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Toxic Effects of Combined Molecule from Novaluron and Diflubenzuron on *Paramecium caudatum*

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Abstract: The struggle against the agricultural ravagers was increased in few last years; many products were appeared and destined to kill the harmful insects, of which we did not know their effects on ecosystem and non-target animals. In present work, we try to investigate the ecotoxicological effects of a combined molecule from Novaluron and diflubenzuron; two Insect growth Regulator (IGR) pesticides, on ecosystem and biomasses by the estimation of the growth and respiratory metabolism of *Paramecium caudatum* as a non-target aquatic organism exposed to these pesticides. The pesticides are tested *in vitro* by the addition of four concentrations (5,10,15 and 20µg/ml) in strictly controlled conditions, to the culture medium before/after apparition of the protozoan. *Paramecium* population growth was evaluated by optic density at 600nm method, for the cells number we use the account under an optical microscope, the generation time and number were evaluated, also we evaluated the respiration of *Paramecium* population by oxygen electrode. Treatment with 5, 10, 15 and 20µg/ml of the molecule affects the growth (proliferation) of *Paramecium caudatum* in concentration-dependent manner; also it increases the generation time and response percentage in concentration-dependent manner. The respiratory metabolism of protozoan is perturbed at four concentrations, noting that the oxygen consumption was increased at 10 and 15µg/ml, contrarily to the other two concentrations, which they had an inhibition effect especially for 20µg/ml of pesticide.

Key words: Cytotoxic Tests · Chitin synthesis inhibitors · *Paramecium caudatum* · Respiratory metabolism

INTRODUCTION

Many of the pesticides used in the modern agriculture have the potential to influence the number and functions of a diverse range of water and soil microorganisms [1]. Benzoylureas are an entirely different class of insecticides that act as insect growth regulators (IGRs). More than being the typical poisons that attack the insect nervous system, they interfere with chitin synthesis and they are more taken up by ingestion than by contact [2]. Their value appeared in the control of caterpillars, [3,4]. The benzoylureas act on the larval stages of most insects by inhibiting or blocking the synthesis of chitin [2], a vital and almost indestructible part of the insect exoskeleton [5]. Typical effects on developing larvae are the cleaving of malformed cuticle or death by starvation [5]. Adult female boll weevils exposed to diflubenzuron lay eggs that do not hatch, and mosquito larvae control can be achieved with as little as 1.0 gram of flucycloxuron per acre of surface water [6], [7] reported that Novaluron exhibited a high level of activity against *Culex* mosquitoes. [8] showed that Novaluron exhibited long-term activity against *Aedes aegypti* in water-storage containers; it caused >80% larval mortality at 10 and 20mg/kg of housefly. Many years after application of the IGRs, insects developed a resistance; [9] found resistance by *Musca domestica* toward Diflubenzuron, and field populations with some resistance to cyromazine. As levels of insecticide resistance continue to increase, it is ever more important to develop alternative methods and insecticides for controlling the insects, but the toxicity toward ecosystem, non-target, and beneficial organisms are increased. The present work investigates the contamination probability of freshwater microorganisms after treatment of water surfaces by pesticides to control mosquito, so we interested in the ecotoxicological activity ofcombined molecule from Novaluron and Diflubenzuron on non-target organism which is *Paramecium caudatum* equipped with a chitinous exoskeleton membrane (like all ciliated microorganisms).

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Novaluron N-[[[3-chloro-4 [1,1,2-trifluoro-2-(trifluoromethoxy) ethoxy]phenyl]amino] carbonyl]-2,6-difluorobenzamide, a relatively new CSI (Chitin synthesis inhibitor), that inhibits the chitin formation on larvae of various insects (Lepidoptera, Coleoptera, Homoptera and Diptera) [10]. It has a potent insecticidal activity against several important foliage feeding insect pests [11], and very low toxicity to mammals, birds and earthworms [12]. By inhibiting chitin formation, Novaluron selectively targets immature insect stages, causing abnormal endocuticular deposition abortive molting. While the incompatibility with natural enemies has been reported [13], the compound generally is selective in favor of non-target organisms [14-16], giving it good potential in integrated pest management (IPM) programs. It had no effect on phytoseiid mite field populations [14], mortality and development of the soil-dwelling predatory mite, *Stratiolaelaps scimitus* (Womersley) [16], and greenhouse populations and percent parasitism of the parasitoid *Encarsia Formosa* Gahan [15]. Novaluron is actually registered for usage against *L. decemlieata* in the US (trade name Rimon), and is undergoing registration in Canada [13]. However, ecotoxicological effects of Novaluron on the ecosystem and especially on non-target microorganisms was not been evaluated. A little number of researches have studied the impact of pesticides on microorganisms [3,4,17], but knowledge about ecotoxicological effects of Novaluron is till now ignored especially toward microorganisms beaded with structural chemical targets for this pesticide.

