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Phytotoxicity of four herbicides on *Ceratophyllum demersum*, *Vallisneria natans* and *Elodea nuttallii*

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Abstract

The physiological effects of 4 herbicides (butachlor, quinclorac, bensulfuron-methyl and atrazine) on 3 submerged macrophytes (*Ceratophyllum demersum*, *Vallisneria natans* and *Elodea nuttallii*) were tested in laboratory. The variables of the relative growth rate and the photosynthetic pigment content showed that all of the tested herbicides affected the growth of the plants obviously, even at the lowest concentration (0.0001 mg/L). Except for the *C. demersum* treated with quinclorac at 0.005 and 0.01 mg/L, the relative growth rates of the plants were inhibited significantly ($p < 0.01$). Statistical analysis of chlorophyll *a* (Chl-*a*) contents was carried out with both the *t*-test and one-way ANOVA to determine the difference between the treatment and control. The results showed that Chl-*a* contents of the plants in all treatment groups were affected by herbicides significantly, except for the *C. demersum* treated with bensulfuron-methyl at 0.0005 mg/L. The decrease in Chl-*a* content was positively correlated to the dosage of the herbicides in most treatment groups. It was suggested that herbicides in water bodies might potentially affect the growth of aquatic macrophytes. Since the Chl-*a* content of submerged macrophytes responded to the stress of herbicides sensitively and directly, it could be used as a biomaker in environmental monitoring or in the ecological risk assessment of herbicide contamination.

Key words: phytotoxicity; herbicides; submerged macrophyte; ecological risk assessment

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Introduction

The presence of herbicides and pesticides in surface and ground water is an increasingly serious environmental problem (Gerecke *et al*., 2002; Moses *et al*., 1993). Numerous researchers suggested that herbicides might cause adverse impacts on non-target aquatic vegetations (Davies *et al*., 2003; Van den Brink *et al*., 1997). Such effects had typically been evaluated from tests with territory plants, floating plants, daphnia, and algae (Weiner *et al*., 2007; Noack *et al*., 2003). However, it is unknown whether these species could be used to indicate potential effects to other vegetation types. Previous articles reported that submerged macrophytes might be more sensitive to herbicides (Belgers *et al*., 2007; Coors *et al*., 2006; Coyner *et al*., 2001; Cunningham *et al*., 1984). But those species were rarely used in toxicity tests, due to the lack of reliable and standard method (Lewis, 1995). Because of significant roles of submerged macrophytes in nutrient cycling, altering sediment chemistry and providing habitats for invertbrates and periphyton in the aquatic ecosystem (Jukka and Leena, 2003; Gerecke *et al*., 2002; Wigand *et al*., 1997), it is important to evaluate the risks of herbicides to submerged macrophytes in aquatic system.

The use of butachlor, quinclorac, bensulfuron-methyl, and atrazine as potent herbicides has been increased dramatically since their findings (Liu *et al*., 1999; Cerejeira *et al*., 1998) and has resulted in surface and groundwater contamination due to their high solubility in water. Because these herbicides are typically applied to paddy fields in China, their contamination to nearby aquatic systems may occur through a variety of pathway, including spray drift, runoff and leaching to field drains (Moses *et al*., 1993). Knowing that the agricultural use of herbicides is based on differential susceptibility of species, varying by a factor of 100–1000 among terrestrial species, it is expected that difference in susceptibility may also occur among aquatic species. The previous research have shown that the dosage even under 1% of the recommended rate can have significant effects on growth, morphology, and reproduction of some nontarget terrestrial plants (Boutin *et al*., 2000). Therefore, effects of herbicides to submerged macrophytes could be expected at low dosage (Grossmann and Kwiatkowski, 2000). However, only a few studies have been reported on direct toxicity of herbicides to submerged macrophytes.

Ceratophyllum demersum, *Vallisneria natans* and *Elodea nuttallii* are completely submerged plants and are commonly distributed in ponds, lakes, ditches and

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quiet streams with moderate to high nutrient levels. As a submerged, rootless and freefloating macrophyte, *C. demersum* is suitable for toxicological test of water soluble herbicides in water column without confounding effects of sediment. *V. natans* and *E. nuttallii* were selected to compare the difference in toxic response among different submerged macrophyte species. Morphological index, relative growth rate and photosynthetic pigment contents were selected as endpoints.

