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Comparing the acute sensitivity of growth and photosynthetic endpoints in three *Lemna* species exposed to four herbicides☆

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ABSTRACT

An ecological impact assessment of four herbicides (atrazine, diuron, paraquat and simazine) was assessed using the aquatic floating vascular plants, *Lemna gibba*, *Lemna minor* and *Lemna paucicostata* as test organisms. The sensitivity of several ecologically relevant parameters (increase in frond area, root length after regrowth, maximum and effective quantum yield of PSII and maximum electron transport rate (ETR_{max}), were compared after a 72 h exposure to herbicides. The present test methods require relatively small sample volume (3 mL), shorter exposure times (72 h), simple and quick analytical procedures as compared with standard *Lemna* assays. Sensitivity ranking of endpoints, based on EC_{50} values, differed depending on the herbicide. The most toxic herbicides were diuron and paraquat and the most sensitive endpoints were root length (6.0–12.3 μg L^{-1}) and ETR_{max} (4.7–10.3 μg L^{-1}) for paraquat and effective quantum yield (6.8–10.4 μg L^{-1}) for diuron. Growth and chlorophyll *a* fluorescence parameters in all three *Lemna* species were sensitive enough to detect toxic levels of diuron and paraquat in water samples in excess of allowable concentrations set by international standards. CV values of all EC_{50}s obtained from the *Lemna* tests were in the range of 2.8–24.33%, indicating a high level of repeatability comparable to the desirable level of <30% for adoption of toxicity test methods as international standards. Our new *Lemna* methods may provide useful information for the assessment of toxicity risk of residual herbicides in aquatic ecosystems.

1. Introduction

Aquatic environments are subjected to contamination by the inundation of a variety of toxicants derived from anthropogenic activities. Herbicides are one of the most widely used groups of organic chemicals, with application particularly prevalent in, agriculture, horticulture and, amenity green spaces such as parks, golf courses and sports fields (Fatima et al., 2007). It has been reported that 99.7% of the applied load is dispersed as residues which enter aquatic environments through run-off and leaching (Kloeppel et al., 1997; Prado et al., 2009) and can lead to both negative direct and indirect effects on aquatic biota that are detectable at multiple levels of biological organization, from the molecular to the ecosystem. There is now increasing public awareness of the potential risks posed by herbicides not just to water quality and non-target organisms but also to human health (Hernández et al., 2013). Therefore, effective monitoring and management strategies need to be developed so that the integrity of aquatic ecosystems can be maintained. For this to happen, policies must be underpinned by meticulous quantitative data on both the detection of herbicides in aquatic ecosystem and their risks to aquatic life.

Conventionally, sophisticated analytical methods using HPLC and Mass Spectrometry have been used for measuring herbicide residues. Chemical analysis as a methodology for herbicide detection is highly specific and sensitive but has several drawbacks

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including the complex procedures for sample preparation, the need for expensive chemicals and equipment, and interference from secondary pollutants during analysis (Park et al., 2012). Moreover, this purely chemical approach does not provide ecologically significant information on temporal changes in exposure or the interactive effects of pollutants (Kumar and Han, 2010). To compensate for these limitations biological assays have been developed and employed to assess pollutant-induced ecological risks. Especially, aquatic bioassay is an important means of assessing the quality of water containing mixtures and unknown contaminants and of providing the safety standards for water management in an ecological context that cannot be expected from the conventional chemical analysis-driven management since the latter method relies on the measurements of single and standardized chemicals. The choice of a model organism for toxicity testing is dependent on sensitivity to specific pollutants, with many species of zooplankton, phytoplankton and macroscopic organisms being used. Amongst them, aquatic macrophytes belonging to the class Lemnaceae are attractive experimental model organisms for a number of reasons including their simple structure, small stature, degree of homogeneity, ease of culture and high growth rate (a doubling time of 2–4 d) (Hillman, 1961; Wang and Williams, 1990; Christen and Theuer, 1996; Kumar and Han, 2010; Lahive et al., 2011). Moreover, these plants have important ecological functions and are widely distributed, and are known to be highly sensitive to organic and inorganic substances, including herbicides, pharmaceuticals and metals (Lahive et al., 2011; Scherr et al., 2008; Wang, 1990). Macrophytes are a major group of primary producers at the base of trophic hierarchies in aquatic ecosystems and have prime importance since any negative impacts on them can have serious consequences higher up food chains, leading to alteration in the diversity and functionality of whole aquatic ecosystems. For these reasons, laboratory toxicity testing with *Lemna* spp. (duckweed) is one of the choice methodologies for assessing impacts on freshwater systems (Moody and Miller, 2005).

1.1. *Lemna spp, particularly Lemna gibba and L. minor are being used for decades in*

prospective risk assessment of pesticides worldwide (USA, Europe). In Europe, for example, *Lemna* spp. were, until 2013, the only standard species of aquatic macrophyte species mandatory for regulatory driven risk assessment of each and every herbicides and plant growth regulators in the process of registration (Giddings et al., 2012).

The ultimate goal of bioassay tests is to provide representative and incorporative criteria of exposure conditions, thereby improving risk assessment and management of water quality. In this respect, multiple, rather than single, endpoint assays may have a greater potential for more comprehensive risk assessment of toxicants. Such an approach makes it possible to gain important insights into the mechanisms of toxicity and obtain information on the relative sensitivity of the measured endpoints to toxicant concentration and/or exposure duration thereby identifying specific endpoints which can effectively detect disturbances caused by particular phytotoxicants (Nestler et al., 2012). Many endpoints have been applied in *Lemna*, including frond number, plant number, root number, dry or fresh biomass, frond diameter or area, root length, carbon uptake, chlorophyll content, etc. (see reviews by Wang, 1990). Recently, Gopalapillai et al. (2014) reported root length of *Lemna minor* as to be the optimal endpoint for biomonitoring of mining effluents. The authors considered average root length (RL) the ideal endpoint for three reasons: accuracy (i.e., toxicological sensitivity to the contaminant), precision (i.e., lowest variance), and ecological relevance (metal mining effluents) (Gopalapillai et al., 2014). A well-defined toxicant concentration-dependent inhibition of root re-growth has also recently been shown with the root re-growth test using three *Lemna* species (Park et al., 2013). Several operational benefits of this method over that of more conventional techniques (ISO20079) were highlighted by the authors, including: completion of the test after 48 h, a test solution of only 3.0 mL and the use of non-axenic plant material.

