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**RESEARCH ARTICLE** **ARAŞTIRMA MAKALESİ**

# Determinations of the effects of cyfluthrin on the hemocytes parameters of freshwater mussel (*Unio delicatus*)

## Tatlı su midyesinin (*Unio delicatus*) hemosit parametreleri üzerine Cyfluthrinin etkilerinin belirlenmesi

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**Abstract:** The overuse of pesticides has been increasing since the 20th century. Depending on this use, non-target organisms are also affected apart from target organisms. Cyfluthrin is a synthetic pyrethroid pesticide used in agriculture, domestic and veterinary medicine against insects. It may also affect non-target aquatic organisms as a result of mixing with aquatic ecosystems. This study was aimed to investigate the effect of cyfluthrin on freshwater mussels, one of the aquatic invertebrate species, with hemocyte parameters. Total hemolymph counts, hemolymph cell morphology, and differential hemocyte counts were performed from hemolymph taken from mussels exposed to different cyfluthrin doses exposures for 24 and 48 hours. Compared to the control group, the total hemocyte counts of the experimental groups were found to increase in 24h and decrease in 48h significantly ($p<0.05$). In the examination of hemocyte morphologies, granular, semi granular, and hyalinocyte cells were observed. Similar values of differential hemocyte counts were found both 24 and 48h exposure times. As a result, in aquatic toxicology studies, besides total hemocyte count, analysis of hemocyte morphologies and differential hemocyte counts are found to be good biomarkers.

**Keywords:** Cyfluthrin, hemolymph, total hemocyte counts, hemocyte morphology, differential hemocyte counts

**Öz:** Pestisitlerin aşırı kullanımı 20. yüzyıldan beri artan bir şekilde devam etmektedir. Bu kullanıma bağlı olarak hedef organizmalar dışında hedef olmayan organizmalar da etkilenmektedir. Cyfluthrin tarımda, evsel alanda ve veteriner sağlığında böceklere karşı kullanılan bir sentetik piretroit pestisittir. Sucul ekosistemlere karışması sonucunda hedef dışı sucul organizmalar üzerinde de etki gösterebilir. Bu çalışmada, sucul omurgasız türlerinden biri olan tatlı su midyelerinde cyfluthrinin etkisi hemosit parametreleri ile incelenmesi amaçlanmıştır. 24 ve 48 saat süre ile farklı cyfluthrin maruziyetine bırakılan midyelerden alınan hemolemften total hemosit sayısı, hemolemf hücre morfolojisi ve diferansiyel hemosit sayımı yapılmıştır. Kontrol grubuna göre, deney gruplarının total hemosit sayıları önemli ölçüde değişkenlik gösterdiği bulunmuştur ($p<0.05$). Hemosit morfolojilerinin incelenmesinde ise granüllü, yarı-granüllü ve hiyalinosit hücreleri gözlenmiştir. Ardından yapılan diferansiyel hemosit sayımı ile 24 ve 48 saatlik maruziyet sonucunda benzer değerler bulunmuştur. Sonuç olarak, sucul toksikoloji çalışmalarında total hemosit sayısının yanı sıra hemosit morfolojilerinin incelenmesi ve diferansiyel hemosit sayıları da iyi bir biyobelirteçtir.

**Anahtar kelimeler:** Cyfluthrin, hemolemf, toplam hemosit sayısı, hemosit morfolojisi, diferansiyal hemosit sayısı

## INTRODUCTION

Pyrethroids, synthetic insecticides, are made of pyrethrins that are naturally produced from the flowers of *Chrysanthemum cinerariaefolium* (Soderlund et al., 2002). The first compound of the pyrethroids is allethrin that was produced in 1949 and commercialized in 1952. Then, tetramethrin, resmethrin, phenothrin, permethrin, fenvalerate, deltamethrin, cypermethrin were synthesized and marketed in the 1970s. During the 1980s, bifenthrin, cyfluthrin, and other second-generation pyrethroids were made and started to use for against corps, public health, and household application (Palmquist et al., 2012). Even though the natural pyrethrums consisting of pyrethrins, cinerins and jasmolins were used against insects in the 1800s (Palmquist et al., 2012), pyrethroids have been widely applied in agricultural and household areas since the 1970s (Soderlund et al., 2002).

