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Acute Toxicity Assessment of 20 Herbicides to the Green Alga *Scenedesmus quadricauda* (Turp.) Breb.

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In the present industrialized world, people use large amounts of herbicides. These herbicides may enter aquatic ecosystems and produce undesirable side effects on biological and functional properties (Kersting and Van den Brink 1997). Adverse effects of herbicides on nontarget plants are particularly of concern because of the annual, increasing worldwide use these chemicals (Van den Brink and Ter Braak 1999). During the past 20 years, annual herbicide application in the United States and Canada has increased by three to five-fold. Algae are an essential component in the aquatic ecosystem, as they produce oxygen and organic substances which are vital for most other life forms and they provide food for other aquatic organisms, including fish and invertebrates (Saenz et al. 1997). Chemical effects on algae can directly affect structure and function of an ecosystem, leading to oxygen depletion, decreased primary productivity, increased surface runoff and soil erosion (Kersting and Van den Brink 1997).

Herbicides can affect the structure and function of aquatic communities by changing the species composition of an algal community (Ma et al. 2003). Contamination of surface waters with herbicides has been reported to have direct toxic effects on populations of phytoplankton (Ma et al. 2002b). Furthermore, when these primary producers are affected, indirect effects on ecosystem functioning and animal populations can also be expected (Tadros et al. 1994). There have been fewer attempts to evaluate the relative vulnerability of algae to toxic stress compared with that of other aquatic organisms. Tests on single species of algae are of limited applicability in assessing the effects of environmental contaminants on algal communities, which are composed of an array of species with different sensitivities. Even at concentrations that are sublethal, toxicants can change the structure of algal communities (Boyle 1984; Abou-Waly et al. 1991; Saenz et al. 1997; Jay 1996; Tadros et al. 1994). A few reports have been published on the comparative toxicity of solvents toward various test organisms (Berard 1996; Abou-Waly et al. 1991; Jay 1996; Tadros et al. 1994), but comparatively few reports involved differential responses of various green algal species to pesticides (Saenz et al. 1997; Kasai et al. 1993; Ma et al. 2002b).

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MATERIALS AND METHODS

In the present study, 20 herbicides were tested to examine their effect on the green alga *Scenedesmus quadricauda* and compare their differential sensitivity with three other green algae *Scenedesmus obliqnus*, *Chlorella vulgaris* and *Chlorella pyrenoidosa*. Within the scope of the present study, 20 herbicides from 8 different chemical classes with 5 different modes of action--acetyl-CoA carboxylase, acetolactate synthase, microtubule process and mitotic process inhibitor, were examined. Herbicides were purchased from Zhejiang Chem-tech Group CO., Ltd. People's Republic of China. Their chemical classes and influenced mechanisms are shown in Table 1 (Retzinger 1997). Herbicides were dissolved in $\leqslant$ 0.05% acetone or in distilled water. The US Environmental Protection Agency recommends maximum allowable limits of 0.05% solvent for acute tests and 0.01% for chronic tests (Jay 1996). This level has no significant effect on the toxicity data (Ma and Liang 2001).

The Chinese National Environmental Protection Agency recommends the green alga *S. quadricauda* as an ecological indicator for acute tests because of its high sensitivity to chemicals (Chinese NEPA 1990). The toxicity tests were carried out with the freshwater unicellular green alga *S. quadricauda* obtained from the Institute of Wuhan Hydrobiology, the Chinese Academic of Science. The alga was kept on agar slants at approximately 4℃.

The medium for the algal growth inhibition test of *S. quadricauda* was prepared according to The Chinese National Environmental Protection Agency Guidelines 201 (The Chinese NEPA 1990), using HB-4 medium (Ma et al. 2001), composed of distilled water and the following chemical ingredients (mg/L): $(NH_4)_2SO_4$ (200), $Ca(H_2PO_4)_2{\cdot}H_2O$+$(CaSO_4{\cdot}H_2O)$(30), $MgSO_4{\cdot}7H_2O$ (80), $NaHCO_3$ (100), KCl(23), $FeCl_3$(1.5) and a A_5 liquid (0.5ml, chemical ingredients of A_5 liquid is H_3BO_3 2.86, $MnCl_2{\cdot}4H_2O$ 1.81, $ZnSO_4{\cdot}7H_2O$ 0.222, $Na_2MoO_4{\cdot}2H_2O$ 0.391, $CuSO_4{\cdot}5H_2O$ 0.079 g/L). The culture medium was sterilized at 121℃, 1.05 kg cm^{-2} for 30 min (Kong et al. 1999).

