ACTA ZOOLOGICA BULGARICA

Acta Zool. Bulg., 75 (4), December 2023: 519-525
Published online 28 December 2023
DOI: https://doi.org/10.71424/azb75.4.002716

Applied Zoology
Research Article

Neurotoxic Effect of New Generation Insecticide with Flubendiamide Active Ingredient in *Gammarus pulex* (Linnaeus, 1758) (Amphipoda: Gammaridae) at Different Temperatures

Hilal Söylemez & Osman Serdar*

Faculty of Fisheries, Munzur University, TR62000 Tunceli, Turkey; E-mail: hilalsoylemez6262@gmail.com, oserdar@munzur.edu.tr

Abstract: This study aimed to determine the relationship between temperature and neurotoxicity of a new generation insecticide, the active ingredient of which is flubendiamide, on non-target aquatic organisms (*Gammarus pulex* (L., 1758)). Firstly, LC_{50} values of flubendiamide on *G. pulex* were determined at 16, 18 and 20 °C as 256.83 ± 19.59 μgL^{-1}, 169.38 ± 34.17 μgL^{-1} and 155.84 ± 17.22 μgL^{-1}, respectively. We found that the acute toxicity sensitivity of the test organism to the pesticide containing flubendiamide increased with increasing temperature. Secondly, *G. pulex* was exposed to non-lethal concentrations of flubendiamide pesticide for 96 hours at the above temperatures. Finally, the changes in acetylcholinesterase (AChE) enzyme activity were determined for each temperature using ELISA kits. The exposure time between the 24th and 96th h at the same temperature was found to be statistically insignificant ($p>0.05$). It was determined that the inhibition value of AChE enzyme activity on the test organism of the pesticide with active ingredient flubendiamide increased depending on the temperature increase. Considering the changes in AChE enzyme activities in *G. pulex* at different temperatures against flubendiamide insecticide, it was concluded that it can be used as a useful biomarker.

Key words: Acetylcholinesterase, biomarker, flubendiamide, *Gammarus pulex*, pesticide

Introduction

In recent years, especially after the 2000s, with the increase in pesticide use, various negative effects were established on the balance in ecosystems, both direct and indirect, affecting natural life and even human health (Özkara & Akyil 2018). The production purpose of pesticides is to combat target organisms or groups of organisms that harm agricultural products. For this reason, synthetic and chemical pesticides are frequently used in homes, fields and gardens and areas where industrial and agricultural activities are performed (Koç et al. 2009, Karaismailoğlu 2016). Single or combination agrochemical compounds have been used to prevent, destroy, mitigate or expel pests from the environment. Nowadays, the negative effects of pesticides are widely discussed (Yu 2008, Demirci & Güngördü 2020).

Flubendiamide is a new-generation pesticide widely used against lepidopteran pests in many annual and perennial plants. In order to meet the needs in parallel with the increasing population density, the need to obtain the highest level of crops from a unit area has become inevitable with the increase in industrial and agricultural activities, and the uncontrolled

*Corresponding author: oserdar@munzur.edu.tr

release of pollutants. The active ingredient of flubendiamide has low toxicity in mammals. In addition, it does not have genotoxic, mutagenic or oncogenic properties. In other words, it can be said that it has an ecological, ecotoxicological and environmental profile (Shane 2006, Das & Mukherjee 2011).

One of the most important environmental problems, especially with climate change in recent years, is global warming. Global warming threatens all living species on Earth, aquatic creatures and people. The temperature increase in aquatic ecosystems impacts vital activities of organisms such as reproduction, growth and nutrition, as well as changes in abiotic parameters, such as pH and dissolved oxygen; it triggers negative synergistic effects on metabolic processes. The negative synergistic effect of pollutants with increasing temperature adversely affects aquatic ecosystems, the biological characteristics of aquatic organisms, habitat ecosystems and stocks. The warming of aquatic ecosystems due to the greenhouse effect of gases, such as excess carbon dioxide (CO_2), carbon monoxide (CO), halocarbons (17 groups containing fluorine, chlorine, bromine or iodine) and methane (CH_4) released into the atmosphere by the products of industrial activities. Poikilothermic species are particularly sensitive to environmental temperature changes. Therefore, temperature is defined as one of the important abiotic factors as it can potentially affect all the metabolic, physiological and ecological and behavioural aspects of the life cycle of these organisms (Almeida et al. 2015). As a result of these temperature increases, it is predicted that many aquatic creatures will leave their habitats where they have lived for many years and, as a result, they will seek new habitats to which they can adapt (Wrona et al. 2006). Alternatively, it may mean the extinction of sensitive species that cannot tolerate all these adverse conditions.

