Bull Environ Contam Toxicol (2007) 78:421–426
DOI 10.1007/s00128-007-9131-9

Testing the Use of the Water Milfoil (*Myriophyllum spicatum* L.) in Laboratory Toxicity Assays

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Received: 11 January 2007 / Accepted: 16 March 2007 / Published online: 11 May 2007
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Abstract Tests aiming to determine the toxic properties of compounds discharged into aquatic systems have relied more on fish or invertebrates than on primary producers and among a number of producers; algae are the most popular test organisms. Macrophytes are important ecological elements in freshwaters and are therefore potentially key organisms for use in toxicity testing of compounds suspected of acting in primary producers. The most common macrophyte used in toxicity testing is *Lemna* sp., but as a floating plant, it has the limitation of being exposed to toxic compounds only through its lower leaf surface, including roots and rhizoids. Therefore, it is questionable whether tests with *Lemna* may accurately predict potential effects on submersed and exposed plant species, which have different routs of exposure and morphology. Few other submersed macrophytes have been tested, notably *Myriophyllum*.

In the Iberian peninsula *M. spicatum* is the most common species within its genus and has been presented as a good bioaccumulator of heavy metals (Wang et al. 1996) and as being sensitive to several toxicants (e.g. Hanson et al. 2003). The aim of this study was to assess the potential of *M. spicatum* as a testing organism in laboratory assays, by obtaining axenic cultures of this plant and exposing them to several reference compounds to determine the sensitive endpoints.

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Materials and Methods

Stems of *M. spicatum* were collected from the relatively pristine Carreiras River, in the Guadiana basin, South Portugal, and were kept in outdoor tanks at the University of Coimbra. To obtain axenic cultures, we followed the modified general procedure described in the American Society for Testing and Materials E1913–97 guide for *M. sibiricum* (2000). Plants were rinsed in deionized water and nodal segments were disinfected in a 3% (w/v) sodium or calcium hypochlorite solution containing 0.01% Tween-20 for periods of 20 minutes for three consecutive days. Between sterilization periods, plants were maintained in the dark under agitation (60 rpm) in Andrews modified medium (Selim *et al*. 1989, in ASTM 2000) with 3% sucrose. Apical segments of 3 cm were cut, weighed (fresh mass ± 0.001 g) and the number of nodes counted. Plant segments were inoculated into 200 ml of sterile Andrew media containing the testing compound in the conditions described by Ferreira and Graça (2002; Table 1).

The following compounds were tested: calcium, iron and copper sulphates, glyphosate and a mining effluent. The sulphates were chosen because of their high concentrations in the tested effluent and other mine effluents (e.g. Coimbra et al. 1996). Copper sulphate has also been related to mining and industrial pollution and the effect of copper on macrophytes and other plants is well documented (Guilizzoni 1991; Roshon et al. 1999). Glyphosate (N-phosphonomethylglycine), a non-selective post-emergent herbicide was tested as its major formulation, Roundup®, in which glyphosate is included as a isopropylamine salt and polyoxyethylene amine is present as a surfactant (Tsui & Chu 2003). We also tested a mine effluent with a low content of heavy metals, but high conductivity, sulphate

Table 1 Summary of the culture conditions for tests on macrophyte *Myriophyllum spicatum*

Agitation	Constant, 100 rpm
Temperature	25°C
Photoperiod	14h light:10h darkness. 96 μm m^{-2} s^{-1}
Test chamber	250 ml Erlenmeyer
Test volume	200 ml
Initial organisms size	3 cm apical shoots
Replicates	5
Control and culture media	Andrews modified medium
Test duration	21 days
Endpoints	Fresh weight, shoot length, node number, root length

Table 2 Concentrations used for four tested compounds and one industrial effluent

Compound tested	Concentration (mg/l or %)
$CaSO_4.2H_2O$	10000; 5000; 2500; 1250; 600; 300; 150; 70; 35
$FeSO_4.7H_2O$	2500; 1250; 600; 300; 150; 110; 70; 45; 25
$CuSO_4.5H_2O$	40; 20; 10; 5; 2.5; 1.8; 1.2; 0.6
Glyphosate	80; 40; 20; 10; 5; 2.5; 1; 0.5; 0.25
Mining effluent	5%; 10%; 20%; 40%; 60%; 80%

and nitrate levels, and high pH (see Ferreira and Graça 2002).

