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Relative sensitivity of duckweed *Lemna minor* and six algae to seven herbicides

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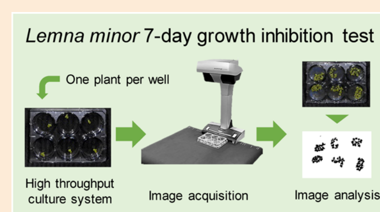
Supplementary material

We investigated the relative sensitivity of duckweed *Lemna minor* and six species of algae to seven herbicides, using an efficient high-throughput microplate-based toxicity assay. First, we assessed the sensitivity of *L. minor* to the seven herbicides, and then we compared its sensitivity to that of previously published data for six algal species based on EC_{50} values. For five herbicides, the most sensitive species differed: *L. minor* was most sensitive to cyclosulfamuron: *Raphidocelis subcapitata* was most sensitive to pretilachlor and esprocarb: *Desmodesmus subspicatus* was most sensitive to pyraclonil; and *Navicula pelliculosa* was most sensitive to pyrazoxyfen. Simetryn was evenly toxic to all species, whereas 2,4-D was evenly less toxic, with only small differences in species sensitivity. These results suggested that a single algal species cannot represent the sensitivity of the primary producer assemblage to a given herbicide. Therefore, to assess the ecological effects of herbicides, aquatic plant and multispecies algal toxicity data sets are essential.



Keywords: aquatic plant, aquatic primary producer assemblages, ecological risk, growth inhibition test, microplate toxicity assay, species sensitivity index.

Introduction

Aquatic primary producers are generally sensitive to herbicides,[1] and herbicides have been found to affect the species composition and community structure of aquatic primary producer assemblages in natural aquatic ecosystems.[2,3] Therefore, when considering the effect of herbicides on non-target organisms, concern for aquatic primary producers in natural ecosystems is important. The green alga *Raphidocelis subcapitata* (Rap) has been widely used as a standard species in conventional ecological effect assessments.[4] In Japan, pesticide registration criteria concerning toxicity to aquatic organisms are set by Japan's Ministry of Environment (MOE) under the Agricultural Chemicals Regulation Law.[5] To account for differences in species sensitivity in the effect assessment of aquatic primary producers, the 50% effect concentration (EC_{50}) for toxicity to Rap is usually divided by an uncertainty factor. Until March 2020, an uncertainty factor of 1 was used for algae because Rap was assumed to be a sensitive species.[5]

Based on algal toxicity assays using five riverine periphytic species (the green alga *Desmodesmus subspicatus* [Des], the diatoms *Achnanthidium minutissimum* [Ach], *Nitzschia palea* [Nit], and *Navicula pelliculosa* [Nav], and the cyanobacterium *Pseudanabaena galeata* [Pse]),[6] Nagai[7,8] showed that algal species are not equally sensitive to different herbicides and that Rap was not always the most sensitive species. Moreover, Nagai[7,8] demonstrated a clear relationship between species sensitivity and herbicide mode of action (MoA). Thus, no specific species is always the most sensitive, and no single standard organism can represent the sensitivity of entire algal assemblages. In Japan, the Agricultural Chemicals Regulation Law was revised in 2018, and the pesticide registration criteria have also been revised.[5] As part of the revised criteria, an algal test using Rap is required, and additional algal species (the green alga Des, the diatom Nav, and the cyanobacteria *Anabaena variabilis* and *Synechococcus leopoliensis*) are optional. The uncertainty factor applied to the lowest EC_{50} to account for differences in species sensitivity has been changed from 1 to 10 by default, but it is reduced depending on the number of algal species tested.

Aquatic plants belonging to the class Lemnaceae are attrac-

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tive experimental model organisms for several reasons, including their simple structure, small size, degree of homogeneity, ease of culture, and high growth rate.[9–13] Moreover, these plants have important ecological functions, are widely distributed, and are known to be highly sensitive to herbicides.[14,15] *Lemna* spp., particularly *L. minor* (Lem) and *L. gibba*, have been used for decades in the prospective risk assessment of pesticides worldwide. *Lemna* spp. is the standard Tier 1 test organism representing aquatic plants in the current risk assessment schemes for herbicides and plant growth regulators in the European Union[16] and the United States.[17] In Japan, toxicity tests using *Lemna* spp. in addition to algae were introduced when the criteria for herbicides were revised.[5] However, the quality and quantity of available data differ markedly among herbicides, and toxicity data sufficient for assessing differences in species sensitivity are available only for herbicides with certain MoAs.[18] Thus, the herbicide ecotoxicity data available for *Lemna* spp. are insufficient to justify their introduction as test organisms in Japan.

