SEPARATION OF DIRECT AND INDIRECT EFFECTS OF TWO HERBICIDES ON THE ASSIMILATORY PIGMENTS CONTENT IN *CHLORELLA VULGARIS* BEIJ.

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ABSTRACT

The *Chlorella vulgaris* Beij. culture was used for the testing of the effect of different concentrations of isoproturon and metribuzin on growth, chlorophyll a, b and carotenoid pigments content after 7 and 14 days from adding of the herbicide in the medium culture. *Chlorella vulgaris* alga was cultivated on Arnon nutritive medium. The isoproturon was added in the nutritive medium of algal suspension in three concentrations: 0.03, 0.08 and 0.13 µM. For the metribuzin the used concentrations were 0.4, 0.7, and 1.0 µM. The results show that metribuzin herbicide induced a quick and sharp limitation of the algal cell growth in liquid suspension (Growth Inhibitory Dose 50% after 2 days was 0.103 µM, GID 50% was 0.282 µM after 7 days, and GID 50% after 14 days was 0.370 µM metribuzin). The concentration of 0.023 µM isoproturon decreased the growth rate at 50% from control level after 7 days. Concomitantly, significant variations in the assimilatory pigments content from the variants treated with herbicides were recorded. The analysis of the regressions between the cell number and pigment content indicates that the variation of the assimilatory pigments content, after one week of cultivation, may be explained through an adaptation reaction to self-shadowing for isoproturon. At 14 days of exposure to isoproturon, only an indirect effect on content of chlorophyll b was visible. In case of metribuzin, the experimental results suggest a direct effect of the herbicide (both for 7 and 14 days).

Key words: *Chlorella vulgaris* Beij., metribuzin, isoproturon, growth, assimilatory pigments, direct effects of herbicides.

INTRODUCTION

The study of the effects of herbicide applications on algae culture represents an easier way for the understanding herbicide interference with physiological processes and a considerable amount of resulting information may be extrapolated for higher plants (Böger and Sandmann, 1990, 1993).

Chlorella vulgaris, which is a unicellular green alga, represents a useful model for the study of the herbicide impact on plant physiology.

This alga was considered by Warburg as a standard species for the research of the physiological processes. That is why we choose this alga for our experimental study. Most papers which refer to the physiological processes of algae used with prevalence this alga.

An extensive study regarding the toxicity of 40 herbicides on *Chlorella vulgaris* pointed out that the acute toxicities of auxin herbicides to this alga were the lowest among all herbicides tested and that of the photosynthesis-inhibiting herbicides was the highest (Ma et al., 2002).

Herbicides play an important role in agriculture and demand for them is increasing. Herbicides are used extensively today to eliminate unwanted competing species of plants in the cultivated environment.

Very often the specific herbicidal effects are still unknown and only become evident generally as growth inhibition (Pfister et al., 1983).

This article presents the effects of a triazine derivative, metribuzin (4-amino-6-1, 1-dimethylethyl-3-(methylthio)-1,2,4-triazin-5-(4H)-one) (Şarpe, 1987) and of an ureic compound, isoproturon (N-4-isopropylphenyl) N', N'-dimethylurea) (Şarpe, 1987) on growth and assimilatory pigments content in *Chlorella vulgaris* Beij.

There are many studies regarding the effects of herbicides on photosynthesis, assimilatory pigments content and their biosynthesis (Böger and Sandmann, 1993), Hill reaction, transfer of electrons in photosynthesis, structure and ultrastructure of chloroplast (Böger and Sandmann, 1990).

Intense studies regarding the physiological effects of herbicides on unicellular alga started in the years '90s (Conrad and Wilhelm, 1994; Perona et al., 1990).

It is well known that the assimilatory pigments content is the subject to adaptation to self shadowing. Self shadowing increases with number of algal cells per unit of volume.

A lot of herbicides are able to reduce the algal population and one of the indirect effects is the reduction of self shadowing, and this

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brings a lower requirement for algal cells to invest in the pigment content.

