Myriophyllum spicatum toxicity test: Design and first results of an inter laboratory ring test using a sediment-free test system.

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Introduction

The eco toxicological laboratory of the German Federal Environment Agency has organized an **inter laboratory ring test** in order to investigate and to optimize a new test method with the dicotyledonous water milfoil *Myriophyllum spicatum* which has been established at the German Federal Environment Agency (Maletzki et al. 2010).

Typical characteristics of the test system include:
- sediment-free, plants grown one by one and under sterile conditions

Twelve laboratories participated in the ring test running from October 2010 to April 2011. The ring test aimed at:

1.investigating the practicability and reproducibility of the sterile, sediment-free test system,
2.identifying the most appropriate endpoints reflecting different modes of action of the test items and
3.optimizing and standardizing the test method in order to obtain a efficient test system for dicotyledonous macrophytes.

In a first step the **general practicability** of the test was investigated and the valid control results were evaluated concerning the intra- and inter-laboratory **reproducibility** and **variability** of the test system and endpoints.

Material and Method

Test conditions:	14 days exposure, plants grown one by one, sterile medium replaced after 7 days, at 23±2°C, alternating 16/8 hour light/dark phases, light intensity 100-150 µE $m^{-2}s^{-1}$
Test items:	• 3,5-dichlorophenol (3,5 DCP, pesticide with narcotic action, reference substance for OECD 201 and OECD 221) • 2,4-dichlorophenoxyacetic acid (2,4 D, auxine herbicide, growth inhibitor) • Isoproturon (IP, photosynthesis inhibitor).
Toxicity parameters:	NOEC, ECx and 95%CI
Statistical design:	Control with 10 replicates, 8 treatments with 5 replicates, each
Response variables:	**Measured:** shoot length (SL), fresh weight (FW), dry weight (DW), number of whorls (W), number and length of lateral branches (LB, LBL), number and length of roots (R, RL). **Calculated:** total shoot length (TSL), total root length (TRL), Yields (YSL, YFW, YDW, YW), Growth Rates (GrSL and GrTSL)
Validity criteria: (preliminary for this ring test)	1. shoot length of the control plants should be at least doubled within test duration 2. number of non-sterile control replicates must not exceed 50% 3. number of non-sterile replicates per treatment must not exceed 50% Definition of non-sterility: visible turbidity

Results

Data base

	3,5 DCP	2,4 D	IP	Total tests	Total number of control replicates
Number of tests performed	12	10	8	30	300 (273 sterile)
Number of tests valid	11	7	7	25	250 (233 sterile)

Practicability

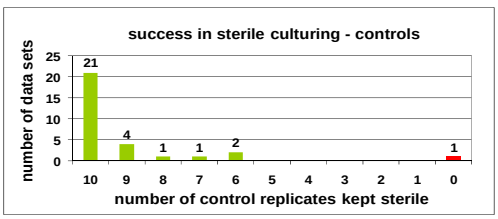

Fig. 1: Success in sterile culturing of controls

Fig. 2: Control group (10 replicates) after 14 days of cultivation

Total test validity: 4 tests with shoot length not doubled; 1 test with > 50% non-sterile replicates in control→ 30 tests performed, 25 valid tests obtained;

Single treatment validity: 14 single treatments in 8 tests with > 50% non-sterile replicates (i.e. not valid), usually observed in high test concentrations.

Reproducibility of response variables

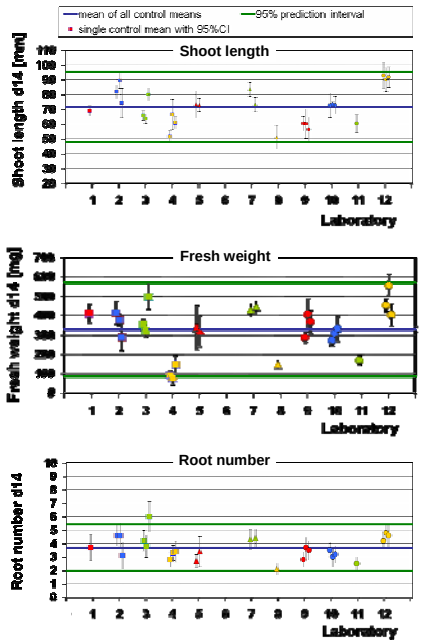

Fig. 3: Single control results per test and laboratory (mean and 95% CI) compared to overall mean and its´ prediction interval (based on all valid control means, n=25); selected response variables shown: shoot length, fresh weight, root number

Inter-laboratory variability exceeds intra-laboratory variability up to a factor of two.

