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Cardiovascular Toxicity in *Daphnia magna*: Heart Rate Analysis Under Exposure to Crystal Violet, Ethanol, and Formaldehyde

Daphnia magna'da Kardiyovasküler Toksisite: Kristal Menekşe, Etanol ve Formaldehit Maruziyette Kalp Atış Hızı Analizi

Türk Denizcilik ve Deniz Bilimleri Dergisi

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ABSTRACT

Water fleas have become valuable model organisms for ecotoxicity studies due to their ease of cultivation, transparent bodies, and high sensitivity to chemical pollutants. In this study, heart rates in Daphnia magna were examined to assess basic behavioral and physiological characteristics under exposure to known toxic chemicals. Crystal violet, ethanol, and formaldehyde selected for their distinct chemical properties and documented toxicity were utilized as toxic solutions. A slow-motion, video-based method was implemented to evaluate cardiovascular performance by monitoring heart rates in water fleas. Data for important parameters like heart rates, body reactions, and heart contraction were extracted from video recordings. It was found that crystal violet increased the heart rates of *Daphnia magna* (489±14.19) more than ethanol (450±40.67) and formaldehyde (445±48.21). Compared to the control group, formaldehyde caused a 28.51% increase in the heart rates of daphnids, while exposure to ethanol (30.54%) and crystal violet (35.89%) resulted in a lower increase. The response of water fleas to each of the three chemicals studied was determined as a statistically significant and noticeable increase in heart rate.

Keywords: Water flea, *Daphnia magna*, Heart rates, Crystal violet, Ethanol.

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ÖZET

Su pireleri, kolay yetiştirilebilmeleri, şeffaf vücut yapıları ve kimyasal kirleticilere karşı yüksek duyarlılıkları sayesinde ekotoksisite çalışmalarında değerli model organizmalar haline gelmiştir. Bu çalışmada, *Daphnia magna*'nın bilinen toksik kimyasallara maruz kaldığında temel davranışsal ve fizyolojik özelliklerini değerlendirmek amacıyla kalp atış hızları incelenmiştir. Kristal menekşe, etanol ve formaldehit, kendilerine özgü kimyasal özellikleri ve belgelenmiş toksisiteleri nedeniyle toksik çözelti olarak seçilmiştir. Su pirelerinin kalp atış hızlarını izleyerek kardiyovasküler performanslarını değerlendirmek için yavaş çekim, video tabanlı bir yöntem kullanılmıştır. Kalp atışları, vücut tepkileri ve kalp kasılması gibi önemli parametreler için veriler video kayıtlarından çıkarılmıştır. Kristal menekşenin, *Daphnia magna*'nın kalp atışlarını (489±14,19), etanol (450±40,67) ve formaldehit (445±48,21) ile kıyaslandığında daha fazla artırdığı bulunmuştur. Kontrol grubuna kıyasla, formaldehit su pirelerinin kalp atışlarında %28,51'lik bir artışa neden olurken, etanol (30,54%) ve kristal menekşenin (35,89%) maruziyeti daha düşük bir artış sonucunu doğurmuştur. Su pirelerinin her üç kimyasala verdiği yanıt, kalp atış hızında istatistiksel olarak anlamlı ve belirgin bir artış olarak tespit edilmiştir.

Anahtar sözcükler: Su piresi, *Daphnia magna*, Kalp atışları, Kristal menekşe, Etanol.

1. INTRODUCTION

Daphnia or daphnids, are zooplanktonic organisms belonging to the class Crustacea and the phylum Arthropoda, typically found in freshwater, with sizes ranging from 0.2 to 5 mm. *Daphnia* are also called water fleas due to their jerky and jumping swimming movements (Chevalier, 2014). *Daphnia* usually has a completely transparent outer shell, which makes its internal organs completely observable from the outside (Johnsen, 2001; Ebert, 2022). In this manner, physiological responses like heart rates in *Daphnia* can be observed within the organism's body without any external interference (Rosenkranz, 2010).

Daphnia reproduces by a method called cyclic parthenogenesis, which allows them to alternate between sexual and asexual reproduction depending on the surrounding environmental conditions (Dukić, 2016; Gerber, 2018). Due to these features, it is possible to generate a significant number of individuals in a lab setting, improving the repeatability of experiments. The feeding habits of *Daphnia* form the basis of the mechanism that makes them sensitive to environmental changes. This sensitivity is particularly crucial in monitoring water quality and assessing the impact of environmental pollutants (Altshuler *et al*., 2011; Kim *et al.* 2015; Rodrigues *et al*., 2022). *Daphnia* filter water by straining algae and small organisms, which they then digest. This behavior makes them extremely sensitive to the quality and content of the water. Due to their sensitivity to various xenobiotics and environmental stress, *Daphnia* have been used as bioindicators in research on toxicity and the bioaccumulation of microplastics since the 1990s (Suman *et al*., 2021; Sönmez *et al*., 2022; Atamanalp, 2024).

