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The morphometric and erythrometric analyses of *Pelophylax ridibundus* living in anthropogenic pollution resources

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Abstract: The health status of *Pelophylax ridibundus* species living in anthropogenic pollution sources in Erzurum (Turkey) were investigated. Firstly, heavy metal concentrations in liver of the frog species, and in water and sediment samples taken from the habitats of the frogs were determined by instrumental analysis. Secondly, morphometric (fluctuating asymmetry-FA, hepatosomatic index-HSI) and erythrometric analyses were performed to reveal the health status of the frogs in the areas. Our study provides the first morphometric datas on the frog in Turkey. Although the metal concentrations in the water were high, it was determined that they did not exceed the limit values. However, Cr, Ni, Zn, and As metal concentrations in the sediment were determined to be above the acceptable level. It was also found that some metals accumulated in the livers of the frog due to heavy metal pollution. On the other hand, the FA, HSI values and the erythrocytic nuclear abnormality frequency increased in the frogs living in polluted stations. Moreover, erythrocyte size decreased and nucleated erythrocyte, mitotic erythrocyte, pycnotic erythrocyte, and immature erythrocyte frequencies increased. It was found that there was a correlation between the presence of pollution and the health status of the frogs, and the frog populations were affected negatively by anthropogenic pollution.

Key words: Anthropogenic pollution, morphometric analyses, erythrometric analyses, *Pelophylax ridibundus*

1. Introduction

Amphibian populations worldwide are known to be decreasing since the 1960s (Houlahan et al., 2000). One of the main reasons for this is anthropogenic pollution (Whittaker et al., 2013). Anthropogenic pollution occurs as a result of human activities such as agricultural and industrial activities (Thammachoti et al., 2012).

Pollution caused by domestic, industrial, and agricultural activities is called anthropogenic pollution. High detergent content, phosphate, and nitrates in sewage wastewater are another source of anthropogenic pollution that causes algae growth in surface waters and disruption of the biological equilibrium in water (Toroğlu et al., 2006). Wastewaters always contain pollutants coming from any anthropogenic industries (Azimi et al., 2017). The industrial waste spreads to a wide area from various sources such as dairy products, sugar, canned products, fat, alcohol, slaughterhouses, flour, yeast, leather, paint, chemicals, fertilizers, coal, iron and steel, textile, paper, metal, salt, oil-based wastes (Özdemir, 2014). Pesticides that can remain in the environment for a long time might have mutagenic, teratogenic, and carcinogenic effects (Chamarthi et al., 2014). Anthropogenic pollution threatens both the freshwater and marine ecosystems (Häder et al., 2020). Pollution in the aquatic environment poses a threat not only to aquatic organisms but also to humans (Moiseenko et al., 2018). This situation requires serious consideration of environmental issues. A good understanding of pollution factors that affect biodiversity, which can lead to a decrease in the population of species or even complete extinction of some, plays a critical role in the protection of species. At this point, appropriate organism selection is often important for monitoring the state of aquatic ecosystems.

In this respect, tailless frogs (Anura) are among chosen aquatic bioindicator organisms. Short- and long-term responses of the frogs to environmental toxicants are easily determined by examining physiological (blood, hormone, immunological and biochemical changes) and morphophysiological parameters (hepatosomatic index, condition factor, asymmetry, etc.) (Zhelev et al., 2013, 2014a, 2015a, 2017a; Pollo et al., 2015; Guo et al., 2017). Another bioindicator in the frogs is erythrometric parameters. These parameters are used during researches on health risks and environmental risk assessments in polluted areas (Pollo et al., 2016). These are the first

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indicators exhibiting the link between the organism exposed to toxic substances and the pollutant (Cajaraville et al., 2000). A few studies have been conducted on changes in erythrometric parameters in *Pelophylax ridibundus* living in different anthropogenic pollution areas (Zhelev et al., 2017b, Şişman et al., 2021). Therefore, studies analyzing erythrometric parameters in frogs are very important in terms of identifying the negative effects of pollution and determining the completion of the cycle of the ecosystem.

Despite the global decrease in tailless frog populations, some species can maintain their viability even under high anthropogenic pollution (Zhelev et al., 2018). One of these species is *Pelophylax ridibundus* (Anura; Ranidae). *P. ridibundus* is one of the oldest identified species among frogs of Western Palearctic (Kuru, 2020). *P. ridibundus* was known as *Rana ridibunda* until recent times. Due to the paraphyletic state of the *Rana* genus, its name was changed to *P. ridibundus* (Mohammadi et al., 2015). However, both names are still known to be used by different researchers. The species is vastly present in Turkey (Arıkan et al., 1998). It is known that the species inhabit wide areas in Erzurum. In our previous field studies (Şişman et al., 2015, Alnoaimi et al., 2021; Şişman et al., 2021), we observed that the number of frogs was gradually decreasing. The main question in this study is; how are frogs in Erzurum province affected by anthropogenic pollution? If so, what is the population and frog health status? To determine these, frogs were collected from three different areas in two different seasons, and erythrometric and morphometric analyzes were performed on the frogs. Also, the concentrations of some heavy metals in water and sediment samples were investigated to detect pollution. It was also determined whether there is a relation between the presence of heavy metal and the results of morphometric and erythrometric analysis. The most important feature of this study is that fluctuating asymmetry (FA) analysis has been done for the first time.

2. Material and methods

2.1. Study area

Three different stations were determined in the study by taking into account the situation of anthropogenic pollution (Figure 1). The first of these is the tributary of Ömertepesuyu creek, which passes through Dereboğazı Village, one of the central villages of Erzurum (1st station: 39°49'49.9"N 41°01'40.4"E). Since there is no urban construction and agricultural activity around this place, it