The purposes of this study were: (1) to investigate the possible effects of the herbicides on 3 submerged macrophytes; (2) to explore sensitive endpoints to indicate the herbicide contamination in water; and (3) to discuss the possible way of using submerged macrophytes to estimate the ecological risk of herbicides to the aquatic ecosystem.

1 Materials and methods

1.1 Materials

Butachlor (96.6%), quinclorac (80.7%), bensulfuron-methyl (95.5%), and atrazine (98.0%) were provided by Nanjing Limin Co., Ltd., China.

C. demersum, *V. natans*, and *E. nuttallii* were obtained from the Taihu Lake and cultured in laboratory. After 1 month culture, plant individuals with similar growth status and modality were selected for experiments. *C. demersum* and *E. nuttallii* were hydroponically cultivated in a 2-L beaker. *V. natans* was cultivated in a glass container with 60 L water and 10 cm sediment. The plants were pre-cultivated for 1 week before being exposed to herbicides.

1.2 Experimental design and sample collection

C. demersum, *V. natans*, and *E. nuttallii* were exposed to herbicides at different concentrations based on their toxicity and pre-tests outcomes. Detailed information is listed in Table 1. Each treatment had a control and each group was in triplicate. The experiments were carried out in a ventilated laboratory with natural lighting. During the experiments, water temperature was in the range of 22–28°C. Evaporative loss of water was replenished daily. Culture medium was supplied every two days. For assessment of the growth of *C. demersum* and *E. nuttallii*, the fresh weight of each group was weighed before exposure and at the end of 21-d experimental period. Leaf length of *V. natans* was recorded. Leaf samples were collected on day 0.5, 1, 2, 4, 11, 18, and 21. Samples were accurately weighed and immediately shock frozen with liquid nitrogen and were stored in the freezer for further tests.

Table 1 Nominal exposure concentration of herbicides to *C. demersum*, *V. natans*, and *E. nuttallii*

Species	Herbicide	Nominal exposure concentration (mg/L)			
C. demersum	Butachlor	0.0001	0.005	0.05	0.5
	Quinclorac	0.005	0.01	0. 5	1.0
	Bensulfuron-methyl	0.0005	0.001	0.05	0.1
	Atrazine	0.0005	0.005	0.05	0.5
V. natans	Butachlor	0.0005	0.005	0.05	–
	Bensulfuron-methyl	0.0005	0.005	0.05	–
	Atrazine	0.0005	0.005	0.05	–
E. nuttallii	Butachlor	0.0001	0.005	0.05	0.5
	Bensulfuron-methyl	0.0005	0.005	0.05	0.5

–: not available.

1.3 Measurement of photosynthetic pigment content

The contents of photosynthetic pigments were measured according to Li (2000). For determination of photosynthetic pigment content, plant material was ground in liquid nitrogen to fine powder and extracted with 95% ethanol in the dark. After centrifugation at 12000 r/min for 20 min at 4°C, absorbance of the supernatant was taken at 710, 665, 649, and 470 nm. The content of chlorophylls was estimated by the method of Arnon (1949). The carotenoid content was calculated by the equation given by Duxbury and Yentsch (1956).

1.4 Statistical analysis

Data were subjected to statistical analysis using SPSS 10.0. The *t*-test (n = 3) was used to compare independent mean values and one-way ANOVA was used to compare mean values between groups. The level of significance was set at 0.05 ($p < 0.05$).

2 Results and discussion

2.1 Effects of herbicides on the growth of submerged macrophytes

C. demersum, *V. natans*, and *E. nuttallii* grew well and no change in modality was observed at the beginning of the experiment. From the day 2, abnormal growth of the plants was observed in all three species. The main symptom in groups treated with butachlor was chlorisis.

C. demersum treated with quinclorac had curled leaves and slim stems, and the color of the whole plants was much light than that of the control. Chlorisis was also observed in *C. demersum* treated with bensulfuron-methyl. Severe fragmentation of *C. demersum* was observed after one week exposure.

