Bull. Environ. Contam. Toxicol. (1991) 46:223–229

Environmental Contamination and Toxicology

Growth Response of Freshwater Algae, *Anabaena flos-aquae* and *Selenastrum capricornutum* to Atrazine and Hexazinone Herbicides

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Atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-s-triazine) is a commonly used herbicide in the USA (DeNoyelles et al. 1982) and is detected in the aquatic habitat due to run-off from agricultural fields (Hallberg et al. 1984). As a result, its aquatic toxicology has been extensively studied (Rohwer and Flückiger 1979; Virmani et al. 1975). Hexazinone (3-cyclohexyl-6-(dimethyl amino)-1-methyl -1,3-5-triazine 2,4(1H,3H)-dione), another triazine herbicide, is used in forestry and in industrial and right-of-way weed control.

Triazine herbicides are toxic to algae by disrupting photosynthesis (Stratton 1987). S-triazine herbicides are often the most algicidal of any of the herbicide groups when tested on freshwater algae (Shehata et al. 1984). Green algae are very susceptible to atrazine (EC_{50} ranges from 0.06 to 0.16 mg/L; Hollister and Walsh 1973; Walsh 1972), while blue-green algae are more tolerant to atrazine (Rohwer and Flückiger 1979; Pillay and Tchan 1972; Kallio and Wilkinson 1977). Although fewer data are available regarding the effects of other triazine herbicides to freshwater algae, they appear to be comparable in toxicity to atrazine (Wright 1978; Goldsborough and Robinson 1983).

The present study compared the toxicity of atrazine and hexazinone to Anabaena flos-aquae (Lyng) and Selenastrum capricornutum (Printz) (freshwater algae) as measured by chlorophyll (a) [Chl(a)] content or dry weight. This study also considered the effect of time for evaluating the toxic effect of herbicides on freshwater algae. The rate of recovery after A. flos-aquae exposure to atrazine was studied.

MATERIALS AND METHODS

A. flos-aquae (blue-green alga) and S. capricornutum (green alga) were obtained from the Algal Culture Collection, University of Texas, Austin, and grown in an aqueous medium (American Public Health Association, 1985, pp. 701–704, 729–730). Aliquots of the cultures were transferred weekly to maintain algal growth in the log-phase.

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Erlenmeyer™ 1-L bioassay flasks were filled with 500 mL algal suspension media, covered with aluminum foil with two small holes for gas exchange and were incubated at 24 ± 2°C with an alternate 16/8 hr light and dark periods using white fluorescent light at 1700 Lux for A. flos-aquae and 2800 Lux for S. capricornutum. Flasks were shaken daily to prevent clumping of the cells. Hexazinone and atrazine (98% and 99.9% purity, respectively) were obtained from Chem Service, Inc. (West Chester, Pennsylvania 19381). Both herbicides were dissolved in methanol 10,000 mg/L. Fortifications were made from each herbicide stock solution to obtain the tested concentrations (Tables 1 and 2). The range of concentrations was determined with preliminary range-finding bioassays. Samples were withdrawn after each herbicide treatment at zero time (control only), 1, 3, 5 and 7 d. Each concentration was replicated three times. For each sample, growth was determined using three methods: (1) dry weight (DW) and (2) Chl(a) content by spectrophotometer and Chl(a) by fluorometer. The pH of each algal culture medium was recorded on sampling days.

For dry weight, each algal sample was filtered with a Whatman GF/C filter and dried at 105°C overnight according to Skowronski et al. (1988). For Chl(a) measurement, a known volume of algal sample was filtered through a 0.45 μ membrane filter. Chl(a) pigment was extracted with hot methanol (Sartory and Grobbelaar 1984). Chl(a) was spectrophotometrically (CAS) measured at three wavelengths. Chl(a) concentration was calculated as: Chl(a) = 11.85 (OD 664) - 1.54 (OD 647) - 0.08 (OD 630), where: Chl(a) = concentrations of Chl(a) mg/L and OD = corrected optical density (with 1 cm light path) at the respective wavelength. The Chl(a) fluorometric (CAF) method was applied by using a standard curve of Chl(a) (99% pure) (Sigma Chemical Co., St. Louis, Missouri). Maximum sensitivity for Chl(a) extract measurements were obtained at an excitation wavelength of 430 nm and an emission wavelength of 663 nm.

The tested herbicide concentrations which reduced algal CAF contents by 50% (EC_{50}) compared with the control were determined using the Probit procedure (SAS Institute 1985). The statistically significant effect of time and herbicide concentrations on growth rates were determined using repeated measurements analysis of variance (GLM Procedure, SAS Institute 1985).

