ACTA ZOOLOGICA BULGARICA

Acta Zool. Bulg., March 2024, 76 (1): 105-114

Aquatic Ecology
Research Article

Published online 28 March 2024
DOI: https://doi.org/10.71424/azb76.1.002674

Evaluation of the Effects of Environmental Pollutants on Populations of the European Pond Turtle *Emys orbicularis* (Linnaeus, 1758) (Testudines: Emydidae) at Çanakkale, Türkiye

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Abstract: We evaluated and compared the effects of different environmental pollutants on haematological, microbiological and genotoxic characteristics of populations of the freshwater turtle *Emys orbicularis* from the Kavak Delta and Dardanos in Çanakkale (NW Türkiye). The haematocrit values of samples of *E. orbicularis* differed considerably between the two localities: they were higher in the Kavak Delta samples. The mean cell haemoglobin concentration was also significantly different between the two localities (higher in the samples of the Dardanos). The water samples of the two localities were within the limit (Class I-II) for all parameters, except Dardanos temperature (Class III). Although it was not statistically significant, the nuclear abnormality formation in the erythrocytes of the sample from Dardanos was numerically higher than that from the Kavak Delta. The study assessed the impact of pollutants on populations of *Emys orbicularis* and conducted on-site evaluations and monitoring of genotoxic pollution.

Key words: Reptilia, Emydidae, genotoxicity, haematology, microbiology

Introduction

The reptiles are one of the animal groups that are highly sensitive to biodiversity loss. Their worldwide reductions, therefore, require large-scale conservation efforts. One of the main threats to reptilian populations is pollution; some reptilian species are good bioindicators of pollution (Lambert 1997). Reptiles can be used as a much more accurate bioindicator in the field of toxicological study since they have a wide variety of feeding and habitat preferences within their long lives compared to classical mammalian model animals (Hall 1980). Turtles differ from other animal species by their high age rates (Gibbons 1987) and long generation times (Hailey & Loumbourdis 1990), which ensures the sustainability of the turtle population. These animals are experiencing problems, which may lead to their extinction as long as factors such as fire, habitat loss, uncontrolled hunting and trade are continuing (Brooks et al. 1991, Hailey 2000). The European pond turtle *Emys orbicularis* (Linnaeus, 1758) is distributed over a vast range in North-Western Africa and much of Europe and Western Asia (Stuckas

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Fig. 1. Location of the studied localities of *Emys orbicularis*.

et al. 2014). This species is threatened throughout its distribution, prompting management of habitats and populations for conservation (Ficetola et al. 2004). *Emys orbicularis* is listed in the NT (Near Threatened) category in the IUCN Red List (IUCN 2022).

Biological monitoring can provide important information in the control and regulation of substances in the environment. It can identify the degree of environmental pollutants in the region and their effects on indicator species and the entire ecosystem, as well as their potential impact on human health (Meyers-Shöne & Walton 1990). Exploring blood profiles in reptiles is especially important for determining the effects of pollution and disease on their health. Haematological analyses are very important for ecotoxicology and ecology studies in vertebrate wild animals (Maceda-Veiga et al. 2015). There are many studies on haematological analyses of aquatic turtles (Tavares-Dias et al. 2009, Yilmaz & Tosunoğlu 2010, Tosunoğlu et al. 2011, Çiçek et al. 2015, Bilgin & Gül 2019). Peripheral blood tissue is the most suitable tissue to analyse the whole organism functions without killing the studied animals (Douglas et al. 2010).

Water systems are ecosystems that are primarily affected by environmental pollution due to their dynamic and variable structures, including many interrelated physical and chemical parameters (Kara & Çömlekçioğlu 2004). Stream water quality is of great environmental concern since it is one of the major available freshwater resources for human consumption. Anthropogenic activities such as urban, industrial, agricultural and natural processes, including precipitation inputs, erosion and weathering of crustal materials, affect stream water quality and determine its use for various purposes (Bhat et al. 2014). The evaluation of freshwater quality is a complex process involving numerous parameters, which contribute with different pressures on water quality (Radu et al. 2014). These parameters are microbial and physicochemical parameters, which could be affected by external and internal factors (APHA 1998). Furthermore, environmental pollutants can cause DNA damage in living organisms. DNA damage can initiate a cascade of biological consequences through mutation, cancer, birth defects, reduced growth, abnormal development and reduced embryo, larval and adult survival rates in cells, organs, whole animals and even populations (Theodorakis et al. 2000, Lee & Steinert 2003). Determining the level of genotoxicity in wild populations allows the assessment of the ecosystem status and the estimation of the risks of environmental pollutants on wildlife (Simonyan et al. 2018).

