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Water quality — Determination of the toxic effect of sediment on the growth behaviour of *Myriophyllum aquaticum*

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National foreword

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Water quality — Determination of the toxic effect of sediment on the growth behaviour of *Myriophyllum aquaticum*

Qualité de l'eau — Détermination de l'effet toxique des sédiments sur la croissance de Myriophyllum aquaticum

Reference number
ISO 16191:2013(E)

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ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2, www.iso.org/directives.

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The committee responsible for this document is ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Introduction

The contact test with *Myriophyllum aquaticum* described in this International Standard allows the measurement of responses of the plant to dissolved and particle-bound substances present in sediment samples within 10 d (References [3][4][5][6][7][8]).

The test plant, *Myriophyllum aquaticum* (Vellosco) Verdcourt (parrot feather), is a dicotyledonous macrophyte. It is a native of the Amazon River in South America, but it has naturalized worldwide, especially in warmer climates. It has been introduced worldwide for use in indoor and outdoor aquaria. For its use as test organism, its capability for emersed growth (no additional liquid as supernatant is needed), its strong regeneration potential, and its vegetative growth are harnessed in the contact test. Furthermore, *Myriophyllum aquaticum* grows without generating side shoots during the test period, which facilitates handling in the laboratory. However, it should be ensured that no live plant material is lost from the laboratory.

Myriophyllum aquaticum can be affected by phytotoxic substances present in sediments (e.g. dredged material). The subsequent inhibition of growth is calculated from the parameter (fresh mass) by a number of defined calculation methods.

INTERNATIONAL STANDARD ISO 16191:2013(E)

Water quality — Determination of the toxic effect of sediment on the growth behaviour of *Myriophyllum aquaticum*

WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This International 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. It shall be ensured that no plant material can elude the laboratory.

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 determining the toxicity of environmental samples on the growth of *Myriophyllum aquaticum*. The method described is applicable to natural fresh water sediment and artificial sediment.

2 Normative references

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

ISO 5667-15, *Water quality — Sampling — Part 15: Guidance on the preservation and handling of sludge and sediment samples*

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

ISO 10523, *Water quality — Determination of pH*

ISO 11465, *Soil quality — Determination of dry matter and water content on a mass basis — Gravimetric method*

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

OECD 218, *OECD Guidelines for the testing of chemicals — Sediment-water Chironomid toxicity test using spiked sediment*

3 Terms and definitions

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

3.1
artificial sediment
defined artificial sediment

[SOURCE: ISO 10872:2010,[2] definition 3.3, modified]

Note 1 to entry: See 6.9.

3.2
chlorosis
loss of pigments (yellowing of plant tissue)

[SOURCE: ISO 20079:2005, definition 3.3, modified]

3.3
control sample
artificial sediment pre-treated according to the need of this test that serves as negative control to which the effect in the respective test material is compared

[SOURCE: ISO 10872:2010,[2] definition 3.6, modified]

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

[SOURCE: ISO 20079:2005, definition 3.9, modified]

Note 1 to entry: To unambiguously denote an EC value deriving from growth rate, it is proposed to use the symbol "E_rC_x".

3.5
emersed growth
morphological habitus of aquatic macrophytes, growing above the water surface

3.6
head-whorl
apical part of a *Myriophyllum* plant

Note 1 to entry: See Figure A.1.

3.7
necrosis
localized dead plant tissue (i.e. brown or white)

[SOURCE: ISO 20079:2005, definition 3.16, modified]

3.8
nutrient solution
solution of nutrients and micronutrients in water which are essential for the growth of *Myriophyllum*

[SOURCE: ISO 20079:2005, definition 3.17, modified]

3.9
pre-culture
culture *of Myriophyllum aquaticum* used for acclimatization of test plants to the test conditions and for the growing of the plants to be used as whorls at test start

[SOURCE: ISO 20079:2005, definition 3.19, modified]

Note 1 to entry: See Figure A.2.

3.10
replicate
one of a selected number of test vessels (containing sample material from one sample and test organisms)

Note 1 to entry: Each vessel is tested.

Note 2 to entry: The replicates mentioned in this International Standard contain sample material (e.g. natural sediment) and three whorls of *Myriophyllum aquaticum.*

3.11
test sample
discrete portion of a sample (e.g. sediment or artificial sediment)

[SOURCE: ISO 10872:2010,[2] definition 3.14, modified]

3.12
whorl
arrangement of leaves that radiate from a single point and surround the stem

Note 1 to entry: See Figure A.1.

4 Principle

Myriophyllum aquaticum whorls are exposed to test samples over a period of 10 d. The growth of *Myriophyllum aquaticum* in a test sample is compared with its growth in the control sample. Phytotoxic effects are quantified as growth inhibition (%) relative to the control growth.