The ability of A. flos-aquae to recover after 7 d of exposure to atrazine was studied. Ten mL from each solution were pipetted into herbicide-free culture media. Algal growth as Chl(a) content was measured spectrophotometrically after 3, 5, 7 and 10 d of growth.

The growth rate was calculated as:

$$\text{Growth rate} = (\ln \text{Chl(a) at } T_2 - \ln \text{Chl(a) at } T_1)/(T_2 - T_1)$$

where T = time in days.

Table 1. Growth rates (slopes) of S. capricornutum treated with atrazine and hexazinone measured as chlorophyll (a) content fluorometrically

Treatment concentration (mg/L)	Growth rate over time (days) 0-1	0-3	0-5	0-7
		Atrazine		
Control	0.940*	0.920	0.634	0.556
0.07	0.530	0.886	0.669	0.549
0.12	0.422	0.807	0.590	0.459
0.19	0.079	0.748	0.532	0.449
0.31	- 0.326	0.607	0.397	0.399
0.50	- 0.742	0.432	0.287	0.228
r^2**	0.965	0.984	0.973	0.953
Intercept	0.808	0.934	0.696	0.568
Slope	- 3.314	- 1.012	- 0.853	- 0.647
		Hexazinone		
Control	1.139	0.924	0.632	0.505
0.035	0.759	0.802	0.603	0.504
0.050	0.673	0.670	0.587	0.503
0.060	0.248	0.617	0.577	0.501
0.076	- 0.110	0.594	0.529	0.499
0.100	- 0.723	0.506	0.416	0.473
r^2**	0.985	0.798	0.966	0.779
Intercept	1.060	0.835	0.640	0.510
Slope	- 3.676	- 0.736	- 0.416	- 0.060

*Slopes are mean of three replicates
**r^2 = correlation coefficient of growth rate vs. treatment rate.

RESULTS AND DISCUSSION

Determining the growth of S. capricornutum using DW, CAS or CAF indicated correlation between DW and both CAS and CAF (r^2 = 0.874 and 0.694, respectively). In the case of A. flos-aquae, values were r^2 = 0.753 and 0.680, respectively. The r^2 values for correlation between CAS and CAF values were 0.752 and 0.874 using S. capricornutum and A. flos-aquae, respectively. CAF was found to be more sensitive for measuring algal growth than CAS or DW (Amer. Publ. Hlth. Assoc. 1985). These results indicated that dry weight or Chl(a) content measured as CAS or CAF can be used as a growth indicator of S. capricornutum or A. flos-aquae.

The growth rates measured as CAF of S. capricornutum and A. flos-aquae to the presence of different concentrations of the two herbicides are presented in Tables 1 and 2, respectively. The expected concentrations of the herbicides to reduce Chl(a) content (CAF) by 50% (EC_{50}) compared to the untreated control are presented in Table 3. The EC_{50} value was calculated for each herbicide at 3, 5 and 7 d after treatment. S. capricornutum was more susceptible to hexazinone than atrazine, while A. flos-aquae was more susceptible to atrazine. The EC_{50} values generally increased with time (Table 2), while the slopes for the relation

Table 2. Growth rates (slopes) of Anabaena flos-aquae treated with atrazine and hexazinone measured as chlorophyll (a) content fluorometricaly

Treatment concentration (mg/L)	Growth rate over time (days) 0–1	0–3	0–5	0–7
		Atrazine		
Control	0.429*	0.716	0.600	0.443
0.50	– 0.209	0.365	0.478	0.383
0.65	– 0.323	0.207	0.427	0.378
0.85	– 0.406	0.061	0.336	0.193
1.10	– 0.504	– 0.013	0.285	0.283
1.40	– 0.696	– 0.142	0.275	0.204
r^2 **	0.731	0.931	0.914	0.908
Intercept	0.272	0.665	0.589	0.460
Slope	– 0.740	– 0.622	– 0.252	– 0.172
		Hexazinone		
Control	0.462	0.591	0.444	0.316
0.70	0.057	0.559	0.442	0.297
0.90	0.033	0.518	0.429	0.294
1.20	– 0.014	0.475	0.422	0.277
1.50	– 0.068	0.421	0.393	0.263
2.00	– 0.209	0.398	0.355	0.260
r^2 **	0.865	0.886	0.677	0.490
Intercept	0.374	0.608	0.459	0.309
Slope	– 0.441	– 0.153	– 0.060	– 0.033

*Slopes are mean of three replications
**r^2 = correlation coeficient of growth rate vs. treatment rate.

