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Investigation of Concentrations of Paddy Herbicides and Their Transformation Products in the Sakura River, Japan, and Toxicity of the Compounds to a Diatom and a Green Alga

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Abstract Four paddy herbicides and their transformation products (TPs) were monitored in the Sakura River, Japan, during the rice growing seasons of 2009 and 2010. Toxicity tests to an attached diatom, *Mayamaea atomus*, and a green alga, *Pseudokirchneriella subcapitata*, were also conducted. Clomeprop propionic acid, which forms from the degrading herbicide, was detected in the river water at much higher concentrations than the parent compound (the maximum concentration of the TP and the parent compound; 0.829–0.925 μg/L and 0.039–0.073 μg/L, respectively). The toxicity of the TPs to the diatom and green alga was relatively low; the 72-h median effective concentration (EC_{50}) value > 1,470 μg/L; for each compound, the maximum concentration in the river did not exceed the EC_{50} value.

Keywords Paddy herbicide · Metabolite · Aquatic organism · River water

Pesticides used on arable lands are degraded by chemical processes and microorganisms. These pesticides and their transformation products (TPs), particularly when applied to paddy fields, can be discharged into water bodies, such as canals, rivers, and lakes, during the growing season. Public concern about the adverse effects of pesticides and their TPs on water quality and aquatic organisms is increasing. A number of surveys of paddy pesticides in rivers have been conducted (Sudo et al. 2002; Kawakami et al. 2005; Ishihara 2008); however, detailed monitoring surveys of pesticide TPs are limited (Iwafune et al. 2010). Iwafune et al. (2010) monitored paddy pesticides and their TPs in the Sakura River, Japan, and detected both the herbicides and their TPs in the river water after transplanting rice seedlings. However, for pesticide TPs, few evaluations on the toxicity to aquatic organisms, such as algae, have been conducted (Stratton 1984; Wei et al. 1998).

In this study, we investigated the changes in concentration of four paddy herbicides and their TPs in the Sakura River during two rice growing seasons (2009, 2010) and tested the toxicity of these compounds to an attached diatom, *Mayamaea atomus* (formerly known as *Navicula atomus*, Lange-Bertalot 2001) and a green alga, *Pseudokirchneriella subcapitata*.

Materials and Methods

Four herbicides (bromobutide, cafenstrol, clomeprop, and pyrazolynate) and four TPs of these herbicides (bromobutide desbromo, cafenstrol descarbamoyl, clomeprop propionic acid, and pyrazolynate destosyl) were selected (Table 1) because our previous work showed that these TPs were frequently detected in our study area (Iwafune et al. 2010). These herbicides are widely used for rice crop protection in Japan and in the rice growing area adjacent to the Sakura River. Standard reagents, all with greater than 98% purity, were purchased from Wako Pure Chemical Industries (Japan) or from the Hayashi Pure Chemical Industrial Company (Japan). We prepared stock solutions of the compounds targeted for this study with acetonitrile for chemical analyses or dimethylsulfoxide (DMSO) for toxicity tests and stored them at 4°C in the dark until use.

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The Sakura River is located in the southern part of Ibaraki Prefecture, Japan, and paddy fields are distributed along the river. Our sampling site is located 41 km downstream from the head of the river. In the river basin, the rice cultivation season is from late April to mid-September, and rice seedlings are transplanted in early May. The herbicides targeted for this study are applied within 2 weeks after transplanting the seedlings. Details of this sampling site and the river basin are described in our previous work (Iwafune et al. 2010). Grab water samples (approximately 3 L per sample) were collected in a stainless steel bucket once a week around 10 a.m. from April to August in both 2009 and 2010 and stored in 1-gallon (approximately 3.8 L) brown glass bottles. Samples were filtered and extracted on the sampling day.

