

DIN AK "Bioteste",

Vorlage für einen ISO/CEN Entwurf zum Lemnatest (ISO/WD 20079)

(Stand: 04.05.2001)

Water quality - Duckweed growth inhibition ; Determination of the toxic effect of water constituents and waste water to duckweed (*Lemna minor*)

General

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 *Arecidae*. Duckweeds are very fast growing higher plants, ranging in the world from tropic to the arctic zone. As primary producers they are a source of food for waterfowl, fish and small animals and serve as physical support for a variety of small invertebrates.

The test is designed for measurement of response of substances dissolved in water, a fixed dilution step, or a concentration of the test sample at which a parameter of calculation is inhibited relative to a control for a defined percentage.

1 Scope

The method should be used to determine the growth inhibiting response of substances and mixtures contained in water, as well as treated municipal and industrial effluents.

2 Normative Reference

The following standard contains dated and undated provisions from other publications. These normative provisions are quoted at the respective places in the text and the publications are subsequently cited. If provisions are dated, later changes or revised editions are only part of this standard if they are integrated by change or revision of it. If provisions are undated the last release of the quoted publication is valid.

EN ISO 5667-16	Water quality – Sampling – Part 16: Guidance on biotesting of samples
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3 Definitions

3.1 Terminology

Must is used to express absolute requirement, unless the purpose of the test requires a different design. *Must* is only used in connection with factors that directly relate to the validity of the test.

Should is used to state that the specific condition or procedure is recommended and ought to be met if possible. Although violation of one *should* is rarely a serious matter, violation of several will often render the results questionable.

May is used to mean „is (are) allowed to“.

Can is used to mean „is (are) able to“.

Might is used to express the possibility that something could exist or happen.

3.2 Test sample

The test sample derives from the sample (i. e. receiving water, waste water, chemical substances or mixtures , products and compounds,) by means of various preparatory steps specific to the sample and to the test, e. g. dissolving, filtering, neutralising.

3.3 Dilution water

Dilution water is added to the test sample to prepare a series of defined dilutions.

3.4 Nutrient medium

Solution of nutrients and micronutrients in water which are essential for the growth of duckweed.

3.5 Test medium

Test medium is the combination of test sample, dilution water and nutrient medium used in the test.

3.6 Test batch

The test batch is obtained by adding test medium and organisms used for testing.

3.7 Frond

Frond is an individual leaf-like structure on a duckweed colony. It is the smallest unit, (i. e. individual) capable of reproducing.

3.8 Colony

Colony means an aggregate of mother and daughter fronds, attached to each other.

3.9 Inoculum

The number of fronds added to the test batch at the beginning of the test.

3.10 Control medium

Control medium is the combination of dilution water and nutrient medium used in the test.

3.11 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*.

3.12 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.

3.13 Growth

Growth is the increase in biomass as the result of proliferation of new tissues. In this test it refers to the increase in any parameter of observation.

3.14 Observation parameters

Observed or measured biomass parameters like frond number, frond area, chlorophyll, dry weight, which are measured or counted once or multiply by observation or measurement. These parameters are relevant for the assessment of growth and vitality of the test organisms (e. g. frond number, frond area or contents of chlorophyll, dry weight).

3.15 Calculation parameters

Parameters for the estimation of toxicity derived from any parameters of observation by different methods of calculation. I. e. growth rates derived from frond number or frond area or chlorophyll are parameters of calculation in this standard.

3.16 Growth rate

Parameter of calculation defined as quotient of the difference of the natural logarithms of a parameter of observation and the respective time period.

If the time period comprises the total duration of the test the term is named 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 12.2.2).

3.17 Doubling time

* Use defined and varified strains, because of possible insecurities in species taxonomy. Address list of suppliers in Annex xx.

The doubling time is the ratio between $\ln 2$ and growth rate

3.17 Effect

An effect is represented as the inhibition of growth rate, calculated from an observation parameter (3.14) with reference to the control (see 10.7).

3.18 Effective concentration

Concentration of the test sample (EC_x) at which there is an effect of x % compared to the control.

To unambiguously denote an EC value, that is derived from growth rate it is proposed to use the symbol " E_rC ". To complete the information this term is followed by the observation parameter used, i. e. E_rC (frond number).

3.19 Frond number

According to this standard all fronds protruding from a mother frond which is directly visible without magnification from above.

