A proposed test method for the rooted aquatic macrophyte, *Myriophyllum sp.*

**Introduction**

Standard (water only) test guidelines are available for aquatic plants, for alga species (OECD 201) and for *Lemna* sp. (OECD 208) as a representative of higher aquatic plants. These methods can be used to generate data to address the risk of substances (in particular substances with herbicidal activity) to aquatic non-target plant species. However, in some cases, these studies may not be sufficient and information on additional macrophytes may be required. This may be the case, when contamination via sediment is a relevant exposure pathway requiring testing of a sediment rooting macrophyte or if there is indication that the standard species (algae and *Lemna*) may not be considered representative for other more sensitive aquatic plant species.

Based on current understanding and experience (Knauer et al., 2008; Kubitza & Dohmen, 2008), *Myriophyllum* sp. (*M. spicatum* and/or *M. aquaticum*) were selected as the preferred additional macrophyte species.

In this document, a standardized test method protocol is proposed, which allows assessment of the toxicity of substances to rooted aquatic plant species of the genus *Myriophyllum* (*M. aquaticum* and *M. spicatum*). The principle approach may be used (adapted as appropriate) for a range of other aquatic macrophyte species. The method presented is based partly on existing guidelines (OECD 221, 219, 201) and takes account of recent research and consultation on a number of key issues (Maltby et al., 2010; Arts et al., 2008; Kubitza & Dohmen, 2008; Knauer et al., 2008) related to the risk assessment of aquatic plants. The proposed method will be tested and validated by a formal international ring-test.

**Materials and Method**

**Principle of the test**

The objective of the test is to assess substance-related effects on the vegetative growth of the genus *Myriophyllum* in standardised media (water, sediment and nutrients) containing different concentrations of the test substance over certain test periods. For this purpose, individual shoot apices of healthy, non flowering plants are potted in artificial standard sediment fertilized to ensure adequate plant growth and are maintained in a nutrient formulated water. After an establishment period, the plants are exposed to a series of test concentrations added to the water column. (Alternatively, it is also possible to simulate exposure via the sediment by spiking the artificial sediment with in the required concentration range¹). The growth of the plants is evaluated for a period sufficient to allow a robust assessment of growth. At the end of the test, the plants are harvested and their biomass, length and other relevant observations are recorded.

To quantify substance-related effects, growth in the test solutions is compared to that of the controls, and the concentration causing a specified x% inhibition of growth (e.g. 50 %) is determined and expressed as the ECx (e.g. EC₅₀).

¹ A different test approach to assess natural contaminated sediments - excluding largely exposure via water - has been proposed using *M. aquaticum* as test species, ISO xy, Feiler et al. 2009.
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**Selection of species**

The principle of the method may be used for testing a range of aquatic macrophyte species; however, several details of the method are designed for testing *Myriophyllum* species, in particular *M. aquaticum* and *M. spicatum*. Other species may need modifications of vessel size, water depth, study duration etc. Species identification must be verified (in North America there is evidence of hybridization between *M. spicatum* and related species, (Moody, M.L. & Les, D.H., 2002)) and the source of the plants should be known and documented.

The plants should be visibly free of any other species (particularly snails or filamentous algae, in some regions also eggs or larvae from the small moth *Paraponyx stratiotata* can be a problem; some level of epiphytes - such as diatoms, but no filamentous algae - may often not be avoidable and will generally not be a problem). Only visibly healthy plants, without flowering shoots, should be used for the study.

If the plants are kept within the laboratory before the test as a maintenance culture, temperature, light and nutrient conditions should be at a low level, i.e. nutrient concentrations reflecting oligotrophic to mesotrophic systems. For this, often standard tap water may be useful. If culturing/maintenance conditions differ significantly from lab conditions (i.e. if plants are taken from outdoor systems at a time when temperature and day length differ significantly from those in the lab or when plants have just arrived from the supplier) then, in order to support good growth, plants should be preferably be cultured/ acclimatized under conditions similar to those in the test for an adequate period before the study.

**Test vessels**

The study using *Myriophyllum* as test species is conducted in 2-L glass beakers (approximately 24 cm high and 11 cm in diameter). Other vessels may be suitable, but they should guarantee a suitable depth of water to allow unlimited growth and keep the plants submerged throughout the study. Small plant pots (approx. 9 cm diameter and 8 cm high and 500 mL volume) are used as containers for potting the plants into the sediment.

The sediment surface coverage should be > 70 % of the test vessel surface; the minimum overlaying water depth should be 12 cm.