Acetylcholinesterase (AChE) is a neurotransmitter; it is an enzyme from the hydrolase group that hydrolyzes acetylcholine (ACh), enabling communication between neurons or between a neuron and another type of cell. AChE, which belongs to the carboxylesterase family of enzymes, is found mainly in neuromuscular and cholinergic brain synapses, where it terminates synaptic transmissions. It is the primary target that inhibits this enzyme with organophosphate derivative compounds such as pesticides and nerve gas (Pohanka 2011, Zilbeyaz et al., 2018, Cavdar et al. 2019). It has a very high activity at a rate that can break up 25,000 ACh molecules per second (Sussman et al. 1991).

The family Gammaridae, one of the most important groups of invertebrates found in clean waters, plays an important role in freshwater ecosystems and food chains (Rosenfeldt et al. 2015, Serdar et al. 2019) titanium dioxide nanoparticles (nano-TiO2). The Gammaridea family, one of the most important groups of invertebrates found in clean water resources, plays an important role in the freshwater ecosystem and food chain (Rosenfeldt et al., 2015; Serdar et al., 2019). *Gammarus* genus amphipods and especially G. pulex are widely used in the evaluation of the toxic effects of pollutants (Adam et al., 2010; Tatar et al., 2020). Amphipods belonging to the genus *Gammarus* are frequently used in ecotoxicological studies due to their abundance in fresh water, high ecological relevance and important role in the food chain (Serdar, 2019).

In this study, it was aimed to investigate the change in AChE enzyme activity of a new generation insecticide with active ingredient flubinamide on non-target model organisms under laboratory conditions.

Materials and Methods

Supply of model organism

Samples of the model organism *G. pulex* (L., 1758) used in the study were collected from the tributaries of the Munzur Stream (Tunceli), from reference points where there is no pollution or domestic waste near water sources (Fig. 1). Collected organisms were quickly transferred alive to Munzur University Faculty of Fisheries Aquatic Toxicology Research Laboratory. Then, healthy male individuals of similar size, who have completed their development, were selected.

Adaptation of the model organism to the experimental environment conditions

The samples with *G. pulex* were placed in aquariums prepared to correspond to their natural environment. Using laboratory lighting, a photoperiod of 12 hours of light and 12 hours of dark was applied (Serdar et al. 2021). The ambient temperature and aquarium water temperature were kept constant at 16±0.2, 18±0.2 or 20±0.2 °C during both adaptation and testing phases and by means of with thermostat air conditioner (48000 BTU, Bosch brand) and by means of chiller (Resun brand, 0-40°C adjustable 3000 l/h), respectively. Willow tree leaves collected from their natural environment for feeding of *G. pulex* were left to rot in water in a glass container (Cold et al. 2004).

Determination of LC_{50} values in flubendiamide-treated *G. pulex* at different temperatures

In the study, 96-hour static LC_{50} test was applied to determine the acute toxicity of flubendiamide active ingredient insecticide on *G. pulex* for each tempera-