The technique of pulse amplitude modulated (PAM) chlorophyll *a* (Chl *a*) fluorescence, which is based on measurements of the fluorescence from Chl *a* in photosystem II (PS II) reaction centers, is considered to be a rapid and sensitive tool for evaluating toxicity in algae and higher plants (Juneau and Popovic, 1999; Ralph and Gademann, 2005; Schreiber et al., 2007). The approach has already been successfully employed with *Lemna* spp. To assess the toxicity of, for example, the phenylurea herbicide linuron in *L. minor* (Hulsen et al., 2002), the wood preservative creotose, sewage treatment plant effluent and copper oxide nanoparticles in *L. gibba* (Marwood et al., 2001; Juneau et al., 2003 and Perreault et al., 2010) and four herbicides in *Lemna paucicostata* (Kumar and Han, 2010).

The four herbicides tested in this study are the most frequently detected herbicides in water bodies. The effects of atrazine, diuron, paraquat and simazine on three species of *Lemna* (*L. gibba*, *L. minor* and *L. paucicostata*) using various endpoints have been investigated in this study. The four herbicides were selected for their common use to control weeds in agricultural activities, and are discharged into aquatic ecosystems through surface runoff, thus potentially causing toxicity to non-target species. While they effectively control targeted weedy species, it is also important to establish their effects on non-targeted species, which are less well known. Specifically, data obtained from root re-growth and Chl *a* fluorescence measurements are compared with those based on a traditional endpoint of frond area. The accuracy and precisions (sensu Gopalapillai et al., 2014) of the three endpoints are evaluated.

2. Materials and method

2.1. *Sample & culture conditions*

Lemna gibba (CPCC 310), *L. minor* (CPCC 490) and *L. paucicostata* were used as research materials in the present study. *L. paucicostata* was collected from a shallow pond in Songjung-dong, Kwangsan-gu, Kwangju, Korea (35.09 N, 125.54 E), and the other two species were obtained from Canadian Phycological Culture Center. Experimental material was cultured in glass tanks (20 cm × 30 cm × 15 cm) containing 1.5 L of Steinberg medium (Steinberg, 1946), adjusted to a pH of 6.9 ± 0.1with 1 M NaOH and 1 M HCl, at 25 ± 1 °C and an irradiance of 30–40 μmol photons m^{-2} s^{-1}, provided by cool white fluorescent lamps (FL 20 SS/18D, Philips Co., Thailand). The growth medium was replaced every week.

2.2. *Toxicity tests*

To compare the relative sensitivities of the three *Lemna* spp. to four herbicides (atrazine, diuron, paraquat and simazine), fronds of each species, consisting of two green leaves of similar size, were selected as test material.

Tests were carried out in a controlled environment chamber at 25 ± 1 °C and continuous light of 100 ± 10 μmol photons m^{-2} s^{-1}. Test vessels were 24-well plastic plates (85.4 mm × 127.6 mm; well dimension 15.6 mm diameter, SPL, Seoul Korea) with 3.0 ml of test solution added to each well. All herbicide stock solutions were prepared from original stock solution (Table 1) in either DMSO (for atrazine, diuron and simazine) or distilled water (for paraquat) and then diluted in a 50% dilution series (five or more concentrations

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Table 1
Final concentration range and mode of action used for testing toxicity of herbicides with three *Lemna* species.

Herbicides (CAS No.)	Physiological site	Molecular targets	Concentrations (μg L^{-1})		
			Lemna gibba	*Lemna minor*	*Lemna paucicostata*
Atrazine (1912-24-9)	Photosynthesis	Qb site of D1 protein	31.25–500.0	31.25–500.0	31.25–500.0
Diuron (330-54-1)	Photosynthesis	Qb site of D1 protein	3.125–100.0	3.125–100.0	6.25–100.0
Paraquat (1910-42-5)	Photosynthesis	Electron acception from PSI	6.25–100.0	6.25–100.0	6.25–100.0
Simazine (122-34-9)	Photosynthesis	Qb site of D1 protein	31.25–500.0	31.25–500.0	31.25–500.0

plus solvent and negative controls; triplicate replication) using the Steinberg medium.

In each of three replicate plates, 24 plants each comprising two fronds were exposed to one of 6 concentrations of herbicide, with 4 plants per concentration.

2.2.1. Frond area and root re-growth

Prior to exposure to test solutions roots were excised from fronds using scissors as described in detail elsewhere (Park et al., 2013). Fronds were then added to wells under the same conditions as described in Park et al. (2013). Following 72 h of exposure to different concentrations of herbicides, plants were harvested to determine changes in surface area using an image analyzer (MV200, Samsung, Seoul, Korea). The relative growth rates (RGR_{area}) were determined according to the formula:

$$RGR_{area}\left(\%d^{-1}\right) = \frac{\ln A_f - \ln A_i}{t_f} \times 100$$

where A_i = initial frond area, A_f = final frond area and t_f = the test duration.

Lengths of longest roots (each frond generally has 1–2 roots) were measured with the same instrument.

2.2.2. Chlorophyll (Chl) a fluorescence

Chl *a* fluorescence was measured simultaneously in all frond samples using a pulse amplitude modulated imaging fluorometer (I-PAM, Walz, Effeltrich, Germany). After exposure, samples were dark adapted for 15 min to obtain the equilibrium of the PSII oxido-reduction state (Ralph, 1997). Maximum quantum yield of PSII in the dark-adapted state (F_v/F_m), derived from $(F_m - F_o)/F_m$, where F_m and F_o are the maximum and minimum fluorescence of dark-adapted fronds, was recorded. This is a very important plant property that indicates how efficient the light reaction is proceeding (Ritchie, 2006). The effective quantum yield of PSII, calculated as $(F'_m - F)/F'_m$, where F'_m is the maximum light-adapted fluorescence yield and F is the lowest fluorescence yield at F_o in dark-adapted samples, was also measured. The effective quantum yield is an actual quantum yield at a point in time and this is generally much lower than the optimal quantum yield (Ritchie, 2006).