Water fleas, particularly species from the *Daphnia* genus, are widely regarded as ideal models organisms for ecotoxicity research (Greene *et al*., 2017; Norambuena *et al*., 2019; Guilhermino *et al*., 2000). Their suitability is due to their ease of cultivation, transparent bodies, and heightened sensitivity to chemical contaminants (Ahmed, 2023). Cardiovascular function indicators are frequently utilized to assess toxicity (Villegas-Navarro *et al*., 1999). However, accurately measuring heart rate and blood flow, while minimizing bias, can be difficult given the unique heart structure, blood flow characteristics, and the rapid nature of the heartbeat. In water fleas, blood is not confined to vessels but is instead pumped into a cavity known as the hemocoel, where it mixes with interstitial fluid and is referred to as hemolymph (Santoso *et al*., 2020). As the heart contracts and the organism moves, hemolymph circulates through the organs and re-enters the heart via small openings called ostia (Offem *et al*., 2008;

Chung *et al.*, 2016; Kundu and Singh, 2018). This circulatory process facilitates the exchange of gases and nutrients throughout the body of *Daphnia* species (Santoso *et al.*, 2020).
The fact that *Daphnia magna*'s heart is myogenic, meaning it can generate its own cardiac contractions without neural input similar to the human heart, makes the studies on this subject particularly noteworthy. This is particularly intriguing because, as an arthropod, *D. magna* typically possesses a neurogenic heart, which requires neural stimuli for contraction, making it an exception among its class (Angus-Whiteoak, 2018; Matveeva *et al.*, 2018). Previous research has examined the structure of the *D. magna* heart, revealing long striated myofibrils and a mostly single-cell-thick wall (Stein *et al.*, 1965). The use of *D. magna* for studying the effects of various substances is further justified by the fact that human cardiac muscle is also striated. Additionally, the organism's relatively transparent body allows for easy measurement of heart rates, providing a model that is comparable to the human heart (Perez *et al.*, 2019).
The study conducted by Villegas-Navarro *et al.* (2003) demonstrated that *Daphnia* is an effective model organism for toxicological and pharmacological research, particularly for examining the effects of substances on the cardiovascular system. The results suggest that analyzing *Daphnia*'s cardiac functions can reveal the toxicological effects of different substances. In the study by Kaas *et al.* (2009), *Daphnia magna* was used to investigate the cardiovascular effects of melatonin and ethanol. When both substances were combined, the decline in heart rates was even greater than expected. These findings confirm that *Daphnia* is a reliable model for cardiovascular pharmacology and toxicology studies.
Most studies in the field of ecotoxicology investigate broader endpoints such as mortality rates, reproduction, growth, and food consumption (Hellou, 2011). Molecular-level endpoints, such as enzyme activity or gene expression, are less frequently examined. However, the behavioral and physiological characteristics of an organism establish the connection between molecular-level endpoints and the organism itself (Connon *et al.*, 2012). The inhibitory effect of a contaminant on the feeding rate of *Daphnia magna* can be analyzed by exploring a sequence of endpoints, ranging from behavioral aspects (such as thoracic limb and mandible activity) to physiological responses (like gut peristaltic activity), and ultimately to molecular levels (including digestive enzyme activity and gene expression) (Lari *et al.*, 2017). This study aimed to investigate heart rates reflecting basic behavioral and physiological characteristics of *Daphnia magna* when exposed to known toxic chemicals.

2. MATERIALS AND METHODS

The research was designed as a three-phase experimental setup. For this purpose, data were obtained with 10 daphnids (n=10) without adding any factors and under control conditions. In the second stage, the reactions of daphnids to the selected chemicals and the changes in their heart rates over time were determined. At this stage, a special video program (Adobe Premiere Rush) and Image J Software were used.