3.20 Frond area

According to this standard the total area of all fronds which is visible from vertically above.

3.21 Chlorosis

Chlorosis is the loss of pigment (yellowing of frond tissue).

3.22 Necrosis

Necrosis is localised dead frond tissue (i. e. brown or white).

3.23 Limit test

Under the circumstances, e. g. when a preliminary test indicates that the test substance is non-toxic at concentrations up to 100 mg/L or up to its solubility in the test medium (whatever is lower), a limit test involving a comparison of responses in a control group and one treatment group (100 mg/L or a concentration equal to the limit of solubility).

3.24 Root

Root is that part of the *Lemna* plant that assumes a root-like structure.

3.27 Axenic cultures

Axenic cultures are monocultures of organisms from a single species. Cultures shall be free of bacteria, fungi, algae and other macrophyte species.

4 Principle

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

The performance of the test and the following evaluation of the results depends on the scope of application respectively.

For the determination of toxic effects of water constituents (e. g. chemicals, plant protection products) the assessment of EC values is necessary. The evaluation should be based on the average specific growth rates.

5 Interferences

Non soluble, poorly soluble or volatile substances or substances that react with the dilution water or the nutrient medium or change their state during the test, may falsify or reduce the reproducibility the results (see EN ISO 5667-16). Special consideration has to be taken for substances which are enriched at the water surface as this may intensify the effects on duckweed.

6 Facilities and apparatus

- cylindrical vessels (glass beakers, crystallising dishes, Petri dishes); a minimum volume of 150 ml is recommended (for 2/3 of total volume, i. e. 100 ml of test solution), Erlenmeyer flasks are not appropriate as a homogeneous illumination of the water surface is not possible
- uniform coverings made of glass
- facilities with constant temperature and lightning (temperature controlled room or water bath, incubator or environmental chamber)
- spectrophotometer to monitor chlorophyll (665 nm and 750 nm) (ISO 12600)
- (lumino)meter should be used to measure light intensity
- pH-meter
- tweezers
- glass ware for the preparation of concentration steps and nutrient medium (volumetric flasks, graduated cylinders, pipettes, Petri dishes)
- image analysis system to measure frond number and frond area

7 Reagents

Use only reagents of recognised analytical grade.

- Hydrochloric acid c (HCl); e. g. 0.1 mol/L
- Sodium hydroxide, c (NaOH); e.g. 0.1 mol/L

7.1 Dilution water

Reagent water such as distilled or deionised water or such of the same degree of purity and a conductivity of 10 µS/cm maximum must be used.

7.2 Nutrient media

For the assessment of waste water the modified Steinberg medium must be used.

Substance		Nutrient medium	
Macroelements	mol weight	mg/l	mmol/l
KNO ₃	101.12	350.00	3.46
Ca(NO ₃) ₂ *4H ₂ O	236.15	295.00	1.25
KH ₂ PO ₄	136.09	90.00	0.66
K ₂ HPO ₄	174.18	12.60	0.072
MgSO ₄ *7H ₂ O	246.37	100.00	0.41
Microelements	mol weight	µg/l	µmol/l
H ₃ BO ₃	61.83	120.00	1.94
ZnSO ₄ *7H ₂ O	287.43	180.00	0.63
Na ₂ MoO ₄ *2H ₂ O	241.92	44.00	0.18
MnCl ₂ *4H ₂ O	197.84	180.00	0.91
FeCl ₃ *6H ₂ O	270.21	760.00	2.81
EDTA Disodium-dihydrate	372.24	1500.00	4.03

For the assessment of water constituents (chemicals) modified Steinberg or if more appropriate SIS should be used (Annex A) For all tests the concentration of the respective nutrient medium must be kept at the same level in all treatments and controls.

7.3 Reference substance

3,5-Dichlorophenole (reagent grade)

8 Test organisms

Lemna minor ST (with a documentation of origin) is recommended for use. If plants are obtained from a wild population, their taxonomy must be confirmed.

9 Stock- and pre-cultures

9.1 Stock cultures

Stock cultures should be 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).

9.2 Pre-cultures

Cultures used for toxicity tests should be initiated at 7 to 10 days prior to the test using test medium and test conditions.^{*1}

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

- Growth near to exponential
- The number of fronds in the pre-culture should have increased by ≥ 8 -fold by the end of 7 days (i. e. $\mu \geq 0,275 \text{ d}^{-1}$ or doubling time ≤ 2.5 days)
- 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 indicative for environmental stress.