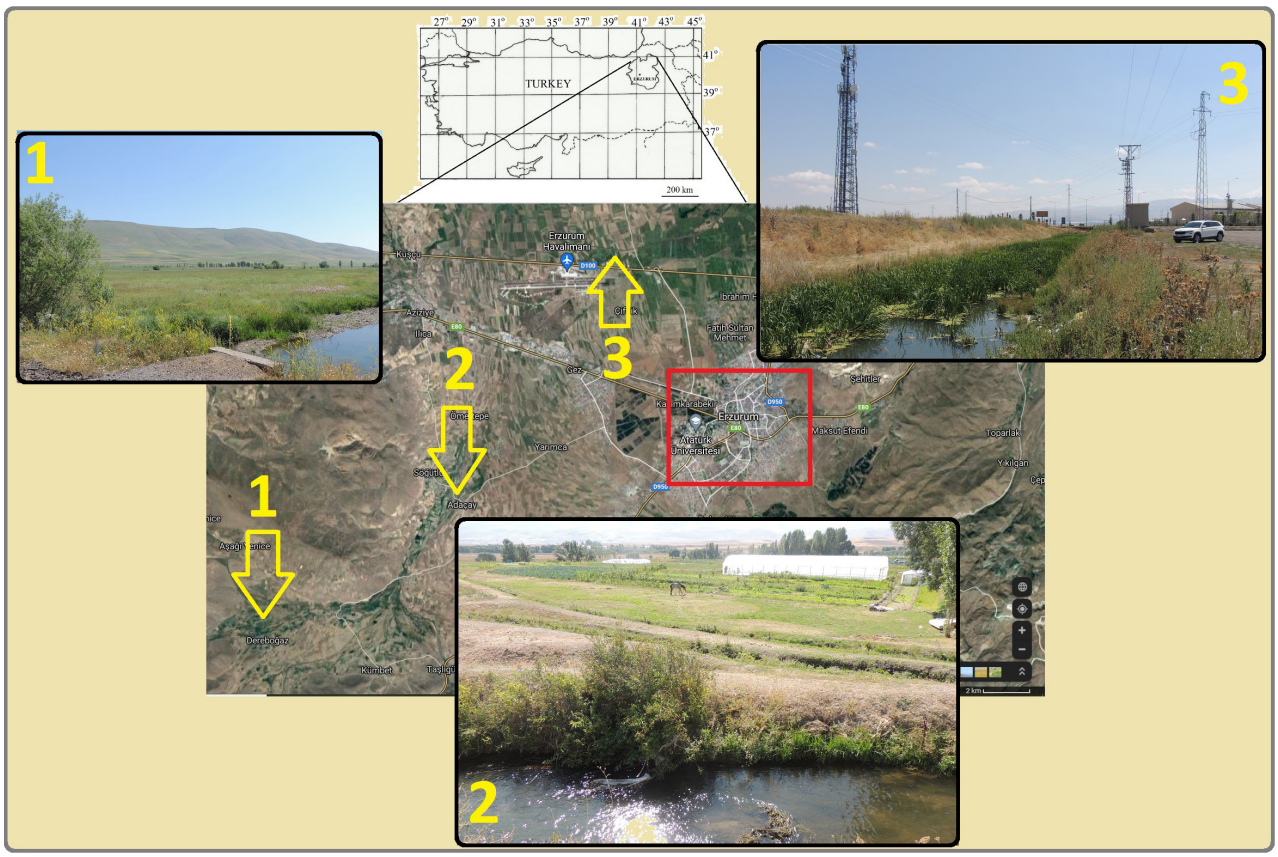

Figure 1. The sampling stations. 1st station (Dereboğazı); Erzurum-Dereboğazı Village, 28.6 km. 2nd Station (Adaçay); Erzurum-Adaçay, 15.7 km. 3rd station (Erzurum State Airport Square); Erzurum-Airport, 12 km (red square; centrum).

is considered a reference region. The 2nd station is located around Adaçay Village, where pesticides are used (2nd station: 39°52'28.9"N 41°07'27.3"E). The 3rd station is the irrigation channel next to the airport, where the highway and domestic wastewater pollution are present (3rd station: 39°57'54.7"N 41°10'35.6"E). There is D100 Highway, which is densely used and which connects Artvin and Erzincan, next to the irrigation canal. The irrigation canal is fed by the water of the Karasu River; and since the river is polluted by the provincial sewage system, the 3rd station was assessed in terms of domestic wastewater as well.

2.2. Water and sediment analyses

Water samples (total of six samples in May and September 2019) were taken from the stations from 1.5 m of depth, and these water samples were reserved in 2 L light-proof plastic containers at +4 °C until the analysis. Ten milliliters of water samples were used for element analysis. Sediment samples (total of six samples) were also taken from the bottom of the water and placed in 250 mL light-proof glass bottles. The sediments were dried at 103 °C, ground in a mortar, sieved, through a 63-micron sieve, and stored at 4 °C until the analysis. Heavy metals were extracted from the sediments using USEPA Method 3051 A. Sediment solutions were diluted to 50 mL with ultrapure water and heavy metals analyzed using inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7800) in DAYTAM (Eastern Anatolia High Technology Application and Research Center). Cr, Mn, Fe, Ni, Cu, Zn, As, Cd, Hg, and Pb were analyzed in water and sediment samples. The heavy metals have been chosen because they have the most negative effects on the environment and they are toxic to aquatic and terrestrial organisms even in minor quantities (Masindi and Muedi, 2018).

2.3. Morphometric analysis

P. ridibundus (Anura; Ranidae) frogs more abundant in the natural fauna of the selected stations were preferred for biomonitoring studies in the study. Necessary official permits were obtained by applying relevant authorities before starting the study (Atatürk University Experimental Medicine Animal Experimentation Local Ethics Committee No: 36643897-132/110, Ministry of Food, Agriculture and Livestock General Directorate of Fisheries and Aquaculture No:67852565/140.03.03-863, Ministry of Forestry and Waterworks, General Directorate of Nature Conservation and National Parks No:72784983-488.04-63471).

Ten adult frogs from each station were caught by using sweep nets in May and September 2019, and the frogs were brought to the laboratory in plastic containers. The technical characteristics and physical conditions of the containers are suitable for the welfare of the animals. Because all populations are considered to be equally affected by pollution, sexual differences in frogs were not taken into account. However, care was taken to ensure that all frogs were the same length (SVL; 5–8 cm, min-max.). Each frog was measured in length and body weight, later their sexes were determined and recorded. In the frogs, sex is determined by the pad in the forefoot (If there is a pad in the forefoot, it is a male).

Biotic indices and physicochemical parameters are frequently used in environmental quality studies. In biotic indexes, especially fluctuating asymmetry (FA) are frequently used in environmental assessments for frog adults and tadpoles (Møller and Swaddle, 1997, Montalvão et al., 2018).

In this study, the FA parameter was used for morphometric analysis. In the FA calculation of *P. ridibundus*, the fluctuating asymmetry characteristics (the number of stripes on the dorsal side of the thigh, the number of spots on the dorsal side of the thigh, the number of stripes on the dorsal side of the shank, the number of spots on the dorsal side of the shank, the number of stripes on the foot, the number of spots on the foot, the number of stripes on the back, the number of spots on the back, the number of white spots on the plantar side of the third finger of the hind leg, the number of white spots on the plantar side of the fourth finger of the hind leg) determined by Zakharov et al. (2001) were used. These characteristics were counted separately for each right and left side and determined for each individual. The FA was calculated using the formula (Zakharov et al., 2001). FA = Σ dL-R/N

In the formula, L; left side, R; the right-hand side show the characteristics and N shows the total number of individuals in the population. The values are range between 0 and 1. Any value close to 1 indicates that asymmetry increased and consequently frogs were affected by anthropogenic pollution. While interpreting obtained data, the criteria developed by Peskova and Zhukova (2007) were used.

2.4. Liver analysis

The livers of dissected frogs were taken and weighed. At first, the liver was used for the determination of hepatosomatic index (HSI). HSI was calculated using the formula: HSI = (LW/BW)100 (Zhelev et al., 2015a). Here, LW refers to the weight of liver and BW refers to total weight of the body. Also, the livers preserved in –80 °C were delivered to DAYTAM for being subjected to element analysis. All elements assessed in water and sediment were also analyzed in the liver samples.

2.5. Erythrometric analysis

The frogs were anesthetized with ether and dissected. A blood sample of about 0.2 mL was taken from the frog's cardiac ventricle by using a hematocrit tube for the genotoxic and cytotoxic analyses. The blood samples were dried after being placed on slides. At least 5 blood smear preparations were prepared from each frog. These

slides were fixed in ethanol (99.8%) for 10 min and then stained with Giemsa (10%) for 30 min. Preparations were examined in a Leica DM750 microscope, and obtained images were analyzed by the Leica LAS EZ Software Program.

Peripheral bloods of the adult frogs were used to examine genotoxic effects. In preparations belonging to each individual, 2000 cells were counted, and the values were assessed per thousand (‰) (Lajmanovich et al., 2014). According to Fenech (2000) and Carrasco et al. (1990), micronuclei (MN) and erythrocytic nuclear abnormalities (ENA) were investigated in mature erythrocytes to determine genotoxicity. MN and ENA such as notched, binucleated, bud, lobed, blebbed, and kidney-shaped nuclei were common abnormalities in frogs (Pollo et al., 2015) and they were analyzed in the slides.

Frog erythrocytes are nucleated and their shape is usually oval. It was reported that the deviations from this shape were detected in the frogs living in anthropogenic pollution sources (Zhelev et al. 2017b). For cytotoxic analysis, peripheral blood erythrocytes of the frogs were assessed in terms of erythrocyte length (EL), erythrocyte width (EW), erythrocyte size (ES), nucleus length (NL), nucleus width (NW), nucleus size (NS) and nucleo-cytoplasmic ratio (NS/ES). Forty out of 600 cells from each prepared preparation were randomly selected for analysis and measured in terms of EL, EW, NL, and NW. ES and NS values are calculated using the formula: ES = ELEWπ / 4, NS = NLNWπ / 4 (Atatür et al., 1999; Arserim and Mermer, 2008). Cell and nucleus shapes were determined according to EL/EW and NL/NW ratios. NS/ES value refers to the nucleo-cytoplasmic ratio. Also, frequencies of enucleated erythrocytes (EE), mitotic erythrocytes (ME), pyknotic erythrocytes (PE), immature erythrocytes (IE) were used to estimate erythrocytic abnormalities (Pollo et al., 2019).