5 Interferences

In case of problems with *Myriophyllum* control growth using artificial sediment, the respective components should be checked, first to exclude contamination with, for example, heavy metals (kaolin) or suitability of peat (if the recommended peat is not used).

6 Reagents

Use, as far as possible, reagents of recognized analytical grade.

6.1 Water, distilled or deionized water or water of equivalent purity, conductivity < 10 µS/cm.

6.2 Kaolin clay, kaolin powder (CAS RN 1332-58-7).

6.3 Calcium carbonate, $CaCO_3$ powder (CAS RN 471-34-1).

6.4 Quartz sand, average grain size 170 µm (see Annex C).

6.5 Reference substance, 3,5-dichlorophenol [$C_6H_4OCl_2$ (purity ≥ 99 %), CAS RN 591-35-5].

6.6 Nutrient solution, use Steinberg medium as specified in Annex B.

6.7 Peat, Sphagnum peat (e.g. Lithuania peat), H2-H5, fine (grain size ≤5 mm) (see Annex C).

6.8 Peat powder, dry peat (6.7) for 7 d at room temperature.

Spread the peat on shallow trays, and turn the peat every 2 d to 3 d. Then grind the peat and sieve it through a 0,5 mm sieve. Determine dry mass of the peat powder by drying a small sub-sample at 60 °C for 3 h in four aliquots, and determine the dry mass by re-weighing until constant mass (see ISO 11465). Store the peat powder in airtight vessels until use. Note down the dry mass on the vessel.

6.9 Artificial sediment, see Table 1

Table 1 — Dry constituents for composition of the artificial sediment

Constituents	% of sediment dry mass	Characteristics
Peat powder	5	see 6.8
Quartz sand	74	average grain size 170 µm
Kaolin clay	20	powder
$CaCO_3$ powder	1	pro analysis

6.9.1 Artificial sediment as control sample.

Preparation of the artificial sediment as control sediment is described below (6.9.1.1 to 6.9.1.3). The dry constituents for preparation of the artificial sediment may be stored separately in closed, airtight vessels in a dry and dark place at room temperature for at least 6 months.

NOTE These requirements (6.9.1.1 to 6.9.1.3) are carried out for establishing stable ambient conditions in the sediment and avoiding separation of the sediment components during the test.

6.9.1.1 Preparation of peat suspension.

Take the required amount of peat powder (6.8, Table 1) and $CaCO_3$ powder (see Table 1) and add nutrient solution (6.6) until the suspension can be stirred easily (at maximum 50 % of total sediment dry mass). Stir carefully. Keep the peat suspension for 3 d to 4 d with continuous gentle stirring at room temperature to stabilize pH. Afterwards, measure pH of the suspension and adjust if necessary to 6,7 ± 0,5 by adding $CaCO_3$ powder.

NOTE Experience has shown that the pH is at 6,7 ± 0,5; therefore, no further addition of $CaCO_3$ is usually necessary.

6.9.1.2 Addition and blending of sediment components.

Mix the peat suspension with the other constituents (quartz sand and kaolin clay, see Table 1) to obtain a homogeneous sediment. Measure the pH of the final mixture again and adjust it to 7,0 ± 0,5 by adding $CaCO_3$ powder if necessary.

NOTE Experience has shown that the pH is at 7,0 ± 0,5; therefore, no further addition of $CaCO_3$ is usually necessary.

6.9.1.3 Conditioning of the mixed sediment suspension.

Add nutrient solution (6.6) as supernatant to the mixed sediment (6.9.1.2) in the ratio: 1 part (mass) mixed sediment plus maximum 0,5 parts (volume) nutrient solution. Condition this mixture for 7 d to 9 d under exposure conditions (see 10.5) to establish a stable microbial component and to avoid separation of the sediment components during the test. Remove the supernatant of nutrient solution carefully after 7 d to 9 d. The sediment is ready for instant use.

NOTE Experience has shown that the artificial sediment can be stored at 4 °C to 8 °C in the darkness for 14 d.

6.9.2 Artificial sediment for pre-culturing.

Mix the dry sediment constituents (see Table 1) and shake the dry mixture for 2 h to 3 h in a rotary shaker at room temperature. The dry sediment powder mixture can be maintained without restraint in an airtight vessel in a dark and dry place at room temperature.

Measure 125 g dry sediment powder mixture into a 1 000 ml pre-culture vessel, add (65 ± 5) ml nutrient solution (6.6), and stir carefully until it is homogeneous. The sediment suspension should be muddy.

Condense the sediment by knocking the vessels on the table to eliminate air bubbles and cavities within the sediment matrix.

