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Assessment of Nuclear Abnormalities in Erythrocytes of Balkan Pond Turtle *Mauremys rivulata* (Valenciennes, 1833) (Testudines: Geoemydidae) from the Biga Stream, Çanakkale, Turkey

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Abstract: Reptilian species are potentially efficient bioindicators of pollution and habitat destruction. Turtles may be especially useful for monitoring the chemical contamination of their habitats. The aim of this study was to assess the genotoxic effect of environmental pollutants on Balkan pond turtle *Mauremys rivulata* (Valenciennes, 1833) from the Biga Streamp Canakkale, Turkey. Healthy mature animals were collected using the hand-capture method from four stations: estuary zone, city centre, buffer zone and industrial zone of the stream. Blood samples of live specimens were obtained in within one day of their capture. The peripheral blood smears for each specimen were prepared, fixed with methanol for 15 min and dyed with Giemsa stain. Micronuclei and other nuclear abnormalities such as blebbed, kidney-shaped, lobed and notched nuclei were identified. Comparing the nuclear abnormalities in the samples, it was determined that their frequency was highest in the samples collected from the station in the city centre (9.16±0.02). The results indicated that the levels of genotoxic pollutants in different regions of the Biga Stream lead to DNA damage in *Mauremys rivulata*.

Key words: *Mauremys rivulata*, micronucleus, nuclear abnormalities, ecotoxicology, Turkey.

Introduction

Freshwaters such as rivers, streams and wetlands provide important habitats for nature conservation. They are also vital to human life and economic growth, recreation and transportation (Kinge et al. 2012). Anthropogenic activities strongly influence the natural status of almost all aquatic ecosystems. Physical, chemical and biological environmental pollutants can alter the quality of the recipient lakes, streams, rivers and coastal marine ecosystems (Gugliandolo et al. 2009). Among the pollutants, heavy metals may cause increasing ecological and global public health concerns. People's exposure to heavy metals has risen dramatically as a result of an exponential increase in use in several industrial, agricultural, domestic and technological applications (Bradl 2002, Tchounwou et al. 2012). Environmental pollutants that threaten the ecosystems of living organisms can cause damage to the genetic material of organisms. As a result of environmental damage caused by environmental pollutants, exposed living beings or cells could be prone to a cascade of biological consequences: mutation, cancer, birth defects, diminished growth, abnormal development and reduced embryo, larval and adult survival of individuals or whole populations (Lee & Steinert 2003).

Blood parameters of reptiles may assist in evaluating physiological and health conditions

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of populations and may be used as an indicator in determining the environmental condition since these species are very susceptible to habitat changes (Jacopson et al. 1991, Raphael et al. 1994, Dickinson et al. 2002, Lopez-Olivera et al. 2003). The increased types and amounts of pollutants have led to develop several bioassay test systems in order to evaluate the genotoxic effects induced by these agents in living organism (Zhelev et al. 2013, 2017, Pollo et al. 2017, Salinas et al. 2017). Short-term tests are widely employed to monitor the genotoxicity of many chemicals to people, animals and plants. Among them, Micronucleus test (MN) can be used for the estimation of biological impacts of water pollutants and the resultant genotoxic damage in organisms inhabiting aquatic environments (Bolognesi & Hayashi 2011, Kousar & Javed 2015). MN ensures continuous and effective evaluation of pollution level in aquatic environments (Obiakor et al. 2012). However, other nuclear abnormalities as a result of the genotoxic effect of pollutants, such as kidney-shaped nuclei (KS), lobed nuclei (LB), notched nuclei (NT) and blebbed nuclei (BL), were recently determined. Determination of the number of micronuclei together with other nuclear abnormalities formed in the cells of different species that are exposed to various environmental pollutants can be used to evaluate these genotoxic effects of pollutants (Strunjak-Perovic 2010). In recent years, nuclear abnormalities have been used with micronucleus to determine the effects of pollutants in various species (Guilherme et al. 2008, Napierska et al. 2009, Strunjak-Perovic 2010, Pollo et al. 2015, 2016). The frequency of micronuclei and nuclear abnormalities caused by environmental pollutants were determined in *Pelophylax ridibundus* (Pallas, 1771) from the Biga Stream (Canakkale, Turkey) by Çördük et al. (2018).

Turtles easily accumulate environmental contaminants in their habitats due to their long life spans and generalist diet, integrating a lifetime of exposure from multiple sources, interactions with sediments and ingestion of invertebrates or aquatic plants. Turtle populations can also persist in areas after other species have disappeared, partly because of their long lives and low generational turnover (Meyer et al. 2016). Thus, they can be used in biomonitoring of genotoxic effects of environmental pollutants. *Mauremys rivulata* (Valenciennes, 1833) is listed in Appendix II of the Convention of European Wildlife and Natural Habitats of 1979. The species was reported as decreasing and endangered on Cyprus and decreasing in number in Bulgaria, European Turkey and Anatolia (Honegger 1981, Kornilev et al. 2017, Popgeorgiev et al. 2017). For *M. rivulata* in the Aegean region, that habitat loss seems to be the main risk for the turtle populations (Broggi 2012).