The deviations from the known growth pattern of the unicellular algal bioassays are used as an established tool in ecotoxicological effect assessment for chemicals and environmental samples (Altenburger et al., 2008).

Besides the herbicides which have a known direct inhibitory effect on synthesis of assimilatory pigments, one may suppose that herbicides with a different main mechanism of action may have also a direct effect on assimilatory pigments content which can not be explained as an adaptation to self shadowing.

Whenever a linear relationship between the number of cells per volume unit from cultures maintained with various concentrations of herbicide and the assimilatory pigments content was found, and this relationship was valid also for control, a hypothesis of an indirect effect of the herbicide may be advanced. On the contrary, „an outlier" status of the control or highest used concentrations may be interpreted as a possible indication for a direct effect.

Of course, these suggestions about the direct effect of an herbicide require an independent confirmation by other methods.

MATERIAL AND METHODS

Chlorella vulgaris alga was cultivated on Arnon nutritive medium (Boldor et al., 1983).

The experiments were carried out in a chamber with artificial illumination of 8000 lux. In order to reduce mutual shadowing, the algal suspensions, in cylindrical glass recipients of 1000 ml, were bubbled with steady stream of air produced by aquarium air pumps. The culture medium was inoculated with an amount of algal biomass producing a 100000 cells/ml suspension in all experiment variants. The ambient temperature varied between 24-25°C. The results represent average values of three replications for each variant.

The herbicide was introduced in the culture medium before inoculation. The isoproturon was added in the nutritive medium of algal suspension in three concentrations: 0.03, 0.08 and 0.13 μM. For the metribuzin the used concentrations were 0.4, 0.7, and 1.0 μM.

The commercial product Sencor was used as a source of metribuzin and Izoguard as a source of isoproturon. The Sencor herbicide has 70% active substance (Şarpe, 1987) and Izoguard herbicide has 50% active substance (Şarpe, 1987).

The growth of algae was observed at each two days within the culture cycle (established at 16 days) with a „Spekol 10" spectrophotometer at $\lambda = 676$ nm. A linear regression was performed in order to study the relationship between the extinction of the algal culture and the number of cells counted with Thoma haemocytometric mount for 24 samples from different treatments. The R squared was significant at a P value = 0.001. This relationship was used to convert the extinction in number of cells.

The areas (A) encompassed under the graphs for the number of cells/ml (X) from each concentration (c) at different days (t) were calculated with the following formula:

$$A_{(c,\, t_n)} = \sum_{i=0}^{n} (t_{i+1} - t_i) \times \frac{(x_{(c,\, t_i)} + x_{(c,\, t_{i+1}})}{2}$$

for t = 2, 7 and 14 days.

For each replicate, several simply nonlinear regressions were calculated between the reductions (% from control) of these areas (X values) and the doses of herbicide as Y values. The equation: $Y = a + bX^3$ was preferred because the confidence intervals were narrower than those obtained by other types of equations which produced better values for R^2. The equations for each replicate were used to interpolate the LD 0, 25, 50, 75, 100% for these replications in order to calculate the standard deviations. The regressions for all the replicates from a given day were used to calculate a common R^2. As it was expected, the values interpolated with these equations were not significantly different from the LD averages based on each replicate. This calculus integrates the relative influence of each herbicide concentration over the whole duration of experiment.

The required biomass for extraction of the assimilatory pigments content was obtained using a centrifuge with 3000 rotations/minute.

The absorbance of the solutions extracted with 100% acetone were measured at 661.6, 644.8 and 470 nm with a „Cecil 1020" spectrophotometer and the calculation of the content in a, b chlorophylls and carotenoids was performed according to Lichtenthaler (1987); the values were expressed in mg/g dry matter (D.M.).

