AMRAP – workgroup

Myriophyllum sp.
Test Methodology for a Rooted Aquatic Macrophyte

AMRAP – workgroup: method development

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Myriophyllum sp. - Test Methodology

Background

Risk Assessment for aquatic plants is usually done using a limited number of tests systems. For pesticides RA, in the US generally 5 aquatic plant tests are required (green alga, blue-green alga, marine and freshwater diatom, higher aquatic plant (Lemna));
in the EU for herbicides two alga species and the higher aquatic plant test.

Concern has been raised whether the information obtained is sufficient (sufficiently protective) for aquatic plant RA.

Brock et al. (2000) showed, that the RA presently used in the EU (lowest endpoint from aquatic plants plus factor of 10) is generally sufficiently protective; however, they observed one exception (auxin type herbicide).

In addition, there was a concern that exposure via sediment might be underestimated from substances with strong adsorption properties.

Myriophyllum sp. - Test Methodology

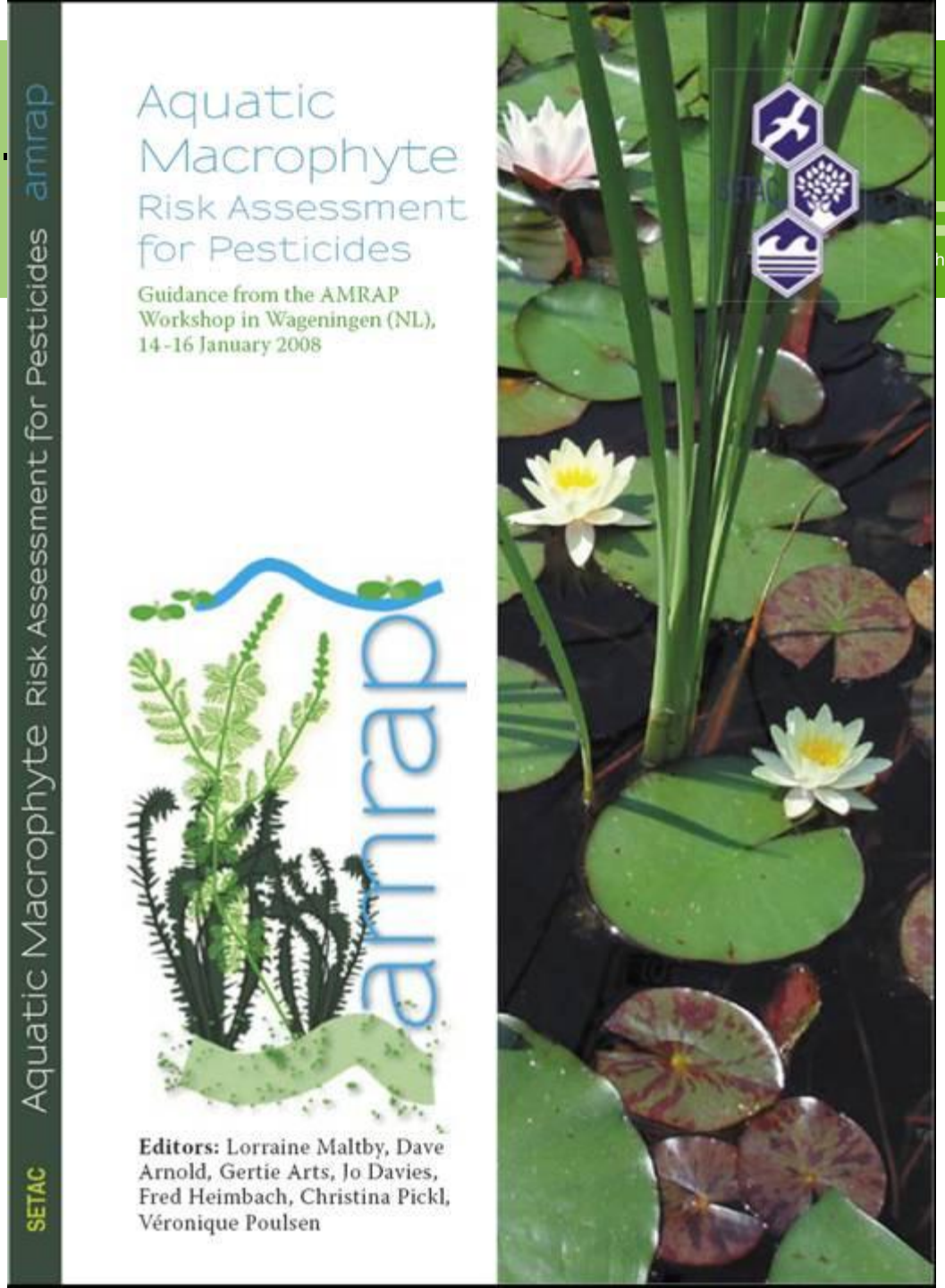
Background

In conclusion: Concerns have been raised that RA based on algae and Lemna may not be protective of other macrophyte species due to potential differences in exposure route, recovery rate, or sensitivity to specific toxic modes of action.