The aim of this study was to monitor potential genetic damage in *Mauremys rivulata* caused by environmental pollutants from four various stations of the Biga Stream (Çanakkale, Turkey) by determination of micronuclei and other nuclear abnormalities.

Materials and Methods

Sampling area

The Biga Stream is one of the most important water bodies located in the Biga District, Çanakkale, Turkey. It is 80 km long: originates from the Ida Mountains and flows into the Sea of Marmara; it has a wide catchment area. The stream has almost no economic contribution to the region but it is species rich (Tosunoğlu et al. 2016, 2017). It is in danger because of excessive pollution due to the leather enterprises, agricultural areas and animal husbandry activities in the area. In this study, four collecting stations with a distance of about 20 km between them were selected along the Biga Stream (Fig. 1). The results of heavy metal analyses and information about stations as presented by Çördük et al. (2018) are given in Table 1. Briefly, the second station had higher values of Al, Cu, Zn and Fe than the values obtained for the other stations. The fourth station had higher concentrations of heavy metals than the second station except Cu and Fe. Two stations (Stations 1 and 3) had low amounts of heavy metals and Station 1 had the lowest heavy metal level except for Fe (Çördük et al. 2018).

Collection of Animal Samples

Specimens of *M. rivulata* (*N*=10 from each station) were captured along the Biga Stream in May 2014. Only healthy mature animals were studied and they were hand-picked, mostly from the water. Blood samples were obtained in the laboratory within one day of their capture. The samples were collected according to the guidelines of the university's ethics committee (2012/05-01).

Haematological analyses

The red blood cell counts (RBC) and white blood cell counts (WBC) were carried out using a Neubauer haemocytometer, where standard Hayem's solution for red blood cells and Turk's solution for white blood cells were used as a diluting

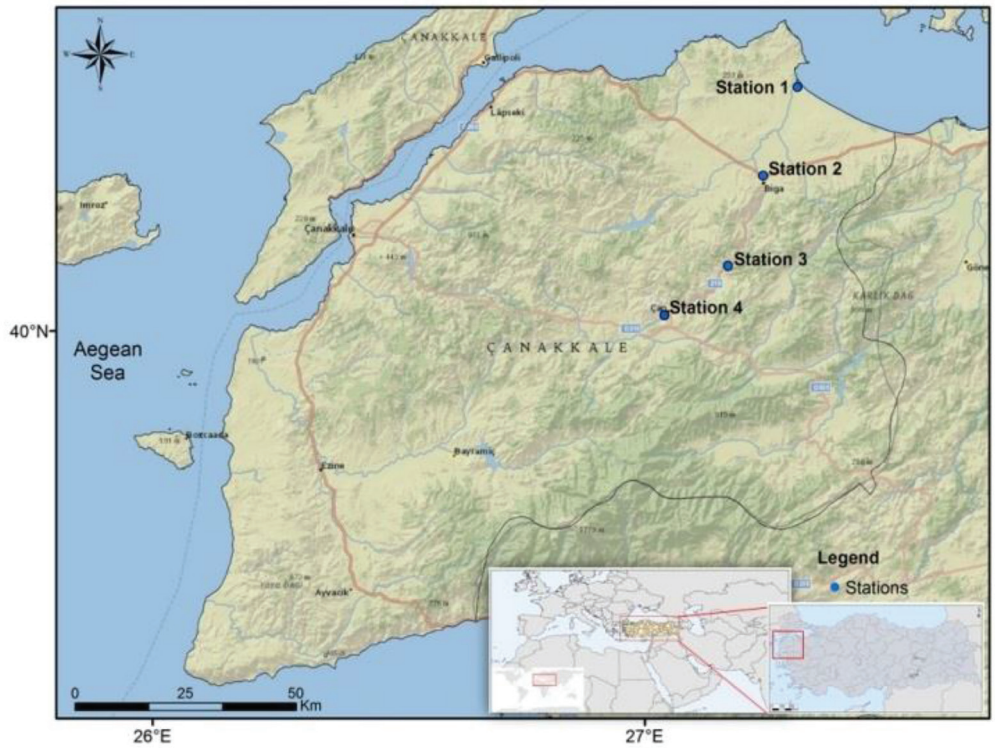

Fig. 1. Locations of the four stations on the Biga Stream, Çanakkale, Turkey

Table 1. The information of each station and levels of heavy metals in the water samples taken from four stations of the Biga Stream