NOTE Growing of the pre-cultures (see 10.1) in artificial sediment for control samples (6.9.1) or in artificial sediment for pre-culturing (6.9.2) has no significant influence on the results of a subsequent test; therefore, both artificial sediments (6.9.1 and 6.9.2) are suitable as pre-culturing sediment.

7 Apparatus

Usual laboratory equipment and, in particular, the following:

7.1 Autoclave.

7.2 Cylindrical or conical vessels, plastic or glass beakers, crystallizing dishes [e.g. for the pre-culture: diameter bottom 10 cm, diameter top 13 cm, height 11 cm; for the test: 250 ml glass beakers, low form; see Figures A.2 b) and A.3 b)].

7.3 Drying oven, approximately 105 °C.

7.4 Temperature-controlled incubator with constant illumination, e.g. climate chamber.

7.5 Light meter, to measure photosynthetically active radiation (PAR), within the photosynthetic range 400 nm to 700 nm with a spherical quantum sensor.

7.6 pH meter.

7.7 Precision balance, required accuracy of 0,1 mg.

7.8 Rotary shaker.

7.9 Scalpel or scissors.

7.10 Sieve, stainless steel, mesh size 0,5 mm.

7.11 Translucent lids, glass or plastic, with openings (e.g. holes) to allow air and humidity exchange [see Figures A.2 b) and A.3 b)].

7.12 Tweezers.

7.13 Stirrer.

7.14 Glass electrode, to measure pH values of aqueous solutions and sediments.

7.15 Grinder, to pulverize peat after drying (e.g. blender).

7.16 Mortar, to homogenize sediments after drying (see Annex D).

7.18 Fume hood (see Annex D).

8 Test with reference substance

To ensure that the laboratory test conditions (including the condition and sensitivity of the test organisms) are adequate and have not changed significantly, a reference substance has to be tested regularly, at least every 6 months, using one concentration near its EC_{50} for growth rate. A suitable reference substance is 3,5-dichlorophenol (DCP), which has been shown to affect the growth of *Myriophyllum aquaticum*.

DCP is tested in artificial sediment according to instructions for spiking (see Annex D) and testing (see Clause 10). The inhibition of growth at a concentration of 90 mg/kg dry mass of artificial sediment compared to the control growth should be in the range of 20 % to 50 %.

NOTE The range of inhibitory effects for 3,5-dichlorophenol is based on the data of the international interlaboratory test of this International Standard (see Annex E).

9 Test organism

Myriophyllum aquaticum (Vellosco) Verdcourt (source of supply, see Annex C) is used as test species in this International Standard. Documentation of its origin is needed. Plants obtained from a wild population need a confirmation of their taxonomy.

10 Procedure

10.1 Pre-culturing of *Myriophyllum aquaticum* for the contact test

Use artificial sediment for pre-culturing (6.9.2). Plant the fresh-cut head-whorls from 5- to 7-week old cultures into the pre-culturing sediment. Close the vessels with translucent lids with openings for aeration and humidity exchange. Incubate the plants in a climate chamber at (24 ± 1) °C at a light intensity of 60 µmol m^{-2} s^{-1} to 75 µmol m^{-2} s^{-1} or 4 100 lx to 5 550 lx (neutral white) with constant light regime. Add nutrient solution (6.6) diluted with equal volumes of water (6.1) to avoid desiccation, and randomize the cultures at least every 3 d to 4 d. After one week, remove the lids to allow the plants to grow without hindrance.

Planting of seven to nine head-whorls per pre-culture vessel (7.2, conical, Ø bottom 10 cm, Ø top 13 cm, height 11 cm) and many pre-culture vessels per test are recommended. The length and the thickness of the stem have influence on the mass of the whorl. To have mostly homogeneous whorls for the test, it is important to have much plant material to select whorls from for the test.

When irrigating, take care that new planted head-whorls do not float out of the sediment. Nutrient solution diluted with water is carefully added until obtaining a thin layer of liquid at the sediment surface. After a few days, when the head-whorls are rooted, more liquid (e.g. 0,5 cm height) can be added.

NOTE Any other pre-culture method is possible as long as pre-cultures are growing emersed, using growth conditions comparable to test conditions and the validity criteria (growth rate ≥ 0,09, variation in the control ≤ 15 %) is met.

10.2 Preparation of the control sample

Use artificial sediment (6.9.1) as control sample. Measure and record the pH. Fill at least three replicate vessels with 80 g of freshly prepared control sample each. Before using the replicates, condense the sediment by knocking the vessels on the table.

10.3 Sampling, storage, and preparation of the test samples

10.3.1 Sampling and storage of the test sample

Examine the material to be tested as soon as possible after sampling. Collect samples as specified in ISO 5667-16 and ISO 5667-15, and store them in the dark at a temperature of 2 °C to 5 °C for not more than two weeks.