To minimise lag-phases caused by interactions between colonies it should be assured that the covering should be less than 50 % of the total available surface (no crowding). All colonies used should originate from the same pre-culture. It is recommended to change the nutrient medium 1 to 2 days before a test.

10 Procedure

The general recommendations according to EN ISO 5667-16 should be followed.

The pH value of the test sample should be adjusted $\pm 0,2$ units to the pH value of the nutrient medium in the control by adding HCl or NaOH. Dilution should be kept at a minimum level. No later adjustment should be made.^{*2}

Neutralisation should be omitted if the effect of pH to be reflected in the test results or if physical modification or chemical reaction is observed due to pH adjustment.

Note: Duckweed has generally no growth problems between pH 5 and 9. Therefore adjustment of pH may be omitted if pH of the sample is between 5 and 8 depending on buffer capacity.

10.1 Preparation of concentration series for E_rC_x value assessment

If the E_rC_x is to be estimated a sufficient number of concentrations has to be used to define the E_rC_x at an appropriate level of confidence. An appropriate test design consists of a geometric series of at least 5 concentrations. At least one measured inhibition value for the intended E_rC_x calculation parameter should be below and above the E_rC_x to be estimated and three or more values should be other than 0 or 100 % inhibition. Otherwise confidence limits might be too large.

10.2 Test

^{*1} Note: Longer adaptation-time may be required in case of change of the nutrient medium between stock- and pre-culture.

^{*2} Note: Duckweed has generally no growth problems between pH 5 and 9. Therefore adjustment of pH may be omitted if pH of the sample is between 5 and 8 depending on buffer capacity.

10.2.1 Test for E_rC_x

At least 3 replicates for the treatments and 6 replicates for the controls should be used at each concentration for E_rC_x assessment (see also EN ISO 5667-16).

10.2.2 Test for $E_rC_{<20}$

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

10.2.3 Limit test

At least 6 replicates for the limit concentration and the controls should be used.

10.2.4 Use of solvents or dispersants

If solvents or dispersants may not be avoided (at a maximum concentration of 0.1 mL/L or 100 mg/L resp.) an additional control with 6 replicates including the solvent or chemical at the same concentration as in all replicate vessels must be used. Solvents or dispersants should be non-toxic at the concentration chosen.

10.2.4 Test with reference substance

3,5-Dichlorophenol

10.3 Number of fronds at the start of the test

At least 10 fronds (2 or 3 fronds per plant) of similar size satisfying the pre-culture conditions must be used as inoculum for each test vessel. To provide a random inoculation it is recommended to place one of the pre-selected colonies in each test vessel and continuing this process until each test vessel contains the required number of fronds.

10.4 Temperature

Temperature should be in the range of at $24\text{ °C} \pm 2\text{ °C}$ during the test and should be maintained at $\pm 1\text{ °C}$ in all vessels. It must be monitored at least at the four observation times but continuous temperature control is recommended. If temperature records are based on measurements other than in the test vessels the relationship must be established.

10.5 Light

Neutral white illumination should be used. Light intensity should be $85\text{-}125\ \mu\text{E m}^{-2}\text{ s}^{-1}$ (400-700 nm) at the level of water in the test vessels. Light intensity should be measured once per test in at least 5 characteristic points of the test area in a realistic test environment and should not vary by more than $\pm 15\%$ of the selected light intensity. Light from the side and bottom must be excluded by test design, i. e. black side covers and a black bottom. Measuring light with a spherical head quantifies all light that would reach the plants if the test solution is clear.

Note:

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 nutrient medium. The coefficient of variation of growth rate should be smaller than 10 %.

10.6 Test duration

The test duration is 168 h (± 2 h).

10.7 Measurements and observations

As basic parameter the frond number must be measured. In addition a second observation parameter (frond area, dry weight or chlorophyll) has to be measured obligatory.

Qualitative observation of any visual sign of phytotoxicity should be recorded for each test vessel at the end of the test as follows: abnormally sized fronds, root length and destruction, local or size specific chlorosis or necrosis, loss of buoyancy, break-up of colonies. Changes of the test medium, bacterial contamination and any other relevant changes have to be recorded as well.

For the observation the test vessels are placed on a white background and light should be directed from the side or from the bottom into the test vessel.

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

Chlorophyll can be quantified according to ISO 12600.