The mechanism of pyrethroids is the disruption of the peripheral nervous system of insects. Their main effects on sodium-voltage channels and hence they cause paralysis. According to their mechanism of action, pyrethroids are divided into two groups: Type 1 and Type 2 (Palmquist et al., 2012; Williams et al,. 2018). Exposure to the first causes restlessness, overstimulation and body shake whereas the last causes hyperactivity, incoordination, and writhing. Among pyrethroids compounds, Type 1 consists of allethrin, bifenthrin, d-phenothrin, permethrin, resmethrin, and tetramethrin while Type 2 includes many of the second generation pyrethroids such as cyhalothrin, cypermethrin, cyfluthrin, and deltamethrin (Palmquist et al., 2012).

Cyfluthrin (Cas Number: 68359-37-5; $C_{22}H_{18}Cl_2FNO_3$) is widely used for various pests in agriculture, household, veterinary, and food industry. After hypersensitiveness of the insects, it causes convulsion and death. It affects either on depolarization of sodium-voltage channel or calcium transport mechanism on the cell membrane (Verma et al., 2021).

Cyfluthrin is detected in different parts of aquatic environments. Duavi et al. (2021) found that cyfluthrin concentration in water and sediment samples of the Ceara River (CE, Brazil) were from 15.60 to 178.35 ng/L and from 5.75 to 55.45 ng/g, respectively. Cryder et al. (2021) found that the mean concentration of cyfluthrin ranged from 13.8 to 455 ng/L in the water, from 0.263 to 52.4 ng/g in the sediment, and from non-detected to 3.18 ng/g in the plant samples of Prado Wetlands (California, USA). Lizotte et al. (2021) found that cyfluthrin was detected 16-18 µg/kg in sediment samples from Mississippi Stream Bayous. They also found that *Hyelalla azteca* were exposed to collected sediment for 28 days and the residues of cyfluthrin in its tissue were found between 179 and 1003 µg/kg. In addition to being detected in the aquatic ecosystem, it has also been found to have toxic effects on aquatic organisms such as green algae (Sáenz et al., 2012), mussels (Serdar, 2021), and fish (Selvi et al., 2008; Sepici-Dinçel et al., 2009; Farag et al., 2021) in laboratory experiments.

Aquatic invertebrates have cellular and humoral defence mechanism against to pathogens, pollutants (Ciacci et al., 2009). The blood tissue of invertebrates is known as hemolymph that is an important bioindicator parameter of the species health status (Günal et al., 2018). Hemocytes, blood cells, are responsible for cellular defence mechanism via phagocytosis, release of lysosomal enzymes and etc (Ciacci et al. 2009).

Aquatic invertebrates have been affected by alterations of abiotic parameters including salinity (Nunes et al., 2021; Pérez–Velasco et al., 2021), temperature (Matoo et al. 2021; Wu et al., 2021), pollutants (Baussant et al., 2009; Tresnakova et al., 2020; Günal et al., 2021) and by biotic factors such as bacteria (Canesi et al., 2002; Ciacci et al. 2009), invasive species (Berber et al., 2018) in the aquatic environment. Therefore, their immune system may be also affected by these factors.

Freshwater mussels have been effectively used for biomonitoring studies of aquatic systems. Due to their filter-feeding characteristics, they reflect the environment where they inhabit (Negishi et al., 2013; Lundquist et al., 2019). Therefore, they are bioindicator organisms many studies including biomonitoring (Wagner and Boman 2004), biodiversity (Bolotov et al., 2020), and aquatic toxicology (Gillis et al., 2004; Machado et al., 2014; Yurdakok-Dikmen et al., 2018; Tresnakova et al., 2020; Arslan et al., 2021a, 2021b).

The freshwater mussel organism in the current study is *Unio delicatus* Lea, 1863. It is one of the most common mussel species in the southwest to east Anatolia in the large river basins. Due to the distribution areas, it is an indicator species in biomonitoring, ecotoxicological, and phylogenetic studies (Lopes-Lima et al., 2021). The current study was aimed to investigate immunological response of non-target organisms, freshwater mussels (*Unio delicatus*) via the total hemocyte counts, differential hemocyte counts, and hemocyte morphology of exposure different concentrations of cyfluthrin.