Rapid light curves (RLC) were produced using 10 s pulses of actinic light increased stepwise from 0 to 1517 μmol photons m^{-2} s^{-1}. The levels of the measuring and saturating light were <0.4 μmol photons m^{-2} s^{-1} and >6000 μmol photons m^{-2} s^{-1} for 0.4–0.8 s, respectively. Maximum electron transport rates (ETR_{max}) were derived from the RLC data using the following equation, with the aid of the program Grapher 3 (Golden Software Inc., USA):

$$ETR = 0.5 \times PAR \times Yield \times AF$$

AF refers to the absorption of light by *Lemna* fronds. Green plants generally exhibit a value of approximately 0.84. However, since this value can vary between species, AF was calculated as the ratio of incident PAR absorbed by the fronds of each *Lemna* species. PAR sources were white light fluorescent tubes and PAR levels were measured using a LiCor quantum sensor (LI 1400) before and after covering with fronds.

2.3. Statistical analysis

Four herbicides and 5 endpoints were included in the statistical analyses. The results are reported as EC_{10}s and EC_{50}s values with 95% CI estimated by the linear interpolation method (ToxCalc 5.0, Tidepool Science, CA, USA).

3. Results

3.1. Frond area

There was a significant decrease in frond area with respect to the type and concentration of herbicide tested ($p < 0.05$). The EC_{50}s of RGR_{area} are shown in Tables 2–4. For this parameter, the rank order of the tested herbicides was: diuron > paraquat > atrazine > simazine for *L. gibba* and *L. minor* and diuron > paraquat > simazine > atrazine for *L. paucicostata*.

Although diuron was found to be most toxic to all three *Lemna* species there were species-specific differences in sensitivity as indicated by the EC_{50} values: *L. paucicostata* (24.3 μg L^{-1}) > *L. gibba* (29.8 μg L^{-1}) > *L. minor* (34.6 μg L^{-1}). The least toxic herbicide for *L. gibba* and *L. minor* was simazine (276.1 μg L^{-1}) but it was atrazine (342.2 μg L^{-1}) for *L. paucicostata*.

CV values for frond area were between 2.28 and 14.82%.

3.2. Root re-growth

The rank order of herbicide toxicity, derived from EC_{50}s of the inhibition of root regrowth was: paraquat > diuron > atrazine > simazine for *L. gibba* and *L. minor*, and paraquat > diuron > simazine > atrazine for *L. paucicostata*. Paraquat was the most toxic herbicide for all three *Lemna* species but their sensitivity differed with EC_{50} values of 7.1 μg L^{-1} for *L. gibba*, 7.9 μg L^{-1} for *L. paucicostata* and 10.6 μg L^{-1} for *L. minor* and (Tables 2–4). The least toxic herbicide was simazine (156.0 and 226.6 μg L^{-1}) for *L. gibba* and *L. minor*, respectively and atrazine (206.2 μg L^{-1}) for *L. paucicostata* as seen in frond area.

Coefficients of variation for root re-growth ranged from 9.86 to 15.06% for *L. gibba*, 2.4–17.96% for *L. minor* and 2.50–22.42% for *L. paucicostata*.

3.3. Chlorophyll a fluorescence

The optimal quantum yield (F_v/F_m) of all *Lemna* species did not change significantly even on exposure to the highest concentrations of three of the four herbicides tested. The exception was atrazine which caused a 50% reduction at 351.0 μg L^{-1} for *L. gibba*, 237.7 μg L^{-1} for *L. minor* and 126.2 μg L^{-1} for *L. paucicostata* (Tables 2, 3, and 4). Coefficients of variation for all the EC_{50} values were less than 17.19%.

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Table 2
EC_{10}, EC_{50}, and CV values for inhibition of various parameters in *Lemna gibba* exposed to 4 herbicides. Mean and 95% CI are shown (n = 3 plates, 24 plants per plate with 4 plants per concentration). (Unit: $\mu g \cdot L^{-1}$).

End point	Herbicides	*Lemna gibba*			
		EC_{10} (95% CI)	CV(%)	EC_{50} (95% CI)	CV(%)
RGR_{area}	Atrazine	45.3 (18.7–63.3)	24.23	149.0 (119.7–171.7)	8.52
	Diuron	7.8 (3.8–11.9)	28.72	29.8 (24.0–34.2)	8.72
	Paraquat	10.5 (9.5–11.8)	5.87	47.7 (41.8–52.1)	6.29
	Simazine	55.6 (17.8–89.9)	39.65	276.1 (213.4–332.5)	11.30
Root length	Atrazine	37.1 (13.0–69.3)	40.30	111.7 (83.6–142.4)	12.18
	Diuron	4.2 (1.7–6.7)	31.26	14.8 (10.7–18.5)	14.20
	Paraquat	1.3 (1.2–1.5)	6.82	7.1 (6.0–8.6)	9.86
	Simazine	58.3 (12.3–82.4)	44.23	156.0 (114.6–200.7)	15.06
F_v/F_m	Atrazine	82.0 (0–99.6)	17.19	352.0 (160.4–462.4)	16.17
	Diuron	10.8 (6.7–14.9)	21.52	>50.0	–
	Paraquat	10.5 (8.0–17.2)	26.36	>100.0	–
	Simazine	157.8 (89.7–193.2)	12.57	>500.0	–
F'_v/F'_m	Atrazine	13.6 (6.9–55.0)	109.41	81.6 (48.3–101.8)	17.08
	Diuron	1.5 (1.2–1.9)	11.81	9.4 (8.6–10.2)	4.19
	Paraquat	8.0 (5.0–11.3)	20.10	39.7 (36.2–46.8)	7.80
	Simazine	12.6 (11.2–14.1)	5.88	79.2 (73.8–83.5)	3.09
ETR_{max}	Atrazine	11.1 (6.0–37.1)	90.45	51.5 (29.8–73.3)	20.35
	Diuron	2.7 (1.4–4.6)	34.87	9.1 (8.2–10.1)	5.72
	Paraquat	1.3 (1.0–2.2)	23.72	7.1 (5.0–10.3)	21.77
	Simazine	22.7 (9.5–43.0)	48.28	80.0 (55.8–98.4)	14.82

Table 3
EC_{10}, EC_{50}, and CV values for inhibition of various parameters in *Lemna minor* exposed to 4 herbicides. Mean and 95% CI are shown (n = 3 plates, 24 plants per plate with 4 plants per concentration). (Unit: $\mu g \cdot L^{-1}$).

