

# Effect of novel phosphoramidate on growth and respiratory metabolism of *Paramecium aurelia*

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## Abstract

The continuous increase in the number of new chemicals as well as the discharges of solid and liquid wastes triggered the need for simple and inexpensive bioassays for routine testing. In recent years, there has been increasing development of methods (particularly rapid tests) for testing environmental samples. This paper describes the quick toxic evaluation of a novel synthetic compound: Phosphoramidate derivative B at different concentrations (2, 4 and 8  $\mu\text{M}$ ) for 72 h on *Paramecium aurelia*. We showed that B concentrations affect the growth of *Paramecium* in concentration- dependent manner; also it decreases the growth rate and increases response percentage in concentration- dependent manner. The value of LC50 obtained for these protozoa was estimated at 4.9693  $\mu\text{M}$  after 24 hours of exposure. The respiratory metabolism of protozoan is perturbed at three concentrations, noting that the oxygen consumption was significantly increased at high concentrations after 18 hours of exposure. The results indicate that the *Paramecium* toxicity assay could be used as a complementary system to rapidly elucidate the cytotoxic potential of insecticides. The major advantages associated with these tests are: inexpensive, simple, rapid and seem to be attractive alternatives to conventional bioassays

**Key words:** Cytotoxic tests, growth kinetics, phosphoramidate B, *Paramecium aurelia*, respiratory metabolism

## INTRODUCTION

The extensive use of organophosphorus insecticides, during the past decades has led to a number of negative effects on terrestrial and aquatic organisms. Phosphoramidates (phosphoryl amides) are important organophosphorus compounds possessing variety of applications such as insecticides, fungicides and herbicides.<sup>[1]</sup> Insecticides are being used in agriculture and they are found to be more hazardous than herbicides and fungicides.

A number of studies were conducted on the toxicity of organophosphorus compounds like acephate on different organisms and indicated as a potent neurotoxicant.<sup>[2]</sup> It is also found to be mutagenic,<sup>[3]</sup> carcinogenic,<sup>[4]</sup> and

cytotoxic.<sup>[5]</sup> Monitoring of aquatic ecosystem pollution represents one of the major activities involved in measures aimed at environmental protection.

Usage of non- targeted organisms in environmental toxicology is needed to understand the wide range of toxic effects caused by the pesticides on different organisms.<sup>[6]</sup> Fish and other aquatic biota that were commonly used as bioindicators of persistent organic pollutants<sup>[7]</sup> have been replaced in recent years successfully by ciliates.<sup>[8]</sup> Protozoan cells are often used as bioindicators of chemical pollution, especially in aqueous environment. The application of unicellular organisms to study the toxic effects of pesticides from contaminated wastewater is relatively new throughout the world. Hence, in the present paper, we have studied the toxic impacts of novel synthetic compound: phosphoramidate derivative B on *Paramecium aurelia* with special emphasis on respiratory metabolism, cells numbers and growth rate.

## MATERIALS AND METHODS

### Cells and culturing methods

*Paramecium aurelia* were used in the logarithmic phase of

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growth. The cells were established by single cell isolation in the Laboratory of Cellular Toxicology at the University of Annaba (Algeria). The cells were cultured at  $27 \pm 3^\circ\text{C}$  in a lettuce medium (pH 6.8), previously inoculated with *Klebsiella pneumonia*, supplemented with  $0.2 \mu\text{l/ml}$  of  $\beta$ -sitotrerol.<sup>[9]</sup>

### Synthesis of N, N' benzylamine phosphoramidate B

In this research N, N' Cyclohexylamine phosphoramidates was prepared in one step by the reaction of phenyl phosphonic dichloride (PPDC) (0.75 g, 5mmole) and primary amine (1.98 g, 20mmole) in 35 mL of anhydrous acetonitrile. The resulting mixture was stirred for less than 12 hr at  $-5^\circ\text{C}$ . The reaction mixture concentrated in vacuum and was washed with water and the organic layer was dried over anhydrous magnesium sulfate and concentrated in vacuum. The residue was purified by chromatography on silica gel (eluted with  $\text{CH}_2\text{Cl}_2/\text{MeOH}$ , 9/1) to give phosphoramidate B as white solid in high yields ( $>80\%$ ).

### *Paramecium aurelia* toxicity test

The cells were exposed to phosphoramidate B (Synthetic compound Cytotoxic) an initial cell density of  $10^3$  cell/ml for 72 hours using culture in Erlenmeyers flasks. B solutions were added of culture medium to obtain B concentration of 2, 4 and  $8 \mu\text{M}$ . These concentrations were selected after series of preliminary finding rang toxicity tests.

### Measurement of growth kinetics and rate

Total number of cells of paramecia exposed to three concentrations of B was measured after: 1, 6, 18, 24, 48 and 72 h of exposure by counting the exact number of living cells in a known volume of culture under a stereomicroscope.