## MATERIALS AND METHODS

The freshwater mussels *Unio delicatus* were obtained from fishermen from Gölbaşı Lake in Adıyaman (Turkey) situated in the southern of Anatolia. It has rich biodiversity and also located in the migration routes of birds between Africa and Europe (Alkan Uçkun, 2018). The species were taken to the laboratory in the aerated water. The mussels were brought to the laboratory and were placed in 15 L aquariums. Before starting the experiments, the mussels were adapted to the laboratory conditions for 2 weeks. The dechlorinated municipal tap water was used in the aquariums and the temperature was kept constant with thermostat heaters. The water parameters are as follows: the mean temperature as 21.4±1.21°C, the mean dissolved oxygen as 5.46±0.17 mg/L, the mean conductivity as 223.15±0.48 mS/cm, the mean pH as 7.70±0.03. During the adaptation period, they were fed by *Chlorella* sp. The aquariums water were changed every two day.

Cyfluthrin was prepared via solvent (dimehtylsulfoxide, DMSO) in stock concentration as 10.05 mg/L. Then, it was diluted with water 1.05, 0.105, and 0.0105 mg/L.

In the experiment, five aquariums consisting 10 L water in 15 L aquariums were used. Ten mussels were randomly placed in the aquarium (n=50). The mean weight, mean length, mean thickness, and mean height of mussels were 31.59±4.8 g, 4.87±0.43 cm, 1.18±0.30, and 1.88±0.17 cm, respectively. Three of the aquariums were the experiment groups (1.05, 0.105, and 0.0105 mg/L cyfluthrin, CF) and two of them were the control groups (control and solvent control (DMSO)). The acute toxicity test was applied for 24 and 48 hours. The cyfluthrin concentrations in the exposed groups were analysed according to AOAC Official Method 2007.01 by LC-MS/MS (AOAC 2007). The results were within acceptable limits of nominal concentrations. At the end of each exposure period, 5 mussels were taken from the aquariums and hemolymph samples (about 1 mL) were taken by stimulating the adductor muscle with the help of a 2.5 mL sterile syringe.

Total hemocyte counts (THCs) were made using hemocytometer according to Yavuzcan and Benli (2004). After taking hemolyph, some of the hemolymph samples (around 200 µL) was mixed with 4% formaldehyde fixative solution (1:1) and then the mixed hemolymph samples were counted in Thoma counting chambers. Every hemolymph specimen was prepared and counted three times.

Among morphological analysis of hemocytes, hemocyte types and differential hemocyte counts were detected. The hemolymph samples were stained Giemsa dye according to

Silva et al., (2002). After the hemocyte types were analyzed under the light microscope, the differential hemocyte counts were carried out to count four different parts of the slides. Lysosomal observations were made Neutral Red staining according to Matozzo and Marin (2010).

The results were evaluated by using a non-parametric statistical method (Kruskal Wallis) via the GraphPad Prism 5 program. The percentage of differential hemocyte counts was calculated via the Microsoft Excel 13 program. The cells diameters were determined by using the ImageJ program.

## RESULTS AND DISCUSSION

The blood parameters of invertebrates are very important to detect the health status of the species as well as the environment where they inhabit. Exposure to environmental pollutants, adverse effects including abnormal offsprings (Saidov and Kosevic 2021), deformation in their morphology (Banumathi et al., 2017) were detected. In the short- and long- term exposure, the blood parameters such as total hemocytes counts, differential hemocyte counts are used for the immunological investigations of the species (Matazzo and Marin 2010; Günal et al., 2018; Ayhan et al., 2021). Due to exposure to pollutants, the immune system of species were effected, and hence their total hemocyte counts increase (Katalay et al., 2019).

The health assessment of aquatic invertebrates generally occurred lethal tissue collection from species for alterations histopathological (Costa et al., 2013; Larguinho et al., 2014; Yee-Duarte et al., 2018; Noleto et al., 2021) and enzymatic (Baussant et al., 2009; Larguinho et al., 2014; Noleto et al., 2021) analysis. These methods have resulted in the impacts on the habitat status of aquatic invertebrates. In some cases, the investigated species may be threatened/endangered due to the decrease in the number of specimens in the habitat. Thus, rapid techniques and the use of a small number of animals are required to study the health status of aquatic organisms (Gustafson et al., 2005). Therefore, in this study, the number of individuals was used to examine the hemolymph parameters, which is one of the physical parameters of the mussels, to be low.

