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ECOTOXICOLOGICAL EFFECTS OF DIFLUBENZURON ON MOTILITY, MORPHOLOGY, AND OSMOREGULATION IN PARAMECIUM CAUDATUM

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Abstract– The freshwater ciliate protozoan *Paramecium caudatum* was used to assess the toxic and functional effects of the insecticide Diflubenzuron. In acute toxicity tests, exposure to lower concentrations of Diflubenzuron led to a slight decrease in cell motility, whereas higher doses induced abnormal rocking behavior, ultimately resulting in complete immobility. The median lethal concentration (LC_{50}) after 3 hours of exposure was determined to be 300 ppm. Cellular deformities, including swelling, an oval shape, and at elevated concentrations, a reduction in body length and darkening of the cytoplasm, were observed, indicating significant toxic effects. Additionally, leakage of cellular contents suggested membrane damage. Changes in the contractile vacuole activity were noted after 20 minutes of exposure to 25, 50, 75, and 100 ppm, indicating potential disruption of osmoregulatory functions. Altered phagocytosis and vacuolar activity further suggest that Diflubenzuron has substantial physiological impacts on *Paramecium caudatum*. Due to their rapid growth, short life cycles, and reproducibility, ciliates like Paramecium are valuable model organisms for evaluating the toxicity and physiological effects of xenobiotics.

INTRODUCTION

Pesticides commonly used in modern agriculture have the potential to impact the abundance and functioning of various aquatic and soil microorganisms (Rouabhi Rachid, 2009). As a result, pesticides contribute significantly to pollution in freshwater ecosystems, posing potential risks to a broad spectrum of non-target species (Nageswara Rao Amanchi and Masood Hussain, 2010). Exogenous chemicals introduced into the environment can lead to long-lasting ecological damage by reducing biodiversity and disrupting the integrity of ecosystems. Among these chemicals, phytosanitary products, including pesticides, have attracted considerable attention due to their harmful effects on living organisms (Fleeger *et al*., 2003; Martina, 2020).

The ciliated protozoan *Paramecium* serves as a crucial component of the heterotrophic eukaryotic food web. It acts as an effective bio-indicator for assessing environmental health and evaluating the impacts of human activities on ecosystems. Additionally, *Paramecium* plays an important role in regulating critical ecological processes (Finlay and Fenchel, 2004). *Paramecium* species are frequently used in cytotoxicity research due to their rapid generation times, efficient cultivation in laboratory settings, and ability to assess the effects of xenobiotics across multiple cell generations. This organism has been employed in various studies investigating the impact of chemical pollutants (Rehman *et al.*, 2008; Amanchi and Bhagavathi, 2009; Helmut Plattner, 2022). Diflubenzuron, a third-generation pesticide also known as Dimilin (1-chlorophenyl)-3-(2,6-difluorobenzoyl)urea), is widely used for pest control in agriculture, forestry, and cereal farming. It is classified as toxic when ingested or upon direct contact. Diflubenzuron operates by inhibiting the synthesis of chitin, disrupting the development of the insect cuticle (exoskeleton), likely by blocking the incorporation of

N-acetylglucosamine into chitin. Studies have also shown that diflubenzuron may interfere with the metabolism of molting hormones, further disrupting the growth and development of target insects (Rouabhi *et al*., 2006; 2009; Rachel *et al*., 2022).

This study aims to evaluate the effects of Diflubenzuron on *Paramecium caudatum* by conducting an *in vitro* series of cytotoxicity tests. The bioassays in this investigation include a fast and straight forward acute toxicity test, assessing the locomotor behavior, morphology, phagocytosis, and contractile vacuole activity of *Paramecium* cells exposed to varying concentrations of Diflubenzuron.

MATERIALS AND METHODS

Test compound used

Commercial grade of Diflubenzuron 25% EC (DITOX) insecticide used in this study was manufactured by Unikil pesticides, Pvt, Ltd.

Experimental model organism

Paramecium caudatum were collected from a freshwater source at Osmania University in Hyderabad, India. The organisms were grown in a sterilized hay infusion medium at room temperature in the lab to establish a pure stock culture. Log phase cultures were utilized for the current studies. Six grams of dried hay were boiled in one liter of distilled water, then cooled and filtered. After that, it was sterilized in an autoclave for 15 minutes at 15 pounds of pressure and set aside for later use. Sterile conditions were strictly observed throughout the research. To culture the organisms, the hay infusion medium was combined with distilled water in a 1:1 ratio and poured into various cavity blocks. Under sterile conditions, ciliates were introduced into the medium to produce a pure stock culture. The ciliates were added to the culture fluid in each cavity block, which were then covered with lids to prevent contamination and evaporation while still allowing gas exchange between the air and the culture medium, and they were sub-cultured every sixth day (Shiny *et al.*, 2005).