Myriophyllum sp.

Background

To address these issues, the SETAC workshop "Aquatic Macrophyte Risk Assessment for Pesticides", AMRAP, was held in 2008.



Myriophyllum sp. - Test Methodology

Background

In conclusion: Concerns have been raised that RA based on algae and Lemna may not be protective of other macrophyte species due to potential differences in exposure route, recovery rate, or sensitivity to specific toxic modes of action.

To address these issues, the SETAC workshop "Aquatic Macrophyte Risk Assessment for Pesticides", AMRAP, was held in 2008. Aims were

- To identify uncertainties for macrophyte RA in the regulatory framework
- Make recommendations how to address uncertainties, e.g. propose
  - if and when to do additional macrophyte testing
  - how to perform such additional macrophyte testing

Myriophyllum sp. - Test Methodology

Background

- Make recommendations how to address uncertainties, e.g. propose
  - if and when to do additional macrophyte testing

Recommendation in short:

"Conduct an additional test with a rooted macrophyte if there is a lack of expected herbicidal activity in Tier 1 algae and Lemna tests or where exposure via sediment may be a critical factor in the risk assessment."

Myriophyllum sp. - Test Methodology

Background

- Make recommendations how to address uncertainties, e.g. propose
  - if and when to do additional macrophyte testing
  - how to perform such additional macrophyte testing

A workgroup was established for:

...the development and ring-testing of a study design using *Myriophyllum* sp. as potential additional test species

Myriophyllum sp. - Test Methodology

Test species

Myriophyllum sp. as potential additional test species

- Fullfills a number of requirements (based on AMRAP identified needs)
- Is a rooted aquatic macrophyte
- Is from a different taxonomic group (dicot)
- Is amenable for laboratory testing
- Is of some ecological relevance
- Is sensitive
- Is sufficiently fast growing

The suitability as test species has been shown in previous investigations (Kubitza & Dohmen, 2002, 2008); Poovey & Getsinger, 2005; Knauer et al., 2006; Knauer & Mohr & Feiler, 2008)

Myriophyllum sp. - Test Methodology

Test species

Myriophyllum sp. as potential additional test species

A number of different *Myriophyllum* species have been previously tested:

- *M. spicatum*
- *M. aquaticum*
- *M. sibiricum*
- *M. heterophyllum*
- *M. verticillatum*

Most (positive) experiences with *M. spicatum* and *M. aquaticum*

Myriophyllum sp. - Test Methodology

Test species

Pros and cons of the test species:

	Pros	Cons
M. aquaticum	- easy to maintain, handle and propagate - fast growing species (biomass doubling in 5-7 days) - less problems with interacting factors and disturbances - readily available	-has been less frequently used as test species before - availability maybe more difficult in some countries (UK) - tends to grow above water surface
M. spicatum	- has been used as test species before in several research publications - will generally grow submersed - sufficiently fast growing (biomass doubling in 10-14 days)	- needs longer test duration - increased potential for disturbances (e.g. unwanted algal growth) - less readily available throughout the year

In principle, both species are suitable !

Myriophyllum sp. - Test Methodology

Test method

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Principle

assess substance-related effects on the vegetative growth of the aquatic plant in standardised media (water, sediment and nutrients) containing different concentrations of a test substance.

Individual shoot tips of healthy plants are potted in an artificial standard sediment 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, simulate exposure via the sediment by spiking the artificial sediment). 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.

Myriophyllum sp. - Test Methodology

Test method

Species:

Myriophyllum aquaticum* or *M. spicatum

Only visibly healthy plants, without flowering shoots, should be used for the study. Plants should be visibly free of other species (particularly snails or filamentous algae can be a problem; some level of epiphytes - such as diatoms - may often not be avoidable and will generally not be a problem).

Myriophyllum sp. - Test Methodology

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Species

Myriophyllum spicatum or *M. aquaticum*

Myriophyllum sp. - Test Methodology

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Species

Myriophyllum aquaticum or *M. spicatum*

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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. Other species may need modifications of vessel size, water depth, study duration etc.

