Original Article

Applicability of ELISA in pesticide monitoring to control runoff of bensulfuron-methyl and simetryn from paddy fields

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The applicability of ELISA kits was evaluated as an alternative to monitor bensulfuron-methyl and simetryn behavior in paddy water under intermittent (Plot 1) and continuous (Plot 2) irrigation schemes. Simetryn concentrations in both plots decreased exponentially from the peak of the first day. However, the simetryn kit systematically underestimated by a factor of 0.79 as compared to the GC method. Bensulfuron-methyl concentrations exhibited similar dissipation kinetics in paddy water and the drainage water. The bensulfuron-methyl kit was capable of distinguishing spatial variations of concentrations in the paddy field. The ELISA kits clearly indicated differences in the loss of both herbicides between the two plots and therefore may be useful for evaluating the water management practice of pesticide runoff control in paddy fields. © Pesticide Science Society of Japan

Keywords: ELISA, paddy field, bensulfuron-methyl, simetryn, runoff.

Introduction

In modern paddy cultivation, pesticides are considered indispensable to protect the crop from damage by pests. Although pesticides are highly beneficial when they remain in the targeted area, problems can be caused when they enter the environment through leaching, runoff and drift from paddy fields. In Japan, a number of pesticides have been detected in river and lake systems especially in the period shortly after herbicide application in the fields.[1–5] The impact of pesticides in surface water on fish, algae and aquatic plants has been reported.[6,7] Therefore, public concern about the low-dose, long-term effect of these pesticides on aquatic flora as well as human health is increasing.[8] The above situation reveals that more research is needed to reduce the discharge of pesticides into the aquatic environment. Monitoring the behavior of pesticides and their loss in paddy fields is an effective approach for environmental risk assessment associated with agricultural production.

Meanwhile, interest in immunochemical assays for monitoring pesticide concentrations has been steadily increasing.[9] Immunoassay techniques now provide a simple, yet powerful and inexpensive screening method with enormous potential including the generation of quantitative data.[10] Therefore, these assays can be a valuable alternative to conventional analytical methods. Moreover, their low cost allows more replicates or more samples to be measured so that the researchers can obtain more information about their targets. In addition, from the environmental prospective, immunochemical method uses almost no toxic organic solvents and hence is more environmental friendly.[11] Immunochemical methods are especially suitable for the analysis of water where matrix effects are seldom observed.[9] Several researches have validated in-house and commercial enzyme-linked immunosorbent assay (ELISA) kits for various pesticides in water.[12,13] Walker *et al.*[14] and Ishii[15] also reported the potential of commercial kits to detect pesticide residue in river water. ELISA results were proven to correlate well with the results from powerful conventional methods such as Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS).[11,16] In some cases, the accuracy of the ELISA method was greater than that of the conventional one.[17] Some researchers also managed to use ELISA success-

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fully for the quantitative analysis of numerous pesticides in water matrices with little or no matrix interference.[18] Recently, Banks *et al.*[19] have reported the use of commercial ELISA test kits for analyzing pesticide residues in water. Without compromising accuracy and precision, the ELISA method can overcome all the disadvantages of conventional instrumental analysis such as: 1) the extremely high cost of initial investment and running costs, 2) longer working-time requirement for chemical extraction and conditioning of instruments, 3) environmental load of the analysis from using significant amounts of toxic solvents, 4) sophisticated extraction and analytical procedures. To monitor pesticide behavior in paddy fields, ELISA may have great advantages, especially for the comparative evaluation of different management practices for reducing pesticide losses from paddy fields.

The objectives of this research are to evaluate the applicability of ELISA in monitoring pesticide concentrations in paddy water, and to discuss important factors for reducing the risk of pesticide loss into the aquatic environment. Simetryn [N^2, N^4-diethyl-6-methylthio-1,3,5-triazine-2,4-diamine] and bensulfuron-methyl [methyl α-(4,6-dimethoxypirimidin-2-ylcarbamoylsulfamoyl)-o-toluate] were monitored, two commonly applied herbicides in experimental paddy fields under two different water management practices. In addition to monitoring the evolution of herbicide concentrations in paddy water with ELISA, conventional gas chromatography (GC) was compared with simetryn. In addition, various herbicide concentrations within a plot were evaluated using an ELISA kit for bensulfuron-methyl.