The formulas used for the calculus of the content in chlorophyllian and carotenoid pigments were:

$Ca = 11.24*A_{661.6} - 2.04*A_{644.8}$

$Cb = 20.13*A_{644.8} - 4.19*A_{661.6}$

$Cx+c = (1000*A_{470}-1.90*Ca-63.14*Cb)/214$

Carotenoid and xanthophylls pigments were measured together.

RESULTS AND DISCUSSION

For each combination alga – herbicide, the counting of cells number per volume unit was followed immediately by measurements of the extinction. For *Chlorella vulgaris* cultures treated with metribuzin, a linear relationship ($R^2 > 0.99$) between the number of cells per ml and extinction was obtained (Figure 1).

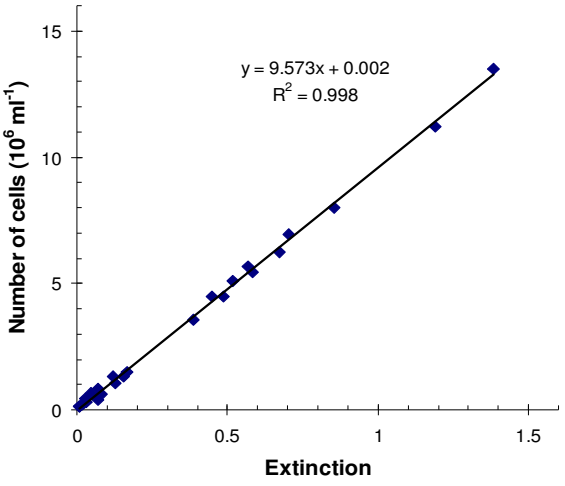

Figure 1. The relationship between extinction and number of cells per volume unit from *Chlorella* cultures treated with metribuzin

For the cultures treated with isoproturon, for the quick estimation of the algal population a quadratic relation based on extinction was preferred (Figure 2).

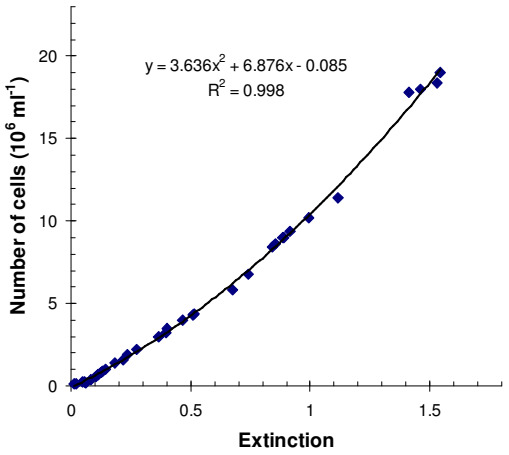

Figure 2. The relationship between extinction and number of cells per volume unit from *Chlorella* cultures treated with isoproturon

In tests with metribuzin, values lower than control were noticed for *Chlorella vulgaris* from the first days of the culture cycle, for all used concentrations (0.4µM, 0.7µM and 1µM). The differences between those variants became evident after a week from the adding of the herbicide in the culture medium, excepting the 1µM metribuzin concentration. For all concentrations the cell number of algae grew till the 11th day. After this moment the growth was obviously affected. The dynamics of number of cells from different metribuzin concentrations are presented in figure 3.

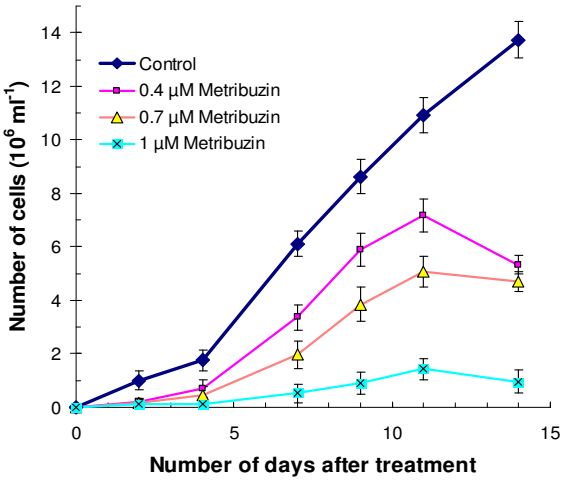

Figure 3. Growth of *Chlorella* population under different metribuzin concentrations

