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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.

For the assessment of water constituents (chemicals), the OECD medium (modified SIS) could also be used (see Annex A). The concentration of the respective nutrient medium shall be kept constant in all treatments and controls.

7.7 Reference substances

7.7.1 3,5-dichlorophenol

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

7.7.2 **Potassium chloride**, KCl.

8 Test organisms

For use in this International Standard, *Lemna minor* (with a documentation of origin) is the recommended species. Plants obtained from a wild population need a confirmation of their taxonomy.

9 Stock cultures and pre-cultures

9.1 Stock cultures

Stock cultures are kept axenically. Addition of glucose (1 %) enables the recognition of microbial infections. For solid medium, about 1 % of agar may be added (approved media are listed in Annex A). Stock cultures may be maintained at low light conditions and ambient temperature for several months without re-inoculation if evaporation is minimized by covering the Erlenmeyer flask with a plug or cap and aluminium foil (see also Annex A).

9.2 Pre-cultures

Initiate cultures used for toxicity tests at 7 d to 10 d prior to the test, using test medium and test conditions.

NOTE 1 A longer adaptation-time may be required in the case of change of the nutrient medium between stock culture and pre-culture.

NOTE 2 Where the modified APHA-medium is to be used as testing medium, specific treatment will be necessary (see Annex A).

Pre-cultured duckweed to be used in toxicity tests should meet the following health criteria:

— exponential growth;

— the number of fronds in the pre-culture should have a seven-fold increase by the end of 7 d (i.e. $r \geqslant 0{,}275$ per d or doubling time $\leqslant$ 2,5 d);

— the culture should consist of young, rapidly growing colonies with bright green colour without visible lesions, chlorosis or necrosis;

— a large number of single fronds or small colonies is an indication of environmental stress and these plants should not be used in testing;

— the depth of the medium should be at least 3 cm.

To minimise lag-phases caused by interactions between colonies, it should be assured that the covering is less than 50 % of the total available surface (no crowding). All colonies used should originate from the same pre-culture.

10 Procedure

10.1 General

The general recommendations as specified in ISO 5667-16 are to be taken into account. Adjust the pH value of the test sample to ± 0,2 of the pH value of the nutrient medium in the control by adding hydrochloric acid (7.2) or sodium hydroxide solution (7.3). Dilution should be kept at a minimum. No later adjustment should be made.

NOTE Duckweed generally has no growth problems between pH 5 and pH 9. Therefore adjustment of pH is usually not necessary as long as the pH of the sample is between 5 and 8, depending on buffer capacity.

Neutralisation is not allowed if the effect of pH is to be reflected in the test results or if physical modification or chemical reaction is observed due to pH adjustment.

10.2 Preparation of concentration series for $EC(r)_x$ value assessment

If the $EC(r)_x$ is to be estimated, a sufficient number of concentrations is used to define the $EC(r)_x$ at an appropriate confidence level. An appropriate test design consists of a geometric series of at least five concentrations. At least one measured inhibition value for the intended $EC(r)_x$ should be below and one above the $EC(r)_x$ to be estimated, and three or more values should be other than 0 % or 100 % inhibition. Otherwise, confidence limits might be too large.

10.3 Test

10.3.1 Test for $EC(r)_x$

For $EC(r)_x$ assessment (see also ISO 5667-16), use at least 3 replicates at each concentration level for the treatments and 6 replicates for the controls.

10.3.2 Test for $EC(r)_{<20}$

The number of replicates strongly depends on the size of the expected coefficient of variation and the tolerated deviations. The coefficient of variation should be calculated from tests with a sufficiently large number of controls (see also ISO 5667-16).

10.3.3 Limit test

A dilution series is unnecessary when it is only desired to ascertain, whether a given concentration or dilution level exhibits an effect (limit test). Use at least 6 replicates for the limit concentration and the controls.

10.3.4 Use of solvents or dispersants

If solvents or dispersants cannot be avoided (at a maximum concentration of 0,1 ml/l or 100 mg/l, respectively), use an additional control with 6 replicates including the solvent or dispersant at the same concentration as in all replicate vessels. Solvents or dispersants shall exhibit no toxic effects at the concentration chosen.

10.3.5 Test with reference substances

Carry out a test using 3,5-dichlorophenol and/or potassium chloride solution (7.7.1, 7.7.2).

$EC(r)_{50}$ (frond number) should be in a range 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.

Reference substances may be tested for checking the test procedure and sensitivity. It is advisable to test the reference substances regularly and to use control charts for measuring within laboratory precision and monitoring culture health. Guidance on preparation of control charts is available [13].