The pH of the water samples ranged from 7 to 8. Analyses of the compounds in the water samples were carried out using solid-phase extraction and high-performance liquid chromatography linked with tandem mass spectrometry (LC–MS/MS; Waters, USA), as described in our previous work (Iwafune et al. 2010). Briefly, each water sample was filtered through glass-fiber filters (GF-B and GF-F; Whatman, UK), and the filtrate was adjusted to pH 6.5 with 10% phosphoric acid. One liter of the filtrate was passed through a Sep-Pak Plus PS-2 cartridge (Waters) and 400 mL was passed through an Oasis HLB Plus cartridge (Waters). Compounds retained in the Sep-Pak Plus PS-2 were eluted with 10 mL dichloromethane and those retained in the Oasis HLB Plus cartridges were eluted with 8 mL methanol and 8 mL acetonitrile. Each eluate was concentrated with a rotary evaporator and dried under a nitrogen stream. The residue was dissolved in 2 mL acetonitrile–water (1:1, v/v) for LC–MS/MS. The limits of quantification for the compounds ranged from 0.001 to 0.003 μg/L. A recovery test for all compounds was carried out in the same manner as our previous work (Iwafune et al. 2010); recovery ranged from 81.8% to 101.9% for the compounds, except for pyrazolynate destosyl (9.1% ± 2.5%), and the coefficients of variations were less than 10% for all compounds.

The test species were axenic cultures of two unicellular freshwater algae, *Mayamaea atomus* (diatom) and *Pseudokirchneriella subcapitata* ATCC22662 (green alga). *M. atomus* is a periphytic diatom that was isolated from the riverbed at the sampling site. Ishihara (2008) reported that *M. atomus* is a dominant diatom species at the sampling site throughout the year. *P. subcapitata* was used as a test species for assessing the toxicity of the test substances to green algae on the basis of its high sensitivity to chemical substances (Lewis 1995). *P. subcapitata* was obtained from the American Type Culture Collection, USA. These species were maintained on modified CSi medium (Watanabe et al. 1988) (pH 7.5; buffering solution was changed from HEPES to tris hydroxymethyl aminomethane) with 1.5% agar slants at 25 ± 1°C under continuous illumination (approximately 4,000 lux) and under static culture.

Algal toxicity tests were conducted according to a growth inhibition test method for *P. subcapitata* with a 96-well flat-bottom microplate based on the test guidelines of Environment Canada (1992). Test solutions were prepared by adding appropriate volumes of the stock solutions to modified CSi medium. The final concentration of the stock solution solvent (DMSO) in the test solution did not exceed 0.1% (v/v). The nominal concentration range of the test substances and number of treatments for toxicity tests are shown in Table 1. For the algal species, inocula were taken from the preculture during the exponential growth phase. The inoculated algae were suspended in approximately 1 mL of modified CSi medium. The initial cell density in the test culture was adjusted to approximately 5×10^3 cells/mL for *M. atomus* and 1×10^4 cells/mL for *P. subcapitata* by adding 5 μL of

Table 1 Nominal concentrations of test substances and numbers of treatments for toxicity tests

Compounds	*Mayamaea atomus*		*Pseudokirchneriella subcapitata*	
	Range[a] (μg/L)	Number of treatments	Range (μg/L)	Number of treatments
Bromobutide	3,500[b]	1	2,000[b]	1
Bromobutide desbromo	3,500[b]	1	5,000[b]	1
Cafenstrol	500[b]	1	1.95–62.5[c]	6
Cafenstrol descarbamoyl	25,000[b]	1	50,000[b]	1
Clomeprop	35[b]	1	35[b]	1
Clomeprop propionic acid	50,000[b]	1	6,250–200,000[c]	6
Pyrazolynate	56[b]	1	56[b]	1
Pyrazolynate destosyl	1,250–40,000[c]	6	156–20,000[c]	8

[a] Nominal concentration

[b] Limit tests were carried out at shown concentrations

[c] The dilution ratio was 2

the suspended solution of the algal inoculum to 195 μL of the test solution. The cell density was determined by measuring the optical density in the culture at 680 nm (OD_{680}), using a microplate reader (BIO-RAD, Model 550, USA). Each of the initial OD_{680} values was approximately 0.007/well. All tests were conducted with more than six replicates at each concentration at 25 ± 1°C under continuous illumination (approximately 4,000 lux) and under static culture for 72 h. After 0, 24, 48, and 72 h, OD_{680} values were measured with the microplate reader. The concentrations of the test substances were measured in all treatment groups at the start and end of each test by LC–MS/MS, as described later. The 72-h median effective concentration (EC_{50}) values were calculated from geometric means of measured concentrations of the test substances by linear regression analysis on the basis of the average growth rate over the entire test duration.