The main objective of the present study was to test the relative sensitivity of aquatic primary producers (Lem and the six algae—Rap, Des, Ach, Nit, Nav, and Pse) to various herbicides. First, we tested the toxicity of seven herbicides that are widely used in rice paddy fields in Japan using an efficient high-throughput microplate-based duckweed toxicity assay. Second, we compared the sensitivities of Lem and the six algae to the seven herbicides, based on EC_{50} values.

Materials and methods

1. Test organism

The strain of duckweed *Lemna minor* (Lem) used here originated from a laboratory culture at Eurofins-GAB GmbH (Pforzheim, Germany). Stock cultures were aseptically maintained in 300 mL polycarbonate boxes with lids (6.5 cm in width, 6.5 cm in length, and 8 cm in height) containing 100 mL Swedish Standard (SIS) medium,[19] with the pH adjusted to 6.5, at 20°C with a light intensity of 1000 lux. Continuous light was provided using a white fluorescent lamp (FL8W, color temperature 4200 K, NEC, Tokyo, Japan). Lem specimens were subcultured every 2 weeks by transferring a plant to 100 mL of fresh growth medium. Preculturing was performed in SIS medium for 7 days at 24°C and 5000 lux to accustom plants to test conditions.

2. Test substances

Test performance was evaluated by conducting bioassays of 3,5-dichlorophenol (DCP), which is used as a reference substance in standardized toxicity testing.[19] Seven herbicides with various MoAs were used as test substances (Table 1). All analytical standards were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Stock solutions of the herbicides were prepared in dimethyl sulfoxide (DMSO; FUJIFILM Wako Pure Chemical), but stock solutions for 2,4-dichlorophenoxyacetic acid (2,4-D) were prepared in SIS medium because of their high water solubility (20.03 g/L at pH 5, 34.2 g/L at pH 9).[20] The effect of DMSO on the growth of Lem was preliminarily assayed using 0%, 0.01%, 0.1%, and 1% DMSO with the same method as that described below. No observed effect concentrations using the growth rate based on the total frond area and frond number as the endpoint were both 1%, indicating that final test solutions should be prepared using <1% DMSO (Supplemental Fig. S1). Here, the DMSO concentration in the test solution was set to 0.1% (v/v).

Table 1. The properties of the seven herbicides studied: CAS number, mode of action (MoA; legacy HRAC code), and 50% effect concentration (EC_{50}) values reported for the green alga *Raphidocelis subcapitata*

Herbicide	CAS No.	MoA[a]	EC_{50}(μg/L)[b]
cyclosulfamuron	136849-15-5	B	3.5
simetryn	1014-70-6	C1	18.9
pyraclonil	158353-15-2	E	5.4
pyrazoxyfen	71561-11-0	F2	>457
pretilachlor	51218-49-6	K3	2.92
esprocarb	85785-20-2	N	66
pretilachlor	51218-49-6	K3	2.92
esprocarb	85785-20-2	N	66
2,4-D	94-75-7	O	63600

[a] Herbicide Resistance Action Committee[28]; B: inhibitor of acetolactate synthase; C1: inhibitor of photosynthesis by photosystem; E: inhibitor of protoporphyrinogen; F2: inhibitor of 4-hydroxyphenyl-pyruvate-dioxygenase; K3: inhibitor of very long-chain fatty acid synthesis; N: inhibitor of lipid synthesis; O: like indole acetic acid. [b] Japan's Ministry of Environment[5]