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

Dry weight can be quantified after drying blotted fronds and roots at 60 °C up to constant weight.

10.8 Sequence of measurement

All observation parameters chosen for measurement must be quantified at the start and the end of the test. For the initial measurement of chlorophyll and dry-weight 6 additional controls shall be inoculated. Controls must be sacrificed randomly for chlorophyll measurement. The frond must be measured at least each 48-72 hours.

11 Validity

Assuming that all recommended procedures and conditions were followed, the mean number of fronds in the control must have increased at least 8-fold by the end of the test. This corresponds to a doubling time of 2.5 days and an average specific growth rate of 0.275 d^{-1} .

3,5-Dichlorophenol was used as reference substance.

$E_r C_{50}$ (frondnumber) should be in a range between 1.8 and 3.6 mg/L.

12 Interpretation and validity of results

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

12.1.1 General

Growth rate and growth inhibition values of each replicate, mean values and standard deviations must be tabled for all observation parameters.

Even if a solvent or dispersant has been used the medium control remains reference for calculating inhibition. Data for solvent control must not be pooled with those for the control even if there is no significant difference between the two data sets.

Data of the solvent or dispersant control are used to demonstrate non-toxic effect of these substances (see also EN ISO 5667-16).

Data from observation parameters measured several times should be plotted as growth curves (natural logarithm of the observation parameter versus time), two point measurements as concentration-effect curve (observation parameter versus log concentration).

The calculation parameters and inhibition values will be calculated from the following formulas for each treatment. Mean values and coefficients of variation of the replicates are plotted as concentration-response curves (Inhibition against log concentration).

12.2.2 Estimation of growth rate

Growth rate r will be calculated by the following formula:

$$r = \frac{\ln x_{t_2} - \ln x_{t_1}}{t_2 - t_1}$$

x_{t_1} value of observation parameter at t_1

x_{t_2} value of observation parameter at t_2

$t_2 - t_1$ period between x_{t_1} and x_{t_2}

Mean values are only used as basic data if the parameter was measured destructively. In all other cases the individual value of the replicate should be used.

The growth rate should be calculated for the entire test period, or a rationale for selecting only a portion of the growth curve provided. For each test concentration and

control, a mean average specific growth rate should be calculated with variance estimates.

Percent inhibition of growth rate I_{μ} may then be calculated for each test concentration according to the following formula:

$$I_{ri} = \frac{rc - ri}{rc} * 100$$

I_{ri} percent inhibition in mean average specific growth rate

rc mean average specific growth rate of the control

ri mean average specific growth rate of the treatment group i

12.2 Assessment of test validity

For all control vessels single values, mean values and coefficients of variation for quantified parameters should be tabled to calculate:

- average specific growth rate
- doubling time
- multiplication factor within 7 days.

For the control of exponential growth the mean value and coefficients of variation of all observation parameters measured several times are tabled and the mean values are plotted as

- growth curves (\ln (observation parameters) versus time) or
- segmented growth rates (r versus time).

13.2.4 Estimation of E_rC_x values

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

Note:

Using non-linear regression can help to deal with tests, where at high concentrations after an initial growth the further growth stopped. As a consequence - almost independently of concentration - inhibition reaches only values of 70 –90 %.

14 Documentation of results

The values calculated for E_rC_1 , E_rC_5 , E_rC_{10} , E_rC_{25} , E_rC_{50} and E_rC_{90} (as far as possible) and the corresponding confidence intervals (95 %) will be displayed with a maximum of two significant numbers. The response of the mass concentration of the tested water constituents or chemicals should be displayed graphically, mathematically or be tabled (see EN ISO 5667-16).

15 Test report

The following data should be recorded in the test report:

- a) name of the laboratory performing the test;
- b) date and period of test;
- c) reference to the test method (international standard number) used;
- d) test organism (e.g. scientific name, strain, source), therapeutic or acclimatory pretreatment (if any);
- e) designation of test material (batch number, origin, date and period of sampling);
- f) sample pretreatment (e.g. preservation, preconcentration, homogenisation, pH adjustment, type of neutralizing agent, pre-aeration);
- g) data, derived, condensed or transformed, including, if appropriate, results of positive controls (reference batch);
- h) chemical and physical data determined during the test (e.g. temperature, CO₂-content, pH, turbidity, precipitation, possible change in substance concentration, etc.);
- i) 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);
- j) method of estimating EC values evaluation;
- k) details of the test results;
- l) comments on the test results, if necessary;
- m) signature of responsible investigator;
- n) signature of quality controller, if appropriate.