Determine the pH of the test material and control sample in accordance with ISO 10523 (aqueous test material, sediments).

NOTE Determination of pH according to ISO 10390[1] is also possible.

10.3.2 Preparation of the test sample

Use natural or artificial sediment as the test sample. The sediments can be used in their original state, or artificial sediments can be spiked with chemicals (spiking procedure is specified in Annex D). Stir each sample thoroughly and record the pH. Fill at least three replicate vessels per test sample with 80 g (wet mass) of test sample each. Further addition of nutrient solution (6.6) to test samples is only necessary for samples where there is no supernatant (1 mm to 2 mm) after packing of the sample.

NOTE The nutrient supply in natural sediments usually is sufficient for plant growth.

10.4 Test procedure

At the start of the test, mark the positions of the three whorls on each test vessel. Use plants from (21 ± 3) d old pre-cultures and cut them into the whorls needed for testing (2 to 4 maximum per shoot, each whorl with five to six leaves; see Figure A.1). The whorls used for the test shall show no signs of side shoots. Collect the cut whorls in a glass vessel in nutrient solution (6.6) for randomization. Before transferring three whorls into the replicate, handle each whorl step by step as follows: Dry the whorl carefully (bottom side up) on tissue paper in a way that ensures the reduction of surplus water without damaging the whorls. Weigh it immediately on a precision balance and record the mass. The mass of each whorl should be (25 ± 6) mg. Transfer this whorl (top side up) immediately to one of the pre-marked positions (1 to 3) of the replicate (see Figure A.3).

This allows a comparison of the fresh mass of the test plants reached at the end of the exposure time to the initial mass of the whorls at the beginning of the test. Once the whorls are introduced into the replicates, close the vessels with translucent lids with openings for aeration and humidity exchange. During the exposure period, irrigate and randomize the control and test samples every 24 h to 72 h. Use nutrient solution (6.6) diluted with equal volumes of water (6.1) for irrigation.

When irrigating, take care that newly planted whorls do not float out of the sediment. Nutrient solution diluted with water is carefully added until obtaining a thin layer of liquid at the sediment surface. Daily randomization is recommended.

NOTE The range of (25 ± 6) mg as whorl mass is recommended.

10.5 Exposure conditions

The test vessels shall be sufficiently covered by translucent lids to reduce water evaporation, but they shall not be airtight in order to allow air exchange into the vessels. Incubate the test vessels under controlled standardized conditions at (24 ± 1) °C, under continuous lighting (neutral white), for 10 d. Measure photosynthetically active radiation (PAR) with a spherical quantum sensor. Light intensity at the sample level in and among the test vessels shall be homogeneous within the range of 60 µmol m^{-2} s^{-1} to 75 µmol m^{-2} s^{-1} (400 nm to 700 nm) or 4 100 lx to 5 550 lx.

Random positioning of test vessels within the incubator with re-randomization is recommended, but does not compensate for high deviations of light intensity and temperature between different places of the test area.

NOTE Randomization can be achieved by using a turntable (1 turn/min).

10.6 Measurements

10.6.1 Visual observation of the plants and pH control

Determine and record physiological adverse effects, such as necrosis or chlorosis, and morphological changes for each plant per replicate at the end of the test. Determine and record the pH of the samples at the end of the test.

10.6.2 Fresh mass of the whole plant

Determine and record fresh mass of each plant at the beginning (whorls, 10.4) and the end (whole plant, including roots) of the test. To measure the final fresh mass of the test plants, pick them carefully from the test samples with tweezers. Wash the plants cautiously, dry them carefully on tissue paper without damaging the whorls, and weigh them immediately to ensure that the results are not impaired by drying of the plant material.

10.6.3 Inactivation of the test plants

After finishing the test, inactivate the plants by an adequate procedure (e.g. by letting them desiccate for 7 d at room temperature or by thermal treatment).

Table 2 — Example for the timetable of the test

Day	Procedure
−21	1. Pre-culturing of *Myriophyllum aquaticum* (see 10.1)
−21	1.1 Preparation of artificial sediment for the pre-culture (6.9.2)
−21	1.2 Transfer of head-whorls into the artificial sediment for pre-culturing (see 10.1)
−11	2. Preparation of artificial sediment as the control sample (6.9.1)
−11	2.1. Preparation of the peat suspension (6.9.1.1)
−8	2.2 Addition and mixing of sediment components (6.9.1.2)
−8 to 0	2.3 Conditioning of the artificial sediment as control sample (6.9.1.3)
0	3. Preparation of the test sample (see 10.3)
0	4. Beginning of the test [d0, weighing of whorls, transfer of whorls into the vessels, incubation at exposure conditions (see 10.4 and 10.5)]
10	5. End of the test [d10, measurement (weighing of whole plants) and visual observation (see 10.6)]

11 Evaluation

11.1 Growth rate *r*

Calculate the growth rate for each plant from the measured fresh mass using Formula (1):

$$r = \frac{\ln m_{t_2} - \ln m_{t_1}}{t_2 - t_1} \quad (1)$$

where

r is the growth rate per day;

m_{t_1} is the fresh mass of a single plant at the time of measurement, t_1;

m_{t_2} is the fresh mass of a single plant at the time of measurement, t_2;

t is the time in days, d;

$t_1 - t_2$ is the time period between t_1 and t_2 in days, d.