In the present study, the acute effects of cyfluthrin on the freshwater mussels were detected via hemocyte parameters. Differential hemocyte parameters were examined after the determination of the total hemocyte counts used in many studies in which pollutant research was carried out. By examining the cells morphologically, it was determined whether the granules in the hemocytes were lysosomes.

The values of THCs of the freshwater mussels exposed to cyfluthrin for 24h and 48h were given in Figure 1. At 24-hour exposure, hemocyte counts significantly increased compared to control groups ($p<0.05$). In the high concentration applied (1.05 mg/L), an increase of THCs occurs almost twice of the control group, while a decrease in the number of THCs occurs when the dose concentration is decreased. During the 48-hour exposure period, the THCs values observed at a high rate in the control group led to a decrease in half at the lowest concentration of CF (0.0106 mg/L). A decrease was observed in the exposure groups from high dose to low dose at 48 hours of exposure ($p<0.05$). However, the difference between exposure times did not affect on THCs ($p>0.05$). On the other hand, the alterations in THCs in this study were agreed with other studies exposed to different pollutants on aquatic invertebrates (Günal et al., 2018; Katalay et al., 2019; Tresnakova et al., 2020; Ayhan et al., 2021).

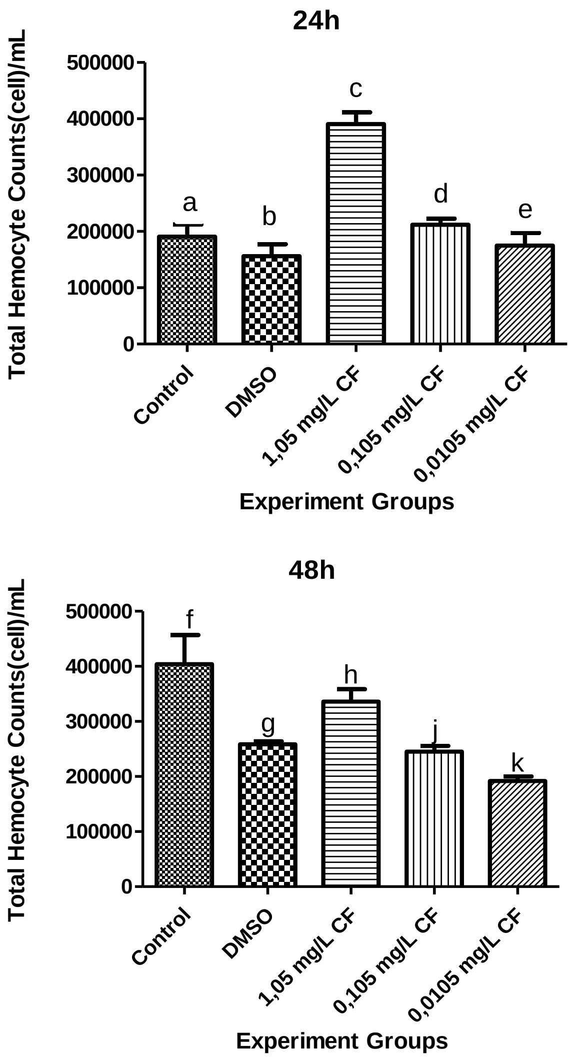


**Figure 1.** The values of THCs (±SEM) of *Unio delicatus* exposed to cyfluthrin for 24 and 48h and control groups. (Different letters shows the significant difference, $p<0.05$)

In the morphological examination of hemocyte types by light microscopy, three types of hemocytes were detected: granulocytes (Figure 2.A), semigranulocytes (Figure 2.B), and hyalinocytes (agranulocytes, Figure 2.C). The first has great cytoplasmic granules in the cells, the second has lots of cytoplasmic granules in the cells, and the third has no cytoplasmic granules. The granules in the cytoplasm of granulocytes and semigranulocytes were lysosomes and they were shown in Figure 2.D. The obtained hemocyte type results in the current study are similar to Matozzo and Marin (2010).

The mean diameters of granulocytes, semigranulocytes, and hyalinocytes in the control group were 15.97±1.42 µm, 10.48±0.87 µm, and 11.04±0.16 µm, respectively. Matozzo and Marin (2010) found that granulocytes (11.94 µm), semigranulocytes (12.38 µm), and hyalinocytes (7.88 µm) in the hemocyte cells of the crab *Carnicus aestuarii*. Bolognesi and Fenech (2012) observed that granular (8-12 µm) and agranular (3-4 µm) hemocytes in the hemolymph of the Mediterranean mussel *Mytilus galloprovincialis.* According to the results obtained from the control group in this study, the difference in hemocyte distribution and size from Matozzo and Marin (2010) and Bolognesi and Fenech (2012) may be due to the difference between species and the marine/freshwater species.