The root activity of *V. natans* treated with bensulfuron-methyl decreased and some plants floated at the end of the experiment. Leaves of *V. natans* treated with atrazine involuted on margins. A great amount of cotton like white matter grew on the leaves after being exposed for a time. Increasing in the density of epiphyte and algae was observed in *V. natans* treated with butachlor and atrazine, which might also be the result of herbicides application.

Except for chlorisis, the leaves of *E. nuttallii* treated with butachlor were blasted and tillering of the plants decreased obviously. The color of *E. nuttallii* treated with bensulfuron-methyl was darker and leaves were blasted. These symptoms would be more obvious with higher exposure concentration and longer duration of exposure.

After a 21-d experimental period, reduction in the relative growth rate was observed in most treatment groups (Table 2). The results demonstrated that butachlor was most toxic among the herbicides tested. It inhibited the growth of *C. demersum* and *E. nuttallii* significantly ($p < 0.05$) even at an extremely low concentration (0.0001

Table 2 Effects of herbicides on the growth of *C. demersum* and *E. nuttallii*

Species	Herbicides	Herbicide concentration (mg/L)	Fresh weight (g)		Relative growth rate (%)
			Day 1	Day 21	
C. demersum		CK	10.41	14.45	38.81
	Butachlor	0.0001	9.62	9.95	3.74**
		0.005	9.18	9.06	–1.31**
		0.05	9.37	8.76	–6.51**
		0.5	9.56	8.13	–14.96**
	Quinclorac	0.005	9.86	15.79	60.14**
		0.01	9.70	14.27	47.11**
		0.5	9.94	13.03	31.09*
		1.0	9.81	12.12	23.55**
	Bensulfuron-methyl	0.0005	9.81	13.18	34.35
		0.001	9.63	13.05	35.51
		0.05	9.32	10.51	12.77**
		0.1	9.43	10.65	12.94**
	Atrazine	0.0005	10.28	13.43	30.64*
		0.005	10.16	12.73	25.30**
		0.05	9.65	11.01	14.09**
		0.5	9.86	11.92	20.89**
E. nuttallii		CK	9.98	11.68	17.03**
	Butachlor	0.0001	9.96	10.05	0.90**
		0.005	9.75	LD	LD
		0.05	9.09	LD	LD
		0.5	9.16	LD	LD
	Bensulfuron-methyl	0.0005	10.23	11.21	9.58**
		0.005	9.87	10.13	2.63**
		0.05	10.12	9.94	–1.78**
		0.5	10.37	9.28	–10.51**

* Significant difference ($p < 0.05$); ** $p < 0.01$; LD: lethal dose; CK: control.

mg/L). *E. nuttallii* treated with butachlor when concentration above 0.005 mg/L and *V. natans* treated with butachlor at all concentrations died after one week. *E. nuttallii* was more sensitive to bensulfuron-methyl than *C. demersum*, whose relative growth rate decreased significantly at a low concentration as 0.0005 mg/L. The data of *V. natans* was not included due to the large data variation, even though it appeared to respond to herbicides.

Evident reduction in biomass accumulation and relative growth rate of submerged macrophytes were observed in this study, which is in agreement with the results in previous publications (Cedergreen *et al.*, 2004; Strandberg and Scott-Fordsmand, 2002). Plants in groups treated with high herbicide concentrations died or partially died, indicating that the herbicides had the similar killing effects to submerged macrophytes as they did to target weeds. It could also be concluded that sensitivity difference was present among species of submerged macrophyte to herbicides. *E. nuttallii* was more sensitive to butachlor and bensulfuron-methyl than *C. demersum* and *V. natans*.

Interestingly, the biomass accumulation of *C. demersum* treated with quinclorac at 0.005 and 0.01 mg/L increased significantly ($p < 0.05$), which have been described as the hormesis phenomenon (Monferran *et al.*, 2007; Calabrese and Baldwin, 2002). This might be the result of the auxin activity of quinclorac at low concentration. This phenomenon should also be considered in evaluating herbicide contamination in water body.