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Test vessels

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 submersed 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.

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Sediment

The following formulated sediment, based on the artificial sediment used in OECD Guideline 219, is recommended for use in this test; the sediment is prepared per the guideline except for the additional nutrients as described below:

- 4-5 % peat, 20 % kaolin clay, 75-76 % quartz sand
- An aqueous nutrient medium is added such that the final sediment batch contains 300 mg/L sediment of both ammonium chloride and sodium phosphate

The sediment is filled into a suitable size container, 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). This is covered with a very thin layer (≤ 5 mm) of an inert material such as quartz sand (or crushed corral) to assist in keeping the sediment in place.

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Test method

Sediment

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Water medium

The test is conducted using **Smart & Barko** medium, which has been shown to provide good plant growth (but not containing phosphate or nitrogen and thus avoiding unwanted alga growth) and which is easy to produce.
(If another water source should be employed, it has to be demonstrated that it will allow sufficient plant growth (without promoting 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.

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Experimental design

In general, 5 test concentrations arranged in a geometric series.

Three replicates for each treatment group and six replicates for the control

Each test vessel contains a plant pot with three individual shoots.

The 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.

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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 culture plants are clipped off at a length of c. 6 cm (+/- 1 cm).

Clippings should be weighed individually, plants within a 30% weight range should be utilized.

Five shoot tips are planted into each pot such that the lower 3 cm, covering two nodes, are beneath the sediment surface.

Shoots are maintained for 3 days for *M. aquaticum* or 7 days for *M. spicatum* to induce root development. Thereafter, two of the five plants are removed to leave three uniform (size, appearance) individuals.

Myriophyllum sp. - Test Methodology

Test method

A: Exposure via the water phase

Plants from five additional pots are harvested at test initiation and plant biomass (wet and dry weight) and length is determined to obtain respective mean biomass data for DAT 0.

The treatment group pots with the three plants are placed into the test vessels (one pot per vessel). 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).

Myriophyllum sp. - Test Methodology

Test method

B: Exposure via sediment

Following the 3, respectively 7 day rooting phase (depending on the test species) 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 sediment (range of concentrations) is filled into the pots. Three individually measured plants are planted into each pot; the position of the individual plant should be marked in order to be able to determine individual plant growth data.

Myriophyllum sp. - Test Methodology

Test method

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Test conditions

Warm and/or cool white fluorescent **lighting** should be used to provide a light intensity in the range of about **160 (+/- 20) µE·m-2*s-1** when measured as a photosynthetically active radiation (400-700 nm) (equivalent to about 8 - 10 klux) at the water surface and using 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).

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Test method

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Biological assessments

The minimum exposure period should be 7 days for *M. aquaticum* and 14 days for *M. spicatum* (the test duration should be long enough to reach at least a doubling of shoot length). During this time, shoot length and any other observations are recorded at least twice during the exposure period. Shoot length is determined, e.g. using a ruler positioned within the vessel close to the plant to be measured.

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Test method

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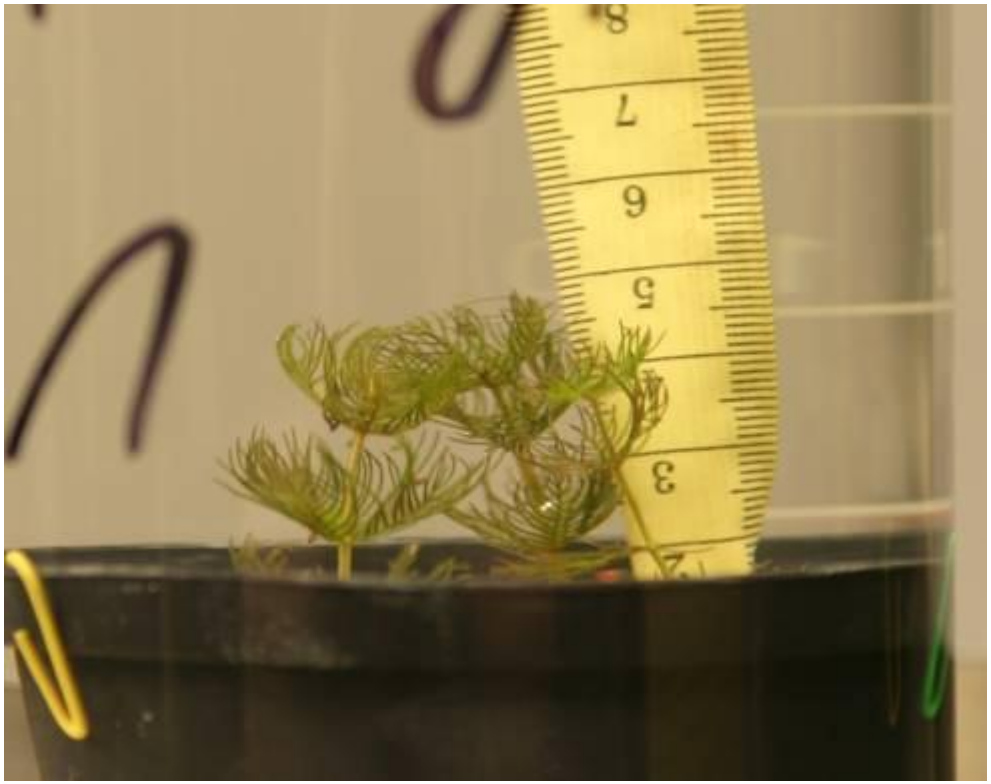
Biological assessments