End point	Herbicides	*Lemna minor*			
		EC_{10} (95% CI)	CV(%)	EC_{50} (95% CI)	CV(%)
RGR_{area}	Atrazine	51.0 (16.1–129.2)	53.51	219.6 (175.9–300.8)	12.66
	Diuron	12.3 (7.5–14.3)	14.0	34.6 (29.8–37.7)	6.07
	Paraquat	17.3 (15.1–18.3)	4.86	56.9 (54.0–58.9)	2.28
	Simazine	51.5 (17.9–90.5)	41.91	229.1 (188.5–313.9)	12.40
Root length	Atrazine	17.9 (8.8–65.5)	83.92	120.7 (61.6–180.7)	2.40
	Diuron	8.0 (1.9–9.5)	28.80	25.0 (20.7–29.3)	9.20
	Paraquat	2.0 (1.5–3.1)	21.03	10.6 (8.8–12.3)	9.43
	Simazine	17.8 (9.8–74.1)	109.18	226.6 (123.0–294.8)	17.96
F_v/F_m	Atrazine	30.6 (0.2–119.0)	58.73	341.5	–
	Diuron	8.2 (0–11.7)	27.69	>50	–
	Paraquat	15.6 (0.9–24.1)	12.72	>100	–
	Simazine	37.0 (24.3–54.5)	22.55	>500	–
F'_v/F'_m	Atrazine	8.9 (6.2–15.8)	28.51	57.3 (30.9–76.5)	17.82
	Diuron	1.6 (1.2–2.1)	15.94	9.9 (9.2–10.4)	2.93
	Paraquat	7.3 (4.2–9.9)	21.39	36.0 (31.6–41.3)	6.70
	Simazine	10.2 (8.9–12.4)	9.06	59.8 (51.5–71.3)	8.30
ETR_{max}	Atrazine	8.8 (5.2–35.9)	101.13	43.3 (26.2–67.3)	24.33
	Diuron	3.1 (1.0–6.0)	48.73	9.5 (8.2–10.5)	6.34
	Paraquat	1.3 (0.9–3.2)	63.57	6.6 (4.7–9.8)	21.86
	Simazine	13.6 (7.7–37.4)	69.41	59.3 (47.9–75.1)	11.39

The effective quantum yield of PSII (F'_v/F'_m) decreased significantly with increasing herbicide concentrations ($p < 0.05$). The rank order, based on EC_{50}s, was diuron > paraquat > simazine > atrazine for *L. gibba*, and diuron > paraquat > atrazine > simazine for *L. minor* and *L. paucicostata*. CV values were between 2.93 and 17.82% for all the tested herbicides in the three *Lemna* species (Table 2).

Maximum electron transport rates (ETR_{max}) of the plants exposed to all herbicides were significantly ($p < 0.05$) inhibited with a rank order of paraquat > diuron > atrazine > simazine for *L. gibba* and *L. minor*, and diuron > paraquat > simazine > atrazine for *L. paucicostata*.

3 correlative relationships between the two endpoints (effective quantum yield vs root regrowth, root regrowth vs frond area and effective quantum yield vs frond area) were summarized in Figs. 1–3.

4. Discussion

Atrazine, diuron, and simazine are all photosystem II (PSII) inhibitors while paraquat inhibits photosystem I (PSI). Atrazine and simazine both compete with the second electron acceptor (Q_B binding site) of the D1 protein in PSII, and exert a similar mode of actions whereas diuron strongly blocks the re-oxidation of the primary electron acceptor (Qa) (Ralph, 2000). These three PSII herbicides inhibit photosynthetic electron flow, leading to reduced CO_2 fixation and growth in plants. Paraquat accepts electrons from Fe–S centers and/or ferredoxin of the PSI complex which disrupts electron transfer to NADP (Eullaffroy and Vernet, 2003). Exposure to all these herbicides also generates reactive oxygen species (ROS) such as superoxide and hydroxyl radicals, and H_2O_2, which causes oxidative damage within plant cells (Dodge, 1975).

The most frequently measured endpoint in *Lemna* toxicity tests

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Table 4
EC_{10}, EC_{50}, and CV values for inhibition of various parameters in *Lemna paucicostata* exposed to 4 herbicides. Mean and 95% CI are shown (n = 3 plates, 24 plants per plate with 4 plants per concentration). (Unit: $\mu g \cdot L^{-1}$).

End point	Herbicides	*Lemna paucicostata*			
		EC_{10} (95% CI)	CV(%)	EC_{50} (95% CI)	CV(%)
RGR_{area}	Atrazine	104.1 (67.2–132.4)	17.70	342.2 (277.5–373.4)	7.33
	Diuron	10.8 (7.1–12.9)	14.08	24.3 (22.7–26.8)	4.12
	Paraquat	23.1 (12.4–27.0)	13.28	45.6 (41.2–51.1)	5.26
	Simazine	43.8 (32.9–59.5)	15.43	209.8 (170.8–291.0)	14.82
Root length	Atrazine	82.6 (19.7–130.6)	33.39	206.2 (181.4–224.2)	5.38
	Diuron	5.9 (2.7–9.5)	35.09	20.0 (19.1–21.0)	2.50
	Paraquat	1.3 (1.2–1.6)	7.81	7.9 (5.8–11.0)	18.99
	Simazine	59.6 (18.9–77.1)	26.19	162.8 (111.5–250.0)	22.42
F_v/F_m	Atrazine	16.4 (11.9–22.4)	10.57	126.2 (112.2–160.1)	6.51
	Diuron	1.3 (0.9–2.0)	12.63	8.3 (6.0–11.0)	9.58
	Paraquat	14.7 (8.6–20.1)	12.52	>100	–
	Simazine	27.2 (11.7–45.9)	23.55	143.2 (112.5–184.6)	8.30
F'_v/F'_m	Atrazine	10.0 (8.5–11.9)	9.60	58.3 (53.5–64.2)	4.46
	Diuron	1.5 (1.3–1.7)	6.09	7.8 (6.8–8.6)	6.06
	Paraquat	7.2 (4.3–9.7)	18.58	30.1 (27.5–32.5)	4.62
	Simazine	14.1 (11.8–17.1)	9.65	66.2 (58.1–74.1)	6.91
ETR_{max}	Atrazine	13.5 (7.4–35.9)	60.26	58.0 (45.2–73.9)	12.17
	Diuron	1.5 (1.1–3.2)	44.72	7.6 (5.6–9.5)	14.91
	Paraquat	1.6 (1.1–3.1)	55.72	8.0 (5.5–9.7)	14.65
	Simazine	17.8 (8.9–37.2)	17.95	55.6 (47.2–63.9)	7.45

