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Lethal and sublethal effects of the pyrethroid insecticide tau-fluvalinate on the non-target organism *Gammarus roeseli*: A study of acute toxicity, genotoxicity and locomotor activity

Fikret Sari

Department of Plant and Animal Production, Tavas Vocational School, Pamukkale University, 20500, Denizli, Turkey

Correspondence: fikretsari1@gmail.com; fsari@pau.edu.tr

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Abstract: Aquatic ecosystems are recipients of various contaminants including pesticides. For many years, pyrethroid insecticides (e.g., tau-fluvalinate) have been used extensively in agricultural activities to control pests. However, they can affect not only target organisms but also non-target organisms. This study was conducted to investigate the lethal and sublethal effects of tau-fluvalinate on the non-target organism *Gammarus roeseli*. To this end, acute toxicity of tau-fluvalinate was determined using a toxicity test with a 96-h exposure period, and the genotoxic effects of different sublethal concentrations on hemocytes of the test organism were assessed at 24-, 96- and 240-h exposure periods using the comet assay. Alterations in locomotor activity of the test organism in response to exposure to sublethal concentrations were evaluated at 120- and 240-h periods. The 96-h median lethal concentration (LC_{50}) was found to be 17.29 μg/L, and tau-fluvalinate was observed to cause a significant increase in DNA damage and a significant reduction in locomotor activity at the tested sublethal concentrations (2.15, 4.30 and 8.60 μg/L). The results of this study suggest that the long-term existence of tau-fluvalinate in aquatic environments at high concentrations is a noteworthy threat to non-target organisms and that its use in agricultural activities should be reconsidered.

Keywords: *Gammarus roeseli*; tau-fluvalinate; DNA damage; comet assay; non-target organism; locomotor activity

INTRODUCTION

Aquatic ecosystems are the ultimate recipients of different contaminants that arise from various anthropogenic activities [1-3] such as urbanization, industrialization and agricultural activities [1]. One type of these contaminants are pesticides (insecticides, herbicides, etc.) used extensively for protecting different crops from diseases and pests [3]. Pesticides can relocate from the target site to other environmental media by transfer processes such as spray drift, leaching, run-off and/or rainfall events, and hence they end up in aquatic ecosystems [4]. Once in aquatic ecosystems, pesticides are known to be capable of reducing environmental quality and affecting essential ecosystem functioning, since they cause many alterations of various components of ecosystems such as species diversity, energy flow and food chain [5]. Their effects on aquatic ecosystems are the result of both long-term exposures to their low concentrations (chronic) and short-term exposures to high concentrations (acute). Therefore, pesticides are of environmental concern.

Pyrethroids are a major class of neurotoxic insecticides affecting both the peripheral and central nervous systems of insects by interacting with voltage-gated sodium channels [6]; they prolong membrane depolarization and eventually cause paralysis and death [6,7]. For many years, pyrethroid insecticides have been used extensively to control arthropod pests of crops, since they have been regarded as a very successful group of compounds. Unfortunately, the long-term use of these insecticides has resulted in the evolvement of resistance to the compounds in many species [7]. This is a serious problem in pest control because they have become less effective due to resistance development. Pesticide manufacturers are consistently replacing older generation pesticides with newly developed ones to overcome such problems. For example, a new group of chemicals, the neonicotinoids, have emerged

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as an environmentally safe alternative to previous generations of insecticides such as organophosphates and carbamates [8], and they have been introduced into the market. However, pyrethroids (e.g., flumethrin and tau-fluvalinate) are still one insecticide group with widespread usage [9]. Although these insecticides are used for controlling target organisms, they also have different toxic effects on exposed non-target organisms, which compromises the ecosystems. There are still little data about their potential consequences on non-target aquatic organisms. Tau-fluvalinate is used extensively because of its effective and extensive role in outbreak control and protection of crops, which leads to its entry into aquatic environments. Due to differences in feeding behavior among invertebrate species, different routes of uptake of environmental contaminants can be expected, resulting in toxic effects that vary depending on the species. Therefore, studying all aspects of tau-fluvalinate toxic effects on a wide range of potential non-target organisms is crucial to obtain robust risk assessment for aquatic ecosystems.

Gammarid species (Crustacea: Amphipoda) are found in a variety of freshwater habitats where they are the dominant component of many benthic macroinvertebrate assemblages [10]. Gammarids have a crucial role in the trophic food chain of aquatic environments as detritivorous species [11]; they decompose organic matter and serve as prey for various organisms, including macroinvertebrates, mammals, birds, amphibians and fish [12]. Additionally, they are sensitive to a variety of pollutants and other disturbances [13], making them valuable indicators of ecosystem health in aquatic ecotoxicology. Thus, different gammarid species have been used in ecotoxicological studies as test organisms to assess the toxicity of many substances for many years [3,13-18]. Given the entry of pesticides into waterbodies through various routes such as surface runoff, direct spray and intentional dumping, it is not surprising that crustaceans of the genus *Gammarus* have come into prominence as aquatic non-target organisms.