The media was changed weekly only in the case of the effluent. Concentrations for each tested compound were established from the literature and previous experiments and are summarized in Table 2. After 21 days, plants were measured for final plant length, node number (except calcium sulphate), fresh mass and root length. The concentration that inhibited 50% of the parameter (IC_{50}) and the percentage of inhibition (%I) were calculated according to American Society for Testing and Materials procedures (2000; E 1913 – 97 procedures). Square root, logarithmic or reciprocal transformations were used where appropriated to guarantee the highest correlation using a linear model.

Results and Discussion

In control conditions, *M. spicatum* grew 0.019 g/day or 0.79 cm/day, which corresponds to an increase of 29% in mass and 26% in length. Under control conditions, all specimens developed roots (n = 1–8) with a total length of 17.8 cm (range 11.8 to 33.8 cm).

Calcium sulphate stimulated root growth of *M. spicatum* in concentrations ranging from 0.07 to 0.60 g/l; concentrations of 0.6 g/l inhibited weight increase while concentrations of 1.25 g/l and higher inhibited length and root growth (Fig. 1).

Iron sulphate was more toxic for *M. spicatum* than calcium sulphate, affecting total length, weight and node

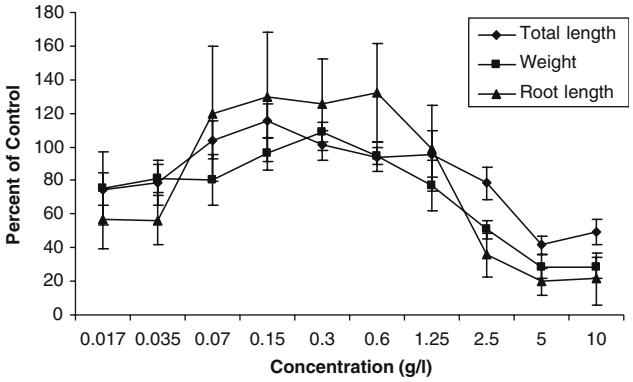

Fig. 1 Total length, weight and root length of *M. spicatum* after 21 days culture with calcium sulphate. Mean and SE; n = 5

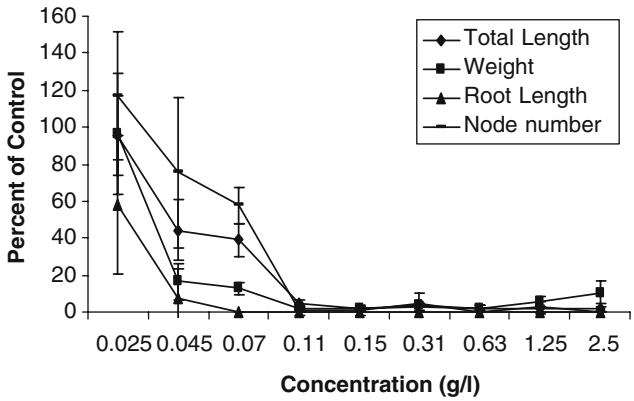

Fig. 2 Plant and root length, weight, and node number of *M. spicatum* after 21 days culture exposed to iron sulphate. Mean and SE; n = 5

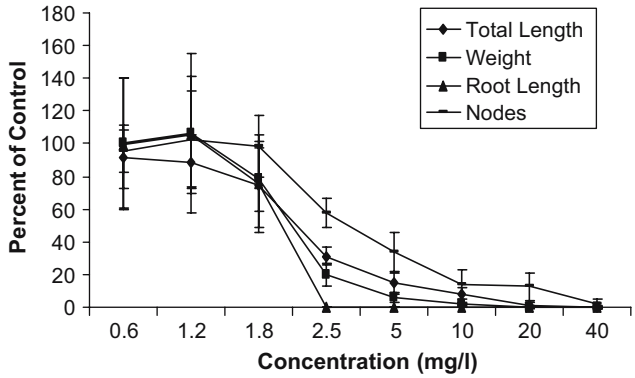

Fig. 3 Total length, weight, number of nodes and root length of *M. spicatum* after 21 days culture with copper sulphate. Mean and SE; n = 5

