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ENVIRONMENT DIRECTORATE

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Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology

DRAFT NEW TEST GUIDELINE 239: WATER-SEDIMENT MYRIOPHYLLUM SPICATUM TOXICITY TEST

ACTION REQUIRED: The Joint Meeting is invited to endorse the Draft New Test Guideline 239: Water-sediment Myriophyllum Spicatum Toxicity Test and agree on its declassification by 27 June 2014.

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English - Or. English

A project proposal for the development of a Test Guideline on "Water-Sediment *Myriophyllum spicatum* Toxicity Test" was submitted by the Netherlands and the United Kingdom in 2011, and included in the Test Guidelines work plan in 2012. A ring test was conducted in 2011-2012. The proposed method is to evaluate the effects of chemicals on a dicotyledonous macrophyte – *Myriophyllum sp.* – in a water-sediment test system. The *Myriophyllum* test will supplement another aquatic macrophyte toxicity test – the existing TG 221: *Lemna sp.* Growth Inhibition Test – for effects assessment of chemicals; such data are required in particular for plant protection products registration. Moreover, this (two-phase) water-sediment *Myriophyllum* test will complement a (one-phase) sediment-free *Myriophyllum* test currently under development.

A draft Test Guideline on a "Water-Sediment *Myriophyllum sp.* Toxicity Test" was circulated twice to the WNT for review in July-October 2013 and December 2013-January 2014 respectively. The revised draft Test Guideline was approved by the 26th Meeting of the WNT in April 2014.

OECD GUIDELINES FOR THE TESTING OF CHEMICALS

DRAFT WATER-SEDIMENT *MYRIOPHYLLUM SPICATUM* TOXICITY TEST (TG 239)

INTRODUCTION

1. OECD Test Guidelines are available for the floating, monocotyledonous aquatic plant, *Lemna* species (1) and for algal species (2). These guidelines are routinely used to generate data to address the risk of test chemicals, in particular chemicals with herbicidal activity, to non-target aquatic plant species. However, in some cases, data for additional macrophyte species may be required. Recent guidance published from the Society of Environmental Toxicology and Chemistry (SETAC) workshop on Aquatic Macrophyte Risk Assessment for Pesticides (AMRAP) proposed that data for a rooted macrophyte species may be required for test chemicals where *Lemna* and algae are known not to be sensitive to the mode of action or if partitioning to sediment is a concern, leading to exposure via root uptake (3). Based on current understanding and experience, *Myriophyllum* spp were selected as the preferred species in cases where data are required for a submerged, rooted dicotyledonous species (4) (5) (6). This test does not replace other aquatic toxicity tests; it should rather complement them so that a more complete aquatic plant hazard and risk assessment is possible. The water- sediment *Myriophyllum spicatum* test method complements the sediment-free *Myriophyllum spicatum* Toxicity Test (7).

2. This document describes a test method, which allows assessment of the effects of a test chemical on the rooted, aquatic plant species *Myriophyllum spicatum*, growing in a water-sediment system. The method is based partly on existing guidelines (1) (2) (8) and takes account of recent research related to the risk assessment of aquatic plants (3). The water-sediment method has been validated by an international ring-test conducted with *Myriophyllum* species grown under static conditions, which were exposed to the test chemical through applications made via the water column (9). However, the test system is readily adapted to allow for exposure via spiked sediment or exposure via the water phase in semi-static or pulsed-dose scenarios, although these scenarios have not been formally ring tested. Furthermore, the general method can be used for other rooted, submerged and emergent species including other *Myriophyllum* species (e.g. *Myriophyllum aquaticum*) and *Glyceria maxima* (10). Modifications of test conditions, design and duration may be required for alternative species. In particular, more work is needed to define appropriate procedures for *Myriophyllum aquaticum*. These options are not presented in detail in this Test Guideline, which describes the standard approach for exposure of *Myriophyllum spicatum* in a static system via the water phase.

3. This Test Guideline applies to substances for which the test method has been validated, see details in the ring test report (9) or to formulations, commercial products, or known mixtures. A *Myriophyllum* test may be conducted to fulfil a Tier 1 data requirement triggered by potential test chemical partitioning to sediment or mode of action/selectivity issues. Equally, a laboratory-based *Myriophyllum* test may be required as part of a higher-tier strategy to address concerns over the risk to aquatic plants. The specific reason for conducting a test will determine the route of exposure (i.e. via water or sediment). Before use of the Test Guideline for the testing of a mixture intended for a regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

PRINCIPLE OF THE TEST

4. The test is designed to assess chemical-related effects on the vegetative growth of *Myriophyllum* plants growing in standardised media (water, sediment and nutrients). For this purpose, shoot apices of healthy, non-flowering plants are potted in standardised, artificial sediment, which is supplemented with additional nutrients to ensure adequate plant growth, and then maintained in Smart and Barko medium. After an establishment period to allow for root formation, plants are exposed to a series of test concentrations added to the water column. Alternatively, exposure via the sediment may be simulated by spiking the artificial sediment with the test chemical and transplanting plants into this spiked sediment. In both cases, plants are subsequently maintained under controlled environmental conditions for 14 days. Effects on growth are determined from quantitative assessments of shoot length, fresh weight and dry weight, as well as qualitative observations of symptoms such as chlorosis, necrosis or growth deformities.

