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Effect of Herbicides in Paddy Runoff on Seed Germination of *Vallisneria asiatica* and *Ammannia multiflora*

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Abstract

Although rice production provides stable environments for aquatic plants, the wide use of herbicides is a concern for wild plants inhabiting the surroundings of rice paddies. Because commercial herbicides are typically a cocktail of chemicals, they may pose a threat to wild plants even when the constituent chemicals do not individually have detrimental effects. We sampled water from a rice paddy and a river receiving the paddy drainage immediately after the transplanting period to identify and compare the concentrations of herbicides. We also examined the effect of the sampled water on the germination of two plants: *Ammannia multiflora* (hygrophyte) and *Vallisneria asiatica* (submerged). We found that the concentrations of glufosinate in the paddy and river waters were 0.0015 and 0.0013 mg L^{-1}, respectively, and those of pyraclonil were 0.0010 and 0.0009 mg L^{-1} in the same waters, indicating that these chemicals persist outside the rice paddy. The germination rate of *A. multiflora* was significantly diminished with exposure to river and paddy water under fluctuating temperature conditions, whereas no difference was observed for *V. asiatica*. For a comprehensive understanding of the influences of residual herbicides on wetland biodiversity, it is necessary to analyse the effects of herbicides on a wide range of aquatic plants and at various stages of growth.

Keywords: Glufosinate, Pyraclonil, Herbicide, River, Rice production

1. Introduction

Since the introduction of rice cultivation in Japan, lowland areas have been managed as paddies for optimal rice growth. Rice production is supported by regional irrigation systems, including ditches and ponds, creating stable environments for colonization by aquatic plants (Watanabe, 2011).

Recent investigations suggest that herbicides discharged into natural aquatic systems, primarily through water runoff, could be one of the highest risk factors for plant species survival (Eullaffroy et al., 2009; Casanova, 2012). Paddy herbicides are a high-risk concern for aquatic plants not only in the paddy system but also in the surrounding wetlands, because these herbicides readily flow out of paddy fields into rivers causing toxic effects (Nagai et al., 2011). A typical case is that of the cosmopolitan species *Marsilea quadrifolia* L., which inhabits paddy fields, irrigation ditches, and ponds (Kadono, 1994; Environment Agency of Japan, 2000). In recent decades, this aquatic fern has been included in several national Red Lists in Western Europe and even in Japan. Experiments conducted to assess the sensitivity of *M. quadrifolia* plantlets to common rice paddy herbicides have revealed that herbicides represent a major threat to the survival of this species (Bruni et al., 2013). The Environment Agency of Japan (2000) has shown that 17 endangered species that grow in rice paddies, rivers, and neighbouring habitats have decreased due to herbicide use over the 40-year period between 1960 and 2000.

In Japan, herbicides are used intensively in rice paddies immediately after the transplantation of rice seedlings (grown for 30–50 days). The annual dose of herbicides is sprayed onto the rice paddies during the rainy season, between June and July, and the herbicides drain into rivers and flow downstream (Yamamuro, 2012). In order to assess the risk posed by paddy herbicides to wild aquatic plants, it is necessary to identify the amounts of herbicides remaining in the neighbouring habitats.

Commercial herbicides are generally composed of several chemicals, and these combinations of chemicals may be effective in weed control, whereas the constituent chemicals may not individually be as effective. The sensitivity of vascular plants is expected to differ at each growth stage (i.e. seed, seedling, juvenile, and mature plant). Germination of seed, as the first stage of plant establishment, is critical to the plant life cycle, and therefore even the slightest sensitivity to herbicides at this stage may result in significant damage.

In this study, we sampled runoff water from paddies and river water connected to the drainage of a paddy field immediately after the transplanting period to compare the change in types and concentrations of herbicides in each water source. We also examined the effect of environmental water on the germination of *Ammannia multiflora* (a hygrophyte) and *Vallisneria asiatica* (a submerged plant).

2. Material and Methods

Distilled water was purchased from Nacalai Tesque Inc. (49506-64; Kyoto, Japan). River water and paddy water were sampled on 22 May 2014 using a clean stainless steel bucket. River water (Hanamuro River) was sampled at Watarido Bridge (36°04′21.2″N, 140°08′26.2″E, Fig. 1). Rice paddies extend on both sides of the river, and water was sampled from the paddy nearest to the bridge. Water was stored in screw-capped glass bottles, which were transported to the laboratory in a cool box containing an ice pack. The transport time was 5 min by car. In the laboratory, the water was divided into two equal volumes and sent via a refrigerated courier service to two institutes for herbicide analysis

(listed in Table 1) and germination experiments, respectively.

