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TOXIC EFFECTS OF ATRAZINE, DEETHYL-ATRAZINE, DEISOPROPYL-ATRAZINE AND METOLACHLOR ON *CHLORELLA FUSCA VAR-FUSCA*

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ABSTRACT

The toxic effects of the herbicide Atrazine, its degradation products deethyl-atrazine and deisopropyl-atrazine, and the herbicide metolachlor were examined in unialgal cultures of *Chlorella fusca var-fusca*. The toxicity of a mixture of atrazine and metolachlor was also evaluated using the same bioassay system. Cell numbers were determined daily and growth rates were calculated for a period of 4 days. The order of toxicity of chemicals was atrazine>metolachlor>deethyl-atrazine>deisopropyl-atrazine. The presence of a mixture of atrazine and metolachlor in toxic concentrations lower than the EC50 resulted in reduced toxicity (antagonism) in comparison with the toxicity caused by the sum of toxic actions of the same levels of concentration from single chemicals.

KEY WORDS: Herbicides, mixtures, toxicity, algae, interaction.

Over the last decades, the extensive agricultural and non-agricultural use of pesticides has elicited extensive research on their effects on non-target organisms. Much of the research has been focused on phytoplankton (microalgae), because these organisms form the basis of food chains in the aquatic environment (Parish, 1985). The majority of the studies only report the effects of the parent compounds, although pesticide degradation products are themselves xenobiotic and could also be inhibitory (Stoermann and Jastorff, 1993). Most bioassays investigate the effects of single compounds under standard test conditions, despite the fact that the toxicity of a substance is also influenced by many biotic and abiotic factors, as well as the presence of other chemicals (Marking, 1985). The toxicity of mixtures of chemicals, could be additive, more than additive (synergistic), or less than additive (antagonistic) compared to the toxicity of a single substance (Marking, 1985).

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) and metolachlor (2-chloro-N-(2-ethyl-methylphenyl)-N-(2-methoxy-1-methyl-ethyl) acetamide) are two herbicides commonly used in agriculture (Meister Publishing Company, 1987) and in many monitoring studies appear to coexist in

the surface waters (Albanis, 1995; Gaynor et al., 1998; Squillace and Thurman, 1992). The main degradation products of atrazine, deethyl-atrazine (2-chloro-4-amino-6-isopropylamino-1,3,5-triazine) and deisopropyl-atrazine (2-chloro-4-ethylamino-6-amino-1,3,5-triazine) are also frequently detected (Thurman et al., 1992).

The risk that the presence of atrazine imposes to the photosynthetic microorganisms of the surface waters has been extensively studied (Abou-Waly et al., 1991; Caux et al., 1996; Francois and Robinson, 1990; Mayasich et al., 1986; Peterson et al., 1994; Stratton, 1984). In comparative studies, atrazine appears to be more toxic than metolachlor towards different species of algae and cyanobacteria (Kotrikla et al., 1997; Peterson et al., 1994). The toxic effects of the degradation products of atrazine are much less studied, although in a number of studies the parent compound appears to be significantly more toxic than the main degradation products (Kotrikla et al.,1997; Stratton, 1984).

Concerning the toxicity of mixtures of the above chemicals, Stratton (1984) reports a variety of responses (additive, synergistic and antagonistic) of atrazine and its main degradation products on phototropic microorganisms. Faust et al (1993, 1994) tested some binary combinations of herbicides, insecticides and fungicides and the majority of the results were in agreement with the additivity concept, including a mixture of simazine and metazachlor, two compounds with similar structure and mode of action with atrazine and metolachlor respectively (Corbet, 1974).

The objectives of this study were to determine the toxicity of the herbicide atrazine, its degradation products, deethyl-atrazine and deisopropyl-atrazine and the herbicide metolachlor towards the green alga Chlorella *fusca var-fusca* using single species algal bioassays. In addition, the toxic effect of a mixture of atrazine and metolachlor is examined using the same bioassay system.

