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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).

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 *L. 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