2.1. Materials

Crystal violet ($C_{25}H_{30}N_3Cl$) is a cationic dye widely used in many industries such as textile, leather, paint, cosmetics and plastic. Studies have shown that crystal violet causes tumor formation in fish, reduces photosynthetic activation in aquatic plants and inhibits seed germination and growth in land plants (Mani and Bharagava, 2016). Crystal violet, which has high toxicity on mammals, has been reported in various studies to damage the skin, digestive and respiratory systems and to have carcinogenic effects in long-term exposure (Mani and Bharagava, 2016). Ethanol (C_2H_5OH) is a substance that is soluble in water and can easily dissolve oils. Thanks to these properties, it has a wide range of uses as a solvent, disinfectant and fuel. Studies have shown that ethanol has both acute and chronic toxicity (Li *et al.*, 2015; Paprocki *et al.*, 2022). Formaldehyde (CH_2O) is a colorless, flammable gas with a characteristic pungent odor. It is used for various purposes in many sectors such as textile, paint, food safety and plastic industry (Zhang, 2018). Formaldehyde, classified as "carcinogenic to humans" by the International Agency for Research on Cancer (IARC), possesses genotoxic properties and contributes to ozone layer depletion (McLaughlin, 1994; Protano *et al*., 2021).

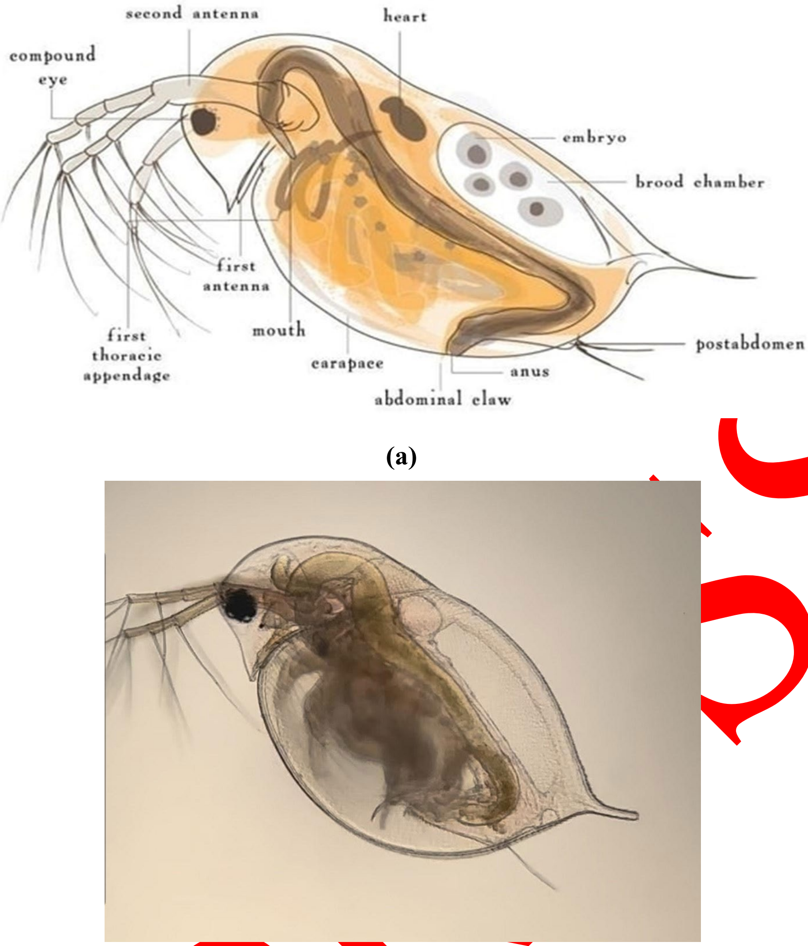
Crystal violet (CV) is a widely used chemical in microbiology studies because it causes bacterial wall destruction in the gram stain apparatus. Ethanol (E) is a toxic chemical commonly used in laboratory studies. It is preferred for environmental cleaning and analysis after microbiological experiments and is widely available for general use. First, behavioral responses and heart rates of water fleas were recorded at different concentrations of both solutions. Formadehyde (F) is frequently preferred in today's laboratories in studies aimed at preserving/preserving organism integrity (Sönmez and Sivri, 2016).

2.2. *Daphnia magna* culturing

A commercial strain of *D. magna* was cultured in freshwater at room temperature (22°C ± 1°C) with a photoperiod of 16 hours of light and 8 hours of darkness. The cultures were fed with $3.0x10^7$ cells/mL of green microalgae (*Spirulina* sp.) three times a week and with 0.1 mg/mL of dissolved baker's yeast once a week. To maintain dissolved oxygen (DO) concentrations above 4 mg/L, the cultures were aerated during the weekly experiments. The reconstituted water had the following physicochemical parameters: pH 7.5–8.5, hardness of 160–180 mg/L, conductivity of 250–600 µS/cm, and a temperature of 22°C. *Daphnid* density was maintained at approximately 40 neonates, 20 juveniles, and 12 to 16 reproducing adults per liter to avoid overcrowding. Under these conditions, daphnids reached maturity within 6 to 8 days after birth.