2.2 Changes in the content of photosynthetic pigment

Photosynthetic pigments in higher plants are chlorophyll *a* (Chl-*a*), chlorophyll *b* (Chl-*b*) and carotenoids (Car). Chl-*a* content of *C. demersum*, *V. natans* and *E. nuttallii* decreased evidently (Figs. 1–3) as herbicides concentration increase. The decrease in the content of Chl-*a* was observed in most treatment groups from day 2. At the end of the experimental period, Chl-*a* content of plants in all treatment groups was significantly lower than that of the control ($p < 0.05$). The decrease in the Chl-*a* content was found to be positively correlated with the dosage of the herbicides in some treatment groups from day 2. Correlation analysis of the data on day 2 are presented in Table 3. Data of *C. demersum* and *E. nuttallii* were in good logarithmic correlation. Data of *V. natans* treated with butachlor was in good linear correlation. There were no significant difference in the content of Chl-*b* and Car of the plants (data omitted).

Photosynthetic pigments in higher plants are the material base for photosynthesis. The content of chlorophyll can indicate the growth status and the photosynthetic ability of the plant (Liu *et al.*, 2006). The positive

Table 3 Correlation analysis of the declining contents in Chl-*a* and the concentration of herbicides

Species	Herbicide	Correlation equation	*R*
C. demersum	Butachlor	$y = 0.0348\ln x + 0.3885$	0.9943
	Quinclorac	$y = 0.0328\ln x + 0.3629$	0.9299
	Bensulfuron-methyl	$y = 0.0397\ln x + 0.3439$	0.8391
	Atrazine	$y = 0.0221\ln x + 0.3794$	0.8885
V. natans	Butachlor	$y = 4.7147x + 0.1161$	0.9888
	Bensulfuron-methyl	Uncorrelated	–
	Atrazine	Uncorrelated	–
E. nuttallii	Butachlor	$y = 0.0254\ln x + 0.3581$	0.9713
	Bensulfuron-methyl	$y = 0.0109\ln x + 0.1825$	0.8356

x: concentration of herbicides; *y*: declining contents in Chl-*a*.
–: not available.