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Test method

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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.

Myriophyllum sp. - Test Methodology

Test method

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Biological assessments

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.

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Test method

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Physical, chemical assessments

Light conditions, pH, oxygen levels and temperature of the water are determined at test initiation. Temperature in the test medium (or/and within the room) should be monitored over the 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.

Correct application should be supported by analytical measurements of test substance concentrations in water at test initiation and termination. Sediment conc. should be determined at test termination unless the test substance is stable in water (> 80% of nominal) in studies where water was spiked. In a spiked sediment test, test substance concentrations in the sediment need to be determined at test initiation and termination (and water concentrations only at test termination).

Myriophyllum sp. - Test Methodology

Test method

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Data evaluation

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:

$$\mu i\text{-}j = \ln(Nj) - \ln(Ni) / t$$

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Test method

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Data evaluation

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. For each test concentration and control, calculate a mean value for average specific growth rate along with the variance estimates.

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

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

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Test method

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Data evaluation

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

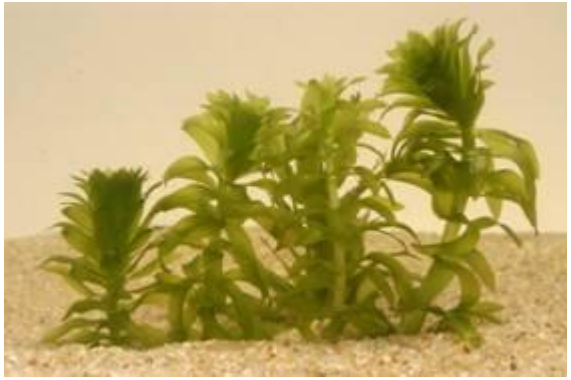
Estimates of the EC50 (EC20) should be based upon average specific growth rates (ErCx), which should in turn be based upon respective biomass data, and where relevant other additional measurement variables.

Myriophyllum sp. - Test Methodology

Test method

- **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.



Myriophyllum sp. - Test Methodology

Test method

- **General Remarks**

Previous studies have shown that both species of *Myriophyllum* 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.

Myriophyllum sp. - Test Methodology

Method test

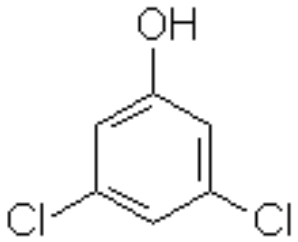
- First orientating ring-test with both Myriophyllum species was conducted last year

  - Alterra WUR NL
  - BASF
  - Bayer Crop Science
  - Biochem Agrar
  - Chemex
  - eurofins-GAB
  - Ibacon
  - MSU
  - NOACK
  - Springborn Smithers
  - UBA
  - USACE

Myriophyllum sp. - Test Methodology

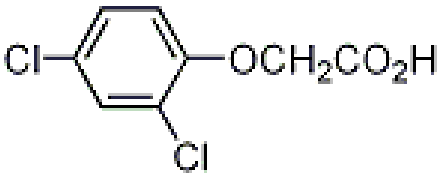
Method test

Two reference compounds:



- 3,5-dichlorphenol, 3,5-DCP
- Unspecific (narcotic) mode of action
- Reference substance for biotesting (e.g. for *Lemna)*