On the other hand, the mean diameters of granulocytes, semigranulocytes, and hyalinocytes in the exposed groups were 19.91±0.96 µm, 23.68±0.31 µm, and 27.30±1.20 µm, respectively. The diameter of the hemocytes in the control groups were smaller than the exposed groups. This may be related to phagocytosis of the contaminant by hemocyte cells.

In the exposure of Cyfluthrin for 24h and 48h, all the groups had semigranulocytes, granulocytes and hyalinocytes (Figure 3). The highest and lowest values of the cells obtained from the experiment groups are same in the 24h and 48h exposure time. For instance, the highest semigranulocytes were 80.24% in the 0.0105 mg/L CF group where the lowest were in the control group (23.73%) in the 24h exposure duration. Likewise, the highest semigranulocytes were 71.42% in the 0.0105 mg/L CF group where the lowest were in the control (31.42%) in the 48h. The highest granulocytes were 58.21% in the 0.105 mg/L CF group where the lowest were in the 0.0105 mg/L CF group (20%). The highest hyalinocytes were 16.9% in the control group where the lowest were in the 1.05 mg/L CF group (5.79%). Besides, the highest granulocytes were 57.89% in the 0.105 mg/L CF group where the lowest were in the 0.0105 mg/L CF group (19.05%). The highest hyalinocytes were 11.43% in the control group where the lowest were in 4.35% the 1.05 mg/L CF group.