In many studies, counts of micronucleus and other nuclear abnormalities in erythrocyte were used as a direct measure of genotoxicity and for an efficient means to detect environmental pollutants (Ergene et al. 2007, Guilherme et al. 2008, Marques et al. 2009, Napierska 2009, Strunjak-Perovic et al. 2010, Josende et al. 2015, Zhelev et al. 2022).

In this study, we aimed to evaluate and compare the effects of different environmental pollutants on populations of *E. orbicularis* distributed in Çanakkale through the analyses of haematological, microbiological and genotoxic differences of the populations.

Materials and Methods

Study area and field studies

The study area consisted of two different localities in Çanakkale Province, NW Turkey (Fig. 1): the Kavak Delta (N40.60077°, E26.84039°, Fig. 2A) and the Dardanos Village (N40.09062°, E26.36316°, Fig. 2B). During the field work between April–July 2019, seven specimens of *E. orbicularis* from the Kavak Delta and nine specimens of *E. orbicularis* from Dardanos Village were caught.

Analyses on animal samples

The necessary procedures were followed for analysing the samples from the collected turtles (Decision No. 2018/06-09, Animal Experiments Ethics Committee, Çanakkale Onsekiz Mart University). Within the scope of morphological analyses, gender was determined, body measurements (carapace length and width; plastron length and width) and body weights (with a balance with 0.001 gr precision) were taken. Two of blood was taken from the dorsal caudal vein of each turtle with the help of a 5 ml syringe with a diameter of 21G needles for genotoxic damage and haematological analyses (Ballard & Cheek 2003, Thrall et al. 2004). All turtles were returned to the area where they were collected after morphological measurements and the blood collections were completed.

Haematological analyses

The erythrocyte count, leukocyte count, haemoglobin and haematocrit value, mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC) and leukocyte types from blood smear preparations of *E. orbicularis* were determined. Haemoglobin determination was done using the Sahli method and the haematocrit value was determined by centrifuging the blood taken into microhaematocrit capillary tubes and measuring the proportion of blood cells. Erythrocyte and leukocyte counts were made with Neubauer haemocytometer and Olympus CX 31 microscope. MCV, MCH and MCHC were calculated mathematically from the results (Tanyer 1985). Blood smear preparations stained with Wright's dye were examined under a 40X magnification and the percentage ratios of leukocyte types were determined (Tanyer 1985).

Fig. 2. Habitats where *Emys orbicularis* were caught. A, Kavak Delta; B, Dardanos Village.

Physical, chemical and microbiological water quality analyses of stations

The physical and chemical parameters of the collected water samples were analysed, i.e. pH, temperature (T), electrical conductivity (EC), dissolved oxygen (DO), as well as the their microbiological parameters through the multiple tube technique, including total coliform (TC), faecal coliform (FC), faecal streptococci (FS) by following the standard methods of the American Health Association (APHA 1998).

Table 1. Descriptive statistics of the morphological and haematological characteristics of *E. orbicularis* collected from the two stations (BW: Body Weight; CL: Carapace Length; CW: Carapace Width; PL: Plastron Length; PW: Plastron Width, RBC: Red Blood Cell Counts, WBC: White Blood Cell Counts, HMG: Haemoglobin, HCT: Haematocrit, MCV: Mean Cell Volume, MCH: Mean Cell Haemoglobin, MCHC: Mean Cell Haemoglobin Concentration, N: Number of samples, SD: Standard deviation, SE: Standard Error).