2.3. Heart rates of water fleas

The water flea *Daphnia*, a key branchiopod crustacean belonging to the order Cladocera, inhabits lotic ecosystems globally and is widely recognized as a model organism in the fields of ecology, evolution, and ecotoxicology (Ebert, 2022; Ngu *et al*., 2022). Additionally, *Daphnid* are commonly employed as environmental indicators in regulatory toxicology and are playing an increasingly significant role in "new approach methodologies" (NAM) for assessing chemical risks (Abdullahi *et al*., 2022). In *D. magna*, the single, small heart is easily visible when viewed under transmitted light using a low-power microscope (Figure 1). Heart rates can be monitored and quantified under various conditions, such as changes in water temperature or variations in the type and concentration of chemicals introduced to the water. Although changes in daphnid heart rates may not directly predict similar changes in the heart rates of humans or other vertebrates under the same conditions, this method provides a valuable approach for studying the impact of various chemicals on metabolic processes.



(a)

(b)

Figure 1. (a) The functional anatomy of an adult *Daphnia magna* (taken from Ebert, 2005; Tomar, 2024). **(b)** Image of *Daphnia magna* used in the experiments (Olympus stereo microscopes, 10x10 magnification) (please access the video taken during the counting from the link https://youtu.be/bj8xFXUydc0)

From the tank where the culture was maintained, random young daphnids were selected and placed on a slide. To prevent changes in the final concentration of the chemicals to which the daphnids would be exposed, the remaining culture medium on the slide, along with the daphnids, was removed using a Pasteur pipette. The daphnids were then exposed to a solution containing a drop of crystal violet (CV), ethanol (E), and formaldehyde (F) at a concentration of 1 ppm. A one-minute waiting period was allowed to ensure the chemicals were incorporated into the daphnid's circulation before counting the reactive heartbeats.

To count the heartbeats of the daphnids, videos were recorded using the camera of a Samsung Galaxy S22 smartphone in conjunction with an Olympus stereo microscope. The recorded videos were edited into 13 segments of 10 seconds each using Adobe Premiere Rush. Each 10-second segment was adjusted to slow motion, and the heart rates were counted. This procedure was repeated separately for each chemical and conducted three times. The heartbeats of the daphnids exposed to the prepared solutions were recorded on video for a total duration of 130 seconds. The video recordings focused on the heart chamber located towards the back of the *Daphnia* sp. (Saputra *et al*., 2023).

The data obtained were analyzed using SPSS 20 to perform a one-way ANOVA to determine if there was a statistically significant difference.

3. RESULTS AND DISCUSSIONS

In this study, the feasibility of using water fleas as indicator species was assessed based on their responses to the selected toxic chemical solutions, in order to facilitate the advancement of diagnostic methodologies and to evaluate various preventive and therapeutic strategies for Cardiovascular diseases. To determine heart rates without any chemical or stress factors, 10 daphnids were randomly selected from the water flea culture tank. The heart rates results of the ten daphnids are presented in Figure 2. It was found that the heartbeat is individual-level but not size-dependent in water fleas. After testing the ten water fleas, it was determined that *D. magna* has a robust heartbeat and blood flow rate, indicating that it is suitable for ecotoxicity testing. The heart rates of 10 daphnids, taken from the culture tank and measured over time without chemical exposure, averaged 313 beats per minute.

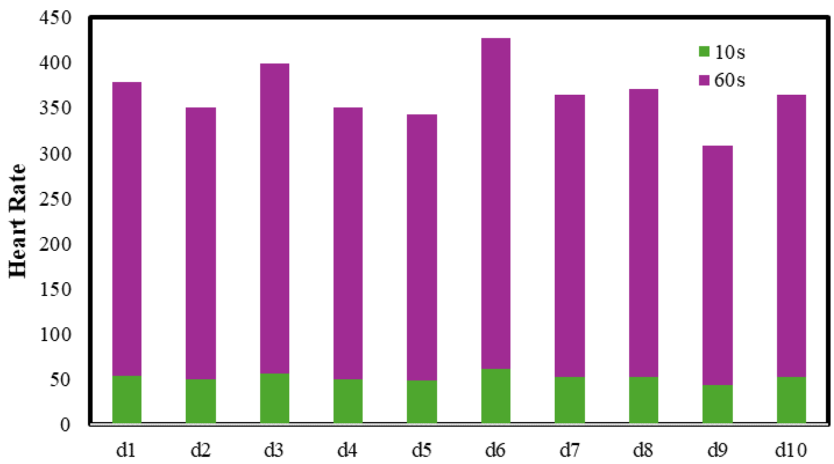


Figure 2. Heart rates results of ten *D. magna* (control group) (heart rates counting was done using Adobe Premiere Rush).