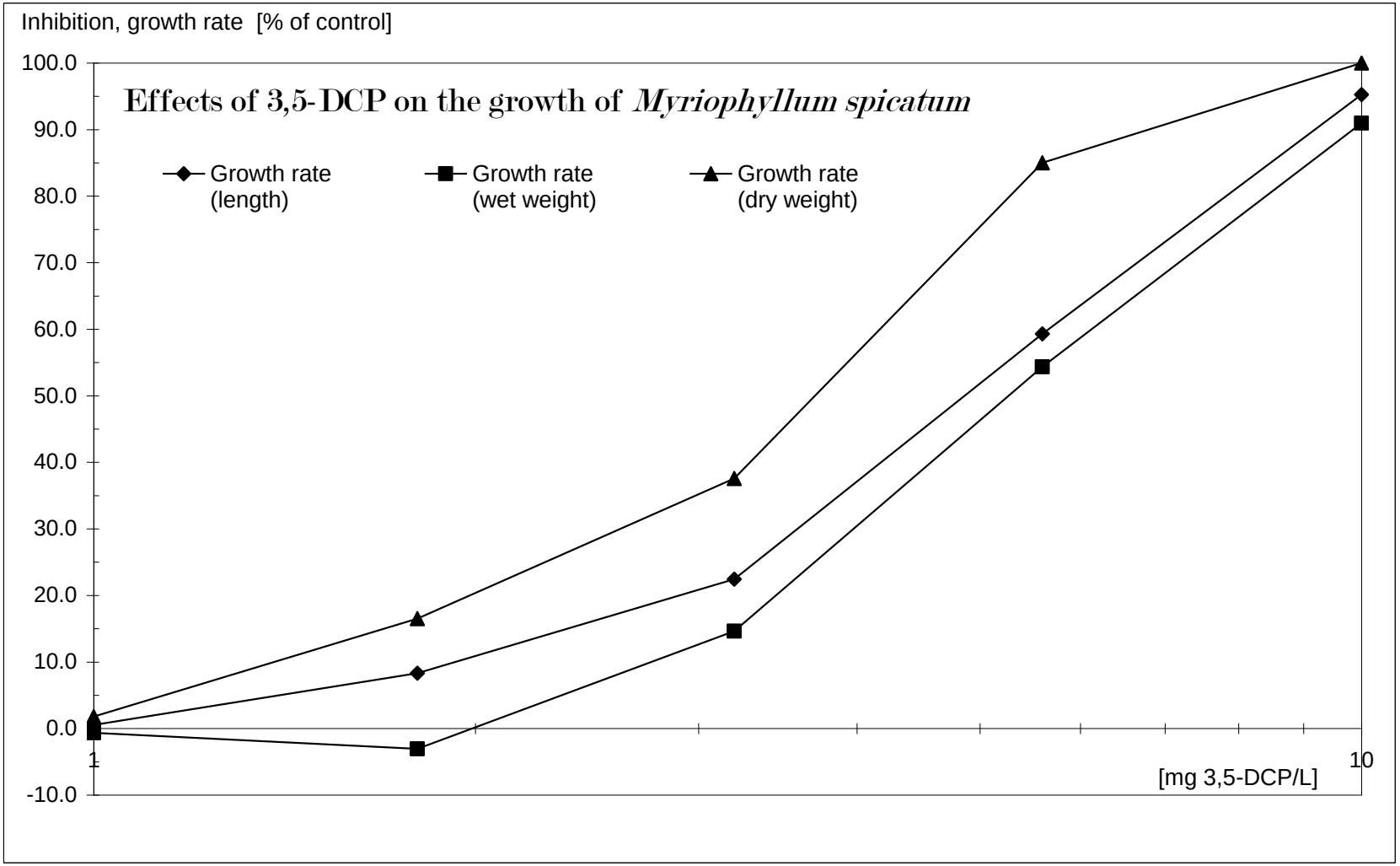
- 2,4-D
- Specific mode of action: synthetic auxin (plant growth hormone)
- selective systemic herbicide (broad-leaved weeds)
- accumulation at meristematic regions
- high water solubility



