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## A novel bioassay using root re-growth in *Lemna*

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### ABSTRACT

A new phytotoxicity test method based on root elongation of three *Lemna* species (*Lemna gibba*, *L. minor*, and *L. paucicostata*) has been developed. Tests with aquatic plants have, typically, favored measurements on fronds (e.g. frond number, area, biomass) rather than on roots, due, in part, to issues associated with handling fragile roots and the time-consuming procedures of selecting roots with identical root lengths. The present method differs in that roots were excised prior to exposure with subsequent measurements on newly developed roots. Results show that there were species-specific difference in sensitivity to the five metals tested (Ag, Cd, Cr, Cu and Hg), with Ag being the most toxic ( $EC_{50} = 5.3\text{--}37.6 \mu\text{g L}^{-1}$ ) to all three species, and Cr the least toxic for *L. gibba* and *L. minor* ( $1148.3$  and  $341.8 \mu\text{g L}^{-1}$ , respectively) and Cu for *L. paucicostata* ( $470.4 \mu\text{g L}^{-1}$ ). Direct comparisons were made with measurements of frond area, which were found to be less sensitive. More generally, root re-growth was shown to reflect the toxic responses of all three *Lemna* species to these five important metals.

The root growth bioassay differs from three internationally standardized methods (ISO, OCED and US EPA) in that it is completed in 48 h, the required volume of test solutions is only 3 ml and non-axenic plants are used. Our results show that the *Lemna* root method is a simple, rapid, cost-effective, sensitive and precise bioassay to assess the toxic risks of metals and has practical application for monitoring municipal and industrial waste waters where metals are common constituents.

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## 1. Introduction

The global importance of detecting toxicants in the environment and assessing their impacts on biota has resulted in the development and adoption of a range of methodologies. Direct chemical analysis has several drawbacks that include the complexity of the procedures for preparing samples, the need for expensive analytical equipment and interference from secondary pollutants during analysis. In addition, this approach does not take account of temporal changes in exposure or the interactive effects of pollutants, nor does it provide ecologically significant information (Kumar and Han, 2010). To compensate for these limitations, various biological assays have been developed, including some that employ aquatic plants, in order to provide information on pollutant-induced toxic effects that can be used to assess environmental risks (Eullaffroy and Vernet, 2003).

Aquatic vascular plants belonging to the genus *Lemna* have gained broad acceptance as bioassays in ecotoxicological research. *Lemna* spp. have many advantages as test organisms, including their simple structure and small size, allowing for small volumes of sample toxicants to be used (Li and Xiong, 2004; Kumar and Han, 2010), rapid rates of growth (a doubling time of 1–4 d), ease of culture and handling, high degree of homogeneity such that most clones are morphologically similar (Lahive et al., 2011), and sensitivity to a wide range of pollutants (Hillman, 1961; Wang and Williams, 1990; Christen and Theuer, 1996). Moreover, *Lemna* plants are appealing test organisms due to their ecological importance as primary producers and their wide geographical occurrence (Drost et al., 2007). Among *Lemna* spp., *L. gibba* and *L. minor* have been most extensively used in phytotoxicity testing and there are several standard methods which have been adopted by major international standardization agencies e.g. U.S. Environmental Protection Agency (USEPA, 1996), Organization for Economic Cooperation and Development (OECD, 2002), and International Standardization Organization (ISO, 2005).

To ensure thorough evaluation of the risks posed by pollutants for environmental and human health, the test methods employed should be sensitive, rapid, simple and of ecological relevance.

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Therefore, a technique that can assess toxicity more rapidly but without loss of sensitivity would be a valuable asset. In the case of *Lemna*, bioassays using traditional endpoints such as number of fronds, wet or dry biomass and growth rates of fronds require standard exposure durations of at least 7 d to detect toxicity. Tests based on root elongation are some of the most widely used phytotoxicity methodologies for terrestrial angiosperms (Wang, 1991) because of their simplicity and rapidity (Munzuroglu and Geckil, 2002). However, despite reports that roots of *L. minor* are highly sensitive to environmental stressors (Panda and Upadhyay, 2003) and that they play important ecological roles by providing stability and facilitate dispersal there have been few studies incorporating root elongation as a test endpoint (Davis, 1981). This may be due, in part, to the fragility of the roots, which makes handling of roots for measurements of length difficult (Davis, 1981). It can also be impractical to obtain sufficient numbers of individual plants with identical root lengths for initiating tests.

The aim of this study was to develop a new root elongation test method using three *Lemna* species (*L. gibba*, *L. minor*, and *L. paucicostata*) and to evaluate the sensitivity and consistency of the method upon exposure to 5 different essential and non-essential metals (Ag, Cd, Cr, Cu and Hg). Metals derived from anthropogenic activities and released into the aquatic environments are not degraded, and represent a serious source of environmental pollution for plant function (Teisseire and Vernet, 2000; Prasad et al., 2001; Zhou et al., 2009) and to higher trophic organisms via bioaccumulation through food chains (Li and Xiong, 2004; Drost et al., 2007; Khella and Zerdaoui, 2009).

The new test method presented in this study differs from the traditional procedures by removing all roots from fronds prior to exposure to toxicants and measuring growth of newly developed roots during the exposure period of 48 h.

## 2. Materials and methods

### 2.1. Sample collection and maintenance

*Lemna gibba* (CPCC 310) and *L. minor* (CPCC 490) were obtained from the Canadian Phycological Culture Center. *L. paucicostata* was collected from an artificial fresh water pond located in a botanical garden in Gwang-ju, Republic of Korea (35.09 N, 125.54 E). Maintenance and cultivation of fronds were carried out under continuous white light ( $20\text{--}30 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) at  $20^\circ\text{C}$  in 10 ml plastic tank with Steinberg growth medium (Steinberg, 1946) under static conditions. Low fluorescent lighting was employed to ensure less likelihood of contamination. The pH of the medium was adjusted to  $7 \pm 0.2$  with solutions of hydrochloric acid (HCl) or sodium hydroxide (NaOH) at strengths  $\leq 1 \text{ M}$ .