Cells were sampled after post exposure of 24 h (because the cellular density was decreased after 24 h of exposure). *Paramecium* cells were characterized by their growth rate ( $\mu$ )<sup>[10]</sup> calculated using the following equation:

$$\mu = \frac{\text{Ln}N_t - \text{Ln}N_0}{\text{Ln}2(t-t_0)}$$

Where  $t_0$  and  $t$  are the initial and final time of the exponential growth period, both expressed as days, and  $N_0$  and  $N_t$  are the number of cells/ml at those times.

### Determination of percentage of response and median lethal concentration (LC50)

Percentage of response was calculated to evaluate the toxicity of xenobiotic via the inhibition of cell growth of protists,<sup>[11]</sup> according to the equation:

$$RP = \frac{CN - EN}{CN} \times 100$$

Where  $RP$  is the Response Percentage of protozoa (%);  $CN$  is the cell control number (cell/ml) and  $EN$  is treated cells number (cell/ml). The positive values of response percentage indicate an inhibition of growth, while negative values indicate a stimulation of growth.

The total number of paramecia in each concentration after 24 h was used to determine  $LC_{50}$  value by a linear regression, defined as the concentration of phosphoramidate B required for 50% inhibition of proliferation.<sup>[12]</sup>

### Quantification of oxygen

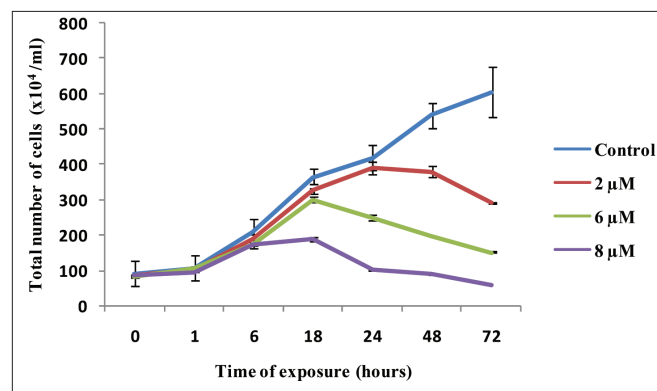
The production or consumption of oxygen in the order of nanomole (nmole) was determined by the polarographic method.<sup>[13]</sup> The equipment used was an oxygen electrode, type HANSATECH.

### Statistical analysis

All the experiments were repeated three times, and the results were expressed as average and standard error (SE) values. Statistical analysis was performed using a one-way ANOVA and the test of Dunnett for comparison between the control and treated cells. The  $\alpha$ -level for significant differences was set at  $P < 0.05$ .

## RESULTS AND DISCUSSION

*Paramecium* that has long been a model organism for cellular aging and clonal lifespan.<sup>[14-19]</sup> and is also used as a rapid bioindicators for the presence of xenobiotic compounds. The impact of B concentrations on the population growth of *P. aurelia* is shown in Figure 1. The molecule B has an inhibitory effect on the population density growth of *P. aurelia* in concentration dependent manner, cells growth was



**Figure 1:** Total number of paramecia cells with the B concentrations assayed. Each data point represents the mean of three independent assays  $\pm$  standard error. Values are significant from control values at  $P < 0.001$

significantly decreased ( $P < 0.001$ ) after 72 h of exposure. Thus our results show that between 1 to 6 h of exposure, cell cultures exposed to different B concentrations illustrate a similar cell growth to that control, this could be due to the adsorption of xenobiotic on the cell membrane and the presence of cuticle in paramecia, which make them resistant but remain nevertheless permeable.<sup>[20]</sup> After 18 h of exposure, the inhibitory effect was detected for those with 4 and 8  $\mu\text{M}$  of B. Those differences became larger as time of exposure increased. This difference proved the inhibitory effect in the growth of paramecia<sup>[21]</sup> reported that toxics may affect the survival of protista in a variety of ways, as the concentration of toxicants in the cell membranes and destroy their integrity causing cell lysis.

The response percentage measurement results are presented in Figure 2. Gradual increase of 51.84 %, 74.87% and 90.58% of response percentage, respectively of 2, 4 and 8  $\mu\text{M}$ . It can be said that the positive evolution of the response percentage confirm the growth inhibition of the treated paramecia and this regardless of the cell concentration.