The Diflubenzuron or Dimilin (1-chlorophenyl)-3-(2,6-difluorobenzoyl) urea) is a pesticide of third generation to large specter, it is used mainly in agriculture: forests, cereal cultures, it is the more used between the Benzoylphenyl ureas. It is considered like a poison of contact and ingestion; it inhibits the synthesis of the chitin and interferes with the formation of the cuticle (exoskeleton), probably by the inhibition of the *N-acetyl glucosamine* incorporation in the chitin. It is shown that the Diflubenzuron could modify the metabolism of the molting hormones, traduced by cuticle deformation [2].

As treatment by pesticides is applied to the wide surfaces of soil and water, fish and other aquatic biota that were commonly used as bio-indicators of persistent organic pollutants [18] are usually exposed, and organisms with structures that incorporate chemical target -in our case chitin- to pesticides are affected. Protozoa, algae and bacteria from broad base of food chains are often used as boindicators of chemical pollution [19] especially in aqueous environment [20, 3, 4 and 21]. In addition, the cilia of *Paramecium* exhibit comparable characteristics with human respiratory epithelia-cells [22], so any impact on cilia could be extrapolated to human cilia. This protozoa facilitates the study of biochemical and biological processes and effects on locomotory behavior by the microtubular system and mitochondria [23-25].

MATERIALS AND METHODS

Cells Culturing and Treatments: Paramecium strain was used in the logarithmic phase of growth. The cells were grown at exponential phase in Proteose Peptone Yeast Medium (PPY), 2% proteose peptone and 5% yeast extract at pH 7.0-7.5, at 24±2 °C. The density of cells cultures was adjusted in fresh PPY in order to obtain at least 10^4 cells per ml. Before the experiments on respiration metabolism, the cells were washed with fresh culture medium and were resuspended at the concentration of $5x10^4$ cells ml^{-1} in 200ml flask; we take 1 ml to test in oxygraph (each time we added the appropriate concentration of DFB/Novaluron to the reactive chamber by microsyringe). The cells were not exposed to combined molecule were used as control, the acetone is used to dissolve the pesticide so we obliged to investigate the impact of acetone on cells by addition of 5µl/ml to the medium cells (acetone-control). For the evaluation of Diflubenzuron/Novaluron effect on *Paramecium* population growth, generation time and number, chitin integrity; we added the pesticide in culture medium before the addition of *Paramecium* cells, the used cells are starved for 96 h to become encysted. After the regeneration in culture medium that contains Novaluron, we investigate the effect on new chitin integrity.

Chemical Preparation: Diflubenzuron/ Novaluron are insoluble in water [26], so they are dissolved in acetone before adding to the distilled water in three concentrations 5, 10, 15 and 20µg of diflubenzuron/ Novaluron/5µl acetone/1ml of culture medium. We are obliged to make an acetone control to eliminate the effect of solvent.

Growth Measurement: The growth of *Paramecium* population density is estimated by [27] method (Optic density at λ=600nm), on aliquots of 2.5ml of cultures for each concentration with 3 repetitions, we used distilled water as white control, the cell number was determined by counting every cell present in 1 ml sample using a microscope and Petri box [28]. We have calculated the response percentage in 7^{th} day, which evaluates the response of cells opposite the pollutant, described in Eq. 1 [29]:

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$$RP = \frac{CN - EN}{CN} x100 \quad (1)$$

Where *RP* is the Response percentage of protozoa (%); *CN* is the cell control number (cell/ml) and *EN* is treated cells number (cell/ml).

Determination of Generation Time and Number: Aliquot of 100µl were immediately taken (T_0) from the control, control acetone and the exposed cultures and subsequently at 24 h and 48 h (3 repetitions). the samples were diluted in distilled water and fixed with neutral-buffered formalin (NBF) containing 10% (V/V) formalin in phosphate-buffered saline (PBS) (ph 7.4) at a final concentration of 2% to 5% for 1 h. the cell number was determined in each 30µl under optical microscope. *Paramecium* cells were characterized by their generation time *(g)* required for doubling the population. Generation time and number were calculated using the following formulae [30].