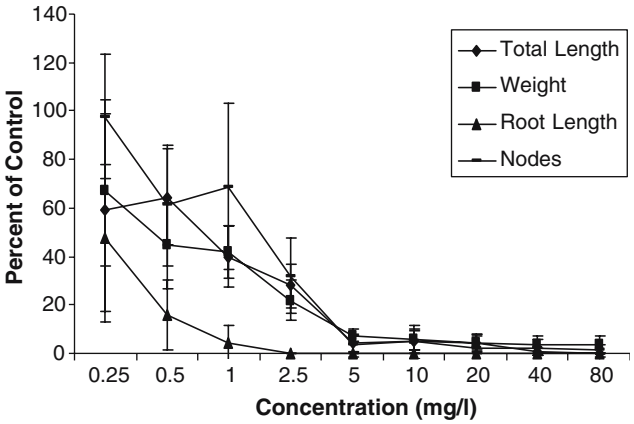

Fig. 4 Total length, weight, root length and node number of *M.spicatum* after 21 days culture in glyphosate. Mean and SE; $n = 5$

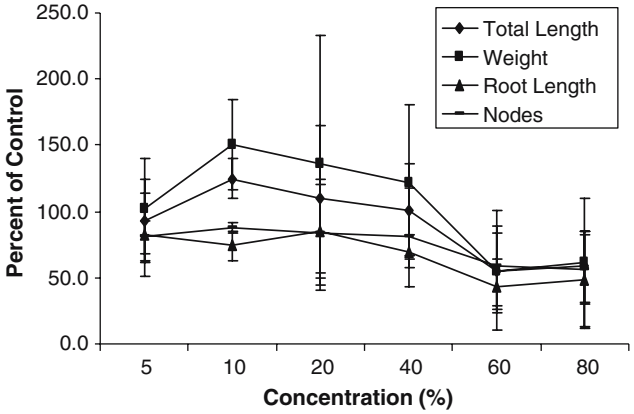

Fig. 5 Total length, weight, root length and node number of *M. spicatum* after 21 days exposition to a mining effluent. Mean and SE; $n = 5$

number at concentrations of 0.045 g/l. Root length was already inhibited by the lowest test concentration (0.025 g/l; Fig. 2). All plants died in concentrations of 0.11 g/l. In all the treatments, roots were necrotic. At concentrations of 0.15 g/l and above the plants had precipitations of iron on their surface, which explains the weight increase. Copper sulphate inhibited length increases at the lowest concentration of 0.6 mg/l. Inhibition of weight increase and root length occurred at concentrations above 1.2 mg/l and the number of nodes was inhibited at concentrations above 1.8 mg/l (Fig. 3).

Glyphosate inhibited all the measured parameters at the lowest concentration (Fig. 4). Nevertheless, due to branch development, total length and node number increased in the 1 mg/l concentration. Glyphosate stimulated lateral growth, inhibiting apical development in non-lethal treatments. It caused plant death at concentrations exceeding 2.5 mg/l.

Total length and weight of plants exposed to the mine effluent were only affected by concentrations of 60% or more (Fig. 5). Node number and root length were always lower in the affluent that in the control conditions. In terms of IC_{50} (Table 3), root growth was consistently the most sensitive endpoint in every test, despite the high variability among explants.

Altogether, our results showed that *Myriophyllum spicatum* was stimulated by low concentrations of calcium sulphate and copper. Calcium is used by plants as a component of the cell membrane and wall, and as a cofactor for several enzymes (Barceló et al. 1995). On the other hand, copper is an essential element for plant growth, since it is a component of enzymes such as tirosinase or fenolase. Copper also plays an important role in photosynthesis (Barceló et al. 1995). However, copper is also a highly effective herbicide (Guilizzoni 1991). In our case an inhibitory effect was measured at concentrations from 1.2 to 1.8 mg/l and above. Stimulatory effects, even with toxic compounds not used by plants, are known to have stimulatory effects at low concentrations occasionally (e.g., McCan et al. 2000), a situation known as hormesis.