The estimation of the effect of metribuzin concentrations on the growth of the *Chlorella* cells was based on regression equations like $Y = a+b*X^3$, where X represent % from control of the areas under the growth curves, Y - the herbicide concentration, and „a" and „b" are the empirical coefficients of the regression equations. The herbicide concentrations which reduced at half the cells growth of *Chlorella vulgaris* were relatively low: 0.1, 0.28 and 0.37 µM metribuzin at 2, 7 and respectively 14 days. The applied statistic model „forecasts" that it is possible to obtain growth inhibition rates of 0 and 25% in the first two days of the culture cycle through the application of some „negative" doses. This behaviour represents a strong hint for the possibility of a stimulating effect for a short period of time for the 0.4 µM concentration. The results regarding the growth aspects under different metribuzin concentrations were analyzed in a previous paper (Lazăr, 2000). The doses of metribuzin inducing different degrees of growth inhibition are shown in table 1.

The calculated concentration for killing all the algae cells in 2 days (1.3 µM) is near the lower limit of the concentration of 2.4-4.8 µM metribuzin reported in literature to control the unicellular algae in 3-14 days (Frank, 1991).

The growth of cell population under different isoproturon concentrations is shown in figure 4. These results were processed with the method described for metribuzin and the doses of isoproturon inducing different degrees of growth inhibition are shown in table 1.

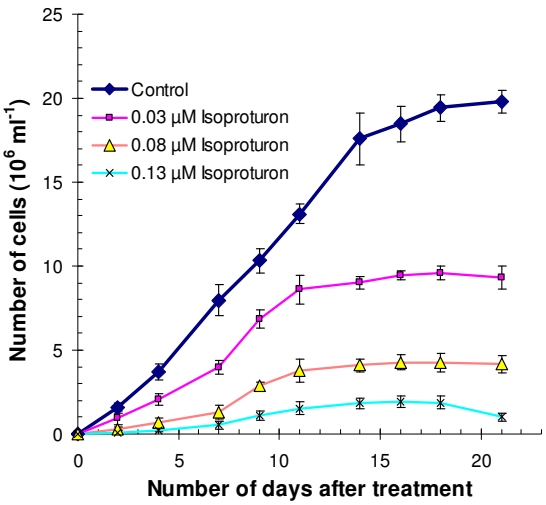

Figure 4. Growth of *Chlorella* population under different isoproturon concentrations

Due to time required for adaptation of the assimilatory pigments content to self shadowing, the relationships with number of cells per volume unit were analyzed separately for 7 and 14 days.

The differences between the content of the *Chlorella* cells in carotenoid pigments after 7 and respective 14 days of growth under different metribuzin concentrations were insignificant (Figure 5). The differences between the content of the *Chlorella* cells in carotenoid pigments after 7 and respective 14 days of growth under different metribuzin concentrations were insignificant (Figure 5).

Table 1. Concentrations (µM) of metribuzin and isoproturon which induce different rates of growth inhibition in *Chlorella vulgaris*, estimated through the $Y = a + b * X^3$ equations

Growth inhibition rate (%)	Metribuzin				Isoproturon			
	7 days		14 days		7 days		14 days	
	averages	SD*	averages	SD*	averages	SD*	averages	SD*
0	0.124	0.045	0.218	0.014	0.002	0.001	0.009	0.004
25	0.144	0.047	0.237	0.016	0.005	0.001	0.011	0.004
50	**0.282**	0.060	**0.370**	0.029	**0.023**	0.003	**0.031**	0.005
75	0.656	0.096	0.733	0.065	0.072	0.007	0.084	0.007
100	1.384	0.166	1.439	0.609	0.168	0.014	0.188	0.012
a	0.124		0.218		0.002		0.009	
b	1.26594E-06		1.2215E-06		1.6549E-07		1.7927E-07	
r^2	0.926		0.880		0.945		0.974	