MATERIALS AND METHODS

A non-axenic unialgal culture of *Chlorella fusca var-fusca* was obtained from the Algal Culture Collection, University of Texas, Austin (UTEX number 343). The tested organism was selected according to its ecological relevance and ease of culture in the laboratory (Lewis, 1995). Cultures were grown in Bold Basal medium, pH 6.6, according to Wayne (1973). The bioassays were performed according to methods of OECD (1981) for testing chemicals soluble in water, with some modifications: In 200 ml cotton-gauge plugged bioassay flasks, an appropriate volume of Bold Basal medium was added and sterilized by autoclaving. The flasks were aseptically inoculated with 100,000 cells ml^{-1} of exponentially growing cells. Stock solutions of the four chemicals were prepared in Bold Basal medium, in concentrations less than one third of the respective water solubility and sterilised by filtration through a 0.2 μm Anodisc filter (Whatman).

The exact concentration of the filtered stock solutions was confirmed by measurements in HPLC, consisted of a Varian 9012 pump, equipped with a Polychrom 9065 diode-array detector and a Rheodyne 7161, 100 μl, loop injector. A Nucleosil 100 C18, 5 μm, 150mmX4.6mm column (Rigas Labs) was used and the mobile phase was HPLC grade acetonitrile (BDH) and water, prepared with a MilliQ/MilliRO system (Millipore).

The stock solutions of chemicals were prepared in Bold Basal medium in order to avoid the use of any organic solvent, which could interfere with the growth of alga (Stratton and Corke, 1981). The dissolution was achieved in an ultrasonic bath. The nominal concentration of the stock solution was less than one third of the water solubility of each chemical. The exact concentration was determined in HPLC as there was a possibility of partial dissolution of the chemicals in Bold Basal medium.

Appropriate volumes of the stock solutions were added to 200 ml bioassay flasks in order to obtain the desired concentrations of chemicals. The final volume of the culture was 100 ml. The cultures were incubated in a temperature-controlled chamber, at 20°C, with continuous illumination of 3,000 Lux. Flasks were shaken daily to prevent clumping of cells**.** The herbicides and degradation products were obtained from The Promochem Group, Germany and the purity of each chemical substance was more than 95 %.

Each single chemical bioassay consisted of 5 treatments: 4 herbicide levels (in duplicate) and 1 control culture (in triplicate). Each bioassay was repeated twice and lasted 96 h. Cell-number was determined every 24 h, in a Palmer-Maloney chamber (Guillard, 1973).

Growth rate (divisions day^{-1}) was calculated as (Guillard, 1973),

$$k=3{,}322\left(\frac{\log N_2 - \log N_1}{t_2 - t_1}\right)$$

where N_1 and N_2 are the cell numbers at periods of t_1=24 h and t_2=96 h respectively. Percentage reduction in growth rate was calculated as

$$\frac{100(k_{\text{control}} - k_{\text{herbicide treatment}})}{k_{\text{control}}}$$

and plotted against the logarithm of concentration. Whenever the highest applied concentration caused an inhibition in growth rate of at least 50%, EC50 and its confidence intervals were calculated using Probit Analysis (SPSS 7.5).

For the study of the interaction of atrazine and metolachlor, a factorial design was used (Cox, 1958), with two factors (concentration of atrazine and concentration of metolachlor) in three levels (absence, low and high concentration). Each combination of factors was repeated twice except for the control (absence of the two chemicals) which was repeated three times. The whole experiment was repeated twice. Growth rate was calculated for every treatment and the results were analyzed using Analysis of Variance (ANOVA) and Least Significant Difference (LSD) at 0.05 level of significance (Statgraphics 4.0).

RESULTS AND DISCUSSION

The influence of chemicals on the growth of algae is plotted against time in Fig. 1. In Table 1, the 96-h EC50 and its 95% confidence intervals, calculated from Probit analysis, for the two replications of each experiment, are presented. EC50's are considered to be different, if their 95% confidence intervals do not overlap. The order of decreasing toxicity of the four chemicals for *Chlorella fusca var-fusca*, was atrazine>metolachlor>deethyl-atrazine>deisopropyl-atrazine (Table 1). The lower toxicity of metolachlor, compared to atrazine, is expected, because the chloroacetamides, which inhibit protein synthesis in plants, are generally less toxic to algae than triazines, which interfere with photosynthesis (Corbet, 1974).

Both degradation products of atrazine are significantly less toxic to *Chlorella fusca var-fusca*, than the parent compound. The removal of the propyl group from atrazine results in a less toxic derivative than the removal of the ethyl group. These results are in general agreement with those of Stratton (1984), who found that atrazine is significantly more toxic than its degradation products towards algae and cyanobacteria. The same order of toxicity was discovered in bioassays with the above chemicals and *Chlorella fusca var-fusca*, using the solvent methanol to introduce the chemicals in the bioassay system (Kotrikla et al., 1997).