16 Test performance

3,5-Dichlorophenol was used as reference substance.

E_rC₅₀(frondnumber) was in a range between 1.8 and 3.6 mg/L.

Annex A

STEINBERG medium (modified)

The nutrient medium has to be prepared from single solutions. The required concentrations of pre-culture and test medium can be obtained by dilution

Table: pH-stabilised STEINBERG medium (modified ref. to Altenburger)

Substance		Nutrient medium	
<i>Macroelements</i>	mol weight	mg/l	mmol/l
KNO ₃	101.12	350.00	3.46

Ca(NO ₃) ₂ *4H ₂ O	236.15	295.00	1.25
KH ₂ PO ₄	136.09	90.00	0.66
K ₂ HPO ₄	174.18	12.60	0.072
MgSO ₄ *7H ₂ O	246.37	100.00	0.41
Microelements	mol weight	µg/l	µmol/l
H ₃ BO ₃	61.83	120.00	1.94
ZnSO ₄ *7H ₂ O	287.43	180.00	0.63
Na ₂ MoO ₄ *2H ₂ O	241.92	44.00	0.18
MnCl ₂ *4H ₂ O	197.84	180.00	0.91
FeCl ₃ *6H ₂ O	270.21	760.00	2.81
EDTA Disodium-dihydrate	372.24	1500.00	4.03

Stock solutions:

1. Macroelements (50-fold concentrated)	g/l
<u>Stock solution 1:</u>	
KNO ₃	17.50
KH ₂ PO ₄	4.5
K ₂ HPO ₄	0.63
<u>Stock solution 2:</u>	
MgSO ₄ *7H ₂ O	5.00
<u>Stock solution 3:</u>	
Ca(NO ₃) ₂ *4H ₂ O	14.75

2. Microelements (1000-fold concentrated)	mg/l
<u>Stock solution 4:</u>	
H ₃ BO ₃	120.0
<u>Stock solution 5:</u>	
ZnSO ₄ *7H ₂ O	180.0
<u>Stock solution 6:</u>	
Na ₂ MoO ₄ *2H ₂ O	44.0

<u>Stock solution 7:</u>	
MnCl ₂ *4H ₂ O	180.0
<u>Stock solution 8:</u>	
FeCl ₃ *6H ₂ O	760.00
EDTA Dinatriumsalz-dihydrat	1500.00

Stock solution 2+3 and 4 to 7 may be pooled

Preparation of nutrient solution (final concentration):

- 20 ml of stock solution 1, 2 and 3
- 1,0 ml of stock solution 4, 5, 6, 7, 8 and 9
- aqua bidest ad 1000 ml
- autoclavation (121°C, 20 min.) or sterile filtration (0.2 µm)

Preparation of 10fold-concentrated testmedium:

- 20 ml of stock solution 1, 2 and 3
- 1,0 ml of stock solution 4, 5, 6, 7, 8 and 9
- aqua bidest ad 100 ml
- autoclavation (121°C, 20 min.) or sterile filtration (0.2 µm)

The pH-value of the medium should be 5,5.

Table A 2: OECD, 1998 – Culture and Test media for *Lemna minor* (SIS growth medium)bb

Substance	Concentration		Element	Stock Solution
	Stock Solution (g/l)	Medium ^a (mg/l)		
MgSO ₄ ·7H ₂ O	15	75	NI ^b	II
NaNO ₃	8.5	85	NI	I
CaCl ₂ ·2H ₂ O	7.2	36	NI	III
Na ₂ CO ₃	4.0	20	NI	IV
KH ₂ PO ₄	1.34	13.4	NI	I
H ₃ BO ₃	1.0	1.0	NI	V
MnCl ₂ ·4H ₂ O	0.2	0.2	NI	V
Na ₂ MoO ₄ ·2H ₂ O	0.010	0.010	NI	V
ZnSO ₄ ·7H ₂ O	0.050	0.050	NI	V
CuSO ₄ ·5H ₂ O	0.005	0.005	NI	V

Co(NO ₃) ₂ ·6H ₂ O	0.010	0.010	NI	V
Na ₂ EDTA	0.28	1.4	NI	VI ^c
FeCl ₃ ·6H ₂ O	0.168	0.84	NI	VI ^c
MOPS (buffer)	488	488	NI	VII ^c

pH Adjustment

pH adjust to 6.5 ± 0.2 by addition of NaOH or HCl.