		Coordinates	Heavy metal analyses (mg/L)							
			Al	Cu	Zn	Fe	Cd	Cr	Pb	Ni
Station 1	Estuary zone	40º22'23.00" N 27º18'37.51" E	0.132	0.022	<0.0006	0.326	<0.0006	<0.002	<0.01	<0.003
Station 2	City centre	40º14'15.75" N 27º14'24.71" E	0.545	0.029	0.066	0.945	<0.0006	<0.002	<0.01	0.004
Station 3	Buffer zone	40º05'59.46" N 27º10'08.19" E	0.154	0.025	0.030	0.032	<0.0006	<0.002	<0.01	0.034
Station 4	Industrial zone	40º01'26.38" N 27º02'22.02" E	0.218	0.021	0.037	0.068	<0.0006	<0.002	<0.01	0.026

solution. The haematocrit (HCT) was determined using the micro-haematocrit method (Tanyer 1985). The tubes were then spun in a micro-haematocrit centrifuge for 5 min at 12000 rpm and HCT was calculated with a total blood level divided by blood cell level. The haemoglobin concentration (HB) was measured using the Sahli method with a Sahli Haemoglobinometer (Tanyer 1985). The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated mathematically taking the above-mentioned results into consideration (Tanyer 1985). Blood smears stained with Wright's stain were used in measuring the blood cells. Cell measurements were performed under a microscope with an ocular micrometer. Forty erythrocytes were randomly chosen on each blood smear; cell lengths (EL) and widths (EW), together with the lengths (NL) and widths (NW) of their nuclei were measured in micrometers and then cell (ES) and nuclei sizes (NS) were computed with the formulas: $EL \cdot EW \cdot \pi / 4$ and $NL \cdot NW \cdot \pi / 4$, respectively. Cell photomicrographs were taken with an Olympus photomicroscope.

The descriptive statistics were obtained using the Statistical Package for the Social Sciences (SPSS Chicago, IL, USA), v. 10.0. The Mann-Whitney U test was used to compare differences between haematological parameters of the four studied sites.

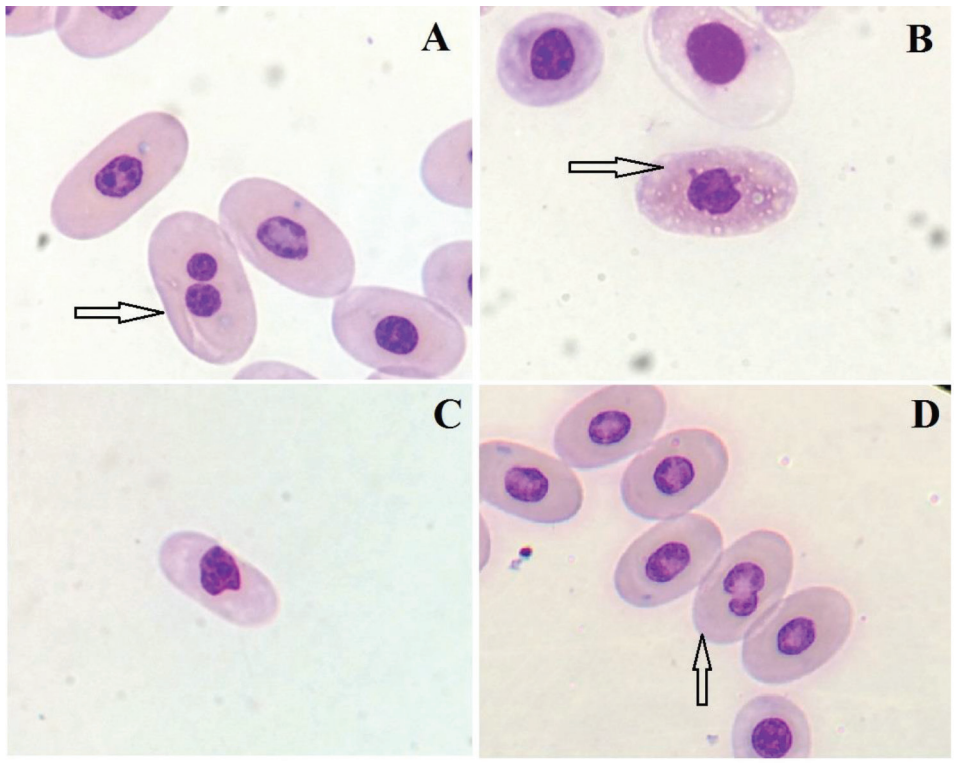

Fig. 2. Nuclear abnormalities in erythrocytes of *Mauremys rivulata* from the Biga Stream. Binucleate (A), Blebbed nucleus (B), Notched nucleus (C), Lobed nucleus (D).