11.2 Means per test and control vessel ($r_{V,T}$, $r_{V,C}$)

Calculate the arithmetic mean of the growth rates per test vessel ($r_{V,T}$) and control vessel ($r_{V,C}$). Note that the vessel represents the statistical replicate and not the single plant (see ISO/TS 20281[9]).

11.3 Means per test and control sample ($r_{S,T}$, $r_{S,C}$)

Calculate the mean growth rates per test sample ($r_{S,T}$) and control sample ($r_{S,C}$) as well as its standard deviation (SD), and the coefficient of variation (CV, %).

11.4 Inhibition I

Calculate the results as % inhibition I from the sample means, $r_{S,T}$, $r_{S,C}$, using Formula (2):

$$I(\%) = \frac{(r_{S,C} - r_{S,T})}{r_{S,C}} \times 100 \quad (2)$$

where

I is the growth inhibition;

$r_{S,C}$ is the mean growth rate per control sample;

$r_{S,T}$ is the mean growth rate per test sample.

11.5 Estimation of E_rC_x values

Use spiked sediments (for spiking method, see Annex D) for the estimation of E_rC_x values of chemicals.

11.5.1 Preparation of concentration series for E_rC_x value assessment

If the E_rC_x is to be estimated, a sufficient number of concentrations are used to calculate the E_rC_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 should be below and one above the E_rC_x to

be estimated, and three or more values should be other than 0 % to 5 % or 95 % to 100 % inhibition. Otherwise, confidence limits might be too large.

11.5.2 Statistical design for E_rC_x

For E_rC_x assessment (see also ISO 5667-16), use at least three replicates in the control(s) and at each concentration level for the treatments.

11.5.3 Use of solvents

If solvents cannot be avoided, use an additional control with at least three replicates including the solvent at the same concentration as in all replicate vessels. Solvents shall exhibit no toxic effects at the concentration chosen.

Table 3 — Example for the calculation of mean growth rates in three replicates of the control sample and in three replicates of the test sample

Control sample	*m* (mg)				**Test sample**	*m* (mg)			
	d0	d10	*r*	$r_{V,C}$		d0	d10	*r*	$r_{V,T}$
Rep-1, pl-1	25	66	0,097		Rep-1, pl-1	25	46	0,061	
Rep-1, pl-2	26	67	0,095		Rep-1, pl-2	29	51	0,056	
Rep-1, pl-3	23	70	0,111	0,101	Rep-1, pl-3	22	45	0,072	0,063
Rep-2, pl-1	24	66	0,101		Rep-2, pl-1	26	46	0,057	
Rep-2, pl-2	23	60	0,096		Rep-2, pl-2	23	49	0,076	
Rep-2, pl-3	29	71	0,090	0,096	Rep-2, pl-3	23	43	0,063	0,065
Rep-3, pl-1	25	67	0,099		Rep-3, pl-1	25	46	0,061	
Rep-3, pl-2	23	68	0,109		Rep-3, pl-2	27	52	0,066	
Rep-3, pl-3	24	69	0,105	0,104	Rep-3, pl-3	23	35	0,042	0,056
$r_{S,C}$				**0,100**	$r_{S,T}$				**0,061**
SD				**0,004**	**SD**				**0,005**
C_V (%)				**4,0**	C_V (%)				**7,6**

Rep	replicate (= vessel)
pl	plant
m	fresh mass
d	day
$r_{V,C}$	mean growth rate of control vessel
r	growth rate
$r_{S,C}$	mean of $r_{V,C}$ (= mean growth rate of the control sample)
SD	standard deviation
CV	coefficient of variation
$r_{V,T}$	mean growth rate of test vessel
$r_{S,T}$	mean of $r_{V,T}$ (= mean growth rate of the test sample)

11.6 Expression of results

Express the results as mean and standard deviation of the growth rate in test and control samples, and as % inhibition relative to the control sample.

Report the observations of the visual observations and the pH values at the beginning and the end of the test.