### 2.2. Experimental procedures

To determine the optimal conditions for frond and root growth of each species, healthy green plants, each with two fronds, were selected and their roots excised using stainless steel scissors. Individual rootless plants were then placed in each cell of a 24-well plate (85.4 mm  $\times$  127.6 mm; well dimension 15.6 mm diameter, SPL, Korea), and exposed for 48 h to various levels of environmental factors: photon irradiance ( $50\text{--}200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ), temperature ( $10\text{--}35^\circ\text{C}$ ) and pH (3–10). While testing each single factor, the other environmental conditions were maintained optimally: photon irradiance at  $150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , temperature at  $30^\circ\text{C}$  and pH 7. The growth medium was not replaced during the incubation period. After 48-h incubation, all plants were harvested and the longest root lengths (each frond generally has 1–2 roots) were measured using an image analyzer (Moticam 2500, Ted Pella

**Table 1**

Final concentration range used for testing toxicity of metals with three species of *Lemna*.

Metal concentrations ( $\mu\text{g L}^{-1}$ )	<i>L. gibba</i>	<i>L. minor</i>	<i>L. paucicostata</i>
Ag	6.25–100	3.125–100	7.8125–125
Cd	31.25–1000	31.25–2000	19.53–625
Cr	93.75–3000	46.875–3000	156.25–2500
Cu	125–4000	62.5–4000	156.25–5000
Hg	15.625–2000	15.625–1000	78.125–2500

Inc., USA). For comparison, frond areas were also measured with the same instrument.

### 2.3. Toxicity test

Toxicity tests were carried out in a growth chamber under the optimal conditions established from the experiment described above, i.e. continuous light of  $150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , pH 7 and  $30^\circ\text{C}$ . The test was a static type, with no renewal of solutions during the period of exposure. The test vessel was a 24-well plastic plate with 3 ml of test solution added to each well. Individual rootless plants were added to cells of the plate; there were 4 plants per concentration, 6 concentrations of metal per plate and three replicate plates were exposed for 48 h. A different range of metal concentrations depending on metal were made by diluting the original stock solutions of  $\text{AgNO}_3$  (CAS no. 7440-22-4, SHOWA, Japan),  $\text{CdSO}_4$  (CAS no. 7440-43-9, SHOWA, Japan),  $\text{K}_2\text{Cr}_2\text{O}_7$  (CAS no. 7789-00-6, SHOWA, Japan),  $\text{CuSO}_4$  (CAS no. 7440-50-8, SHOWA, Japan), and  $\text{HgCl}_2$  (CAS no. 7439-97-6, SHOWA, Japan) with Steinberg medium. Final metal concentration range used is present in Table 1. In carrying out the experimental procedures, a fully randomized design was used to account for any variability in environmental conditions within the culture cabinet.

### 2.4. Statistical analysis

Analysis of variance was performed to confirm significant differences in response. Multiple comparison tests by the least significance difference (LSD) were then carried out to find significant differences from the controls and between treatments. Results are reported as  $\text{EC}_{50}$ s (the effective concentrations at which 50% inhibition occurs) with 95% confidence intervals estimated by the linear interpolated method (ToxCalc 5.0, Tidepool Science, CA, USA).

## 3. Results and discussion

Several environmental factors can influence the toxicity of contaminants to *Lemna* species, and so standardization of the toxicity test was established by determining the optimal conditions for root and frond growth of all three *Lemna* species. The growth of three species incubated for 48 h under different photon irradiances, pH and temperatures is shown in Figs. 1–3. In all the tested species, growth of roots and fronds was saturated at irradiances higher than  $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  while there was no evidence of growth in the dark. *Lemna* is known to be a low light plant, with an adequate requirement of photon irradiances for rapid growth at  $60\text{--}100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  (Greenberg et al., 1992). The maximal growth of the three *Lemna* species occurred at irradiances higher than  $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for *L. gibba* and *L. paucicostata* and  $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for *L. minor*. Therefore, quantum irradiance was set at  $150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for the toxicity tests. As shown in Table 2, it is notable that *Lemna* tests have been conducted at photon irradiances of  $40\text{--}129 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ .

Optimum growth temperatures for *Lemna* are known to be between 25 and  $31^\circ\text{C}$  and the temperature minima and maxima are

**Table 2**List of metal toxicity tests using various endpoints in three *Lemna* species (*L. gibba*, *L. minor* and *L. paucicostata*).