Materials and Methods

1. Field experiment

The pesticide fate and transport monitoring was conducted in two 27.9×49.0 m paddy plots at the experimental farm of Tokyo University of Agriculture and Technology (TUAT) in Fuchu, Tokyo from May 12th to June 30th, 2003. The soil in these plots is a light clay according to the classification of the International Society of Soil Science (37.6% sand, 31.8% silt and 30.6% clay) having 3.96% of organic carbon.

One plot was assigned to an intermittent irrigation scheme with a high drainage gate (denoted as Plot 1) representing the good management practice using an automatic irrigation system (Rakutaro®, Nihon System Kaihatsu Co., Ltd., Saitama). The other was assigned to a continuous irrigation scheme with a lower drainage gate (denoted as Plot 2) as an inappropriate management scenario.

A commercial preparation of granule herbicide Weedless® (3.0% cafenstrole, 6.0% daimuron, 0.51% bensulfuron-methyl) was applied at a rate of 10 kg/ha, 5 days after transplanting the rice seedlings, as a source of bensulfuron-methyl. Then, 21 days after transplanting, KumishotSM® (4.5% simetryn, 4.5% mefenacet, 15.0% thiobencarb, 2.4% MCPB) was applied at a rate of 10 kg/ha as a source of simetryn.

The water balance was monitored for precipitation, irrigation, surface runoff/drainage, evapotranspiration, and percolation. Precipitation data were collected from the meteorology station in TUAT. The volume of irrigation water in each treatment was monitored with a flow meter connected to a data logger. The depth of paddy water was monitored with a water level sensor (LSP-100, UIJIN Co. Ltd., Tokyo) and the volume of surface runoff/drainage through a 90° V-notch weir was calculated using the paddy water level data. For Plot 1, the height from the paddy soil to the bottom of the notch was set at 7.5 cm to promote water retention and to minimize paddy water runoff. The corresponding height for Plot 2 was 2.5 cm. Evapotranspiration (ET) was observed by a water level sensor in a lysimeter box (35×50×30 cm) containing 15 cm of a paddled soil layer in flooded conditions with four growing rice plants. Total percolation including lateral seepage was calculated from the remaining monitored hydrological data.

2. Sampling and analysis

2.1. Sampling

Composite paddy water samples from 5 spots (one center and four corner spots) in the plot and at the drainage gate water were taken as the 1st, 3rd, 7th, 14th, 21st, and 35th days after herbicide application (DAHA) for each active ingredient. The 1st day drainage sample of simetryn and 35th day sample of bensulfuron-methyl were not available. Water samples for investigating the spatial variation of herbicide concentration in paddy water were taken from 9 spots in Plot 2 at 40 DAHA (Fig. 4). The samples were kept frozen until chemical analysis. Bensulfuron-methyl was analyzed by ELISA whereas simetryn was analyzed by ELISA and GC in parallel.

2.2 ELISA test

The ELISA test kit for simetryn was supplied by Horiba Biotechnology (Osaka, Japan). The test kit for bensulfuron-methyl was provided by Iatoron Laboratories, Inc. (Tokyo, Japan) and Otsuka Chemical Co., Ltd. (Osaka, Japan). These two kits are in form of competitive immunoassays. Each kit has 96 antibody-coated wells in an 8×12-well plate. All kits were kept at 4°C before use.

Water samples were filtered with 1.2 μm glass micro-fiber filters (GF/C, Whatman) then diluted to achieve a concentration in the working range of the kits (0.03–0.3 μg/l for bensulfuron-methyl and 3–50 μg/l for simetryn). The target concentrations for the dilution were set at 0.1 μg/l and 10 μg/l for bensulfuron-methyl and simetryn, respectively. The dilution factor for the sample preparation was determined by the published data of Takagi *et al.*[20] for bensulfuron-methyl, and by Inao *et al.*[21] for simetryn. All reagent solutions were prepared according to the kit instructions and the analytical procedures of the kits were followed. Samples and standards were first mixed with the conjugate at 1 : 1 ratio (v/v) then 100 μl of the mixtures were added to each well. After 1 h, the solution was removed and the well was washed 3 times with washing solution provided in the kit. Then, 100 μl of substrate solution was added and allowed to incubate for 10 min. Finally, 100 μl of stopping agent was added.

