Sensitivity differences among seven algal species to 12 herbicides with various modes of action

Takashi NAGAI
Institute for Agro-Environmental Sciences, NARO, 3–1–3 Kannondai, Tsukuba, Ibaraki 305–8604, Japan
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Seven algal species were used to conduct toxicity assays with 12 herbicides to determine differences in species sensitivity. A fluorescence microplate toxicity assay was used as an efficient and economical high-throughput assay. The obtained toxicity data were standardized based on the species sensitivity distribution concept. The most-sensitive individual species differed among herbicides: Desmodesmus subspicatus was most sensitive to chloronitrofen and pendimethalin; Achnanthidium minutissimum was most sensitive to chlorpropham; Nitzschia palea was most sensitive to diquat, glyphosate, and dichlobenil; Navicula pelliculosa was most sensitive to trilhuralin; and Pseudanabaena foetida was most sensitive to glufosinate, asulam, and 2,4-D. Surprisingly, Raphidocelis (formerly Pseudokirchneriella) subcapitata, a standard green alga, was not the most sensitive to any of the herbicides. The results clearly showed that a single algal species cannot represent the algal assemblage in terms of sensitivity. Therefore, multispecies algal toxicity data sets are essential for assessing the ecological effect of herbicides.

Keywords: algae, difference in species sensitivity, herbicide.

Electronic supplementary materials: The online version of this article contains supplementary materials (Supplemental Tables S1 and S2), which are available at http://www.jstage.jst.go.jp/browse/jpestics/.

Introduction

Algae are a taxonomic group generally sensitive to herbicides,1) and herbicides have been found to affect the species composition and community structure of benthic algal assemblages in natural aquatic ecosystems.2) Therefore, algae in natural ecosystems are an important concern regarding the effect of herbicides on non-target organisms. The green alga Raphidocelis subcapitata (formerly Pseudokirchneriella subcapitata) has been widely used as a standard species in conventional ecological effect assessments.3) 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.4) In effect assessment for algae, a 50% effect concentration (EC50) of a toxicity test using R. subcapitata is divided by an uncertainty factor that considers the species sensitivity difference. An uncertainty factor of 1 for algae is currently used because R. subcapitata is assumed to be a sensitive species.5)

Recently, it was determined that the differences in algal species sensitivity to herbicides are remarkably large.6–8) In our previous study, an efficient and economical high-throughput algal toxicity assay using five riverine periphytic species was developed.9) The use of a microplate assay, in which periphytic algae are attached to the bottom of a microplate,10,11) was combined with fluorometric measurement of algal growth with high measurement sensitivity. Moreover, the five candidate test species were selected according to their suitability for microplate assay and their ecological relevance.9) Then we used this developed method with five species of periphytic algae to conduct toxicity assays of 20 herbicides and demonstrated a clear relationship between sensitive species and the herbicide mode of action (MoA).12) In addition to our tested toxicity data, obtained toxicity data from open literature and databases were analyzed, and more robust evidence of the relationship between sensitive species and herbicide MoA was found.7,8) However, the quality and quantity of available data differ markedly among herbicides, and herbicides with only some MoAs have sufficient toxicity data to assess differences in species sensitivity.8) Thus, there are many herbicides with MoAs whose sensitivity differences are not yet well understood. The main objective of the present study is the collection of toxicity data of herbicides for several algae to assess the difference in species sensitivity. For that purpose, toxicity assays were conducted for 12 herbicides that are used in Japan with seven species of algae.
Materials and Methods

1. Test organisms

The seven algal strains used for the toxicity assay were green alga *R. subcapitata* strain NIES-35 (Rap), green alga *Desmodesmus subspicatus* strain NIES-797 (Des), diatom *Achnanthidium minutissimum* strain NIES-71 (Ach), diatom *Navicula pelliculosa* strain UTEX-B673 (Nav), diatom *Nitzschia palea* strain NIES-487 (Nit), cyanobacterium *Pseudanabaena foetida* strain NIES-512 (Pse), and cyanobacterium *Synechococcus leopoliensis* strain NIES-3277 (Syn). These included five species (Des, Ach, Nav, Nit, and Pse) selected in our previous study\(^9\) plus two species (Rap and Syn) recommended in the Organization for Economic Cooperation and Development (OECD) test guideline.\(^13\) The NIES strains were obtained from the Microbial Culture Collection of the National Institute for Environmental Studies, Japan. The Nav strain was obtained from the Culture Collection of Algae at the University of Texas at Austin.