3. Microplate toxicity assay

The toxicity assays with Lem were conducted in accordance with the Organisation for Economic Cooperation and Development (OECD) Test Guideline 221.[19] Although glass beakers are recommended,[19] 6-well polystyrene transparent microplates with lids (Falcon #351146, BD Biosciences, Franklin Lakes, NJ, USA) were used as test vessels to increase throughput. Each well was filled with 10 mL of test solution. Precultured plants were randomly selected and transferred to test solutions in microplates, one plant per well. The initial number of fronds per plant was three to six. Plants were exposed to a geometric sequence of six concentrations with a common ratio of 2.5. The experiments proceeded for 7 days under the preculture temperature, light intensity, and medium conditions, with three replicates for exposures and six replicates in the control tests (without test substances).[19]

Three endpoints (frond area, frond number, and root length) were evaluated. However, root length was not measured for the tests of DCP, pretilachlor, and 2,4-D. Images for determining total frond area were captured using a scanner (ScanSnap SV600, Fujitsu, Tokyo, Japan) on days 0, 3, 5, and 7 and analyzed using the image analysis software ImageJ ver. 1.51 (National Institutes of Health, Bethesda, MD, USA). When the original color image was split into RGB (red, green, and blue) channel images, preliminary analysis revealed that the leaf's green color was well characterized by the difference between the green- and blue-

channel images. Thus, the frond area was measured as a green area by subtracting the blue-channel image from the green-channel image.[21] The fronds were visually counted at the end of the experiment (7 days). To measure root length at the end of the experiment, plants were transferred from the test microplate to a glass staining dish (3.5 cm in width, 8.5 cm in length, and 8.5 cm in height) filled with 200 mL tap water with one millimeter paper. Images for determining root length were captured using a USB document camera (Ziggi-HD Plus, IPEVO, Sunnyvale, CA, USA) from the side and analyzed as for determining the frond area, using ImageJ. At the start of the assay, the root lengths of six plants were measured separately from the culture experiments, and the averaged root length was used as the initial root length.

4. *Chemical analysis*

At the start and end of the assay, the concentrations of herbicides in the culture medium were analyzed. A series of test solutions for chemical analysis were made in a microplate separate from the growth experiment and without Lem inoculation. The microplate was incubated under the same conditions as the other microplates in the growth experiment. Subsamples (400 μL) from this microplate were taken on days 0 and 7, and then acetonitrile was added. These subsamples were stored at −20°C in darkness until analysis. Details of the analytical conditions are shown in Supplemental Table S1. The geometric means of the measured concentrations on days 0 and 7 were calculated. If the geometric mean values were all within ±20% of the nominal concentration, a concentration–response analysis was conducted based on nominal values[19]; otherwise, geometric mean concentrations were used for concentration–response analysis (Supplemental Table S2).

5. *Concentration–response analysis*

During the growth experiments, the growth rate (per day) from day t' to day t was calculated as follows:

$$\text{growth rate} = \frac{\ln(x_t) - \ln(x_{t'})}{t - t'} \quad (1)$$

where x_t is the frond area, the frond number, or the root length at time t. Growth rates were calculated during the period from day 0 to day 7. Then, the relative growth rate at each test concentration was calculated by dividing by the average growth rate of the control tests (without test substances).

Concentration–response functions were determined using statistical regression analysis; that is, the relative growth rate and herbicide concentrations were fitted to a two-parameter log-logit model using nonlinear least squares regression. The model can be expressed as follows:

$$\text{relative growth rate} = \frac{1}{1 + \exp(f_a + f_b \cdot \ln(C_{her}))} \quad (2)$$

where C_{her} is the herbicide concentration (geometric mean or nominal, μg/L), and f_a and f_b are coefficient values. The 50% and 10% effect concentrations (EC_{50} and EC_{10}, respectively, μg/L) are expressed as follows:

$$EC_{50} = \exp(-f_a/f_b) \quad (3)$$

$$EC_{10} = \exp([-2.197 - f_a]/f_b) \quad (4)$$

Statistical analyses were conducted using R software ver. 3.4.4 (R Foundation for Statistical Computing).