NOTE 1 The range for 3,5-dichlorophenol (tested with *modified STEINBERG* medium) is based on data of the international interlaboratory test for this International Standard. The range for potassium chloride (tested with *modified STEINBERG* medium and APHA medium) is based on Canadian and German data according to 95 % confidence limits of $EC(r)_{50}$ (frond number).

NOTE 2 In recent research studies, nickel sulfate ($NiSO_4$) has been proved to be an additional reference substance.

10.4 Number of fronds at the start of the test

Each test vessel should contain a total of 10 to 16 fronds (2 or 3 fronds per colony) satisfying the pre-culture conditions. The number of fronds and colonies should be the same in each test vessel. Generally the total size of all colonies together has significant influence on the coefficient of variation at the end of the test for all observation parameters. For this reason, keep the total size of all colonies as homogeneous as possible.

To provide a random inoculation, it is recommended to place one of the pre-selected colonies in each test vessel and to continue this process until each test vessel contains the same required number of fronds. A minimum volume of 100 ml is recommended. The diameter of the vessel should be chosen in such a way that overlapping of the fronds (more than about 50 % covering) at the end of the test is avoided.

10.5 Temperature

The temperature in the test vessels should be 24 °C ± 2 °C. Maintain this temperature throughout the test allowing a deviation of not more than ± 1 °C in all vessels. Measure at least at the four observation times; however, continuous temperature control is recommended. If temperature records are based on measurements other than in the test vessels, the relationship should be established.

10.6 Light

Continuous warm or cool white fluorescent lighting should be used. Measure photosynthetically active radiation (PAR) with a spherical quantum sensor. Light intensity should be 85 $\mu E \cdot m^{-2} \cdot s^{-1}$ to 135 $\mu E \cdot m^{-2} \cdot s^{-1}$ (400 nm to 700 nm) at the water level in the test vessels.

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. Exclude light from the side and bottom by test design, i.e. black side covers and a black bottom. 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. The coefficient of variation of growth-rate should be less than 10 %.

10.7 Test duration

The test duration is 7 d.

10.8 Measurements and observations

Measure the basic parameter frond number. In addition, a second observation parameter (frond area, dry weight or chlorophyll) shall be measured.

Record qualitative observations of any visual signs of phytotoxicity for each test vessel at the end of the test as follows:

a) abnormally sized fronds;

b) root length and destruction;

c) local or size specific chlorosis or necrosis;

d) loss of buoyancy, break-up of colonies;

e) changes of the test medium, bacterial contamination and any other relevant changes;

f) pH value of the medium at the end of the test.

For observations, place the test vessels on a white background. Illuminate from the side or the bottom of the vessel.

Count all fronds protruding from a mother frond visible without magnifying device irrespective of colour. If necessary, fronds with altered colour (yellow, brown or white) of at least 50 % are counted separately.

Chlorophyll may be quantified according to ISO 10260.

Frond area and frond number may be quantified using an image analysis system.

Dry weight may be quantified after drying blotted colonies at 60 °C to constant mass.

10.9 Sequence of measurement

Quantify all observation parameters chosen for measurement at the start and at the end of the test. For the initial measurement of chlorophyll or dry weight, inoculate 6 additional controls. Carry out random control measurements for chlorophyll or dry weight. Measure the frond number in the controls at least every 48 h to 72 h.

11 Validity criteria

Assuming that all recommended procedures and conditions were followed, the mean number of fronds in the control shall have at least an average specific growth-rate of 0,275 per d. This corresponds to a doubling time of about 2,5 d and a 7-fold increase of the mean number of fronds by the end of the test.

3,5-dichlorophenol and/or potassium chloride should be used as reference substances. An international interlaboratory test for this International Standard with 3,5-dichlorophenol was performed in 2004 with results as presented in Table 4 (Clause 15). As a result, the $EC(r)_{50}$ (frond number) should be in a range between 2,2 mg/l and 3,8 mg/l for 3,5-dichlorophenol in *modified STEINBERG* medium. For the APHA medium only EC-values for potassium chloride between 5,5 g/l and 10 g/l are applicable [4].

For formal accreditation, requirements of ISO/IEC 17025 [14] should be fulfilled. For internal quality control, reference toxicant tests should be completed regularly. $EC(r)_{50}$ should be within $\pm 2\,s$ (standard deviation) as described in the range above. Documentation of the results using quality control charts for one or more test endpoints are recommended (see 10.3.5).

12 Expression of results

12.1 Test results – Inhibition value of water constituents (chemicals)

12.1.1 General

Record growth-rate and growth inhibition values of each replicate, mean values and standard deviations for all observation parameters.