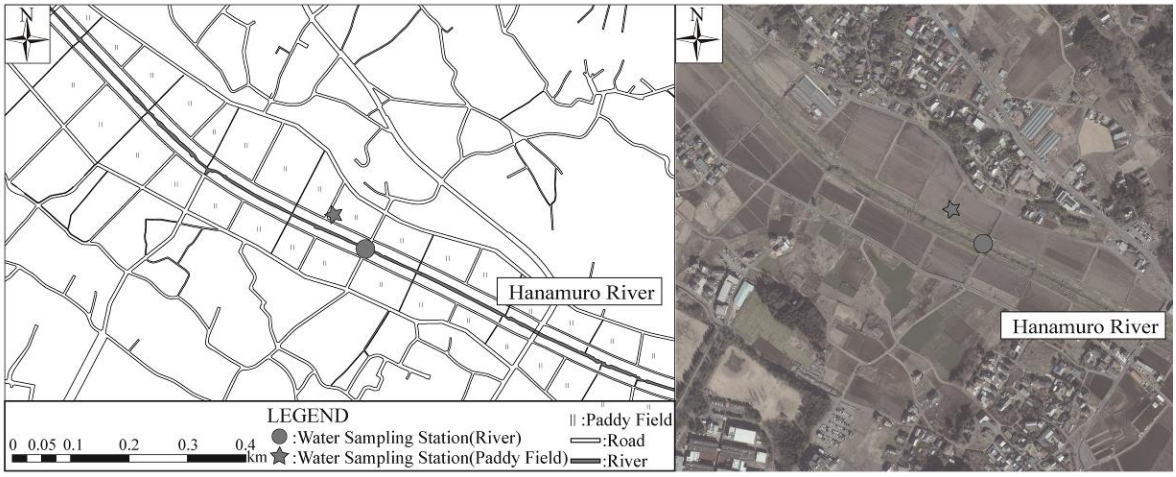

Figure 1. Map and aerial photograph of the water sampling stations

Note: The photograph was taken by the Geospatial Information Authority of Japan in February 2016.

Herbicides were analysed as follows. Sample Nos. 1–7 were measured using the official standard method for detection of the first set of designated herbicides listed in Table 1 (Ministry of Health, Labour and Welfare, 2015a) by using solid-phase extraction (SPE) cartridges and liquid chromatography/mass spectrometry (LC/MS). Each cartridge was successively rinsed with 10 mL of acetonitrile, 10 mL of methanol, and 10 mL of water. Next, 500 mL of water sample spiked with EDTA solution (10% (w/v), 10 mL) and adjusted to pH 3.5 with (1 + 10) HNO_3 was passed through a rinsed SPE cartridge at a flow rate of 10–20 mL min^{-1}. The cartridge was dried using N_2 gas and eluted using 5 mL of acetonitrile. The eluted solution was concentrated with N_2 gas until its liquid volume was reduced to ≤0.2 mL. The concentrated eluted solution was diluted with water to 1 mL, and the sample was analysed using LC/MS (Table 2).

Sample No. 8 was measured using the official standard method for the second set of herbicides listed in Table 1 (Ministry of Health, Labour and Welfare, 2015b) by using LC/MS (Table 2). For this method, 100 mL of sample was filtered, and the first 10 mL of filtrate was rejected.

Sample Nos. 9–21 were measured using the official standard method for the second set of herbicides listed in Table 1 (Ministry of Health, Labour and Welfare, 2015c) by using LC/MS (Table 2). For this method, 100 mL of sample was filtered, and the first 10 mL of filtrate was rejected.

Sample Nos. 22 and 23 were measured using the official method for the third set of herbicides listed in Table 1 (Ministry of Health, Labour and Welfare, 2015d) by using SPE cartridges and LC/MS. Each cartridge was successively rinsed with 3 mL of methanol and 3 mL of water. Next, 50 mL of the water sample was passed through the cartridge at a flow rate of 2–3 mL min^{-1}. The cartridge was rinsed with 3 mL of water, then with 1 mL of methanol, and finally eluted with a mixed acetonitrile/formic acid solution (2.5 mL). The eluted solution was concentrated using N_2 gas until its liquid volume was reduced to ≤0.2 mL. The concentrated eluted solution was diluted with water to 1 mL. The sample was analysed using LC/MS (Table 2).