Measurement of Micronucleus and Nuclear Abnormalities

In this study, 1 ml of blood from the dorsal caudal vein of each specimen was taken for blood smears using a 5 ml syringe with needles with a diameter of 21mm (Ballard & Cheek 2003, Thrall et al. 2004). The peripheral blood smears for each sample were prepared on clean slides, after fixation in ethanol for 20 min; the slides were air-dried at room temperature, fixed with methanol for 15 min and then stained with Giemsa stain (10% v/v) (Josende et al. 2015). Three slides were prepared for each sample and a total of 1000 erythrocytes per slide were scored from each slide under an optic microscope (1000x magnification). The micronucleus was defined according to the criteria as follows: a) MN must not touch the main nuclei, b) MN should be smaller than one-third of the main nuclei) and c) MN must not be refractive and should be the same colour and intensity as the main nuclei (Heddle et al. 1976, Titenko-Holland et al. 1997, Fenech 2000). Nuclear abnormalities, such as kidney-shaped nuclei, lobed nuclei, and notched nuclei and blebbed nuclei, were also determined and scored from the slides (Carrasco et al. 1990, Strunjak-Perovic et al. 2009, Josende et al. 2015).

The data were tested for normality using the Kolmogorov–Smirnov test. Since the data showed normal distribution, parametric tests were used to detect differences at the 0.05 level of significance. One-way analysis of variance (ANOVA) was used to evaluate significant differences in the mean values of different groups using SPSS, v. 20.0. The differences in total nuclear abnormalities between the four groups were compared using the Tukey's test ($p \leq 0.05$).

Results

Nuclear abnormalities such as binuclei, blebbed nuclei, kidney-shaped nuclei, lobed nuclei, notched nuclei and micronuclei (Fig. 2) were observed in erythrocytes from *Mauremys rivulata* from the Biga Stream.

We found no statistical difference among stations for haematological parameters of *M. rivulata* (Mann-Whitney U test, $p>0.05$; Table 2).

The frequencies of micronuclei and nuclear abnormalities in erythrocytes varied from 4.42% to 9.16% (Table 3). The frequency of total nuclear abnormalities was highest at Station 2 (9.16±0.02), followed by Station 4 (8.44±0.01). Station 1 and Station 3 had similar frequency: 4.84±0.01 and 4.49±0.01, respectively. Micronuclei were observed with low frequencies at all stations. The frequency of blebbed nuclei was higher than the other abnormalities at all station.

Table 2. Haematological values for *Mauremys rivulata* at each station. (N: Number of specimens, SD: Standard Deviation, RBC: Red Blood Cell Count, WBC: White Blood Cell Count, HB: Haemoglobin, HCT: Haematocrit, MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Haemoglobin, MCHC: Mean Corpuscular Haemoglobin Concentration)

	Parameters	N	Minimum	Maximum	Mean	SD
Station 1	RBC (1mm^3)	4	380000.00	620000.00	483750.00	113971.85
	WBC (1mm^3)	4	4700.00	8500.00	6075.00	1705.62
	HB (g/dl)	4	4.00	8.20	5.90	1.74
	HCT (%)	4	14.00	33.00	21.50	8.88
	MCV (fl)	4	350.00	616.82	437.16	121.35
	MCH (pg)	4	100.93	150.00	122.11	23.18
	MCHC (%)	4	16.36	42.86	30.01	11.25
	Erythrocyte size (ES)	4	146.48	178.51	159.98	13.74
	Nucleus size (NS)	4	21.98	27.79	24.06	2.70
	NS/ES	4	0.13	0.16	0.15	0.01
Station 2	RBC (1mm^3)	15	410000.00	860000.00	582666.66	131391.81
	WBC (1mm^3)	15	3300	8000	5980	1325.68
	HB (g/dl)	14	4.00	8.30	6.35	1.33
	HCT (%)	15	16.00	37.00	24.66	6.79
	MCV (fl)	13	305.08	902.43	468.98	152.95
	MCH (pg)	14	62.50	170.73	113.84	30.87
	MCHC (%)	14	12.19	37.50	26.49	7.76
	Erythrocyte size (ES)	4	136.75	182.91	161.82	24.23
	Nucleus size (NS)	4	23.55	29.83	26.72	3.16
	NS/ES	4	0.16	0.18	0.16	0.007
Station 3	RBC (1mm^3)	4	470000.00	690000.00	566750.00	98702.83
	WBC (1mm^3)	4	5000.00	6200.00	5750.00	525.99
	HB (g/dl)	4	5.00	8.00	6.35	1.39
	HCT (%)	4	19.00	24.00	21.00	2.16
	MCV (fl)	4	347.83	404.26	374.13	29.41
	MCH (pg)	4	98.62	116.67	111.53	8.63
	MCHC (%)	4	25.00	33.33	30.02	4.07
	Erythrocyte size (ES)	4	148.68	182.91	163.75	16.00
	Nucleus size (NS)	4	21.51	29.83	23.98	3.93
	NS/ES	4	0.13	0.16	0.14	0.01
Station 4	RBC (1mm^3)	6	450000.00	720000.00	579666.66	97171.32
	WBC (1mm^3)	6	5000.00	8100.00	6000.00	1082.58
	HB (g/dl)	6	6.40	9.00	7.78	0.84
	HCT (%)	6	20.00	30.00	25.00	4.19
	MCV (fl)	6	303.03	622.22	443.56	116.82
	MCH (pg)	6	96.97	200.00	138.69	35.33
	MCHC (%)	6	27.00	38.10	31.57	4.14
	Erythrocyte size (ES)	4	143.81	185.73	167.44	19.30
	Nucleus size (NS)	4	21.35	33.44	26.25	5.18
	NS/ES	4	0.13	0.19	0.15	0.02

