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## Growth Behavior of *Protosiphon* Alga under the Impact of Thiobencarb Herbicide

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**Abstract:** Thiobencarb herbicide toxicity to growth of *Protosiphon botryoides* (Chlorococcal alga) isolated from an Egyptian paddy was studied using batch culture technique. After sixteen days of incubation in growth medium in presence of 2 ppm thiobencarb, *Protosiphon* cells count, chlorophyll a content and growth rate were significantly reduced. On the other hand, generation time elevated to be 10.8 day<sup>-1</sup> respect to control 6.9 day<sup>-1</sup>. Electron microscope examination of *Protosiphon* cells exposed to sublethal concentration (2 ppm) revealed that spores liberation from mother cell wall was delayed; this phenomenon may be attributed to changes in the cell wall structure of the treated cells or physiological reasons induced by the used herbicide or both factors. In conclusion, ultrastructural result implies that reduction in growth of the treated *Protosiphon* was due to the effect of thiobencarb herbicide high concentration on algal reproduction through retardation on liberation of spores from the mother cell.

**Key words:** *Protosiphon*, herbicide, thiobencarb, spores

### INTRODUCTION

Thiobencarb is a thiocarbamate herbicide widely used for weed control in Egyptian paddy fields. It interferes with protein and fatty acids synthesis and inhibits photosynthesis (Corbett *et al.*, 1984; Tomlin, 2003). Beside the use of thiobencarb by direct application for weed control, it may enter to aquatic ecosystem through off site movement from treated fields, by surface run off or adsorbed to suspended particles.

Because of their short generation times, microalgae respond rapidly to environmental changes and thus, may report impacts on higher organisms, which generally respond on longer time scales (McCormick and Cairns, 1994). Furthermore, microalgal tests are generally sensitive, rapid and low-cost effective (Sosak-Swidarska *et al.*, 1998).

There are many studies about the effect of herbicides on algal growth, biochemical composition, metabolic activities and the ultrastructure morphology (Liu and Xiong, 2009; Vendrell *et al.*, 2009; Gonzalez-Barreiro *et al.*, 2006; Rioboo *et al.*, 2002; Kobbia *et al.*, 2001; Caux *et al.*, 1996; El-sheekh *et al.*, 1994). Xia (2005) concluded that, high thiobencarb concentration (10 mg L<sup>-1</sup>) significantly reduced the biomass yield, protein content and photosynthetic rate in *N. sphaeroides* colonies. Bhunia *et al.* (1991) found that thiobencarb at concentrations ranging from 2 to 6 mg L<sup>-1</sup> led to a significant decrease in growth, DNA, RNA and total protein content of *Nostoc muscorum*. Some researchers had been reported that cellular Chl a content was significantly reduced under thiobencarb treatment in *Protosiphon botryoides* (Eladel *et al.*, 1998) and *Anabaena variabilis* (Battah *et al.*, 2001).

Since, algae are the primary producers of aquatic ecosystem; the damage to algae may affect both function and structure of the whole ecosystem. Hence, it is of great importance

evaluating the impact of thiobencarb to non-target algal organisms. The addition of thiobencarb herbicide to *Protosiphon* growth medium will certainly bring some changes in its growth. The present study aimed at evaluating the response of *Protosiphon botryoides* alga to thiobencarb herbicide.

## MATERIALS AND METHODS

### Organism and Culture Conditions

*Protosiphon botryoides* (Kutezing) Klebs, a chlorococcal alga, was isolated from paddy field soil samples of Benha, Egypt (2004). Isolation and purification was made by dilution and plating technique. Cultures were grown in 250 mL flasks containing 100 mL of Bold's basal medium (Bold and Wynne, 1985) and incubated in a controlled growth chamber at  $24 \pm 2^\circ\text{C}$  and  $70 \mu\text{mol/m}^2/\text{sec}$  photosynthetically active radiation provided by cool white fluorescent lamps set on 16:8-light:dark photoregime for sixteen days. All cultures were shaken twice daily to prevent cells from clumping.