5. To quantify chemical-related effects, growth in the test solutions is compared with the growth of the control plants, and the concentration causing a specified x% inhibition of growth is determined and expressed as the EC_x; "x" can be any value depending on the regulatory requirements, e.g. EC_{10} EC_{20} and EC_{50}. It should be noted that estimates of EC_{10} and EC_{20} values are only reliable and appropriate in tests where coefficients of variation in control plants fall below the effect level being estimated, i.e. coefficients of variation should be <20% for robust estimation of an EC_{20}.

6. Both average specific growth rate (estimated from assessments of shoot length, shoot fresh weight and shoot dry weight) and yield (estimated from the increase in shoot length, shoot fresh weight and shoot dry weight) of untreated and treated plants should be determined. Specific growth rate (r) and yield (y) are subsequently used to determine the E_rC_x (e.g. E_rC_{10}, E_rC_{20}, E_rC_{50}) and E_yC_x (e.g. E_yC_{10}, E_yC_{20}, E_yC_{50}), respectively.

7. If required, the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) may be statistically determined from estimates of average specific growth rates and yield.

INFORMATION ON THE TEST CHEMICAL

8. An analytical method with adequate sensitivity for quantification of the substance(s) in the test medium should be available.

9. Information on the test substance(s) which may be useful in establishing the test conditions includes the structural formula, composition in the case of mixtures or formulations, purity, water solubility, stability in water and light, pK_a, K_{ow}, if available K_d to sediments, vapour pressure and biodegradability. Water solubility and vapour pressure can be used to calculate Henry´s Law constant, which will indicate whether significant losses of the test chemical during the test period are likely. If losses of the test chemicals are likely, the losses should be quantified and the subsequent steps to control such losses should be documented. Where information on the solubility and stability of the test substance(s) are uncertain, it is recommended that these properties are assessed under the conditions of the test, i.e. growth medium, temperature, lighting regime to be used in the test. *Note*: when light dependent peroxidising herbicides are tested, the laboratory lighting used should contain the equivalent presence of solar ultraviolet light found in natural sunlight.

10. The pH should be measured and adjusted in the test medium as appropriate. The pH control of the test medium is particularly important, e.g. when testing metals or chemicals which are hydrolytically unstable. Further guidance for testing chemicals with physical-chemical properties that make them difficult to test is provided in the OECD Guidance Document, series on Testing and Assessment, No. 23 (11).

VALIDITY OF THE TEST

11. For the test results to be valid, the mean total shoot length and mean total shoot fresh weight in control plants must at least double during the exposure phase of the test. In addition, control plants must not show any visual symptoms of chlorosis and should be visibly free from contamination by other organisms such as algae and/or bacterial films on the plants, at the surface of the sediment and in the test medium.

12. The mean coefficient of variation for yield based on measurements of shoot fresh weight (i.e. from test initiation to test termination) in the control cultures must not exceed 35% between replicates.

REFERENCE SUBSTANCE

13. A reference substance(s), such as 3,5-dichlorophenol used in the ring test (9), should be periodically tested in order to check the performance of the test procedure over time. The ring test data indicate that the mean EC_{50} values of 3,5-DCP for the different response variables were between 4.7 and 6.1 mg /L (see the ring-test report for details of anticipated confidence interval around these values). It is advisable to test a reference substance at least twice a year or, where testing is carried out infrequently, in parallel with the definitive toxicity tests. A guide to expected EC_{50} values for 3,5-DCP is provided in the Statistical Report of the International Ring-test (9).

DESCRIPTION OF THE METHOD

Test apparatus

14. The test should be conducted under controlled environmental conditions, i.e. in a growth chamber, growth room or laboratory, with controllable day length, lighting and temperature (see section "Test conditions", paragraphs 55-57). Stock cultures should be maintained separately from test vessels.

15. The study should be conducted using glass test vessels such as aquaria or beakers; 2-L glass beakers (approximately 24 cm high and 11 cm in diameter) are commonly used. However, other (i.e. larger) vessels may be suitable provided that there is sufficient depth of water to allow unlimited growth and keep the plants submerged throughout the test duration.