The absorbance of the final sample was measured by a UV-VIS spectrometer (Emax, Molecular Devices, USA) at the wavelength $\lambda = 450$ nm. The standards and samples were analyzed in triplicate. Concentrations were calculated from the standard curves (3-point standard curve for bensulfuron-methyl and 2-point standard curve for simetryn). The limits of detection for bensulfuron-methyl and simetryn were 0.03 μg/l and 3 μg/l, respectively.

2.3 Gas chromatography analysis

Simetryn was extracted by liquid–liquid extraction. Water samples were filtered with 1.2 μm glass micro-fiber filters (GF/C, Whatman). High concentration samples (1, 3 and 7 DAHA samples) were diluted with de-ionized water before extraction. Thirty grams of sodium chloride was added to 500 ml of sample, and the sample was extracted twice with 400 ml dichloromethane. Dichloromethane solution was dehydrated by sodium sulfate and filtered with silicon-treated filter paper (1PS, Whatman). The filtrate was concentrated using a rotary evaporator up to 1 ml and then dried with a gentle nitrogen stream. The residue was reconstituted with 5 ml acetone using an ultrasonic bath. The solution was transferred into a test tube and kept at 4°C until GC analysis. A GC system (SHIMAZU GC-17A) was used for analysis. The column was a DB-5 (J&W) column (30 m×0.25 μm×0.32 mm). The temperature was programmed as follows: 60°C (2 min) ramped up to 140°C at 10°C/min then to 270°C at 5°C/min. Temperature was held at 270°C for 4 min. The splitless injection mode was used with an injected volume of 4 μl. Carrier gas pressure was set at 40 kPa for 2 min then increased to 64 kPa at 3 kPa/min and continued to ramp at 1.5 kPa/min to 103 kPa which was maintained for 4 min. The herbicide was detected by a Flame Thermoionic Detector (FTD). The determination limit of this analysis was 1.0 μg/l.

Results and Discussion

1. Water balance monitoring

The monitoring result of the water balance for 2 experimental plots is shown in Table 1. With continuous irrigation, an overflow drainage scheme and a low drainage gate, Plot 2 required approximately 50% more irrigation water and drained almost 8 times more than Plot 1. The intermittent irrigation scheme and the high drainage gate retained paddy water and prevented significant paddy water runoff in Plot 1. Generally, a large amount of irrigation affects herbicide concentrations due to dilution and intensive surface drainage affects the transport of herbicide from the paddy field. Watanabe and Maruyama[22] observed about 0.35 cm/day as the average surface drainage from paddy fields in Japan. However, this is highly dependent on the farmer's practice. The total percolation in the two plots was similar, giving daily percolation rates of 1.0 cm/day. Nakagawa[23] reported percolation rates from 0.5 to 3.0 cm/day depending on the soil type for typical Japanese paddy fields. Also, an experimental paddy plot monitored in Tsukuba indicated a similar water balance with average irrigation, sum of drainage, seepage and percolation, precipitation and evapotranspiration of 0.97, 1.17, 0.47, 0.28 cm/day, respectively.[24]

Table 1. Water balance in the paddy plots

		Plot 1		Plot 2	
		Total (cm)	Average (cm/day)	Total (cm)	Average (cm/day)
Input	Irrigation	49.6	1.0	74.4	1.49
	Precipitation	24.2	0.48	24.2	0.48
	Total	73.8	1.48	98.6	1.97
Output	Drainage	3.5	0.07	27.2	0.54
	Percolation	50.8	1.0	51.2	1.0
	Evapotranspiration	19.7	0.39	19.7	0.39
	Total	73.9	1.48	98.1	1.96

2. Behavior of Simetryn in paddy water

As a quality control measure for the ELISA analysis, a precision test was executed beforehand. The relative standard deviation of a sample's absorbance was in accordance with the recommendation of less than 10%.[9] The control blank sample included in the kit was undetectable. For ELISA analysis in simetryn, since only 2 standard solutions (3 and 50 ppb) were included in the kit, a linearity check was carried out including 2 more external standards with concentrations of 5 and 10 ppb All experimental samples were detected in the linear segment of the standard curve.