### Treatment with Thiobencarb

Thiobencarb is a thiocarbamate herbicide with moderate water solubility ( $30 \text{ mg L}^{-1}$  at  $20^\circ\text{C}$ ). Concentrated herbicide stock prepared from thiobencarb herbicide (technical grade, 95%) by appropriate dilution in acetone. Aliquots of the stock added to each culture to obtain a final concentration of 0, 0.5, 1, 2, 4 and 8 ppm. The carrier solvent acetone completely evaporated to dryness, the culture medium dispensed, equilibrated and the flasks left for one day to obtain aqueous solutions. All cultures (three per treatment) received identical inocula and incubated under the prescribed growth conditions.

### Growth Measurements

*Protosiphon botryoides* algal cells were counted using haemocytometer (Levy and Levy-Hausser corpuscle counting chamber). Chlorophyll a was determined fluorometrically according to APHA (1992).

Algal growth inhibition (I%) respect to the control through cells count (US Environmental Protection Agency, 1989) was calculated as follows:

$$I\% = \frac{T-C}{C} \times 100$$

Where:

T = Number of cells  $\text{mL}^{-1}$  in treated cultures

C = Number of cells  $\text{mL}^{-1}$  in control cultures

Growth rate ( $\mu$ ) and the generation time (GT) of the cultures in the presence and absence of thiobencarb determined by the following equations (Reynolds, 1984):

$$\mu = \frac{\ln(X_2/X_1)}{T_2 - T_1} (\text{day}^{-1}) \quad GT = \frac{\ln 2}{\mu} (\text{day}^{-1})$$

Where:

$X_2$  = Cells number at end of selected time interval

$X_1$  = Cells number at beginning of selected time interval

$T_2 - T_1$  = Elapsed time per day between selected intervals

### Transmission Electron Microscopy

After sixteen days of treatment with thiobencarb, *Protosiphon botryoides* cells exposed to sublethal concentration (2 ppm) of thiobencarb and control cells were centrifuged at 1500 rpm for 15 min, washed in thiobencarb-free culture medium and then fixed with 3% glutaraldehyde in phosphate buffered, they were postfixed in 1% osmium tetroxide, dehydrated by series of ethanol and embedded in Araldite resine. Ultrathin sections stained with uranyl acetate and lead citrate and examined using electron microscope (Corradi *et al.*, 1995).

## RESULTS

Growth curves of *Protosiphon botryoides* incubated in growth media containing thiobencarb for sixteen days were clearly different from the control. At 0.5 and 1 ppm *Protosiphon* growth exceeded control values after 4-6 days of growth till the end of the experiment (Fig. 1). Percent of inhibition, which designated with negative sign plotted down the zero line, replaced with percent of stimulation, which plotted in the positive part over the zero line (Fig. 2). The stimulation percent were 49 and 45% for both 0.5 and 1 ppm, respectively at the end of incubation period.

The growth curves of the cultures exposed to thiobencarb doses above 2 ppm showed a different pattern of growth from control, being more flattened instead of the characteristic exponential curve (Fig. 1). Complete growth inhibition was recorded at 4 and 8 ppm thiobencarb concentrations, where *Protosiphon* was treated for sixteen days (Fig. 2).

After sixteen days growth, cell number and chlorophyll a content were higher than the control at thiobencarb low concentrations (0.5 and 1 ppm). However, *Protosiphon* cultures treated with 2, 4 and 8 ppm thiobencarb produced cell number and chlorophyll a content clearly lower than that produced within control (Fig. 3, Table 1).

Growth rate ( $\mu$ ) of *Protosiphon* cultures exposed to 2 ppm dose of thiobencarb was  $0.06 \text{ day}^{-1}$ , being nearly half of that recorded within the control ( $0.1 \text{ day}^{-1}$ ) (Table 1). Growth rate at 2 ppm dose and above were decreased significantly as respect to the control. On the other hand, at 0.5 and 1 ppm growth rate was higher than the control (Fig. 4).

