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Determination of Growth Rate Depression of Some Green Algae by Atrazine

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A common contaminant of surface waters of agricultural regions is the triazine herbicide, atrazine (2-chloro-4-ethylamino-6-isoproplyamino-s-triazine) (Hallberg et al. 1984). Atrazine effectively inhibits growth and photosynthesis of most plants, including freshwater algae. Both depression of growth rate and reduced yield have been used as parameters in studies of the effects of atrazine on algal growth (Walsh 1983; Stratton 1984; Mayasich et al. 1986).

Considerable variation exists among algal toxicity methods despite attempts at standardization. Experimental endpoints range from percent inhibitions to EC50s. Payne and Hall (1979) proposed that the algalstatic concentration (the concentration of toxin which initially causes no growth, yet upon reculture with toxin-free medium, growth does occur) be used to report the results of toxicity tests with some chemicals, because the algalstatic concentration is easily determined and environmentally meaningful.

Algae from two different Iowa springs were the subjects of a study of naturally occurring atrazine tolerance (Hersh 1986). We report here the results of two aspects of that study: development of a quick method of assessing toxin effects on algal growth, and investigation of a ecologically meaningful endpoint for toxin-growth experiments.

MATERIALS AND METHODS

Samples of water and surficial sediments were taken from Big Spring, Clayton County, Iowa (42°54'38"N, 91°28'17"W), and Osage Spring, Mitchell County, Iowa (43°15'09"N, 92°53'48"W), on 21 August 1985. Standard

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techniques and procedures were used to establish axenic, clonal cultures of algae from the samples (Hoshaw and Rosowski 1973). Identifications were done using cells taken from actively growing cultures preserved with 1% gluteraldehyde. Cultures were maintained on 2% agar slants. In addition to the isolates from field samples, two strains of Chlamydomonas reinhardii were used as references in the study. One strain (2137 mt+) is a "wild type" laboratory strain (Spreitzer and Mets 1981), and is susceptible to atrazine. The other strain (Ar 204) is an atrazine resistant alga and a mutant of 2137 mt+ (Galloway and Mets 1984). Originally, "WC" medium was used for isolation and maintenance (Guillard 1975). In order to increase the medium's buffering capacity, the sodium bicarbonate ($NaHCO_3$) concentration was increased from 12.6 mg/L to 98.6 mg/L. To keep the Na^+ concentration near that of "WC" medium, calcium nitrate ($Ca(NO_3)_2 \cdot 4H_2O$) was used as the nitrogen source. The final Ca^{2+} concentration in the modified medium was similar to that found in Iowa waters (Jones 1972). The two strains of Chlamydomonas reinhardii were cultured in the high [$NaHCO_3$] medium except that ammonium chloride (NH_4Cl) was used as the nitrogen source.

Each isolate was grown in the modified medium with atrazine additions to assess the effect of atrazine on growth rate. Spectrophotometrically matched 13x100-mm glass culture tubes were autoclaved, and 3.2 mL culture medium was aseptically dispensed into each. Stocks of 108, 1080, and 10800 ug/L atrazine were prepared from medium saturated with atrazine (33 mg/L), and were, along with a control stock, filter sterilized. The stocks were added to the tubes in order to give treatment concentrations of 0, 21.6, 216, and 2160 ug/L atrazine. For each isolate and treatment, triplicate tubes were inoculated with 50 uL of cell suspension from an actively growing culture.

Each tube was covered with a sterile Teflon membrane designed for use on a Clark type oxygen electrode (Yellow Springs Instruments, Yellow Springs, Ohio), and the membrane was secured with a rubber O-ring. This allowed gas exchange while preventing contamination or leakage of medium. These procedures were conducted in a sterile hood. A piece of cellophane tape around the tube secured the O-ring without obstructing the membrane.

A holder that accommodated the 13x100-mm tubes was constructed of clear acrylic (Figure 1). A length of narrow sewing elastic riveted to the long sides of the rotating box held each tube in place. In order to