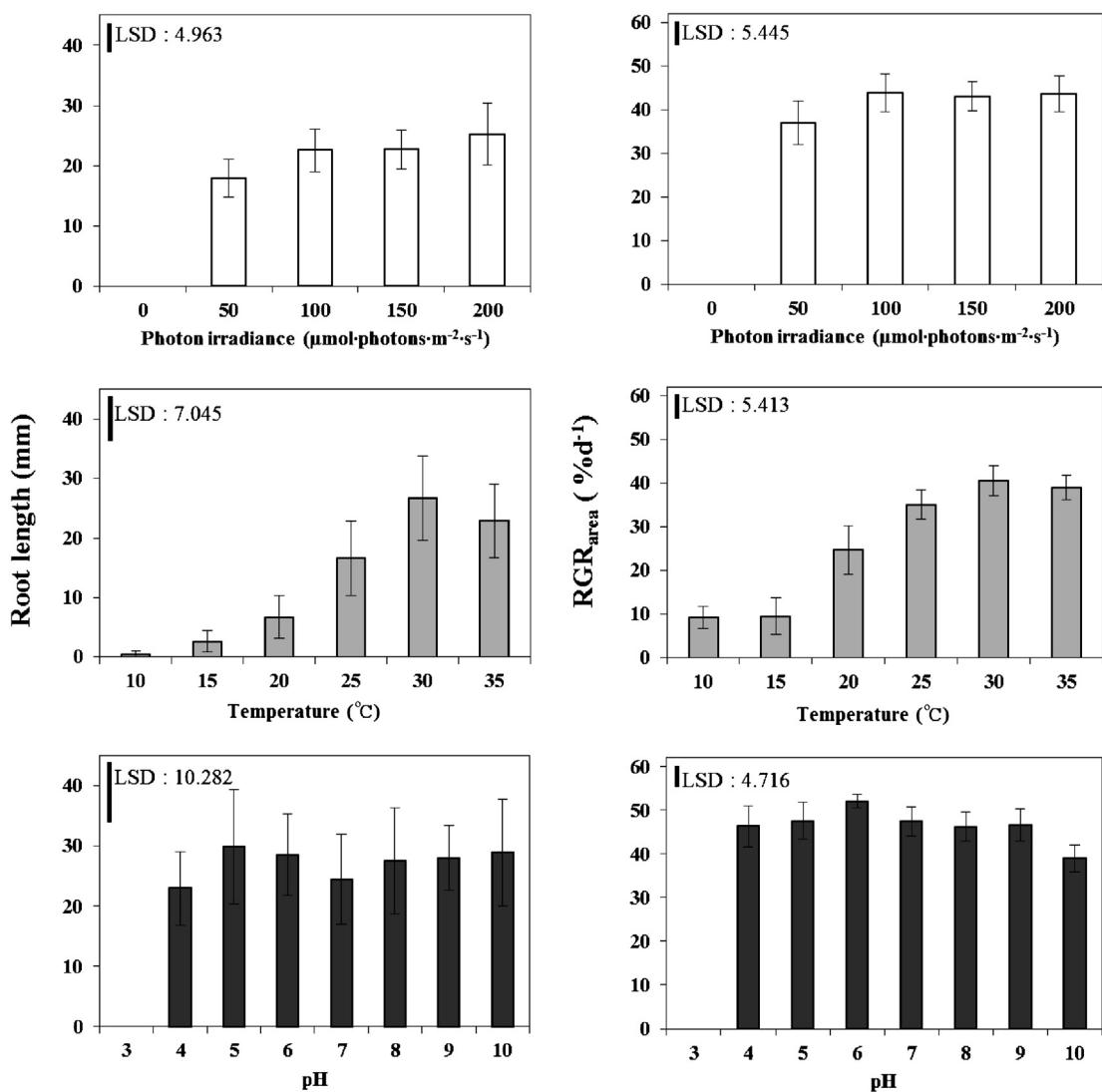
Toxicant	Test organism	End-point	Exposure time	EC <sub>50</sub> ( $\mu\text{g L}^{-1}$ )	Test volume (ml)	Medium	Light ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	Light duration (h)	Temperature (°C)	pH	References
Ag	<i>Lemnagibba</i>	RL	2 d	27.6 (21.3–34.2)	3	Steinberg	150±10	24	30±1	7	This study
AgNO <sub>3</sub>	<i>L. minor</i>	FN	7 d	81 (66–98)	300	Steinberg	100±15	24	25±1	5.5	Naumann et al. (2007)
		FW		30 (24–37)							
		DW		31 (26–37)							
		CHL		37 (17–65)							
AgNO <sub>3</sub>	<i>L. minor</i>	CAR		24 (18–31)							Topp et al. (2011)
		FAB	7 h	78 (56–108)		Steinberg	100±15	24	25±1	5.5	
AgNP <sub>1</sub>	<i>L. minor</i>	FN	7 d	140		SIS			24±2	5.5±1.4	Gubbins et al. (2011)
		DW		640							
		FN	14 d	38							
AgNP <sub>2</sub>	<i>L. minor</i>	DW		19							This study
		FN	7 d	125							
		DW		62							
		FN	14 d	43							
		DW		19							
Ag	<i>L. minor</i>	RL	2 d	5.3 (2.6–23.1)	3	Steinberg	150±10	24	30±1	7	This study
AgNO <sub>3</sub>	<i>L. paucicostata</i>	FN	7 d	100–1000	25	Bonner-Devirian	120	24	25±1	4.1–7.1	Nasu and Kugimoto (1981)
Ag	<i>L. paucicostata</i>	RL	2 d	37.6 (28.6–44.3)	3	Steinberg	150±10	24	30±1	7	This study
Cd	<i>L. gibba</i>	RL	2 d	363.5 (257.4–407.6)	3	Steinberg	150±10	24	30±1	7	This study
Cd	<i>L. minor</i>	FN	4 d	200	200	Plant nutrition Solution (Standard Methods 1980)			27±2	7.5	Wang (1986)
CdCl <sub>2</sub>	<i>L. minor</i>	FN	7 d	323 (232–450)	300	Steinberg	100±15	24	25±1	5.5	Naumann et al. (2007)
		FW		110 (72–189)							
		DW		241 (151–384)							
		CHL		102 (86–125)							
		CAR		92 (75–112)							
CdSO <sub>4</sub>	<i>L. minor</i>	FN		213	150	Steinberg	85–125	24	25±2	5.5±0.2	Drost et al. (2007)
3CdSO <sub>4</sub> ·8H <sub>2</sub> O	<i>L. minor</i>	FN		2500	200	Duckweed nutrient solution	42	16	25±1	5	Ma et al. (2007)

Table 2 (Continued)