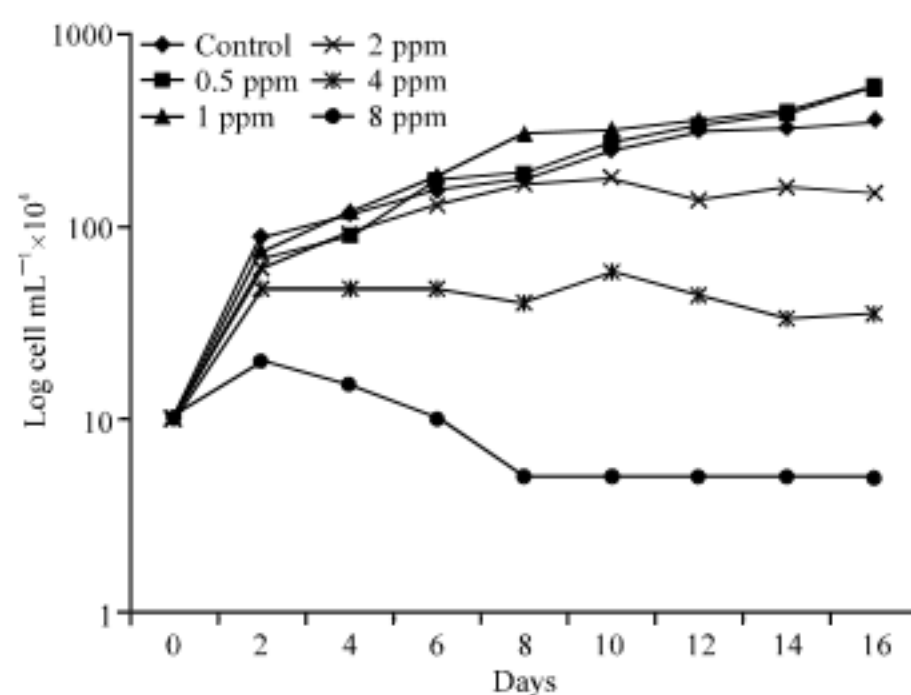


Fig. 1: Growth curves of *Protosiphon botryoides* incubated in different thiobencarb concentrations for 16 days

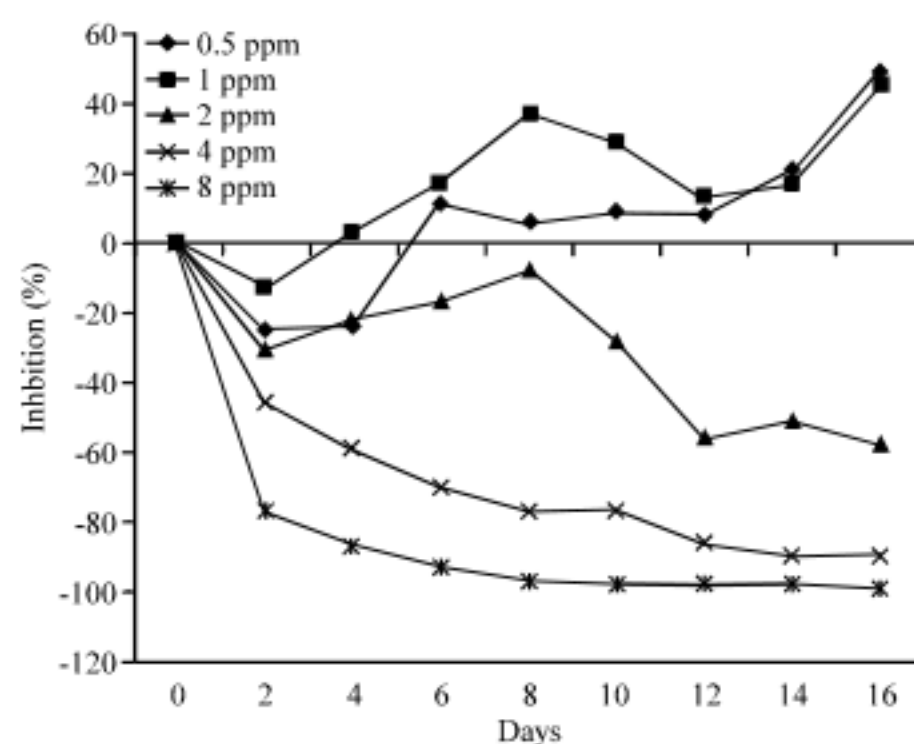


Fig. 2: Inhibition % of *Protosiphon botryoides* incubated in different thiobencarb concentrations for 16 days