6. *Analysis of species sensitivity index*

For comparison with EC_{50} values determined for Lem, algal toxicity data (Rap, Des, Ach, Nav, Nit, and Pse) were compiled from published literature[7,8] and assessment reports of pesticide registration criteria for toxicity by Japan's MOE (Supplemental Table S3).[5] The obtained toxicity data for the seven species were standardized based on the species sensitivity distribution (SSD)[22] concept to compare differences in species sensitivity among herbicides. The standardized toxicity was defined as the species sensitivity index (SSI).[8,18] Differences in species sensitivity to environmental contaminants can be described by the statistical distribution (often a log-normal distribution), and the SSD has been used as a key concept for higher-tier ecological effect assessment.[22]

The SSI was calculated according to the method reported by Nagai.[8,18] First, SSD analysis was conducted using the EC_{50} data for the seven species. All data were converted to confidence interval (CI) data. The EC_{50} data were treated as CI data using the upper and lower CIs. If CIs could not be calculated (see Results), for example, an EC_{50} value of 15 was treated as an interval of 14.5–15.5 (considering two significant digits) because each value within the interval could be rounded to 15. If the data were reported as "greater than" values, they were treated as CI data from the minimum value to 10 times the minimum value. For example, an $EC_{50} > 10{,}000$ was treated as a CI data of 10,000–100,000. Differences in the quality of toxicity data among herbicides, such as the number of "greater than" data, were compensated for as much as possible by this treatment. Treatment of $EC_{50} > 10{,}000$ as 10,000 (ignoring the inequality sign) would bias the results toward higher toxicity. The CI dataset was fitted to a log-normal distribution using the maximum likelihood method.[23] The maximum likelihood parameters of the distribution, logarithmic mean, and logarithmic standard deviation were obtained by fitting. The 50th percentile value of the analyzed SSD (hazardous concentration for 50% of the species, HC_{50}, which is equivalent to the geometric mean converted from the logarithmic mean) was regarded as the standardized toxicity of the herbicide. Then, the SSI was calculated as the difference in toxicity value from HC_{50} after taking the common logarithm.

$$SSI = \log_{10} HC_{50} - \log_{10} EC_{50} \quad (5)$$

The SSI is a relative index of the difference in species sensitivity: a higher SSI indicates higher sensitivity, and a difference of one unit in the SSI indicates a tenfold difference in EC_{50}. The relationships of SSI values with the tested species were tested using

a one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test.

Results

1. Lemna minor growth inhibition test

The EC_{50} and EC_{10} of DCP based on total frond area were 2.4 and 2.3 mg/L, respectively (Fig. S1). The CI could not be calculated due to the steep concentration–response relationship. The EC_{50} and EC_{10} of DCP based on frond number were 2.9 (95% CI: 2.7–3.1) and 1.8 (95% CI: 1.6–2.1) mg/L, respectively (Fig. S1).

Seven herbicide toxicity tests were conducted, and images of each microplate at the end of the experiment are shown in Supplemental Fig. S1. The EC_{50} and EC_{10} values for Lem determined based on the endpoints of frond area, frond number, and root length are shown in Table 2. Of the three endpoints, root length was the most sensitive to cyclosulfamuron, simetryn, pyraclonil, and esprocarb. Frond area was the most sensitive to pretilachlor and 2,4-D. Frond number was not the most sensitive to any of the tested herbicides. For all herbicides, sensitivity differences between endpoints were within 2.5-fold, suggesting that sensitivity differences between endpoints were small. The EC_{50}s based on total frond area were used for comparison of sensitivity with algal species because frond area can be easily measured non-destructively at the beginning, during, and at the end of the test. The fresh weights were also measured for reference and showed high correlations with the green areas (Supplemental Fig. S2).

2. Species sensitivity index

The SSIs for the seven herbicides differed widely between species (Fig. 1). Lem was the most sensitive to cyclosulfamuron and 2,4-D, whereas Rap was the most sensitive to pretilachlor and esprocarb. Average SSI values for the seven herbicides were calculated for each species: the highest value was 1.01 for Rap, followed by 0.91 for Lem, and the lowest was −0.69 for Ach. The ANOVA showed significant differences in SSI among the seven tested species ($p=0.0014$). Specifically, the average SSI for Rap was significantly higher than those of the diatoms Ach and Nit (Table 3). Additionally, the average SSI for Lem was significantly higher than that of the diatom Ach.