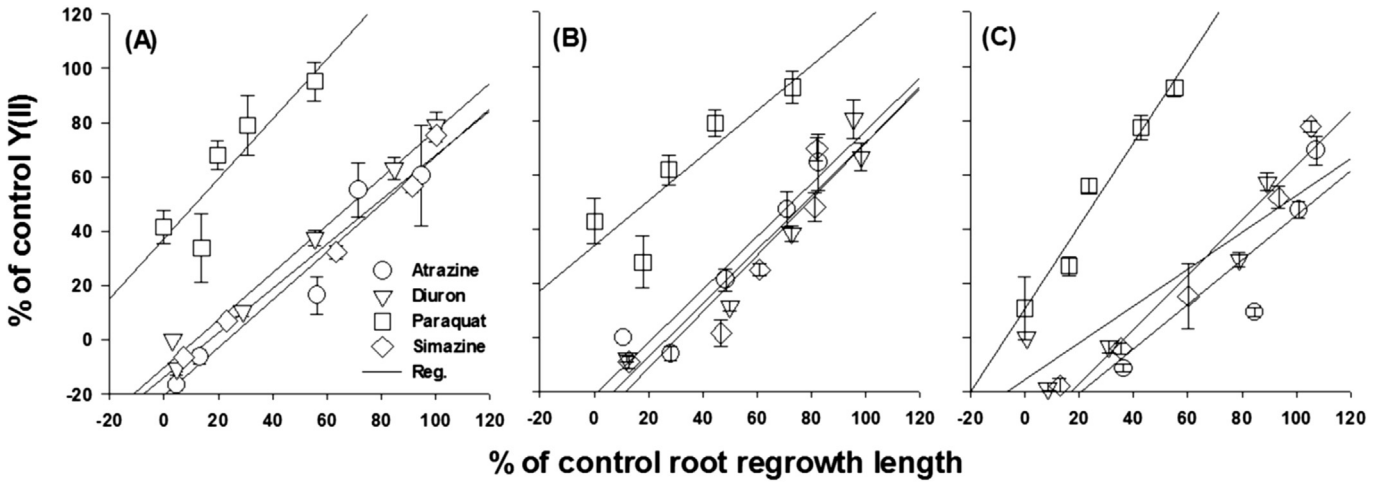

Fig. 1. Relationship between root re-growth length and the effective quantum yield [Y(II)] responses to herbicides after 72 h exposure,. (A) *Lemna gibba*, (B) *Lemna minor*, and (C) *Lemna paucicostata*.

is frond area. In this study, the sensitivity of frond area was compared with that of two other endpoints, but measured after 3 days of exposure instead of the typical 7 days. It is usually accepted that the longer exposure time, the greater sensitivity to toxicants (Mohammad et al., 2010) and therefore, the use of shorter exposure periods should be taken into account when interpreting the present results. Based on RGRs derived from frond area, the most toxic herbicide was diuron with EC_{50} values of 24.3–34.6 $\mu g\ L^{-1}$ for the three *Lemna* species tested, which did not differ significantly in their responses. These values obtained after 3 days of exposure are similar to the reported EC_{50} values (16.0–102.0 $\mu g\ L^{-1}$) for vegetative growth endpoints including frond number, frond area, fresh and dry weight in *Lemna* species exposed to diuron for 7 days (Table 5). Values obtained for paraquat (EC_{50}s of 45.6–56.9 $\mu g\ L^{-1}$) and simazine (209.8–276.1 $\mu g\ L^{-1}$) were also within previously reported ranges of the two herbicides (EC_{50} 17–617 $\mu g\ L^{-1}$, paraquat; 100–550 $\mu g\ L^{-1}$, simazine); differences between species were not significant. It was notable that the toxicity of atrazine was species-specific with the ranking order of *L. gibba* > *L. minor* > *L. paucicostata*. A literature search provided evidence for a similar toxicity ranking for the same three species (Table 5).

The relatively low CV values of the frond area endpoint (2.28–14.82%) indicate good repeatability and stability of the method (Table 2).

Changes to the methodology of the test procedure did not alter the sensitivity to the four herbicides previously reported using conventional tests.

4.1. Root re-growth

In the past, little attention has been paid to the roots in *Lemna* since it was generally considered that root fragility made their handling for measurements difficult and that it was impractical to obtain sufficient numbers of individual plants with identical root lengths to initiate tests. However, more recently the ecotoxicological significance of the root endpoint has been re-evaluated and root length was shown to be a sensitive, precise and ecologically

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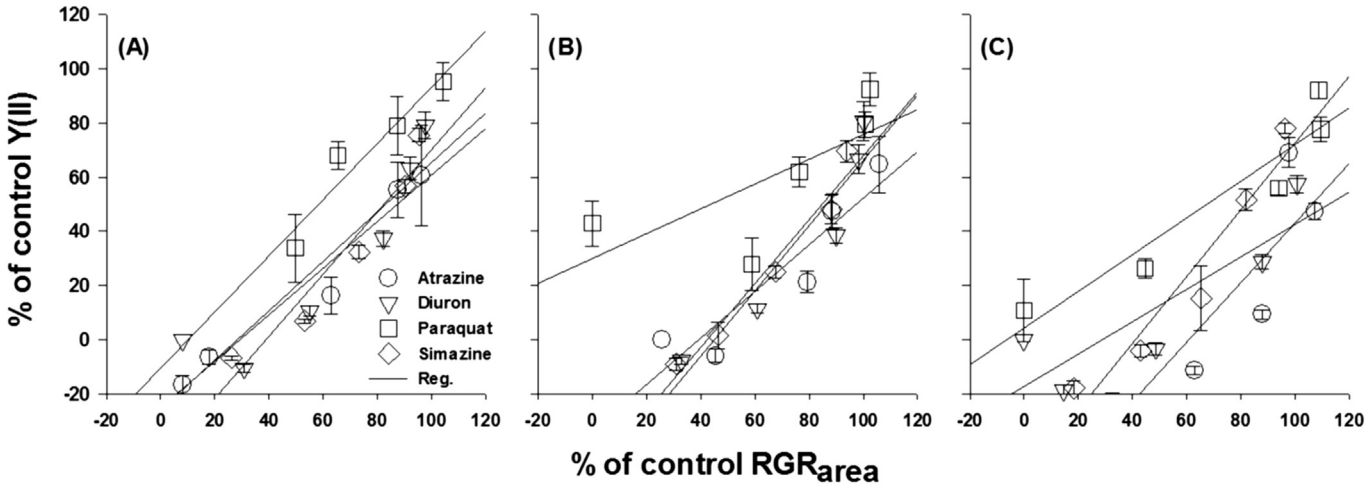