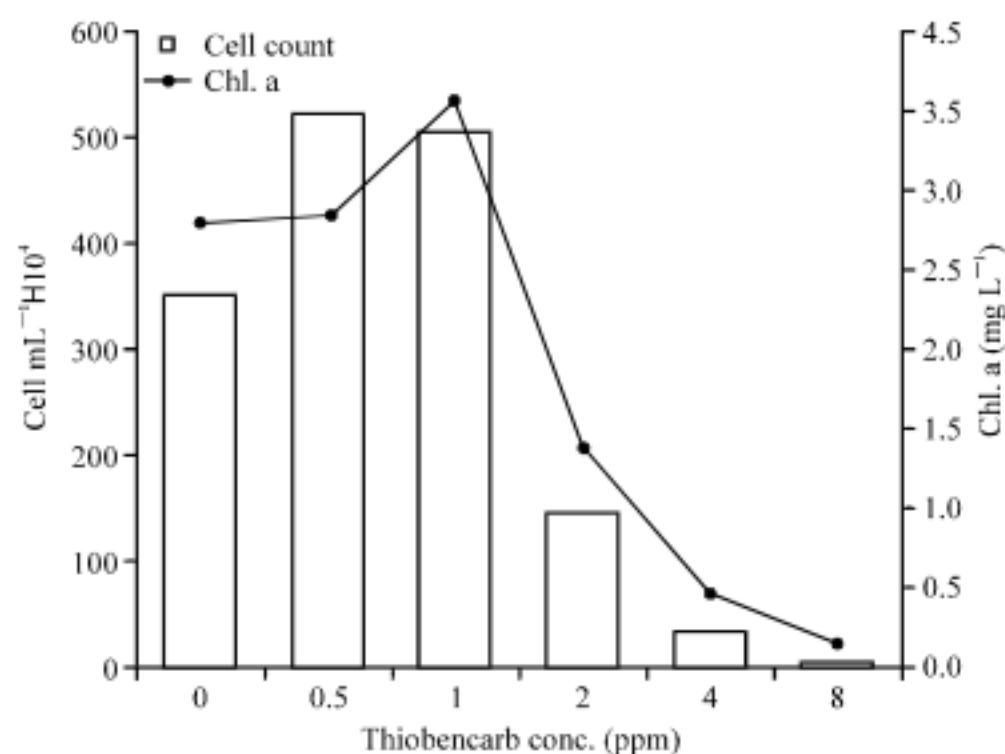


Fig. 3: *Protosiphon botryoides* cell count and chlorophyll a content after incubation in different thiobencarb concentrations for 16 days

Table 1: *Protosiphon botryoides* cells count, chlorophyll a, growth rate ( $\mu$ ) and Generation Time (GT) after 16 days incubation with different thiobencarb concentrations

Thiobencarb conc. (ppm)	Start cells No.×10 <sup>4</sup>	Cell No.×10 <sup>4</sup>	Chl. a (mg L <sup>-1</sup> )	$\mu$	GT
Control	15	350	2.79	0.10	6.9
0.5	15	522	2.83	0.15	4.6
1	15	507	3.56	0.14	5.1
2	15	147	1.37	0.06	10.8
4	15	35	0.46	NG*	NG*
8	15	5	0.15	NG*	NG*

NG\*: no growth developed

After sixteen days *Protosiphon* generation time curve in (Fig. 4) showed remarkable increase as thiobencarb concentration elevated. At 2 ppm exposed *Protosiphon* culture, generation time was high (10.8 day<sup>-1</sup>) as compared with the control 6.9 day<sup>-1</sup> (Table 1).