Simetryn concentrations in the paddy water of Plots 1 and 2 analyzed by ELISA are shown in Fig. 1. The concentration of simetryn peaked at 1 day after herbicide application (DAHA) and an exponential decline of the concentration during the early period was observed thereafter. The concentration in Plot 1 decreased from 748 to 3.4 μg/l while Plot 2 fell from 670 to 3.4 μg/l. Simetryn concentrations in Plot 1 were

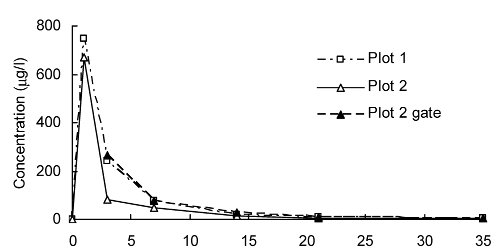

Fig. 1. Observed simetryn concentrations in paddy water for Plots 1 and 2, and that at the drainage gate before the application and until 35 days after by ELISA.

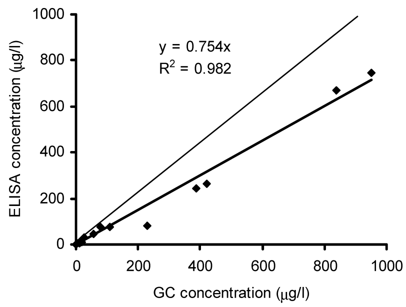

Fig. 2. Correlation between ELISA and GC values for simetryn.

Table 2. DT_{50} of simetryn in paddy water calculated by ELISA and GC data

	ELISA			GC		
	R^2	k (day^{-1})	DT_{50} (day)	R^2	k (day^{-1})	DT_{50} (day)
Plot 1	0.97	0.37	1.90	0.99	0.35	1.96
Plot 2	0.76	0.40	1.75	0.97	0.43	1.60

always higher than those in Plot 2 during the monitoring period, which reflects significant dilution from the irrigation practice in Plot 2. The dilution effect caused by irrigation and precipitation affects the concentration of the applied herbicide in paddy water. Higher simetryn concentrations near the drainage gate were also observed as compared to the averaged concentration of 5 sampling spots, including the concentration near the irrigation inlet which was affected by considerable dilution. In addition to degradation processes, herbicide loss through percolation and surface runoff/drainage can promote the dissipation process. From 7 DAHA toward the end of the monitoring period, herbicide concentrations in the two plots were lower and the dissipation rate of the herbicide was slower than in the earlier period.

Comparing ELISA and conventional GC analyses, both results plotted similar curves. However, the peak concentrations detected by the GC system were 950 and 840 μg/l in Plot 1 and Plot 2, respectively, which suggested that ELISA may underestimate the presence of the herbicide in the paddy water matrix. The signed-rank test confirmed the difference between these 2 sets of data with 99% significance. Consequently, linear regression of the detected concentrations by ELISA against GC gives a slope of 0.754 with R^2 of 0.982 (Fig. 2). Note that the intercept was forced to be 0 because the concentration ratio by ELISA over the GC method below 20 μg/l still gives 0.74. This result was similar to the study by the National Institute of Agro-Environmental Sciences (NIAES) of Japan in which the ELISA kit underestimated the mefenacet and triazine concentrations by about 20% in comparison with the conventional method.[28)]

From the above data, concentrations in the period from 1 to 7 DAHA were used to calculate the DT_{50} of simetryn in two plots using first order kinetics (Table 2). The calculated DT_{50} values were similar between the two methods of analysis and were also comparable with the estimated DT_{50} of less than 2 days from the data at Tsukuba, Japan reported by Inao *et al.*[21)] This confirmed that ELISA could be used in quantitative studies to measure the actual half-life of herbicides despite the systematical deviation of ELISA results from the conventional reference method.

Concerning the fate of simetryn in the field, the high application rate and high solubility of simetryn resulted in its high concentration in paddy water, which is vulnerable to loss by runoff. The quick dissipation of simetryn immediately after its application, especially in Plot 2, indicates significant herbicide loss along with a dilution effect from irrigation input, and consequently the importance of controlling pesticide loss from paddy fields in this period. Ross and Sava[29)] reported that a 6-day water holding period after herbicide application can facilitate the dissipation of highly soluble molinate within paddy plots. During the monitoring period, both ELISA and GC methods confirmed the remarkably different simetryn concentrations between the two plots with a higher concentration in Plot 1. This consolidated the observation in two similar water management scenarios by Inao *et al.*[21)] and considerable loss of herbicide through runoff water occurred in the plot with the continuous irrigation scheme.