If the observed effects shall be statistically evaluated, use appropriate ANOVA methods, provided the prerequisites (normal distribution, homoscedasticity) are fulfilled, e.g. use the one-sided two-sample *t*-test with the test vessel means ($r_{V,T}$) and the control vessel means ($r_{V,C}$) as replicates in order to compare each test sample with the control. In case the aforementioned prerequisites cannot be met, use the two-sample Mann-Whitney U-test (see also ISO/TS 20281[9]).

If EC_x values are required, use the test vessel means ($r_{V,T}$) and the control vessel means ($r_{V,C}$) and the normal or logistic sigmoid function combined with linear or nonlinear regression (see also ISO/TS 20281[9]).

Table 4 — Example for expression of the results

	$r_{S,T;S,C}$	SD	CV %	*n*	Inhibition %	*t*-test	Damage (necrosis/chlorosis)	pH (start/end)
Control	0,101	0,002 9	2,9	3			no chlorosis, no necrosis	6,80/7,22
Sample 1	0,061	0,005 0	7,6	3	39,2	P <0,01	chlorosis observed, no necrosis	6,80/6,90
Sample 2	0,065	0,024 6	37,7	3	35,1	P <0,05	chlorosis observed, no necrosis	7,21/6,90
Sample 3	0,070	0,018 5	26,5	3	30,6	P <0,01	no chlorosis, no necrosis	6,84/7,15
Sample 4	0,070	0,008 0	11,4	3	30,3	P <0,001	chlorosis observed, no necrosis	7,47/7,38

12 Validity criteria

The growth performance of the plants in the control sample must meet a minimum mean growth rate $r_{S,C}$ of 0,090 per day and a maximum variance of CV ≤ 15 %.

13 Test report

This test report shall contain at least the following information:

a) test method used, together with a reference to this International Standard (ISO 16191:2013);

b) name of the laboratory performing the test;

c) date and period of test;

d) test organism (e.g. scientific name, source);

e) sample identification and characteristics (e.g. sample number, sample origin, date and period of sampling, storage temperature and duration, water content of sediment, pH, redox potential, pre-treatment);

f) control sample used;

g) expression of the results in accordance with Clause 12 and Table 4;

h) results from the tested reference substance;

i) any deviations from this method and information on all circumstances which might affect the results.

Annex A
(informative)

Figures

The following figures show the culturing and the testing of *Myriophyllum aquaticum* by illustrating the state of the plants in the pre-culture and under exposure in the sediment.

Figure A.1 — Example for a 21-d-old pre-culture plant [example of shoot cut into two whorls (for exposure) and one head-whorl]

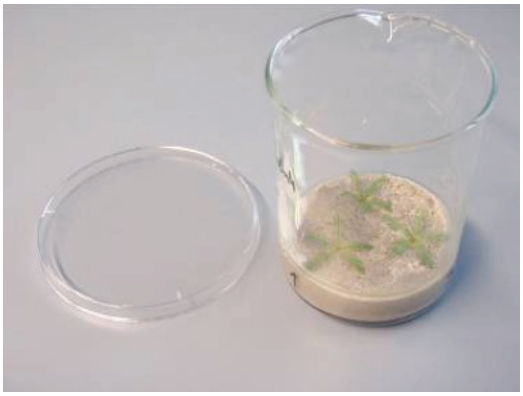
a) Alignment of the planted head-whorls

b) Conical vessel and cover with holes

Figure A.2 — Example for pre-culture: head-whorls planted in artificial sediment

NOTE Sometimes, if humidity in the pre-culture vessels is too high, the plants change shape: the whorls become fragile and brownish. In this case, the plants are probably growing too close to each other and/or the pre-culture vessel is too high; thus, aeration cannot occur properly.

a) Three whorls planted in natural sediment



b) Three whorls planted in spiked artificial sediment

Figure A.3 — Example for test samples (day 0)

Annex B
(normative)

Preparation of nutrient solution (Steinberg medium, following ISO 20079)

B.1 Concentrations and stock solutions

Prepare the Steinberg medium from single solutions. The required concentrations of test medium are then obtained by dilution.

Table B.1 — Composition of pH-stabilized Steinberg medium

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

Table B.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 B.3 — Stock solutions (Microelements)

Microelements (1 000-fold concentrated)	mg/l
Stock solution 4:	
H_3BO_3	120,0
Stock solution 5:	
$ZnSO_4$ $7H_2O$	180,0
Stock solution 6:	
Na_2MoO_4 $2H_2O$	44,0
Stock solution 7:	
$MnCl_2$ $4H_2O$	180,0
Stock solution 8:	
$FeCl_3$ $6H_2O$	760,00
EDTA Disodium salt·$2H_2O$	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 is strongly recommended.