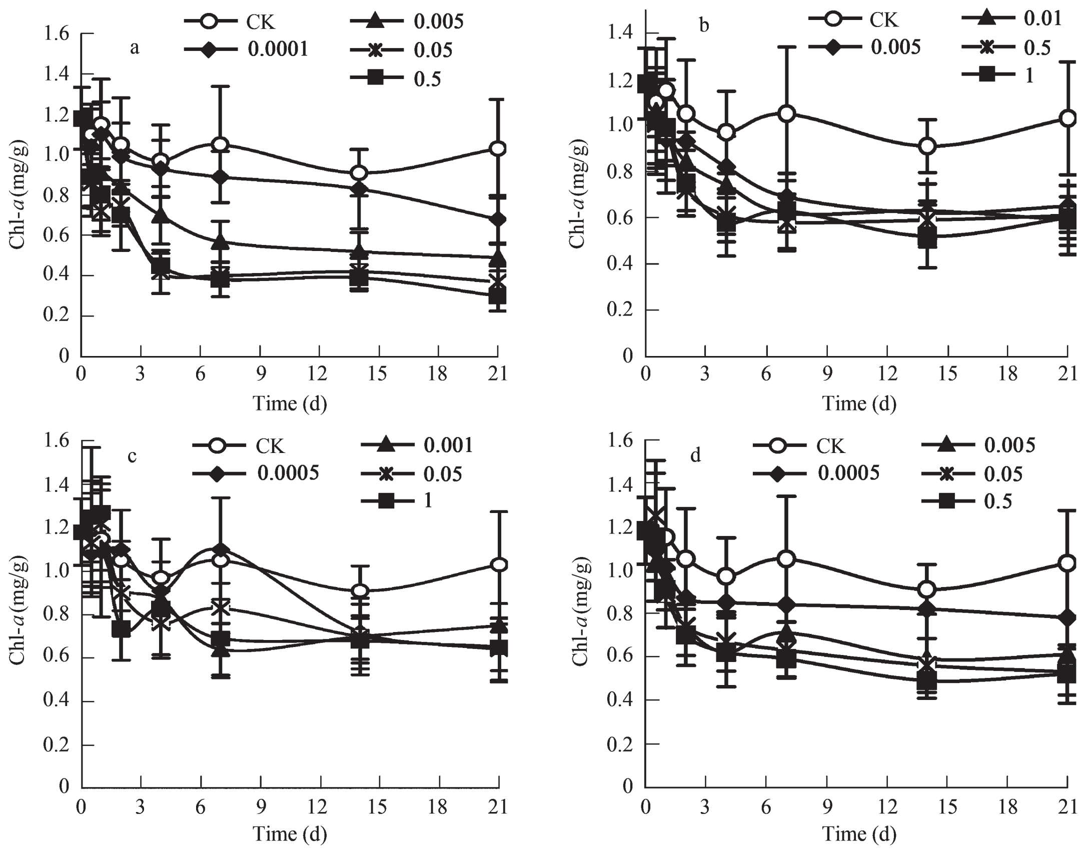

Fig. 1 Chl-*a* content of *C. demersum* exposed to butachlor (a), quinclorac (b), bensulfuron-methyl (c), and atrazine (d).

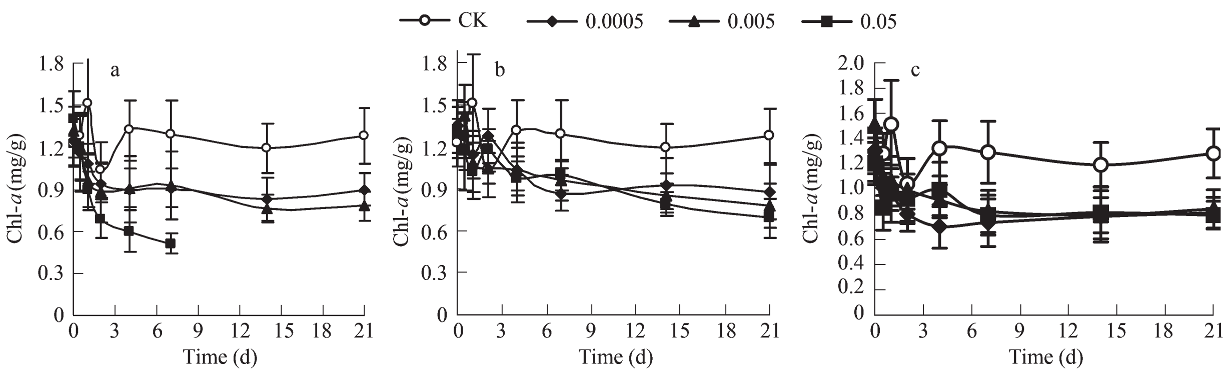

Fig. 2 Chl-*a* content of *V. natans* exposed to butachlor (a), bensulfuron-methyl (b) and atrazine (c).

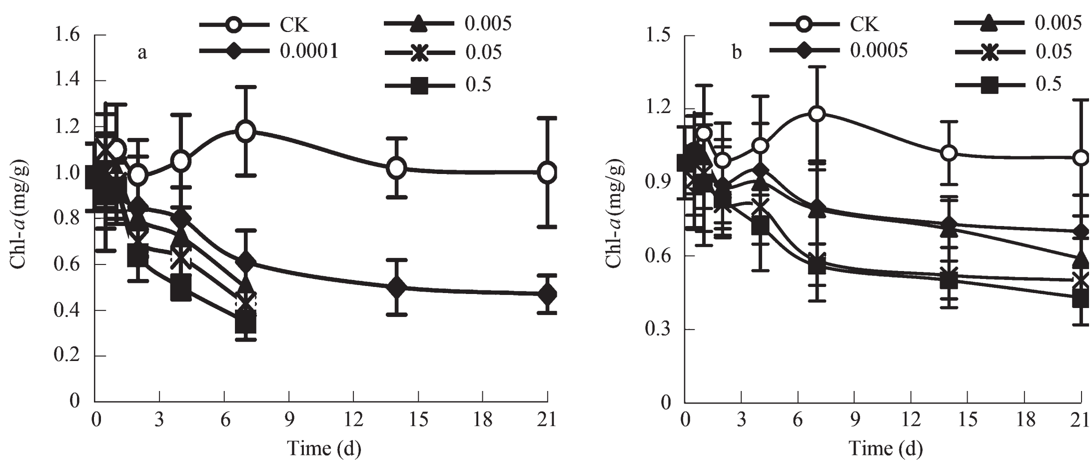

Fig. 3 Chl-*a* content of *E. nuttallii* exposed to butachlor (a) and bensulfuron-methyl (b).

correlation between the decrease of Chl-*a* content and the exposure concentration of the herbicides implied that herbicides might have harmful effects to the growth of aquatic macrophytes.