**Sediment**

The following formulated sediment, based on the artificial soil used in OECD Guideline 219, is recommended for use in this test; the sediment is prepared per the guideline except with the additional 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 (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 per cent of the particles between 50 and 200 µm).

An aqueous nutrient medium is added such that the final sediment batch contains 300 mg/L sediment of both ammonium chloride and sodium phosphate and a moisture of the final mixture in a range of 30% - 50%

(f) Calcium carbonate of chemically pure quality (CaCO₃) is added to adjust the pH of the final mixture of the sediment to 7.0 +/- 0.5.
The source of peat, kaolin clay and sand should be known and documented. If the origin is unknown or gives level of concern, then the respective components should be checked for the absence of chemical contamination (e.g. heavy metals, organochlorine compounds, organophosphorous compounds).

The constituents of the sediment should be mixed homogenously; afterwards water, respectively the nutrient solution, should be mixed thoroughly into this sediment. The moist sediment should be prepared at least two days before use to allow proper soaking of the peat (to prevent hydrophobic peat particles floating to the surface when the sediment is overlaid with media). The moist sediment may be stored in the dark.

For the test, the sediment is filled into a suitable size contain, such as standard planting pots of a diameter which just fit into the glass vessels (the sediment should cover a minimum of 70% of the vessel bottom surface). In cases where container has holes at the bottom, a piece of filter paper in the bottom of the vessel will help to keep the sediment. The pots are filled with the nutrient containing sediment. This is covered with a very thin layer (≤ 5 mm) of an inert material such as sand or crushed corral to assist in keeping the sediment in place. standard sediment without fertilizer (in order to provide sufficient nutrients to the plants via the sediment without enriching the water with further nutrients).

Water medium
The test is conducted using Smart & Barko medium, which has been shown to provide good plant growth (but not containing phosphate and thus avoiding unwanted alga growth) and which is easy to produce (see Appendix 1 for its composition). (If another water source should be employed, it has to be demonstrated that it will allow sufficient plant growth (without disturbing algal growth) and does not interfere with the test substance.)

The pH at test initiation should be between 7.5 and 8.0 to allow optimum plant growth.

Experimental design
Three replicate test vessels are prepared for each treatment group (in general 5 test concentrations arranged in a geometric series) and six replicate test vessels are prepared for the control. Each test vessel contains one plant pot with three shoots. The individual test vessels should be impartially (respectively randomly) assigned to the different treatment groups.

A randomized design for the location of the test vessels in the growth chamber is required to minimize the influence of spatial differences in light intensity or temperature. A repositioning of the vessels in an impartial way needs also be taken into account after observations are made.

Test procedure
Due to the different inherent growth rates of *M. spicatum* and *M. aquaticum*, the test procedure varies according to the species selected for testing.

Healthy shoot tips from the culture plants are clipped off at a length of 6 cm (+/- 1 cm). As the shoot tips may differ significantly in weight, all clippings should be weighed individually and, for the test, only plants within a 30% weight range should be utilized. Five shoot tips are planted into each pot containing the sediment such that the lower 3 cm, covering two nodes, are beneath the sediment surface. Shoots are then maintained for 3 days for *M. aquaticum* or 7 days for *M. spicatum* in a nutrient-poor water to induce root development. Thereafter, two of the five plants are removed to leave three uniform (size, appearance) individuals.
A  Exposure via the water phase

Plants from five pots are harvested at test initiation (again, only using the three most homogenous individuals) and plant biomass (wet and dry weight) and length is determined to obtain respective mean biomass data for DAT 0.

The pots with the three plants are placed into the test vessels. Standard Smart & Barko medium will be added very carefully (i.e. via a funnel) in order to avoid any disturbance of the sediment. The shoot length above sediment is measured thereafter (DAT 0).

The relevant amounts of the test substance may be added to the test medium before it is added to the test vessels. Alternatively, the test substance may be introduced into the media after it has been added to the test vessels. In this case, care should be taken to ensure that the test substance is homogeneously distributed throughout the test system (providing the same volume to all vessels) without disturbing the sediment. The final water volume (1.8 L has been proven to work for the kind of test vessels described above) must be recorded and the concentrations are set up accordingly. Beakers may be loosely covered (by a transparent cover such as a glass plate) during the study to prevent evaporation. If water evaporates during the test by more than 10% (it may be helpful to mark the water level at test initiation), the water level should be adjusted with distilled water.

B  Exposure via sediment

Following the 3 or , respectively 7 day rooting phase, plants are removed from their culture vessels and shoot length and wet weight are determined individually. For the test homogenous plants (wet weight within a range of +/- 30%) are selected. Three plants with known weight and shoot length are assigned to each replicate.