Residue analyses of each test solution were carried out just before the start of each test and immediately after its end, and the concentration of the test substance was determined. For analysis of test solutions, 20 and 1.2-mL test solutions for the start and end of each test, respectively, were used. The test solutions were loaded onto a Chem Elut column (CE 1020; VARIAN, USA) and allowed to soak for 10 min. The column was eluted with 100 mL hexane for bromobutide and its TP, with 100 mL ethyl acetate for cafenstrol and pyrazolynate and their TPs, and with 200 mL ethyl acetate for clomeprop and its TP. Each eluate was stored at 4°C in the dark until LC–MS/MS analysis. Prior to LC–MS/MS analysis, each eluate was concentrated with a rotary evaporator and dried under a nitrogen stream. The residue was dissolved in 2 mL acetonitrile–water (1:1, v/v) for LC–MS/MS. All samples were analyzed with LC–MS/MS procedures modified slightly as noted in our previous work (Iwafune et al. 2010). A recovery test of the test substances was conducted with duplicate water samples spiked at 5 μg/L. Recovery ranged from 82.0% to 103.9% for all test substances except for pyrazolynate destosyl (63.8% ± 3.9%); coefficients of variations were less than 10% for all test substances.

Results and Discussion

Bromobutide, cafenstrole, clomeprop, and pyrazolynate and their TPs were detected in the river water (Table 2). Figure 1 shows the changes in the concentrations of these herbicides and their TPs during the rice growing season in 2009 and 2010. The peaks of the herbicides in the river were observed immediately after transplanting (from early May to mid-May) in 2009 (Fig. 1a, c, e) and 2010 (Fig. 1b, d, f). A similar trend has been reported in other studies (Sudo et al. 2002; Kawakami et al. 2005). Changes in the concentrations of the parent compound and the

Table 2 Toxicity of selected herbicides and their transformation products to *Mayamaea atomus* and *Pseudokirchneriella subcapitata* and maximum concentrations in the Sakura River, Japan, in 2009 and 2010

Compounds	*M. atomus*		*P. subcapitata*		2009		2010	
	72-h EC_{50} (μg/L)	95% Confidence interval (μg/L)	72-h EC_{50} (μg/L)	95% Confidence interval (μg/L)	Max concn. (μg/L)	Frequency (%)[a]	Max concn. (μg/L)	Frequency (%)
Bromobutide	>3,480[b] (4.5%)[c]	–[d]	>1,580 (7.2%)	–	13.7	100	11.9	100
Bromobutide desbromo	>3,480 (2.7%)	–	>4,810 (3.9%)	–	0.318	100	0.223	100
Cafenstrol	>478 (2.9%)	–	15.5	9.7–25.9	0.578	100	0.815	77
Cafenstrol descarbamoyl	>23,600 (14.6%)	–	>48,900 (13.6%)	–	0.859	100	0.766	100
Clomeprop	>15.1 (2.3%)	–	>15.2 (−0.9%)	–	0.073	18	0.039	27
Clomeprop propionic acid	>30,200 (6.3%)	–	74,300	39,200–139,000	0.925	100	0.829	82
Pyrazolynate	>39.4 (4.1%)	–	>38.9 (7.8%)	–	0.008	27	0.066	32
Pyrazolynate destosyl	21,900[e]	15,000–33,300[e]	1,470[e]	1,010–2,150[e]	0.469[e]	100[e]	0.629[e]	100[e]

[a] Number of times detected at more than the limit of quantification divided by the number of sampling events ($n = 22$ in both 2009 and 2010)