Stock cultures were maintained using CSi medium,\(^14\) with the pH adjusted to 7.3, under conditions of 22°C and light intensity of 1000 lux, except for Pse and Syn which were subjected to 500 lux. Light irradiation was continuous using a white fluorescent lamp (color temperature 3500 K).

2. Fluorescence microplate toxicity assay

Twelve herbicides covering various MoAs were used as test substances (Table 1). All analytical standards were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) and Kanto Chemical Co. Inc. (Tokyo, Japan).

Fluorometric algal toxicity assays were conducted using 96-well microplates, according to the standardized algal growth inhibition test method.\(^9,13\) Stock solutions of the herbicides were prepared in dimethyl sulfoxide (DMSO; FUJIFILM Wako Pure Chemical Corporation), but stock solutions for glyphosate, glufosinate, asulam, and 2,4-D were prepared in CSI medium because of their high water solubility. The final concentration of DMSO in test solutions was 0.1% (v/v), a concentration at which no adverse effects have been observed.\(^9\) Preculturing was performed in CSI medium in borosilicate glass tubes for 5 day at 22°C and 3000 lux. Then precultures were inoculated into the medium to give fixed initial fluorescence intensity, which corresponds to an approximate cell density of 0.3–2.8×10⁴ cells/mL (depending on the species). Algae were incubated for 4 day in 200 µL of culture medium per well in 96-well polystyrene transparent microplates (Falcon 35-1172, BD Biosciences, Franklin Lakes, NJ, USA). Cells were exposed to a geometric sequence of nine concentrations (with some exceptions) with a common ratio of 3.2. Each culture experiment, including the control test (without herbicides and DMSO), was conducted with six replicates. The temperature, light intensity, and medium used in the tests were the same as for the preculture. Algal growth was measured by *in vivo* fluorescence every day using a microplate reader (Gemini EM, Molecular Devices, San Jose, CA, USA) with SoftMax Pro Software for analysis. Excitation and emission wavelengths were 435 and 685 nm, respectively, except those for Pse and Syn, which were 600 and 650 nm.

3. Chemical analysis

At the start (0 day) and end (4 day) 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 algal cell inoculation. The microplate was incubated under the same conditions as the other microplates in the growth experiment. Subsamples (500 µL) from this microplate were taken at 0 and 4 day, and then an appropriate organic solvent (depending on herbicides; see sample treatment in Supplemental Table S1) was added. These were stored at −20°C in darkness until analysis. Details of the analytical conditions are shown in Supplemental Table S1. The geometric mean of the measured concentrations at 0 and 4 day was then calculated. If the geometric mean values were all within ±20% of the nominal concentration, a concentration–response analysis was conducted based on nominal values;\(^13\) otherwise, geometric mean concentrations were used for concentration–response analysis.

Trifluralin is a highly adsorptive substance and thus could not be detected in any test solutions at 4 day. Instead, the 100 µg/L test solution was incubated under the same conditions as in the toxicity test, and the concentrations were monitored at 0, 4, 10, and 24 hr. Then a first-order reaction model was fitted to the measured concentrations. After that, geometric mean concentrations were calculated using estimated concentrations at 0 and 4 day by the model:

\[
\text{EC}_{50} = \frac{\text{conc} \times t}{C_{0} - C_{t}}
\]

where EC₅₀ is the concentration producing 50% of the toxicity effect, conc is the concentration of the herbicide in the test solution, t is the time of measurement, and C₀ and Cₜ are the initial and final concentrations, respectively.

### Table 1. The properties of the 12 herbicides studied: CAS number, mode of action (MoA), and reported EC₅₀ values for *R. subcapitata*

| Herbicide  | CAS No. | MoA⁹ | EC₅₀ (µg/L)⁹ |
|------------|---------|------|-------------|
| sethoxydim  | 74051-80-2 | A    | >100000     |
| diquat     | 85-00-7  | D    | 19          |
| chloronitrofen | 1836-77-7 | E⁵   | 9.82⁶       |
| glyphosate | 1071-83-6 | G    | 180000      |
| glufosinate| 77182-82-2 | H  | 80000       |
| asulam     | 2302-17-2 | I    | 49000       |
| pendimethalin | 40487-42-1 | K₁  | 14.6        |
| trifluralin| 1582-09-8 | K₁  | 532         |
| chlorpropham | 101-21-3  | K₂  | 1100        |
| dichlobenil| 1194-65-6 | L    | 2700        |
| 2,4-D      | 94-75-7  | O    | 33200       |
| MCPA       | 94-74-6  |      | >392000     |