2.3 Statistical analysis of changes in content of Chl-*a*

Although Chl-*a* content of submerged macrophytes decreased with exposure time, the fluctuation of the data were evident at some sampling points. To make the statement more reliable, the data were subjected to statistical analysis with *t* test and one-way ANOVA. Since Chl-*a* content of the plants usually decreased significantly from day 2 and did not change much after 14 d, the data of day 2, 4, 7 and 14 were selected for statistical analysis.

First, data of the treatment groups and the control were all subjected to one-way ANOVA to avoid the disturbance from other environmental factors. If there were significant difference between them, the effect was confirmed. If not, the value of each sample day was subjected to *t*-test. This step further explored the differences between the data to ensure the sensitivity of the method. If at least two of them were significantly different, the effect was confirmed also. Otherwise, no effect could be confirmed.

According to this method, Chl-*a* content of the plants in all treatment groups was affected significantly, except for the *C. demersum* treating with bensulfuron-methyl at 0.0005 mg/L.

3 Discussion

Some studies showed that chlorisis was a common symptom after plants were exposed to herbicides (Sunohara and Matsumoto, 2004) and herbicides could lead to oxidative damages to aquatic plants (Menone and Pflugmacher, 2005). This was particularly crucial for photosynthetic organisms which generate reactive oxygen species (ROS) constantly during normal photosynthesis. The loss in chlorophyll content could be due to peroxidation of chloroplast membranes mediated by herbicides via increased production of free radicals (Mishra *et al*., 2006; Sharma and Dubey, 2005). Although herbicides acted on weeds via different mechanism, the reduction in the content of chlorophyll was observed in all treatment groups. Significant effect on it could be observed before any visual symptoms of toxicity were shown and significant decreases in plants growth were observed. It could be concluded that Chl-*a* content of submerged macrophytes has the potential to act as a biomarker of herbicide contamination in aquatic environment.

Compared to terrestrial plants, fish, plankton and invertebrate, submerged macrophytes might be more sensitive to the contamination of herbicides (Noack *et al*., 2003). Butachlor and bensulfuron-methyl are effective herbicides through internal absorbance and conduction. They are absorbed into the weeds and acted on their photosynthetic system. Submerged macrophyte live under water, has more opportunities to contact with contaminants than territory plants. Therefore, herbicides in water might be more harmful to submerged macrophytes.

The present study showed that the growth of aquatic plants could be affected by herbicides at chronic exposure. Submerged macrophytes showed diverse response when exposed to herbicides, from structural to biochemical changes. The species-specific sensitivity of them might result in the alteration of plant community and accelerating the retardation of the submerged macrophytes in eutrophic lakes. Current water quality standard of China only includes a few kinds of herbicides, and the threshold value is fairly high. The environmental quality standard of surface water (GHZB1-1999) set 0.003 mg/L for atrazine, which is much higher than the concentration tested here. Based on the toxicity data obtained in this study, it is reasonable to say that current water quality standard could not protect aquatic plants from herbicides effectively. Further research is needed to provide scientific base for the protection of the aquatic plants, especially submerged macrophytes, which should be included in the ecological risk assessment.

4 Conclusions

Toxic effects caused by 4 herbicides (butachlor, quinclorac, bensulfuron-methyl, atrazine) were evident in reduction of relative growth rate and Chl-*a* content in 3 submerged macrophytes. The experiment showed that herbicides inhibited the growth of *C. demersum*, *V. natans*, and *E. nuttallii* by decreasing their fresh weight and the relative growth rate, which was a sensitive index to indicate the severe hurt of herbicides to aquatic plants. As an apparent indicator, morphological symptoms of senescence phenomena were also observed. Chl-*a* content of submerged macrophytes could be a sensitive endpoint to herbicide stress.

In conclusion, herbicides in freshwater posed potential risk to submerged macrophytes. Studies of the physiological effects of herbicide on submerged macrophytes, especially test *in situ*, will enhance the understanding of degradation mechanism for submerged macrophytes and are beneficial to the ecological risk assessment. It is necessary to make long term and field-based tests to monitor the potential risk of the herbicides to freshwater ecosystem in the future study.

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