16. Plastic or glass plant pots (approximately 9 cm diameter and 8 cm high and 500 mL volume) may be used as containers for potting the plants into the sediment. Alternatively, glass beakers may be used and are preferred in some cases (e.g. testing hydrophobic substances or substances with high K_{ow}).

17. The choice of pot/beaker size needs to be considered alongside the choice of test vessels and the preferred test design (see below). If using Test Design A (one shoot per pot with three pots per vessel) then smaller pots or larger vessels may be needed. If using Test Design B (three shoots per pot and one pot per vessel) then the stated pot and vessel sizes should be adequate. In all cases, the minimum overlaying water depth should be 12 cm above the top of the sediment and the ratio of sediment surface area/volume to water surface area/volume should be recorded.

Test organism

18. The general approaches described in this test method can be used to test a range of aquatic plant species. However, the conditions outlined in this guideline have been tailored for testing the water milfoil species, *Myriophyllum spicatum*. This species belongs to the dicotyledonous family, Halogoraceae.

19. *Myriophyllum spicatum* (Eurasian water milfoil) is a submerged, rooted species which tolerates a wide range of conditions and is found in both static and flowing water bodies. *M. spicatum* is a perennial which dies back to the roots over winter. Plants usually flower and set seed freely although vegetative propagation from axillary buds or stem fragments that detach naturally or after disturbance, is often the primary method of colonization.

Cultivation of the test organism

20. Plants may be obtained from natural populations or via aquatic plant suppliers. In both cases, the source of the plants should be documented and species identity should be verified. Great care should be taken to ensure that the correct species is obtained when collecting *Myriophyllum spicatum* from the field, especially in regions where it can hybridise with other *Myriophyllum* species. If in doubt, use of verified laboratory cultures from known sources is recommended. Plants that have been exposed to any chemical contaminants, or collected from sites known to be contaminated, should not be used in this test.

21. In regions where *M. spicatum* is not readily available during the winter months, long-term maintenance of stock cultures may be necessary under glasshouse or laboratory conditions. Stock cultures should be maintained under conditions similar to the test conditions although irradiance and temperature may be reduced in order to reduce the frequency of culture maintenance (e.g. when *Myriophyllum* tests are not planned for a period). Use of larger aquaria and plant pots, than would be used in tests, is recommended in order to allow space for proliferation. Sediment and water-media composition should be the same as would be used for tests although alternative methods of sediment fertilization may be adopted (e.g. use of commercial slow-release fertiliser formulations)

22. Stock plants should be visibly free of contamination with any other organisms, including snails, filamentous algae, fungi and insects, e.g. eggs or larvae of the moth *Paraponyx stratiotata* and larve or adults of the curculionidae *Eubrychius velutus*. Rinsing plant material in fresh water may be necessary to eliminate visible contamination. In addition, efforts should be made to minimise the development of unicellular algae and bacterial contamination although complete sterility of the plant material is not necessary. Stock cultures should be monitored and transplanted as necessary to avoid development of algal and bacterial contamination. Aeration of stock cultures may be beneficial should algal or bacterial contamination become problematic.

23. In all cases, plants are cultured/ acclimatized under conditions that are similar, but not necessarily identical, to those used in the test for an adequate period (i.e. > 2 weeks) before their use in a test.

24. Flowering stock cultures should not be used in a test as vegetative growth rates generally decline during and after flowering.

Sediment

25. The following formulated sediment, based on the artificial sediment used in OECD Test Guideline 219 (8), is recommended for use in this test. The sediment is prepared as described in TG 219, except for the addition of nutrients as described below:

a) 4-5% peat (dry weight, according to 2 ± 0.5% organic carbon) as close to pH 5.5 to 6.0 as possible; it is important to use peat in powder form, finely ground (preferably particle size < 1 mm) and only air dried.

b) 20% (dry weight) kaolin clay (kaolinite content preferably above 30%).

c) 75-76 % (dry weight) quartz sand (fine sand should predominate with more than 50% of the particles between 50 and 200 μm).

d) An aqueous nutrient medium is added such that the final sediment batch contains 200 mg/Kg dry sediment of both ammonium chloride and sodium phosphate and the moisture content of the final mixture is in a range of 30-50 %.

e) Calcium carbonate of chemically pure quality ($CaCO_3$) is added to adjust the pH of the final mixture of the sediment to 7.0 ± 0.5.

26. The source of peat, kaolin clay and sand should be known and documented. If the origin is unknown or gives some level of concern, then the respective components should be checked for the absence of chemical contamination (e.g. heavy metals, organochlorine compounds, organophosphorous compounds).