Toxicant	Test organism	End-point	Exposure time	EC <sub>50</sub> ( $\mu\text{g L}^{-1}$ )	Test volume (ml)	Medium	Light ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	Light duration (h)	Temperature (°C)	pH	References
Cd	<i>L. minor</i>	FA	4 d	910	100	¼ Coïc & Leisant (1973)	101	16	22	6.1 ± 0.1	<a href="#">Khellaf and Zerdaoui (2009)</a>
Cd	<i>L. minor</i>	RL	2 d	226.4 (200.7–248.5)	3	Steinberg	150 ± 10	24	30 ± 1	7	This study
CdCl <sub>2</sub> ·2½H <sub>2</sub> O	<i>L. paucicostata</i>	FN	7 d	20–50	25	Bonner-Devirian	120	24	25 ± 1	4.1–7.1	<a href="#">Nasu and Kugimoto (1981)</a>
Cd	<i>L. paucicostata</i>	RL	2 d	77.0 (61.1–118.1)	3	Steinberg	150 ± 10	24	30 ± 1	7	This study
K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub>	<i>L. gibba</i>	No plants	7 d	49,000 (12,000–85,000)	100	Hoagland	129	24	25 ± 2	4.6–5.4	<a href="#">Cowgill et al. (1991)</a>
		FN		45,000 (16,000–75,000)							
		DW		48,000 (17,000–79,000)							
Cr (VI)	<i>L. gibba</i>	RL	2 d	1148.3 (276.1–1403.6)	3	Steinberg	150 ± 10	24	30 ± 1	7	This study
K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub>	<i>L. minor</i>	FN	14 d	5000	–	Btistol	40	12	21	7.2	<a href="#">Mangi et al. (1978)</a>
Cr (VI)	<i>L. minor</i>	FN	4 d	35,000	200	Plant nutrition solution (Standard Methods 1980)			27 ± 2	7.5	<a href="#">Wang (1986)</a>
K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub>	<i>L. minor</i>	No plants	7 d	40,000 (8000–204,000)	100	Hoagland	108	24	25 ± 2	4.6–5.4	<a href="#">Cowgill et al. (1991)</a>
		FN		30,000 (6000–151,000)							
		DW		28,000 (7000–106,000)							
Na <sub>2</sub> CrO <sub>4</sub> ·4H <sub>2</sub> O	<i>L. minor</i>	FN	7 d	2300 (870–8500)	300	Steinberg	100 ± 15	24	25 ± 1	5.5	<a href="#">Naumann et al. (2007)</a>
		FW		584 (33–1060)							
		DW		2300 (870–8500)							
		CHL		160 (30–2770)							
		CAR		80 (10–6620)							
Cr (VI)	<i>L. minor</i>	RL	2 d	341.8 (163.0–559.7)	3	Steinberg	150 ± 10	24	30 ± 1	7	This study
K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub>	<i>L. paucicostata</i>	FN	7 d	>1000	25	Bonner-Devirian	120	24	25 ± 1	4.1–7.1	<a href="#">Nasu and Kugimoto (1981)</a>
Cr (VI)	<i>L. paucicostata</i>	RL	2 d	237.9 (195.0–280.5)	3	Steinberg	150 ± 10	24	30 ± 1	7	This study
CuSO <sub>4</sub> ·5H <sub>2</sub> O	<i>L. gibba</i>	FA	4 d	450	100	¼ Coïc & Leisant (1973)	–	12	21	6	<a href="#">Khellaf and Zerdaoui (2010)</a>
Cu	<i>L. gibba</i>	RL	2 d	310.0 (236.7–391.3)	3	Steinberg	150 ± 10	24	30 ± 1	7	This study
Cu	<i>L. minor</i>	FN	4 d	1100	200	Plant nutrition solution (Standard Methods 1980)			27 ± 2	7.5	<a href="#">Wang (1986)</a>

Table 2 (Continued)

Toxicant	Test organism	End-point	Exposure time	EC <sub>50</sub> ( $\mu\text{g L}^{-1}$ )	Test volume (ml)	Medium	Light ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	Light duration (h)	Temperature (°C)	pH	References
CuSO <sub>4</sub> ·5H <sub>2</sub> O	<i>L. minor</i>	FN	7 d	160 (110–210)	50	Inorganic growth medium (Chollet 1993)	40±2	24	25±1	6.5	Teisseire et al. (1998)
CuCl <sub>2</sub>	<i>L. minor</i>	FN	7 d	616	150	Steinberg	85–125	24	25±2	5.5±0.2	Drost et al. (2007)
CuCl <sub>2</sub>	<i>L. minor</i>	FN	7 d	330 (236–474) 95 (54–150)	300	Steinberg	100±15	24	25±1	5.5	Naumann et al. (2007)
		FW		157 (129–181)							
		DW		136 (121–153)							
		CHL		132 (113–154)							
		CAR									
Cu	<i>L. minor</i>	FA	4 d	470	100	¼ Colc& Leisant (1973)	101	16	22	6.1±0.1	Khellaf and Zerdaoui (2009)
Cu	<i>L. minor</i>	RL	2 d	221.0 (123.9–385.0)	3	Steinberg	150±10	24	30±1	7	This study
CuSO <sub>4</sub> ·5H <sub>2</sub> O	<i>L. paucicostata</i>	FN	7 d	50–100	25	Bonner-Devirian	120	24	25±1	4.1–7.1	Nasu and Kugimoto (1981)
Cu	<i>L. paucicostata</i>	RL	2 d	470.4 (420.8–509.0)	3	Steinberg	150±10	24	30±1	7	This study
Hg	<i>L. gibba</i>	RL	2 d	222.2 (189.4–259.2)	3	Steinberg	150±10	24	30±1	7	This study
HgCl <sub>2</sub>	<i>L. minor</i>	FN	5 d	2100	50	Hoagland	40	24	25±2		Subhadra et al. (1991)
HgCl <sub>2</sub>	<i>L. minor</i>	FN	7 d	683 (536–881)	300	Steinberg	100±15	24	25±1	5.5	Naumann et al. (2007)
		FW		191 (155–235)							
		DW		221 (162–301)							
		CHL		135 (92–205)							
		CAR		123 (75–212)							
HgCl <sub>2</sub>	<i>L. minor</i>	FW	4 d 7 d	640 480	200	Jacobs	40	24	25±2	6	Dirilgen (2011)
Hg	<i>L. minor</i>	RL	2 d	196.2 (93.1–286.8)	3	Steinberg	150±10	24	30±1	7	This study
Hg	<i>L. paucicostata</i>	RL	2 d	106.6 (65.6–330.4)	3	Steinberg	150±10	24	30±1	7	This study



**Fig. 1.** Effects of photon irradiance, temperature and pH on root and frond growth in *L. gibba*. The bar denotes least significance difference (LSD) at 5% level and each error bar indicates 95% confidence interval ( $n=3$ ).