Table 1. 96-h EC50 (μg l^{-1}) and its 95 % confidence interval (μg l^{-1}) for the toxic effect of atrazine, metolachlor, deethyl-atrazine, deisopropyl-atrazine on growth of *Chlorella fusca var-fusca* (replications A and B).

Chemical substance		EC50	95 % C.I.
Atrazine	A	68.2	58.7-80.0
	B	76.9	56.6-120.2
Metolachlor	A	156.5	134.5-191.4
	B	177.8	143.4-269.2
Deethyl-atrazine	A	1,043	754.5-1,506
	B	821	580.2-1,057
Deisopropyl-atrazine	A	3,824	3,348-4,556
	B	4,504	3,474-6,237

Atrazine is a common contaminant of surface waters, as a result of agricultural nonpoint surface and subsurface runoff, and is usually detected in levels less than 0.5 μg l^{-1} (Albanis, 1995; Squillace and Thurman, 1992) and in maximum concentrations of up to 100 μg l^{-1} (Thurman et al., 1992). Metolachlor usually occurs in lower concentrations with maximum values of 40 μg l^{-1} (Squillace and Thurman, 1992; Thurman et al., 1992). Only recently have the metabolites of atrazine been included in large monitoring surface water schedules: Thurman et al (1992), reported median and maximum concentrations of deethyl-atrazine 0.28 and 4.4 μg l^{-1} and of deisopropyl-atrazine 0.09 and 3.2 μg l^{-1}, respectively.

The results of the toxicity experiments suggest that atrazine, metolachlor and the dealkylated products of atrazine would not normally be present in the aquatic environment at levels inhibitory to algae