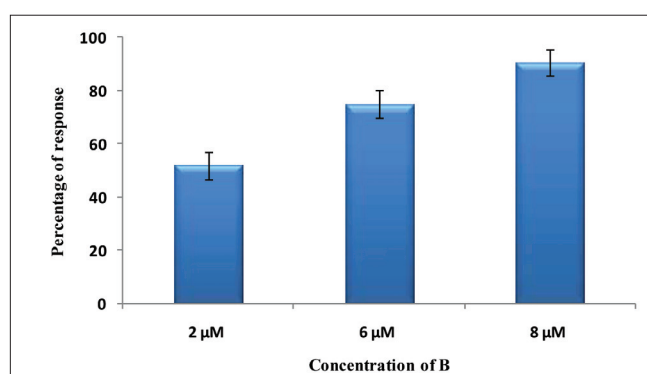
The data of growth rate obtained for the cultures exposed to the different B concentrations assayed, also proved the toxic effect of B on the growth of paramecia, since this value decreased as the B concentration increased [Table 1]. The ANOVA test ( $P < 0.05$ ) applied to the growth rates confirmed this significant effect of the xenobiotic, and the data obtained using the Dunnett test showed that this toxic effect could be expressed as Control  $>$  2  $\mu\text{M}$   $>$  4  $\mu\text{M}$   $>$  8  $\mu\text{M}$  [Table 1]. The value of LC50 obtained for these protozoa was estimated at 4.9693  $\mu\text{M}$  after 24 hours of exposure.

The  $\text{O}_2$  consumption of paramecia was significantly affect ( $P < 0.001$ ) by the action of B concentrations [Figure 3]. It should be noted that the cells treated with the lowest B concentration (2  $\mu\text{M}$ ) between 1 to 24 h of exposure present the same evolutions of the control cells. After 18 h of exposure, cell cultures treated with strongest concentration (4 and 8  $\mu\text{M}$ ) present a significantly deceleration of their respiratory activity. The perturbation of the respiratory activity obtained in our work shows that the concentrations of B generates an oxidative stress.<sup>[22,23]</sup> Our results are consistent with those of<sup>[24]</sup> who tested the effect of gossypol on the morphology, mobility and metabolism of *Dunaliella bioculata* (flagellate protists) regarded as a cell model of human sperm. This result is explained if we base on the detoxification/metabolisation mechanisms by mono- oxygenases enzymes, where the cells consummate  $\text{O}_2$  to make the substrate more hydrophilic so eliminated by water. These enzymes are coupled with substrate in the cells treated with highest B concentrations (4 and 8  $\mu\text{M}$ ), but this reaction occur after 18 h of exposure in cells treated

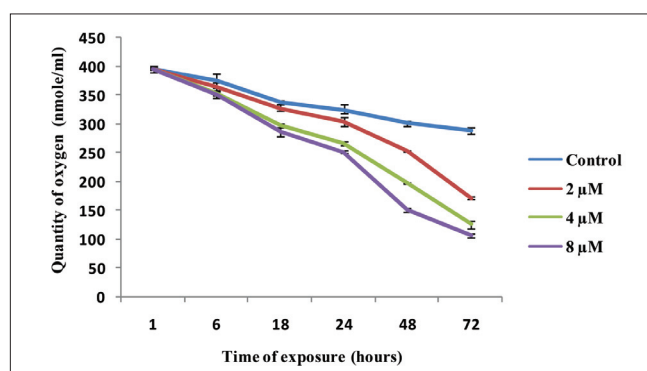
with 2  $\mu\text{M}$ . The decrease of oxygen consumption in the highest concentration of B molecule is also a signification of the reduced number of cells because we started from the same number of cells.

## CONCLUSIONS

The prime focus of the present paper was to develop a simple and reliable evaluation method to detect the toxic effects of insecticides at laboratory conditions. The effect of toxicant on several biological properties can be studied on paramecia considered as a single cell cum organism, where as such wide range tests may not be possible to perform with human cell lines.<sup>[25]</sup>



**Figure 2:** Evolution of the response percentage of paramecia in presence of different concentrations of B. Each value is mean  $\pm$  standard error of three replicates



**Figure 3:** Impact of B (2, 6 and 8  $\mu\text{M}$ ) on respiratory metabolism of *Paramecium aurelia*. Each value is mean  $\pm$  standard error of three independent observations

**Table 1: Growth rate  $\pm$  standard error of *Paramecium aurelia* cultures exposed to different B concentrations after 24 h.**

B concentration ( $\mu\text{M}$ )	2	4	8	
Control	0.397 $\pm$ 1.414	0.395 $\pm$ 1.626	0.286 $\pm$ 1.838	0.042 $\pm$ 2.241

In the present study, we have shown that B concentrations molecule caused a dose- dependent growth inhibition of *Paramecium aurelia* population. It can be said that the positive evolution of the response percentage confirm the growth inhibition of the treated paramecia and this regardless of the cell concentration. Molecule B has a the cell concentration. Iso toxic effects on *Paramecium* by increasing of generation time and disturb respiratory metabolism.

After considering all the experimental data obtained throughout the study, it appears that the ciliate protists used in our work is a material of choice for studies in toxicology and occupies a privileged position in aquatic ecosystems because it is one of the basic elements of food chain, and hence, the need for a deep study of the impact of pollution on our environment.

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