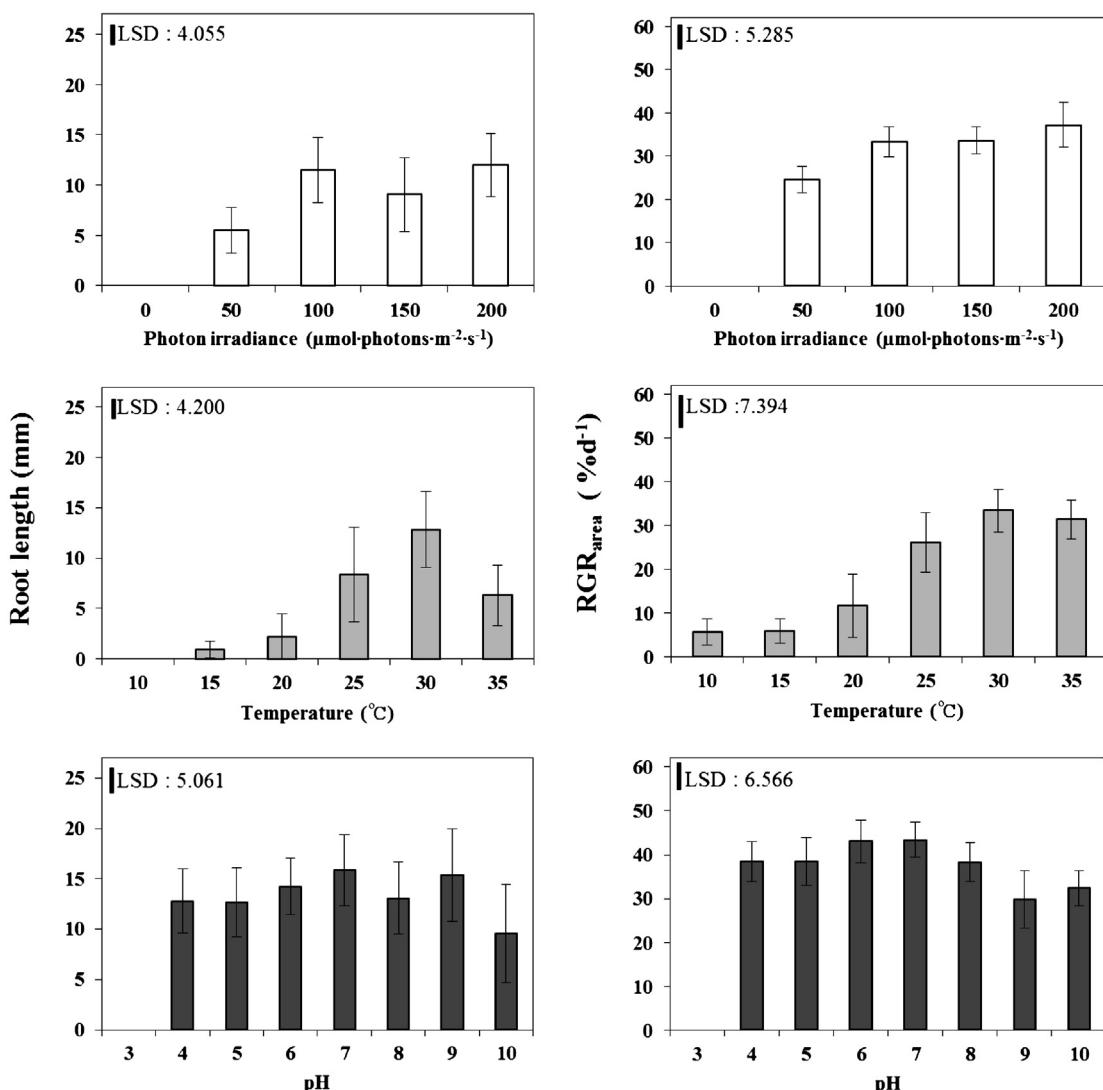
reported to be 7 °C and above 31–35 °C, respectively (Iqbal, 1999). Toxicity of *L. minor* has been shown to be independent of temperatures between 20 and 30 °C (Nasu and Kugimoto, 1981) although, in other species, toxicity decreases with decreasing temperature (Das et al., 1997). In our study, temperature was set at 30 °C as this was the temperature for maximal growth of roots and areas of fronds in all three *Lemma* species. This temperature is at the higher limit of optimal temperatures (22–30 °C) employed for most *Lemma* tests.

The pH of the test medium can influence the growth of *Lemma* plants. For example, the higher the pH value, the greater the inhibition of frond multiplication in *L. paucicostata* (Nasu and Kugimoto, 1981). The pH can also affect the bioavailability of metals by altering speciation, and hence can influence toxicity. The desirable pH range for *Lemma* toxicity testing of Ag, Cd, Cr and Cu was suggested to be 6.1–7.1 (Nasu and Kugimoto, 1981). In the present study, pH was set at  $7 \pm 0.2$  since growth of both roots and fronds of three *Lemma* species was recorded between 4 and 10. Table 1 shows that a wide range of pH values (4.1–7.5) have generally been employed for *Lemma* toxicity tests.

From comparisons of the results obtained from the many studies on *Lemma* spp. it is apparent that there are considerable inconsistencies in the reporting of the sensitivities of the various measured

endpoints. For instance, a study of metal toxicity using 5 endpoints (frond number, fresh weight, dry weight, chlorophyll (Chl) and carotenoid (Car) concentrations) in *L. minor* revealed that Chl and Car were the most sensitive endpoints, while frond number was the least sensitive (Naumann et al., 2007). Contrary to this, Mackenzie et al. (2003) reported that frond number was the most sensitive of the endpoints measured while others (e.g. Teisseire et al., 1998; Lahive et al., 2011), have shown that growth rates, based on changes in biomass, were more sensitive than photosynthetic pigment concentrations. Differences in sensitivity may occur between *Lemma* species and clones of the same species (Lahive et al., 2011).

Root growth following excision prior to exposure to metals proved to be a sensitive measure of toxic effects. Coefficients of variation of the root test method ranged from 4.22 to 12.82% for *L. gibba*, from 0.83 to 15.80% for *L. minor* and from 1.60 to 19.63% for *L. paucicostata*. CV values of the frond area test method were between 3.64 and 32.12% (Table 3). These ranges of CV are comparable to those obtained from internationally recognized bioassays carried out on the same metal toxicants. The rank order of metal toxicity, in terms of EC<sub>50</sub>s, was species-specific; for *L. gibba* and *L. minor* it was Ag > Hg > Cu > Cd > Cr whereas it was Ag > Cd > Hg > Cr > Cu for *L. paucicostata* (Table 3). It is notable that Ag was the most toxic metal for all three species, whereas Cr was least toxic for *L. gibba*.