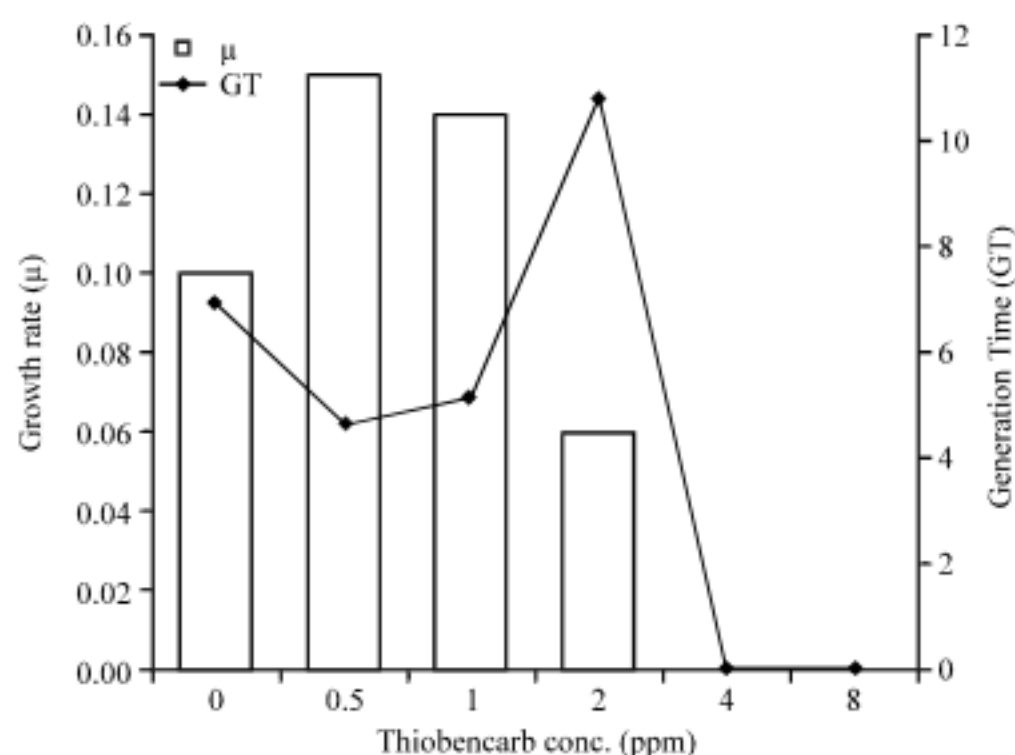


Fig. 4: *Protosiphon botryoides* growth rate ( $\mu$ ) and Generation Time (GT) after incubation in different thiobencarb concentrations for 16 days