B.2 Preparation of the final concentration of Steinberg medium (modified)

Add to about 900 ml of water (6.1) 20 ml of stock solutions 1, 2, and 3 (see Table B.2) each.

Add 1,0 ml of stock solutions 4, 5, 6, 7, and 8 (see Table B.3).

The pH should be to 5,5 ± 0,2 [adjust by addition of minimized volume of sodium hydroxide solution (e.g. c(NaOH) = 0,1 mmol/l) or hydrochloric acid (e.g. c(HCl) = 0,1 mmol/l)].

Adjust with water (6.1) to 1 000 ml.

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

Annex C
(informative)

Suppliers

For *Myriophyllum aquaticum* [1)]

Institut für Gewässerschutz MESOCOSM GmbH
Neu-Ulrichstein 5
35315 Homberg/Ohm
Germany
Tel: +49 - 6633 - 642 – 740
Fax: +49 - 6633 - 642 – 790
www.mesocosm.de

For Quartz sand: F36 [1)]

Quarzwerke GmbH
Kaskadenweg 40
50226 Frechen
Germany
Tel: + 49 (0) 22 34 101-0
Fax: + 49 (0) 22 34 101-400

Quartz sand F36 product data (HS-No.: 2505 1000, from 2003 to 02-24)

Grain size (mm)	Residue in % (mass)
Average grain size	0,17
> 0,710	—
0,710 to 0,500	—
0,500 to 0,355	—
0,355 to 0,250	4
0,250 to 0,180	34
0,180 to 0,125	50
0,125 to 0,090	11
< 0,090	1

1) This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this supplier.

For peat: Lithuanian peat moss[2)]

Klasmann-Deilmann GmbH
Georg-Klasmann-Str. 2-10
49744 Geeste
Germany
Tel: +49 (0)59 37-31-0
Fax: +49 (0)59 37-31-279
www.klasmann-deilmann.com

Lithuanian peat moss (data sheet 12/2007)	
Structure	extra fine (0 mm to 5 mm)
pH value (H_2O, volume fraction 1:2,5)	3,0 to 3,5
Salt level (volume fraction 1:3,6)	< 150 mg/l
Nitrogen	< 50 mg/l
Phosphate	< 30 mg/l
Potassium	< 30 mg/l
Magnesium	< 80 mg/l
Organic substances in dry mass (mass fraction in %)	94 to 99
Ash in dry mass (mass fraction in %)	1 to 6
Water capacity (volume fraction in %)	80 to 85

2) This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this supplier.

Annex D
(normative)

Preparation of spiked artificial sediments

D.1 General

The spiking procedure described here follows the OECD Guideline 218. All test concentrations are calculated as concentration of the test substance in the sediment (mg/kg sediment dry mass).

If dilution series will be performed, the different concentration(s) of the test substance will be prepared by spiking a defined solution of the test substance into the sediment, not via dilution of the sediment. Only the stock solution of the test substance will be diluted in the appropriate concentration steps.

If scarcely soluble substances which are dissolved in an organic solvent are to be investigated, a solvent control is needed.

Split up the original sediment sample into the required number of portions by taking into account the number of concentration levels and solvent controls needed for the test.

A control sample (see 3.3 and 6.9.1) is needed for all test series and is performed according to 6.9.1.

D.2 Spiking procedure

Use freshly prepared (6.9.1) artificial sediment.

Determine the dry mass of the sediment (ISO 11465).

Calculate the required amount of spiking substance needed for the test concentration.

D.2.1 Preparation of the sediment aliquot for spiking

Weigh out 10 % (wet mass) of the required amount of sediment and dry it according to ISO 11465. If necessary, pulverize the dried material, so that it consists of possibly small particles. Keep in mind to compensate the lost water content later (see below).

NOTE Experience has shown that drying the sediment aliquot 24 h at 105 °C is sufficient.

D.2.2 Preparation of the test substance solution for spiking

Dissolve the chosen test substance in a suitable solvent [e.g. water (6.1), nutrient solution (6.6), or organic solvent (e.g. 2-propanone), depending on the solubility of the test substance] in as small a volume as possible [maximum 4 % (volume/wet mass) of the required amount of wet sediment].

D.2.3 Spiking of the prepared sediment aliquot

Add the test substance solution to the dried sediment aliquot (D.2.1). Let the solvent evaporate under a fume hood until it has been totally removed from the sediment aliquot {up to 3 d in case of inorganic solvents [e.g. water (6.1)]}. If lumps or crusts form during drying, crush them with a stainless-steel spatula or, if they are persistent, in a mortar.

D.2.4 Spiking of the total sediment

Weigh out 90 % (wet mass) of the required amount of sediment.