Discussion

The genotoxicity of water resources may largely be based on heavy metals present in aquatic ecosystems (Salles et al. 2016). Heavy metals have toxic effects and can be accumulated in organisms (Barcellos et al. 1988, Topashka-Ancheva et al. 2003a, 2003b, Gajalakshmi & Ruban 2014). Many studies have reported that heavy metals affect reproductive, immune and nervous systems, behaviour and carcinogenesis in terrestrial species (Mazliah et al. 1989, Burger & Gochfeld 2000, Fair & Ricklefs 2002). Excessive exposure to some heavy metals during the developing stage

Table 3. Frequencies (%) of micronuclei and nuclear abnormalities with standard deviation in erythrocytes of *Mauremys rivulata* from the Biga Stream, Çanakkale, Turkey

	Station 1	Station 2	Station 3	Station 4
Micronuclei	0	0	0.1±0.007	0
Lobed nuclei	0.41±0.07	0.34±0.06	0.14±0.004	0.29±0.01
Notched nuclei	1.07±0.1	1.22±0.2	0.85±0.01	1.29±0.02
Blebbed nuclei	3.34±0.05	7.45±0.03	3.40±0.2	6.75±0.21
Binuclei	0.02±0.006	0.05±0.007	0	0
Kidney-shaped nuclei	0	0.10±0.004	0	0.09±0.001
Total nuclear abnormalities (%)	4.84±0.01[b]	9.16±0.02[d]	4.49±0.01[a]	8.44±0.01[c]

*Means with the different letter within the columns are significantly disparate at $p \leq 0.05$

can change the level of gonadal steroid hormones, adrenal and thyroid hormones (Colborn 2002, Dawson 2000), which could be the cause of potentially serious effects on the survival of sensitive or vulnerable populations (Garciá-Fernández et al. 2009, Lopez et al. 2010).

The concentrations of pollutants in the Biga Stream vary according to the pressures it is exposed to and are different at each station. These pollutants have genotoxic potential on genetic materials of organisms living in these habitats. Heavy metals are the most important source of inorganic pollutants in the water. The highest percentage of the total nuclear abnormalities and each kind of abnormality in erythrocytes of *M. rivulata* at Station 2 could be likely owing to the highest content of heavy metals as demonstrated by Çördük et al. (2018).

Based on our data, the amount of micronuclei alone was found to be insufficient to determine the genotoxic effect of the environment on *M. rivulata.* Similarly, Zúñiga-González et al. (2000, 2001) reported that turtles might not be good indicators of genotoxic exposure based on their results of MN test examining one specimen of *Macrochelys temminckii* (Troost in Harlan, 1835) and two specimens of *Kinosternon subrubrum* (Lacépède, 1788). Our results suggested that the other nuclear abnormalities should be used together with micronuclei to determine the levels of genetic damage in erythrocytes of *M. rivulata*. Guilherme et al. (2008) specified that due to its higher responsiveness, nuclear abnormalities assay represents an alternative to MN test, overcoming a possible lack of sensitivity related to the low MN frequency in wild fish.

When compared with the haematological values provided by previous studies (Pages et al. 1992, Metin et al. 2008, Yilmaz & Tosunoğlu 2010, Tosunoğlu et al. 2011), it was determined that the data we obtained for *M. rivulata* were similar.

Understanding the effects of adverse environmental conditions is important for managing the sustainability of ecosystems and maintaining species. Biomonitoring is a particularly useful tool for identifying pollutants affecting human and environmental health (Silva et al. 2003). Zapata et al. (2016) indicated that turtle species were often used as indicators of pollution levels but only a few reports assessed genotoxic effects through the application of the MN test or Comet Assay (CA) on erythrocytes of species inhabiting impacted environments. Meyers-Schöne et al. (1993) reported that *Trachemys scripta* (Schoepff, 1792) and *Chelydra serpentina* (L., 1758) may be useful as a bioindicator for genotoxic monitoring using CA.