Table 1 List of analysed herbicides and their detection limit (LOQ)

No.	Pre-treatment method	Pesticide	LOQ(mg L^{-1})
1	1	2,4-Dichlorophenoxyacetic acid (2,4-D)	0.0003
2		Asulam	0.002
3		Diuron	0.0002
4		Daimuron	0.008
5		Triclopyr	0.00006
6		Bentazone	0.002
7		Mecoprop	0.0005
8	2	2,2-Dichloropropionic acid (2,2-DPA)	0.0008
9		Indanofan	0.00009
10		4-Chloro-2-methylphenoxy acetic acid (MCPA)	0.00005
11		Oxaziclomefone	0.0002
12		Cumyluron	0.0003
13		Clomeprop	0.0002
14		Cyanazine	0.00004
15		Pyraclonil	0.0001
16		Pyrazoxyfen	0.00004
17		Pyrazolynate	0.0002
18		Fentrazamide	0.0001
19		Benzobicyclon	0.0009
20		Benzofenap	0.00004
21		Metribuzin	0.0003
22	3	Diquat	0.00005
23		Paraquat	0.00005
24	4	Glyphosate	0.02
25		Glufosinate	0.0002

Sample Nos. 24 and 25 were measured for the fourth set of herbicides listed in Table 1, in accordance with the standard official method (Ministry of Health, Labour and Welfare, 2015e), by using derivatized SPE cartridges and LC/MS. Sodium borate (5% w/v, 1 mL) and [(9-fluorenylmethyl)oxy]carbonyl (FMOC) solution (0.1% w/v, 2 mL) were added to 20 mL of sample, and the sample was heated at 50 ℃ for 20 min. After cooling the sample, phosphate solution (2% v/v, 1.2 mL) was added. The cartridge was successively rinsed with 3 mL of acetonitrile and 3 mL of water. The treated sample was passed through the cartridge at a flow rate of 2–4 mL min^{-1}. The cartridge was then rinsed with ammonium acetate (0.005 M, 1 mL) and the sample was eluted using a mixed acetonitrile/ammonium acetate solution (1.5 mL). The eluted solution was diluted with acetonitrile/ammonium acetate solution to 2 mL. The sample was then measured using LC/MS (Table 2).

For the germination experiments, we assessed the effects of environmental water on the germination of two local plant species. *Vallisneria asiatica* Miki (Hydrocharitaceae), which is listed in the Red Data Books of 29 of the 47 prefectures in Japan, was selected as a representative species of the locally endangered submerged plants in water courses or ponds. *Ammannia multiflora* Roxb. (Lythraceae), which is listed in 13 of the 47 prefectures, was selected as a representative species of locally endangered emergent plants in paddies.

Vallisneria asiatica is a perennial, deciduous, submerged plant that flowers from July to

November but generally propagates asexually by stolons. It grows in lakes, irrigation ponds, water courses, and rivers (Kadono, 1994). Its local status in Japan is as follows: extinct in two prefectures, endangered in 15, and not threatened in 11 of 47 prefectures; although it is an extraordinarily rapidly declining species, it has yet to be listed in the Red Data Book of Japan (Ministry of the Environment of Japan, 2015). This species formerly grew in Lake Kasumigaura, which is ca 8 km downstream of the Hanamuro River, but is now extinct in this locality.

Ammannia multiflora is an annual hygrophyte that grows in wet lowlands, particularly in paddies (Ohashi, 1999). Its local status is as follows: endangered in five and not threatened in four of 47 prefectures; it has a low estimated risk of extinction. In the study area, *A. multiflora* is a common plant.

For statistical evaluations of germination rate, data were analysed for significant differences using the Kruskal–Wallis and Mann–Whitney U tests using IBM SPSS Statistics Ver.21 for Windows.