For each treatment, calculate the parameters and inhibition values from the equations given below. Plot mean values and coefficients of variation of the replicates as concentration-response curves (inhibition against log concentration in a figure).

If dispersants have been used, appropriate test design should be established and suitable statistical treatment of data should be carried out. Requirements may be followed according to ISO 5667-16. General recommendations are made in the statistical paper of ISO/TS 20281 [13].

12.1.2 Estimation of growth-rate

Calculate the growth-rate r from Equation (1):

$$r = \frac{\ln x_{t2} - \ln x_{t1}}{t_2 - t_1} \quad (1)$$

where

r is the growth-rate per day;

x_{t1} is the value of observation parameter at t_1 days;

x_{t2} is the value of observation parameter at t_2 days;

$t_2 - t_1$ is the time period between x_{t1} and x_{t2}, in days.

Use only mean values as basic data if the parameter is measured destructively. In all other cases, use the individual value of the replicates.

Calculate the growth-rate for the total test period, or a rationale for selecting only a portion of the growth curve provided. For each test concentration and control, calculate the average specific growth-rate with variance estimates.

Calculate the percent inhibition of growth-rate for each test concentration, according to Equation (2):

$$i_r = \frac{r_c - r_t}{r_c} \times 100 \quad (2)$$

where

i_r is the inhibition of the average specific growth-rates, in percent, %;

r_c is the average specific growth-rate of the control, in d^{-1};

r_t is the average specific growth-rate of the treatment groups, in d^{-1}.

12.2 Assessment of test validity

For all control vessels, record single values, mean values and coefficients of variation for quantified parameters in order to calculate:

— average specific growth-rate;

— doubling time;

— multiplication factor within 7 d.

For the control of exponential growth, record the mean value and coefficients of variation of all observation parameters measured several times and plot the mean values as:

— growth curves [ln (observation parameters) versus time]; or

— segmented growth-rates (r versus time).

For additional quality control purposes:

— statement of compliance that validity criteria for reference toxicant tests have been met (e.g. using control charts).

13 Estimation of EC(r)$_x$ values for frond number and the second observation parameter

A non-linear regression of the concentration-response curve with a suitable model is recommended.

NOTE Applying a non-linear regression can help to interpret tests, in which, at high concentrations, after an initial growth the further growth has stopped. As a consequence - almost independently of concentration - inhibition reaches only values of 70 % to 90 %.

14 Documentation of results

The values calculated for EC(r)$_x$ [e.g. EC(r)$_5$, EC(r)$_{10}$, EC(r)$_{25}$, EC(r)$_{50}$ and EC(r)$_{90}$ as far as appropriate] and the corresponding confidence intervals (95 %) for frond number and the second observation parameter are displayed with the required significant precision (digits). The (inhibitory) effect of the mass concentration of the tested water constituents or wastewater on the growth of *Lemna minor* should be displayed graphically, mathematically or be tabled (see ISO 5667-16 and references in the Bibliography).

15 Precision

International interlaboratory tests based on the test described in this International Standard were carried out in 2004. The results with the reference substance 3,5-dichlorophenol for EC(r)$_{10}$, EC(r)$_{20}$ and EC(r)$_{50}$ for the observation parameters (frond number, frond area and dry weight) are shown in Table 4.

Table 4 — Interlaboratory test results for EC(r)$_{50}$

Parameter	EC(r)$_x$	l	n	n_{AP} %	$\bar{\bar{x}}$	s_R	CV$_R$ %	s_r	CV$_r$ %
Frond-number FN	EC(r)$_{10}$	23	61	10,3	1,70	0,353	20,7	0,270	15,8
	EC(r)$_{20}$	24	65	4,4	2,01	0,390	19,4	0,254	12,6
	EC(r)$_{50}$	25	68	0,0	2,98	0,367	12,3	0,281	9,4
Dry weight DW	EC(r)$_{10}$	11	29	12,1	1,67	0,268	16,0	0,280	16,8
	EC(r)$_{20}$	11	29	12,1	1,91	0,260	13,6	0,267	13,9
	EC(r)$_{50}$	12	33	0,0	2,39	0,379	15,9	0,292	12,2
Frond-area FA	EC(r)$_{10}$	13	35	0,0	1,67	0,348	20,8	0,207	12,4
	EC(r)$_{20}$	12	33	5,7	1,98	0,268	13,5	0,169	8,5
	EC(r)$_{50}$	11	32	8,6	2,60	0,242	9,3	0,197	7,6

l is the number of laboratories;
n is the number of valid measured values;
n_{AP} are the outliers;
$\bar{\bar{x}}$ is the overall mean;
s_R is the reproducibility standard deviation;
CV$_R$ is the reproducibility variation coefficient;
s_r is the repeatability standard deviation;
CV$_r$ is the repeatability variation coefficient.

