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Toxicity of Seven Herbicides to the Three Cyanobacteria *Anabaena flos-aquae, Microcystis flos-aquae* and *Mirocystis aeruginosa*

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ABSTRACT: The toxicity of 7 herbicides to the three cyanobacteria was tested in this work. The results indicated that: (1) There was a highly significant relationship between dried weight or chlorophyll-a and OD680nm for tested cyanobacteria; (2) the toxicity of the tested herbicides with the order from high to low was: photosynthesis-inhibiting > ACCase inhibitor > protox inhibiting herbicides; (3) the sensitivity of various species exposed to cyanazine, diclofop, prometryn, simazine and simetryn varied by over one order of magnitude. The decreasing order of sensitivity of cyanobacteria to the selected herbicides was: *M. Flosaquae* > *M. Aeruginosa* > *A. flos-aquae*. Cyanobacteria can produce toxins including hepatotoxins e.g. microcystins and endotoxins e.g. lipopolysaccharides. Therefore, the research on comparing the differential sensitivity of cyanobacteria and green algae is of important scientific significance and realistic value

Key words: Acute Toxicity, Berbicides, Cyanobacteria, Sensitivity

INTRODUCTION

The pollution of aquatic systems by pesticides has attracted public concerns, especially in herbicides. A few studies have been conducted to determine the harm of these pollutants to living organisms in the aquatic systems (Kasai, *et al.*, 1993; Ma, 2005). Herbicides may enter freshwater ecosystems by spray drift, leaching, run-off, or accidental spills and present potential risks for aquatic flora. However, little is known about the toxicity of these herbicides against the aquatic flora, despite the importance of aquatic plants in the functioning of ecosystems (Ma, *et al.*, 2004; Naito, *et al.*, 2003; Wong, 2000). Alterations of the species composition of an aquatic community as a result of toxic stress may affect the structure and function of the whole aquatic ecosystem. Cyanobacteria (blue-green algae) are known to be comparatively sensitive to many chemicals and the inclusion of these organisms in test batteries has been shown to improve the capacity of battery to predict the most sensitive responses of ecosystem (Ma, *et al.*, 2006; Real, *et al.*, 2003). Their ecological position at the base of most aquatic food webs and the essential roles in the nutrient cycling and oxygen production are critical to all ecosystems (Breitholtz, *et al.*, 2006). A great deal of information on toxicological aspects of pesticides on green algae, especially on *Chlorella*, *Scenedesmus* and *Selenastrum* is available (Ma, *et al.*, 2007; Sabater & Carrasco, 2001). However, little is known on the toxicological aspects of pesticides on cyanobacteria (Abou-waly, *et al.*, 1991; Ma & Chen, 2005). Cyanobacteria can produce algal toxins, but also can fixate atmospheric nitrogen, which has important application for humans and aquatic organism (An & Kampbell, 2003; Saker

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& Neilan, 2001). In order to compare the differential sensitivity of herbicides to cyanobacteria, the toxicity test has been devised. In the present study, 7 herbicides were tested to examine their effects on the cyanobacteria *Anabaena flos-aquae, Microcystis flos-aquae* and *Mirocystis aeruginosa* and then compare their different sensitivity.

MATERIALS & METHODS

Tested herbicides were purchased from People's Republic of China and their chemical classes and mode of actions (Retzinger & Smith, 1997) are shown in Table 1. The tested herbicides were dissolved in a small volume of 99.5% acetone. The concentration of acetone in the medium was kept minimizing in response to the solubility of the tested herbicides.The concentration of the acetone in the medium was less than 0.05%. The US Environmental Protection Agency recommends the allowable maximal limits of 0.05% solvent for acute tests and 0.01% for chronic tests, this level was not significant with regard to toxicity (Jay, 1996).

The toxicity tests were carried out with the freshwater cyanobacteria *Anabaena flos-aquae, Microcystis flos-aquae* and *Mirocystis aeruginosa* obtained from the Wuhan Institute of Hydrobiology, the Chinese Academy of Science. The medium for cyanobacterial growth inhibition test was HGZ medium which is composed of distilled water and the following chemical ingredients (mg/L): $NaNO_3$ 1500, K_2HPO_4 39, $MgSO_4 \cdot 7H_2O$ 75, Na_2CO_3 20, $CaCl_2$ 27, $Na_2SiO_3 \cdot 9H_2O$ 58, EDTA 1, Citric acid 6, Fe-Citric 6, and A_5 liquid 1 mL/L (ingredients of A_5 liquid are H_3BO_3 2860, $MnSO_4$ 2060, $ZnSO_4 \cdot 7H_2O$ 222, $Na_2MO_4 \cdot 2H_2O$ 391 and $CuSO_4 \cdot 5H_2O$ 79). The medium was sterilized at 121°c, 1.05 kg /cm^2 for 30 min.