2.6. Statistical analysis

The general assessment of all obtained data was carried out using one-way analysis of variance (ANOVA). Duncan's test was applied for multiple comparisons in equal variances assumed analyses. The data were interpreted considering the significance level as $p < 0.05$. SPSS 21.0 (IBM Corporation, Armonk, NY, USA) was used for the assessment of all statistical data.

3. Results

3.1. Water and sediment

The water and sediment samples were analyzed in terms of some element contents. When the results obtained throughout the year 2019 were compared with reported acceptable heavy metal ratios in water resources according to Surface Water Quality Regulation (SWQR, 2015), it was seen that standard values were not exceeded (Table 1). Between all stations, significant variation was observed among all stations except the reference station (station 1). Although metal concentrations appear within the acceptable limits, it was seen that the metal concentrations were high in the 3rd station.

The sediment quality of stations was shown in Table 2 according to international sediment quality criteria. None of the metal levels in sediments obtained from the 1st station (except Ni) exceeded determined limit values. However, Cr, Ni, Zn, and As metal concentrations in the 2nd and 3rd stations were determined to be above the acceptable level. In parallel with the results of water analysis, the results of sediment analysis also indicated that the 3rd station was the most polluted area.

3.2. Liver

The mean values of some metals detected in the frog livers were presented in Table 3. A significant increase in concentrations of some metals (Cr, Mn, Fe, Cu, Zn, As, Cd, Hg) was observed in the liver of frogs from the 2nd and the 3rd stations ($p < 0.05$). The most accumulated heavy metal was Cu in livers of frogs from the 3rd station. It was followed by Fe, Zn, Cd, and Mn. Currently, the results of metal accumulation in livers, except for Cd, were quite consistent with sediment metal levels. As an indicator of the health status of frogs, HSI values were summarized in Table 4. The highest value in terms of mean HSI was found in frogs from the 3rd station and the lowest value was found in frogs from the 1st station. It was also seen that the difference between values was statistically significant ($p < 0.05$).

3.3. Morphometric results

FA values were calculated separately for each frog and the following results were obtained (Table 5). FA value increased in the 3rd station compared to other stations. This condition was also identified morphologically in the frogs.

While asymmetry was rarely observed in the frogs living in the reference station (Figure 2A), little asymmetry was observed in some frogs living in the 2nd station (Figure 2B). However, whole asymmetry was observed in almost all of the frogs living in the 3rd station (Figures 2C and 2D). There was no statistically significant difference between the sexes (Table 5). According to these results 1st station was considered as clean water basin, the 2nd station was considered as slightly polluted water basin and finally 3rd station was considered as polluted water basin.

3.4. Erythrometric results

When peripheral blood cells of the frogs were examined, it was found that mature erythrocytes and the nuclei were in elliptical shapes, and the nucleus was at or near the cell center (Figure 3A). Besides, various ENA at various rates were detected depending on the stations. Especially

Table 1. The Surface Water Quality Regulation (SWQR, 2015) and water quality class status of water taken from stations according to the regulation.

	Water quality classes (µg/L)				Mean values of the stations (µg/L)		
Metals	I	II	III	IV	1. station	2. station	3. station
Cr	20	50	200	>200	0.058 ± 0.00	0.107 ± 0.00	0.306 ± 0.026*
Mn	100	500	3000	>3000	0.264 ± 0.05	0.723 ± 0.20	91.93 ± 10.00*
Fe	300	1000	5000	>5000	5.136 ± 1.00	15.196 ± 2.85*	39.15 ± 9.00*
Ni	20	50	200	>200	1.275 ± 0.02	2.089 ± 0.08*	3.055 ± 0.06*
Cu	20	50	200	>200	0.259 ± 0.01	1.130 ± 0.10*	1.949 ± 0.40*
Zn	200	500	2000	>2000	<0.000	0.903 ± 0.10*	2.930 ± 0.90*
As	20	50	100	>100	1.193 ± 0.09	6.609 ± 1.0*	18.36 ± 0.90*
Cd	≤2	5	7	>7	0.097 ± 0.01	0.108 ± 0.00	0.131 ± 0.03
Hg	≤0.1	0.5	2	>2	0.004 ± 0.00	0.004 ± 0.00	0.005 ± 0.00
Pb	10	20	50	>50	0.094 ± 0.00	0.114 ± 0.01	0.119 ± 0.00

Values are the mean of two months (May, September 2019) and expressed as mean ± standard errors. Asterisk shows a statistical difference compared to the reference station (station 1) ($p < 0.05$).

Table 2. The status of some metal concentrations in sediment samples according to various sediment quality criteria (mg/kg) (MacDonald et al., 2000).

Metals	1. station	2. station	3. station	TEL	PEL	ERM
Cr	32.20 ± 4.20	69.49 ± 10.50[a]	72.90 ± 9.90[a]	37.3[a]	90	145
Ni	22.27 ± 4.70[b]	45.61 ± 5.01[b,c]	47.47 ± 7.40[b,c]	18[b]	36[c]	50
Cu	12.24 ± 2.09	22.56 ± 5.60	26.92 ± 9.80	35.7	197	390
Zn	18.96 ± 3.50	48.93 ± 9.70	211.87 ± 38.78[d]	123[d]	315	270
As	1.80 ± 0.10	7.17 ± 1.80[e]	14.26 ± 2.26[e]	5.9[e]	17.0	85.0
Cd	<0.000	<0.000	<0.000	0.6	3.53	9.0
Hg	<0.000	<0.000	0.005 ± 0.00	0.17	0.486	1.3
Pb	3.71 ± 0.29	4.41 ± 0.40	6.13 ± 0.48	35.0	91.3	110

Values are the mean of two months (May and September 2019) and expressed as mean ± standard errors. The letters a, b, d, and e indicate that the metal concentration exceeds the TEL value, the letter c indicates that the metal concentration exceeds the PEL value. TEL; threshold effect level, PEL; probable effects level, ERM; effect range median (MacDonald et al., 2000).

nuclear abnormalities such as micronucleus (Figure 3B), lobed (Figure 3C), notched (Figure 3D), bud (Figure 4A), blebbed (Figure 4B), kidney-shaped (Figure 4C), and binucleated (Figure 4D) were observed. Moreover, vacuolized erythrocytes (Figure 5A) and erythrocytes with distorted elliptical shapes (Figure 5B) were detected.

Frequencies of ENA and cellular abnormalities of stations were found to be different (Table 6). The ENA and cellular abnormalities were found to be the highest in the 3rd station, whereas the lowest was found to be in the 1st station. Besides, the mean values of ENA of the groups were found to be statistically significant ($p < 0.05$). Blebbed and notched nuclear frequencies were found to be higher compared to the frequency of other nuclear abnormalities (Table 6). These abnormalities indicated that frogs living in polluted stations were negatively affected by anthropogenic pollution.

The mean ES values were shown in Table 7. It was determined that EL/EW, and ES values of stations were statistically different. The 3rd station was found to have the least EL, EW and ES values in erythrocytes.

The mean NS values were given in Table 8. NL, NW, and NS values of the stations were different and a statistically significant difference was found between the groups. It was

Table 3. The mean concentrations of metals detected in the frog liver (ppb).