⁹ HRAC;\(^20\) A: inhibition of acetyl CoA carboxylase; D: photosystem-I–electron diversion; E: inhibition of protoporphyrinogen oxidase; G: inhibition of EPSP synthase; H: inhibition of glutamine synthetase; I: inhibition of dihydropterotate synthase; K₁: microtubule assembly inhibition; K₂: inhibition of mitosis/microtubule organisation; L: inhibition of cell wall (cellulose) synthesis; O: action like indole acetic acid.\(^9\) Japan Plant Protection Association (2016).\(^24\) Japan Plant Protection Association (1994).\(^25\) Hatakeyama et al. (1994).\(^26\)
trifluralin concentration (μg/L)  
= nominal concentration × 0.339 × exp(-1.76 × day)  \quad (1)

The OECD test guideline allows the use of models describing the decline in concentration of the test substance.\(^{13}\)

4. Concentration–response analysis
During the growth experiments, the growth rate (per day) from day \(t'\) to day \(t\) was calculated as follows:

\[
growth\ rate = \frac{\ln(x_t) - \ln(x_{t'})}{t - t'}  \quad (2)
\]

where \(x_t\) is the fluorescence intensity or the cell density at time \(t\). Growth rates were calculated during 1–4 days, because a lag time for algal growth was observed prior to 1 day. Then, relative growth rates at each test concentration were 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.\(^9\) The model can be expressed as follows:

\[
\text{relative growth rate} = \frac{1}{1 + \exp(f_a + f_b \cdot \ln(C_{\text{her}}))}  \quad (3)
\]

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

\[
\begin{align*}
\text{EC}_{50} &= \exp(-f_a / f_b)  \\
\text{EC}_{10} &= \exp([-2.197 - f_a] / f_b)
\end{align*} \quad (4)\quad (5)
\]

Statistical analyses were conducted using software R ver. 3.4.4 (R Foundation for Statistical Computing). Concentration–response analysis was not conducted when the effect was less than 10% in the experiments with maximum concentrations. In this case, EC\(_{50}\) and EC\(_{10}\) were regarded as greater than the maximum concentration of the test.

5. Analysis of species sensitivity index
The obtained toxicity data for seven species were standardized based on the species sensitivity distribution (SSD)\(^{15}\) concept to compare the difference in species sensitivity among herbicides. The standardized toxicity was defined as the species sensitivity index (SSI).\(^9\) 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.\(^7\)

First, SSD analysis was conducted using the obtained EC\(_{50}\) data for seven species. All data were converted to interval data according to Nagai.\(^7\) The EC\(_{50}\) data were treated as interval data using the upper and lower confidence intervals. If confidence intervals could not be calculated (see Results), for example, an EC\(_{50}\) value of 15, the data were treated as interval data 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 interval data from the minimum value to 10 times the minimum value. For example, for EC\(_{50}\) > 10,000, the data was treated as interval data of 10,000–100,000. The difference in the quality of toxicity data among herbicides, such as the number of “greater than” data, could be compensated for as much as possible by the treatment. Treatment of EC\(_{50}\) > 10,000 as 10,000 (ignoring the inequality sign) would bias the results toward higher toxicity. The interval dataset was fitted to a log-normal distribution using the maximum likelihood method.\(^{16}\) The maximum likelihood parameters of the distribution, logarithmic mean, and logarithmic standard deviation, were obtained by fitting.

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**Fig. 1.** Comparison of EC\(_{50}\) values for *Raphidocelis subcapitata* using the conventional test method and the microplate assay. Arrows indicate “greater than” values.
Table 2. 50% and 10% effect concentrations (EC$_{50}$ and EC$_{10}$ µg/L) with 95% confidence intervals (CI) of each tested species.