Cells of cyanobacteria were propagated in a 250 mL Erlenmeyer flask containing 100 mL HGZ medium and kept on a rotator shaker (100rpm) at 24°c, and illuminated with cool-white fluorescent lights at a continuous light intensity of 5000 Lx (Verdisson, *et al.*, 2001). 20 mL HGZ medium containing cyanobacterial cells (initial concentration OD_{680nm}=0.008) were distributed to sterile 50 mL Erlenmeyer flasks. The medium was then treated with a variety of herbicide concentrations, and incubated at the same temperature and light intensity (Verdisson, *et al.*, 2001). Cyanobacterial medium was scanned at 400-800 nm wavelength after 96 hours incubated with a Shimadzu UV-2401PC spectrophotometer. The most suitable wavelength for monitoring medium was 680 nm. Strong linear relationships between dry weight concentration (DWC) or Chlorophyll-a (Chl-a) concentration of the cyanobacteria cultures and OD680nm were approved in the work (see result part). The growth of cyanobacterial biomass was calculated indirectly using OD680nm data. Appropriate control systems containing no herbicide were included in each experiment. Three replicates were made for every herbicide concentration and control. And the percent inhibition values, relative to the growth in the control systems, were also calculated in each experiment by using OD680nm data. Chl-a analysis comes after the filtration of 20 ml medium samples through the filtration (0.45-

Table 1. Selected herbicides, chemical classes and mode of action

Herbicides	Reg. No.	Formulation[a]	Chemical family	Mode of action
Diclofop	40843-25-2	97%TC	Aryloxyphenoxy propionate	ACCase inhibitor
Triclopyr	55335-06-3	95%TC	Quinoline carboxylic acid	Synthetic auxins
Ametryne	834-12-8	95.5%TC	Triazines	Inhibition of photosynthesis at PSII
Simazine	122-34-9	98%TC		
Prometryne	7287-19-6	96%TC		
Cyanazine	21725-46-2	97.8%TC		
Simetryn	1014-70-6	95%TC		

[a]**TC denotes technical grade product**

μm-pore-size Whatman GF/C membranes) and extraction with cold 90% acetone. Chl-a content in samples was estimated using spectrophotometer as a trichromatic method. Dry weight of cyanobacteria was determined with a digital balance after cells filtered on a 0.45μm membrane and dried at 105! for 8h. The EC_{50} values were calculated by using linearregression analysis of transformed herbicide concentration as natural logarithm data versus percent inhibition (Ma, *et al.*, 2006). All raw data was analyzed under SPSS version 11.0.

RESULTS & DISCUSSION

The medium of the three cyanobacteria was scanned respectively by using a Shimadzu UV-2401PC spectrophotometer. The most suitable wavelength for monitoring culture growth was 680 nm. There was a highly significant relationship between dried weight or chlorophyll-a and OD_{680nm} for three tested cyanobacteria. Their linear regression equations were shown in Table 2. All coefficient of correlation R>0.97 and significance level P<0.001.Therefore, growth of cyanobacterial biomass was calculated indirectly using OD_{680nm} data in this work.

The acute toxicity of 7 herbicides to the three cyanobacteria *A. flos-aquae*, *M. flos-aquae* and *M. aeruginosa*, was shown in Table 3. The 96 h EC_{50} values of ACCase inhibitor diclofop-p varied around 9-311mg/L. Synthetic auxins triclopyr varied around 32-109 mg/L. The 96 h EC_{50} values of inhibition of photosynthesis at PSII such as ametryne, simazine, prometryne, cyanazine and simetryn varied around 0.0002-1.2425 mg/L. Comparing the acute toxicity of 7 herbicides with various primary modes of action to cyanobacteria, the acute toxicity of photosynthesis-inhibiting herbicides was the highest among the tested herbicides with the order from high to low as follows: photosynthesis-inhibiting herbicides > ACCase inhibitor > protox inhibiting herbicides. Similar results also have been obtained when green algae were used as tested organism (Ma, 2005).

Wide variations occurred in response to the tested herbicides among three individual species of cyanobacteria (Table 4). Compared with *M. aeruginosa*, *M. flos-aquae* was moresensitive to 6 herbicides— cyanazine, diclofop, prometryn, simazine, ametryn, and was less sensitive to one —triclopyr. Sensitivity of various species of cyanobacteria exposed to cyanazine, diclofop, simetryn varied over one order of magnitude. However, *A. flos-aquae* was less sensitive to the tested 7 herbicides while compared with *M. aeruginosa.* The sensitivity of various species of cyanobacteria exposed to simetryn varied over one order of magnitude. In contrast with *A. flos-aquae*, *M. flos-aquae* was more sensitive to the tested 7 herbicides. Whereas the sensitivity of various species of cyanobacteria exposed to cyanazine, diclofop, prometryn, simazine and simetryn varied by over one order of magnitude. The decreasing order of average sensitivity of 3 dissimilar cyanobacteria to the selected herbicides was: *M. flos-aquae*> *M. aeruginosa*> *A. flos-aquae*.