Fig. 2. Relationship between RGR_{area} and the effective quantum yield [Y(II)] responses to herbicides after 72 h exposure,. (A) *Lemna gibba*, (B) *Lemna minor*, and (C) *Lemna paucicostata.*

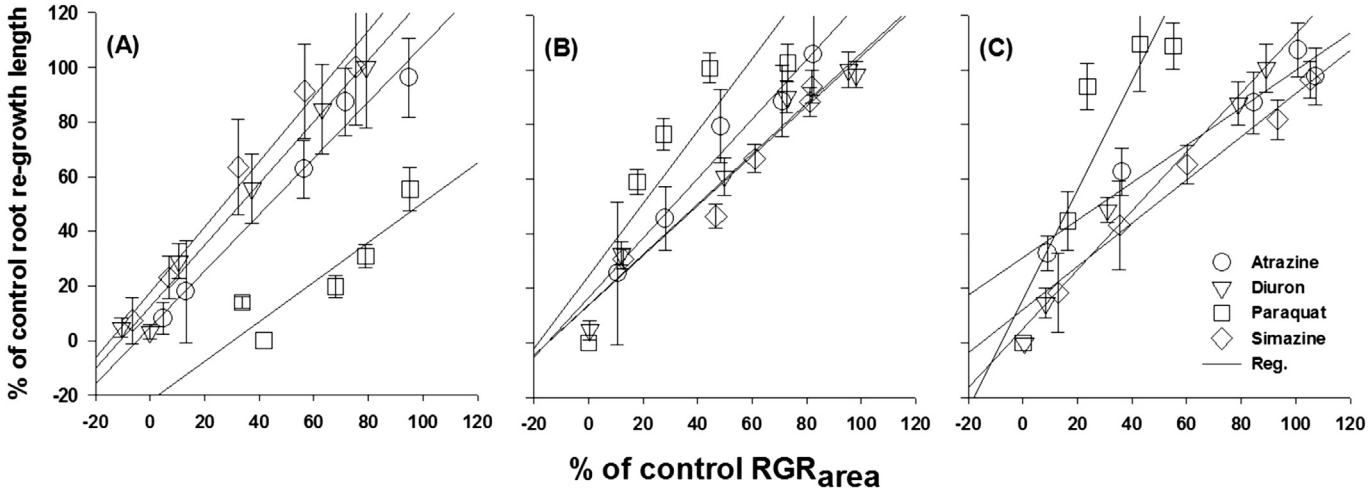

Fig. 3. Relationship between RGR_{area} and root re-growth length responses to herbicides after 72 h exposure,. (A) *Lemna gibba*, (B) *Lemna minor*, and (C) *Lemna paucicostat.*

significant endpoint in comparison with more traditional frond growth and biomass endpoints (Park et al., 2013; Gopalapillai et al., 2014).

Compared with results obtained from measurements of frond area, root re-growth showed paraquat to be the most toxic of the tested herbicides to all three *Lemna* species, with mean EC_{50} values of 7.1, 7.9 and 10.6 μg L^{-1} for *L. gibba*, *L. paucicostata* and *L. minor*, respectively. Diuron was the second most toxic herbicide to root re-growth although it was most toxic to frond growth. It is however notable that the EC_{50} values of diuron toxicity were similar between these two endpoints, indicating similar mechanism of diuron toxicity to vegetative growth processes. The sensitivity of root lengths to diuron (EC_{50} = 14.8–20.0 μg L^{-1}) was similar to or higher than that of other growth endpoints (EC_{50} = 15–450 μg L^{-1}, Table 5). The range of coefficients of variation of the root test method was between 2.4 and 22.42%, which was again within the recommendable levels of variation for standard test methods (Table 2).

4.2. Chlorophyll a fluorescence

One of the most frequently used methods for monitoring the status of the photosynthetic apparatus in plants is *in vivo* chlorophyll *a* fluorescence, a non-destructive, straightforward and rapid technique that is applicable in both laboratory and field studies. It is used as a potential indicator of exposure to environmental and chemical stresses, including herbicides.

The impact of certain herbicides, such as commonly used ones like diuron, atrazine and simazine, on the photochemical activity of PSII has long been recognized (Beaumont et al., 1976; Merlin et al., 1993; Küster and Altenburger, 2007; Kumar and Han, 2011). Such PSII inhibitors restrict photosynthetic activity through their binding to the D1 protein in thylakoids and blocking electron transport through the PSII reaction center with effects being manifested by changes in various chlorophyll fluorescence parameters (Murata et al., 2007).

The maximum quantum yield of PS II chemistry, which is provided by the ratio F_v/F_m (Genty et al., 1992), is consistent across higher plants species which typically have F_v/F_m maxima of about 0.84; this is also true for the *Lemna* spp. (0.68 ± 0.04) in this study. F_v/F_m assesses the intrinsic photochemical efficiency of PS II, reflecting the intactness of the PS II/LHC II complex or the intactness of thylakoid membranes Björkman and Demmig 1987; DeEll et al. 1999), which may not become apparent when the site of

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Table 5
List of herbicides toxicity tests using various endpoints in *Lemna* sp.