A search of the literature reveales that *M. spicatum* has a medium sensitivity to copper sulphate; the 21d-IC_{50} values were among the least sensitive of the reported studies, but were comparable to those reported for *Lemna* (Table 4). However, these comparisons should be

Table 3 Concentration of the test compound that inhibited 50% of the parameter (IC_{50}) (and its confidence intervals) weight, length, root length and node number

IC_{50}	Effluent (%)	$CaSO_4$ (g/l)	$FeSO_4$ (g/l)	$CuSO_4$ (mg/l)	Glyphosate (mg/l)
Weight	80.30	3.96	0.038	2.47	1.00
	(31–100)	(1.44–7.19)	(0.028–0.048)	(1.340–4.437)	(0.90–1.10)
Length	87.30	7.63	0.043	3.73	2.86
	(34–100)	(3.40–11.84)	(0.037–0.052)	(1.86–5.60)	(1.25–5.10)
Roots	69.50	2.24	0.023	2.19	0.33
	(40–99)	(0.64–3.84)	(0.017–0.037)	(1.024–4.682)	(0.25–0.47)
Nodes	89.30	–	0.057	4.49	5.01
	(60–100)		(0.047–0.073)	(2.70–6.74)	(1.79– 4.04)

Table 4 Summary of copper toxicity. Results are referred mg $CuSO_4$/l (mg Cu^{2+}/l)

Organism	Group	Parameter	Method	mg l^{-1} $CuSO_4$ (Cu^{2+})	Author
Myriophyllum spicatum	Macro	Weight	21d-IC_{50}	2.47 (0.98)	a
	Macro	Shoot length	21d-IC_{50}	3.73 (1.48)	a
	Macro	Root length	21d-IC_{50}	2.19 (0.87)	a
Lemna minor	Macro	Root length	20d-EC_{50}	(0.037)	b
	Macro	Biomass	5d-EC_{50}	2.3 (0.92)	c
Elodea canadiensis	Macro	O_2 production	10d-EC_{50}	(0.040)	b
Sinapsis alba	Other	Root length	8d-IC_{50}	(4.3)	d
Chlamydomonas	Algae	Growth	72h-EC_{50}	(0.079)	b
reinhardi	Algae	Growth	96h-EC_{50}	(0.047)	b
	Algae	Growth	10d-EC_{50}	(0.032)	b
Scenedesmus subspicatus	Algae	Growth	72h-EC_{50}	(0.12)	b
Euglena gracilis	Algae	Growth	72h-EC_{50}	(18)	b
	Algae	Growth	5d-EC_{50}	(7.9)	b
Selenastrum capricornutum	Algae	Growth	4d-EC_{50}	(0.008)	e
	Algae	Growth	5d-EC_{50}	0.031 (0.012)	c
Skeletonema costatum	Algae	Growth	5d-EC_{50}	0.25 (0.995)	c
Tethahymena pyriformis	Protoz	Growth	48h-EC_{50}	(8.0)	b
	Protoz	Growth	96h-EC_{50}	(10)	b
Hydra vulgari	Protoz	Mortality	96h-LC_{50}	(0.056)	f
Hydra oligartis	Protoz	Mortality	96h-LC_{50}	(0.084)	f
Hydra viridissima	Protoz	Mortality	96h-LC_{50}	(0.025)	f
Cambarus robustus	Crust	-	24h-LC_{50}	(3.48)	g
Gammarus pulex	Crust	Mortality	48h-LC_{50}	(0.047)	b
	Crust	Mortality	10d-LC_{50}	(0.033)	b
Ceriodaphnia sp	Crust	Mortality	48h-LC_{50}	(0.035)	e
Orconectes rusticus	Crust	-	24h-LC_{50}	(2.5)	g
Daphnia magna	Crust	Immobility	48h-EC_{50}	0.18 (0.072)	c
Chironomus riparius	Dipt	Mortality	48h-LC_{50}	(1.2)	b
	Dipt	Mortality	96h-LC_{50}	(0.70)	b
	Dipt	Mortality	10d-LC_{50}	(0.20)	b
Brachionus calyciflorus	Rotif	Mortality	24h-LC_{50}	(0.026)	b
	Rotif	Feeding	5h-EC_{50}	(0.033)	b
Lepomis macrochirus	Fish	Mortality	96h-LC_{50}	0.892 (0.355)	c