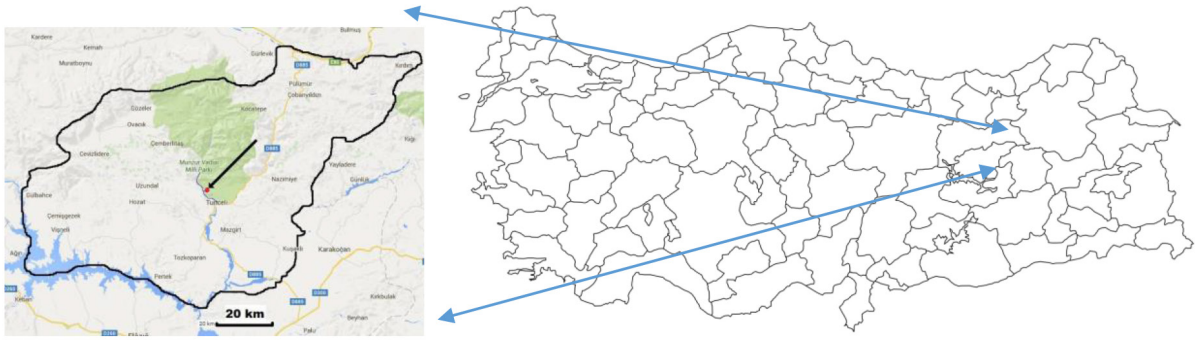


Fig. 1. The area where *G. pulex* specimens were collected (URL-1, 2023)

ture. For this purpose, the range determination test was first applied to the organisms. Values of LC_{50} of flubendiamide pesticide in *G. pulex* were calculated using Probit analysis. All steps of acute toxicity testing, including range determination tests, were performed in triplicate. Each of the experiments was carried out in one-litre glass aquariums and using ten *G. pulex*. In this study, approximately 1000 test organisms were used in total.

Experimental Design

The following bioassay groups were formed according to the LC_{50} values of the flubendiamide active ingredient insecticide at the determined temperatures (16, 18 and 20 °C):

- **A group (Control) –** 16 °C, organisms not exposed to any substance,
- **B group** – 16 °C, organisms exposed to flubendiamide active substance at a rate of 1/20 of LC_{50} values,
- **C group** – 16 °C, organisms exposed to flubendiamide active substance at a rate of 1/10 of LC_{50} values,
- **D group** – 16 °C, organisms exposed to flubendiamide active substance at a rate of 1/5 of LC_{50} values,
- **E group (Control) –** 18 °C, organisms not exposed to any substance,
- **F group** – 18 °C, organisms exposed to flubendiamide active substance at a rate of 1/20 of LC_{50} values,
- **G group** – 18 °C organisms exposed to flubendiamide active substance at a rate of 1/10 of LC_{50} values,
- **H group** – 18 °C, organisms exposed to flubendiamide active substance at a rate of 1/5 of LC_{50} values,
- **I group (Control)** – 20 °C, organisms not exposed to any substance,
- **J group** – 20 °C, organisms exposed to flubendiamide active substance at a rate of 1/20 of LC_{50} values,
- **K group** – 20 °C, organisms exposed to flubendiamide active substance at a rate of 1/10 of LC_{50} values,
- **L group** – 20 °C, organisms exposed to flubendiamide active substance at a rate of 1/15 of LC_{50} values.

A total of ten groups were formed as three application groups and control groups for each temperature. AChE activity responses of 24 and 96 hours to organisms in each group were determined.