2,4-D

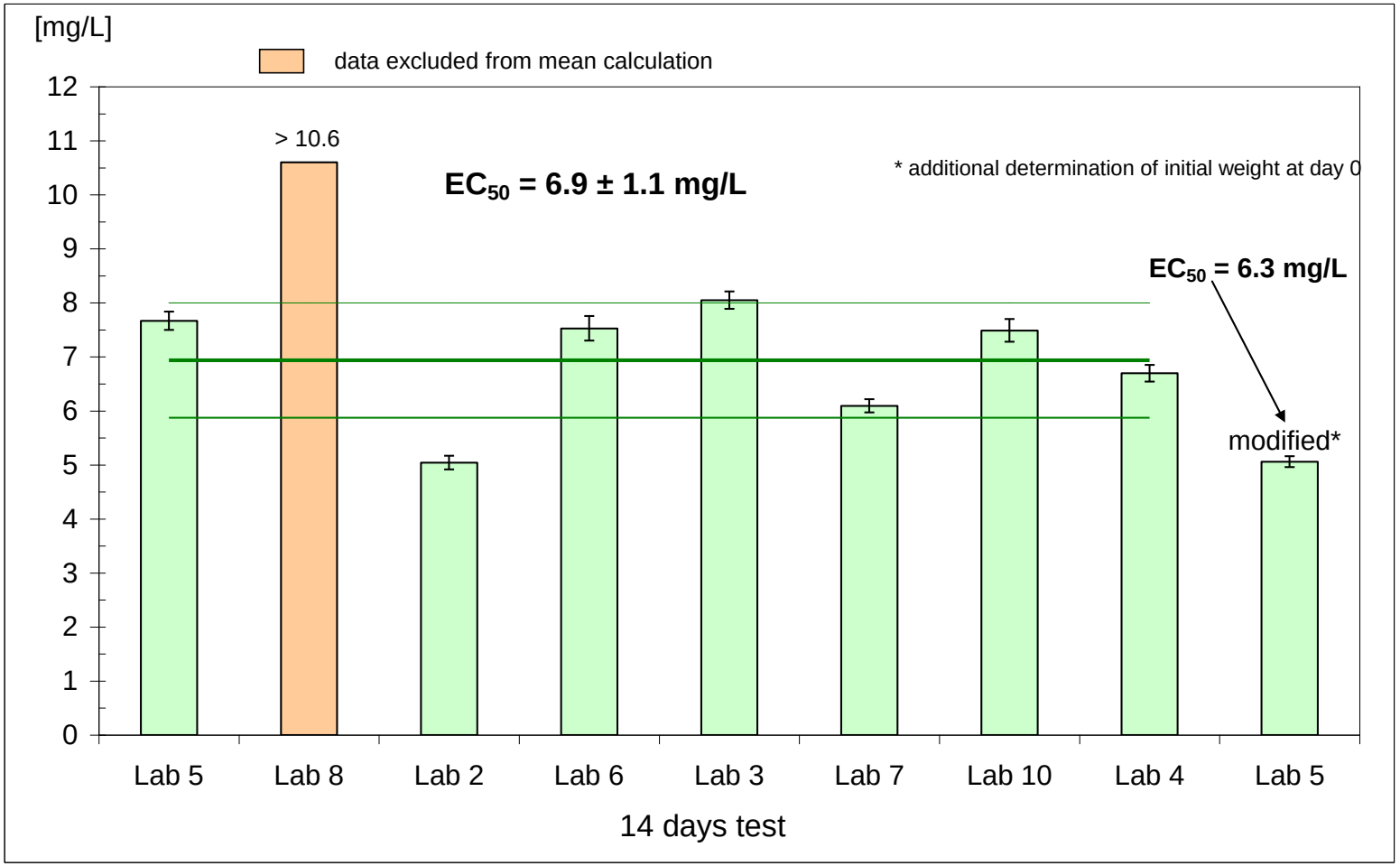
Myriophyllum sp. - Test Methodology

Method test



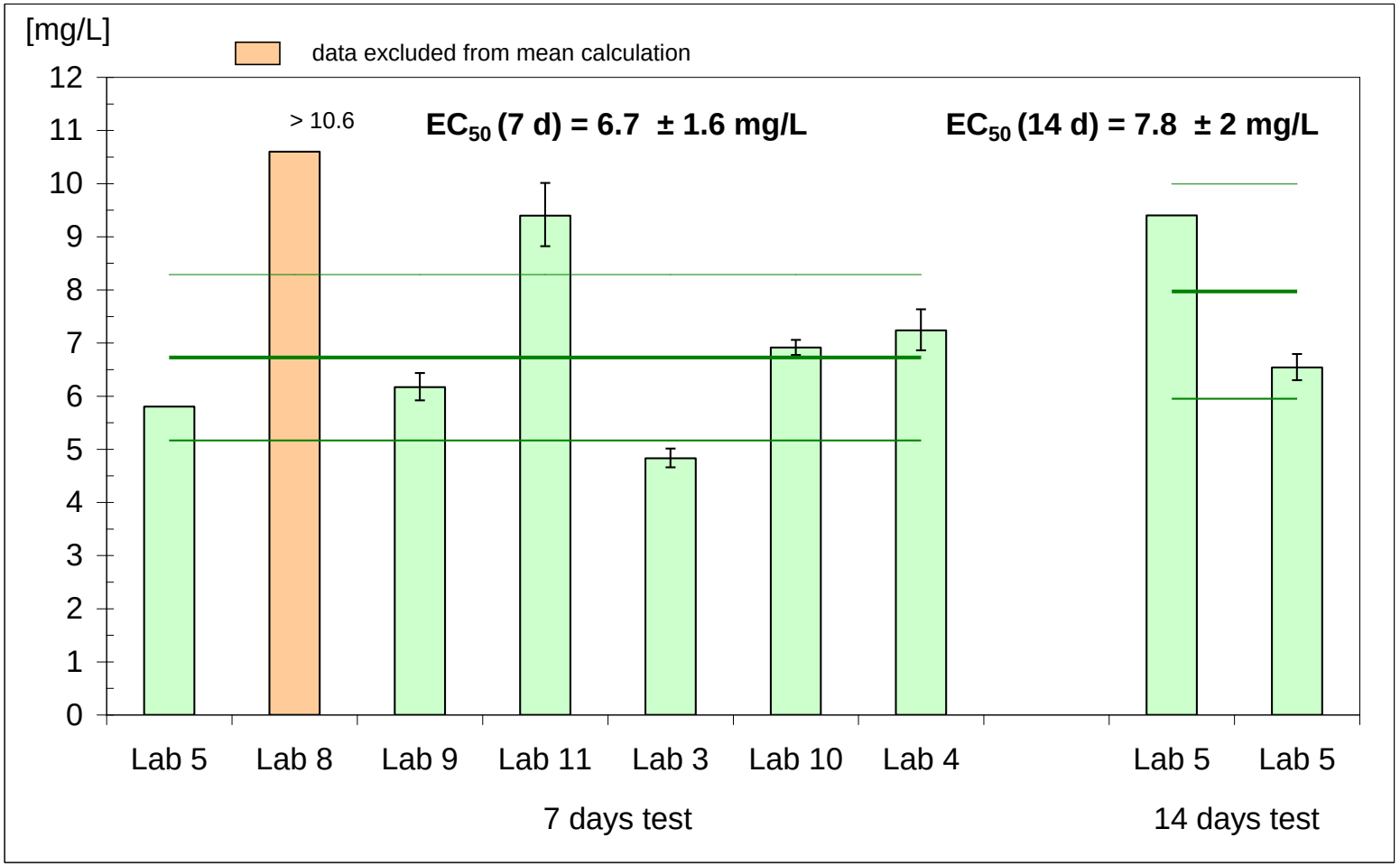
Myriophyllum sp. - Test Methodology

3,5-DCP; *Myriophyllum spicatum* with rooting phase - shoot length, growth rate



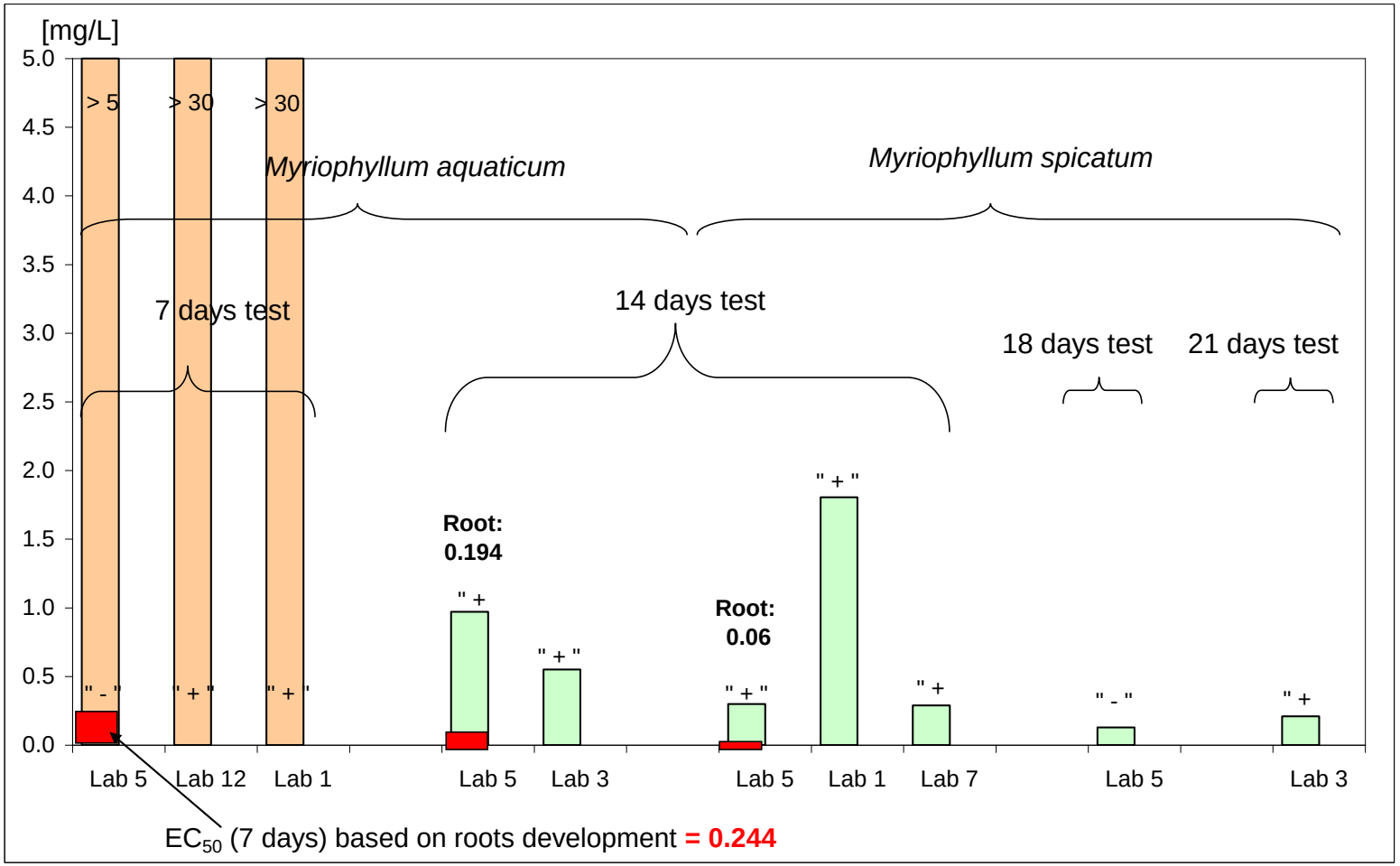
Myriophyllum sp. - Test Methodology

3,5-DCP; *Myriophyllum aquaticum* with rooting phase - shoot length, growth rate



Myriophyllum sp. - Test Methodology

2,4-D; *Myriophyllum aquaticum* and M. spicatum - shoot length, growth rate



Myriophyllum sp. - Test Methodology

Method test

Summary of results – *Myriophyllum spicatum*: EC50values (14 d) for 3,5-DCP

Parameter	mean EC_{50} value - rooting phase	mean EC_{50} value - no rooting phase
shoot length (growth)	6.9 (5.0 – 8.1)	5.4 - 6.9