The SSI patterns differed among all other herbicides, suggesting that differences in species sensitivity are specific to the MoA. In particular, cyclosulfamuron (the aquatic plant Lem was highly sensitive), pretilachlor, esprocarb (the green alga Rap was highly sensitive), pyraclonil (the green alga Des was highly sensitive), and pyrazoxyfen (the diatom Nav was highly sensitive) showed remarkable SSI patterns that differed widely among species. On the other hand, simetryn was evenly toxic to all species, whereas 2,4-D was evenly less toxic, with only small differences in species sensitivity.

Discussion

EC_{50} values for DCP, based on frond number and determined using a conventional method, ranged from 2.7 to 3.4 mg/L.[24] Our result (2.9 mg/L) was within this range, supporting the validity of our microplate assay method with Lem. The microplate toxicity assay has been widely used in algal growth inhibition testing, and it has also been applied in *Lemna* spp. growth inhibition testing.[25,26] The advantages of microplate toxicity assays include 1) a small sample volume requirement, 2) economy of incubator space, 3) use of disposable microplates, and 4) increased bioanalytical output.[27]

Here, we showed that the aquatic primary producer species tested differed in their patterns of sensitivity to the various her-

Table 2. The 50% and 10% effect concentrations (EC_{50}s and EC_{10}s, μg/L) with 95% confidence intervals (CIs) for the aquatic plant *Lemna minor* using the endpoints frond area, frond number, and root length. Values are reported to two significant digits.

Herbicides		Frond area		Frond number		Root length	
		EC	95% CI	EC	95% CI	EC	95% CI
cyclosulfamuron	EC_{50}	0.28	0.22–0.35	0.27	0.22–0.33	0.20	0.16–0.24
	EC_{10}	0.076	0.041–0.14	0.047	0.027–0.082	0.046	0.025–0.083
simetryn	EC_{50}	35	29–43	38	31–45	27	21–35
	EC_{10}	13	8.2–20	11	7.0–16	13	7.8–21
pyraclonil	EC_{50}	6.2	—[a]	8.9	8.2–9.6	3.5	2.6–4.7
	EC_{10}	4.9	—[a]	3.8	5.0–2.5	1.6	0.92–2.8
pyrazoxyfen	EC_{50}	>1200	—	>1200	—	>1200	—[a]
	EC_{10}	>420	—	>420	—	370	98–1400
pretilachlor	EC_{50}	4.7	3.8–5.9	9.2	6.1–14	n/a[b]	n/a[b]
	EC_{10}	1.0	0.57–1.6	1.1	0.42–2.9	n/a[b]	n/a[b]
esprocarb	EC_{50}	1900	880–4200	3000	280–32000	1800	760–4400
	EC_{10}	470	290–750	750	450–1200	500	300–850
2,4-D	EC_{50}	16000	7200–35000	>10000	—	n/a[b]	n/a[b]
	EC_{10}	390	95–1600	—	—	n/a[b]	n/a[b]

[a] The CI could not be calculated due to the steep concentration–response relationship. [b] n/a = data not available.