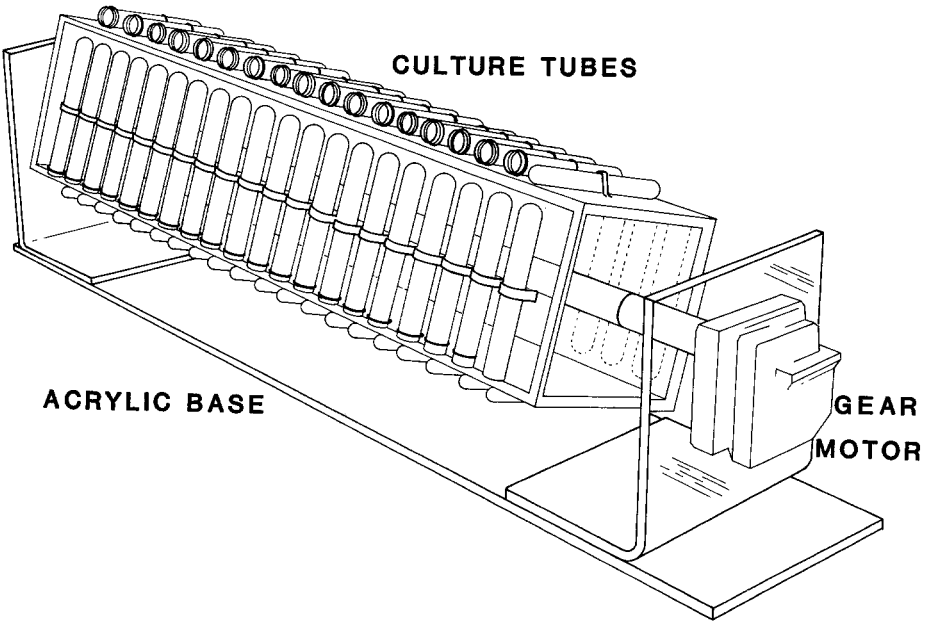

Figure 1. Rotating cell culture tube holder used in assays of growth rate.

minimize wall growth, the holder was continually rotated by a low rpm DC motor.

The tubes were placed onto the holder, and the apparatus placed into a growth chamber held at 20±1°C. The two end tubes on each side were filled with 4.0 mL distilled water so that each tube containing cells had at least one tube on either side of it. The holder was turned at 25 rpm. Continuous light was provided by 12 40W cool white fluorescent bulbs at an intensity of 193 uE/m^2/sec. Daily, the rotator was removed and optical densities determined for each tube at 678 nm (Sorokin 1973) using a Perkin-Elmer Lambda 3a spectrophotometer fitted with a 13x100-mm tube holder.

Growth rates were determined by the logistic growth equation $N_t=N_0e^{rt}$, where:

N_t = the number of cells at time t

N_0 = the number of cells at time 0

e = the base of the natural logarithms

r = the growth rate between times t and 0

t = the difference in time between times t and 0.

Optical densities were used as estimates of cell numbers (Sorokin 1973). Within an isolate, the growth rate (r) was calculated between the first two successive days when logarithmic growth was apparent in control tubes. With the mean growth rate of the three control tubes serving as the basis for comparison, the percentage of control growth rate for each treatment tube was calculated. Averages and standard deviations were then determined.

RESULTS AND DISCUSSION

Eight clonal cultures (order Chlorococcales), were isolated from the samples. The Big Spring samples yielded four isolates (BSC), as did the Osage Spring samples (OSA).

Wall growth was apparent in four of the eight field isolates, so reliable data were generated for four isolates, plus the two laboratory strains. Based on a comparison with the appropriate control growth rate, the BSC and OSA isolates along with Chlamydomonas reinhardii 2137 mt+ exhibited low tolerance to atrazine (Table 1), although the response was not the same for each isolate. In contrast, Ar 204, the C. reinhardii atrazine resistant mutant, showed little inhibition by even 2160 ug/L atrazine.

The results are similar to those found by other investigators. Inhibition of growth rate by low concentrations of atrazine was reported by Stratton (1984) and Mayasich et al. (1986), and in both studies, the growth rates of different isolates were dissimilarly affected. Stratton (1984) reported growth rate EC50s from 100 to 5000 ug/L for five different algae. Mayasich et al. (1986) found that while the growth rate of Phaeodactylum tricornutum was relatively unaffected by 50 ug/L atrazine, the same concentration inhibited the growth rate of Nannochloris oculata by about 35%. The differences observed by any one investigator for different algae is most likely a function of the natural variation in tolerance among genotypes, and it is likely that this is the case with the results of our growth assays. Differential responses also occurred when photosynthetic response of these isolates to atrazine was tested (Hersh 1986). No correlation between photosynthesis sensitivity and growth sensitivity seems to exist, however. Stratton (1984) also demonstrated that those isolates that were the most sensitive when photosynthesis was tested were not necessarily the most sensitive when growth rate or final yield was tested.

Table 1. Percentage of control growth rate for the algal cultures grown with atrazine.