Number of generation *n* is given by

$$n = \frac{\log N_1 - \log N_0}{\log 2} \quad (2)$$

Generation time *g* is given by:

$$g = \frac{Time\ of\ growth}{n} \quad (3)$$

where N_1 is the number of cells at 24 h

Respiratory Metabolism: Protistes respiration is estimated using Clark's electrode (oxygraph), described by [31], which go until nanomol of oxygen consumption. N_0 is the number of cells at T_0 and time of growth is 24 h. *P. caudatum* at logarithmic phase ($5x10^4$ cells/ml) from homogenous culture were obtained and 1ml of culture is put inside reaction chamber of oxygraph, then we added the pesticide at appropriate concentrations 1, 10 or 20µg, each trial was repeated 3 times, the respiration kinetic is followed for 20 minutes (after that time, cells become no stables) [31]. The results are registered directly as graphs on computer screen linked to the oxygraph.

Statistical Study: All the experiments were repeated three times or more, and the results were expressed as mean and standard deviation (SD) values. We use Minitab 15.1.30 software to make simple two-way ANOVA test with two criteria (treatment and time) and the test of Dunnett for comparison between the control and treated cells.

RESULTS AND DISCUSSION

The impact of DFB/Novaluron on the population growth of *P. caudatum* is shown in (Fig. 1). Indeed, combined molecule has an inhibitory effects on the population density growth of protiste in concentration-dependent manner, the highest concentration inhibits strongly the growth of *Paramecium* ($p<0.001$). This results is confirmed by [3,4] results on *Paramecium sp.* and *Tetrahymena pyriformis* treated with DFB, this due probably to the toxic effects on chitin synthesis by the combined molecule which incorporate on *N-glucosamine* [33], it is to note that Dunnett test shows a total difference between treated cells and the controls. The response percentage measurement results are presented in (Fig.2). Gradual increase of 45.50 and 82% of response percentage, respectively of 5, 10, 15 and 20µg/ml. Microorganisms are highly sensitive to chemicals in aqueous environment. However, paramecia cells are relatively resistant to high concentration of acrylamide [34]. [35] found an increase in protein level of marine protozoa *Tetraselmis suecica* caused by the resistance phenomenon against aquatic pollutants, which confirm our results about response percentage. It is to note that acetone has not any toxic effect on protiste number and response ($p>0.05$), Dunnett test revealed a difference between treated cells and control.

Under the culture conditions used in this study, the initial cell density was $1.5x10^4$ cells/ml and the normal generation time of *Paramecium caudatum* was about 7.2 h. the experiments showed that the addition of DFB/Novaluron affects gradually generation time (Table 1) in dose-dependent manner. The acetone and

Tab 1: Effect of DFB/Novaluron on generation time of *Paramecium* after 24 h of growth. Each value is means±SD of three independent observations

Samples	Generation time / h
Control	7.24±0.04
Acetone-control	7.27±0.35
DFB/Novaluron (5µg/ml)	7.59±0.61*
DFB/Novaluron (10µg/ml)	8.71±1.03***
DFB/Novaluron (15µg/ml)	9.01±1.02***
DFB/Novaluron (20µg/ml)	9.50±1.52***

*** $p<0.001$ ** $p<0.01$ Ss* $P<0.05$

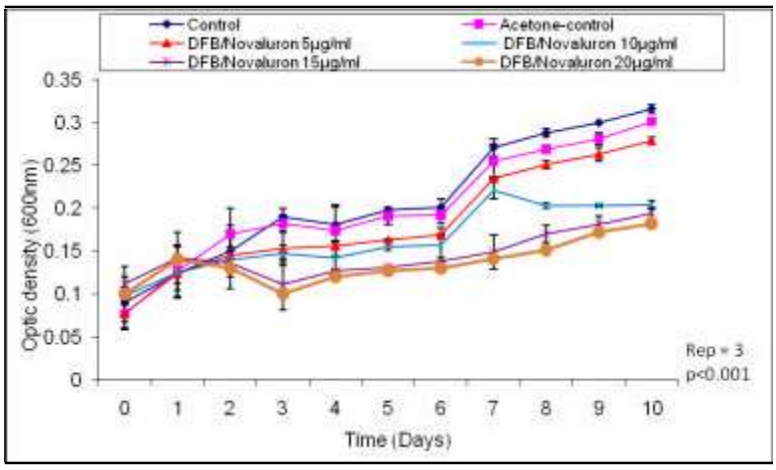

Fig. 1: Impact of DFB/Novaluron on growth of *Paramecium caudatum* population number according the time ($p<0.001$). Each value is means ± SD of three independent observations