| Herbicide | Species | EC$_{50}$ | 95%CI   | EC$_{10}$ | 95%CI   |
|-----------|---------|----------|---------|----------|---------|
|           | Rap     | >6000    | —       | >6000    | —       |
|           | Des     | >6000    | —       | >6000    | —       |
|           | Ach     | >6000    | —       | 1100     | 400–2900|
|           | Nit     | >6000    | —       | >6000    | —       |
|           | Nav     | >6000    | —       | >6000    | —       |
|           | Pse     | >6000    | —       | >6000    | —       |
|           | Syn     | >6000    | —       | >6000    | —       |

| Herbicide | Species | EC$_{50}$ | 95%CI   | EC$_{10}$ | 95%CI   |
|-----------|---------|----------|---------|----------|---------|
|           | Rap     | 55       | 50–60   | 11       | 8.8–13  |
|           | Des     | 3200     | 2900–3600 | 1200  | 950–1600|
|           | Ach     | 7.3      | —       | 6.1     | —       |
|           | Nit     | 5.2      | 3.7–7.1 | 2.2     | 1.1–4.5 |
|           | Nav     | 330      | 250–450 | 120     | 60–240  |
|           | Pse     | 230      | 200–260 | 28      | 21–38   |
|           | Syn     | 290      | 210–390 | 94      | 44–200  |

| Herbicide | Species | EC$_{50}$ | 95%CI   | EC$_{10}$ | 95%CI   |
|-----------|---------|----------|---------|----------|---------|
|           | Rap     | 12       | —       | 9.9     | —       |
|           | Des     | 2.5      | —       | 2.0     | —       |
|           | Ach     | 460      | —       | 380     | —       |
|           | Nit     | 90       | 76–110  | 27      | 19–39   |
|           | Nav     | 140      | —       | 120     | —       |
|           | Pse     | 150      | —       | 130     | —       |
|           | Syn     | >1400    | —       | >1400   | —       |

| Herbicide | Species | EC$_{50}$ | 95%CI   | EC$_{10}$ | 95%CI   |
|-----------|---------|----------|---------|----------|---------|
|           | Rap     | 59000    | 52000–6700 | 13000   | 11000–1500|
|           | Des     | >32000   | —       | 18000   | 12000–2900|
|           | Ach     | 45000    | 42000–48000 | 16000 | 14000–18000|
|           | Nit     | 37000    | 35000–40000 | 6500   | 5700–7500 |
|           | Nav     | >100000  | —       | >100000 | —       |
|           | Pse     | >32000   | —       | 33000   | 28000–39000|
|           | Syn     | >32000   | —       | 30000   | —       |

| Herbicide | Species | EC$_{50}$ | 95%CI   | EC$_{10}$ | 95%CI   |
|-----------|---------|----------|---------|----------|---------|
|           | Rap     | 13000    | 11000–16000 | 410   | 280–610 |
|           | Des     | >100000  | —       | >100000 | —       |
|           | Ach     | 480000   | 290000–820000 | 90000 | 81000–99000 |
|           | Nit     | >100000  | —       | >100000 | —       |
|           | Nav     | >100000  | —       | >100000 | —       |
|           | Pse     | 25       | 21–30   | 13      | 7.7–21  |
|           | Syn     | 54000    | 50000–580000 | 18000 | 16000–21000 |

| Herbicide | Species | EC$_{50}$ | 95%CI   | EC$_{10}$ | 95%CI   |
|-----------|---------|----------|---------|----------|---------|
|           | Rap     | 10000    | 9200–11000 | 1300 | 1100–1600 |
|           | Des     | 20000    | 17000–24000 | 5100 | 3400–7600 |
|           | Ach     | 7400     | 6800–8000 | 4100    | 3300–5100 |
|           | Nit     | 12000    | —       | 9700    | —       |
|           | Nav     | 110000   | —       | 77000   | —       |
|           | Pse     | 3700     | 2100–6600 | 84     | 23–310  |
|           | Syn     | 210000   | 130000–340000 | 8100 | 4700–14000 |
Table 2. 50% and 10% effect concentrations (EC50 and EC10, µg/L) with 95% confidence intervals (CI) of each tested species.

| Herbicide | Species | EC50 | 95%CI | EC10 | 95%CI |
|-----------|---------|------|-------|------|-------|
| pendimethalin | Rap | 42 | 38–45 | 8.7 | 7.1–11 |
| | Des | 18 | 15–21 | 1.3 | 0.98–1.9 |
| | Ach | >140 | — | >140 | — |
| | Nit | 61 | —(a) | 53 | —(a) |
| | Nav | 26 | —(a) | 22 | —(a) |
| | Pse | >140 | — | >140 | — |
| | Syn | >140 | — | >140 | — |

| pendimethalin | Rap | 310 | 160–600 | 1.5 | 1.2–1.9 |
| | Des | 16 | 12–20 | 0.45 | 0.31–0.64 |
| | Ach | >10 | — | >10 | — |
| | Nit | >10 | — | >10 | — |
| | Nav | 6.5 | 5.9–7.3 | 1.8 | 1.4–2.3 |
| | Pse | >10 | — | >10 | — |
| | Syn | >10 | — | >10 | — |