The study investigated the effects of CV, E, and F on the heart rates of water fleas. In Figure 3-a, the heart rates of three different *Daphnia magna* exposed to CV at a concentration of 1 ppm are shown in 10-second intervals. The highest average heart rate (84 beats per 10 seconds) was recorded for *Daphnia* sp. exposed to crystal violet, representing the highest heart rate count obtained in the study.
The average heart rate of *Daphnia* sp. exposed to formaldehyde in 10-second intervals was found to be 73.71 beats per 10 seconds. During exposure to formaldehyde, the lowest heart rates were observed in period t13 (61 beats per 10 seconds). It was noted that during the 3rd beat of interval t7, the heart of the daphnids contracted less. During ethanol exposure, the heart rates of daphnids ranged from a minimum of 66 beats per 10 seconds to a maximum of 81 beats per 10 seconds. In all ethanol exposure experiments, the heart rates of *D. magna* were found to be higher than that of the control group *D. magna* ($t = 15.65$; $p < 0.05$). The heart rates of *Daphnia magna* exposed to crystal violet, ethanol, and formaldehyde were found to be 489±14.19, 450±40.67, and 445±48.21 per minute, respectively. As shown in Figure 4-a, 62% increase in heart rate was observed in *Daphnia magna* exposed to crystal violet. The change in heart rate following exposure to ethanol in the three different *Daphnia* sp. is presented in Figure 4-b. This change shows an increase in average heart rate to 76 beats per 10 seconds, indicating a 51% increase. Similarly, the effect of formaldehyde exposure on *Daphnia* heart rate is illustrated in Figure 4-c. Although formaldehyde, with a 28.51% increase in heart rate compared to the control, exhibited a lower increase than ethanol (30.54%) and crystal violet (35.89%). The one-way ANOVA test conducted between

the groups F, CV, and E revealed a statistically significant difference among the groups (F(2.39) = 437.06, p < 0.001). The study demonstrates that crystal violet has a more pronounced effect on heart rate compared to ethanol and formaldehyde (p<0.001). The impact of crystal violet was found to be markedly higher than that of the other substances (p<0.001). The reliability of the results is enhanced, as the analyses were conducted using three different daphnids from the same genus.