KAVAK DELTA					
	N	Minimum	Maximum	Mean	SE
BW (g)	7	220.00	680.00	385.7143	69.65415
CL (cm)	7	112.46	152.62	129.2871	6.06012
CW (cm)	7	84.88	111.88	96.5400	4.14379
PL (cm)	7	99.80	149.42	119.5171	8.01056
PW (cm)	7	64.64	87.45	74.0657	3.50541
RBC (mm^3)	7	360000.00	640000.00	492857.1429	44439.05863
WBC (mm^3)	7	2200.00	3400.00	2742.8571	155.62033
HMG (g/dL)	7	4.00	8.00	6.0571	0.47552
HCT (%)	7	23.00	36.00	29.5714	1.74379
MCV (μ^3)	7	359.38	1000.00	633.8391	74.15676
MCH (μμg)	7	83.87	188.89	128.9479	15.51731
MCHC (%)	7	11.11	27.59	21.1882	2.31432
Lymphocyte (%)	7	30.00	41.00	35.4286	1.50961
Monocytes (%)	7	13.00	19.00	16.0000	0.81650
Eosinophils (%)	7	14.00	27.00	21.8571	1.56492
Heterophils (%)	7	10.00	16.00	12.8571	0.82890
Basophils (%)	7	9.00	17.00	12.0000	1.04654
DARDANOS VILLAGE					
	N	Minimum	Maximum	Mean	SE
BW (g)	9	200.00	520.00	351.7778	35.78865
CL (cm)	9	105.94	132.91	122.3067	3.19144
CW (cm)	9	85.90	103.23	95.4944	1.92216
PL (cm)	9	98.03	127.02	114.3344	3.62666
PW (cm)	9	70.59	87.17	81.2711	1.84432
RBC (mm^3)	9	300000.00	560000.00	435555.5556	30051.39630
WBC (mm^3)	9	1000.00	8000.00	3200.0000	862.97290
HMG (g/dL)	9	4.80	9.20	7.2667	0.46904
HCT (%)	9	17.00	26.00	21.5556	1.05556
MCV (μ^3)	9	314.81	633.33	509.5630	32.77210
MCH (μμg)	9	88.89	242.11	176.1430	18.65972
MCHC (%)	9	23.20	43.81	33.9662	2.09551
Lymphocyte (%)	7	35.00	46.00	40.8571	1.54963
Monocytes (%)	7	10.00	15.00	13.0000	0.61721
Eosinophils (%)	7	19.00	24.00	22.0000	0.61721
Heterophils (%)	7	9.00	17.00	12.1429	0.93678
Basophils (%)	7	9.00	17.00	12.0000	1.04654

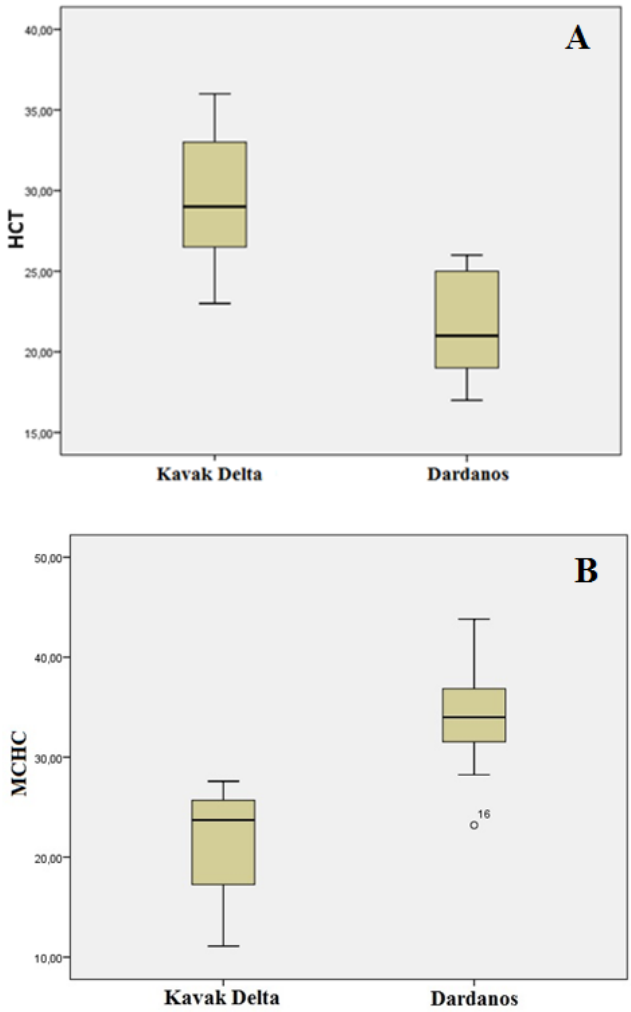

Fig. 3. A comparison between the haematocrit (A) and MCHC (B) values of *Emys orbicularis* in the studied localities.