Cells of *S. quadricauda* were propagated photoautotrophically in a 250 mL Erlenmeyer flask containing 100 mL liquid and kept on a rotator shaker (100 rpm) at 25℃, and illuminated with cool-white fluorescent lights at a continuous light intensity of 5000 lux. For cell experiments, 15 mL aliquots of the HB-4 medium containing single algal cells (initial cell concentration 2.5×10^5/L) were distributed to sterile 50 mL Erlenmeyer flasks. The media of *S. quadricauda* were then treated with various herbicide concentrations ranging from zero to 300 mg/L, and incubated for 96 hr on an orbital shaker (100 rpm) at a temperature of 25℃

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

No.	Herbicides	Formulations	Chemical class	Influenced mechanisms
1	Diclofop-P	99.5%TC[a]	Aryloxyphenox propionates	Acetyl-CoA carboxylase (ACCase)
2	Quizalofop-p	5% EC[b]		
3	Haloxyfop-R	10.8%EC		
4	Fenoxaprop	6.9% EC		
5	Fluazifop-p	53%EC		
6	Clethodim	12%EC	Cyclohexene oxime	
7	Nicosulfuron	4%SC[c]	Sulfonylureas	Acetolactate synthase (ALS)
8	Metsulfron-methyl	90%TC		
9	Cyclosulfamuron	10%WP[d]		
10	Tribenuron	95%TC		
11	Bensulfuron-methyl	88.5%TC		
12	Chlorimuron-ethyl	92.6%TC		
13	Pyrazonsulfuron -ethyl	95%TC		
14	Ethametsulfuron	25% WP		
15	Flumetsulam	80%SL[e]	Triazolopyrimidines	
16	Bispyribac-sodium	10% SC	Dimethoxypyrimidinylsalicylic acids	
17	Anilofos	30%EC	Organophosphorus	Unknown
18	Trifluralin	48%EC	Dinitrophenols	Microtubule process
19	Pendimethalin	33%EC		
20	Cinmethylin	10%EC	Benzylether	Mitotic process

[a]TC (technical product); [b]EC (emulsible concentrate); [c]SC (suspension concentrate); [d]WP (wettable powder); [e]SL (soluble concentrate)

with a continuous light intensity of 5000 lux. A wide range of concentrations was examined in a previous test in order to find the adequate range of toxicity for each herbicide. Then, similar concentrations were tested according to results of the previous test (Moreno-Garrido et al. 2000). Cell counts were correlated with absorbance over time for 96 hr on a Shimadzu UV-2401PC spectrophotometer. The most suitable wavelength to use for monitoring culture growth was 680 nm. Growth of algal cells was calculated indirectly using spectrophotometric data.

Each herbicide concentration was tested in triplicate. Appropriate controls containing no pesticide were included in each experiment. Control and treated cultures were grown under the same temperature, photoperiod and shaking conditions as the stock cultures. In each experiment, percent inhibition values,

relative to growth in control systems, were calculated using spectrophotometric data (Ma et al. 2001).

For the growth inhibition tests with the green alga, the EC_{50} values (pesticide concentration required to cause a 50% reduction in growth) were calculated using linear regression analysis of the transformed pesticide concentration as natural logarithm data versus percent inhibition (Ma 2002). All correlation coefficients were >0.95.

RESULTS AND DISCUSSION

When cells of *S. quadricauda* were counted under a microscope, the count of algal cells is proportional to the absorbance at 680nm (linear regression equation is $C=0.9912+47.0461 \times A$, coefficient of correlation r=0.9912, P=0.0001). Our previous works have confirmed this for *S. obliqnus*, *C. vulgaris* and *C. pyrenoidosa* (Ma et al. 2001; Ma and Liang 2001; Ma et al. 2002a;b). Thus, growth of algal cells was calculated indirectly using spectrophotometric data in this work.

The acute toxicity of 20 herbicides to the green alga *S. quadricauda* is shown in Table 2. The 96 h EC_{50} values of herbicides which block the *de novo* sysnthesis of fatty acids by inhibiting the activity of acetyl-CoA carboxylase (ACCase) varied around 0.9-77mg/L (10^{-4}-10^{-6} M), haloxyfop-R and clethodim were about 10^{-4} M, diclofop-p, quizalofop-p and fluazifop-p were about 10^{-5} M, and fenoxaprop was about 10^{-6} M. The decreasing order of the average acute toxicity to green alga *S. quadricauda* of these 6 herbicides as: fenoxaprop > quizalofop-p > diclofop-p > fluazifop-p > haloxyfop-R > clethodim.