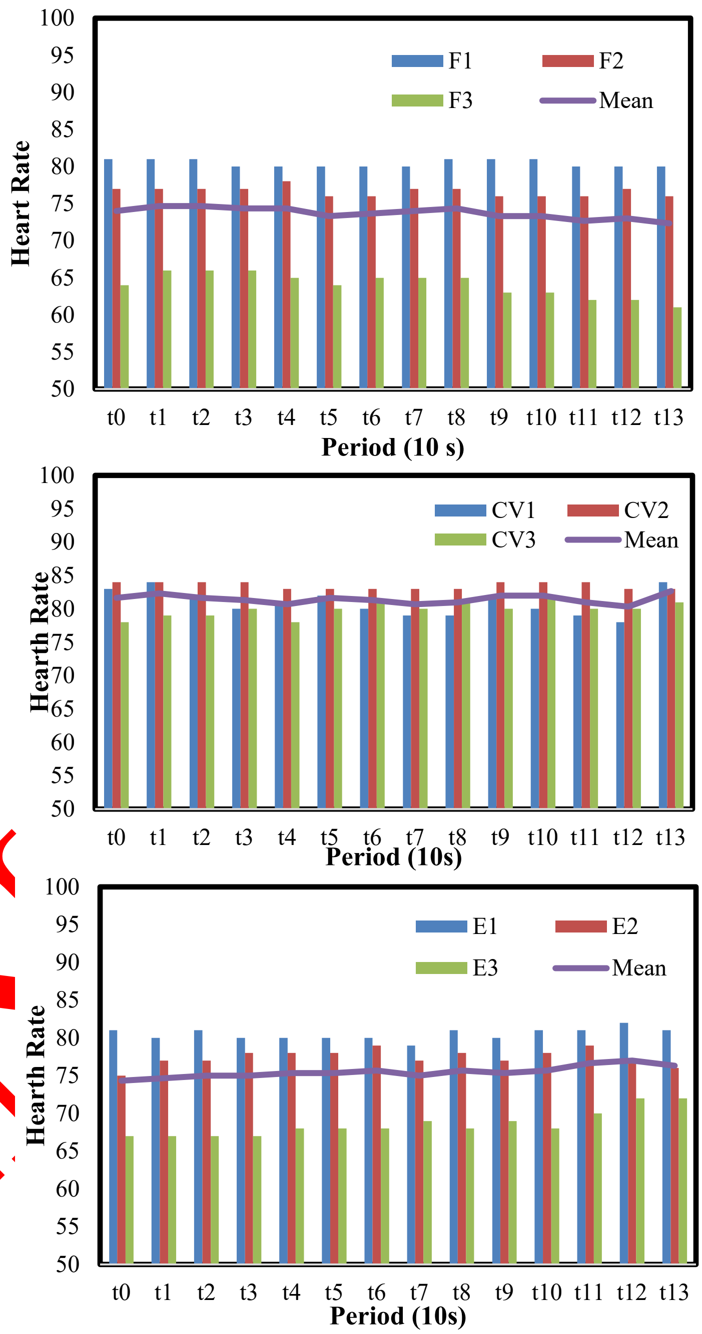


Figure 3. Heart rates of *D. magna* (n=3) exposed to different chemicals (CV, E, and F) depending on the periods (t=10 seconds)

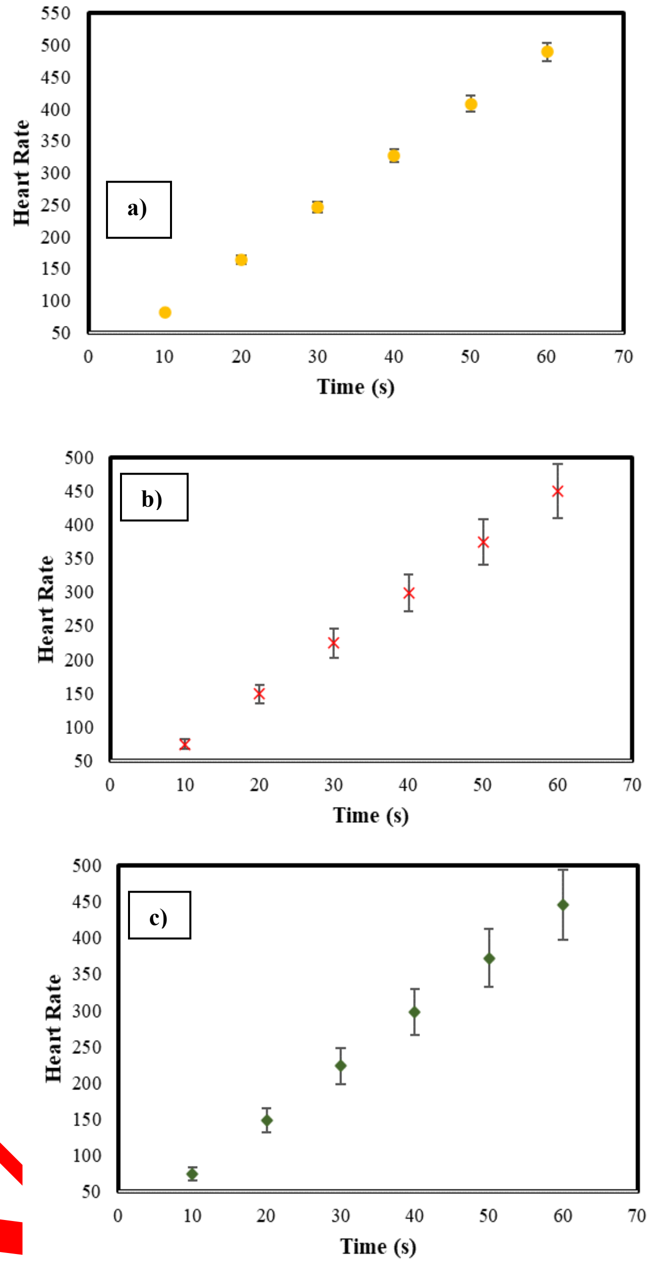


Figure 4. Heart rate of *Daphnia magna* exposed to different during the first 60 seconds chemicals (a-CV, b-E, and c-F)