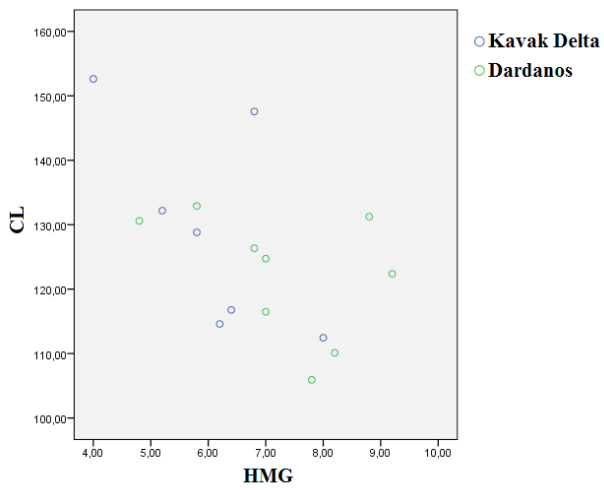

Fig. 4. Correlation between carapace length and haemoglobin values of *Emys orbicularis* from the two localities.

Heavy metals analyses

Blood samples (1 ml), 65% nitric acid (8 ml) and 30% hydrogen peroxide (1 ml) were digested in a microwave (Milestone Start D) for 15 min at 180ºC. Heavy metals (aluminium, barium, cadmium, chromium, cobalt, copper, iron, lead, manganese, nickel and zinc) in water and blood samples were measured using ICPOES (Perkin Elmer Optima-8000) at Scientific and Technological Application and Research Centre of Mehmet Akif Ersoy University, Burdur, Türkiye.

MN and other nuclear abnormalities analyses

The blood taken from each animal was spread on three separate slides and allowed to air dry for 48 hours. At the end of the period, the cells were fixed with pure ethanol and stained with 10% Giemsa for 10 minutes (Zapata et al. 2016). The preparations were examined under light microscope and the number of micronuclei and other nuclear abnormalities (notched nuclei, bud nuclei, lobed nuclei, binucleate) was calculated and evaluated in 1000 erythrocytes in each preparation. Totally, 3000 erythrocytes were randomly selected for each sample.

Statistical analyses

The Mann-Whitney U test was used for the data that did not show normal distribution in haematology, morphology and genotoxicology analyses. The basic descriptive statistics were performed and the Spearman's correlation test was applied to some data. The SPSS 20.0 program (IBM) was used for these analyses. In all cases, a value of $p<0.05$ was considered statistically significant.

Results

Morphology and haematology of *E. orbicularis*

The morphological and haematological analyses of 16 specimens of *E. orbicularis* are given in Table 1. Since there was no significant difference between males and females, the data were evaluated together. Based on the statistical comparison, a statistically significant difference was found in the haematocrit (U = 3.5; W = 48.5; Z = -2.973; p = 0.003) and MCHC (U = 4.00; W = 32.00; Z = -2,911; p = 0.004) (Fig. 3). In our study, the haematocrit value was found to be higher in the samples from the Kavak Delta with larger individuals. In addition, it was determined that there was a negative correlation between the haemoglobin value and the carapace length (r: -0,570; p: 0,021) (Fig. 4). It was determined that the percentage of agranulocytes was high in both stations.

Table 2. Physical, chemical and microbiological results for the water samples of the two stations.

Parameter	Kavak Delta	Dardanos Village
pH	7.53 (I-II)	8.02 (I-II)
T (°C)	14.0 (I)	29.8 (III)
EC (µS/cm)	787 (II)	3.01 (I)
DO (mg/(L)	9.98 (I)	7.82 (I)
TC (MPN/100 mL)	$6x10^2$ (I-II)	$4x10^2$ (I-II)
FC (MPN/100 mL)	0 (I)	0 (I)
FS (MPN/100 mL)	0	$4x10^2$

Physicochemical and microbiological analysis of water samples

The water samples taken from the Kavak Delta and Dardanos Village were evaluated in terms of physicochemical and microbial pollution (Table 2). We found that the waters of the two stations were within the limit of Class I-II for all parameters (except for the temperature in Dardanos, where it corresponded to Class III) based on the comparison with data in WPCR (1988). This is a proof that the exposure of the two stations to anthropogenic pressure is quite low. In this study, both stations were classified as I-II, corresponding to High-quality Low-polluted water.