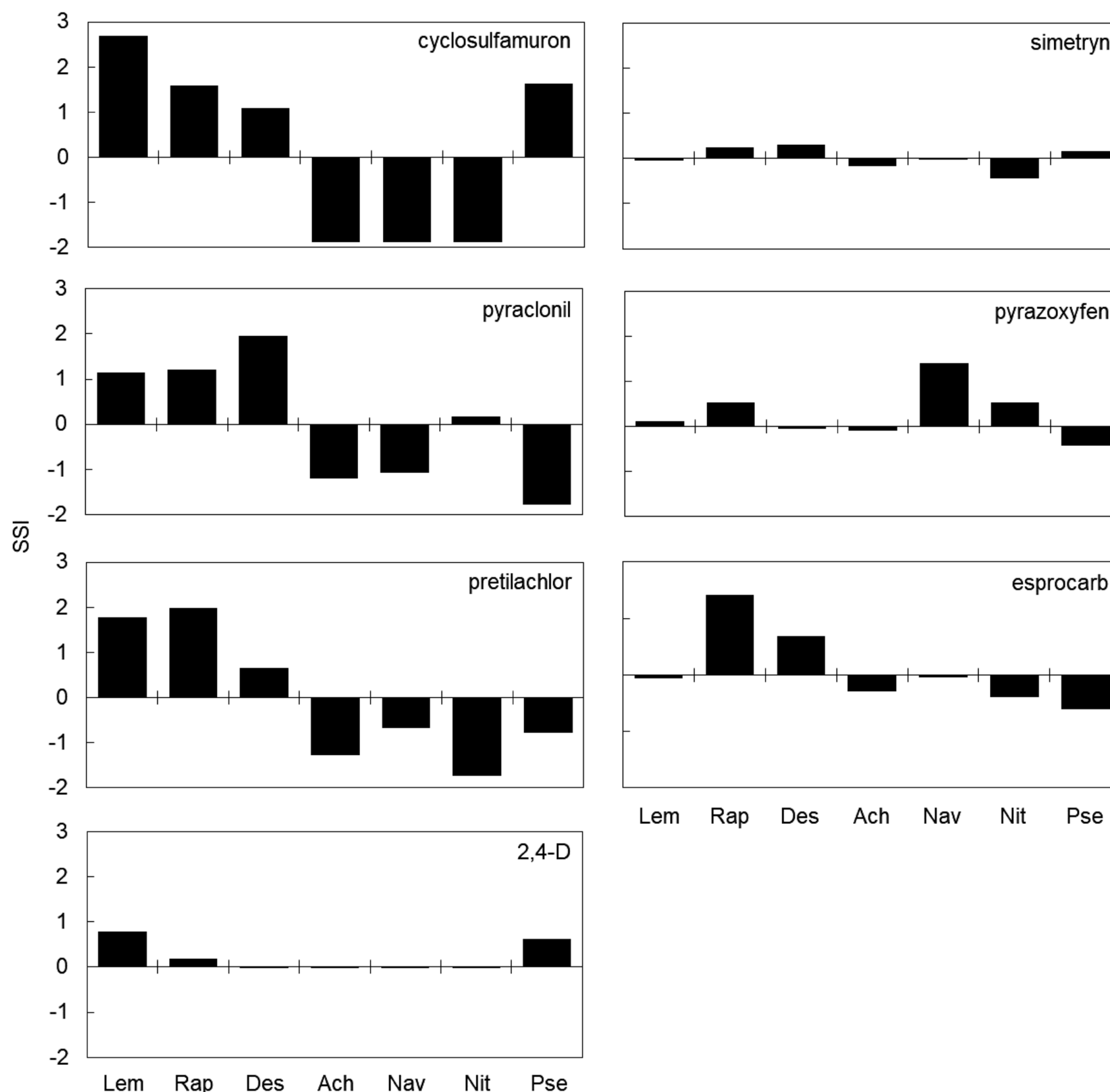

Fig. 1. The species sensitivity index (SSI) for the seven herbicides. Lem, *Lemna minor*; Rap, *Raphidocelis subcapitata*; Des, *Desmodesmus subspicatus*; Ach, *Achnanthidium minutissimum*; Nav, *Navicula pelliculosa*; Nit, *Nitzschia palea*; Pse, *Pseudanabaena galeata*.

bicides (Fig. 1), although Lem was one of the most sensitive species. Because no single species was consistently the most sensitive, no single standard organism could represent the sensitivity of the full primary producer assemblage. These results suggest that multispecies toxicity data, including data for Lem, are essential for appropriate ecological effect assessment.