Table 2. Measurement conditions of herbicides

Item		Sample number				
		1-7	8	9-21	22, 23	24, 25
LC		Agilent 1260 Infinity				
MS		Agilent 6460				
Ionization		Agilent jet stream electrospray				
Column		Zorbax SB-C18 (2.1mm, 30mm, 3.5μm)	Inertsil ODS3 (150mm, 2.1mm, 3μm)	Zorbax SB-C18 (2.1mm, 30mm, 3.5μm)	Poroshell 120 Hilic (2.1mm, 100mm, 2.7μm)	Zorbax Eclipse Plus C18 (2.1mm, 150mm, 3.5μm)
Mobile phase	(A)	0.1% formic acid 10mM ammonium formate	1% formic acid	0.1% formic acid 10mM ammonium formate	150mM ammonium formate (pH3.6)	5mM ammonium acetate
	(B)	0.1% ammonium formate/acetonitrile	0.1% ammonium formate/acetonitrile	0.1% ammonium formate/acetonitrile	Acetonitrile	Acetonitrile
Gradient (ratio of A/B)		0–0.5min (98/2) → 5min (0/100)	0-1min (98/2) → 2min (50/50) → 8–10min (10/90)	0–0.5min (98/2) → 5min (0/100)	(60/40) isocratic solution	0min (95/5) → 9.3min (37/63) →13min (5/95)
Flow rate (mL min^{-1})		1	0.2	1	0.3	0.25
Injection (μL)		5	50	50	15	20
Drying gas (N_2)	Temperature (°C)	300	300	300	300	300
	Flow rate (mL min^{-1})	12	10	12	10	10
Sheath gas (N_2)	Temperature (°C)	250	250	250	400	400
	Flow rate (mL min^{-1})	11	11	11	11	11
Nebulizer gas (N_2) pressure (psi)		50	45	50	50	50
Capillary volt (V)		3500	1500	3500	2500	3500
Nozzle volt (V)		0	0	0	0	0

Seeds of *V. asiatica* were collected from Ikoma-shi, Nara Prefecture (34°45′42″N, 135°42′45–50″E), on 1 November 2014, and were maintained in water at 7°C in a refrigerator. Seeds of *A. multiflora* were gathered from To-on-shi, Ehime Prefecture (33°47′38″N, 132°57′08″E), on 25 October 2014, and were maintained under dry conditions in the laboratory.

The germination experiment began during the period 26–28 May 2015. Seeds were maintained under thermostatic (constant temperature at 20 ℃ with a 12 h light:dark cycle in a growth chamber) or fluctuating (temperature varying between 22 ℃ and 42 ℃ with window-side sunlight in the laboratory) conditions to simulate the actual conditions of the paddy fields. We used a temperature of 20 ℃ because, for the past 30 years, the average temperature of Ikoma-shi in late May has been 20 ℃. Seeds were subjected to one of six experimental water treatments (listed in Table 3): distilled water (control), river water, or a solution of paddy water (full-strength or diluted 1/10, 1/50, or 1/100 with distilled water). For the experiment, 10 seeds were placed on absorbent cotton in a Petri dish containing one of the water treatments; 10 replicates were conducted for each treatment. Supplies of each experimental water treatment were stored in a dark place in the laboratory. When the water level in any Petri dish decreased, it was refilled with the appropriate experimental water. Seeds were considered germinated when the hypocotyl emerged after breaking through the seed coat. The cumulative number of seeds germinating over 5 weeks (35 days) was recorded. Table 3 summarizes the experimental plants and conditions.

Table 3. Conditions and plants used for the sensitivity test

<table>
<tr><th>Experimental conditions</th><th>Seeds</th><th colspan="2">Experimental water</th></tr>
<tr><td rowspan="2">20 ℃, 12 h light:dark</td><td rowspan="2">V. asiatica</td><td colspan="2">Distilled water
River water</td></tr>
<tr><td>Paddy water</td><td>Untreated
Diluted 1/10
Diluted 1/50
Diluted 1/100</td></tr>
<tr><td rowspan="2">22–42 ℃, window-side in the laboratory</td><td>V. asiatica or A. multiflora</td><td colspan="2">Distilled water
River water</td></tr>
<tr><td>V. asiatica</td><td colspan="2">Paddy water</td></tr>
</table>

3. Results

None of the 25 herbicide compounds examined was detected in the distilled water, whereas two compounds, glufosinate and pyraclonil, were detected in paddy and river waters. The concentrations of glufosinate in the paddy and river waters were 0.0015 and 0.0013 mg L^{-1}, respectively, whereas those of pyraclonil were 0.0010 and 0.0009 mg L^{-1} in the same waters. A mecoprop concentration of 0.00007 mg L^{-1} (which is lower than the standard detection limit of 0.0005 mg L^{-1}) was also observed in both the paddy and river waters.