Heart dimensions were compared during heart contraction in all chemical exposures (CV, E, and F) and significant differences were found in heart contraction ratios compared to the control group ($F(3,39)=1044.21$, $p<0.001$). The mean heart contraction ratio in the control group was found to be 4.28%. In contrast, a 23.19% increase in heart contraction ratio was observed in daphnids exposed to CV (Figure 5). Furthermore, differences of 38.59% and 12.77% were found in daphnids exposed to ethanol and formaldehyde, respectively. The highest difference compared to the control group was observed with ethanol exposure. Ethanol, isopropanol, and carbon dioxide can easily pass through cell membranes due to their lipophilic properties (Major *et al.*, 2010). Therefore, the effects on the heart of daphnids are thought to be primarily caused by changes in cell membrane viscosity. Allen *et al*. (2019) tested the hypothesis that nicotine would raise the *Daphnia* sp. heart rate, while cannabidiol water would lower it, similar to how these drugs affect humans, because *Daphnia* sp. hearts have been shown to respond similarly in the presence of specific drugs. Their results neither supported nor contradicted the original hypothesis.

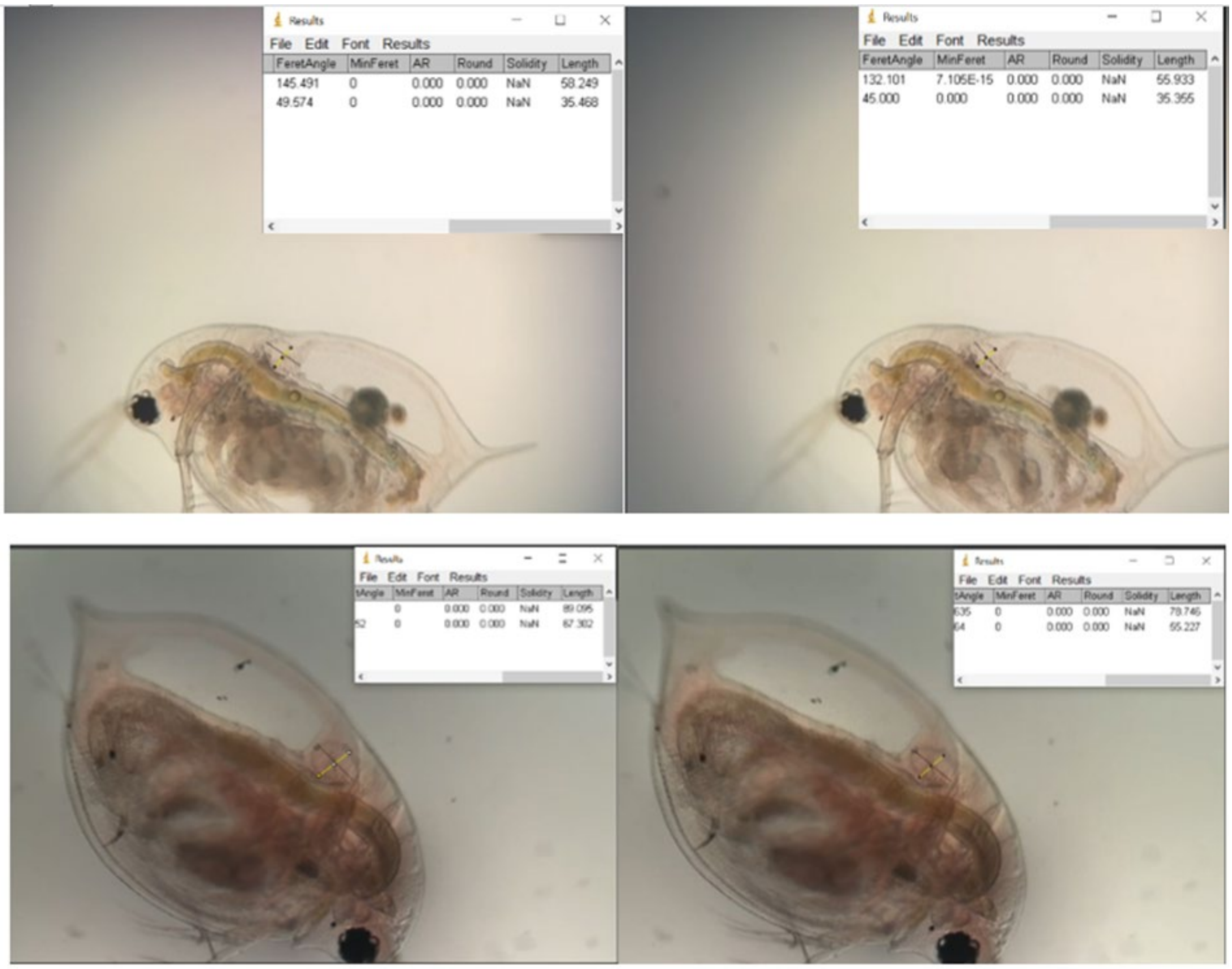


Figure 5. Detection of heart rates in daphnids using images obtained with the ImageJ software for a) control group and b) the group exposed to CV.