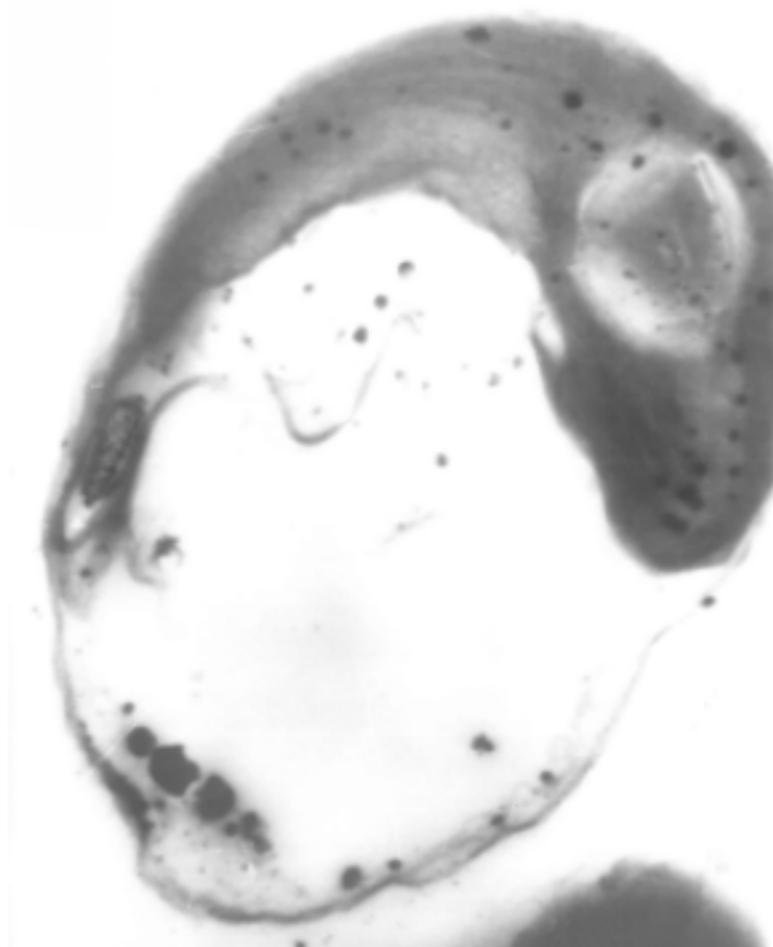


Fig. 5: Transmission electron micrograph of *Protosiphon botryoides* control cell showing a typical structure

Ultrastructure investigation of *Protosiphon* cells grown in the used medium free from thiobencarb herbicide (control) by transmission electron microscopy revealed that, control cells maintained their normal characters, the parietal reticulate chloroplast with many scattered pyrenoids and a large vacuole both were surrounded by the cell wall (Fig. 5). Nevertheless, inspection of Fig. 6 one may notice changing in cell wall structure of the treated *Protosiphon* cells with sublethal concentration (2 ppm) moreover herbicide treated cells undergoing cell division to multispores, which hardly to seen in control cells. The



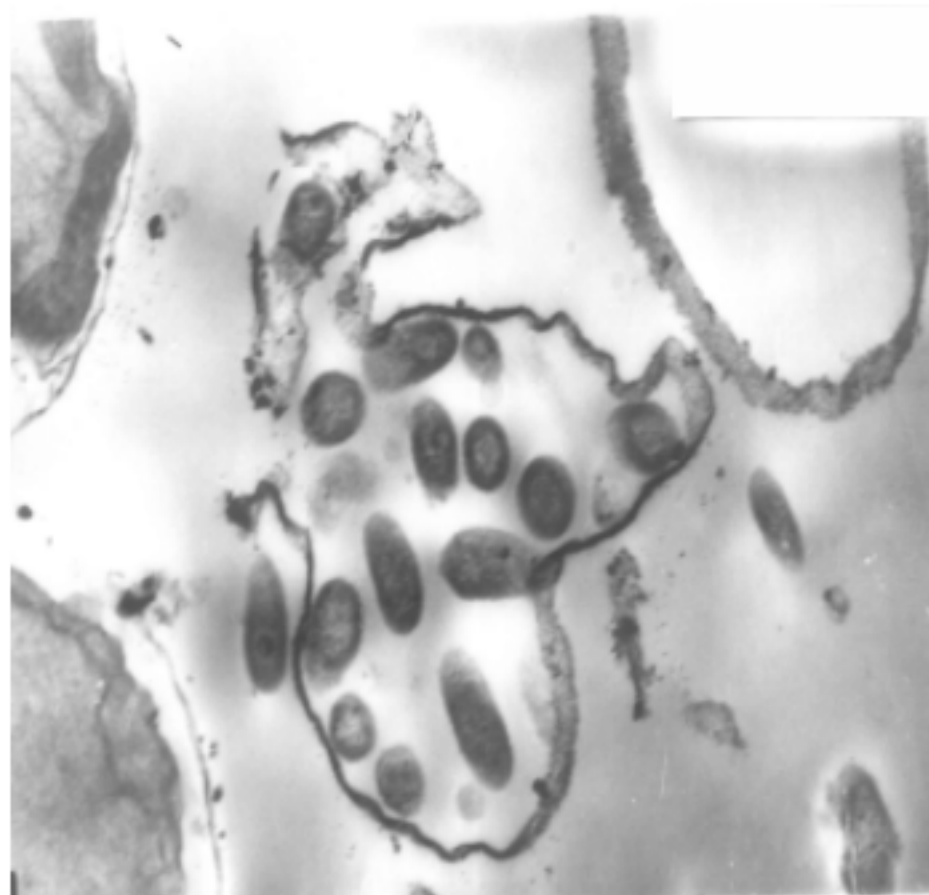


Fig. 6: Transmission electron micrograph of *Protosiphon botryoides* cell exposed to 2 ppm thiobencarb, showing spores still enclosed in the mother cell wall

produced spores still enclosed within the treated cells. The result may reveal that retardation of spores liberation from mother cell wall was one of the modes of action of the used herbicide on *Protosiphon* alga.