As many xenobiotics and newly developed substances released in the aquatic environment are genotoxic for living organisms [19], the genotoxic effects of these environmental pollutants including pesticides are a growing concern. Genotoxicity is the ability of an agent to exert harmful effects on DNA and/or other cellular targets responsible for genetic material integrity and involves strand breaks, induction of DNA adducts, point mutations, and structural and numerical chromosomal changes [20]. Among a variety of methods used in genotoxicity studies, the single-cell gel electrophoresis (or comet) assay represents a rapid, sensitive, reliable, robust and relatively inexpensive way of investigating DNA damage [21]. The comet assay can be performed on different cell types such as blood cells, hemocytes, oocytes and spermatozoa for assessing DNA damage. Hemocytes are free-floating somatic cells that are closely exposed to environmental agents through their crucial roles both in the transport of toxicants and in different defense mechanisms. Furthermore, they are easy to obtain and require very little manipulation to prepare microscopic slides; this keeps at minimum the possibility of their damage [22]. Therefore, hemocytes are frequently chosen as single-cell suspensions in genotoxicity assessments of pollutants including pesticides in invertebrate test organisms using the comet assay. Hemocytes of different gammarid species have been used in genotoxicity studies [19,23-27]. However, no genotoxicity study has been carried out so far using *Gammarus roeseli*, a species that is distributed widely throughout the northern hemisphere [28], to assess the genotoxic potential of pesticides or other environmental pollutants. In addition, in the last decades, several studies have been carried out to examine behavioral responses of gammarid species exposed to different toxicants including pesticides [29-31], but there is no study on the behavioral responses of *G. roeseli* exposed to pesticides. Behavioral responses provide an important tool for ecotoxicity testing and water quality monitoring [29], and assessing these responses enables researchers to link the toxic effects of toxicants at the individual level to their impacts at the population and ecosystem levels [29,32]. Determining which behavioral endpoints are sensitive indicators of exposure to different toxicants is of great importance, providing further evidence that exposure can lead to ecologically relevant sublethal effects [32].

The present study aimed to investigate the lethal and sublethal effects of the pyrethroid tau-fluvalinate on the non-target organism *G. roeseli*. To this end, acute toxicity of tau-fluvalinate was determined using an acute toxicity test with a 96-h exposure period, and the genotoxic effects of different sublethal

concentrations on hemocytes of the test organism were assessed comprehensively at 24-, 96- and 240-h exposure periods using the comet assay. In addition, alterations in locomotor activity of the test organism in response to exposure to sublethal concentrations were evaluated at 120- and 240-h periods. This study is the first report of the acute toxic, genotoxic and locomotor activity-reducing effects of tau-fluvalinate on the non-target organism *G. roeseli*.

MATERIALS AND METHODS

Collection and acclimatization of test animals

G. roeseli was selected as the test organism mainly because of its environmental importance and ease of collection from the field and culturing in the laboratory. *G. roeseli* individuals were collected in June 2022 from an unpolluted natural water source of the Büyük Menderes River in Denizli (Turkey), the Işıklı-Akgöz spring (38.32339 N, 29.85041 E), using a sieve with a mesh size of 500 μm. Collected individuals were then transported to the laboratory in plastic tanks containing water taken from the sampling site. In the laboratory, gammarids were placed in aerated aquaria (10 L) initially filled with water taken from the sampling site and allowed to acclimatize at 20±1°C and a photoperiod of 16-h light/8-h dark for at least 10 days before use in exposure experiments. During this acclimatization period, about half of the water volume in the aquaria was daily replaced with dechlorinated tap water (hereafter referred to as tap water), and the gammarids were fed *ad libitum* with TetraMin fish food.

Tested pesticide

The model insecticide tau-fluvalinate was bought as a commercially available mixture (Mavrik® 2F), which is widely used in pest control in the Aegean region of Turkey, and hence there was no need to use other solvents. Considering that 1 L of the commercial pesticide mixture consists of 240 g tau-fluvalinate, the stock solutions (at concentrations of 1 g/L for the preliminary range-finding test and 1 mg/L for acute toxicity and sublethal exposure tests) were prepared by diluting the commercial tau-fluvalinate with distilled water and stored at 4°C in the dark when not in use. Test solutions for acute toxicity testing and sublethal effect examinations were prepared by adding a necessary volume of the stock solution in tap water to achieve final concentrations.