**Fig. 2.** Effects of photon irradiance, temperature and pH on root and frond growth in *L. minor*. The bar denotes least significance difference (LSD) at 5% level and each error bar indicates 95% confidence interval ( $n=3$ ).

and *L. minor* and Cu for *L. paucicostata*. In terms of frond growth, Ag was also found to be most toxic metal for the three species while the second most toxic metal was either Hg for *L. gibba*, or Cd for *L. minor* and *L. paucicostata*.

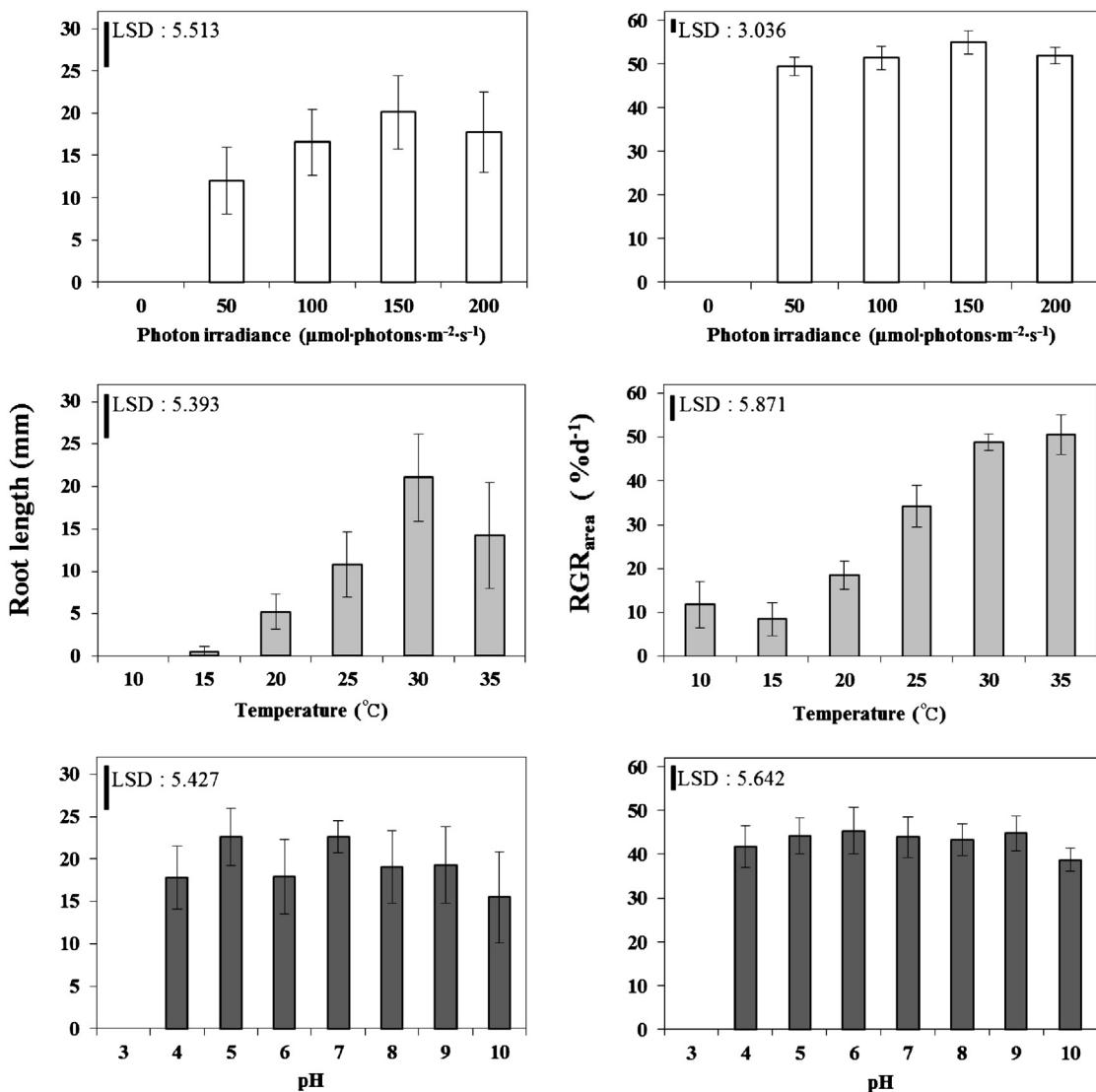
Silver is a relatively rare element occurring naturally in the earth's crust, and the principal sources of the occurrence of silver

and silver compounds are from human uses such as photographic materials, electroplating, electrical conductors, dental alloys, solder and brazing alloys, paints, jewelry, coins, and mirror production. It is also used for seeding clouds, as an antibacterial agent, and has been used for water purification (<http://cira.ornl.gov/documents/SILVER.pdf>).

**Table 3**

48 h EC<sub>50</sub> and CV for the inhibition of surface growth rate (RGR<sub>area</sub>), and root length in three *Lemna* species exposed to 5 metals. Mean and 95% CI are shown ( $n=3$  plates, 24 plants per plate with 4 plants per concentration).

End point	Heavy metals	<i>L. gibba</i>		<i>L. minor</i>		<i>L. paucicostata</i>	
		EC <sub>50</sub> (95% CI)	CV (%)	EC <sub>50</sub> (95% CI)	CV (%)	EC <sub>50</sub> (95% CI)	CV (%)
RGR <sub>area</sub>	Ag	42.6 (36.7–55.6)	9.71	50.8 (37.6–60.1)	8.30	74.5 (66.4–81.1)	19.35
	Cd	361.6 (323.2–410.4)	17.49	516.6 (314.8–620.9)	5.51	403.6 (381.7–429.1)	32.12
	Cr <sup>6+</sup>	>3000	–	>3000	–	>2500	–
	Cu	486.2 (421.2–599.3)	10.42	1774.9	–	2448.7000	–
	Hg	210.6 (160.5–290.2)	3.64	>1000	–	913.5 (808.3–1036.9)	16.71
Root length	Ag	27.6 (21.3–34.2)	7.25	5.3 (2.6–23.1)	0.83	37.6 (28.6–44.3)	10.07
	Cd	363.5 (257.4–407.6)	7.91	226.4 (200.7–248.5)	15.80	77.0 (61.1–118.1)	5.10
	Cr <sup>6+</sup>	1148.3 (267.1–1403.6)	4.22	341.8 (163.0–559.7)	3.38	237.9 (195.0–280.5)	10.99
	Cu	310 (236.7–391.3)	6.91	221 (123.9–385.0)	3.47	470.4 (420.8–509.0)	19.63
	Hg	222.2 (189.4–259.2)	12.82	196.2 (93.1–286.8)	3.96	106.6 (65.6–330.4)	1.60



**Fig. 3.** Effects of photon irradiance, temperature and pH on root and frond growth in *L. paucicostata*. The bar denotes least significance difference (LSD) at 5% level and each error bar indicates 95% confidence interval ( $n=3$ ).