shoot length (yield)	6.2 (3.9 – 7.4)	4.9 – 6.9
wet weight (growth)	6.8 (4.0 – 8.8)	5.3 – 5.9
wet weight (yield)	7.1 (4.0 – 9.7)	4.2 – 5.7
dry weight (growth)	5.1 (3.3 – 6.2)	6.2
dry weight (yield)	5.7 (3.2 – 9.4)	5.4 – 6.7

Myriophyllum sp. - Test Methodology

Method test

Summary of results – *Myriophyllum aquaticum*: EC50values (7 d) for 3,5-DCP

Parameter	mean EC_{50} value - rooting phase	mean EC_{50} value - no rooting phase
shoot length (growth)	7.3 (4.8 – >10.6)	10
shoot length (yield)	5.2 (4.8 – >10.6)	6.8 – 8.3
wet weight (growth)	5.8 (2.8 – 9.8)	6.5 - 10
wet weight (yield)	4.4 (2.8 – >10.6)	5.8 - >10.
dry weight (growth)	2.7 (1.3 – 5.3)	5.5 – 6.7
dry weight (yield)	3.3 (1.2 – 4.9)	6.0 – 6.6

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Test method

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- **Conclusions**

The studies (pre-ring test) have shown that the proposed test methodology apparently is well applicable providing very reasonable results with comparatively low variability already at the pre-ring testing phase.

Both species of *Myriophyllum* provide comparable results and appear to be equally suitable. The quality of the test actually depends more on the quality of the plant material used than on the selection of either species.

Myriophyllum sp. - Test Methodology

Test method

- **Outlook**

We are planning a final full ring-test (further participants and very welcome)

The results of the final ring-test will indicate if further changes / modifications of the proposed test method are indicated.

It is further planned to develop this method into an OECD test guideline.