Variability of response variables

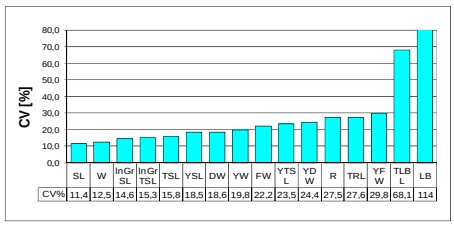

Fig. 4: Coefficients of variation (CV) for different response variables; means of single CVs obtained in all valid controls (n=25)

Coefficients of variation ranged between 10- and 30%, except for lateral branches (LB). LB seemed to be produced by chance – occurring only in about 50% of all control replicates; from these 70% produced only 1 or 2 LB.

In contrast, **roots** were produced in all control replicates and root number was close to normal distribution (Shapiro Wilks Test, p = 0,743; mean = 3,7; CV% = 27,5%).

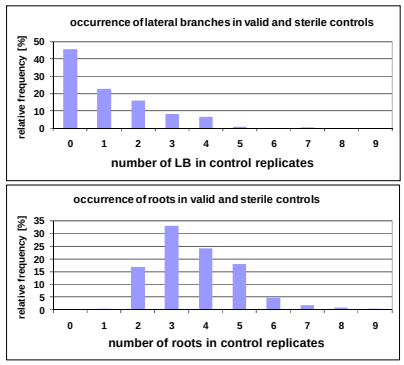

Fig. 5: Occurrence of lateral branches (5a) and roots (5b) in controls

Discussion and Conclusion

A sediment-free test system was chosen with a low degree of methodical and analytical complexity to get toxic threshold and effect concentrations being independent of the water sediment-partition of the test item. Also, a direct comparison of the results obtained with the duckweed *Lemna spec.* (OECD 221) is possible.

Practicability: Although 11 of the 12 participants didn´t manage to perform *Myriophyllum spicatum* culturing under sterile conditions previously, a critical number of non-sterile replicates (> 50 %, see validity criteria) only occurred in one control data set (of 30 controls cultured) and in 14 treatment data sets (of 240 cultured). With respect to the latter it still remains to be clarified whether the turbidity in higher concentrations is due to plant degradation, i.e. to a toxic effect rather than to contamination. Summarizing, even under the assumption that in some laboratories tests have been repeated to fulfil the sterile conditions, the results indicate that generally it is possible to conduct the test under sterile conditions.

In 4 of 30 tests performed doubling of shoot length failed. This is possibly due to insufficient growth-conditions (irradiation, temperature). It remains to be investigated whether varying growth conditions affect the toxicological parameters (ECx, NOEC) and whether the validity criteria hold under these circumstances (e.g. total shoot length instead of shoot length or reducing the shoot length factor).

Response variables variability and reproducibility: For all variables the inter-laboratory variability clearly exceeds the intra-laboratory variability. This indicates effects of (slightly) varying test conditions (irradiation, temperature…) and variations in handling (e.g. fresh weight measurement). Although this does not necessarily affect the toxicological results (yet to be evaluated). It appears that the test procedure and conditions could be optimized in order to further reduce the inter- and intra-laboratory variability.

Except for the number and length of lateral branches (LB, LBL), in all remaining variables the intra-laboratory coefficients of variation ranged from 10 to 30%. Thus the given statistical design allows to detect effects in the range of 15% to 35%. LB and LBL are seen as not appropriate as separate response variables, but are essential to calculate total shoot length and therefore are worth to be measured.

Concluding, the comprehensive data set of endpoints under control conditions, obtained from an inter-laboratory ring test, allowed to demonstrate the general practicability of the test system and to show an acceptable reproducibility.

The final evaluation will include a more detailed assessment of the test system with respect to test conditions, validity criteria, appropriate endpoints, sensitivity to various test items and reproducibility of toxicological parameters (NOEC, ECx).

Acknowledgment
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References

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