Sterilization

Stock solutions I to V are sterilized by autoclaving (120°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 NI = Not indicated.

^c Added after autoclaving.

^d 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).

cited from: Report EPS 1/RM/37; Dec. 1998,

Biological Test Method: Test for Measuring the Inhibition of Growth using the Freshwater Macrophyte, *Lemna minor*

Method Development and Application Section

Environmental Technology Centre

Environment Canada

Ottawa, Ontario

Annex B

(informative)

Measurement of waste water with lowest ineffective Dilution (LID)

In toxicity testing of waste water by means of defined dilutions (D), the lowest ineffective dilution (LID) expresses the most concentrated test batch at which no inhibition, or only effects not exceeding the test-specific variability, had been observed. D is expressed as the reciprocal value of the volume fraction of waste water in the test batch.

Example: $\frac{1}{4}$ waste water (volume fraction of 25 %) is dilution level $D = 4$.

Defined dilution D

D is expressed as the reciprocal value of the volume fraction of waste water in the test batch. If only the concentrated nutrient medium is added to the test sample this dilution is defined as D1.

Lowest Ineffective Dilution (LID)

In toxicity testing of waste water by means of defined dilutions (D), the lowest ineffective dilution (LID) expresses the most concentrated test batch at which no inhibition, or only effects less than 10 % inhibition of growth rate, are observed.

Principle

Duckweed may be damaged by water constituents and effluents. The subsequent inhibition of growth should be calculated from the observation parameters (frond number, frond area, chlorophyll) by a number of defined calculation methods.

The performance of the test and the following evaluation of the results depends on the scope of application respectively:

For the determination of the non-toxic effect of waste water on the growth of duckweed the assessment of D values is prescribed. Waste water will be diluted according to a defined scheme of dilutions. The evaluation of results should be based on the average specific growth rate. A toxic effect is assessed, if the fixed level of 10

% growth inhibition relative to the control is surpassed by the most sensitive observation parameter at the end of the test.

Preparation of dilutions for LID assessment

At first, all test vessels are filled with the identical volume of concentrated nutrient medium. Dilution water and subsequently sample volumes are added referring to table 1. The order of addition must be kept to minimise precipitation. The volumes listed in table 1 derive from the recommended minimum test volume of 100 ml and must be multiplied for higher volumes.

Controls must be prepared analogously without test sample.

A growth inhibition of >10% compared to control is defined as statistical significant. The lowest ineffective dilution (LID) where all calculation parameters are not extending the 10% inhibition level, has to be reported as D value.

Table 1: Scheme for the defined dilutions according to LID-values

Nutrient medium concentrate:	10-fold
Final volume:	100 ml

D-value	dilution	Test sample	Nutrient med. conc.	Dilution water
	l/l	ml	ml	
1	0,9*	90,00	10	0,00
2	0,5	50,00	10	40,00
3	0,3333	33,33	10	56,67
4	0,2500	25,00	10	65,00
6	0,1667	16,67	10	73,33
8	0,1250	12,50	10	77,50
12	0,0833	8,33	10	81,67
16	0,0625	6,25	10	83,75
24	0,0417	4,17	10	85,83
32	0,0313	3,13	10	86,87
48	0,0208	2,08	10	87,92
64	0,0156	1,56	10	88,44
96	0,0104	1,04	10	88,96
128	0,0078	0,78	10	89,22
192	0,0052	0,52	10	89,48
256	0,0039	0,39	10	89,61
384	0,0026	0,26	10	89,74
512	0,0020	0,20	10	89,80

D-value	dilution	Test sample	Nutrient med. conc.	Dilution water
	l/l	ml	ml	
768	0,0013	0,13	10	89,87
1024	0,0010	0,10	10	89,90

*The lowest possible dilution caused by adding of nutrient medium is defined as $D = 1$

Test for LID

At least 3 replicates for the treatments and 6 replicates for the controls must be used at each concentration for LID testing.

Assessment of results – LID value, effluents

From a series of defined dilutions the LID has to be determined where the respective growth calculated as the average specific growth rate is reduced less than 10 % compared to control.

Documentation of results

14.1 Waste water

Only full numbers of D-values are reported, e.g. $D_w = 2$.

Literature