Acute Toxicity Studies

The stock solution and experimental concentrations of Diflubenzuron were prepared in accordance with the guidelines established by APHA (2002). A stock solution with a concentration of 1000 ppm of Diflubenzuron was formulated using distilled water as the solvent. Initial dose-finding trials were conducted to identify appropriate stock solutions and test concentrations. These solutions were freshly prepared prior to the toxicity evaluations. The experimental protocols followed the procedures outlined by Apostol (1973). An acute toxicity assessment was conducted over a 3-hour period. In these tests, 0.5 ml of the pesticide solution was combined with 4.5 ml of culture medium to achieve the desired test concentration. A total of 100 organisms were introduced into each cavity block, with all test concentrations repeated in triplicate. Following the addition of the pesticide, each cavity block was observed under a binocular microscope, and counts of affected organisms were recorded at 10-minute intervals during the first hour and at 20-minute intervals over the subsequent two hours. The LC50 value and lethal concentration (LC) were calculated based on the mortality data collected over the 3-hour exposure period. Control groups containing the same number of organisms but without pesticide exposure were also established for comparison.

Phagocytosis

Phagocytic activity was assessed after exposure to non-lethal concentrations of Diflubenzuron, specifically at concentrations of 25 ppm, 50 ppm, 75 ppm, and 100 ppm. The organisms were exposed to these concentrations for duration of 1 hour. After exposure, 10 treated cells from each concentration were collected using a micropipette, mixed with a 1 M carmine suspension, and allowed to incubate for 20 minutes. From each of the four exposed groups, 10 organisms were selected, immobilized on slides using methyl cellulose, and the number of food vacuoles formed was counted. Similarly, control Paramecium organisms were exposed to the carmine suspension, immobilized, and the number of food vacuoles was recorded. The preparation of the carmine suspension and the subsequent counting of food vacuoles followed the procedure outlined by Nilsson (2003a).

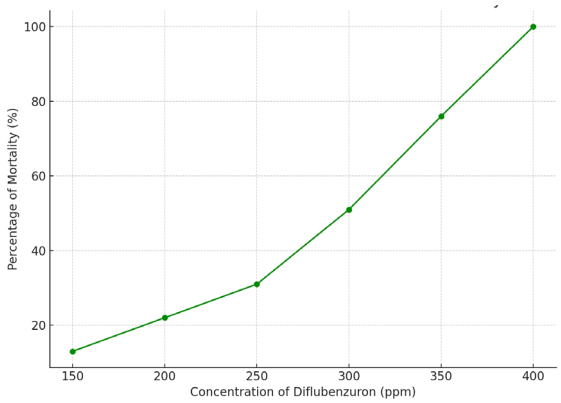
Contractile Vacuole Activity

The function of the contractile vacuole was evaluated in cells after they were immobilized on slides, following the methodology described by Marsot and Couillard (1973). For cells exposed to sub-lethal concentrations of Diflubenzuron for 20 minutes, a single organism that appeared

morphologically normal was selected using a micropipette. The pulsation rate of one contractile vacuole was then measured. Observations were made on cells at each concentration, with an equal number of observations conducted on control cells for comparison.

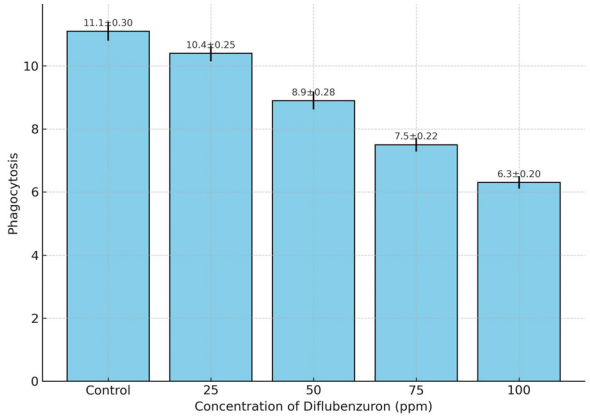
RESULTS AND DISCUSSION

Exposure to 400 ppm of Diflubenzuron resulted in immediate cell death, which was evidenced by the complete cessation of ciliary movement, contractile vacuole activity, and food vacuole function. The LC50 value, determined after a 3-hour exposure period, was found to be 300 ppm (see Graph 1). At concentrations exceeding this threshold, significant morphological and physiological changes were observed, including a reduction in the length of the longitudinal axis, darkening of the cytoplasm, and an increase in the size of the contractile vacuole. No cytotoxic effects were evident at concentrations of 25 ppm, 50 ppm, 75 ppm, and 100 ppm after a 3-hour exposure period.