Metals	1. station	2. station	3. station
Cr	0.41 ± 0.02	0.58 ± 0.02*	0.76 ± 0.02*
Mn	1.37 ± 0.40	2.30 ± 0.30	3.90 ± 0.40*
Fe	189.66 ± 15.20	360.64 ± 26.30*	408.86 ± 35.50*
Ni	0.21 ± 0,01	0.31 ± 0.01*	0.36 ± 0.01*
Cu	100.72 ± 10.30	108.77 ± 12.50	439.15 ± 32.50*
Zn	2.03 ± 0.50	6.47 ± 1.20	86.39 ± 5.40*
As	0.0	1.65 ± 0.05*	0.47 ± 0.10*
Cd	0.0	0.0	12.42 ± 1.50*
Hg	0.03 ± 0.00	0.06 ± 0.00*	0.07 ± 0.00*
Pb	0.0	0.0	0.0

Values are expressed as mean ± standard errors. * shows a statistical difference compared to the reference station (station 1) ($p < 0.05$). 0 shows that the technique is below the quantification limit. For the analysis, the livers of 5 randomly caught frogs from each station were used (n = 5).

Table 4. The HSI values detected at the stations (n = 10).

Stations	May 2019	September 2019
1. station	2.86 ± 0.06[b]	2.92 ± 0.08[b]
2. station	2.55 ± 0.05[b]	2.75 ± 0.05[b]
3. station	3.46 ± 0.07[a]	5.02 ± 0.10[a]

The statistically significant differences between the mean values are shown with different letters (a and b) on the same column. Data are given as mean ± SE. [a] Significantly different than stations 1 and 2 ($p < 0.05$). [b] Significantly different than station 3 ($p < 0.05$).

Table 5. The fluctuating asymmetry (FA) values of the frogs, and the status of the stations according to Peskova and Zhukova (2007).

Station/Sex	May 2019	September 2019	Criteria	Status
1. station ♀	0.11 ± 0.02	0.12 ± 0.04	≤ 0.4[a]	Universal ratio (clean water basin)
1. station ♂	0.08 ± 0.02	0.10 ± 0.02		
2. station ♀	0.38 ± 0.01	0.44 ± 0.02[b]	0.41–0.50[b]	Minimal impact on organisms (slightly polluted water basin)
2. station ♂	0.46 ± 0.02[b]	0.50 ± 0.02[b]		
3. station ♀	0.60 ± 0.02[c]	0.65 ± 0.04[d]	0.51–0.60[c]	Satisfactory condition of organisms (average polluted water basin)
3. station ♂	0.66 ± 0.02[d]	0.70 ± 0.03[d]	0.61–0.70[d]	The unsuitable place for organisms to live (polluted water basin)

Data are given as mean ± SE. The letters a, b, c, and d indicate that the FA value exceeds the criteria.

seen that NS/ES ratio and NS value had the least values in erythrocytes in the 3rd station. NL/NW and NS/ES ratios did not differ statistically.

EE, ME, PE, and IE (Figure 6) were assessed by counting 600 cells in each slide to measure cytotoxicity (Table 9). It was found that the difference between some values from different stations was statistically significant, and these values also increased in parallel with the increase in pollution.

Results can be summarized: According to FA results, the 1st station was considered as clean water basin, the 2nd station was considered as slightly polluted water

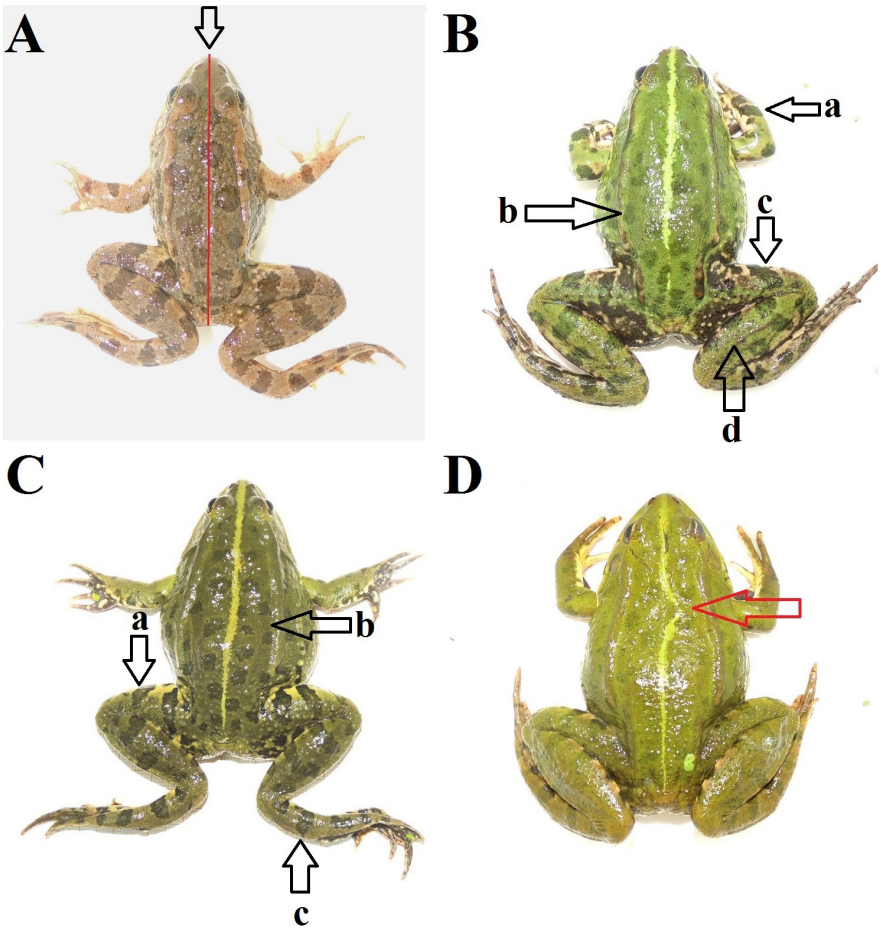

Figure 2. A- Normal symmetry of the frogs, symmetry line (arrow), (1. station). B- Abnormal symmetry of the frogs, radioulna spot (a), dorsal spots (b), femur spots (c), and tibia spot (d) asymmetries, (2. station). C- Abnormal symmetry of the frogs, femur spot (a), dorsal spots (b), and tarsus spot (c) asymmetries, (3. station). D- Asymmetry in the frog's dorsal midline (arrow) (3. station). SVL: 6,2 cm.

basin and the 3rd station was considered as polluted water basin. This situation was confirmed by heavy metal analyses (water, sediment, and liver). The MN, ENA, and cellular abnormalities were found to be the highest in the 3rd station, whereas the lowest was found to be in the 1st station. It was clearly shown that there was a correlation between pollution and the results of morphological and erythrometric analyses. It is concluded that the health status of frogs living in these stations is different from each other.

4. Discussion

It is known that amphibian populations are decreasing all over the world. Overfishing, habitat modification, infectious diseases, climate changes, ultraviolet radiation (Grant et al., 2016), and pollution caused by humans are the main causes of this situation (Whittaker et al., 2013). Human-induced pollution occurs mostly as a result of industrial and agricultural activities (Thammachoti et al., 2012). The main goal of the study is to determine the health status of frogs living in anthropogenic pollution areas by using morphometric and erythrometric parameters. At first, water and sediment samples taken from the stations were analyzed in terms of heavy metal. It was determined that concentrations of Mn, Fe, Ni, Cu, Zn, and As in water were high in the 3rd station but they did not exceed limit values. In sediment samples, it was determined that Cr, Ni, Zn, and As concentrations in the 2nd and 3rd stations exceeded the limits. This clearly shows that there was pollution in the waters of the 3rd station joining the Karasu River. In previous studies on the Karasu River and its tributaries, it was reported that various wastes from different sources reach the sampling areas (Sönmez et al., 2013). In a study conducted by Dane and Şişman (2020), the heavy metal analysis was performed in water and sediment samples were taken from different parts of the Karasu River in 2015–2016, and the levels of some elements (V, Mn, Fe, Co, Ni, Cu, Se, Sr, Ti, Br, Pb) were found to be high according to national standards. The transport of fertilizers and pesticides used in agricultural activities to