27. The dry constituents of the sediment should be mixed homogenously prior to mixing the aqueous nutrient solution thoroughly into the sediment. The moist sediment should be prepared at least two days before use to allow thorough soaking of the peat and to prevent hydrophobic peat particles floating to the surface when the sediment is overlaid with media; before use, the moist sediment may be stored in the dark.

28. For the test, the sediment is transferred into a suitable size containers, such as plant pots of a diameter which fit into the glass vessels (the sediment surface area should cover approximately 70% or more of the vessel surface area). In cases where the container has holes at the bottom, a piece of filter paper in the bottom of the container will help to keep the sediment within the container. The pots are filled with the sediment such that the sediment surface is level, prior to covering with a thin layer (~ 2 to 3 mm) of an inert material such as sand, fine horticultural grit (or crushed coral) to keep the sediment in place.

Test medium

29. Smart and Barko medium (12) is recommended for culturing and testing *Myriophyllum spicatum*. Preparation of this media is described in the Annex. The pH of the media (water phase) at test initiation should be between 7.5 and 8.0 for optimum plant growth.

Experimental design

30. The test should incorporate a minimum of six replicate test vessels for the untreated control and a minimum of four replicate test vessels for each of a minimum of five concentration levels.

31. If determination of NOEC is not required, the test design may be altered to increase the number of concentrations and reduce the number of replicates per concentration.

32. Each test vessel represents a replicate containing three shoots. There are two options for growing three shoots in each test vessel:

- Test Design A: one shoot per pot and three pots per vessel.
- Test Design B: three shoots per pot and one pot per vessel.
- Alternative test designs of one shoot per pot per test vessel are acceptable provided that replication is adjusted as required to achieve the required validity criteria.

33. The individual test vessels should be randomly assigned to the treatment groups. A randomized design for the location of the test vessels in the test area is required to minimize the influence of spatial differences in light intensity or temperature.

Test chemical concentrations and control groups

34. Concentrations should typically follow a geometric series; the separation factor between test concentrations should not exceed 3.2. Prior knowledge of the toxicity of the test chemical from a range-finding test will help to select suitable test concentrations.

35. To determine an EC_x, test concentrations should bracket the EC_x to ensure an appropriate level of confidence. For example, if estimating the EC_{50}, the highest test concentration should be greater than the EC_{50} value. If the EC_{50} value lies outside of the range of test concentrations, associated confidence intervals will be large and a proper assessment of the statistical fit of the model may not be possible. The use of more test concentrations will improve the confidence interval around the resulting EC_x value.

36. To determine the LOEC/NOEC (optional endpoint), the lowest test concentration should be sufficiently low such that growth is not significantly different from growth in control plants. In addition, the highest test concentration should be sufficiently high such that growth is significantly lower than that in the control. The use of more replicates will enhance the statistical power of the no effect-concentration/ ANOVA design.

Limit test

37. In cases where a range-finding test indicates that the test chemical does not have an adverse effect at concentrations up to 100 mg/L or in the case of a substance, up to its limit of solubility in the test medium or in the case of a formulation up to the limit of dispersibility, a limit test may be undertaken to facilitate comparison of responses in a control group and one treatment group – 100 mg/L or in case of a substance a concentration equal to the limit of solubility, or 1000 mg/Kg dry sediment. This test should follow the general principles of a standard dose-response test, with the exception that an increase in the minimum number of replicates to six test vessels per control and concentration is advised. Growth in the control and treatment group may be analysed using a statistical test to compare means, e.g. a Student's t-test.

Test solutions

38. Test solutions are usually prepared by dilution of a stock solution, prepared by dissolving or dispersing the test chemical in Smart and Barko media, using demineralized (i.e. distilled or deionised) water (see Annex).

39. The highest test concentration should normally not exceed the water solubility of the test substance or, in the case of formulations, the dispersibility under the test conditions.

40. For test substances of low water solubility, it may be necessary to prepare a concentrated stock solution or dispersion of the chemical using an organic solvent or dispersant in order to facilitate the addition of accurate quantities of the test chemical to the test medium and aid in its dispersion and dissolution. Every effort should be made to avoid the use of such solvents or dispersants. There should be no phytotoxicity resulting from the use of auxiliary solvents or dispersants. For example, commonly used solvents, which do not cause phytotoxicity at concentrations up to 100 µL/L, include acetone and dimethylformamide. If a solvent or dispersant is used, its final concentration should be reported and kept to a minimum (≤100 µL/L). Under these circumstances all treatments and (solvent) controls should contain the same concentration of solvent or dispersant. Untreated control replicates that do not contain a solvent

or dispersant are also incorporated into the test design. Further guidance on the use of dispersants is given in the OECD Guidance Document, series on Testing and Assessment No. 23 (11).