To compensate the lost water content of the 10 % sediment aliquot (see D.2.1), add the respective aliquot of water (6.1) needed for compensation, and thoroughly mix the dried, spiked sediment aliquot (D.2.3), the aliquot of compensating water, and the 90 % sediment portion until visible homogeneity.

For equilibration, keep the mixed sediment at 20 °C to 24 °C in the dark for at least 7 d. The vessel should be closed but not air tight.

After this equilibration period, the sediment is ready for use and should not be stored.

Perform the same procedural steps for solvent control and concentration levels in case of dilution series.

NOTE 1 The recommended duration of this equilibration was not timed to achieve a complete equilibrium between the substances adsorbed to particles and those dissolved in the pore water. The proposal is a compromise between the time when the equilibrium is reached, possible microbial decomposition processes, and practicability.

NOTE 2 DCP-spiked artificial sediments can be stored for 14 d at 4 °C to 8 °C in the dark.

D.2.5 Example

500 g wet sediment are needed.

Determine the dry mass (= 65 % dry sediment).

Take 10 % aliquot of the wet sediment (= 50 g) and dry it overnight (24 h).

Calculate the test substance (e.g. DCP) concentration with respect to the sediment dry mass (i.e. for 100 mg DCP/kg dry mass → 32,5 mg DCP is needed for 500 g wet sediment).

Dissolve the test substance (32,5 mg DCP) in the minimum volume of suitable solvent (e.g. 2-propanone) but a maximum 4 % of final sediment wet mass (= maximum 20 ml for preparation of 500 g wet sediment).

Add dissolved DCP (at maximum 20 ml) to the dried sediment aliquot.

Let solvent evaporate (e.g. 2-propanone overnight).

Take 90 % aliquot of required wet sediment (= 450 g).

Add the air-dried, spiked sediment aliquot.

Add 10 % water (6.1) to compensate lost water content during drying, with respect to the dry mass of the sediment [e.g. 65 % sediment dry mass corresponds to 35 % water mass fraction; therefore, 500 g wet sediment contains 35 % water (= 175 ml); therefore, 10 % water to be added (= 17,5 ml)].

Mix all (wet sediment, air-dried sediment, water) until visible homogeneity.

Equilibrate.

Annex E
(informative)

Performance data

An international interlaboratory test based on the procedure described in this International Standard was carried out in 2012. A total of 21 laboratories from seven countries (CH, D, F, NL, S, SRB, UK) participated in the interlaboratory test. Four sediment samples were analysed [sample 1: artificial sediment; sample 2: artificial sediment prepared by adding solvent (2-propanone); sample 3: artificial sediment prepared by adding 3,5-dichlorophenol; sample 4: native sediment prepared by adding nickel chloride]. The results are shown in Tables E.1 and E.2.

Table E.1 — Interlaboratory test results — Toxicity parameters (growth rate)

Sample	Parameter	l^a	n^a	o^a %	$\bar{\bar{X}}$	s_R	$C_{V,R}$ %	s_r	$C_{V,r}$ %
1	Growth rate	14	14	33	0,135	0,028	21,2	0,009	7,0
2	Growth rate	14	14	33	0,133	0,030	22,4	0,012	9,7
3	Growth rate	14	14	33	0,090	0,023	25,0	0,009	10,6
4	Growth rate	14	14	33	0,102	0,025	24,6	0,013	13,7

l number of laboratories after outlier[a] rejection

n number of individual test results after outlier[a] rejection

o percentage of outliers[a]

$\bar{\bar{X}}$ overall mean of results (without outliers), in days^{-1}

s_R reproducibility standard deviation, in days^{-1}

$C_{V,R}$ coefficient of variation of reproducibility

s_r repeatability standard deviation, in days^{-1}

$C_{V,r}$ coefficient of variation of repeatability

[a] Outlier = sum of non-valid data (3 of 21 = 14 %) and valid data not conforming to ISO procedure (4 of 21 = 19 %). Outliers are not included in the calculations.

Table E.2 — Interlaboratory test results — Toxicity parameters (inhibition)

Sample	Parameter	l^a	n^a	o^a	Median %	MAD[b] %
3	Inhibition	14	14	33	33,7	26,7
4	Inhibition	14	14	33	24,3	39,9

l number of laboratories after outlier rejection

n number of individual test results after outlier rejection

o percentage of outliers

Median median of inhibition values

MAD median absolute deviation

[a] Outlier = sum of non-valid data (3 of 21 = 14 %) and valid data not conforming to ISO procedure (4 of 21 = 19 %). Outliers are not included in the calculations

[b] MAD = median absolute deviation from median was calculated considering non-normally distributed data showing no homogeneity of variance like inhibition values. MAD% = percentage median deviation, measure of reproducibility (equates to $C_{V,R}$ %)

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