| trifluralin | Rap | 960 | —(b) | 780 | —(b) |
| | Des | 3300 | 3000–3700 | 1300 | 1000–1700 |
| | Ach | 590 | 460–740 | 180 | 110–290 |
| | Nit | 5000 | 4800–5200 | 2200 | 2100–2300 |
| | Nav | 3300 | —(b) | 2700 | —(b) |
| | Pse | 5300 | 4900–5800 | 2400 | 2100–2800 |
| | Syn | 4800 | 4500–5200 | 1100 | 960–1300 |

| chlorpropham | Rap | >2600 | — | >2600 | — |
| | Des | >2600 | — | >2600 | — |
| | Ach | 3400 | 3100–3700 | 700 | 620–800 |
| | Nit | 1200 | —(b) | 1000 | —(b) |
| | Nav | 4100 | 3400–4800 | 2100 | 1900–2200 |
| | Pse | >2600 | — | >2600 | — |
| | Syn | >2600 | — | >2600 | — |

| dichlobenil | Rap | >10000 | — | >10000 | — |
| | Des | >10000 | — | >10000 | — |
| | Ach | >10000 | — | >10000 | — |
| | Nit | >10000 | — | >10000 | — |
| | Nav | >10000 | — | >10000 | — |
| | Pse | 24000 | 21000–27000 | 11000 | 8400–15000 |
| | Syn | >100000 | — | >100000 | — |

| 2,4-D | Rap | >10000 | — | >10000 | — |
| | Des | >10000 | — | >10000 | — |
| | Ach | >10000 | — | >10000 | — |
| | Nit | >10000 | — | >10000 | — |
| | Nav | >10000 | — | >10000 | — |
| | Pse | >10000 | — | >10000 | — |
| | Syn | >10000 | — | >10000 | — |

| MCPA | Rap | >10000 | — | >10000 | — |
| | Des | >10000 | — | >10000 | — |
| | Ach | >10000 | — | >10000 | — |
| | Nit | >10000 | — | >10000 | — |
| | Nav | >10000 | — | >10000 | — |
| | Pse | >10000 | — | >10000 | — |
| | Syn | >10000 | — | >10000 | — |

(a) The values were indicated by 2 significant digits. (b) CI could not be calculated due to steep concentration–response relationship.
The 50th percentile value of the analyzed SSD (hazardous concentration for 50% of the species, \( \text{HC}_{50} \), 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 \( \text{HC}_{50} \) after taking the common logarithm:

\[
\text{SSI} = \log_{10}\text{HC}_{50} - \log_{10}\text{EC}_{50}
\]

(6)

The SSI is a relative index of the difference in species sensitivity: a higher SSI indicates higher sensitivity, and the difference of one unit in the SSI indicates a 10-fold difference in \( \text{EC}_{50} \). The relationships of SSI values with the tested species were tested using a one-way analysis of variance (ANOVA).

**Results**

1. **Comparison of \( \text{EC}_{50} \) values between conventional and microplate assays**

The \( \text{EC}_{50} \) values for Rap were also previously determined using a conventional test method with flasks as test chambers (Table 1). Therefore, the \( \text{EC}_{50} \) values obtained for Rap in the present study using microplates were compared with those reported values to validate the microplate test method (Fig. 1). Differences in \( \text{EC}_{50} \) values between the microplate assay and the conventional test method were at most 6.2-fold, except for “greater than” values, indicating good consistency. Therefore, the microplate assay method used in the present study is a valid method.

2. **Herbicide toxicity**

Eighty-four toxicity tests (12 herbicides \( \times 7 \) species) were conducted, and the raw data (growth rates of each replicate and measured concentrations) are shown in Supplemental Table S2. The determined \( \text{EC}_{50} \) and \( \text{EC}_{10} \) values for each herbicide are shown in Table 2. The toxicities for sethoxydim and MCPA could not be determined because of low toxicity, so these data were not used in further analysis. The most sensitive species differed among the herbicides: Des was most sensitive to chloronitrofen and pendimethalin; Ach was most sensitive to chlorpropham; Nit was most sensitive to diquat, glyphosate, and dichlobenil; Nav was most sensitive to trifluralin; and Pse was most sensitive to glufosinate, asulam, and 2,4-D. Surprisingly, Rap, a standard green alga, was not the most sensitive for any of the 10 herbicides.