Toxicant	Test organism	End-point	Exposure time	EC_{50} ($\mu g \cdot L^{-1}$)	References
Atrazine	*L. gibba*	FN	14d	1600	Mohammad et al. (2010)
			28d	89	
	L. gibba	NR	14d	37 (19–72)	Hoberg (1993)
				50 (22–80)	
				22 (4.8–100)	
				45 (15–140)	
	L. gibba	FN	3d	102 (51–127)	Brain et al. (2012)
			5d	86 (52–111)	
			7d	65 (52–79)	
			9d	>77	
			14	67 (62–71)	
		GR	3d	>137	
			5d	>137	
			7d	124 (90–150)	
			9d	>77	
			14d	>75	
		BM	3d	90 (59–110)	
			5d	66 (56–75)	
			7d	57 (48–67)	
			9d	>77	
			14d	64 (58–68)	
	L. gibba	FN	7d	120.0 (32.9–207.1)	Rentz (2009)
		PN		128.4 (0–365.6)	
		WM		64.3 (0–174.9)	
		DM		93.0 (0–295.9)	
		RGR(FN)		187.9 (119.2–256.7)	
		RGR(PN)		292.2 (0–590.6)	
	L. minor	FN	7d	133.6 (83.5–183.7)	
		PN		146.9 (76.1–217.7)	
		WM		84.5 (55.9–113.1)	
		DM		79.9 (63.6–96.1)	
		RGR(FN)		218.2 (182.1–254.3)	
		RGR(PN)		321.0 (260.1–381.8)	
	L.minor	FN	10d	56	Kirby and Sheahan (1994)
		FW		60	
		CHL		62	
	L.minor	FN	4d	153 (89–217)	Fairchild et al. (1997)
	L.minor	FN	14d	92 (80–104)	Fairchild et al. (1998)
	L.minor	FW	7d	121 (102–136)	Teodorovic et al. (2011)
		FN		215 (172–234)	
		FA		188 (162–210)	
	L.minor	$\Delta F/F'_m$	1h	323	Küster and Altenburger (2007)
			5h	138	
			24h	131	
	L. minor	Photosynthesis	10,15,20d	1000	Beaumont et al. (1976)
	L.minor	NR	7d	197.42	Blackburn (1988)
				86.3	
	L.paucicostaa	FA	7d	929	Michel et al. (2004)
	L. perpusilla	RGR	7d	13,487 (16,000-32,000)	Phewnil et al. (2012)
	Lemna sp.	F_v/F_m	4d	107 (92–122)	Kumar and Han (2011)
Diuron	*L. gibba*	FN	7d	41.6 (12.1–71.2)	Rentz (2009)
		PN		61.8 (0–145.2)	
		WM		30.5 (13.6–47.4)	
		DM		32.0 (18.8–45.2)	
		CHL		2.3×10^5 ($0–9.1 \times 10^6$)	
		RGR(FN)		74.7 (25.0–124.3)	
		RGR(PN)		115.7 (0–255.4)	
	L. minor	FN	7d	54.9 (22.3–87.5)	
		PN		51.7 (24.5–78.8)	
		WM		44.1 (15.1–73.2)	
		DM		41.8 (12.1–71.4)	
		RGR(FN)		102.0 (54.2–149.7)	
		RGR(PN)		97.7 (70.8–124.6)	
	L. minor	FN	7d	25.0 (22.0–28.0)	Teisseire et al. (1999)
	L. perpusilla	FN	7d	15	Liu and Cendeno-Maldonado (1974)
	Lemna sp.	RGR(FN)	7d	20.0 (15.0–28.0)	Kumar and Han (2010)
		RGR(FA)		16.0 (10.0–22.0)	
		RGR(FW)		11.0 (5.0–15.0)	
		F_v/F_m		9.0 (8.0–10.0)	
		ETRmax		9.0 (6.0–10.0)	
	Lemna sp.	NR	1d	450	Knauf and Schulze (1972)
Paraquat	*L. gibba*	RGR	7d	31	Mohammad and Itoh (2011)
	L. gibba	RGR	14d	27.3 (17–85.8)	U.S. EPA (2013)

(*continued on next page*)

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Table 5 (*continued*)

Toxicant	Test organism	End-point	Exposure time	EC_{50} ($\mu g \cdot L^{-1}$)	References
	L. minor	FN	5d	107	Tokousbalides et al. (2007)
	L. minor	FN	4d	51 (25–77)	Fairchild et al. (1997)
	L. paucicostata	FA	7d	617	Michel et al. (2004)
simazine	*L.gibba*	FN	14d	310–620	Mazzeo et al. (1998)
		DW		260–550	
		CHL		290–530	
		SA		330–530	
	L.minor	$\Delta F/F'_m$	10d	2670 (2170-3290)	Merlin et al. (1993)
		FN		550 (430–700)	
		$\Delta F/F'_m$	4d	>3000	
		FN		350 (250–510)	
	L. minor	FN	7d	100-1000	Okamura et al. (2000)
	L.minor	FN	4d	166 (102–230)	Fairchild et al. (1997)

*FN; Frond number, RGR; Relative growth rate, GR; Growth rate, BM; Biomass, PN; Plants number, WM; Weight mass, DM: Dry mass, FW; Fresh weight, FA; Frond area, $\Delta F/F'_m$: Effective quantum yield, Fv/Fm; Optimal quantum yield, ETRmax; The maximum electron transport rate, CHL; Chlorophyll, SA; Surface area, NR; Not reported

herbicide action is in another part of the plant (Murchie and Lawson, 2013). Of the four herbicides tested, only atrazine caused a significant reduction in F_v/F_m within the tested concentration range (6.25–500 $\mu g\ L^{-1}$). These results imply that the ratio of PS II/LHC II or thylakoid membranes may have been affected by atrazine. In barley (*Hordeum vulgare* L. cv Boone), sub-lethal concentrations of atrazine induced the redistribution of light-harvesting Chl from Photosystem I to Photosystem II with no effect on the number of thylakoid membrane-protein complexes associated with electron transport (De la Torre and Burkey, 1992). An increase in quantum funneling may be the reason for decreased efficiency of energy transfer in the PSII/LHC complex, thereby lowering F_v/F_m in *Lemna* exposed to higher concentrations of atrazine.

EC_{50} values for the inhibition of F_v/F_m by atrazine were 351.0 $\mu g\ L^{-1}$ for *L. gibba*, 237.7 $\mu g\ L^{-1}$ for *L. minor* and 126.2 $\mu g\ L^{-1}$ for *L. paucicostata* which were comparable to the inhibition of biomass (57.0 $\mu g\ L^{-1}$) and plant number (292.2 $\mu g\ L^{-1}$) for *L. gibba*, dry weight (79.9 $\mu g\ L^{-1}$) and plant number (321.0 $\mu g\ L^{-1}$) for *L. minor* and frond area (929.0 $\mu g\ L^{-1}$) for *L. paucicostata* (Tables 2, 3, and 4).

Relatively low coefficients of variation (6.51–16.67 $\mu g\ L^{-1}$) for the EC_{50} values may indicate confidence of repeatability of F_v/F_m measurements.

The effective quantum yield of PSII, as measured by F'_v/F'_m, dropped significantly with increasing herbicide concentrations ($p < 0.05$) showing the first and second toxic herbicides based on EC_{50}s being diuron (7.8–9.9 $\mu g\ L^{-1}$) and paraquat (30.1–39.7 $\mu g\ L^{-1}$), respectively in all *Lemna* species tested.