Lari *et al*. (2017) studied the effects of cadmium (Cd), suspended particles, and food on the beating rate of thoracic limbs and the frequency of mandible rolling in *Daphnia magna*. Their objective was to create an efficient method for simultaneously measuring all three endpoints on a single individual, which would enhance the reliability of the results. They presented an effective tool for assessing heart rate, thoracic limb movement, and mandible rolling with minimal stress for the test animals, enabling easy handling and adjustment of the test *Daphnia*'s position while reducing the time needed to perform the tests. Kwon *et al*. (2021) discovered that monitoring heart rate in real-time under aquatic conditions could enhance the accuracy of toxicity evaluations.

Bedrossiantz *et al*. (2023) exposed zebrafish, Japanese medaka, and *Daphnia magna* to sub-NOAEC concentrations of carbaryl and fenitrothion for 24 hours to evaluate their effects on heart rate (HR), basal locomotor activity (BLA), visual motor response (VMR), startle response (SR), and habituation. Both pesticides caused an increase in heart rates across all species, with the magnitude of the effect varying depending on the chemical, concentration, and organism.

In a study conducted by Kaas *et al*. (2009), ethanol was found to significantly reduce the heart rate of *Daphnia* sp. In the present study, a lower ethanol concentration was used compared to the previous study, and the exposure duration was extended. It has been noted that ethanol exhibits a stimulatory effect at low doses and an inhibitory effect at high doses, a phenomenon known as "biphasic" (Earleywine and Martin, 1993). The stimulatory effect of the low dose used in this study, which increased the heart rates of *Daphnia* sp., contrasts with the inhibitory effect of the high dose used in Kaas *et al*. (2009), which significantly reduced heart rates. This suggests that the findings of the previous studies are complementary and supportive of the results of this study.

A similar observation was made in a study by Bownik and Stępniewska (2015), where *Daphnia* species exposed to formaldehyde for longer durations (24-48 hours) exhibited a significant reduction in heart rates. As seen in Figure 3, this study, which reflects entirely acute (sudden) changes, demonstrated that formaldehyde at a concentration of 1 ppm significantly increased heart rate within the first 130 seconds. Considering this, it can be suggested that formaldehyde exhibits a stimulatory effect during short-term exposure. Additionally, as formaldehyde is known to exhibit a biphasic effect similar to ethanol, it may have acted as a stimulant during low-dose and short-term exposure. The inhibitory effects observed in previous studies involving long-term and high-dose exposure further support this conclusion.

4. CONCLUSIONS

In environmental toxicity tests, it is possible to employ endpoints related to reproduction, physiological, and behavioral changes in addition to other commonly used endpoints such as immobilization and lethality. Heart rate is a direct indicator of stress levels in water flea species. The results of this study demonstrate that the *D. magna* model offers promising and sensitive endpoints for assessing environmental toxicity, including non-lethal effects. The potential adverse outcomes observed after short-term exposure to low environmental concentrations of chemicals (i.e., below NOAEC values) underscore the importance of incorporating additional toxicological endpoints. These results highlight the necessity of integrating parameters such as behavioral responses or cardiac activity into predictive risk assessment methodologies. The wide range of endpoints presented concerning water flea applications further emphasizes its future potential.

AUTHORSHIP CONTRIBUTION STATEMENT

Arda Sarp KARADEMİR: Conceptualization, Methodology, Microscopy, Formal Analysis, Visualization. **Melisa CAN:** Methodology, Formal Analysis. **Vildan Zülal SÖNMEZ:** Validation, Writing - Original Draft, Writing-Review and Editing**. Nüket SİVRİ:** Resources, Writing-Review and Editing, Supervision.

CONFLICT OF INTERESTS

The author(s) declare that for this article they have no actual, potential or perceived conflict of interests.

ETHICS COMMITTEE PERMISSION

No ethics committee permissions is required for this study.

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