Table 3. Mean values (mg/dm^{-3}) of heavy metals in blood and water samples.

	Pb	Cd	Zn	Cu	Cr	Co	Al	Ni	Mn	Ba	Fe
Kavak Delta water sample	-	-	-	0.004	-	-	0.618	-	0.162	0.040	0.500
Dardanos Village water sample	-	-	-	0.003	-	-	0.11	-	0.160	0.100	0.130
Kavak Delta blood sample	0.0875	-	4.520	1.055	0.009	-	-	0.025	-	-	69.9
Dardanos Village blood sample	-	-	1.755	0.827	-	-	-	0.062	-	0.165	-

* "-" means not detected

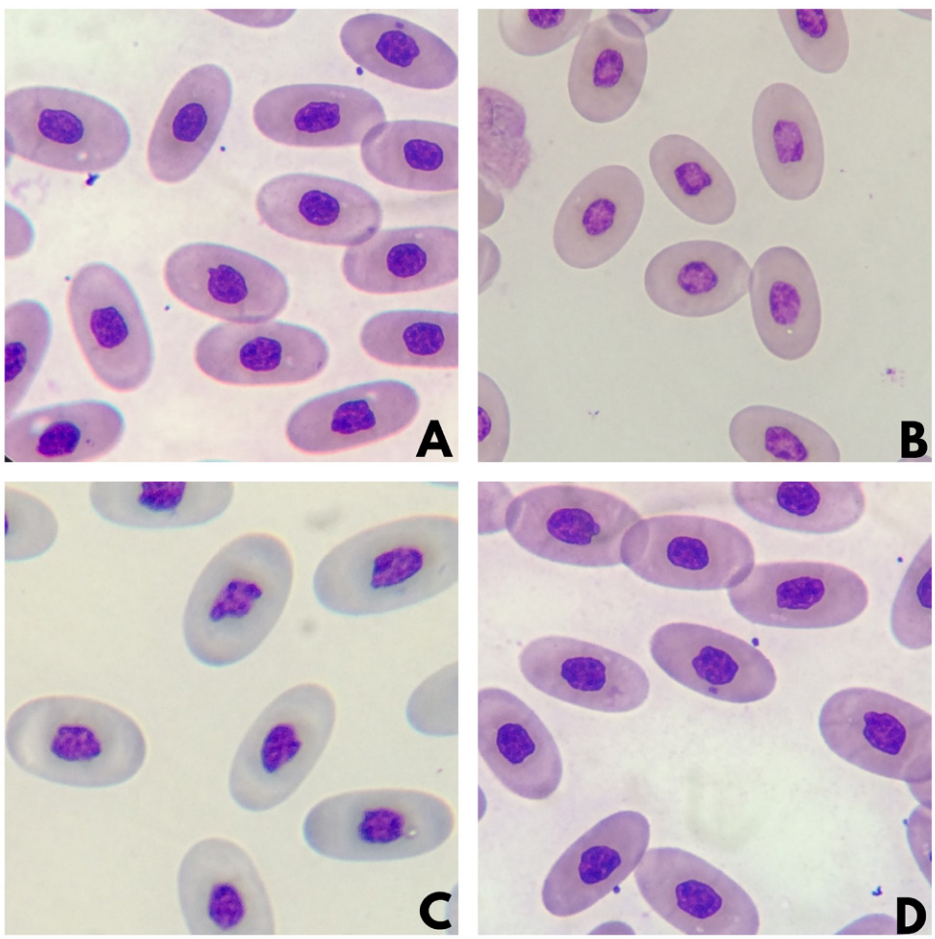

Fig. 5. Micronucleus and nuclear abnormalities in erythrocytes of *Emys orbicularis* from Kavak Delta and Dardanos Village. (A) Blebbed nucleus. (B) Notched nucleus. (C) Lobed nucleus. (D) Micronucleus.