Spiked sediments of the chosen concentration are usually prepared by addition of a solution of the test substance directly to the sediment (containing nutrients, see above). A stock solution of the test substance 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 substance 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 10 g of fine quartz sand for one test vessel. The solvent is allowed to evaporate and it has to be totally removed from sand; 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 substance. It should be born in mind that the volume/weight of sand used in the application of the test substance provided by the test substance and sand mixture, has to be taken into account when preparing the sediment (i.e. the sediment should thus be prepared with less sand). Care should be taken to ensure that the test substance added to sediment is thoroughly and evenly distributed within the sediment.

The spiked sediment is filled into the pots (as described above). Three individually measured plants are planted into each pot; the position of the individual plant should be marked in order to determine individual plant growth data.

Test conditions

Warm and/or cool white fluorescent lighting should be used to provide a light intensity selected from the range of about 120 (+/- 20) μE·m⁻²·s⁻¹ when measured in a photosynthetically active radiation (400-700 nm) (equivalent to about 8 -10 klux)² at the water surface and using

² 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 above.
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a light:dark ratio of 16:8 h. Any differences from the selected light intensity over the test area should not exceed the range of ± 15%. The temperature in the test vessels should be 20 ± 2 °C. 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 validity criteria are met).

Biological assessments

The minimum exposure period should be 7 days for *M. aquaticum* and 14 days\(^3\) for *M. spicatum*.\(^4\) During this time, shoot length and any other observations are recorded at least twice during the exposure period (i.e. on days 3 and 5 for *M. aquaticum* and on days 5 and 10 for *M. spicatum*). Shoot length is determined, e.g. using a ruler positioned within the vessel close to the plant to be measured. It may be necessary to straighten shoots (obviously this needs to be done without inflicting any damage to the plant) for more accurate length measurements. If side shoots are present, their numbers and length should also be measured (the value of the interim measures is mainly to show constant plant growth over time, respectively to indicate lag phases or potential recovery phases; to that extent a lower precision of the interim measurements using a ruler or visual observations within the system is comprehensible and acceptable; in general, these data will not be used for statistical evaluations).

At the end of the test, all plants are measured again (shoot length above sediment) and any growth anomalies are recorded; thereafter the whole plants are harvested. Any symptoms (such as chlorosis or necrosis) or other observations are recorded. Total plant wet weights (after carefully blotting off remaining test medium) and subsequently, total plant dry weights are determined. A visual assessment of the roots is made and any unusual findings should be recorded. A summary of the minimum biological assessments required over the test duration is provided in Table 1.

### Table 1: Assessment schedule

<table>
<thead>
<tr>
<th>Day after treatment (DAT)</th>
<th><em>Myriophyllum spicatum</em></th>
<th><em>Myriophyllum aquaticum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shoot length</td>
<td>Shoot weight</td>
</tr>
<tr>
<td>0</td>
<td>X (all plants)</td>
<td>X</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

X indicates that measurements are made on these occasions
- Indicates that measurements are not required

Environmental assessments

Light conditions, pH, oxygen levels and temperature of the water are determined at test initiation. Temperature in water (or and within the room) should be monitored over the whole test period. The pH and oxygen concentration of the test medium (water) should be checked at test initiation, at least once during the study (each 3-4 days) and at the end of the study in all replicate vessels.

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\(^3\) For compounds known to show a slow or delayed response it may be appropriate to increase the test duration by one week; the growth rate over time may indicate such a delayed response.

\(^4\) In the case of *Myriophyllum sp.* the test duration should be long enough to reach at least a doubling in shoot length.
Analytical measurements of test substance

The correct application of the test substance should be supported by analytical measurements of substance concentrations in water at test initiation and termination. Concentrations in sediment should be determined at test termination unless the test substance is stable in water (> 80% of nominal). In a spiked sediment test, test substance concentrations need to be determined at test initiation, too (and water concentrations only at test termination, in this case).

Data evaluation

Note: The following is a standard text as provided in several more recently developed OECD test guidelines. Specific recommendations considering the test details of this macrophyte study will be provided at a later stage following consultation with respective experts in this area. It will then also be discussed if and how to incorporate hormesis into the concentration/response functions.