Acute toxicity

Acute toxicity tests were conducted in 500-mL glass beakers under static non-renewal test conditions. Tests included nine different tau-fluvalinate concentrations. Nominal test concentrations (based on the results of the 24-h preliminary range-finding test) were 0.5, 1, 2, 5, 10, 25, 50, 100 and 200 μg/L (Supplementary Fig. S1). In addition to these test concentrations, tap water was used in the control group (tau-fluvalinate concentration of 0 μg/L) to confirm the accuracy of the tests. After adding 250 mL of the test solution and 10 active and healthy-looking *G. roeseli* adults (9-10 mm in length) to each beaker, the test beakers were covered loosely with parafilm to prevent water evaporation. Tests were performed at 20±1°C under a photoperiod of 16-h light/8-h dark (without feeding and aeration), with each test consisting of three replicates. Live and dead individuals per beaker were counted and their numbers were noted at 24-h intervals throughout the test period. An individual was considered dead if no movement of the appendages was observed after gentle prodding three times. To prevent contamination, the dead individuals were removed from the beakers using forceps. A test was considered valid if the survival rate was >90% in the control group. Using the obtained mortality data, the median lethal concentration (LC_{50}) values for different exposure periods (24, 48, 72 and 96 h) were calculated.

Sublethal exposure

Based on the calculated 96-h LC_{50} value, three sublethal concentrations of tau-fluvalinate were selected (1/2, 1/4 and 1/8 of the 96-h LC_{50} value), and four experimental groups were designed as follows: group 1 was the control group containing tau-fluvalinate-free tap water, whereas groups 2, 3 and 4 were exposure groups containing sublethal concentrations of tau-fluvalinate (2.15, 4.30 and 8.60 μg/L, respectively) (Supplementary Fig. S1). Sublethal exposure experiments for genotoxicity and locomotor activity assessments were conducted in 1-L glass beakers containing

500 mL of the solutions representing the four experimental groups, with an exposure period of 10 days (240 h). Throughout this period, test organisms were fed *ad libitum* with TetraMin fish food to ensure that they received the right amount of food, and test solutions of the groups were replaced daily with freshly prepared solutions to ensure that water quality and insecticide concentration were stable. The details of the sublethal exposure experiments (temperature, photoperiod, aeration and cover) were identical to those described for acute toxicity.

Genotoxicity

For genotoxicity testing, five replicates were used per group. Each replicate consisted of 12 *G. roeseli* adults (9-10 mm in length) selected randomly from the stock aquaria. After the sublethal exposures started, random samples of four gammarids were taken from each experimental group at 24-, 96- and 240-h exposure periods.

Hemolymph was sampled from each gammarid by inserting an insulin syringe (30G needle) between the cephalon and first mesosomite of the animal [24] and placed in a 200-µL Eppendorf tube containing 25 µL of chilled phosphate-buffered saline (PBS). A pool of four gammarids was required to obtain enough hemocytes per replicate. The viability of the hemocytes was observed by the trypan blue exclusion method. All the cell suspensions with viability of more than 90% were used for further genotoxicity testing.

The alkaline version of the comet assay developed by Singh et al. [33] was performed with slight modifications. Briefly, fully frosted slides were coated with 1% normal melting agarose (NMA) in PBS and dried overnight at room temperature. Following the collection of cells, 20 µL of 1% low melting agarose (LMA) in PBS (37°C) was mixed with 20 µL cell suspension, pipetted onto the slides coated with NMA and covered with a coverslip. The slides were cooled on an ice pack for 5 min. After removing the coverslip, the slides were placed in a freshly prepared lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 1% Triton X-100, 10% dimethyl sulfoxide (DMSO), pH 10) for 60 min at 4°C. The slides were then gently placed in a horizontal electrophoresis chamber filled with freshly prepared chilled buffer (300 mM NaOH, 1 mM EDTA, pH>13) for 30 min. After electrophoresis in the same buffer for 25 min at 4°C (1 V/cm, 300 mA), the slides were neutralized in 0.4 M Tris-HCl, pH 7.5 for 5 min and washed with deionized water two times for 2 min each. After drying for 60 min at room temperature, the slides were fixed in a solution containing trichloroacetic acid (15%), zinc sulphate (5%) and glycerol (5%) for 10 min at room temperature and stained with silver nitrate solution containing solution A (5% sodium carbonate) and solution B (0.2% silver nitrate, 0.2% ammonium nitrate, 0.5% tungstosilicic acid, 0.15% formaldehyde, and 5% sodium carbonate) [34,35]. After the appearance of a light grey color, the slides were washed with deionized water four times and exposed to a stop solution containing 1% acetic acid for 5 min, washed with deionized water and stored in opaque boxes at 4°C until microscopical analysis.