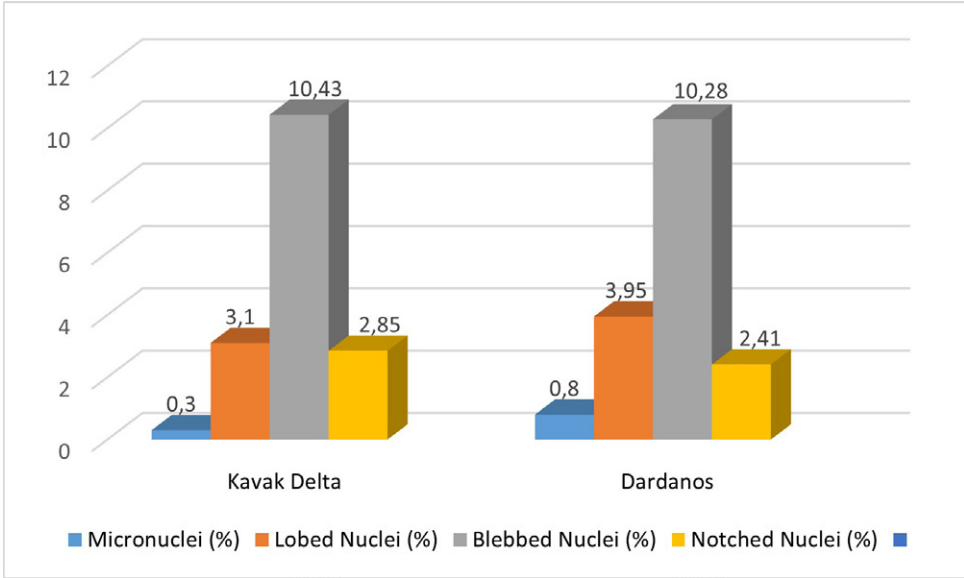

Fig. 6. Frequency (%) of micronuclei and other nuclear abnormalities in erythrocytes of *Emys orbicularis* from Kavak Delta and Dardanos Village.

Heavy metal accumulation in blood and water Samples

When the heavy metal contents of the water samples of the two studied localities were compared, aluminium, chromium, copper, manganese and iron were found to be higher in the Kavak water sample (Table 3). In the blood samples, chromium, iron and lead were not detected in the samples from the Dardanos Village, while they were found in the samples from the Kavak Delta. Copper and zinc were detected in higher amounts in the blood samples from the Kavak Delta and barium was detected only in the Dardanos samples.

Nuclear abnormalities in erythrocytes of *E. orbicularis*

The frequency of micronuclei and other nuclear abnormalities in erythrocytes were separately observed and calculated in the samples of *E. orbicularis* (Fig. 5). While micronuclei, notched nuclei, lobed nuclei and blebbed nuclei were observed in both samples, the frequency of each nuclear abnormality was different (Fig. 6). The frequency of micronuclei was higher in the erythrocytes of *E. orbicularis* in the Dardanos region than that in the samples from the Kavak Delta. The frequency of blebbed nuclei was higher than the other nuclei in both samples. The total abnormality was observed at rate of 16.68±2.5% in the erythrocytes of the animals from the Kavak Delta and 17.45±4.2% in the animals from the Dardanos Village, but the differences were not statistically important (U = 19.00; W = 34.00; Z = -0.146; p = 0.884).

Discussion

The haematocrit value was lower in the samples from the Dardanos Village, whereas the MCHC value was lower in the samples from the Kavak Delta. Haematological and plasma biochemistry values are used to evaluate the health status of reptiles, but these values are affected by many internal and environmental factors, such as season, metabolic activity, reproductive cycle, age, sex, habitat, photoperiod and climate (Allemen et al. 1999, Hidalgo-Vila et al. 2007). Tavares-Dias et al. (2009), in a biochemical study of *Podocnemis expansa* (Schweigger, 1812), found a decrease in erythrocyte count, haematocrit, glucose, total protein, cholesterol, urea and WBC values in malnourished and undernourished individuals. Bilgin & Gül (2019) compared the haematological and biochemical parameters of juvenile and adult individuals in *Mauremys rivulata* (Valenciennes, 1833) and reported a positive correlation between body size and erythrocyte count. Hofmeyr et al. (2017) found that juvenile individuals of *Psammobates geometricus* (Linnaeus, 1758) had a lower erythrocyte count than adult individuals. In our study, the haematocrit value was found to be higher in the samples from the Kavak Delta, where the individuals were larger. In addition, it was determined that there was a negative correlation between the haemoglobin value and the carapace length. Çiçek et al. (2015) studied the blood cell morphologies of specimens of *E. orbicularis* and *M. rivulata* distributed in Türkiye and reported the erythrocyte count as 430667 in *E. orbicularis*.