*) = standard deviation

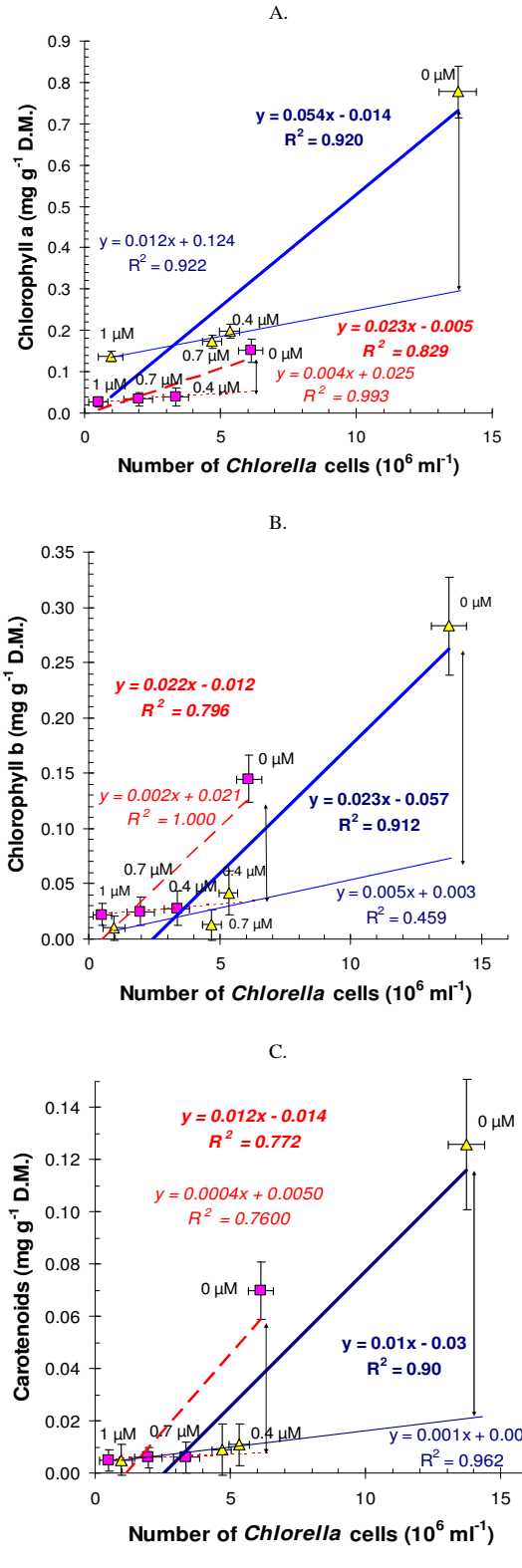

Figure 5. Relationships between the number of cells and assimilatory pigments content after 7 and 14 days of cultivation of *Chlorella* under various concentrations of metribuzin