In conclusion, the correlation between water pollutants and the percentage of the total nuclear abnormalities show that monitoring nuclear abnormalities in erythrocytes of *Mauremys rivulata* may provide baseline data for evaluating its role as a valuable biological marker for biomonitoring environmental pollutants.

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References

Ballard B. M. & Cheek R. 2003. Exotic animal medicine for the veterinary technician. Blackwell Publishing, Iowa State Press, Ames. 520 p.

Bolognesi C. & Hayashi M. 2011. Micronucleus assay in aquatic animals. Mutagenesis 26 (1): 205-213.

Bradl H. (ed.) 2002. Heavy Metals in the Environment: Origin, Interaction and Remediation. Vol 6. Academic Press, London. 282 p.

Broggi M. F. 2012. The Balkan Terrapin *Mauremys rivulata* (Valenciennes, 1833) in the Aegean islands. Threats, conservation aspects and the situation on the island of Kea (cyclades) as a case study (Testudines: Geoemydidae). Herpetozoa 24 (3/4): 149-163.

Carrasco K., Tilbury K. L. & Myers MS. 1990. Assessment of the piscine micronucleus test as an in situ biological indicator of chemical contaminant effects. Canadian Journal of Fisheries and Aquatic Sciences 47: 2123–2136.

Çördük N., Hacioğlu Doğru N., Gül Ç. & Tosunoğlu M. 2018. Monitoring of micronuclei and nuclear abnormalities in *Pelophylax ridibundus* erythrocytes from the Biga Stream (Canakkale, Turkey). Fresenius Environmental Bulletin 27 (1): 147-153.

Dickinson V. M., Jarchow J. L. & Trueblood M. H. 2002. Hematology and Plazma Biochemistry Reference Range Values for Free-Ranging Desert Tortoises in Arizona. Journal of Wildlife Diseases 38 (1):143-153.

Fenech M. 2000. The in vitro micronucleus technique. Mutation Research 455: 81-95.

Gibbons J. W. 1987. Why do turtles live so long? BioScience 37: 262-269.

Guilherme S., Válegab M., Pereirab M. E., Santosa M. A. & Pachecoa M. 2008. Erythrocytic nuclear abnormalities in wild and caged fish (*Liza aurata*) along an environmental mercury contamination gradient. *Ecotoxicology and Environmental Safety* 70 (3): 411–421.

Gugliandolo C., Lentini V., Fera M. F., Camera E. & Maugeri T. L. 2009.Water quality and ecological status of the Alcantra River Estuary (Italy). New Microbiologica 32: 77–87.

Heddle J. A. & Countryman R. I. 1976. The production of micronuclei from chromosome aberration in irradiated cultures of human lymphocytes. Mutation Research 41: 321-332.

Honegger R. E. 1981. Threatened Amphibians and Reptiles in Europe. Wiesbaden: Akademische Verlagsgesellschaft, 123 p.

Jacopson E. R., Gaskin J. M., Brown M. B., Harris H. K., Gardiner C. H., Lapointe J. L., Adams H. P. & Reggiardo C.1991. Chronic upper respiratory tract disease of free-ranging desert tortoises (*Xerobates agasizii*). Journal of Wildlife Diseases 27: 296-316.

Josende M. E., Tozetti A. M., Alalan M. T., Filho V. M., Ximenez S., Junior F. M. R. & Martins S. E. 2015. Genotoxic evaluation in two amphibian species from Brazilian subtropical wetlands. Ecological Indicators 49: 83–87.

Kinge C. W. & Mbewe M. 2012. Bacterial contamination levels in river catchments of the North West Province, South Africa: Public health implications. African Journal of Microbiology Research 6 (7): 1370–1375.

Kornilev Y., Popgeorgiev G., Naumov B., Stoyanov A. & Tzankov N. 2017. Updated distribution and ecological requirements of the nativ e freshwater turtles in Bulgaria. Acta Zoologica Bulgarica, Supplement 10: 65-76.

Kousar S. & Javed M. 2015. Studies on Induction of Nuclear Abnormalities in Peripheral Blood Erythrocytes of Fish Exposed to Copper. Turkish Journal of Fisheries and Aquatic Sciences 15: 879-886.

Lee R. F. & Steinert S. 2003. Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. Mutation Research 544: 43–64.