[b] Values with ">" indicate that the 72-h median effective concentration (EC_{50}) was more than what is shown because the inhibition was −0.9%–14.6% at the nominal concentration shown in Table 1

[c] Percentage in parentheses indicate the percentage of inhibition in the limit test

[d] No value could be calculated because only a limit test was perfomed

[e] Provisional values because of low recoveries with the analytical method

corresponding TP differed among herbicides (Fig. 1). The maximum concentration of bromobutide desbromo (0.318 and 0.223 μg/L in 2009 and 2010, respectively) was much lower than that of bromobutide (13.7 and 11.9 μg/L, respectively). A similar change in the concentrations of bromobutide and its TPs was reported by Mitobe et al. (1999). We attribute this difference in concentration to the fact that bromobutide can be degraded not only to bromobutide desbromo but also to many other TPs in natural water by sunlight (Takahashi et al. 1985) and that bromobutide has a long half-life (DT_{50}) with respect to degradation by sunlight in natural water (DT_{50} = 11–13 weeks; Takahashi et al. 1985) and in soil (DT_{50} = 25–34 days; Food and Agricultural Materials Inspection Center 2008). The maximum concentration of cafenstrol descarbamoyl (0.859 and 0.766 μg/L in 2009 and 2010, respectively) was similar to that of the parent compound (0.578 and 0.815 μg/L, respectively). Cafenstrol degrades primarily to cafenstrol descarbamoyl by photolysis in water and by degradation in soil (DT_{50} in water is 10.7–19.1 days and in soil is 8.9–13.9 days; Food and Agricultural Materials Inspection Center 2009). In contrast, the maximum concentration of clomeprop propionic acid (0.925 and 0.829 μg/L in 2009 and 2010, respectively) was much higher than that of clomeprop (0.073 and 0.039 μg/L, respectively). The detected frequency of clomeprop propionic acid was also much higher than that of clomeprop (Table 2). Clomeprop is rapidly hydrolyzed to clomeprop propionic acid in soil (DT_{50} = 3 days; Kobayashi et al. 1999); therefore, the rapid degradation of clomeprop may have been the reason for its low maximum concentration in the river water. These behavioral patterns of the herbicides and the TPs agree with our previous results (Iwafune et al. 2010).

The EC_{50} values for most of the test substances in the toxicity tests to the two algae could not be calculated (Table 2). The percentage of inhibition in limit tests was less than 14.6% (Table 2). The calculated EC_{50} value to *M. atomus* was 21,900 μg/L for pyrazolynate destosyl and the values to *P. subcapitata* were 15.5 μg/L for cafenstrol, 74,300 μg/L for clomeprop propionic acid, and 1,470 μg/L for pyrazolynate destosyl (Table 2). The toxicities of cafenstrol and pyrazolynate destoytosyl to *M. atomus* were lower than those to *P. subcapitata*; the difference in