Table 3. Toxic effect of triazine herbicides on freshwater algae expressed as EC_{50} over time (mean of 3 replicates)

Herbicide	Algal species	Calculated EC_{50} (mg/L) after 3 d	5 d	7 d
Atrazine	S. capricornutum	0.283 a	0.218 a	0.214 b
	A. flos-aquae	0.058 a	0.469 a	0.766 a
Hexazinone	S. capricornutum	0.056 b	0.085 b	0.126 b
	A. flos-aquae	2.014 a	2.375 a	2.752 a

EC_{50}: Concentration effecting 50% reduction of chlorophyll (a) content compared with the control (fluorometrically). Values in the same column having the same letter are not significantly different ($P < 0.05$)

between growth rate and herbicide concentration declined over time (Tables 1 and 2).

Reduction in growth was observed with an increase of atrazine concentration. Hexazinone-treated cultures of S. capricornutum

Table 4. Growth rates (slopes) of Anabaena flos-aquae recultured in atrazine free media after 7 d of exposure measured as chlorophyll (a) content spectrophotometrically

Previous treatment Concentration (mg/L)	Time (days) 0–3	0–5	0–7	0–10
Control	0.921*	0.808	0.627	0.410
0.50	1.088	0.952	0.787	0.577
0.65	0.713	0.888	0.824	0.612
0.85	0.900	1.021	0.887	0.644
1.10	0.959	1.065	0.833	0.658
1.40	0.813	1.137	0.984	0.727
r^2 **	0.026	0.794	0.859	0.929
Intercept	0.947	0.801	0.653	0.445
Slope	- 0.062	0.236	0.227	0.213

*Slopes are mean of three replications
**r^2 = correlation coefficient of growth rate vs. previous treatment rate

had substantially recovered by 7 d after treatment. The 1st day showed a sharp decrease in the growth rate of Chl(a) content with an increase in herbicide concentration as compared to the growth rate of the control culture. Hersh and Crumpton (1987) suggested that growth rates and effects caused by toxins must be determined when nutrient and toxin concentrations are less affected by cell number. This is best accomplished early in the experiment, as soon as log phase growth is apparent in controls. Walsh (1983) noted that growth rate depressions must be determined early in assays, usually by the 2nd day, before toxicant fate affected toxicity. On the 3rd day, our algal cells displayed a higher growth rate. This indicates that the herbicide tested may have had an algistatic effect, and the algal cells recovered their normal vitality after 3 d of exposure. Lower concentrations slightly affected the growth rate during the experiment. Reduction of the growth rate by low concentrations of atrazine was reported by Stratton (1984) and Mayasich et al. (1986). In both studies, the growth rates of different isolates were dissimilarly affected. Mayasich et al. (1986) found that while the growth rate of Phaeodactylum tricornutum was relatively unaffected by 50 µg/L atrazine, the same concentration inhibited the growth rate of Nannochloris oculate by about 35%.

The pattern of growth rate differences of both tested organisms over time was fairly consistent (Tables 1 and 2). Using repeated measurement analysis of variance for the growth rates over time and concentrations revealed statistical significant importance for both factors.

Because of the negative values of the growth rate, especially at higher concentrations after one day exposure (Tables 1 and 2), and the ability of the algae to recover with time, the effect of herbicides on algae may be more accurate if determined at least 3 d after exposure.

Nyholm (1985) concluded that practical experience as well as theoretical considerations reveal appreciable differences between EC values obtained from algal toxicity tests depended on whether biomass or growth rate was taken as the end point. The growth rate of the recultivated A. flos-aquae, after exposure to atrazine, exceeded the control over the first 5 d (Table 4). After 7 and 10 d, the treated algae had a statistically significant higher rate of growth than the control. A strong relationship ($r^2 = 0.93$) existed between the previous concentration and the growth rate after 10 d (Table 4). The inoculum used showed no effect on high correlations between previous concentrations and time when applying the repeated measurement analysis of variance ($P \leq 0.05$). This phenomena indicated induced higher activity in the exposed algae. This may be explained through selectivity towards more vigorous, surviving cells or an alteration of the vigor of the surviving algal cells. The untreated culture reached its carrying capacity (K-value) on the 7th day and the amount of Chl(a) decreased on the 10th day. The treatment cultures continued to grow up to the 10th day. The treatment of 0.65 mg/L reached 1.2 times the Chl(a) content by the 10th day compared to the control at its K-value (7th day) with the same amount of nutrient. Goldsborough and Robinson (1983) showed that recovery of communities of freshwater marsh periphyton subjected to triazine herbicide treatment began after 1 wk, with a growth rate equal to or greater than the control. These recovery data suggest a reason for the increase in algae in herbicide-treated aquatic ecosystems and perhaps also in aquatic ecosystems subjected to toxicants.