The 96 h EC_{50} values of acetolactate synthase (ALS) inhibitors which block the biosynthesis of the branched-chain amino acids leucine, isoleucine, and valine varied around 0.1-20mg/L (10^{-5}-10^{-7} M). Metsulfron-methyl, tribenuron, ethamet -sulfuron, bensulfuron-methyl and flumetsulam were 10^{-5} M, pyrazonsulfuron -ethyl and bispyribac-sodium were 10^{-6}M, while chlormuron-ethyl and cyclosulfamuron were 10^{-7} M. The decreasing order of the average acute toxicity to green alga *S. quadricauda* of those 10 herbicides as: cyclosulfamuron > chlormuron-ethyl > pyrazonsulfuron-ethyl > bispyribac-sodium > nicosulfuron > metsulfron-methyl > ethametsulfuron > tribenuron > bensulfuron-methyl > flumetsulam. The acute toxicity for these herbicides was higher thanthat of ACCase inhibiting-herbicides. The same results have been obtained using *C. pyrenoidosa* as a tested organism (Ma et al. 2001), but different results were obtained using *S. obliqnus* and *C. vulgaris* as the test organism (Ma and Liang

Table 2. Dose response relationship of 20 herbicides to *S. quadricauda*.

No.	Regression equation [a]	Significance level	Coefficient correlation	EC_{50} (mg/L)	EC_{50} (mol/L)
1	P=-102.7716+36.2897lnC	0.02	0.98	16.9	4.9×10^{-5}
2	P=-5.4058+40.7273lnC	0.02	0.98	3.8	1.1×10^{-5}
3	P=4.6767+10.9456lnC	0.05	0.95	62.8	1.4×10^{-4}
4	P=52.6243+53.7496lnC	0.01	0.97	0.9	2.8×10^{-6}
5	P=-69.7961+41.2065lnC	0.03	0.97	18.3	5.5×10^{-5}
6	P=-127.1689+40.7882lnC	0.00	0.99	76.9	2.1×10^{-4}
7	P=8.7192+30.9725lnC	0.02	0.98	3.7	9.2×10^{-6}
8	P=0.5084+28.4755lnC	0.01	0.97	5.6	1.4×10^{-5}
9	P=89.9660+17.3660lnC	0.01	0.99	0.1	2.3×10^{-7}
10	P=-18.0323+29.4448lnC	0.04	0.96	10.0	2.6×10^{-5}
11	P=-49.2300+34.5540lnC	0.05	0.95	17.6	4.6×10^{-5}
12	P=100.8771+29.2520lnC	0.00	0.98	0.1	4.2×10^{-7}
13	P=75.1955+50.7604lnC	0.02	0.98	0.6	1.4×10^{-6}
14	P=-10.4402+28.0840lnC	0.00	0.98	8.6	2.1×10^{-5}
15	P=-48.7000+33.3862lnC	0.03	0.97	19.2	4.6×10^{-5}
16	P=18.2681+27.9205lnC	0.01	0.95	3.1	7.2×10^{-6}
17	P=-39.6564+37.3261lnC	0.02	0.98	11.0	3.0×10^{-5}
18	P=-133.0276+42.1466lnC	0.00	0.99	76.9	2.2×10^{-4}
19	P=-158.7488+44.2146lnC	0.05	0.95	112.3	3.9×10^{-4}
20	P=31.2260+24.7789lnC	0.04	0.96	2.1	7.7×10^{-6}

[a] P and C stand for percent inhibition and herbicide concentration separately.

2001; Ma et al. 2001). This demonstrates that various algal species vary widely in their response, not only to single chemical, but to the group chemicals which have the same chemical classes or influenced mechanism.

The 96h EC_{50} values of pendimethalin and trifluralin were 76-113 mg/L (10^{-4} M level). Their toxicity was lower than that of ALS and ACCase inhibiting -herbicides, but different results have been obtained using *S. obliqnus*, *C. pyrenoidosa* and *C. vulgaris* as the test organism (Ma and Liang 2001; Ma et al. 2001). The acute toxicity of anilofos was 11 mg/L (10^{-5} M).

Cinmethylin had on EC_{50} of 2.1 mg/L (10^{-6} M level), an acute toxicity was higher than ALS inhibiting-herbicides. The same results have also been obtained using *S. obliqnus*, *C. vulgaris* and *C. pyrenoidosa* as a test organism (Ma and Liang 2001).