Graph 1. Acute toxicity effects of Diflubenzuron on *Paramecium caudatum*

Food vacuole activity was notably inhibited at 100 ppm, with average counts of food vacuoles decreasing in a dose-dependent manner: 10.4, 8.9, 7.5, and 6.3 at 25 ppm, 50 ppm, 75 ppm, and 100 ppm, respectively, after one hour of exposure (see Graph 2). The formation and movement of food vacuoles are dependent on ciliary motility, as the cilia are responsible for both the movement of food vacuoles and their transport to the cytostome. As such, any disruption in ciliary function is likely to affect the rate of food vacuole formation. The significant decrease in food vacuole formation following exposure to Diflubenzuron suggests

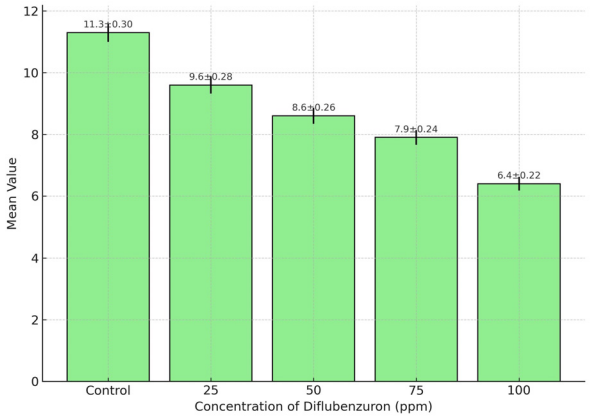


Graph 2. Effect of Diflubenzuron on food vacuole of *Paramecium caudatum*

potential damage to the cell membrane and other internal cellular structures. It is plausible that Diflubenzuron interferes with cytoplasmic contractions, which are crucial for the detachment and subsequent movement of food vacuoles.

Furthermore, while the ciliates retained the ability for suspension feeding, this process appeared to be somewhat suppressed at higher concentrations of the pesticide, as evidenced by the decrease in food vacuole activity. These findings align with the work of Ouissem Moumeni (2016), who also reported that chemical exposure can impair the suspension feeding ability of ciliates. The current study underscores the broader potential effects of Diflubenzuron on key cellular processes in aquatic organisms, emphasizing the need for further investigation into its sub-lethal impacts on ecosystem health.

Paramecium is commonly used as a model organism to study the structure and function of



Graph 3. Effect of Diflubenzuron on Contractile Vacuole of *Paramecium caudatum*

contractile vacuoles, and has been the focus of extensive research by numerous scholars over the years. Stock *et al.* (2002) highlighted that the pressure generated at the pulsating vacuole membrane may play a critical role in facilitating the expulsion of water from the vacuole through the pulsating vacuole pore. The frequency of expulsion from the pulsating vacuole can be influenced by various external factors. Additionally, it was concluded that the osmolarity of the contractile vacuole fluid is consistently hypertonic relative to the cytoplasm, while the osmolarity of the cytoplasm remains hypertonic in comparison to the surrounding environments. When *Paramecium* was exposed to 100 ppm of Diflubenzuron for 20 minutes, a significant reduction in contractile vacuole activity was observed, with a marked decrease in the average number of pulsations per minute in both control and exposed specimens. A noticeable decline in vacuolar activity was evident at all tested concentrations (25, 50, 75, and 100 ppm; see Graph 3).

CONCLUSION

The acute toxicity assessment of Diflubenzuron on *Paramecium* revealed significant dose-dependent cytotoxic effects. A concentration of 400 ppm caused immediate mortality, while the LC50 value was determined to be 300 ppm after a 3-hour exposure. Sub-lethal concentrations (25–100 ppm) did not produce observable cytotoxicity within this timeframe but did impair key physiological processes. Notably, food vacuole formation was progressively inhibited with increasing concentrations, indicating disruption of ciliary function and intracellular transport. Additionally, a marked reduction in contractile vacuole activity suggests interference with osmoregulatory mechanisms, potentially due to membrane damage. These findings highlight that even non-lethal levels of Diflubenzuron can compromise essential cellular functions in *Paramecium*, suggesting broader ecological implications for aquatic micro fauna exposed to this pesticide.

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Conflict of Interest: The authors declare that there is no conflict of interest associated with this research. All work was conducted with scientific integrity.

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