The purpose of the test is to determine the effects of the test substance on the vegetative growth of the test species. The average specific growth rate for a specific period is calculated as the logarithmic increase in the growth variables - plant wet (and dry) weight, shoot length, numbers of side shoots and one other measurement variable - using the formula below for each replicate of control and treatments:

\[ \mu_{ij} = \ln(N_j) - \ln(N_i) / t \]

where \( \mu_{ij} \) is the average specific growth rate from time \( i \) to \( j \), \( N_i \) is the measurement variable in the test or control vessel at time \( i \), \( N_j \) is the measurement variable in the test or control vessel at time \( j \) and \( t \) is time period from \( i \) to \( j \).

For each treatment group and control group, calculate a mean value for growth rate along with variance estimates.

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. In addition, the interim growth rates during the exposure period should be assessed based on shoot length data in order to evaluate effects of the test substance occurring during the exposure period and to check whether sufficient growth occurred throughout the exposure period.

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

\[ \% Ir = \left( \frac{\mu_C - \mu_T}{\mu_C} \right) \times 100 \]

where: \( \% Ir \) is the percent inhibition in average specific growth rate, \( \mu_C \) is the mean value for \( \mu \) in the control and \( \mu_T \) is the mean value for \( \mu \) in the treatment group.

Plotting concentration-response curves

Concentration-response curves relating mean percentage inhibition of the response variable (\( l_i \)) calculated as shown above and the log concentration of the test substance should be plotted.
EC<sub>x</sub> estimation

Estimates of the EC<sub>50</sub> (EC<sub>20</sub>) should be based upon average specific growth rates (E<sub>Cx</sub>), which should in turn be based upon respective biomass data, and where relevant other additional measurement variables.

The biomass data from the additional plants measured at DAT 0 provide the initial data, which are compared to the mean values per test vessel at the end of the study.

Remarks to statistical procedures

The aim is to obtain a quantitative concentration-response relationship, which is usually done 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, 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. It should be noted that standard methods of analysis using probit, logit, or Weibull transforms are usually intended for use on quantal (e.g. mortality or survival) data; in that case they should be modified to accommodate growth rate or yield data.

For each response variable to be analysed, use the concentration-response relationship to calculate point estimates of EC<sub>x</sub> values. When possible, the 95% confidence limits for each estimate should be determined. Goodness of fit of the response data to the regression model should be assessed either graphically or statistically. Regression analysis should be preferably performed using individual replicate responses, not treatment group means.

EC<sub>50</sub> estimates and confidence limits may also be obtained using linear interpolation with bootstrapping, if available regression models/methods are unsuitable for the data.

General Remarks

This test method gives the option to test different species (similar to other OECD guidelines, such as the other aquatic plant tests, OECD 201 and OECD 221). The specific details of the method apply to the named Myriophyllum species. However, it has been shown that the principle of the method also works for other aquatic plant species and even for emerged aquatic plants such as Glyceria sp.. However, certain modifications of vessel size and test duration may be needed.

Previous studies have shown, that both species of Myriophyllum (M. aquaticum and M spicatum) provide comparable results. However, in order to achieve the required growth during the study period, the plants used for the test have to be of good, healthy quality (before flowering as growth is limited in the flowering phase) and should be in an actively growing phase. The quality of the test depends more on the quality of the plants used than on the selection of either species.

Previous studies have also shown, that a pre-adaptation phase may not be necessary for the spiked water test. Allowing the pre-adaptation and prior root development will make the study more realistic and often also more robust. However, if this is not required, the study may also be conducted without the prior-rooting phase.
Reference substance
As reference substance 3,5-DCP is proposed. The expected endpoint based on weight should be in the range of $E_{C_{50}} = 2 - 8 \text{ mg/L}$.

Appendices

Appendix 1
Preparation of Smart & Barko Medium (1985)

<table>
<thead>
<tr>
<th>Amount of reagent added to water</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl$_2 \cdot 2$ H$_2$O</td>
</tr>
<tr>
<td>MgSO$_4 \cdot 7$ H$_2$O</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
</tr>
<tr>
<td>KHCO$_3$</td>
</tr>
</tbody>
</table>

pH (air equilibrium) – 7.9

Appendix 2, Pictures of set up

Appendix 3
Potential plant suppliers:
Jörg Petrowsky, Anschautiche, D-29348 Eschede, Germany (petrowsky.wasserpflanzen@t-online.de, www.repo-pflanzen.de)
Zoo Schnack Genin, Wakmühlenweg 1-3, D-23560 Lübeck, Germany (info@zoo-schnack.de)
Aquaflora B.V., Uitweg 25, 3645 TA Vinkeveen, The Netherlands
Telefon: +31-297-286-709, Fax: +31-297-289-256, E-mail: info@aquafloia.nl, www.aquafloia.nl.

Please, provide further addresses (particularly for the US, too)!