TEST PROCEDURE

41. The test procedure varies according to the application route of the test chemical (i.e. via the water or sediment phase). The likely behaviour of the test chemical in a water-sediment system should be considered to inform the choice of exposure regime used in the test (i.e. static or static renewal, spiked water or spiked sediment). Spiked sediment tests may be preferred in some cases for chemicals that are predicted to significantly partition to sediment.

Establishment phase

42. Healthy shoot apices/tips, *i.e.* without side shoots, are cut from the culture plants to give a shoot length of 6 cm (± 1 cm). For Test Design A (one shoot per pot and three pots per vessel) single shoot tips are planted into each pot. For Test Design B (three shoots per pot and one pot per vessel) four to five shoot apices are planted into each pot containing the sediment.

43. In both cases surplus pots should be planted to allow for selection of uniform plants at test initiation, as well as to provide spare plants to be used for inspection of root growth immediately prior to treatment and spare plants to be harvested for shoot biomass and length measurements on Day 0.

44. Shoots are inserted such that approximately three cm, covering at least two nodes, are beneath the sediment surface.

45. Pots are then transferred to test vessels under the same environmental conditions as for the exposure phase and maintained for seven days in Smart and Barko medium to induce root development.

46. After this time, several plants in spare pots should be removed for inspection of root growth. If root growth is not visible (i.e. root tips are not visible), then the establishment phase should be extended until root growth is visible. This step is recommended to ensure that plants are actively growing at the time of test initiation.

Selection of uniform plant material

47. For Test Design A (one shoot per pot and three pots per vessel) pots are selected for uniformity prior to test initiation. For Test Design B (three shoots per pot and one pot per vessel), surplus plants are removed to leave three plants that are uniform in size and appearance.

Exposure via the water phase

48. Pots, selected for uniformity, are placed into the test vessels as required for the experimental design. Smart and Barko medium is then added to the test vessels. Care should be taken to avoid disturbance of the sediment. For this purpose, media may be added using a funnel or a plastic disc may be used to cover the sediment while the medium is poured into the test vessels provided that the disc is removed immediately afterwards. Alternatively, plant pots may be placed in the test vessels after the addition of the media. In both cases, fresh media may be used at the beginning of the exposure phase, if necessary to minimise the potential build-up of algae and bacteria or to allow preparation of single batches of test solution across replicates.

49. The shoot length above sediment is measured, either prior to or after the addition of the medium.

50. The relevant amounts of the test chemical may be added to the test medium before it is added to the test vessels. Alternatively, the test chemical may be introduced into the medium after it has been added to the test vessels. In this case, care should be taken to ensure that the test chemical is homogeneously distributed throughout the test system without disturbing the sediment.

51. In all cases, the appearance (e.g. clear, cloudy, etc.) of the test media is recorded at test initiation.

Exposure via sediment

52. Spiked sediments of the chosen concentration are prepared by addition of a solution of the test chemical directly to fresh sediment. A stock solution of the test chemical dissolved in deionised water is mixed with the formulated sediment by rolling mill, feed mixer or hand mixing. If poorly soluble in water, the test chemical can be dissolved in as small a volume as possible of a suitable organic solvent (*e.g.* hexane, acetone or chloroform). This solution is then mixed with ca. 10 g of fine quartz sand for one test vessel. The solvent is allowed to evaporate and the sand is then mixed with the suitable amount of sediment per test beaker. Only agents that volatilise readily can be used to solubilise, disperse or emulsify the test chemical. It should be borne in mind that the volume/weight of sand spiked with the test chemical has to be taken into account in the final preparation of the sediment (i.e. the sediment should thus be prepared with less sand). Care should be taken to ensure that the test chemical added to sediment is thoroughly and evenly distributed within the sediment.

53. The spiked sediment is filled into the pots (as described above). Plants, selected for uniformity and an adequate root system, are removed from the pots used during the establishment phase and transplanted into the spiked sediment as described above.

54. Pots are placed into the test vessels as required for the experimental design. Smart and Barko medium is then added carefully (i.e. using a funnel) in order to avoid disturbance of the sediment. The shoot length above sediment is measured, either prior to or after the addition of the media.

Maintenance of water levels over the test duration

55. The final water volume must be recorded and the water level marked on each test vessel. If water evaporates during the test by more than 10%, the water level should be adjusted with distilled water. If necessary, beakers may be loosely covered by a transparent cover such as transparent plastic lids to minimise evaporation and contamination with algal spores.