3. **Species sensitivity index**

The SSIs for 10 herbicides, based on the \( \text{EC}_{50} \) values of the seven species, were analyzed (Fig. 2) and clearly showed differences in...
species sensitivity in the uneven pattern. The average SSI values for 10 herbicides were calculated for each species: the highest value was 0.27 for Pse, and the lowest was −0.50 for Syn. However, the ANOVA showed no significant differences in SSI among the seven tested species (p=0.29).

Pendimethalin and trifluralin have the same MoA (K1, microtubule assembly inhibition) and showed the same SSI pattern, with Des and Nav more sensitive than other species. Chlorpropham has a slightly different MoA (K2, inhibition of mitosis/microtubule organization) than pendimethalin and trifluralin. Because Rap and Ach were sensitive to chlorpropham, the SSI showed different patterns between K1 and K2 herbicides. The SSI patterns differed among all other herbicides, suggesting that differences in species sensitivity were specific to the MoA. In particular, chloronitrofen (the green alga Des was highly sensitive), diquat (diatoms Ach and Nit were highly sensitive), and glufosinate (the cyanobacterium Pse was highly sensitive) have remarkable SSI patterns.

**Discussion**

The present study showed the validity of the fluorescence microplate toxicity assay for a wide range of herbicides by comparison with reported EC50 values derived using a conventional test method (Fig. 1). The advantages of fluorescence microplate algal assays have been discussed previously.11,17,18 Simultaneous assays with seven species using the same test medium and culture conditions were better for comparing toxicity data among species. The most sensitive species differed among herbicides (Table 2), indicating the importance of multispecies toxicity testing. The toxicity data for those seven species were then applied to SSI analysis. I previously proposed an SSI as a relative index of the differences in species sensitivity8); however, in that previous study the species and number of data in the toxicity dataset for SSI analysis were not aligned among pesticides. In contrast, the same seven species toxicity datasets were aligned among all herbicides in the present study, resulting in a more appropriate SSI.

It has been reported that the variation in species sensitivity greatly depends on the chemical MoA.6–8,12,19 For herbicides with MoA E (inhibition of protoporphyrinogen oxidase), sensitivity significantly decreased in the order of Des>Rap>Nav>Syn.8) The toxicity of chloronitrofen showed exactly the same order (Fig. 2). A previous study of herbicides with MoA O (action like indole acetic acid) showed no significant differences in species sensitivity,9) consistent with the result of the present study. The toxicity of 2,4-D and MCPA to seven algal species was weak, and the difference in species sensitivity was not clear (Table 2). In herbicides with other MoAs tested in the present study, there were insufficient toxicity data to find the differences in species sensitivity.8) Therefore, the results of the present study provide valuable information to fill data gaps concerning differences in species sensitivity.

The present study showed that most sensitive species differed among the herbicides and that Rap, a standard green alga for toxicity testing, was not the most sensitive for any of the 10 herbicides. Thus, a specific species was not always the most sensitive species, and any single standard organism could not represent algal assemblages in terms of sensitivity. These results suggest that multispecies toxicity data are essential for appropriate ecological effect assessment. For European Union pesticide registration, toxicity testing of additional algae (not green alga, e.g., the diatom Navicula pelliculosa and/or cyanobacteria) is required in Tier 1 risk assessment for herbicides and plant growth regulators.20 For United States pesticide registration, four algal species are usually tested for herbicides: the green alga R. subcapitata, a freshwater diatom, usually Navicula sp., the marine diatom Skeletonema costatum, and the freshwater cyanobacterium Anaabaena flos-aquae.21 In Japan, the Agricultural Chemicals Regulation Law was revised in 2018, and the pesticide registration criteria have also been revised.22 As part of the revised criteria, the algal test using Rap is required, and additional algal species (Des, Nav, Syn, and Anabaena) can be optionally subjected to testing. The uncertainty factor applied to the lowest EC50 regarding the difference in species sensitivity is changed from 1 to 10 by default, which is reduced depending on the number of tested algal species. The microplate assay used in the present study would be useful because multispecies tests can be performed economically and efficiently. However, it should be noted that it is unclear at present whether the microplate test results will be accepted by regulatory authorities.

Toxicity tests using aquatic plants such as Lemna sp. in addition to algae will be introduced in the setting of revised criteria for herbicides.21 Both algae and aquatic plants should be included in the analysis of species sensitivity differences in future research.

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