In the same study, they reported that agranulocytes were dominant, the nuclei were not differentiated due to the dense granules in the cytoplasm of eosinophils and basophils in blood smear preparations as well as neutrophils were very rare leukocytes. Çiçek et al. (2015) determined that the percentage of agranulocytes was high at both stations. Yilmaz & Tosunoğlu (2010) found the value of MCHC was 31.06% (24.39-38.34) for the same species and the MCHC value obtained by us was in the same range.

Tosunoğlu et al. (2011) reported that erythrocyte and leukocyte count in *E. orbicularis* differed according to the quality of the water and found that both haematological parameters were higher in highly polluted water. The average number of erythrocytes was 414333 in I class quality (high quality) water and 455555 in IV class quality (very polluted) water; the average number of leukocytes was 4940 in I class quality (high quality) water and 7433 in IV class quality (very polluted) water. Gül et al. (2015) compared haematological parameters in samples of *M. rivulata* at two different stations. They found the MCH value to be higher in the samples from the station with high water quality class and the erythrocyte value to be higher at the station with polluted water class; however, they did not find a statistically significant difference. In this study, both stations were classified as I-II Quality (High quality-Low polluted) water and no difference was detected between the stations in terms of the number of erythrocytes and leukocytes. Due to the physical and chemical and microbiological pollution, changes occur in the quality and properties of water. These changes affect aquatic organisms; therefore, water pollution causes damage to aquatic ecosystems and the loss of the self-cleaning capacity of waters (Gidirişlioğlu et al. 1998). In our study, when the water qualities of both stations were examined, it was determined that there was no pressure in terms of pollution, contrary to the literature (Yayintaş et al. 2007, Hacioğlu & Dulger 2009, Hacioğlu & Dulger 2010).

Nuclear abnormalities are indirect indicators of numerical and structural chromosome irregularities that occur in cells because of the effect of various environmental pollutants (Hacioğlu Doğru et al. 2022). In this study, although there was no statistical difference between the two populations, it was revealed that the environmental pollutants that the organisms were exposed to in their habitat caused micronuclei and other nuclear abnormalities in both populations. While Dardanos Village is exposed to anthropogenic effects, the part of the Kavak Delta on the shores of the Gulf of Saros shows the characteristics of coastal dune and saltwater ecosystems and does not directly suffer from anthropogenic pressure. However, in the rest of the area, pollutants originating from grazing activities, paddy farming and settlements, such as Evreşe Town and Kavak Village, threaten the area through the Kavak Creek (Kelkit & Öztürk 2005). Besides these pollutants, various concentrations of heavy metals (Ba, Cd, Cr, Cu, Fe, Li, Ni, Pb, Se, Sr and Zn) have been determined in the salt marsh soil of the Kavak Delta (Sungur & Özcan 2015). All pollutants, particularly heavy metals, in the Kavak Delta may have caused the higher frequency of micronuclei and other nuclear abnormalities in erythrocytes of the population from the Kavak Delta than expected and as compared to the population from the Dardanos Village. Heavy metals are considered the most important inorganic pollutants in water and can cause DNA damage. The genotoxicity of a water source may be largely due to heavy metals in aquatic ecosystems (Ergene et al. 2007, Salles et al. 2016). To examine and evaluate the DNA damage potential of different environmental pollutants, it is important to understand the status of the exposed organism, other wildlife and humans. In the study evaluating whether environmental pollutants caused genetic damage in *E. orbicularis* and *Mauremys caspica* in three locations in Azerbaijan, it was reported that the environmental degradation of Sumgayit caused chromosomal damage in at least one of the two species and that similar damage was likely to occur in other wildlife and possibly humans (Matson et al. 2005).

Conclusion

In this study, the effects of different environmental pollutants were monitored by analysing physical and chemical and microbiological water quality and by comparing the haematological status, the occurrence of signs indicative of genotoxic effects of two different populations of *Emys orbicularis*. Understanding the effects of adverse environmental conditions is important for managing the sustainability of ecosystems and maintaining the survival of species. Therefore, the parameters can be used for monitoring the health conditions of this species and for obtaining information on the control and regulation of substances in their environment.

Acknowledgment: This study was financially supported by the Scientific Research Project Coordination Unit of Çanakkale Onsekiz Mart University, Türkiye (FBA-2018-2633).

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Received: 14.11.2022
Accepted: 20.09.2023