Among four green algal species examined, wide variations occurred in response to the tested herbicides (Table 3). Compared to *S. obliqnus*, *S. quadricauda* was

Table 3. Differential sensitivity of four green algae to 20 herbicides.

No.	[a]Ratio of *SQ / SO*	[b]Orders	[a]Ratio of *SQ /CY*	[b]Orders	[a]Ratio of *SQ /CV*	[b]Orders
1	0.1	-	24.6	++	1.6	+
2	1.0	+	0.6	-	0.7	-
3	2.4	+	11.7	++	5.7	+
4	0.6	-	0.9	-	0.6	-
5	0.6	-	1.1	+	0.8	-
6	1.3	+	0.85	-	1.9	+
7	0.8	-	1.7	+	0.8	-
8	7.8×10^{-2}	--	0.4	-	6.1×10^{-2}	--
9	3.6×10^{-2}	--	0.7	-	0.3	-
10	0.2	-	0.3	-	0.2	-
11	1.3	+	0.9	-	0.9	-
12	1.5×10^{-2}	--	1.1×10^{-2}	--	9×10^{-3}	---
13	5.1×10^{-2}	--	4.1×10^{-2}	--	3.2×10^{-2}	--
14	5.5×10^{-2}	--	4.7	+	0.1	-
15	7.1×10^{-2}	--	0.2	-	1.7	+
16	0.5	-	1.1	+	0.5	-
17	1.1	+	1.5	+	1.1	+
18	14.0	++	42.4	++	17.6	++
19	229.2	+++	285.0	+++	399.6	+++
20	1.1	+	4740	++++	0.7	-

[a] SQ, SO, CY and CV stand for *Scenedesmus quadricauda*, *S. obliquus*, *C. pyrenoidosa* and *C. vulgaris*; their EC_{50} refer to Ma et al. (2001; 2002; 2003).
[b] order denotes EC_{50} ratio, +, ++ and +++ stand for 2-10×, 10-99× and 100-999× in separately; -,-- and --- stand for 0.1-0.5×, 0.01-0.1× and 0.001-0.01× separately.

more sensitive to 8 herbicides— diclofop-p, chlorimuron-ethyl, pyrazonsulfuron -rthyl, flumetsulam, metsulfron-methyl, clethodim, tribenuron and ethamet -sulfuron. Only 3 herbicides-- haloxyfop-R, trifluralin and pendimethalin had less sensitive than *S. quadricauda* and the other 9 herbicides were close to *S. quadricauda.* Compared to *C. pyrenoidosa, S. quadricauda* was less sensitive to 7 herbicides—diclofop-P, haloxyfop-R, ethametsulfuron, anilofos, trifluralin, pendimethalin and cinmethylin, only 4 herbicides— tribenuron, chlorimuron-ethyl, pyrazonsulfuron-ethyl and flumetsulam were more sensitive than *C. pyrenoidosa and* the other 9 herbicides were close to *C. pyrenoidosa.* Compared to *C. vulgaris, S. quadricauda* was less sensitive to 6 herbicides— metsulfron-methyl, cyclosulfamuron, bensulfuron-methyl, tribenuron, chlorimuron-ethyl and pyrazonsulfuron-ethyl, more sensitive to 5 herbicides—

diclofop-P, haloxyfop-R, flumetsulam, trifluralin and pendimethalin, and the other 9 herbicides had similar sensitivity. *Scenedesmus* proved to be the more tolerant genera. While, algal species vary widely in their response to toxic chemicals, the results demonstrate that there was a differential response to various herbicides among the four species of green alga. The sensitivity of various species of alga exposed to diclofop-P, haloxyfop-R, metsulfron-methyl, cyclosulfamuron, ethametsulfuron, pyrazonsulfuron-ethyl, flumetsulam and trifluralin varied by over one orders of magnitude, chlorimuron-ethyl and pendimethalin varied by two orders and cinmethylin varied by three orders of magnitude. However, quizalofop-p, fenoxaprop, fluazifop-p, clethodim, bensulfuron-methyl nicosulfuron, and bispyribac-sodium showed no large-scale variations.

Investigations in which different algal species are used as test organisms have shown that algae vary greatly in their response to herbicides. Differential sensitivity of the algal species to the compounds could induce species shifts within communities (Boyle 1984; Tadros et al. 1994), thereby may be altering the structure and function of aquatic ecosystems.

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