Dissection procedures and preparation of supernatants

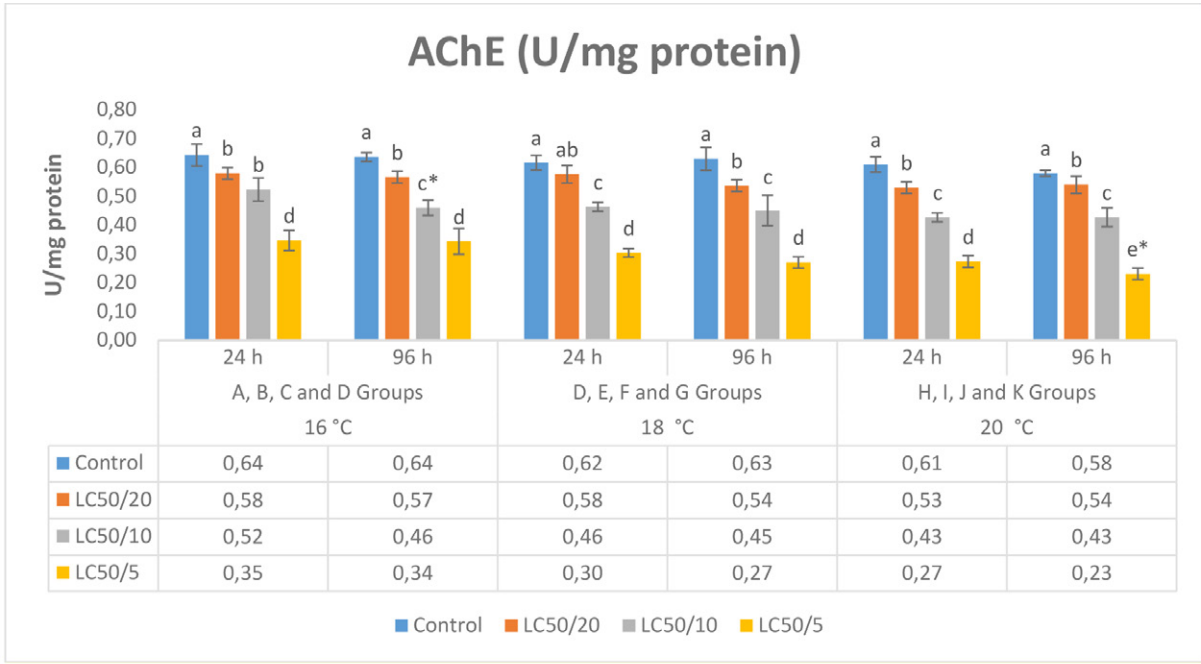
A pool of 0.5 g of test organism individuals was weighed and a PBS buffer (phosphate-buffered saline solution) was added at a rate of 1/1 w/v and homogenised using an ultraturrax with ice. These homogenised samples were centrifuged at 17000 rpm for 15 minutes in a refrigerated centrifuge and the resulting supernatants were stored in a deep freezer at -86 °C until the measurement procedures were completed.

Determination of Neurotoxicity Responses

To determine the neurotoxicity response at the temperatures determined in the study, AChE activities were determined using a commercial ELISA kit with catalogue number (CSB-E17001Fh) purchased from CUSABIO company.

Statistical Analysis

Statistical analyses of the data obtained at the end of the study were carried out using the SPSS 24.0 statistical program. The LC_{50} value in *G. pulex* for each temperature was calculated using Probit analysis. Statistical difference between different groups (A, B, C, D, E, F, G, H, I and control) in the same application period was determined by DUNCAN test.



	16 °C 24 h	16 °C 96 h	18 °C 24 h	18 °C 96 h	20 °C 24 h	20 °C 96 h
Control	0,64	0,64	0,62	0,63	0,61	0,58
LC50/20	0,58	0,57	0,58	0,54	0,53	0,54
LC50/10	0,52	0,46	0,46	0,45	0,43	0,43
LC50/5	0,35	0,34	0,30	0,27	0,27	0,23

Fig. 2. AChE activity in *G. pulex* at 16, 18, and 20 °C. The letters a, b, c, d and e in superscript indicate statistical differences between different groups in the same column. Asterisk (*) sign shows the statistical differences between different hours (24 and 96 hours) of the same group.

Table 1. Average LC_{50} values in *G. pulex* exposed to flubendiamide insecticide at different temperatures.

Temperature	LC_{50} value (μgL^{-1})
16 °C	256.83 ± 19.59
18 °C	169.38 ± 34.17
20 °C	155.84 ± 17.22
Average value	193.99 ± 54.77

The difference between the application times (24 and 96 hours) was determined using an independent t-test.

Results

The average weight of the model organism *G. pulex* used in the experiments was 0.073 ± 0.009 g, while the average length was recorded as 9.3 ± 0.8 mm.

After range tests, LC_{50} values of flubendiamide active ingredient insecticide were determined for *G. pulex* individuals at 16, 18 and 20 °C (Figure 1).

When all temperature groups and application times subjected to flubendiamide were examined, it was determined that the inhibition of AChE enzyme activity increased in all groups exposed to flubendiamide, that is, the enzyme activity decreased. It was determined that the highest inhibition was in the group exposed to the highest flubendiamide concentration, at the 96th application time and at the highest temperature (20 °C) (Fig. 2).