## DISCUSSION

Pollution affected algae in different ways. They may be inhibited from growing because of being deprived of sunlight; toxicity of pollutants or may ecologically modify the physical or chemical environment sufficiently to retard or prevent growth.

Thiobencarb low concentrations stimulated *Protosiphon* growth in terms of increase in cell count and chlorophyll a content. In accordance to the obtained results, stimulation effects are often recorded in algal bioassays with different species and at low concentrations of different pesticides (El-Dib *et al.*, 1991; Haglund, 1997; Franqueira *et al.*, 1999). Other researchers observed the stimulatory phenomenon with lower concentrations of some herbicides (Vendrell *et al.*, 2009; Rioboo *et al.*, 2002; Kobbia *et al.*, 2001; Wong, 2000; Saenz *et al.*, 1993; Aly *et al.*, 1991). It seems that at lower concentration of herbicides, the stimulatory effect may occur because of utilization of the herbicide or its degradation products as an energy source. Other investigator return the stimulation of growth, obtained at the lowest terbutryn concentrations, because the ability of algal cells and especially of *C. vulgaris*, to adapt and resist the inhibitory effect of herbicides. This may explain the increase observed in algae in herbicide-treated aquatic ecosystems and perhaps in aquatic ecosystems subjected to toxicants (Shehata *et al.*, 1997).

Because of the effect of high thiobencarb concentrations on *Protosiphon* growth, the cells needed much time to reproduce themselves, which explain the elevation of generation time value and depression in growth rate value. Growth rate of the chlorophyte *Chlorella saccharophila* also was inhibited slightly at 3 mg L<sup>-1</sup> thiobencarb, but *Scenedesmus acutus* was inhibited even at 0.01 mg L<sup>-1</sup> (Sabater and Carrasco, 1996). Growth pattern, as well as

growth rate and generation times exhibited by the cultures of *Scenedesmus acutus* exposed to 0.05 mg L<sup>-1</sup> paraquate and above, were significantly affected (Saenz *et al.*, 1993).

On the bases of the obtained results from electron micrograph, one may attribute the reduction of the growth, growth rate and increasing of the generation time of the treated *Protosiphon* due to retardation effect of the used herbicide on liberation of spores from the mother cell. This phenomenon seemed to be connected with mechanical reason such as rupture of cell wall, which was not easy to tack place under the used conditions. However, other physiological effects of the herbicide should not be neglected. In accordance to the obtained results, transmission electron microscopy analysis of *Chlorella pyrenoidosa* cells grown in the presence of two metolachlor herbicides revealed that metolachlor herbicides affected cell morphology, cell division, cell wall, chloroplasts, starch granules and cytoplasm compartments. There were more cells undergoing division that were treated by the two herbicides than the control. This result implies that metolachlor might have some effect on reproduction of this alga (Liu and Xiong, 2009).

*Protosiphon* zoospores under environmental stress condition may function as isogametes that, upon fusion develop into zygote, which germinate after a period of dormancy into a new *Protosiphon* (Bold and Wynne, 1985). In agreement with the our results from ultrastructure figures, Corradi *et al.* (1995) recorded that sublethal concentration of hexavalent chromium induces gametogenesis in *Scenedesmus acutus*, also Abou-waly (1987) stated that Patron and gardoprim herbicides induces spore formation in *Scenedesmus acutus*. Cultures exposed to triazine concentrations that affected microalgal growth showed cells larger than normal cells, due to the impossibility of completing their cell division. This effect was reported previously in *S. elongatus* cultured with atrazine (Gonzalez-Barreiro *et al.*, 2004, 2006). Thus, it was revealed that herbicides have the potential to suppress algal growth, as shown with many algae and force it to respond by spores production.

In conclusion, the present study showed that, the ultrastructural morphology of *Protosiphon botryoides* cells was affected by thiobencarb herbicide in high concentration.

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