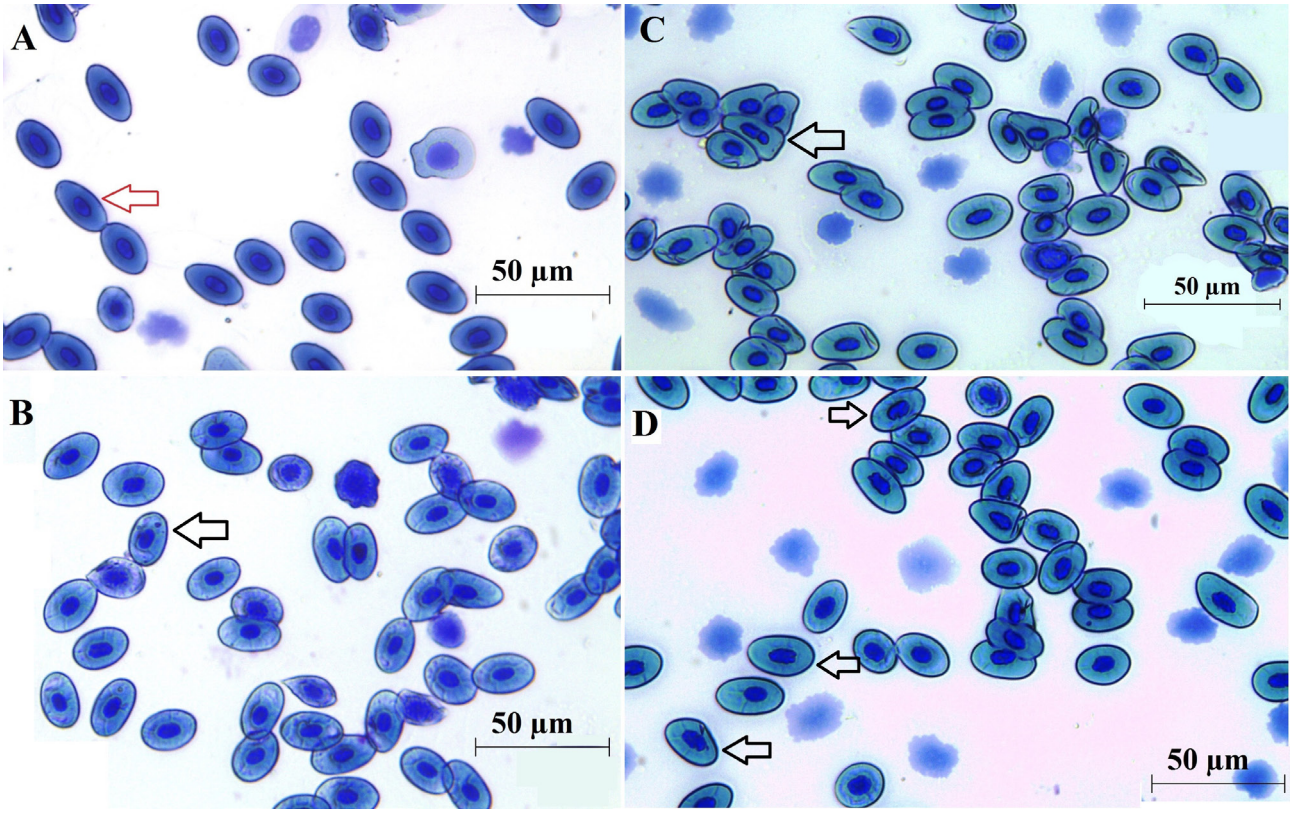

Figure 3. The erythrocytes of *P. ridibundus*. A- Normal erythrocyte (arrow) (1. station), B–D- Erythrocytic nuclear abnormalities (2. and 3. stations); B- Micronucleus (arrow), C- Lobed nucleus (arrow), D- Notched nuclei (arrows). Giemsa.

the river by surface flows and the mixing of wastewater of industrial enterprises and sewage treatment plants into the river, unfortunately, changes the nature of the river water (Dane, 2018). Similar results were reported in previous studies conducted in an area close to our study area. In the study, it was reported that quantities of some heavy metals in water (Pb, As, Cr, Cu, Co, Ni) samples taken from the river were above acceptable levels according to water quality categories (Aydoğan et al., 2016). We see that As, Mn, Fe, Ni, and Cu heavy metals determined in the highest concentration in the 3rd station water are common in the two studies.

The frog livers were analyzed for metal accumulation, and a significant increase in all elements was found in the frogs living in the 2nd and 3rd stations. The most accumulated metals were Cu, Fe, Zn, Cd, and Mn. Similar results were obtained from other studies. In a study that was carried out by Qureshi et al. (2015), high levels of Cu and Cd concentrations were detected in the liver and kidney of *Rana tigrina* and *Euphlyctis cyanophlyctis* frogs inhabiting in a water source polluted industrial wastes in Sialkot District (Pakistan). In another study, Fe, Mn, Pb, Zn, Cu, Cr, and Cd metals were detected in the livers, skin, and digestive systems of *Rana esculenta* living in the Guma River Wetland (in Nigeria), and it was reported that Cd, Cu, and Zn exceeded the limits set by the WHO (Shaapera et al., 2013). The common point of the studies presented here with our study is that Cu and Cd are the most accumulated metals in the livers of frogs. It is already known that these two metals are among the most toxic heavy metals to the environment, humans, and animals (Borković-Mitić et al., 2016).

HSI provides information about the health status of the animal and the quality of the water. Aquatic environmental pollution causes an increase in HSI. Therefore, HSI is considered a good indicator of water pollution (Pyle et al., 2005). The researchers tried to explain HSI status with the increased metabolism rate of frogs living in the polluted biotope (Spirina, 2009). Also, the increased HSI value was caused by the bioaccumulation of xenobiotics (industrial wastes, pesticides, heavy metals) (Thammachoti et al., 2012). Zhelev et al. (2015a) reported that HSI values of frogs from *P. ridibundus* species living in different sources of anthropogenic pollution were higher than that of clean area frogs. In our study, significant differences in the HSI values were found between the stations, and it can be seen that HSI values of frogs taken from the 3rd station polluted by heavy metals were high in September 2019. The results appear to be consistent with other studies.