Discussion

Toxicology studies the damage, destructive effects and levels of physical or chemical agents on living organisms. With this definition, aquatic toxicology aims to determine the effect of a substance that is directly or indirectly involved in the aquatic ecosystem on aquatic organisms with various biomarkers, and at what concentration it harms the organisms by toxicological tests (Karataş 2005, Serdar et al. 2021).

Qiu & Qian (1999) found in their study that temperature was significant for both the survival and development of larvae of *Amphitrite amphitrite*. Bat et al. (2000), determined the LC_{50} values of zinc (Zn), copper (Cu) and lead (Pb) toxicity in freshwater amphipod *G. pulex* at three different temperatures (15, 20 and 25 °C). They reported that LC_{50} values decreased with temperature, while the sensitivity of the organism to the exposed substance increased with increasing temperature. Zauke (1982), examined the relationship between acute toxicity of Cd heavy metal and abiotic variables in natural populations of *Gammarus tigrinus* and reported a relationship between Cd concentration and water temperature. Nasrolahi et al. (2013) reported that

low temperature and low salinity stress of the model organism affected larval growth after 7 and 40 days, thus abiotic factors being directly related. In this study, we obtained similar results, determining that the LC_{50} acute toxicity of the flubendiamide active ingredient pesticide interacted with the temperature increase.

AChE is an important regulatory enzyme that hydrolyses the excitatory acetylcholine (ACh) and controls the passage of nerve impulses across cholinergic synapses. Since this enzyme is suppressed by chemicals, it can be used as a biomarker of neurotoxicity (Müller et al. 2002). Bocquené & Galgani (1998) claimed that temperature is the most important regulatory factor on natural changes in AChE activity. Temperature can affect both pollutant concentrations and physiological activities of organisms (Kopecka et al. 2006). Environmental pollutants, together with global warming, accelerate negative impacts on organisms. In some studies, it has been reported that AChE activity increases with increasing water temperature. Hogan (1970) reported that an increase in water temperature caused an increase in the AChE activity of *Lepomis macrochirus*. Pfeifer et al. (2005) reported significant differences of the AChE activity in *Mytilus* sp. depending on temperature and salinity. Similarly, there are several studies investigating the changes in AChE enzyme activity on aquatic organisms combining xenobiotics and temperature pressure (Kozlovskaya et al. 1993, Moulton et al. 1996, McLoughlin et al. 2000, Varó et al. 2002, Barata et al. 2004, Pfeifer et al. 2005, Kristoff et al. 2006, Bueno-Krawcayk et al. 2015, Gauthier et al. 2016, Pala & Serdar 2018). In this study, a statistically significant inhibition was detected in AChE activity after the application of flubendiamide, which also increased with the increase of water temperature.

Conclusion

We aimed to determine the direct and indirect effects of chemical and synthetic substances on non-target organisms and the combined effect of global warming (increasing temperature). For this reason, the neurotoxic effect of a new generation pesticide with flubendiamide active ingredient was determined in association with temperature. It has been determined that flubendiamide has a neurotoxic effect on *G. pulex* due to the decrease in AChE activity in the test organism exposed to the effect of flubendiamide. Our study demonstrated that AChE activity can be used as a biomarker for the determination of pesticide contamination. The negative synergistic effect of increasing temperature with pesticide with active ingredient flubendiamide was found to be important in terms of LC_{50} value and AChE activity neurotoxic on *G. pulex*.

Declarations

Ethical Approval: The authors have confirmed that this work is original and has not been published elsewhere, nor is it currently under consideration for publication elsewhere. Plagiarism and slicing have been avoided. There are not data belonging to other researchers included in this manuscript.

Author contributions: Both authors conceived and designed the study on the acute toxicity. Tulpar carried out the laboratory analysis and studied the enzyme activity. Both authors were involved in the manuscript preparation, contributed to the statistical analyses and to writing. There is no conflict of interest between the authors.

Funding: This study was supported by the Scientific Research Projects Coordination Unit of Munzur University. Project No:YLMUB021-07.

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Received: 30.03.2023
Accepted: 12.10.2023