Five slides per group for each exposure period were microscopically examined using the B-600Ti Optika light microscope equipped with a 4083.B5 OptikamB5 digital camera (Optika Microscopes, Italy), and cells were classified visually from class 0 (cell with no damage) to class 4 (maximally damaged cell) according to the size of the head (nucleus) and the length and intensity of the tail (Fig. 1). Class 0 (normal nucleus with no tail) and Class 1 (halo around the nucleus) corresponded to cells without significant DNA damage. Classes 2-4 corresponded to a gradual increase in the length and intensity of the

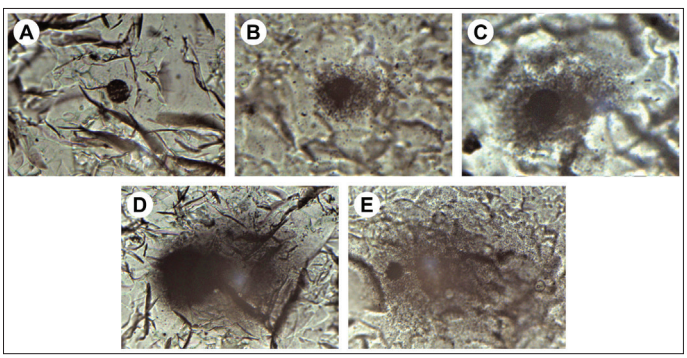

Fig. 1. Classification of *G. roeseli* hemocytes in the comet assay according to the size of the head (nucleus) and the length and intensity of the tail. Class 0 – no damage (**A**); class 1 – minimal damage (**B**); class 2 – moderate damage (**C**); class 3 – high damage (**D**); class 4 – maximal damage (**E**). Stain is silver nitrate. Original magnification is 400 ×.

comet tail evolving in parallel with a decrease in the nuclear DNA content, pointing to a gradual increase in DNA damage. One hundred cells from randomly selected microscopic fields were independently classified by each of two blinded observers per slide. The results from two independent classifications were then averaged to obtain more accurate results. DNA damage was expressed in arbitrary units (AU) calculated based on the classification of cells according to the formula proposed by Collins [36]:

$$Arbitrary\ Units\ (AU)=\sum N_i\ x\ i$$

where N_i is the number of cells classified in each class and i is the class number (0, 1, 2, 3 and 4).

Locomotor activity

For locomotor activity evaluation, three replicates were used per group and each replicate consisted of 10 *G. roeseli* adults (9-10 mm in length) selected randomly from the stock aquaria. At 120- and 240-h periods of sublethal exposure, the moving *G. roeseli* individuals in each beaker in which a piece of the net was added to provide a resting surface for the animals [29] were counted 20 times for 2 s each. Special attention was devoted so that the counting process was performed during the same period of day with similar light conditions and without disturbing the animals [29,30].

Statistical analysis

The LC_{50} values of tau-fluvalinate for different exposure periods (24, 48, 72 and 96 h) and their 95% confidence limits were calculated in SPSS 21 (SPSS Inc., Chicago, IL, USA) using the probit analysis method. Other statistical analyses were conducted in Minitab 19 (Minitab Inc., State College, PA, USA). The Anderson-Darling test was performed to test for normality of DNA damage and locomotor activity data. As the data were not normally distributed (for both DNA damage and locomotor activity), the non-parametric Kruskal-Wallis test followed by the pairwise Mann-Whitney test were used to evaluate the statistical differences among the groups (concentrations) and the exposure periods. Multiple regression analysis was performed to explain how independent variables (concentration and exposure period) are numerically associated with the dependent variable (DNA damage or locomotor activity). Correlations between DNA damage and both the concentration and exposure period, as well as between locomotor activity and concentration, were tested by Pearson's correlation. The significance for all statistical analyses was defined as $P<0.05$.

RESULTS

Acute toxicity

In the current study, acute toxicity of tau-fluvalinate to *G. roeseli* was evaluated at concentrations of 0.5-200 μg/L. Because the mortality rate in the control beaker did not exceed 10% after the test period (96 h), all the tests were considered valid. The mortality rate was observed to increase with both increasing concentration and increasing exposure period (Supplementary Fig. S2). Using the obtained mortality data, the 24-, 48-, 72- and 96-h LC_{50} values were calculated (Table 1). Accordingly, the 96-h LC_{50} value of tau-fluvalinate for *G. roeseli* was found to be 17.29 μg/L.

Table 1. The LC_{50} values of tau-fluvalinate and their 95% confidence intervals at different exposure periods for *G. roeseli*.

Exposure period	LC_{50} (μg/L)	The 95% confidence interval
24 h	34.82	7.08-68.11
48 h	28.38	13.03-50.875
72 h	21.79	9.65-37.55
96 h	17.29	6.15-30.78

Genotoxicity

DNA damage values of the groups at different exposure periods are presented in Table 2. Increased DNA damage ranging from 19.40 to 121.11% in groups 2, 3 and 4 compared to group 1 was detected at three exposure periods. When DNA damage values of the groups at equivalent exposure periods were statistically analyzed, significant differences among the groups were found ($P<0.001$) (Table 2). At each of the exposure periods, the highest DNA damage was observed in group 4 and the lowest in group 1 ($P<0.05$). When the values for the exposure periods in equivalent groups were statistically analyzed, significant differences among the exposure periods were found in groups 2,

Table 2. DNA damage in hemocytes of *G. roeseli* from different groups at different exposure periods.