F'_v/F'_m represents the ability of a phototroph to move electrons beyond PSII and is generally considered a more sensitive indicator of herbicide impact than F_v/F_m (Macinnis-Ng and Ralph, 2003). The greater sensitivity of F'_v/F'_m is thought to relate to processes during the period of dark adaptation, a requirement before taking F_v/F_m but not F'_v/F'_m measurements, which reduce non-photochemical quenching and the pressure on the PS II reaction centers (Maxwell and Johnson, 2000). The photosynthetic endpoint F'_v/F'_m in response to diuron and paraquat appears to be more sensitive than the frond area endpoint (24.3–34.6 $\mu g\ L^{-1}$ for diuron and 45.6–56.9 $\mu g\ L^{-1}$ for paraquat), but similar to or less sensitive than root re-growth (14.8–25 $\mu g\ L^{-1}$ for diuron and 7.1–10.6 $\mu g\ L^{-1}$ for paraquat).

Acceptable CV values for the inhibition of F'_v/F'_m recorded with the range between 2.93 and 17.82% for all the tested herbicides in the three *Lemna* species.

The rate of photosynthetic electron transport (ETR) depends on the rate of photon absorption and the efficiency of PS II (Snel et al., 1998) meaning that the efficiency of PS II electron transport describes the probability that a photochemical event will result in electron transport upon absorption of a photon by the antennae of PS II. Species-specific differences in the inhibition of maximum photosynthetic electron transport rates were observed with the most toxic herbicide being paraquat for *L. gibba* (7.1 $\mu g\ L^{-1}$) and *L. minor* (6.6 $\mu g\ L^{-1}$) and diuron for *L. paucicostata* (7.6 $\mu g\ L^{-1}$) (Tables 2, 3, and 4). The second toxic compound for inhibition of ETR_{max} was diuron for *L. gibba* (9.1 $\mu g\ L^{-1}$) and *L. minor* (9.5 $\mu g\ L^{-1}$), but it was paraquat for *L. paucicostata* (8.0 $\mu g\ L^{-1}$).

After a survey on 24 references reported for herbicide toxicity to *Lemna* species, we found that EC_{50} values of diuron- and paraquat-induced inhibition of ETR_{max} were lowest, indicating that the endpoints were most sensitive (Table 5).

In this study, ETR_{max} values showed a clear concentration-dependent decline, and their EC_{50} values were much lower than those of any other endpoints tested. In PAM instrumentation, calculation of ETR_{max} based on Chl *a* fluorescence measurement is simple and rapid, and this can be considered as the most sensitive endpoint at least for testing herbicide toxicity. The CV ranges were found to lie between 5.72 and 24.33% for all the herbicides tested with the three *Lemna* species.

The root re-growth bioassay also differs from three internationally standardized methods (ISO, OECD and US EPA) in that it is completed in 72 h, the required volume of test solutions is only 3.0 mL and non-axenic plants are used. There are some operational advantages in being able to complete a test in 3 days, thus alleviating the need for axenic cultures. However, the sensitivity of the method is unknown when compared to the 7-d or 14-d methods. This might be a key performance characteristics and cannot be outweighed by operational advantages like speed. Tests using *Lemna* are most widely used for the evaluation of pesticides (especially herbicides) so a comparison of sensitivity to herbicides would await further investigations.

5. Conclusions

The comparative studies of the sensitivity of photosynthetic endpoints with that of growth endpoints have been made but have only shown mixed results. In this study, PAM fluorescence method was found to be more sensitive than the bioassay based on frond area inhibition. Photosynthetic electron transport events support the biochemical reactions needed for plant growth since the electron transport rate is closely related to the photosynthetic activity including oxygen evolution or CO_2 uptake (Beer et al., 1998). Therefore, a direct or an indirect effect of a pollutant on photosynthetic processes is observed prior to an effect on the growth process (Juneau et al., 2003). The relationship between PSII inhibition and growth may exist for short-term studies, but detoxification of cells would be possible and recovery of growth could be

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observed, thereby the inhibition of PSII inhibition no longer reflecting the inhibition of growth (Hayat et al., 2012).

Current guidelines for the allowable concentrations in drinking waters set by Australia, Canada, New Zealand, USA, and WHO are 0.5–5 μg L^{-1} for atrazine, 20–150 μg L^{-1} for diuron, 10 μg L^{-1} for paraquat and 0.5–10 μg L^{-1} for simazine. Sensitivity of bioassay methods is important for determination of whether to use them or not for water quality risk assessment. Effective bioassays should produce results within the relevant environmental ranges. Given that environmentally allowable concentrations of herbicides are low, this study shows that both endpoint root length and ETR_{max} in all three *Lemna* species are sensitive enough to detect toxic impacts of water samples containing diuron and paraquat in excess of allowable guidelines, and would successfully be employed for management decision. In the case of *L. paucicostata*, F_v/F_m was also found to be a possible indicator of diuron toxicity at internationally allowable levels. In contrast, the *Lemna* bioassays would not be employed to determine whether water samples are within the environmentally allowable concentrations for atrazine and simazine. The *Lemna* methods show a high level of precision and reproducibility which are essential for adoption of toxicity testing methods. A desirable level of repeatability expressed by CVs is 30% or less according to Environment Canada (2007). For all bioassays with five different endpoints CVs for EC_{50} values were found to lie within this acceptable range (Park et al., 2016). The present microplate method may have limitations including pH shifts and speciation due to small testing volume and the under estimation of some substance properties (Küster and Altenburger, 2007). However, levels of EC_{50}s with the microplate method using the frond area of three species of *Lemna* were found to be comparable to those reported for 7-day *Lemna* toxicity tests, suggesting that the *Lemna* root and ETR_{max} measurement method may be a simple, rapid, cost-effective, sensitive and precise bioassays to assess the toxic risks of herbicides in aquatic environments.

To ensure thorough evaluation of the risks posed by pollutants for environment and human health, the test methods employed should be sensitive, simple, precise and ecologically relevant (Park et al., 2012). Therefore, a technique that can assess toxicity more rapidly, simply, but without loss of sensitivity would be a valuable asset. In this respect, our 3-day frond growth test may be considered as a modified test method of 7-day standardized frond test in that testing time is important factor for determination of a bioassay since management decision should be made timely just in cases of unexpected pollution events.

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