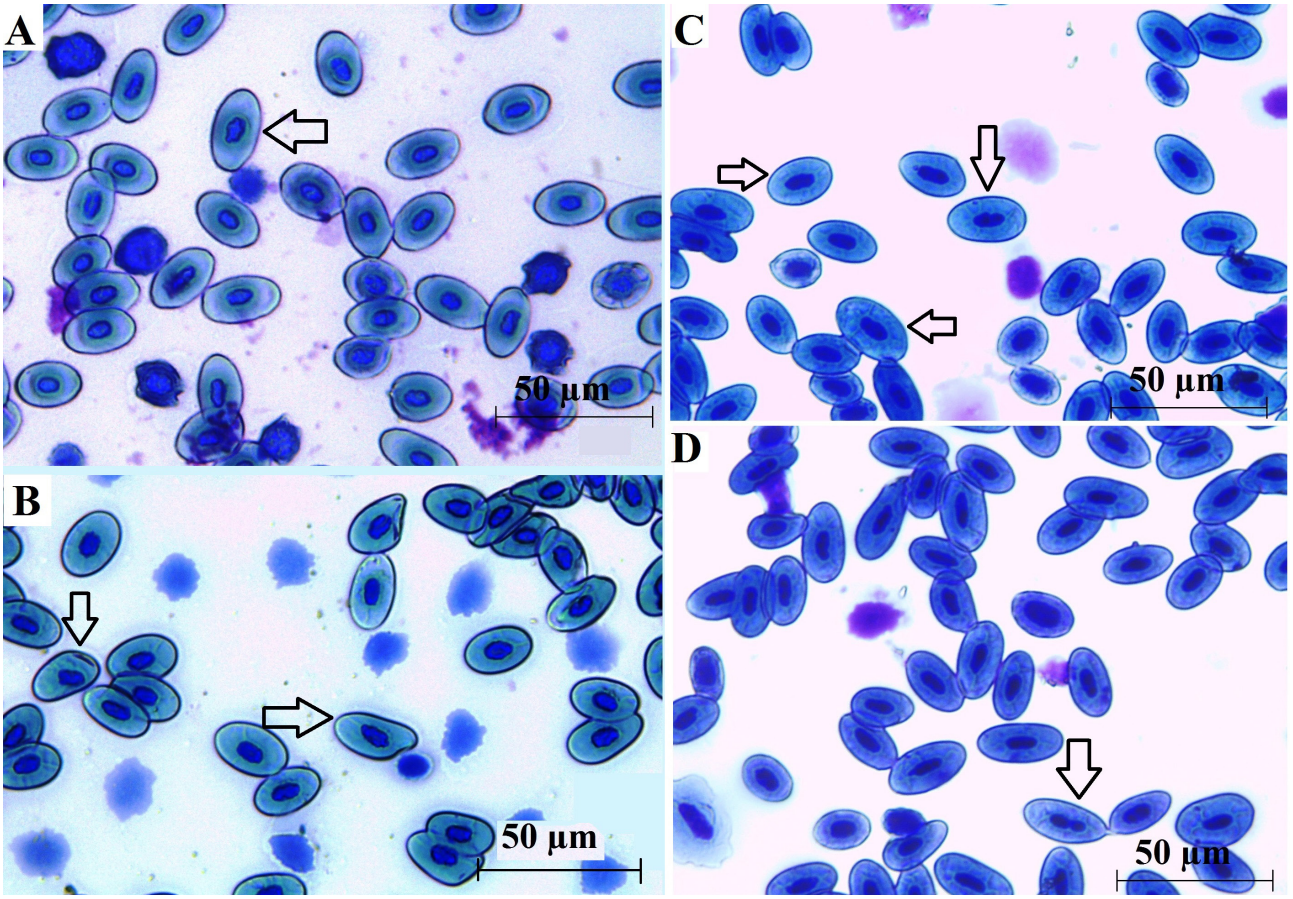

Figure 4. Erythrocytic nuclear abnormalities (2. and 3. stations). A- Bud nucleus (arrow), B- Blebbed nuclei (arrows), C- Kidney shaped nuclei (arrows), D- Binucleated erythrocyte (arrow). Giemsa.

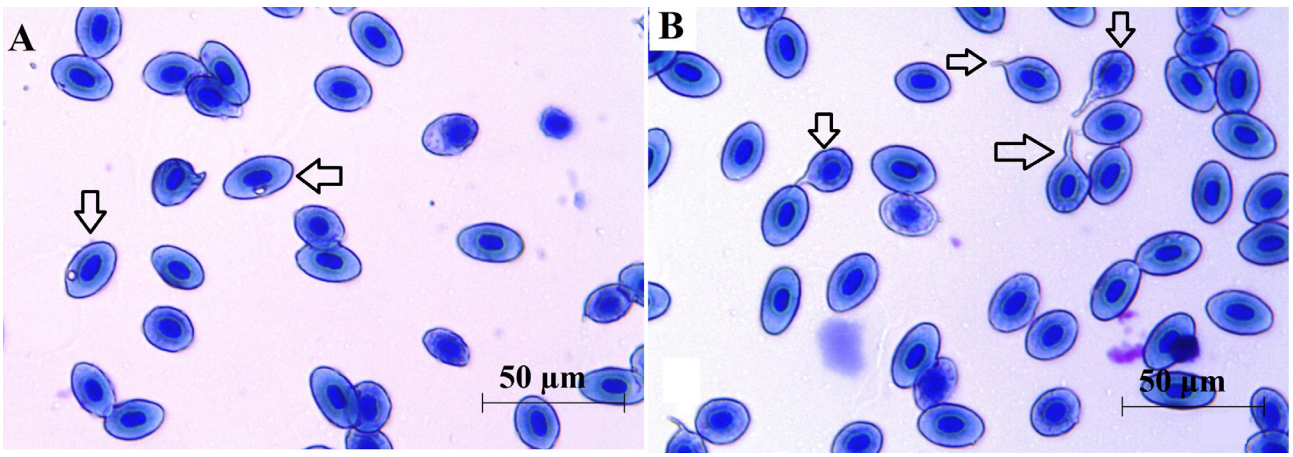

Figure 5. Cellular abnormalities of *P. ridibundus* (3. Station) A- Vacuolized erythrocytes (arrows), B- Erythrocytes with distorted elliptical shape (arrows). Giemsa.

The FA value showed a remarkable increase in the 3rd station. According to the literature, there are no FA data determined for frogs living in Turkey. Our study provides the first FA data on the frog in Turkey. Some other studies support the results. For example, it was reported that there was a significant increase in FA in frogs living in cultivation areas in Bulgaria (Zhelev et al., 2017a). In another study that was carried out between 2009 and 2011

Table 6. The mean values of erythrocytic nuclei and cellular abnormalities detected in peripheral blood cells of the frogs (‰ ± SE).

	Erythrocytic nuclear abnormalities							Cellular abnormalities	
Stations	Blebbed	Notched	Bud	Micronucleus	Kidney shaped	Lobed	Binucleated	Vacuolization	Shape deformity
1.	0.9 ± 0.1^{c}	0.3 ± 0.0^{c}	1.0 ± 0.1^{c}	0.7 ± 0.1^{c}	0.5 ± 0.0^{c}	0.1 ± 0.0^{c}	0.1 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
2.	7.0 ± 0.8^{b}	6.0 ± 0.8^{b}	5.2 ± 0.4^{b}	4.6 ± 0.6^{b}	2.5 ± 0.3^{b}	2.0 ± 0.1^{b}	0.7 ± 0.2^{b}	3.5 ± 0.5^{b}	6.2 ± 0.4^{b}
3.	11.5 ± 1.9^{a}	8.1 ± 1.2^{a}	7.0 ± 0.5^{a}	6.6 ± 0.9^{a}	5.2 ± 0.3^{a}	4.7 ± 0.5^{a}	3.8 ± 0.2^{a}	6.2 ± 0.8^{a}	7.5 ± 0.7^{a}

The statistically significant differences between the mean values are shown with different letters (a–c) on the same column ($p < 0.05$ Duncan's test). Data are given as mean ± SE.

Table 7. The mean values of erythrocyte size (ES) of the frogs (n = 1000 cells).

Stations	Erythrocyte length (EL)	Erythrocyte width (EW)	EL/EW	ES
1.	23.326 ± 1.854^{a}	13.235 ± 0.655^{a}	1.762 ± 0.05^{a}	242.344 ± 1.214^{a}
2.	22.837 ± 1.303^{b}	13.573 ± 0.663^{a}	1.683 ± 0.04^{b}	243.324 ± 1.411^{a}
3.	21.760 ± 1.701^{c}	13.178 ± 0.528^{a}	1.651 ± 0.03^{b}	225.118 ± 1.356^{b}

The differences between the means shown with different letters (a–c) on the same column are statistically significant (p <0.05 Duncan's test). Data are given as mean ± SE.

Table 8. The mean values of nucleus size (NS) of frogs caught from the sampling stations (n =1000 cells).