3. *Behavior of bensulfuron-methyl in paddy water*

3.1. *Bensulfuron-methyl dissipation in paddy water*

The standard curve of the bensulfuron-methyl kit was also confirmed to be linear with a high correlation value (0.999). All experimental samples were detected within the linear segment of the standard curve. Bensulfuron-methyl concentrations in the paddy water of Plots 1 and 2 analyzed by ELISA are shown in Fig. 3.

In general, the evolution of bensulfuron-methyl concentration in both plots during the monitoring period was comparable to the previous report.[25)] Bensulfuron-methyl in paddy water quickly reaches its peak only one day after application with concentrations of 77, 74 and 81 μg/l respectively for Plot 1, Plot 2 and the drainage of Plot 2. The bensulfuron-methyl concentrations decreased exponentially thereafter. The major

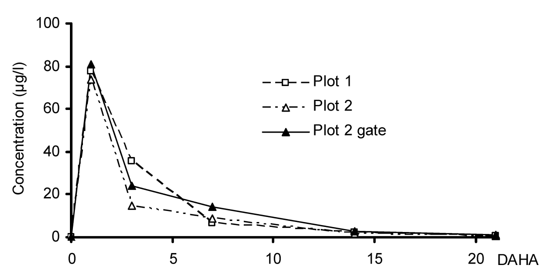

Fig. 3. Observed bensulfuron-methyl concentrations in paddy water for Plots 1 and 2, and that at the drainage gate before the application and until 22 days after.

Table 3. Comparison of bensulfuron-methyl concentrations in paddy water analyzed by HPLC and ELISA

	Concentration (μg/l)				
	Days after herbicide application				
	1	3	7	14	21
HPLC[a]	106	15.4	7.0	0.4	0.7
ELISA-plot 1[b]	113	52.1	9.5	2.8	1.0
ELISA-plot 2[b]	108	21.5	15.7	2.3	1.0

[a] Data from Okamoto *et al.*[25)]

[b] Monitored concentrations were adjusted to an equivalent application rate with experiment of Okamoto *et al.*[25)]

dissipation mechanisms of bensulfuron-methyl include photolysis and dilution. Bensulfuron-methyl was reported to hydrolyze slowly at neutral pH but to degrade rapidly by direct photolysis in natural water with a half-life (DT_{50}) of 3–4 days.[26)] In contrast in the simetryn results, no clear difference in concentration between the two plots was observed with the exception of the third day in the case of bensulfuron-methyl, although Plot 2 had an appreciable amount of irrigation and drainage. This situation is supposedly caused by the cross-reaction of solutes in the paddy water sample matrix. This outcome was also experienced by Newman *et al.*[27)] in their comparison between GC and ELISA for 3 herbicide residues (atrazine, alachlor, and metolachlor) in well-water samples in the US.

Table 3 compares bensulfuron-methyl concentrations in paddy water by Okamoto *et al.*[25)] who monitored the Oppe river watershed (Japan) using HPLC with Plots 1 and 2 with an equivalent application rate to this study. Although the evolution of pesticide concentrations depends upon the soil, hydrological and management conditions, the bensulfuron-methyl concentrations in paddy water from two studies followed the similar dissipation patterns. Linear regression between HPLC and ELISA results with mixed data of two plots gives the slope and intercept of 0.936 and -4.53 μg/l with R^2 of 0.940. The values of DT_{50} calculated from ELISA data were 1.67 and 2.1 days for Plot 1 and plot 2, respectively. These values were comparable to the reported DT_{50} of 1.5 to 2.9 days by Okamoto *et al.*[25)] In this sense, it can be said that the ELISA method can be used to track the general trend of bensulfuron-methyl concentrations in paddy water. Nevertheless, validation of this ELISA kit with a conventional method for real and spiked samples for bensulfuron-methyl should be carried out in order to confirm the accuracy and reproducibility of the ELISA kit.

Similar to simetryn, the concentrations of bensulfuron-methyl in paddy water taken at the drainage gate of Plot 2 were always higher than the concentrations of composite samples of Plot 2. With the supposition that the matrix effect was the same for these 2 series of samples, this phenomenon was probably caused by inhomogeneous dilution of paddy water due to significant irrigation water near the inlet as compared to near the outlet of the plot as discussed above.