Test conditions

56. Warm and/or cool white fluorescent lighting are used to provide light irradiance in the range of about 140 (± 20) $\mu E \cdot m^{-2}\ s^{-1}$ when measured as a photosynthetically active radiation (400-700 nm) at the water surface and using a light:dark ratio of 16:8 h. Any differences from the selected light irradiance over the test area should not exceed the range of ± 15%.

57. The temperature in the test vessels is 20 ± 2°C.

58. The pH of the control medium should not increase by more than 1.5 units during the test. However, deviation of more than 1.5 units would not invalidate the test when it can be shown that the validity criteria specified previously are met. See the OECD Guidance Document, series on Testing and Assessment, No. 23 (11).

Test duration

59. The exposure period is 14 days.

Measurements and analytical determinations

60. After the establishment phase and immediately prior to treatment (i.e. on Day 0), spare plants from five randomly selected pots for the three plants per pot design or 15 pots for the one plant per pot design, are harvested for assessment of shoot length and fresh and dry weight as described below.

61. For plants transferred into the exposure phase, the following assessments are made as shown in Table 1:

- Assessments of main shoot length, side shoot number and side shoot length are recorded at least at the beginning and end of the exposure period (e.g. on days 0 and 14).
- Visual assessments of plant health are recorded at least three times during the exposure period (e.g. on days 0, 7 and 14).
- Assessments of shoot fresh weight and dry weight are made at the end of the test (i.e. on Day 14).

62. Shoot length is determined using a ruler. If side shoots are present, their numbers and length should also be measured.

63. Visual assessments of plant health are made by recording the appearance of plants and the general condition of the test medium. Observations to be noted include:

- Necrosis, chlorosis or other discoloration such as excessive reddening relative to control plants.
- Development of bacterial or algal contamination;
- Growth abnormalities such as stunting, altered internodal length, distorted shoots/leaves, the proliferation of side shoots, leaf loss, loss of turgor and stem fragmentation.
- Visual assessments of root health are made at test termination, by carefully washing sediment from roots to enable observation of the root system. A proposed scale for assessment, relative to control plants, is shown below:
    1) roots absent
    2) few roots
    3) moderate root development
    4) very good root development, similar to controls

64. Assessments of fresh weight are made at the beginning and end of the test by cutting the shoot at sediment level and then blotting dry prior to weighing. Care should be taken to remove sediment particles that may adhere to the base of the shoot. Shoot material is then placed in a drying oven at ca. 60°C and dried to a constant weight, prior to re-weighing and recording the dry weight.

65. A summary of the minimum biological assessments required over the test duration is provided in Table 1.

Table 1: Assessment schedule

Day after treatment (DAT)	*Myriophyllum spicatum*			
	Shoot length, side shoot length and number	**Visual assessment of shoots**	**Shoot fresh and dry weight, Visual assessment of roots**	**pH O_2**
0	A (all plants)	A	A	A
4	-	-	-	-
7	-	A	-	A
14	A	A	A	A

A : indicates that assessments are required on these occasions
- : indicates that measurements are not required

Frequency of measurements and analytical determinations

66. The temperature of the medium in a supplementary vessel held under the same conditions in the growth chamber, incubator or room should be recorded at least daily (or continuously with a data logger).

67. The pH and dissolved oxygen concentration of the test medium should be checked at test initiation, at least once during the study and at the end of the study in all replicate vessels. On each occasion, measurements should be taken at the same time of the day. If bulk solutions are used to prepare all replicates at each test concentration, then a single measurement of each bulk solution is acceptable on Day 0.

68. Irradiance should be measured in the growth chamber, incubator or room at points equivalent to level of the water surface. Measurements should be made at least once at test initiation or during the test. The method of light detection and measurement, in particular the type of sensor, will affect the measured value. Spherical sensors (which respond to light from all angles above and below the plane of measurement) and "cosine" sensors (which respond to light from all angles above the plane of measurement) are preferred to unidirectional sensors, and will give higher readings for a multi-point light source of the type described here.

Analytical measurements of test chemical

69. The correct application of the test chemical should be supported by analytical measurements of test chemical concentrations.

70. Water samples should be collected for test substance analysis shortly after test initiation (i.e. on the day of application for stable test substances or one hour after application for substances that are not stable) and at test termination for all test concentrations.

71. Concentrations in sediment and sediment pore-water should be determined at test initiation and test termination, at least in the highest test concentration, unless the test substances are known to be stable in water (> 80% of nominal). Measurements in sediment and pore-water might not be necessary if the partitioning of the test substance between water and sediment has been clearly determined in a water/sediment study under comparable conditions (e.g. sediment to water ratio, application method, sediment type).