There appear to be difficulties with the techniques used to measure the response of algae to toxicants.

1. Neither cell counts nor any other technique can determine if individual algal cells are actually dead.
2. The length of time an assay should be conducted dependent on log or stationary phase growth has not been precisely determined.
3. The application of these techniques to an ecosystem response needs to be determined.
4. Are algal species are being selected for resistance to toxicants in longer test periods?

Acknowledgment. Florida Agricultural Experiment Station Journal Series No. R-00715.

REFERENCES

American Public Health Association (1985) Standard methods for the examination of water and wastewater, 16th ed. Amer Publ Hlth Assoc, Washington, DC

DeNoyelles F, Kettle WD, Sinn DE (1982) The responses of plankton communities in experimental ponds to atrazine, the most heavily used pesticide in the United States. Ecology 63:1285-1293

Goldsborough LG, Robinson GGC (1983) The effect of two triazine herbicides on the productivity of freshwater marsh periphyton. Aquat Toxicol 4:95-112

Hallberg GR, Libra RD, Bettis EA, Hoyer BE (1984) Hydrogeologic

and water quality investigations in the Big Spring basin, Clayton County, Iowa. Iowa Geol Surv, Open-File Rep 84-4
Hersh CM, Crumpton WG (1987) Determination of growth rate depression of some green algae by atrazine. Bull Environ Contam Toxicol 39:1041-1048
Hollister TA, Walsh GE (1973) Differential responses of marine phytoplankton to herbicides: oxygen evaluation. Bull Environ Contam Toxicol 9:291-295
Kallio S, Wilkinson RE (1977) The effects of some herbicides on nitrogenase activity and carbon fixation in two subarctic lichens. Bot Gaz 138(4):468-473
Mayasich JM, Karlander EP, Terlizzi DE (1986) Growth responses of Nannochloris oculata Droop and Phaeodactylum tricornutum Bohlin to the herbicide atrazine as influenced by light intensity and temperature. Aquat Toxicol 8:175-184.
Nyholm N (1985) Response variable in algal growth inhibition tests--biomass or growth rate? Water Res 19(3):273-279
Pillay AR, Tchan YT (1972) Study of soil algae VII. Adsorption of herbicides in soil and prediction of their rate of application by algal methods. Plant Soil 36:571-594
Rohwer F, Flückiger W (1979) Effect of atrazine on growth, nitrogen fixation and photosynthetic rate of Anabaena cylindrica. Angew Bot 59:53
Sartory DP, Grobbelaar JU (1984) Extraction of chlorophyll a from freshwater phytoplankton for spectrophotometric analysis. Hydrobiologia 114:177-187
SAS Institute (1985) SAS user's guide: Statistics. SAS Institute Inc., Cary, North Carolina
Shehata Salwa A, Aly OA, Hoda Farag (1984) Effect of bladex, etazine, dicryl and baylucide on the growth of Scenedesmus. Environ Intl 10:225-234
Skowronski T, Pawlick Barbara, Jakubowski M (1988) Reduction of cadmium toxicity to green microalga Stichococcus bacillaris by manganese. Bull Environ Contam Toxicol 41:915-920
Stockner JG, Antia NJ (1976) Phytoplankton adaptation to environmental stresses from toxicants, nutrients and pollutants--a warning. J Fish Res Board Can 33:2089-2096
Stratton GW (1984) Effects of the herbicide atrazine and its degradation products, alone and in combination, on phototrophic microorganisms. Arch Environ Contam Toxicol 13:35-42
Stratton GW (1987) The effects of pesticides and heavy metals towards phototrophic organisms. In: Hodgson E (ed) Reviews in Environ Toxicol 3:71-147.
Virmani M, Evans JO, Lynn RI (1975) Preliminary studies of the effects of s-triazine, carbamate, urea and karbutilate herbicides on growth of freshwater algae. Chemosphere 2:65-71
Walsh GE (1972) Effects of herbicides on photosynthesis and growth of marine unicellular algae. Hyacinth Control J 10:45-48
Walsh GE (1983) Cell death and inhibition of population growth of marine unicellular algae by pesticides. Aquat Toxicol 3:209-214
Wright SJL (1978) Interactions of pesticides with microalgae. In: Hill IR, Wright SJL (eds) Pesticide microbiology, Academic Press, New York, 535 pp

Received June 1, 1990; accepted August 9, 1990.