Lopez-Olivera J. R., Montane J., Marco I., Martinez-Silvestre A., Soler J. & Lavin S.2003. Effect of venipuncture site on hematologic and serum biochemical parameters in marginated Tortoise (*Testudo marginiata*). Journal of Wildlife Diseases 39 (4): 830-836.

Metin K., Basimoğlu Koca Y., Kargin Kiral F., Koca S. & Türkozan O. 2008. Blood Cell Morphology and Plazma Biochemistry of Captive *Mauremys caspica* (Gmelin, 1774) and *Mauremys rivulata* (Valenciennes, 1833). Acta Veterinaria, Brno 77: 163-174.

Meyer E., Eskew E. A., Chibwe L., Schrlau J., Simonich S. L. M. & Todd B. D. 2016. Organic contaminants in western pond turtles in remote habitat in California. Chemosphere 154: 326-334.

Meyers-Schöne L., Shugart L. R., Beauchamp J. J. & Walton B. T. 1993. Comparison of two freshwater turtle species as monitors of chemical contamination: DNA damage and residue analysis. Environmental Toxicology and Chemistry 12: 1487-1496.

Napierska D., Barŝienė J., Mulkiewicz E., Podolska M. & Rybakovas A. 2009. Biomarker responses in flounder *Platichthys flesus* from the Polish coastal area of the Baltic Sea and applications in biomonitoring. Ecotoxicology 18: 846–859.

Obiakor M. O., Okonkwo J. C., Nnabude P. C. & Ezeonyejiaku C. D. 2012. Eco-genotoxicology: Micronucleus assay in fish erythrocytes as in situ aquatic pollution biomarker: a review. Journal of Animal Science Advances 2: 123-133.

Pages T., Peinado V. I. & Visco G. 1992. Seasonal Changes in Hematology and Blood Chemistry of the Freshwater Turtle *Mauremys caspica leprosa*. Comparative Biochemistry and Physiology 103A (2): 275-278.

Pollo F. E., Bionda C. L., Salinas Z. A., Salas N. E. & Martino A. L. 2015. Common toad *Rhinella arenarum* (Hensel, 1867) and its importance in assessing environmental health: test of micronuclei and nuclear abnormalities in erythrocytes. Environmental Monitoring and Assessment 187 (9): 581. https://doi.org/10.1007/s10661-015-4802-1

Pollo F. E., Grenat P. R., Otero M. A., Salas N. E. & Martino A. L. 2016. Assessment in situ of genotoxicity in tadpoles and adults of frog Hypsiboas cordobae (Barrio 1965) inhabiting aquatic ecosystems associated to fluorite mine. Ecotoxicology and Environmental Safety 133: 466-474. https://doi.org/10.1016/j.ecoenv.2016.08.003

Pollo F. E., Grenat P. R., Salinas Z. A., Otero M. A., Salas N. E., & Martino A. E. 2017. Evaluation in situ of genotoxicity and stress in South American common toad *Rhinella arenarum* in environments related to fluorite mine. Environmental Science and Pollution Research 24 (22): 1-9. https://doi.org/ 10.1007/s11356-017-9479-2

Popgeorgiev G., Kornilev Y. V., Natchev N., Naumov B., Ivanchev I., Slavchev M., Stoyanov A. & Tzankov N. 2017. Spatial distribution of *Emys orbicularis* (L., 1758) and *Mauremys rivulata* (Valenciennes, 1833) in the lower Veleka River, Bulgaria: First observations. Acta Zoologica Bulgarica, Supplement 10: 129-132.

Raphael B. L., Klemens M. W., Moehlman P., Dterenfeld E. & Karesh W. B.1994. Blood values in free-ranging pancake tortoises (*Malacochersus tornieri*). Journal of Zoo and Wildlife Medicine 25: 63-67.

Salinas Z. A., Baraquet M., Grenat P. R., Martino A. L. & Salas N. E. 2017. Morphology and size of blood cells of *Rhinella arenarum* (Hensel, 1867) as environmental health assessment in disturbed aquatic ecosystem from central Argentina. Environmental Science and Pollution Research 24 (32): 24907-24915. https://doi.org/10.1007/s11356-017-0107-y

Salles F. J., Toledo M. C. B., César A. C. B., Ferreira G. M. & Barbério A. 2016. Cytotoxic and genotoxic assessment of surface water from São Paulo state, Brazil, during the rainy and dry seasons. Ecotoxicology 25: 633–645.

Shugart L. R. 1998. Structural damage to DNA in response to toxicant exposure. In: Forbes V. (Ed.) Genetics and Ecotoxicology, Washington, Taylor & Francis. pp. 151-167.

Silva J., Heuser V. & Andrade V. 2003. Biomonitoramento ambiental. In: Silva J, Erdtmann B, Henriques J, editors. Genética Toxicológica. Porto Alegre: Alcance. pp. 167–178.