Even in its bulk form, silver is reported to be extremely toxic to plants and algae, although the mode of action remains poorly understood (Eisler, 1996). Silver, or silver nanoparticles, can cause abscission of fronds and inhibit multiplication of fronds and frond growth of *Lemna* (Nasu and Kugimoto, 1981; Naumann et al., 2007; Topp et al., 2011). In *L. minor* derived EC<sub>50</sub> values, from growth rates, based on frond number and biomass, and photosynthetic pigment concentrations, varied between 24 and 81  $\mu\text{g L}^{-1}$  (Naumann et al., 2007), while Topp et al. (2011) reported that EC<sub>50</sub>s, for frond abscission differed between clones of the species e.g. values between 51 and 1296  $\mu\text{g L}^{-1}$  for clone 7868 (from Ireland) and clone 7022 (from Spain), respectively (Table 2). Based on root growth measurements the most sensitive species to Ag was *L. minor* (EC<sub>50</sub> of 5.3  $\mu\text{g L}^{-1}$ ) followed by *L. gibba* (27.6  $\mu\text{g L}^{-1}$ ) and *L. paucicostata* (37.6  $\mu\text{g L}^{-1}$ ) (Table 3). For frond growth (area) EC<sub>50</sub> values were 42.6  $\mu\text{g L}^{-1}$  for *L. gibba*, 50  $\mu\text{g L}^{-1}$  for *L. minor* and 74.5  $\mu\text{g L}^{-1}$  for *L. paucicostata*. Therefore, the sensitivity of root growth of *L. minor* to Ag was ca. 5–245 times higher than that of other more conventional endpoints (Table 2). The sensitivity of root growth in *L. gibba* and *L. paucicostata* to Ag was also similar to that of the most sensitive endpoints that have so far been reported. Generally, responses of roots to Ag were more sensitive than fronds.

Cd occurs in natural and waste waters and originates from many industrial sources such as processing, smelting and mining ores, reclamation of scrap metals, incineration for disposal of waste products, run-off carrying fertilizers and fungicides etc. (Schmitz, 1996). Cadmium poses toxic effects on plants by interfering with chlorophyll pigment formation, enzymatic activity, and CO<sub>2</sub> fixation, leading to an overall reduction in growth (Fodor, 2002). In *Lemna*, Cd can reduce cellular protein, carbohydrate and, chlorophyll contents, some antioxidant activity and to inhibit multiplication of frond number, area and biomass (Nasu and Kugimoto, 1981; Smith and Kwan, 1989; Mohan and Hosetti, 1997; Naumann et al., 2007; Megateli et al., 2009; Malec et al., 2010). In the present study, the EC<sub>50</sub> of Cd toxicity, based on root growth measurements, was significantly lower ( $P<0.05$ ) in *L. paucicostata* (77  $\mu\text{g L}^{-1}$ ) than in *L. gibba* (363.5  $\mu\text{g L}^{-1}$ ) or *L. minor* (226.4  $\mu\text{g L}^{-1}$ ) (Table 3). In contrast, effects of Cd on frond growth did not differ significantly between the species. The EC<sub>50</sub> values obtained for *L. minor* and *L. paucicostata* were lower than the corresponding values for root growth. In the wild-type *L. paucicostata* colony break-up was observed more frequently on exposure to Cd than to the other 6 metals tested (Li and Xiong, 2004). When compared with other published reports (Table 2), Cd toxicity to *L. paucicostata*, based on EC<sub>50</sub>

**Table 4**

Comparison of 3 internationally standardized *Lemna* test methods with the root test method developed in this study.

	ISO	OECD	EPA	This study
Test species	<i>Lemna minor</i>	<i>L. gibba, L. minor</i>	<i>L. gibba G3, L. minor</i>	<i>L. gibba, L. minor, L. paucicostata</i>
Test duration	7 days	7 days	7 days	2 days
Temperature (°C)	24 ± 2 °C	24 ± 2 °C	25 ± 2 °C	30 ± 1 °C
Salinity	Fresh water	Fresh water	Fresh water	Fresh water
Light intensity	85–135 μmol photons m <sup>-2</sup> s <sup>-1</sup>	6500–10,000 lux	4200–6700 lux	150 μmol photons m <sup>-2</sup> s <sup>-1</sup>
Photoperiod	Continuous light	Continuous light	Continuous light	Continuous light
Test vessel type	Beaker	Flasks, Petri dish	Beaker, flask	24-well plate
Medium	Steinberg medium	Swedish Standard (SIS) lemma medium for <i>L. minor</i> 20× AAP growth medium for <i>L. gibba</i>	M-Hoagland's medium or 20×-AAP nutrient medium	Steinberg medium
Test solution volume	100 ml (minimum)	100 ml (minimum)	150 ml	3 ml
Test solution pH	5.5	6.5 ± 0.2	7.5 ± 0.1	7 ± 0.2
Test organism size	10–16 fronds per test vessel (2 or 3 fronds per colony)	9–12 fronds per test vessel	12–16 fronds per test vessel	1 colony per test vessel (2 fronds per colony)
Endpoint	Growth rate (frond number, frond area, dry weight, chlorophyll contents)	Average specific growth rate, final biomass, area under the growth curve	Total frond number, growth rate (as number of fronds per day), mortality (% of dead fronds to total number of fronds) and dry weight, chlorophyll and pheophytin pigment analyses	Root length
Test type	Static none-renewal	Static none-renewal	Static none-renewal	Static none-renewal
Condition	Axenic culture	Axenic culture	Axenic culture	Non-axenic culture

values derived from root and frond growth, was similar to or greater than those from measurements of biomass (110–241 μg L<sup>-1</sup>) and frond number (200–2500 μg L<sup>-1</sup>) in *L. minor* (Drost et al., 2007; Khellaf and Zerdaoui, 2009). The present results appear to agree with the previous findings that *L. paucicostata* may be a particularly sensitive species to Cd.