Groups	DNA damage (AU)			Kruskal-Wallis test		
	24-h exposure	96-h exposure	240-h exposure	H	df	P value
Group 1	154.60±6.95^{d}	155.70±2.68^{d}	151.60±2.10^{d}	3.84	2	>0.05
Group 2	184.60±14.11$^{c, C}$	216.30±16.80$^{c, B}$	246.60±5.50$^{c, A}$	11.58	2	0.003
Group 3	264.20±4.63$^{b, B}$	299.70±7.60$^{b, A}$	300.90±14.41$^{b, A}$	9.38	2	0.009
Group 4	321.00±9.66$^{a, B}$	324.60±4.87$^{a, B}$	335.20±6.70$^{a, A}$	8.66	2	0.013
H	17.86	17.90	17.87			
df	3	3	3			
P value	<0.001	<0.001	<0.001			

Values are expressed as the mean±SD of five replicates. Means that do not share a superscript lowercase letter within the same exposure period are significantly different (P<0.05). Means that do not share a superscript uppercase letter within the same group are significantly different (P<0.05).

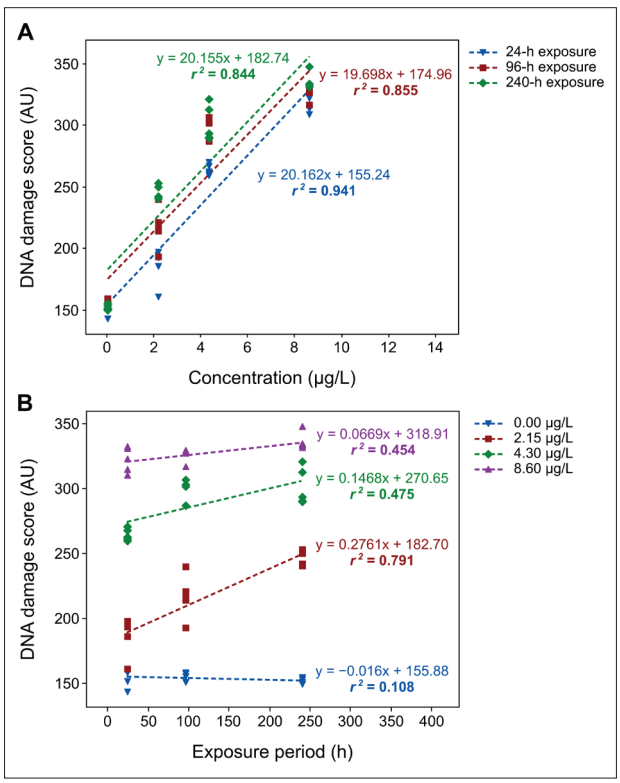

Fig. 2. Correlation between DNA damage score and tau-fluvalinate concentration (**A**) and exposure period (**B**).

3 and 4 (P<0.05) but not in group 1 (P>0.05) (Table 2). The highest DNA damage was observed at 240-h exposure for each of groups 2, 3 and 4; this was statistically significant for groups 2 and 4 (P<0.05) but not for group 3 in which DNA damage at 240-h exposure was not significantly different from that at 96-h exposure (P>0.05). The lowest DNA damage was observed at 24-h exposure for each of groups 2, 3 and 4; this case was statistically significant for groups 2 and 3 (P<0.05) but not for group 4 in which DNA damage at 24-h exposure was not significantly different from that at 96-h exposure (P>0.05).

Multiple regression analysis revealed statistical significance between the DNA damage and both the concentration and the exposure period (P<0.001 for the concentration and P<0.01 for the exposure period), indicating a linear relationship between dependent and independent variables with a strong effect size (r^2=0.878). The multiple regression equation for DNA damage based on the concentration and exposure period was found as: D=156.77+0.1185 E+20.01 C, where D is the DNA damage, E is the exposure period and C is the concentration. DNA damage was found to increase with increasing concentration and lengthened exposure period; however, concentration had a larger effect on DNA damage than exposure period. Also, there was a positive correlation between DNA damage in hemocytes and tau-fluvalinate concentration at all exposure periods (P<0.001) (Fig. 2A) and the exposure period at all concentrations (P<0.01) except for 0 µg/L (P>0.05), at which a temporal decrease in DNA damage was observed (Fig. 2B), indicating a significant increase in DNA damage with increasing concentration and lengthened exposure period. The correlation between DNA damage and concentration was very strong at all exposure periods (r=0.970 at 24-h exposure, r=0.925 at 96-h exposure and r=0.918 at 240-h exposure). The correlation between DNA damage and the exposure period was weak at 0 µg/L (r=-0.329), very strong at 2.15 µg/L (r=0.889) and strong at 4.30 µg/L (r=0.689) and 8.60 µg/L (r=0.673).