(When more than one experiment is reported by a same author, only the lowest and highest values are indicated. Macro = macrophyte; Protoz = protozoa; Crus = crustacean; Dip = diptera; Rotif = rotifera. References: (a) Present work; (b) Girling et al., 2000; (c) Environmental Fate and Effects Division. US EPA, Washington, DC, 2000; (d) Fargašová et al., 1998; (e) Deanovic et al., 1999; (f) Karntanut and Pascoe, 2002; (g) Sherba et al., 2000)

interpreted with caution because of differences in methodology. For instance, in our case and in the EPA report the tests were run with culture media, whereas Guirling et al. (2002) worked on mesocosms. *M. spicatum* was revealed by our tests to be me most sensitive testing organism for glyphosate (see Table 5 for a comparison with the literature).

Our data suggests that *M. spicatum* could be very sensitive to some pollutants, especially herbicides. This species has great potential to be used in toxicological assays because of its sensitivity, its easy culture in the laboratory and its consistent growth among explants. As with other plants, root growth was a suitable endpoint, but showed high variability among explants.

Table 5 Summary of glyphosate toxicity (mg active ingredient/l)

Organism		Parameter	Concentration mgl^{-1}		Author
Myriophyllum spicatum	Macro	Weight	21d- IC_{50}	1.00	a
		Shoot length	21d- IC_{50}	2.86	a
		Root length	21d- IC_{50}	0.33	a
		Shoot number	5d-EC_{50}	1.6	b
Myriophyllum sibiricum	Macro	Shoot length	14d- EC_{50}	28.79	c
		Root number	14d- EC_{50}	3.35	c
		Root length	14d- EC_{50}	1.22	c
Lemna gibba	Macro	Biomass	14d-EC_{50}	21.5	d
Lemna minor	Macro	Biomass	48h-EC_{50}	2–16.91	d
Selenastrum capricornutum	Algae	Biomass	96h-EC_{50}	5.81	e
		Biomass	7d-EC_{50}	12.54	d
Skeletonema costatum	Algae	Biomass	96h-EC_{50}	1.85	e
		Biomass	7d-EC_{50}	0.77	d
Anabaena flosaquae	Algae	Biomass	7d-EC_{50}	38.5	d
Tethahymena pyriformis	Protoz	Biomass	40h-EC_{50}	29.5	e
Euplotes vannus	Protoz	Biomass	48h-EC_{50}	23.5	e
Daphnia magna	Crust	Immobility	48h-EC_{50}	61.72	f
		Immobility	48h-EC_{50}	24–42	f
		Immobility	48h-EC_{50}	3	d
		Immobility	48h-EC_{50}	5.3–310	d
Daphnia spinulata	Crust	Immobility	48h-EC_{50}	66.18	f
Daphnia pulex	Crust	Immobility	48h-EC_{50}	7.9	f
		Immobility	96h-EC_{50}	25.5	f
		Immobility	48h-EC_{50}	7.9–242	d
Ceriodaphnia dubia	Crust	Mortality	48h-LC_{50}	5.39	e
Arcartia tonsa	Crust	Mortality	48h-LC_{50}	1.77	e
Gammarus pseudolimnaeus	Crust	Mortality	96h-LC_{50}	42	d
Chironomus plumosus	Dipt	Mortality	48h-LC_{50}	18	d
		Mortality	48h-LC_{50}	55	d
Crassotrea virginica	Moll	Mortality	48h-LC_{50}	10	d
Ictalurus punctatus	Fish	Mortality	96h-LC_{50}	13–16	d
Lepomis macrochirus	Fish	Mortality	96h-LC_{50}	5.8–140	d
Pimephales promelas	Fish	Mortality	96h-LC_{50}	9.4	d

(When more than one experiment is reported by a same author, only the lowest and highest values are indicated. Macro = macrophyte; Protoz = protozoa; Crus = crustacean; Dip = diptera; Rotif = rotifera; Moll = mollusca. References: (a) Present work; (b) Bird, 1993; (c) Roshon et al., 1999; (d) Environmental Fate and Effects Division. US EPA, Washington, DC, 2000; (e) Tsui & Chu, 2003; (6) Alberdi et al., 1996)

Acknowledgments The technical assistance of Mrs. Maria Ludovina Lopes is gratefully acknowledged. This research was founded by the IMAR, the Institute of Marine Sciences and the IAV, Institute of Environment and Life.

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