No significant difference in germination rate was observed for *V. asiatica* subjected to the different water treatments (i.e. distilled, paddy, or river water) under constant (20 ℃) or varying (22–42 ℃) temperature conditions (Kruskal–Wallis test: $p > 0.05$; Figs. 2 and 3). The level of paddy water dilution (1/10, 1/50, or 1/100) had no effect under constant temperature conditions of 20 ℃ (Kruskal–Wallis test: $p > 0.05$; Fig. 2). Under both constant and varying temperature conditions, the germination rate was lower in the paddy and river water than in the distilled water treatments. A significant difference might, however, have been observed if more replicates of the experiment had been performed. In addition, under thermostatic

conditions, the germination rate was lower in the undiluted paddy water than in the 1/10 and 1/50 diluted paddy water treatments.

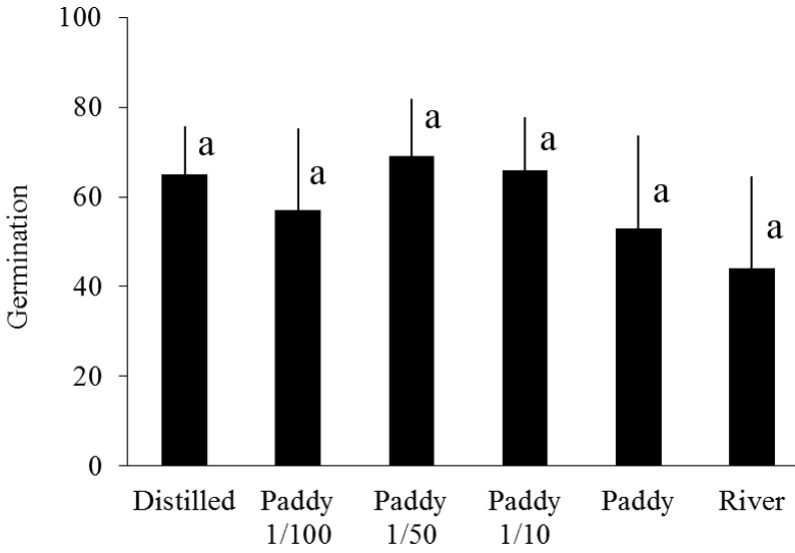

Figure 2. Germination of *Vallisneria asiatica* (20 ℃, 12 h light:dark)

Note: Error bars show standard deviation.

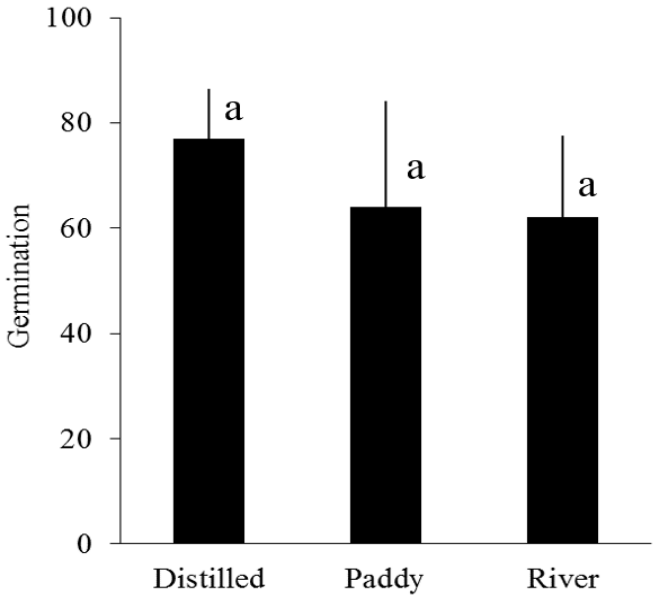

Figure 3. Germination of *Vallisneria asiatica* (22–42 ℃, in the laboratory)

Note: Error bars show standard deviation.

A significant difference (Mann–Whitney *U* test: $p < 0.05$) in *A. multiflora* germination rate was observed under different water treatments and varying temperature conditions, although the rate varied considerably (Fig. 4). The mean germination rates in the distilled and river water treatments were 68% and 41%, respectively.