72. Sampling of sediment at test initiation is likely to disrupt the test system. Hence, additional treated test vessels may be required to facilitate analytical determinations at test initiation and test

termination. Similarly, where intermediate assessments are considered necessary, i.e. on day 7, and analyses require large samples of sediment that cannot be easily removed from the test system, analytical determinations should be performed using additional test vessels treated in the same way as those used for biological assessments.

73. Centrifugation at, for example, 10 000 g and 4ºC for 30 minutes is recommended to isolate interstitial water. However, if the test substance is demonstrated not to absorb to filters, filtration may also be acceptable. In some cases, it might not be possible to analyse concentrations in the pore water if the sample size is too small.

74. In semi-static tests (i.e. exposure via the water phase) where the concentration of the relevant test substance(s) is not expected to remain within 20% of the nominal concentration over the test duration without renewal of test solutions, used and freshly prepared test solutions should be sampled for analyses of test chemical concentration at each renewal.

75. In cases where the measured initial concentration of the test chemical is not within 20% of nominal but where sufficient evidence can be provided to show that the initial concentrations are repeatable and stable (i.e. within the range of 80-120% of the initial concentration), chemical determinations may be carried out on only the highest and lowest test concentrations.

76. In all cases, determination of test chemical concentrations need only be performed on one replicate vessel at each test concentration. Alternatively, the test solutions of all replicates for each concentration may be pooled for analyses.

77. If there is evidence that the test chemical concentration has been maintained within 20% of the nominal or measured initial concentration throughout the test, then analysis of the results and subsequent derivation of endpoints can be based on nominal or measured initial values.

78. In these cases, effect concentrations should be based on nominal or measured water concentrations at the beginning of the test.

79. However, if there is evidence that the concentration has declined (i.e. is not maintained within 20% of the nominal or measured initial concentration in the treated compartment) throughout the test, then analysis of the results should be based on the geometric mean concentration during exposure or models describing the decline of the concentration of the test chemical in the treated compartment (11).

DATA EVALUATION

80. In cases where use of a solvent / dispersant is required, data from solvent and untreated controls may be pooled for the purposes of statistical analyses provided that the responses of the solvent and untreated controls are not statistically significantly different.

Response variables

81. The purpose of the test is to determine the effects of the test chemical on the vegetative growth of the test species, using two response variables, average specific growth rate and yield, as follows:

Average specific growth rate

82. This response variable is based on changes in the logarithms of total shoot length, total shoot fresh weight and total shoot dry weight, over time in the controls and each treatment group. This variable is calculated for each replicate of each control and treatment group. The mean length and weight of the three

plants per test vessel (replicate) and, subsequently, the growth rate for each replicate, should be calculated using the following formula:

$$\mu_{i-j} = (ln(N_j) - ln(N_i)) / t$$

where:

μi-j : average specific growth rate from time i to j

Ni : measurement variable in the test or control vessel at time i

Nj : measurement variable in the test or control vessel at time j

t : time period from i to j

83. From the replicate responses, a mean value for growth rate along with variance estimates should be calculated for each treatment and control group.

84. The average specific growth rate should be calculated for the entire test period (time "i" in the above formula is the beginning of the test and time "j" is the end of the test). For each test concentration and control, calculate a mean value for average specific growth rate along with the variance estimates.

85. Percent inhibition of growth rate (Ir) may then be calculated for each test concentration (treatment group) according to the following formula:

$$\%Ir = \frac{\mu c - \mu t}{\mu c} x100$$

where:

% Ir : percent inhibition in average specific growth rate

μc : mean value for μin the control

μt : mean value for μin the treatment group

Yield

86. This response variable is based on changes in total shoot length, total shoot fresh weight and total shoot dry weight, over time in the controls and each treatment group. The mean percent inhibition in yield (% Iy) may be calculated for each treatment group as follows:

$$\%Iy = \frac{bc - bt}{bc} x100$$

where:

% Iy : percent reduction in yield

bc : final biomass minus starting biomass for the control group

bt : final biomass minus starting biomass in the treatment group

Plotting concentration-response curves

87. Concentration-response curves relating mean percentage inhibition of the response variable (Ir, or Iy), calculated as shown above and the log concentration of the test chemical should be plotted.

EC_x estimation

88. Estimates of the EC_x (e.g., EC_{50}) should be based upon both average specific growth rate (E_rC_x) and yield (E_yC_x), each of which should in turn be based upon total shoot fresh weight, total shoot dry weight and total shoot length.

89. It should be noted that EC_x values calculated using these two response variables are not comparable and this difference is recognised when using the results of the test. EC_x values based upon average specific growth rate (E_rC_x) will in most cases be higher than results based upon yield (E_yC_x) – if the test conditions of this Guideline are adhered to – due to the mathematical basis of the respective approaches. This difference should not be interpreted as a difference in sensitivity between the two response variables, simply the values are different mathematically.