Locomotor activity

When locomotor activity data of the groups at equivalent exposure periods were statistically analyzed, significant differences among the groups were found (P<0.001) (Table 3). At 120-h exposure, locomotor activity decreased significantly in groups 2, 3 and 4 (by 6.87, 29.67 and 35.71%, respectively) compared to group 1. At 240-h exposure, these decreases were more pronounced: locomotor activity decreased significantly

in groups 2, 3 and 4 (by 41.64, 53.42 and 61.64%, respectively) compared to group 1. When the data for the exposure periods in equivalent groups were statistically analyzed, significant differences between the two exposure periods were found in groups 2, 3 and 4 ($P < 0.001$) but not in group 1 ($P>0.05$) (Table 3). Accordingly, the locomotor activity of *G. roeseli* individuals in group 1 remained unchanged between 120- and 240-h exposure periods. However, locomotor activity at the 240-h exposure period decreased significantly in groups 2, 3 and 4 (by 37.17, 33.59 and 40.17%, respectively) compared to the 120-h exposure period.

Table 3. Locomotor activity of *G. roeseli* from different groups at 120- and 240-h exposures.

	Locomotor activity (%)	
Groups	**120-h exposure**	**240-h exposure**
Group 1	60.67±11.63[a]	60.83±11.54[a]
Group 2	56.50±10.05[b]	35.50±9.64[b*]
Group 3	42.67±10.23[c]	28.33±8.06[c*]
Group 4	39.00±9.51[c]	23.33±8.37[d*]
H	105.31	147.27
df	3	3
P value	<0.001	<0.001

Values are expressed as the mean±SD of three replicates. Means that do not share a superscript lowercase letter within the same exposure period are significantly different ($P<0.05$). *Means within the same group that are significantly different ($P<0.001$).

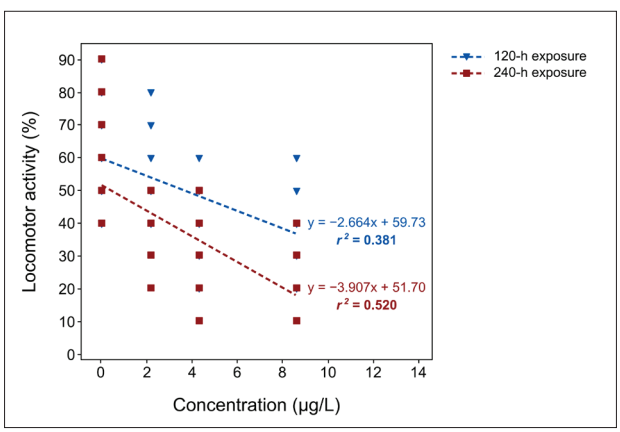

Fig. 3. Correlation between the locomotor activity of *G. roeseli* individuals and the concentration of tau-fluvalinate.

Multiple regression analysis revealed statistical significance between the locomotor activity of *G. roeseli* individuals and both the concentration and the exposure period ($P<0.001$), indicating a linear relationship between dependent and independent variables with a moderate effect size ($r^2=0.528$). The multiple regression equation for locomotor activity based on the concentration and the exposure period was found as follows: $L=74.78-0.1059\ E+3.286\ C$, where L is the locomotor activity, E is the exposure period and C is the concentration. Locomotor activity was found to decrease with increasing concentration and longer exposure period; however, concentration had a larger effect on locomotor activity than the exposure period. Also, for two exposure periods there was a strong negative correlation between the locomotor activity of individuals and the concentration of tau-fluvalinate ($P<0.001$; $r=-0.617$ at 120-h exposure and $P<0.001$; $r=-0.721$ at 240-h exposure), indicating a significant decrease in locomotor activity with increasing concentration (Fig. 3).

DISCUSSION

Pesticides are of environmental concern because of their widespread use in agricultural activities. The pyrethroids were developed in the mid-1970s to enhance the effectiveness of pyrethrins [6]. Despite their similarity in structure to the pyrethrins, the pyrethroids are generally more toxic to insects as well as to mammals and last longer in the environment than the pyrethrins [37]. Non-target species inhabiting water catchments of agricultural areas are potentially at risk if they have similar toxicant receptors as target organisms. The pyrethroids are very toxic to non-target aquatic organisms, and arthropods are particularly sensitive to them [38]. Given that insects are more closely related to crustaceans than they are to any other arthropods [39], crustaceans are likely to present a potentially suitable non-target organism group for investigating the potential effects of the pyrethroids. In this regard, this study provides valuable information regarding the possible non-target effects of tau-fluvalinate on *G. roeseli*.