Stations	Nucleus length (NL)	Nucleus weight (NW)	NL/NW	NS	NS/ES
1.	9.482 ± 0.992^{a}	4.936 ± 0.774^{a}	1.920 ± 0.07^{a}	36.740 ± 0.563^{a}	0.152^{a}
2.	8.487 ± 0.512^{b}	4.689 ± 0.435^{b}	1.809 ± 0.08^{a}	31.239 ± 0.455^{b}	0.139^{a}
3.	8.459 ± 0.410^{b}	4.562 ± 0.448^{b}	1.854 ± 0.09^{a}	30.293 ± 0.487^{c}	0.135^{a}

The statistically significant differences between the mean values are shown with different letters (a–c) on the same column (p <0.05 Duncan's test). Data are given as mean ± SE.

in six different wetlands in the southern part of Bulgaria, it was reported that FA values of *P. ridibundus* species showed a significant increase in some areas polluted with heavy metals and domestic wastes (Zhelev et al., 2015b). Zhelev et al. (2014b) reported that FA values of *P. ridibundus* and *Pseudepidalea viridis* species living in areas close to iron and tin mines were high compared to frogs living in clean biotopes. Similar high FA values were determined in Voronezh River in Russia (Nikashin, 2005), where wastes from metal industry were mixed; in Hadazhka River (Peskova and Zhukova, 2007), where big farms were located, and in Sviyaga River (Spirina, 2009), where wastes of chemical industry were mixed.

MN is preferred as a biomarker in many studies, and they are considered as indicators of exposure to genotoxic agents in amphibians (Pollo et al., 2015). Generally, the frequency of ENA must be higher than MN; and both of them are considered as analog nuclear abnormalities (Guilherme et al., 2008). High ENA frequency is interpreted as more DNA damage (Gómez Meda et al., 2006). In the current study, both ENA and MN abnormalities were detected, and ENA was found to be more than MN. Many studies indicate that water pollution has genotoxic effects. It has been reported in previous studies that ENA and MN frequencies in frog erythrocytes increase due to heavy metal contamination in two polluted areas, which are different in terms of the study area in Erzurum, Turkey (Şişman et al., 2015, 2021). In another study, Corduk et al. (2018) reported that MN and ENA frequencies increased in *P. ridibundus* inhabiting a polluted area in Biga River,

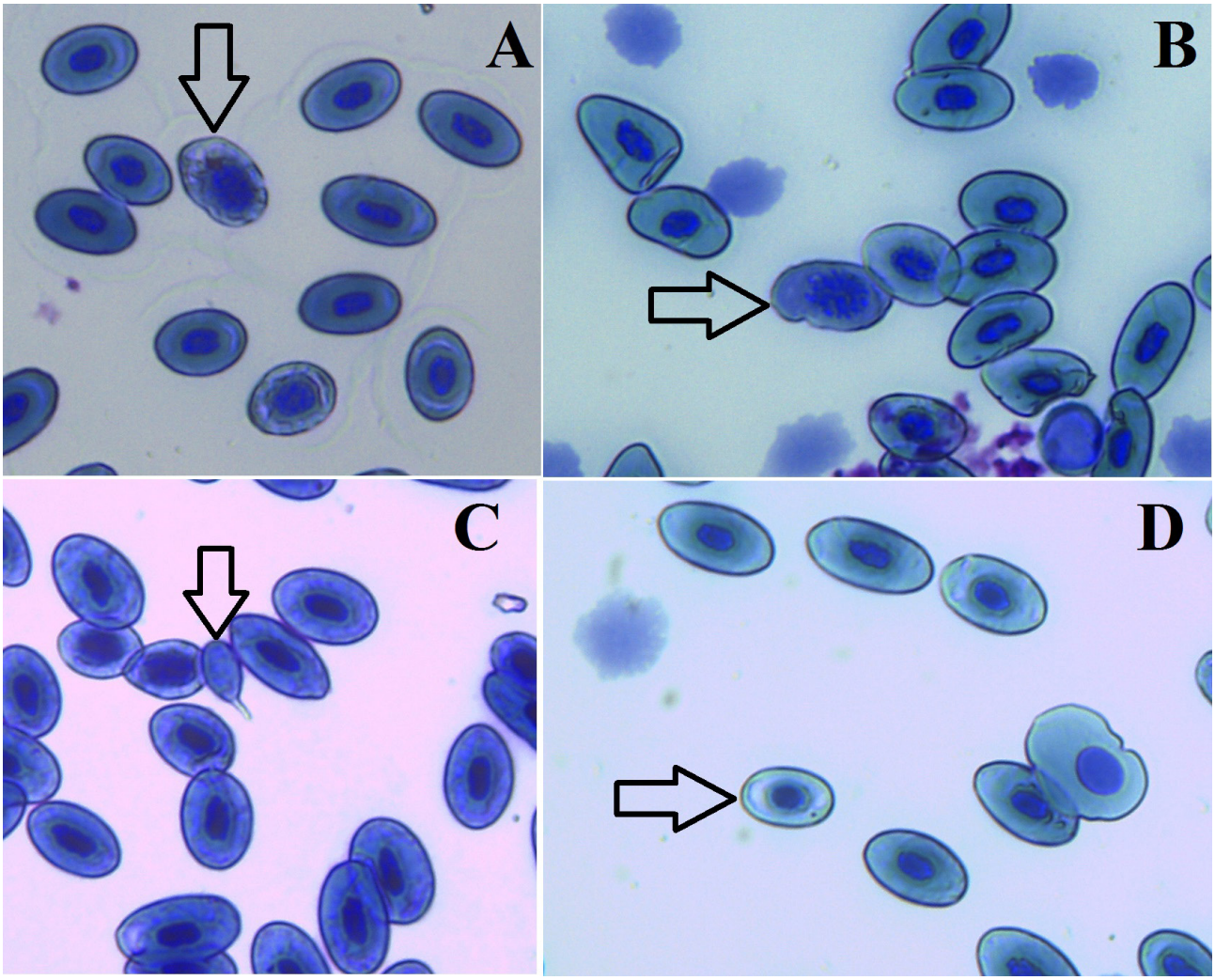

Figure 6. Other erythrocytic abnormalities: A- IE; immature erythrocyte (arrow), B- ME; mitotic erythrocyte (arrow), C- EE; enucleated erythrocyte (arrow), D- PE; picnotic erythrocyte (arrow), Giemsa.

Table 9. The mean values of abnormal erythrocytes of frogs caught from the sampling stations (n = 600 cells).

Stations	Enucleated erythrocyte (EE)	Mitotic erythrocyte (ME)	Immature erythrocyte (IE)	Picnotic erythrocyte (PE)
1.	0.22 ± 0.03^{b}	1.16 ± 0.16^{b}	32.55 ± 4.56^{c}	1.03 ± 0.14^{b}
2.	0.37 ± 0.05^{b}	2.06 ± 0.32^{a}	43.80 ± 6.63^{b}	2.26 ± 0.21^{a}
3.	1.55 ± 0.10^{a}	2.91 ± 0.66^{a}	59.08 ± 9.12^{a}	2.76 ± 0.87^{a}

Values are given as ‰ frequency for 600 cells (mean ± SE). The differences between the means shown with different letters (a–c) on the same column are statistically significant ($p < 0.05$ Duncan's test).

Çanakkale, Turkey. Similar results were found in two different frog species (*P. ridibundus* and *Bufotes variabilis*) caught from Çan wetland of the same province (Özgül et al., 2020). In both studies, the aquatic environment living the frog species was found to be polluted by various pollutants. It was reported by various researchers that ENA increased in frogs living not only in agricultural areas but also in wastewaters from mine processing

facilities (Corduk et al., 2018; Pollo et al., 2015, 2016). Toxicants with oxidative stress potential are known to cause molecular and clastogenic damage by affecting DNA (Jha, 2008). ENA that increases due to pollution in *P. ridibundus* frogs might be explained by the increase in reactive oxygen radicals and disappearance of antioxidant system protection. The results appear to be consistent with other studies.