Statistical procedures

90. The aim is to obtain a quantitative concentration-response relationship by regression analysis. It is possible to use a weighted linear regression after having performed a linearising transformation of the response data, for instance into probit or logit or Weibull units (14), but non-linear regression procedures are preferred techniques that better handle unavoidable data irregularities and deviations from smooth distributions. Approaching either zero or total inhibition such irregularities may be magnified by the transformation, interfering with the analysis (14). It should be noted that standard methods of analysis using probit, logit, or Weibull transforms are intended for use on quantal (e.g. mortality or survival) data, and should be modified to accommodate growth rate or yield data. Specific procedures for determination of EC_x values from continuous data can be found in (15) (16) (17) (18).

91. For each response variable to be analysed, use the concentration-response relationship to calculate point estimates of EC_x values. The 95% confidence limits for each estimate are determined and goodness of fit of the response data to the regression model should be assessed either graphically or statistically. Regression analysis should be performed using individual replicate responses, not treatment group means.

92. EC_{50} estimates and confidence limits may also be obtained using linear interpolation with bootstrapping (19), if available regression models/methods are unsuitable for the data.

93. For estimation of the LOEC and hence the NOEC, it is necessary to compare treatment means using analysis of variance (ANOVA) techniques. The mean for each concentration is then compared with the control mean using an appropriate test method (e.g. Dunnett's, Williams'tests) (20) (21) (22) (23). It is necessary to assess whether the ANOVA assumption of normal distribution (ND) and variance homogeneity (VH) of variance holds. This assessment should be performed by Shapiro-Wilks-test (ND) or Levene's test (VH). Failure to meet the assumption of ND and homogeneity of variances can sometimes be corrected by logarithmic transformation of the data. If heterogeneity of variance and/or deviation from ND is extreme and cannot be corrected by transformation, analysis by methods such as Bonferroni-Welch-t-test, step-down Jonkheere Terpstra test and Bonferroni-Median-Test should be considered. Additional guidance on determining the NOEC can be found in (17).

REPORTING

94. The test report includes the following details:

Test chemical

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVBCs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Test species

- scientific name and source.

Test conditions

- duration and conditions of establishment phase;
- test procedure used (static, semi-static, pulsed);
- date of start of the test and its duration;
- test medium, *i.e.* sediment and liquid nutrient medium;
- description of the experimental design: growth chamber/room or laboratory, test vessels and covers, solution volumes, length and weight of test plants per test vessel at the beginning of the test, ratio of sediment surface to water surface, sediment and water volume ratio;
- test concentrations (nominal and measured as appropriate) and number of replicates per concentration;
- methods of preparation of stock and test solutions including the use of any solvents or dispersants;
- temperature during the test;
- light source, irradiance ($\mu E \cdot m^{-2}\ s^{-1}$) -pH values of the test and control media as well as appearance of test media at test initiation and end;
- oxygen concentrations;
- the method of analysis with appropriate quality assessment data (validation studies, standard deviations or confidence limits of analyses);
- methods for determination of measurement variables, *e.g.*, length, dry weight, fresh weight;
- all deviations from this Guideline.

Results

- raw data: shoot length and shoot weight of plants/pot and other measurement variables in each test and control vessel at each observation and occasion of analysis according to the assessment schedule provided in Table 1;
- means and standard deviations for each measurement variable;
- growth curves for each concentration;
- doubling time/growth rate in the control based on shoot length and fresh weight including the coefficient of variation for yield of fresh weight;
- calculated response variables for each treatment replicate, with mean values and coefficient of variation for replicates;
- graphical representation of the concentration/effect relationship;
- estimates of toxic endpoints for response variables *e.g.* EC_{50}, and associated confidence intervals. If calculated, LOEC and/or NOEC and the statistical methods used for their determination;
- if ANOVA has been used, the size of the effect which can be detected (*e.g.* the minimum significant difference);
- any stimulation of growth found in any treatment;
- any visual signs of phytotoxicity as well as observations of test solutions;
- discussion of the results, including any influence on the outcome of the test resulting from deviations from this Guideline.

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ANNEX

SMART AND BARKO MEDIUM COMPOSITION

Component	Amount of reagent added to water* (mg/L)
$CaCl_2 \cdot 2\ H_2O$	91.7
$MgSO_4 \cdot 7\ H_2O$	69.0
$NaHCO_3$	58.4
$KHCO_3$	15.4
pH (air equilibrium)	7.9

* demineralized (i.e. distilled or deionised) water