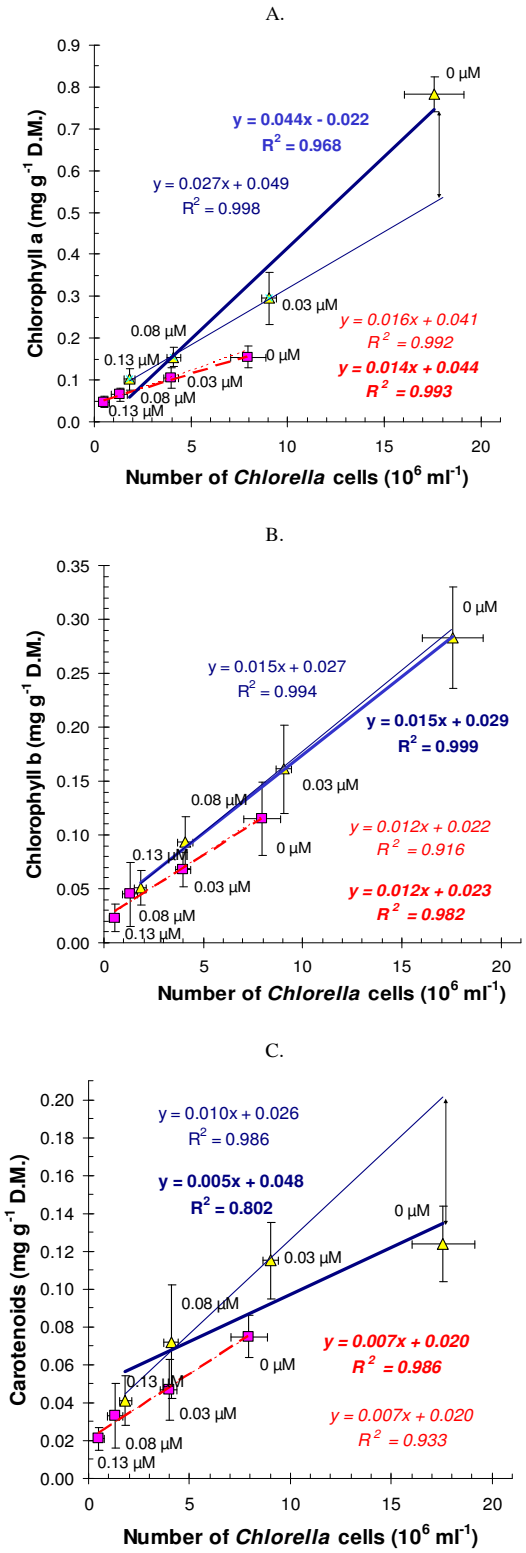

Figure 6. Relationships between the number of cells and assimilatory pigments content after 7 and 14 days of cultivation of *Chlorella* under various concentrations

of isoproturon

The obvious outlier status of the control in the relationships between the *Chlorella* population and the content of chlorophyll a, b or carotenoid pigments is a strong suggestion for a direct effect of metribuzin on the assimilatory pigments content. These findings may be corroborated with the studies reporting the photochemical system II as main action situs of metribuzin, with effects on chlorophyll b and carotenoid pigments (Nikolic et al., 1996). In the studies mentioned above, the metribuzin concentrations affecting the soybeans seedlings were 2.4µM. This is a proof for the very good sensitivity of the algal bioassays. In addition our experiment pointed out an effect on metribuzin on chlorophyll a after 14 days.

A slightly increased content in assimilatory pigments was recorded for the *Chlorella* cells cultivated with isoproturon at the end of the second week as compared with the end of the first week (Figure 6).

The analysis of the regressions between the cells number and the pigments content indicates that the variation of the assimilatory pigments content, after one week of cultivation, may be explained through an adaptation reaction to self-shadowing for isoproturon.

At 14 days of exposure to isoproturon, only an indirect effect on content of chlorophyll b was visible. For the chlorophyll a and carotenoid pigments at the end of the week, a difference was noticed between the controls and the „predicted" values based on the regression equations between the cell populations and pigments content, but, the real differences may be smaller due large variations between some replicates.

An independent confirmation by other methods is required for the content of these two pigments in *Chlorella* after two weeks of exposure.

CONCLUSIONS

The concentration of 0.023 µM isoproturon and 0.282 µM metribuzin decreased the growth rate of *Chlorella* at 50% from control level after 7 days.

Concentrations higher or equal with 0.4 µM of metribuzin induced a strong direct reduction of assimilatory pigments content in *Chlorella vulgaris*. This direct effect was visible both at 7 and 14 days.

Isoproturon, in tested concentrations had only an indirect effect (adaptation to self-shadowing) of on assimilatory pigments content of *Chlorella* after one week of cultivation. This effect was also visible for chlorophyll b after two weeks of cultivation with isoproturon.

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