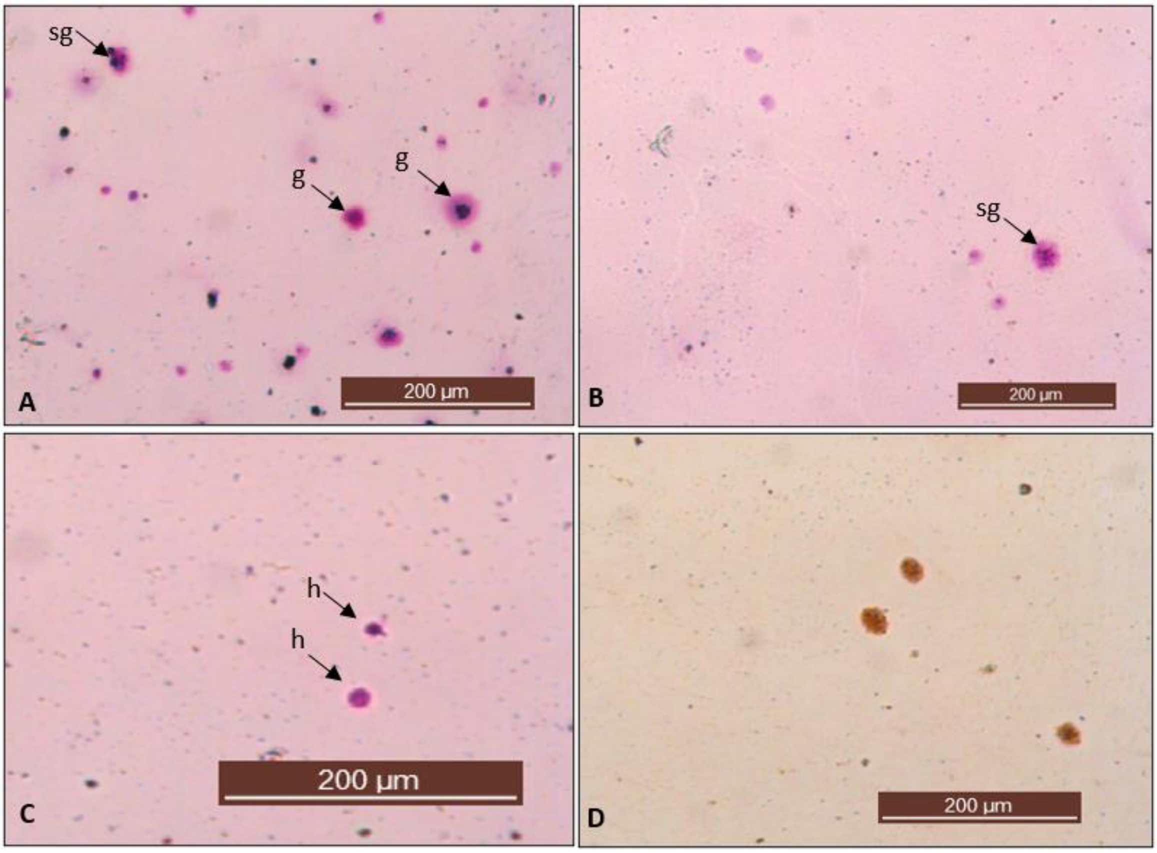


**Figure 2.** *Unio delicatus* hemocytes stained with Giemsa's dye (A-C; g: granulocytes, sg: semigranulocyte and h: hyalinocyte) and Neutral Red dye showing lysosomes in the cell (D).

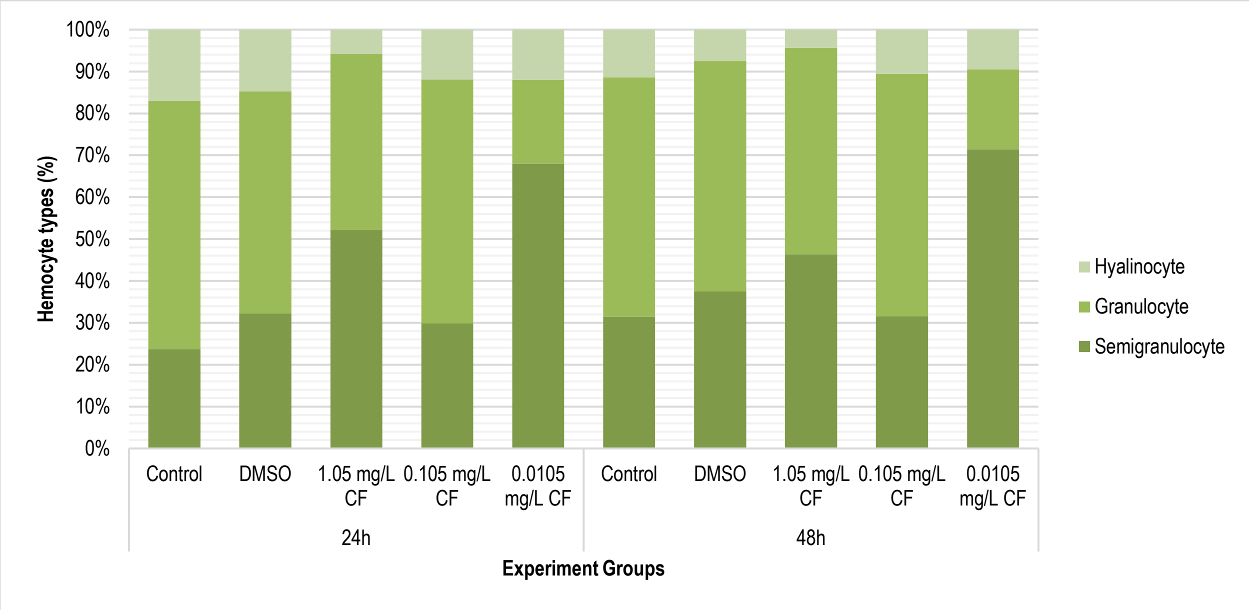


**Figure 3.** The percentage of hemocyte types in the experiment groups for 24h and 48h

## CONCLUSION

It has been found appropriate to use changes in the total hemocyte counts (THCs), which is one of the important physiological markers, in many studies investigating the effects of environmental pollutants in the aquatic toxicology. In this study, the decrease in the THCs due to cyfluthrin exposure indicates that the THCs parameter is a good indicator. The hemocyte cell types and differential hemocyte cell counts (DHCs) in hemolymph tissue were also investigated in the current study. The fact that hemocyte cells with granules, semigranules and agranules are of the same type as the cells in other studies in the literature. When the DHCs results between the experimental groups were examined, it was found that the cell counts were high or low within the same groups depending on the exposure times. Thus, it has been shown that differential hemocyte counts can be used in addition to the total hemocyte counts parameter in investigating the effects of environmental pollutants on aquatic invertebrates.

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