Siu W. H. L., Cao J., Jack R. W., Wu R. S. S., Richardson B. J., Xu L. & Lam P. K. S. 2004. Application of the comet and micronucleus assays to the detection of B[a]P genotoxicity in haemocytes of the green-lipped mussel (*Perna viridis*). Aquatic Toxicology 66: 381-392.

Strunjak-Perovic I., Coz-Rakovac R., Topic Popovic N. & Jadan M. 2009. Seasonality of nuclear abnormalities in gilthead sea bream *Sparus aurata* (L) erythrocytes. Fish Physiology and Biochemistry 35: 287–291.

Strunjak-Perovic I., Lisicic D., Coz-Rakovac R., Popovic N. T., Jadan M., Benkovic V. & Tadic Z. 2010. Evaluation of micronucleus and erythrocytic nuclear abnormalities in Balkan whip snake *Hierophis gemonensis*. Ecotoxicology 19: 1460–1465.

Tanyer, G. 1985. Hematoloji ve Laboratuar, Ayyıldız Matbaa A.Ş. Turkey. (in Turkish). pp. 111-122.

Tchounwou P. B., Yedjou C. G., Patlolla A. K. & Sutton D. J. 2012. Heavy Metals Toxicity and the Environment. Molecular, Clinical and Environmental Toxicology 101: 133–164.

Thrall A., Baker D. C., Campbell T. W., DeNicola D., Feetman M. J., Lassen E. D., Rebar A. & Weiser G. 2004. Veterinary hematology and clinical chemistry. Philadephia: Blackwell Publishing. 776 p.

Titenko-Holland N., Windham G., Kolachana P., Reinisch F., Parvatham S., Osorio A. M. & Smith M. T. 1997. Genotoxicity of malathion in human lymphocytes assessed using the micronucleus assay in vitro and in vivo: a study of malathion-exposed workers. Mutation Research 388 (1): 85-95.

Topashka-Ancheva M., Metcheva R. & Teodorova S. 2003a. Bioaccumulation and damaging action of polymetal industrial dust on laboratory mice *Mus musculus alba*. II. Genetic, cell, and metabolic disturbances. Environmental Research 92 (2): 152-160. DOI:10.1016/S0013-9351(02)00034-8

Topashka-Ancheva M., Metcheva R. & Teodorova S. 2003b. A comparative analysis of the heavy metal loading of small mammals in different regions of Bulgaria II: chromosomal aberrations and blood pathology. Ecotoxicology and Environmental Safety 54 (2): 188-193.

Tosunoğlu M., Yilmaz N. & Gül Ç. 2011. Effects of varying ecological conditions on the blood parameters of freshwater turtles in Çanakkale (Turkey). Ekoloji 20 (78): 7-12.

Tosunoğlu M., Erdoğan A. & Uysal İ. 2016. Çanakkale Kuşları. T.C. Orman ve Su İşleri Bakanlığı, 2. Bölge Müdürlüğü, Çanakkale Şube Müdürlüğü, Özen Reklam, Çanakkale, Turkey, (in Turkish). 304 p.

Tosunoğlu M., Gül Ç. & Uysal İ. 2017. Çanakkale Amfibi ve Sürüngenleri. T.C. Orman ve Su İşleri Bakanlığı, 2. Bölge Müdürlüğü, Çanakkale Şube Müdürlüğü, Özen Reklam, Çanakkale, Turkey, (in Turkish). 81 p.

Yilmaz N. & Tosunoğlu M. 2010. Hematology and Some Plasma Biochemistry Values of Free-Living Freshwater Turtles (*Emys orbicularis* and *Mauremys rivulata*) From Turkey. North-western Journal of Zoology 6 (1): 109-117.

Zapataa L. M., Bockb B. C., Orozcoa L. Y. & Palacioa J. A. 2016. Application of the micronucleus test and comet assay in *Trachemys callirostris* erythrocytes as a model for *in situ* genotoxic monitoring. Ecotoxicology and Environmental Safety 127: 108-116.

Zhelev Z., Popgeorgiev G., Ivanov I. & Boyadzhiev P. 2017. Changes of erythrocyte-metric parameters in *Pelophylax ridibundus* (Amphibia: Anura: Ranidae) inhabiting water bodies with different types of anthropogenic pollution in Southern Bulgaria. Environmental Science and Pollution Research 24 (21): 17920-17934. https://doi.org/10.1007/s11356-017-9364-z

Zhelev Z. M., Popgeorgiev G. S. & Angelov M. V. 2013. Investigating the changes in the morphological content of the blood of *Pelophylax ridibundus* (Amphibia: Ranidae) as a result of anthropogenic pollution and its use as an environmental bioindicator. Acta Zoologica Bulgarica 65 (2): 187-196.

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