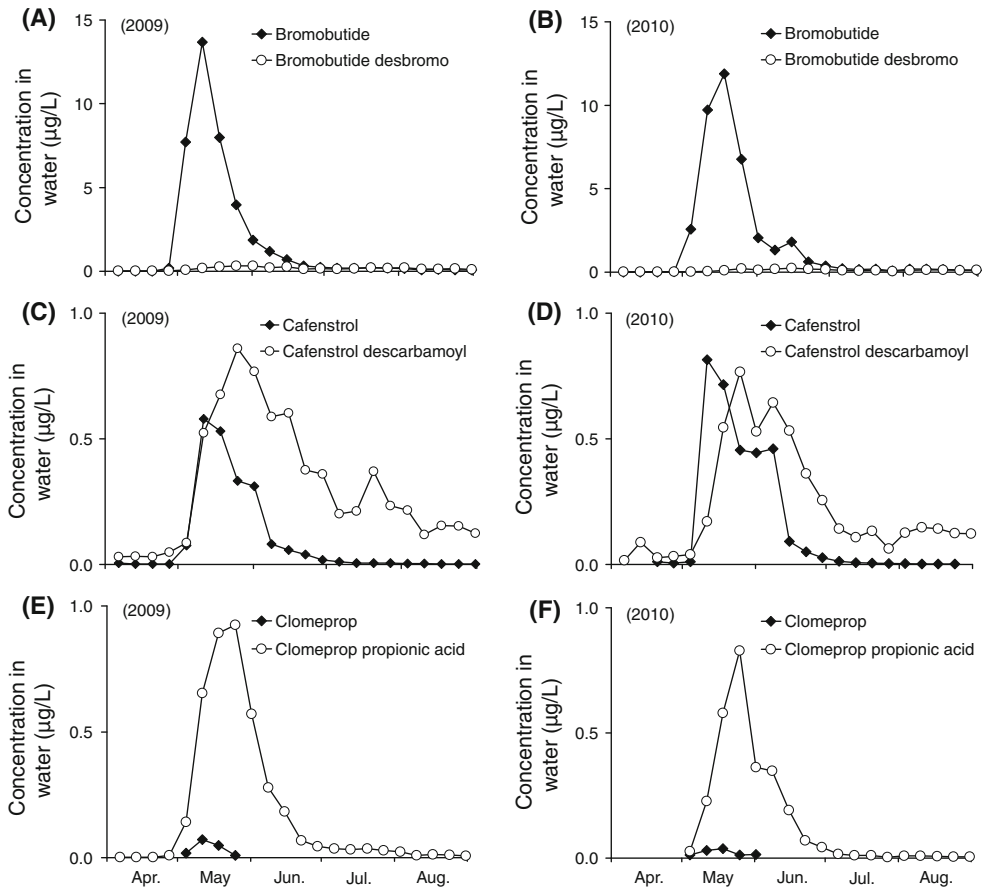

Fig. 1 Concentrations of the selected herbicides and their transformation products (TPs) in Sakura River (Japan) water in 2009 (**a**, **c**, **e**) and 2010 (**b**, **d**, **f**). The results of pyrazolynate and its TP were omitted because of the low recovery of the TP with the analytical method used

sensitivity to the compounds between the two algae was more than 30 and 15 times, respectively (Table 2). All the TPs had EC_{50} values greater than 1,000 μg/L (Table 2), indicating that the toxicity of the TPs to these two algae was relatively low. The toxicity of the TPs of some herbicides, such as atrazine (Stratton 1984), metsulfuron methyl, chlorsulfuron, and bensulfuron methyl (Wei et al. 1998), to green algae or cyanobacteria was found to be lower than the toxicity of the parent compounds. Day (1991) also reported that the toxicities of these TPs were usually less than the parent compound, especially for herbicides. To our knowledge no studies have investigated the toxicity of herbicide TPs to diatoms. Our study indicates that the toxicity of certain herbicide TPs to the diatom, *M. atomus,* and to the green alga, *P. subcapitata,* is relatively low.

To evaluate the effects of selected herbicides and their TPs on a diatom and a green alga in the Sakura River, we compared maximum concentrations of the compounds in the river water in 2009 and 2010 with the EC_{50} values of the corresponding compounds to *M. atomus* and *P. subcapitata*. The EC_{50} values of all the compounds with respect to these algae were much higher than the maximum concentrations in the river water (Table 2; ratio <0.05, maximum concentration in the river water to the EC_{50} value). Even the ratios of cafenstrol descarbamoyl and clomeprop propionic acid, whose maximum concentrations in the river water were similar to or higher than those of the parent compounds, were much less than 1 (<0.01). The results in this study indicate that selected herbicides and their TPs, which are run off from paddy fields after rice transplanting, may have negligible effects on the growth of a dominant diatom, *M. atomus*, in the Sakura River during the rice growing season. Auxinic herbicides (e.g., clomeprop) have a higher toxicity to higher aquatic plants (e.g., watermilfoil) than to green algae (Maltby et al. 2010). However, it is not known how the TPs of such herbicides affect higher aquatic plants in rivers. Further investigation on the effects of these herbicide TPs on higher plants is required.

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