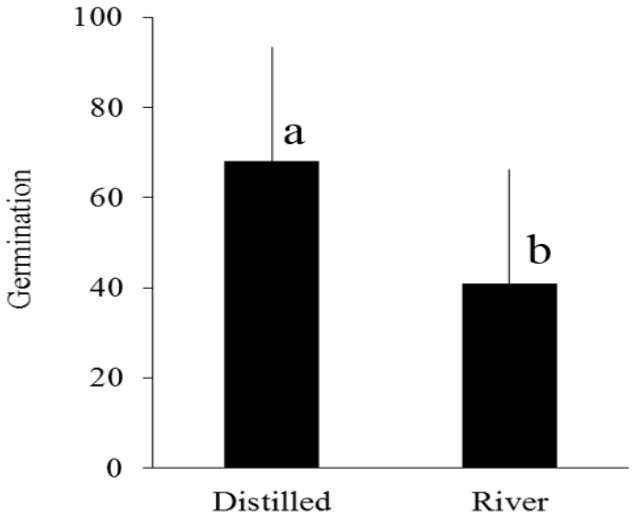

Figure 4. Germination of *Ammannia multiflora* (22–42 ℃, in the laboratory)

Note: Error bars show standard deviation.

4. Discussion

The glufosinate and pyraclonil concentrations were similar in the paddy water into which herbicides were sprayed and the river water that received a large volume of upstream water. Therefore, this study shows that reduction in the concentrations of herbicides is minimal in the environment. Residual concentrations of herbicides released into the environment may be diminished through dilution, absorption by soil, and/or environmental degradation (Kusanagi, 2006). However, as our results indicate, glufosinate and pyraclonil appear to persist in the study area. The mechanisms that prevent a decline in glufosinate and pyraclonil concentrations in river water need to be clarified.

In the experiment examining germination sensitivity, herbicides did not significantly affect the germination of *V. asiatica*, whereas they did affect that of *A. multiflora*. The paddy water contained two herbicide components, indicating that reduction in the germination rate of *A. multiflora* was possibly caused by these substances. This experiment also showed that *V. asiatica* is not highly sensitive to the observed concentrations of the detected herbicides.

The major weed species in paddy fields in Japan include *Lemna* spp., *Potamogeton distinctus* A. Benn, *Sagittaria* spp., *Schoenoplectiella* spp., and noxious *Echinochloa* spp. (Kusanagi, 2006). Herbicides used immediately after seedling transplantation act mainly during the growth phase: they are only effective immediately after germination until the early seedling stage. Therefore, manuals for herbicides typically suggest that they should not be applied to seeds in the soil. Egley and Williams (1978) reported that glufosinate does not affect the germination of weed species. These observations are consistent with the unchanged germination rates of *V. asiatica* exposed to herbicides in our study.

However, germination of *A. multiflora* was significantly affected by herbicide treatments, indicating that the growth of this species could be suppressed by environmental water drained from paddy fields, even at the germination stage, during which herbicides are not considered to have a significant effect. Suppression of germination, a particularly important event in the

plant life cycle, may have a critical influence on plant colonization (Bewley and Black, 1994). Therefore, to assess the effects on the diversity of organisms inhabiting wetlands adjacent to paddy fields, it is necessary to determine the relationships between germination of wild plant species and residual herbicides.

Vascular plants show different sensitivity to stressors depending on several factors, including difference of community, growth stage (such as seed, seedling, and mature plant), and environmental factors (Thai et al., 1985; Marrs et al., 1991; Ratnayake and Shaw, 1992; Vidottoa et al., 2007; Dugdale et al., 2010). For a comprehensive understanding of the influences of residual herbicides in the environment on wetland biodiversity, it is necessary to analyse additional species exposed to herbicides, as well as examining the sensitivity of the species used in the present study at different growth stages. Marrs et al. (1991, 1993) investigated the sensitivity of wild plants to herbicides in order to evaluate buffering zones for conservation. Studies using plants other than the experimental plants used frequently in previous studies (crop plants, meadow grasses, *Azolla* spp., *Lemna* spp., etc.) are required, and the development of new techniques is essential.

Herbicide use is highly profitable in agriculture; however, considering the adverse influences of herbicides on biodiversity, it is necessary to establish guidelines for acceptable herbicide levels in wetlands adjacent to paddy fields. Continued, systematic accumulation of information is necessary for the establishment of such guidelines.

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