16 Test report

The test report shall include information on at least the following aspects of the test:

a) a reference to this International Standard (ISO 20079);

b) name of the laboratory performing the test;

c) date and period of test;

d) reference to the test method (international standard number) used;

e) test organism (e.g. scientific name, strain, source), therapeutic or acclimatory pre-treatment (if any);

f) designation of test material (batch number, origin, date and period of sampling);

g) sample pre-treatment (e.g. preservation, pre-concentration, homogenisation, pH adjustment, type of neutralizing agent, pre-aeration);

h) data, derived, condensed or transformed, including, if appropriate, results of positive controls, control chart;

i) chemical and physical data determined during the test (e.g. temperature, pH, turbidity, precipitation, possible change in substance concentration, etc.);

j) any deviation from the test protocol (nature of dilution water, nutrient solution, aeration, temperature etc., number of organisms or density of inoculum, number of replicates and controls);

k) method of estimating $EC(r)_x$ values;

l) details of the test results;

m) comments on the test results, if necessary.

Annex A
(informative)

Preparation of the nutrient media

A.1 Modified APHA medium for testing metal compounds and metal contaminated effluents using *Lemna minor*

Where the modified APHA medium is to be used as testing medium, *STEINBERG* medium should be used for the pre-culture, according to pre-culture requirements. *Lemna minor* plants from the pre-culture to be used for testing shall be acclimatized for 16 h to 24 h in modified APHA medium under test conditions. Longer acclimation would negatively affect growth performance during the test.

A.1.1 Concentrations and stock solutions

Table A.1 — Modified APHA medium

Substance	Concentration		Stock solution
	Stock solution [a] g/l	Medium [b, c] mg/l	
$MgSO_4 \cdot 7H_2O$	14,7	147	C
$NaNO_3$	25,5	255	A
$CaCl_2 \cdot 2H_2O$	4,41	44,1	B [d]
KCl	1,01	10,1	A
$NaHCO_3$	15,0	150	A
K_2HPO_4	1,04	10,4	A
H_3BO_3	0,186	1,86	C
$MnCl_2 \cdot 4H_2O$	0,414 9	4,149	B
$MgCl_2 \cdot 6H_2O$	12,17	121,7	B
$Na_2MoO_4 \cdot 2H_2O$	0,007 26	0,072 6	C
$ZnCl_2$	0,003 27	0,032 7	C
$CuCl_2$	$9,0 \times 10^{-6}$	$9,0 \times 10^{-5}$	C
$CoCl_2$	0,000 78	0,007 8	C
$FeCl_3 \cdot 6H_2O$	0,16	1,6	B
pH adjustment	Adjust to pH 8,30 immediately before testing		
Sterilization	None		

[a] To prepare medium, add 10 ml of each stock solution to 970 ml dilution water (7.1) and aerate vigorously at least 1 h to 2 h.

[b] *Lemna* to be used for testing shall be acclimated for 18 h to 24 h in modified APHA medium under test conditions.

[c] Concentrations of substance in medium.

[d] Acidify solution B to pH 2,0 to prevent precipitation. Protect the solution from light by storing in a dark amber bottle.

A.1.2 Preparation of the final concentration of modified APHA medium

To prepare the final concentration of 1 l of nutrient medium, add 10 ml of each stock solution to 970 ml dilution water (7.1) and aerate vigorously at least 1 h to 2 h. If a larger volume (> 4 l) of medium is prepared, overnight aeration is recommended to stabilize the pH of the medium.

Dilute to volume (1 000 ml) with water (7.1).

Adjust the pH to 8,3 ± 0,1 by addition of a minimised volume of NaOH solution (7.3) or HCl (7.2) immediately before testing.

The nutrient medium is not sterilised.

A.1.3 Preparation of test medium (modified APHA medium)

An aliquot of each of the three nutrient stock solutions (A, B and C) is added to the test sample in a ratio of 10 ml aliquot per 1 000 ml of test medium diluting the sample to 97 %.

Using a tube with small aperture (e.g. 0,5 mm), gently aerate the spiked samples for 20 min to stabilize the pH. Make sure that about 100 bubbles per minute are not exceeded in order to avoid stripping of volatile chemicals.

Where necessary, filter through a glass fibre filter (pore size of 0,2 µm) to exclude algae or bacteria.

Further dilutions can be achieved with the nutrient media or similarly spiked dilution water.