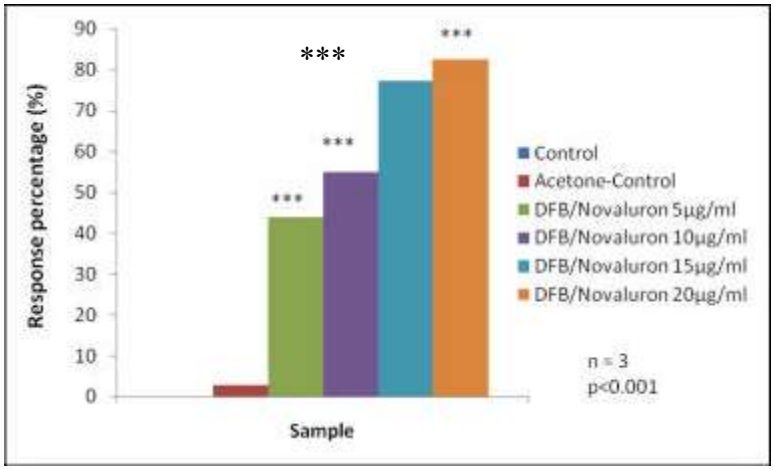

Fig. 2: Response percentage of *P. caudatum* in presence of 5, 10, 15 and 20µg/ml of DFB/novaluron ($p<0.001$) at 7^{th} day. Each value is means ± SD of three independent observations

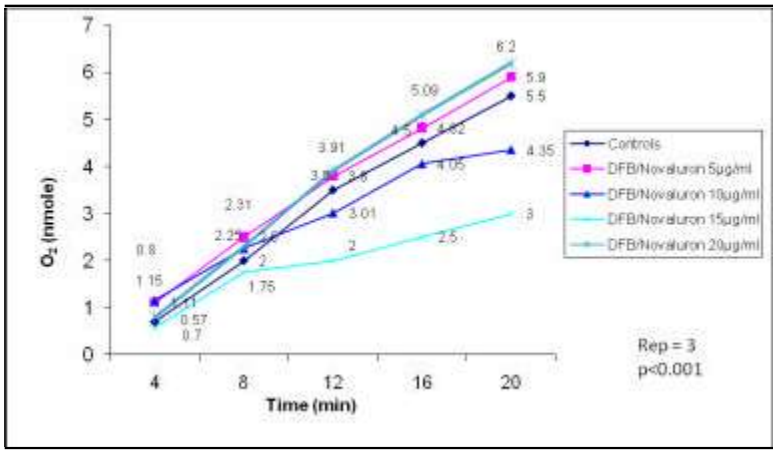

Fig. 3: Impact of DFB/Novaluron (5, 10, 15 and 20µg/ml) on respiratory metabolism of *P.caudatum.* Each value is means ± SD of three independent observations

DFB/Novaluron in lower concentration (5µg/ml) did not have a significant effect ($p > 0.01$) on generation time of *Paramecium*. However, generation time was increased at higher concentrations (10, 15 and 20µg/ml) of DFB/Novaluron, indicating that this molecule of higher concentration inhibits the growth of *Paramecium* ($p<0.001$), Dunnett test showed a difference of the cells treated by 10, 15 and 20µg/ml to the control. [3,4] reported that the Benzoylphenyl ureas inhibit the growth of protozoa by the reduction of chitin thickness, also this reduction it may be due by the incorporation of Benzoylphenyl ureas in RNA of protozoa [36]. [37] 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 lysis, or by effect on enzymes, inactivating them by binding to sulphydryl, amino and amino groups of enzyme protein.

The effect of DFB/Novaluron on the respiratory metabolism of *Paramecium* was investigated for 20 minutes (acute toxicity) using Hansatech electrode described by [31], the results are illustrated in (Fig. 3). Indeed, 20µg/ml of molecule inhibits *paramecium* respiration ($p<0.001$), dunnett test revealed that cells treated with 5µg/ml of DFB/Novaluron is not different to control. Wherever, the treatment by 10 and 15µg/ml increases the oxygen consumption ($p<0.001$) according to the control (from 4 to 20nmol). This result is explained if we base on the detoxification/metabolisation mechanisms by mono-oxygenases enzymes, where the cells consummate O_2 to make the substrate more hydrophilic so eliminated by water. These enzymes are coupled with substrate in the cells treated with 10 and 15µg/ml of DFB/Novaluron, but saturated or blocked in cells treated with the two other concentrations (5 and 20µg/ml) [4]. The decrease of oxygen consumption in the highest concentration of combined molecule is also a signification of the reduced number of cells because we started from the same number of cells.

In conclusion, our study showed that the highest concentrations of this combined molecule caused a dose-dependent growth inhibition of *Paramecium* population. The results suggest that the antiproliferative effect of DFB/Novaluron may be mediated by reducing the chitin and cuticle of cells, also by the effect on mitochondria and lashes, so DFB/Novaluron has toxic effects on *Paramecium* by reduction of growth, increasing of generation time, perturbation of respiratory metabolism, and high response percentage. It is to note that the used concentrations are high compared with concentrations found to effect aquatic crustaceans and insects, this due to the response and the physiology of *Paramecium* cells which are comparable to human and high organisms.

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