3.2. *Spatial variation of bensulfuron-methyl concentrations in paddy water*

According to the above observation, concentrations of herbicides within the plot seem to be inhomogeneous as indicated by the difference between the plot-averaged concentrations and those at the drainage gate. Dilution effects by irrigation water may influence the spatial variability of herbicide concentrations within paddy plots. Figure 4 shows the spatial variation of herbicide concentrations in the paddy plots at 40 DAHA. Differences were clearly observed among samples taken at 9 spots evenly distributed around the plot. The lowest concentration of 0.08 μg/l was observed at the spot next to the irrigation inlet. The concentration increased with the distance from the inlet. Higher concentrations up to 0.4 μg/l were detected on the opposite side of the inlet. The mean and the coefficient of variation of detected concentrations at 9 spots were 0.242 μg/l and 49%, respectively. This result implies that the inhomogeneous dilution by irrigation significantly influences the distribution of herbicide concentrations in paddy fields. Therefore, it is suggested that an optimal sampling design is necessary considering the structure of irrigation and the drainage system.

The results again demonstrated the capability of ELISA to implement not only qualitative but also quantitative analysis. This method clearly distinguished the concentrations of different magnitudes monitored at low concentrations. However, quantitative analysis of such samples with concentrations lower than 0.4 μg/l usually requires sophisticated analytical systems or significant concentrations in the case of HPLC analysis.[25)]

4. *Herbicide loss from paddy fields and its control*

In general, ELISA successfully produced a graph showing the behavior of herbicides in paddy fields. Furthermore, the great potential of ELISA kits for the evaluation of water management practice in paddy fields was indicated as shown in Fig. 5. ELISA kits were able to distinguish losses between the two management practices used in this study. Cumulative herbi-

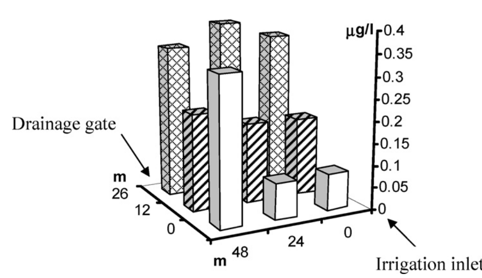

Fig. 4. Distribution of bensulfuron-methyl concentrations in Plot 2 at 40 DAHA; position of the bars correspond to the cross points of lines 0 m, 24 m and 48 m from the irrigation inlet on the long side and 0 m, 12 m and 26 m on the short side.

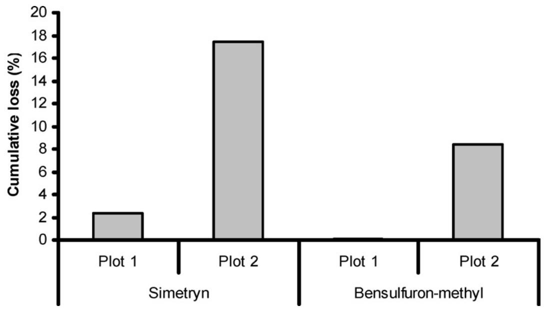

Fig. 5. Cumulative simetryn and bensulfuron-methyl losses as % mass applied during the monitoring period.

cide losses as % of applied mass were 2.4% and 17.4%, respectively, for Plots 1 and 2 for simetryn, and the corresponding values for bensulfuron-methyl were 0.1% and 8.4%, respectively. For both herbicides, the treatment in Plot 2 had a significant volume of paddy water runoff (Table 1) with the loss of a large amount of herbicide. Although the absolute value of the loss may be different from that obtained using a conventional method of analysis, ELISA kits stipulated the magnitude of loss occurring under each management. This information could be very useful for large scale monitoring with a limited budget and time, and it is crucial for the design of appropriate management practices to reduce pesticide losses from paddy fields. In addition, it should be noted that inhomogeneous herbicide concentrations throughout the paddy field depending on the irrigation practice affect the evaluation of herbicide loss as discussed in the previous section.

Concerning the saving of irrigation water and preservation of water quality, the importance of water management in paddy rice production was elucidated in this study. The combination of an intermittent irrigation scheme with a high drainage gate to promote paddy water retention is recommended as the best management practice for the expectation of no significant drainage and herbicide runoff. However, paddy fields managed by continuous irrigation and an overflow-drainage scheme may cause significant water and herbicide losses depending on the volume of irrigation and precipitation.

Acknowledgments

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