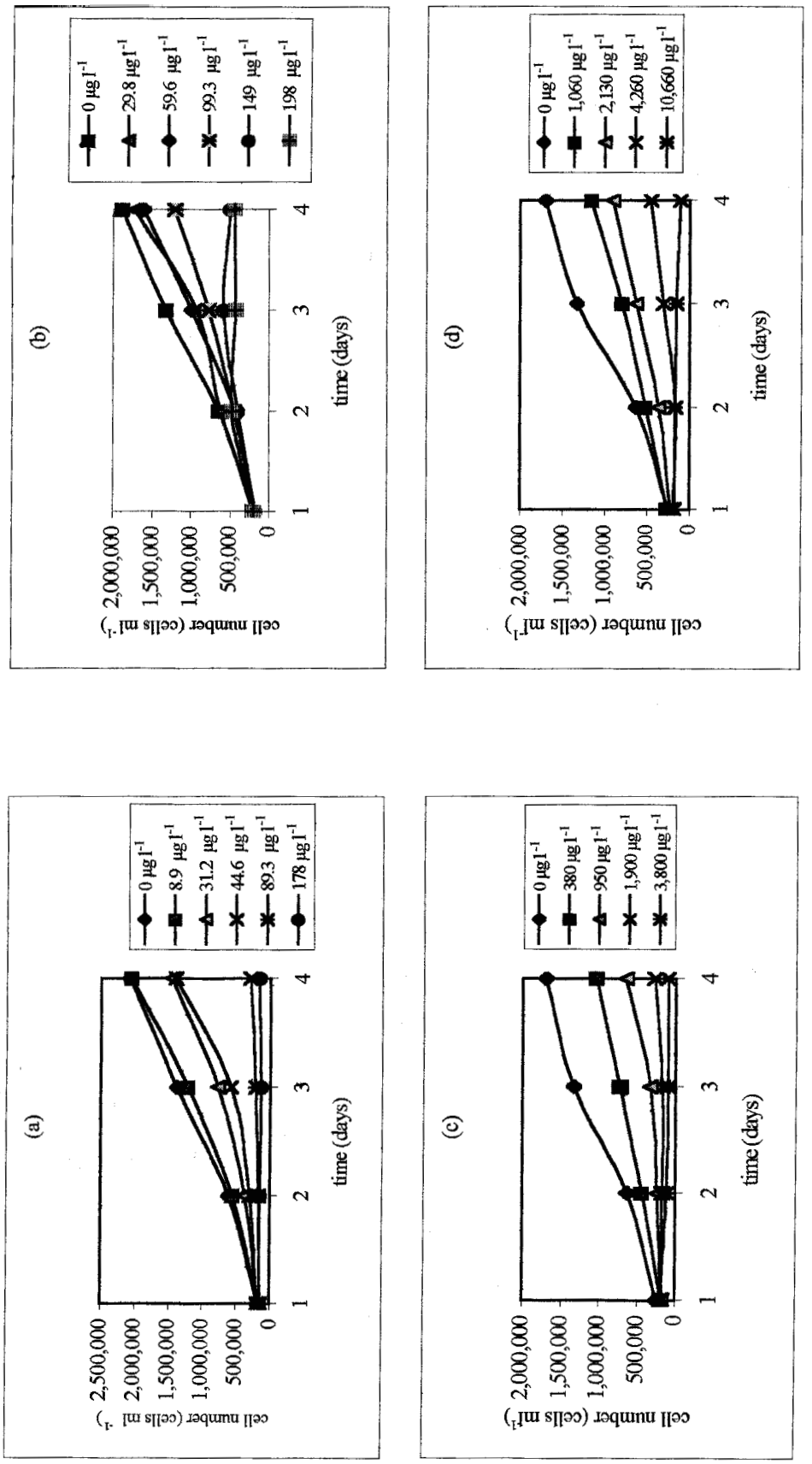

Figure 1. The toxic effect of (a) Atrazine, (b) Metolachlor, (c) Deethyl-atrazine, (d) Deisopropyl-atrazine on growth of *Chlorella fusca var-fusca* (replication A).

Table 2. Influence of a mixture of atrazine and metolachlor on the growth rate of *Chlorella fusca var-fusca* (replication A). Entries are the mean growth rate k (in divisions day^{-1}), standard deviation and the % reduction of growth rate compared to the control.

	Metolachlor (μg l^{-1})		
Atrazine (μg l^{-1})	0	80.2	131
0	0.99^{a} (0.091)	0.78^{b} (0.028)	0.55^{c} (0.007)
		21.2 %	44.4 %
23.8	0.83^{b} (0.071)	0.76^{b} (0.042)	0.73^{b} (0.035)
	16.2 %	23.2 %	26.3 %
47.4	0.56^{c} (0.078)	0.53^{c} (0.049)	0.48^{c} (0.036)
	43.4 %	46.5 %	51.5 %

Means denoted by the same letter belong to the same homogenous group at the 0.05 level (LSD test).

(Table 1). However, the maximum concentration of atrazine reported (100 μg l^{-1}) is higher than that of the EC50 value that was derived from the *Chlorella fusca var-fusca* bioassay. A concentration of 100 μg l^{-1} induced an average 65 % reduction in the growth rate of the alga. The maximum environmental concentrations of metolachlor and the degradation products of atrazine do not cause any significant toxic effect to the algae. The results of single species algal bioassays should be used as indices of what might happen to the complex environmental conditions (La Point, 1989; Lewis, 1995).

In Fig. 2 and Table 2 the results of the experiment of the toxic effect of a mixture of atrazine and metolachlor on *Chlorella fusca var-fusca* are presented. Each chemical was added to the bioassay system in two concentrations, lower than its respective EC50. The highest concentration had a significant toxic effect and the lowest a marginal toxic effect on the alga, as revealed by the previous experiments for the estimation of EC50. In Table 3 the significance level of the ANOVA for the main effects and interactions of atrazine and metolachlor on *Chlorella fusca var-fusca* is presented.