Chromium has multifarious industrial uses for leather processing, the production of refractory steel, drilling muds, electroplating cleaning agents, catalytic manufacture, chromic acid and specialty chemicals, metal plating, cooling tower water treatment, and wood preservation (Shanker et al., 2005). Chromium compounds are reported to be highly toxic to plants, being detrimental to physiological mechanisms including CO<sub>2</sub> fixation, electron transport, photophosphorylation, chlorophyll biosynthesis, enzyme activities, leading to negative impacts on their essential life processes such as growth and development (Vajpayee et al., 2000; Shanker et al., 2005). Plants of the Lemnaceae appear to be relatively more tolerant of Cr than other metals (Landolt and Kandeler, 1987). The maximal EC<sub>50</sub>s of Cr toxicity recorded (Table 2) are 1148.3 μg L<sup>-1</sup> for *L. gibba*, 341.8 μg L<sup>-1</sup> for *L. minor*, and 237.9 μg L<sup>-1</sup> for *L. paucicostata*, and these values are, for example, 6–10 times higher than the EC<sub>50</sub>s of Ag toxicity for the corresponding species (Table 2). The EC<sub>50</sub> values derived from frond growth were significantly higher than those for root growth. The newly developed root system appears to be more sensitive to Cr exposure than fronds as is evident from a comparison of EC<sub>50</sub>s from measurements of root growth with more traditional endpoints (Table 2); for *L. gibba*, values are 1148.3 μg L<sup>-1</sup> vs 48,000–49,000 μg L<sup>-1</sup> for roots and fronds respectively, 341.8 μg L<sup>-1</sup> vs 584–40,000 μg L<sup>-1</sup> for *L. minor* and 237.9 μg L<sup>-1</sup> vs 1000 μg L<sup>-1</sup> for *L. paucicostata* (Tables 2 and 3).

There are various anthropogenic sources of Cu entering aquatic ecosystems including mine drainage, industrial, domestic, agricultural waste, agrochemicals and antifouling paints (Ma et al., 2003; Andrade et al., 2004). Although Cu is an essential micronutrient and structural and catalytic component for metabolic pathway in plants, exposure to Cu at concentrations beyond a certain threshold induces phytotoxic symptoms in *Lemna*, including the inhibition of growth, photosynthesis, respiration, pigment synthesis and enzymatic activity (Teisseire and Vernet, 2005; Kanoun-Boule et al., 2009; Khellaf and Zerdaoui, 2010).

The present study shows that Cu was significantly ( $P < 0.05$ ) more toxic to *L. minor* (EC<sub>50</sub> 221.0 μg L<sup>-1</sup>) than both *L. gibba* (310.0 μg L<sup>-1</sup>) and *L. paucicostata* (470.4 μg L<sup>-1</sup>) (Table 2). The EC<sub>50</sub> values obtained for *L. gibba* and *L. minor* based on root growth measurements are similar to those (95–1100 μg L<sup>-1</sup>) from measurements of frond number, frond weight and pigment concentrations (Table 2). In the case of *L. paucicostata*, however, the EC values are greater in root (470.4 μg L<sup>-1</sup>) than in frond growth (50–100 μg L<sup>-1</sup>) (Table 2). A direct comparison of EC<sub>50</sub>s from measurements of root with frond growth showed that root was significantly ( $P > 0.05$ ) more sensitive to Cu than frond for all three *Lemna* species (Table 3).

Anthropogenic sources of Hg come mostly from stationary coal combustion, gold and non-ferrous metal prospecting, cement production, waste disposal, human crematoria, caustic soda production, pig iron and steel production, mercury production (mostly for batteries), and incineration of biomass (Pacyna et al., 2006; Dirilgen, 2011).

Hg is known to be one of the most toxic metals because of its mobility, bioaccumulation and methylation process. In plants, Hg has harmful effects on various physiological and biochemical processes, such as vegetative growth, germination, oxygen evolution, chlorophyll *a* fluorescence, pigmentation, and enzymatic activities (Patra and Sharma, 2000). In the present study, there was no significant difference between species in the sensitivity of root growth to Hg; the EC<sub>50</sub> values were 222.2 μg L<sup>-1</sup> for *L. gibba*, 196.2 μg L<sup>-1</sup> for *L. minor* and 106.6 μg L<sup>-1</sup> for *L. paucicostata* (Table 3). Root length was a more sensitive endpoint to Hg than frond area for *L. minor* and *L. paucicostata* but was the same for *L. gibba*. More generally, the EC<sub>50</sub>s obtained from root growth measurements were similar to or higher (68.3–106.6 μg L<sup>-1</sup>) than those based on the measurements of frond parameters (Table 2).

#### 4. Conclusions

The measurements of root re-growth following their excision showed species-specific differences in sensitivity to five important metal species (Ag, Cd, Cr, Cu and Hg) in three *Lemna* species (*L. minor*, *L. gibba*, *L. paucicostata*). The EC<sub>50</sub> values indicated that root growth was a more sensitive measure of toxicity (Ag, Cd, Cr) or of similar sensitivity (Cu, Hg) compared with the more commonly

measured parameters on fronds. Table 4 compares the present *Lemna* root test method with 3 internationally standardized methods (ISO, OCED and US EPA). The root growth bioassay differs in several key respects: the test can be completed in only 48 h, the test vessel is a 24-well cell plate, the required volume of test water samples is 3 ml, and axenic cultures, which are costly and difficult to maintain, are not required. Furthermore, the technique of excising roots prior to exposure means that there is no requirement to pre-select roots of uniform length and it reduces the amount of handling of these fragile roots.

The results of this investigation clearly show that *Lemna* root method is a simple, rapid, cost-effective and precise bioassay that can be used to assess the toxic risks of metals. It has practical applications for monitoring municipal and industrial waste waters where metals are common constituents. Further investigations are currently underway to evaluate the sensitivity of the method to a wider range of toxic chemicals.

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