It has been reported that the variation in species sensitivity greatly depends on the chemical MoA.[18] Our results were generally consistent with this statement. We previously reported that for herbicides with MoA B (inhibitor of acetolactate synthase), sensitivity significantly decreased in the order of Lem>Rap>Des>Nav,[18] and here cyclosulfamuron, which has MoA B, showed an identical pattern of toxicity (Fig. 1). For herbicides with MoA C1 (inhibitor of photosynthesis by photosystem), sensitivity significantly decreased in the order of Rap≈Nav>Lem,[18] and here simetryn showed an identical pattern of toxicity (Fig. 1). For herbicides with MoA E (inhibition of protoporphyrinogen), sensitivity significantly decreased in the order of Des>Rap>Lem>Nav,[18] and the toxicity pattern of pyraclonil was identical (Fig. 1). For herbicides with MoA K3 (inhibitor of very long-chain fatty acid synthesis), sensitivity significantly decreased in the order of Rap≈Des ≈ Lem>Nav.[18] Here, pretilachlor showed roughly the same pattern of toxicity (Fig. 1). For herbicides with MoA N (inhibitor of lipid synthesis), sensitivity significantly decreased in the order of Rap>Des>Lem≈Nav.[18] Here, esprocarb showed an identical pattern of toxicity (Fig. 1). For herbicides with MoA F2 (inhibitors of 4-hydroxyphenyl-pyruvate-dioxygenase), sensitivity significantly decreased in the order of Lem>Nav≈Rap>Des.[18] However, here the toxicity of pyrazoxyfen to Lem was lower than expected and decreased in the order of Nav>Rap>Lem>Des (Fig. 1). We attribute this difference to

Table 3. Tukey's multiple comparison test among the aquatic plant *Lemna minor* (Lem) and six algae.

Comparisons	Difference Means	Standard Error	*t*	*p*
Lem-Rap	−0.10	0.48	−0.21	1.0
Lem-Des	0.25	0.48	0.53	1.0
Lem-Ach	1.6	0.48	3.3	**0.027**
Lem-Nav	1.2	0.48	2.6	0.16
Lem-Nit	1.4	0.48	3.0	0.062
Lem-Pse	1.1	0.48	2.2	0.30
Rap-Des	0.36	0.48	0.74	0.99
Rap-Ach	1.7	0.48	3.5	**0.016**
Rap-Nav	1.3	0.48	2.8	0.10
Rap-Nit	1.5	0.48	3.2	**0.036**
Rap-Pse	1.2	0.48	2.5	0.20
Des-Ach	1.3	0.48	2.8	0.098
Des-Nav	0.98	0.48	2.0	0.40
Des-Nit	1.2	0.48	2.5	0.19
Des-Pse	0.82	0.48	1.7	0.61
Ach-Nav	−0.36	0.48	−0.76	0.99
Ach-Nit	−0.16	0.48	−0.33	1.0
Ach-Pse	−0.53	0.48	−1.1	0.93
Nav-Nit	0.21	0.48	0.43	1.0
Nav-Pse	−0.16	0.48	−0.34	1.0
Nit-Pse	−0.37	0.48	−0.77	0.99

Rap, *Raphidocelis subcapitata*; Des, *Desmodesmus subspicatus*; Ach, *Achnanthidium minutissimum*; Nav, *Navicula pelliculosa*; Nit, *Nitzschia palea*; Pse, *Pseudanabaena galeata*

the fact that the F2 group contains triketone herbicides (such as mesotrione) and pyrazole herbicides (such as pyrazolynate and pyrazoxyfen), which differ in chemical structure and can, therefore, be expected to show different patterns of species sensitivity; further research on this question is necessary. The toxicity of 2,4-D to Lem and all six algal species was weak, and differences in species sensitivity could not be discerned (Fig. 1). This is consistent with previous results, in which herbicides with MoA O (like indole acetic acid) showed no significant differences in species sensitivity.[18] Herbicides with low toxicity to Lem and algae require additional testing, using different methods with higher sensitivity. For example, we developed the seed-germination and seedling-growth test method to determine differences in species sensitivity among five species of vascular plants simultaneously, using hydroponics.[21] All five species of vascular plants were more sensitive to 2,4-D (EC_{50}: 100–2100 μg/L) than Lem in the present study (EC_{50}: 16,000 μg/L). These differences in sensitivity among the species studied here and other vascular plant species warrant further research.

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Electronic supplementary materials

The online version of this article contains supplementary materials(Supplemental Figs. S1, S2 and Supplemental Tables S1–S3), which is available at https://www.jstage.jst.go.jp/browse/jpestics/.

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