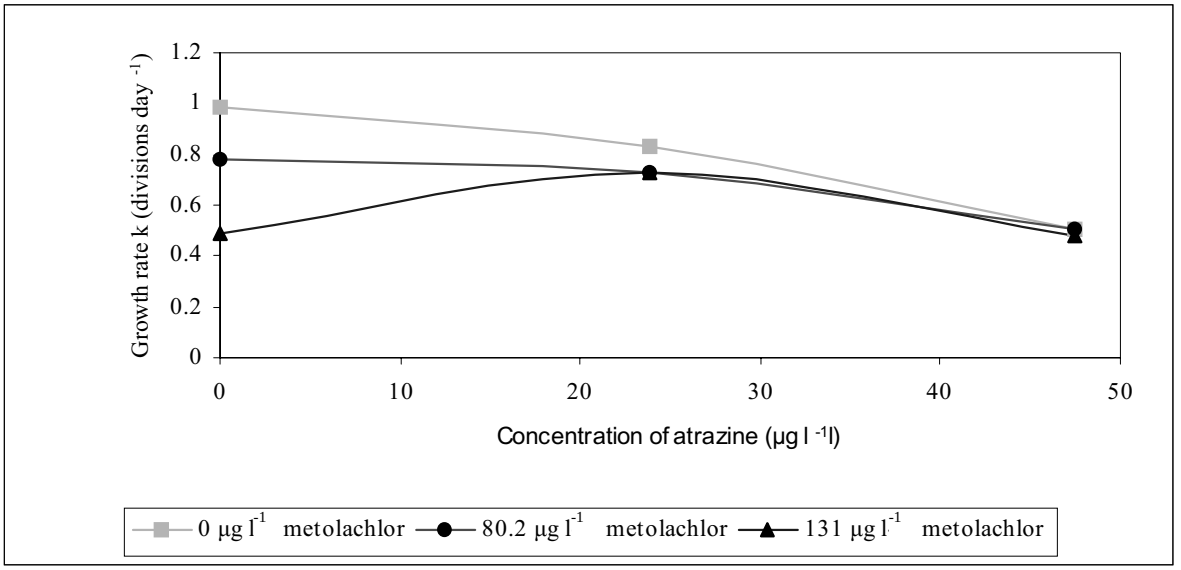

Figure 2. Toxic effect of atrazine on *Chlorella fusca var-fusca* for different concentrations of Metolachlor (replication A).

Table 3. Significance Level for the main effects and interactions of atrazine and metolachlor on *Chlorella fusca var-fusca* (replication A).

Main Effects	Significance Level
Atrazine	0.0000
Metolachlor	0.0002
Interaction	Significance Level
Atrazine, metolachlor	0.0021

Application of each chemical in the bioassay system always resulted in inhibition of the growth rate with the concentration (Table 2). The addition of the mixtures of chemicals also caused inhibition compared to the control. However, the resulting inhibition was not equal to the sum of the toxic action of every single chemical. At the lowest concentration of atrazine there was an average inhibition of the growth rate equal to 100*(0.99-0.83)/0.99=16.2 %, which increased to 23.2 % at the lowest concentration of metolachlor. However, it remained the same at the highest concentration of metolachlor. At the highest concentration of atrazine, the percentage reduction remained unchanged, as concentration of metolachlor increased. The highest concentration of metolachlor caused 44.4 % reduction in the growth rate, but the combination of the highest concentration of metolachlor with the lowest of atrazine resulted only in a 26.3 % reduction of the growth rate. Similarly, although the combination of the highest concentrations of both chemicals should give an additive response of 88 % inhibition, the actual measured response was only 51.5 %.

Interaction is statistically defined as the dependence of the effect of one factor on the level of another factor (Sokal and Rohlf, 1969). From the experimental data (Table 2, Figure 2) and from the statistical tests (Table 3), it is apparent that there was an antagonistic interaction between atrazine and metolachlor, for the concentration range of the test. The presence of one chemical inhibited the toxic action of the other. Similar results were obtained by the replication B of the same experiment.

Reasons for the antagonistic action could be, a decrease in the rate of the uptake of the chemicals, formation of non-toxic metabolites, enhancement of excretion rates, alteration of distribution and reinforcement of detoxification mechanisms (Marking, 1985).

CONCLUSION

Atrazine is one of the most frequently detected herbicides in the surface waters and is also the most toxic to *Chlorella fusca var-fusca.* Its presence could be hazardous for microalgae in heavily polluted agricultural areas. Metolachlor is significantly less toxic than atrazine and is usually detected in lower concentrations. The dealkylated degradation products of atrazine obey to the general concept of toxicology, that is that degradation usually detoxifies the toxic agents.

Antagonism in the toxic action of two chemicals is a very important phenomenon from an environmental point of view, because it reduces the possible damage that a single chemical can induce to an organism.

Additionally, more detailed studies should be performed in order to explain and further evaluate the interaction between the atrazine and metolachlor or other herbicides of the same chemical classes.

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