A.2 SIS medium as used by OECD

A.2.1 Concentrations and stock solutions

Table A.2 — OECD TG 221 Draft, April 2004 — Culture and test media for *Lemna minor* [modified Swedish Standard (SIS) growth medium]

Substance	Concentration		Stock solution
	Stock solution g/l	Medium [a] mg/l	
$MgSO_4 \cdot 7H_2O$	15	75	II
$NaNO_3$	8,5	85	I
$CaCl_2 \cdot 2H_2O$	7,2	36	III
Na_2CO_3	4,0	20	IV
KH_2PO_4	1,34	13,4	I
H_3BO_3	1,0	1,0	V
$MnCl_2 \cdot 4H_2O$	0,2	0,2	V
$Na_2MoO_4 \cdot 2H_2O$	0,010	0,010	V
$ZnSO_4 \cdot 7H_2O$	0,050	0,050	V
$CuSO_4 \cdot 5H_2O$	0,005	0,005	V
$Co(NO_3)_2 \cdot 6H_2O$	0,010	0,010	V
Na_2EDTA	0,28	1,4	VI [b]
$FeCl_3 \cdot 6H_2O$	0,168	0,84	VI
MOPS (buffer) [c]	490	490	VII
pH adjustment	Adjust to 6,5 ± 0,2 by addition of NaOH solution (7.3) or HCl (7.2).		
Sterilization	Sterilize stock solutions I to V by autoclaving (121 °C, 15 min) or by membrane filtration (pore diameter 0,2 µm); stock solutions VI (and optional VII) are sterilised by membrane filtration only (i.e. these should not be autoclaved).		

[a] Concentration of substance in medium.

[b] Added after autoclaving.

[c] MOPS buffer is only required when pH control of the test medium is particularly important (e.g. when testing metals or substances which are hydrolytically unstable).

A.2.2 Preparation of the final concentration of SIS medium

Add the following stock solutions to about 900 ml water (7.1):

— 10 ml of stock solution I;

— 5 ml each of stock solutions II, III, IV, VI;

— 1 ml of stock solution V;

— 1 ml of stock solution VII (optional).

Adjust the pH to 6,5 ± 0,2 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 carried out with the final medium, stock solution VI (and optional VII) should be added after autoclaving (at 121 °C for 20 min).

A.3 Solid medium for axenic stock cultures

A.3.1 Concentrations and stock solutions

Table A.3 — Solid medium according to Jungnickel (1986) (P+ medium modified by Jungnickel by adding glucose and agar)

Substance		Nutrient medium	
Macroelements	**Molecular mass**	mg/l	mmol/l
KNO_3	101,12	809,0	8,00
$Ca(NO_3)_2 \cdot 4H_2O$	236,15	236,0	1,00
KH_2PO_4	136,09	204,0	1,50
$MgSO_4 \cdot 7H_2O$	246,37	246,0	1,00
H_3BO_3	61,83	0,31	0,05
$MnCl_2 \cdot 4H_2O$	197,84	2,57	0,13
$Na_2MoO_4 \cdot 2H_2O$	241,92	0,097	0,000 4
$FeCl_3 \cdot 6H_2O$	270,21	38,9	0,14
EDTA disodium-dihydrate	372,24	48,4	0,13
Glucose monohydrate		10 000	
Agar		9 000	

Table A.4 — Stock solutions (20-fold concentrated)

Stock solution	20-fold concentrated	g/l
1	KNO_3	16,18
1	KH_2PO_4	4,08
2	$MgSO_4 \cdot 7H_2O$	4,92
3	$Ca(NO_3)_2 \cdot 4H_2O$	4,72
		mg/l
4	H_3BO_3	6,20
4	$Na_2MoO_4 \cdot 2H_2O$	1,94
4	$MnCl_2 \cdot 4H_2O$	51,40
5	$FeCl_3 \cdot 6H_2O$	778
5	EDTA disodium-dihydrate	968

Prepare all solutions with dilution water (7.1) and autoclave 20 min at 121 °C.

All solutions can be stored for three months at a temperature of 4 °C ± 2 °C.

A.3.2 Preparation of the solid medium for axenic stock cultures

Add 50 ml each of stock solutions 1, 2, 3, 4, 5 to about 500 ml of dilution water (7.1).

Add 10 g of glucose monohydrate (7.4) and 9 g of agar (7.5).

Adjust with water (7.1) to 1 000 ml. Partition into final containers and treat in an autoclave at 121 °C for 20 min.

Addition of glucose is not obligatory but helps to detect contamination and allows the cultures to be kept at extremely low light conditions.

Stock-cultures may be maintained at low light conditions and ambient temperature for several months without re-inoculation if evaporation is minimised by covering the Erlenmeyer flasks with plug or cap and aluminium foil.