Toxicological bioassays can track the susceptibility of test organisms to chemical materials. In these bioassays, the LC_{50} values of the chemicals are determined, and the tested chemicals are classified based on their detected LC_{50} value. For this classification, toxicologists generally use five toxicity categories. These categories are as follows: practically non-toxic (the LC_{50} values of more than 100 mg/L), slightly toxic (the LC_{50} values of 10-100 mg/L), moderately toxic (the LC_{50}

values of 1-10 mg/L), highly toxic (the LC_{50} values of 0.1-1 mg/L), and extremely toxic (the LC_{50} values of less than 0.1 mg/L) [40]. The LC_{50} value does not indicate an acceptable concentration in the environment; it is generally used as an indicator of relative acute toxicity [15]. It is well known for any given substance that there is a negative relationship between the LC_{50} value and acute toxicity. In the current study, the LC_{50} value of tau-fluvalinate was observed to decrease with lengthened exposure period. This finding is in agreement with the general pattern for insecticides [41] and shows that the toxicity of tau-fluvalinate considerably increases with time, making the long-term existence of tau-fluvalinate in aquatic ecosystems a noteworthy threat. The LC_{50} value of tau-fluvalinate at all exposure periods was determined to be lower than 0.1 mg/L. Considering the five toxicity categories mentioned above, tau-fluvalinate could be classified as extremely toxic to *G. roeseli*. Consistent with this, the high toxicity of tau-fluvalinate has been previously observed in aquatic organisms [42,43]. However, chronic exposure to sublethal concentrations can also produce toxic effects, and these effects may pose a hazard for aquatic organisms as well. Therefore, chronic toxicity tests at sublethal concentrations should be conducted for *G. roeseli*, and endpoints such as growth and reproduction should also be studied for not only this species but other non-target species; this could contribute to an acceptable prediction of the total impact of this insecticide on any given environment and reliable risk assessment in receiving aquatic ecosystems.

Aside from the acute lethal effect of tau-fluvalinate, its genotoxic effect on hemocytes of *G. roeseli* was also investigated for the first time in the current study. DNA damage caused by DNA single- and double-strand breaks, DNA adduct formations, DNA-DNA and DNA-protein cross-links resulting from the interaction of a pesticide or its metabolites and DNA is an important index in genotoxicity assessment of environmental agents to an organism [44]. Evaluation of DNA damage in hemocytes is employed extensively to assess the impact of environmental factors/pollutants on invertebrates, since hemocytes are closely exposed to environmental agents due to their important role in the immunological response. The alkaline comet assay used in this study enables the evaluation of wide types of DNA damage, such as DNA single- and double-strand breaks, oxidatively induced base damage, DNA cross-links, alkali-labile sites and incomplete DNA repair sites [33,45]. Taken together, the DNA damage results of this study are comprehensive and reliable.

In the present study, the use of tau-fluvalinate was found to cause a significant increase in DNA damage in hemocytes of *G. roeseli*, pointing to the genotoxic potential of this insecticide for the species. Consistent with this finding, several previous studies have reported genotoxic effects of various pyrethroids such as tau-fluvalinate (in hemocytes of honey bee *Apis mellifera*) [9], cypermethrin (in hemocytes of silkworm *Philosamia ricini*) [46], permethrin (in human erythrocytes and lymphocytes) [47] and phenothrin (in human blood peripheral lymphocytes and hepatocytes) [48]. The detailed evaluation undertaken in this study showed a concentration-dependent increase in DNA damage. The effect of increasing concentration of tau-fluvalinate on DNA damage was the greatest at the 24-h exposure period. Also, the effect of a longer exposure period on DNA damage is the most pronounced at the concentration of 2.15 μg/L. Based on these inferences, tau-fluvalinate appears to be a genotoxic insecticide even at low concentrations and short-term exposures. However, for a better understanding of all aspects of toxicity mechanisms of tau-fluvalinate in the potential non-target organisms, further studies are necessary to explore its effects and action mechanisms, as well as to characterize its genotoxic properties at the cellular level.

For decades, different animal behaviors have been used increasingly as a sensitive and integrated indicator of sublethal exposure to toxicants [29,30,49,50]. In this respect, determining the sensitivity of different behavioral endpoints as indicators of exposure to various toxicants comes into prominence. Locomotor activity as a behavioral endpoint in *G. roeseli* was evaluated comprehensively, and a significant reduction in locomotor activity with increasing concentration of tau-fluvalinate and a longer exposure period was detected. Also, the concentration-dependent reduction observed in locomotor activity was more pronounced at 240-h exposure. This study demonstrates the locomotor activity-reducing effect of tau-fluvalinate in *G. roeseli*, which is in agreement with several previous studies in which locomotor activity of gammarids was reported to be decreased by the pyrethroid insecticide cypermethrin [31] and several chemicals/toxicants

such as certain pharmaceuticals [51], heavy metals [29,32] and nanoparticles [30,52].