Species/Designation	Day	Atrazine (ug/L) 21.6	216[a]
Chlorella sp./BSC-2	1-2	106 ± 13	14 ± 23
Chlorococcales/BSC-4	1-2	22 ± 37	0 ± 0
Chlorella sp./OSA-2	2-3	45 ± 21	0 ± 0
Chlorococcales/OSA-4	3-4	48 ± 15	0 ± 0
Chlamydomonas reinhardii/ 2137 mt+	1-2	108 ± 7	0 ± 0
Chlamydomonas reinhardii/ Ar 204	1-2	87 ± 10	87 ± 26[b]

[a]Unless otherwise noted, no isolate showed any growth at the highest concentration (2160 ug/L).

[b]Growth rate of cultures grown with 2160 ug/L atrazine was 73 ± 7% of the control growth rate.

The inhibitions of growth rate for the two *Chlamydomonas reinhardii* laboratory strains were not unexpected. Galloway and Mets (1984) found that 2137 mt+ was sensitive to atrazine, reporting that the lowest concentration that completely inhibited growth on agar plates was 1.08 mg/L, while the analogous concentration for Ar 204 was 21.6 mg/L.

The method, besides being sufficiently sensitive, was neither difficult nor unduly time consuming. The Teflon membrane and O-ring arrangement allowed gas exchange, but effectively prevented leakage of medium. After the initial set-up, an experiment's only requirement was the daily determination of cell density (approximately 1.5 h were needed to determine the optical densities of seventy-two tubes). The fact that wall growth took place for some isolates was expected, as periphyton probably dominated the original samples. The flagellated *Chlamydomonas reinhardii*, and some of

the field isolates (particularly Chlorella) grew readily in the tubes.

Growth rate proved to be relatively simple to calculate. Once log phase growth was apparent in control tubes, any lag shown by treatment tubes was an affect caused by the atrazine, even if the tubes later reached optical densities (cell numbers) similar to control tubes. To detect such an effect, accurate estimates of control growth rates were needed, so optical densities had to be determined at least every 24 h. Growth rate of control tubes rose rapidly but declined as quickly. At the lowest concentration of atrazine tested, log phase growth was often delayed by approximately 1 day (compared to controls). By this time, however, the growth rate of control tubes was relatively stable, and the resultant division of treatment growth rate by control growth rate could lead to a conclusion of no effect or even stimulation (Table 2). Growth rates (and effects caused by toxins) must be determined early in the experiment, as soon as log phase growth is apparent in controls, when nutrient and toxin concentrations are less affected by cell number. Walsh (1983) also noted that growth rate depressions must be determined early in assays, usually by the second day, before toxicant fate affected toxicity. In this case, experiments could be terminated as soon as control tube growth rates began to level off or decrease, a maximum of 4 days.

The manner in which the assays were conducted (three tubes per atrazine concentration, three concentrations plus a control) and the fact that none of the field

Table 2. Comparison of mean growth rates (r) for two successive days for two algal isolates.

Isolate	Atrazine (ug/L)	r 1-2	% of control	r 2-3	% of control
BSC-2	0	2.24	---	0.08	---
	21.6	2.37	106	0.27	338
	216.0	0.31	14	0.75	938
BSC-4	0	2.12	---	1.37	---
	21.6	0.46	22	1.68	123

isolates grew at the highest atrazine concentration, did not allow for calculation of EC50s by probit analysis. This could have been remedied by adjusting the assay set-up or using a different statistical model. Direct comparisons of the treatment growth rates to the appropriate control growth rate, however, showed that most of the field isolates were susceptible to low concentrations of atrazine (21.6 ug/L), and that the isolates responded differentially.

The differential sensitivity to toxins can have serious implications regarding algal community structure and seasonal successional patterns. Differential sensitivity to toxins means that low concentrations will affect certain genotypes more than others. If this occurs on a large enough scale, the result will be noticeable changes in community structure and seasonal succession. In regard to atrazine, such changes have been noted in artificial streams and experimental ponds (deNoyelles et al. 1982; Kosinski 1984). In both studies, the atrazine doses were one-time additions, and changes were noted at concentrations lower than the lowest concentration used here (21.6 ug/L). A predominance of atrazine tolerant genotypes isolated from a spring during a period of high atrazine loading was attributed to the atrazine contamination of the spring (Hersh 1986).

Because of possible effects on algal community structure and seasonal succession, a depression of growth rate at any concentration of a toxin should be considered environmentally meaningful. Effects may be detected at concentrations that are low and environmentally realistic. For example, concentrations higher than the lowest concentration used in these assays (21.6 ug/L) have been reported for surface waters in North America (Hallberg et al. 1984). Determinations of toxicity that are based on algalstatic or algalcidal concentrations may lead to erroneous conclusions if the effects caused by lower, more realistic concentrations are ignored. An assessment that includes the effects caused by such concentrations will give more realistic information concerning the implications of trace contamination and the potential hazard of new chemicals.

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