in which all test vessels contain control medium (see also ISO 20079, 10.6; EPS 1/RM/25]

7.3 pH meter, for the adjustment of pH during the preparation of cultures and test solutions and to measure pH at the beginning and end of a test.

7.4 Tweezers, for handling fronds.

7.5 Stainless scissors, for excising roots.

7.6 Beakers.

7.7 Graduated cylinders.

7.8 Pipettes.

7.9 Capacities of conical tubes.

7.10 Plastic tank.

7.11 Exposure dishes, for example 24-well cell plates with 3,0 ml per well (a diameter of 15,6 mm may be suitable).

Cell plates shall be sealed with sealing tape for prevention of evaporation of medium and test solution. In the case of volatile organic compounds, separate cell plates should be used to avoid transfer of volatile compounds between the wells.

NOTE Cross contamination is possible, if the test substance is highly volatile.

7.12 Sealing tape, to seal around the exposure dishes.

7.13 Microscope slide glasses, for putting fronds on for taking measurements of root length.