Evaluation of locomotor activity at the individual level may provide indirect information on the potential effects of exposure to tau-fluvalinate on ecologically important behaviors such as feeding and locomotor activity that are closely associated. The reduction in locomotor activity observed in the present study may be attributed to reduced feeding activity due to tau-fluvalinate. It is known that physiological impairments in individuals reduce their ability to move and feed. Given that reduced feeding possibly alters energy provision, reduced locomotor activity may result from a reduction of energy allocation to the locomotor activity of *G. roeseli*. Monitoring locomotor activity could allow us to make interpretations both at the individual level and at the population level. Locomotion is an ecologically relevant behavioral marker and necessary for essential activities such as foraging, finding a mate and fleeing predators. It is suggested that tau-fluvalinate, by decreasing locomotor activity, could reduce the fitness and survival of *G. roeseli* individuals, posing a noteworthy threat to the species at the population level, possibly resulting in the extinction of this species when exposed to high concentrations, especially at long-term exposures. Taken together, the results of this study indicate that locomotor activity could be an effective and sensitive behavioral marker in *G. roeseli* to assess the health of organisms and freshwater ecosystems under the risk of contamination with pesticides like tau-fluvalinate. However, additional behavioral endpoints should be tested for their sensitivity in the indication of exposure to tau-fluvalinate, and further comprehensive studies on behavioral responses of *G. roeseli* exposed to this insecticide should be conducted; this could contribute to a better and broader assessment of the potency of tau-fluvalinate.

CONCLUSIONS

In this study, potential harmful lethal and sublethal effects of the pyrethroid tau-fluvalinate on the non-target organism *G. roeseli* were examined for the first time. It was found that the 96-h LC_{50} value is 17.29 μg/L and that tau-fluvalinate causes a significant increase in DNA damage and a significant reduction in locomotor activity at the tested sublethal concentrations. In addition, both increasing concentration and a longer exposure period result in an increase in DNA damage but a decrease in locomotor activity; however, insecticide concentration has a larger effect on both DNA damage and locomotor activity than the exposure period. The results suggest that long-term existence of tau-fluvalinate in aquatic environments at high concentrations is a noteworthy threat for this species. Therefore, the use of tau-fluvalinate in agricultural activities should be reconsidered to minimize the risk of exposure and subsequent health effects. For a better understanding of the toxicity mechanisms of this insecticide in the potential non-target organisms, further studies, particularly at the cellular level, are recommended. With an overall approach, this study provides compelling evidence regarding the suitability of *G. roeseli* for toxicological and genotoxic tests as well as the effectiveness and sensitivity of locomotor activity as a behavioral marker to assess the health of organisms and freshwater ecosystems under the risk of contamination with pesticides such as tau-fluvalinate.

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Data availability: All data underlying the reported findings have been provided as part of the submitted article and is available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Sari_8068_Data.Report.pdf

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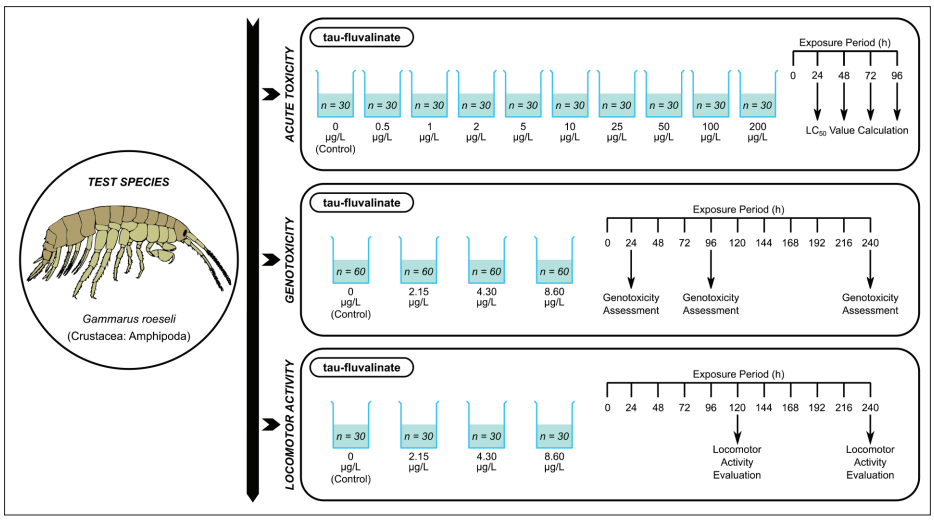
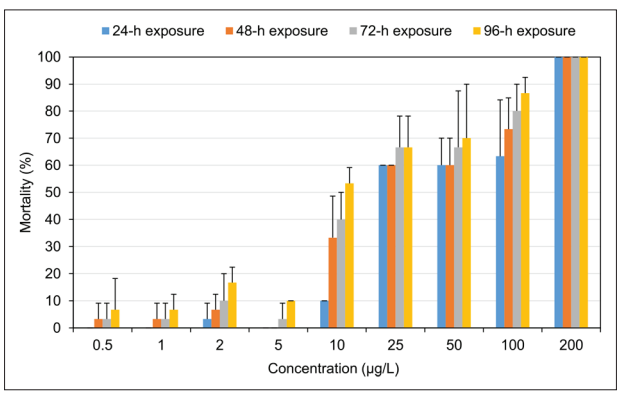
Supplementary material



Supplementary Fig. S1. Schematic outline of the experimental design and procedures applied in the study.



Supplementary Fig. S2. Effects of different concentrations of tau-fluvalinate on the mortality of *G. roeseli* at different exposure periods.