Chlorophyll-a content or dry weight was usually used as an indicator of green algal biomass. However, the assessment method for chlorophyll-

Table 2. Relationship between dried weight or Chla-a and optical density of three cyanobacteria

Cyanobacteria	Regression equation*	Correlation coefficient	Significance level
A. flos-aquae	$DW= 0.0268+0.4050 \times OD_{680nm}$	0.995	<0.001
	$ChlA=-0.3676+5.0436 \times OD_{680nm}$	0.972	<0.001
M. flos-aquae	$DW= 0.0542+0.3920 \times OD_{680nm}$	0.993	<0.001
	$ChlA-0.2229+4.6502 \times OD_{680nm}$	0.993	<0.001
M.aeruginosa	$DW= -0.0111+0.2881 \times OD_{680nm}$	0.996	<0.001
	$ChlA=0.1023+2.9115 \times OD_{680nm}$	0.975	<0.001

***DW, ChlA, and OD_{680nm} stand for three cyanobacterial dried weight (g/L), Chlorophyll-a (mg/L) and optical density at 680 nm respectively**

Table 3. The effects of various herbicides on *A. flos-aquae* (1), *M. flos-aquae* (2) and *M. aeruginosa* (3)

Herbicides	Regression equation[a]	Coefficient correlation	Significance level	EC_{50} (mg/L)
Cyanazine	(1) Y=5.1371+0.2908X	0.9618	0.0089	0.1185
	(2) Y=3.9698+0.1773X	0.9190	0.0030	0.0032
	(3) Y=3.7866+0.1922X	0.9680	0.0003	0.0376
Triclopyr	(1) Y=3.7848+0.3611X	0.9476	0.0143	108.9693
	(2) Y=3.1399+0.2646X	0.9604	0.0023	46.4056
	(3) Y=1.2030+0.0681X	0.9680	0.0020	32.8666
Diclofop	(1) Y=2.2118+0.2120X	0.9967	0.0033	310.8884
	(2) Y=2.1031+0.1388X	0.9830	0.0010	9.6388
	(3) Y=2.1202+0.1978X	0.9930	0.0030	277.1083
Prometryn	(1) Y=3.4502+0.1877X	0.9710	0.0020	0.1493
	(2) Y=6.6370+0.3334X	0.9715	0.0058	0.0102
	(3) Y=4.0072+0.1960X	0.9875	0.0000	0.0169
Simazine	(1) Y=3.4637+0.2179X	0.9707	0.0060	1.2425
	(2) Y=4.738+0.2576X	0.9873	0.0017	0.0718
	(3) Y=3.5491+0.1903X	0.9735	0.0010	0.1100
Ametryn	(1) Y=3.4874+0.1726X	0.9724	0.0055	0.0304
	(2) Y=4.8878+0.2369X	0.8320	0.0110	0.0090
	(3) Y=4.5796+0.2288X	0.9337	0.0065	0.0180
Simetryn	(1) Y=4.2283+0.1979X	0.9762	0.0008	0.0066
	(2) Y=5.4442+0.2217X	0.9620	0.0002	0.0002
	(3) Y=6.1839+0.2846X	0.9634	0.0084	0.0021

[a] **Y and X denote percent inhibition and natural logarithm of concentration respectively**

Table 4. Differential sensitivity of three cyanobacteria to tested herbicides

Herbicide	MA/MF[a]	AF/MF	AF/MA	AF/CP
Cyanazine	11.75	37.03	3.15	0.51
Triclopyr	0.71	2.35	3.32	Δ[b]
Diclofop	28.75	32.25	1.12	450.56
Prometryn	1.66	14.64	8.83	12.44
Simazine	1.53	17.31	11.30	15.15
Ametryn	2.00	3.378	1.69	101.33
Simetryn	10.50	33.00	3.14	Δ[b]

[a] **MA, MF, AF and CP stand for *M. flos-aquae*, *M. aeruginosa*, *A. flos-aquae* and *C. pyrenoidosa* respectively.** [b] **Δ denotes no data**

a content or dry weight was too complicated to assess quickly. With respect to green algae, a great many works have been published revealing the correlation between biomass and absorbance (Cetin & Mert, 2006) while few concerning cyanobacteria. In the present work, we think that cyanobacterial suspension absorbance could take the place of the biomass. Therefore, the absorbance data instead of Chl-a or dried weight could be used indirectly to express the biomass of cyanobacterial suspension. It could be a quick, simple and accurate method to assess toxicity of tested contamination to cyanobacteria.

CONCLUSION

Compared with our previous works, wide variation occurred in response to the tested 5 herbicides among individual species of green algae and cyanobacteria. Contrasting with the green alga *C. pyrenoidosa*, Cyanobacteria *A. flos-aquae* was less sensitive to diclofop, prometryn, simazine and ametryn. The sensitivity of various species of cyanobacteria and green alga *C. pyrenoidosa* that were exposed to prometryn or simazine varied by over one order of magnitude and that exposed to diclofop or ametryn varied by over two orders of magnitude (see Table 4). It may also be important for sustaining cyanobcterial bloom during special period in the aquatic ecosystem. Cyanobacteria can produce toxins including hepatotoxins e.g. microcystins and endotoxins e.g. lipopolysaccharides. Therefore, the research on comparing the differential sensitivity of cyanobacteria and green algae is of important scientific significance and realistic value.

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