In recent years, it was discovered that amphibian erythrocyte sizes differ between species and genus (Zhelev et al., 2017b). For example, it was reported that aquatic frog species (such as *P. ridibundus* and *Bombina bombina*) have larger erythrocytes than terrestrial frogs (such as *Bufo bufo, Bufotes viridis, Hyla arborea, Pelobates syriacus*) (Atatür et al., 1999). According to Arıkan et al. (2001), erythrocyte size (ES) in *Rana macrocnemis* is larger in both all aquatic amphibians and *Rana holtzi*. This difference in erythrocyte size is interpreted as a result of frog adaptation to its environment (Zhelev et al., 2017b). In anthropogenic pollution sources, frogs are directly exposed to some contaminants that are not dissolved in water. These substances penetrating the skin enter the body of the frog (Duellman and Trueb, 1994). According to Wojtaszek and Adamowicz (2003), these types of pollutants absorbed into the bodies of frogs disrupt the mechanism of hematopoiesis by changing the size and volume of erythrocytes. In our study, NL, NW, NS, EL, and ES values were found to decrease, and the frequencies of EE, ME, PE, and IE were found to increase. The results are very consistent with the other studies. For example, Zhelev et al. (2017b) reported that EL, EW, ES, NL, NW, and NS values in frogs living in anthropogenic polluted areas (especially metal pollution) decreased. In another study, it was expressed that the ES and NS values of frogs living in the mine waste wetland decrease (Pollo et al., 2016). Studies also show that NR obtained through ES and NS values in frogs are being affected and changed by anthropogenic pollution (Zhelev et al., 2016, 2017b). According to Pollo et al. (2019), the frequencies of EE, ME, PE, and IE increased in frogs (*Odontophrynus*, Anura: Odontophrynidae) living in agricultural areas. EE refers to increased erythropoiesis, and PE refers to the activation of apoptosis, and these two conditions are interpreted as responses that circulating cells develop against stress (Saquib et al., 2012). It was reported that small erythrocytes have a larger surface area and provide more effective oxygen exchange under hypoxic conditions in frogs living in polluted areas. However, it is estimated that this situation will be valid for a short time and then the organism will be adversely affected by pollution (Peltzer et al., 2013). Changes in the erythrometric parameters in *P. ridibundus* populations living in different water basins depend on the pollutant type, the pollutant concentration, the type of toxic substance, and the geographical characteristics of the basin.

Some studies show that the life span of the amphibian population changes significantly with the increase of pollution in water basins. The life of the animals decreases by 2 to 3 years (Zhelev et al., 2014a). Xenobiotics can selectively reduce genetic diversity in the population. They can also lead to population decline through the effects of somatic and hereditary mutations (Bickham et al., 2000). Xenobiotics not only cause toxicity, but can also impair processes such as growth, development, survival, and reproduction in amphibian species (Pollo et al., 2019). The number of amphibians may decrease. The biggest threat is that frogs in our study area will be affected by the pollution, and the population and their lifespan will be decreased.

4.1. Conclusion

This research shows that the health status of *P. ridibundus* populations living in anthropogenic pollution conditions can be evaluated using the FA. The FA values in *P. ridibundus* populations living in river areas contaminated by heavy metals and domestic sewage pollution also allow interpretation of the environment. It was also found that some metals accumulated in the livers of *P. ridibundus*, the frequency of ENA and MN increased, the size of erythrocytes decreased, and the frequency of abnormal erythrocytes increased. All these results show that anthropogenic pollution negatively affects the health of frogs. No frog deaths have been encountered in the area. However, we believe that the adult frogs living here may experience reproductive and behavioral changes, which may result in long-term population declines. Therefore, new studies are needed to evaluate the survival, fertility, and reproductive success of frogs living in polluted environments.

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References

Alnoaimi F, Dane H, Şişman T (2021). Histopathologic and genotoxic effects of deltamethrin on marsh frog, Pelophylax ridibundus (Anura: Ranidae). Environmental Science and Pollution Research 28 (3): 3331-3343. doi: 10.1007/s11356-020-10711-5

Arikan H, Olgun KI, Çevik EC, Tok V (1998). A taxonomical study on the Rana ridibunda Pallas, 1771 (Anura: Ranidae) population from Ivriz-Eregli (Konya). Turkish Journal of Zoology 22 (3): 181-184.

Arıkan H, Çevik İE, Kaya U, Mermer A (2001). Erythrocytes measurements on Anatolian mountain frogs. Anadolu University Journal of Science Technology 2: 387-391.

Arserim S, Mermer A (2008). Hematology of the Uludağ frog Rana macrocnemis Boulenger, 1885 in Uludağ National Park (Bursa, Turkey). Turkish Journal of Fisheries and Aquatic Sciences 25 (1): 39-46.

Atatür MK, Arıkan H, Çevik İE (1999). Erythrocyte sizes of some anurans from Turkey. Turkish Journal of Zoology 23 (2): 111-114.

Aydoğan Z, Gürol A, İncekara Ü (2016). The investigation of heavy element accumulation in some Hydrophilidae (Coleoptera) species. Environmental Monitoring and Assessment 188 (4): 204. doi: 10.1007/s10661-016-5197-3

Azimi A, Azari A, Rezakazemi M, Ansarpour M (2017). Removal of heavy metals from industrial wastewaters: a review. ChemBioEng Reviews 4 (1): 37-59.

Bickham JW, Sandhu S, Hebert PD, Chikhi L, Athwal R (2000). Effects of chemical contaminants on genetic diversity in natural populations: implications for biomonitoring and ecotoxicology. Mutation Research/Reviews in Mutation Research 463 (1): 33-51.

Borković-Mitić SS, Prokić MD, Krizmanić II, Mutić J, Trifković J et al. (2016). Biomarkers of oxidative stress and metal accumulation in marsh frog (Pelophylax ridibundus). Environmental Science and Pollution Research 23 (10): 9649-9659. doi: 10.1007/s11356-016-6194-3

Cajaraville MP, Bebianno MJ, Blasco J, Porte C, Sarasquete C et al. (2000). The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Science of the Total Environment 247 (2-3): 295-311. doi:10.1016/s0048-9697(99)00499-4

Carrasco KR, Tilbury KL, Myers MS (1990). Assessment of the piscine micronuclei test as an in situ biological indicator of chemical contaminant effects. Canadian Journal of Fisheries and Aquatic Sciences 47 (11): 2123-2136. doi: 10.1139/f90-237

Chamarthi RR, Bangeppagari M, Gooty JM, Mandala S, Tirado JO et al. (2014). Histopathological alterations in the gill, liver and brain of Cyprinus carpio on exposure to quinalphos. American Journal of Life Sciences 2 (4): 211-216. doi: 10.11648/j.ajls.20140204.14

Corduk N, Hacioglu-Dogru N, Gul C, Tosunoglu 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.

Dane H (2018). Bio-cological, toxicological and histological investigations on some Cyprinidae species from the fish fauna of Karasu River. Phd, Atatürk University, Erzurum, Turkey (in Turkish).

Dane H, Şişman T (2020). Effects of heavy metal pollution on hepatosomatic ındex and vital organ histology in Alburnus mossulensis from Karasu River. Turkish Journal of Veterinary and Animal Sciences 44 (3): 607-617. doi: 10.3906/vet-1904-50

Duellman WE, Trueb EL (1994). Biology of Amphibians. 1st ed. Baltimore, USA: John Hopkins University Press.

Erhunmwunse NO, Ekaye SA, Ainerua MO, Ewere EE (2014). Histopathological changes in the brain tissue of Africa catfish exposure to glyphosate herbicide. Journal of Applied Science and Environmental Management 18 (2): 275-280. doi: 10.4314/jasem.v18i2.19

Fenech M (2000). The in vitro micronucleus technique. Mutation Research 455 (1-2): 81-95. doi: 10.1016/s0027-5107(00)00065-8

Gómez Meda BC, Zamora Pérez AL, Luna Aguirre J, González Rodríguez A, Ramos Ibarra ML et al. (2006). Nuclear abnormalities in erythrocytes of parrots (Aratinga canicularis) related to genotoxic damage. Avian Pathology 35 (3): 206-210. doi: 10.1080/03079450600711003

Grant EHC, Miller DA, Schmidt BR, Adams MJ, Amburgey SM et al. (2016). Quantitative evidence for the effects of multiple drivers on continentalscale amphibian declines. Scientific Reports 23 (6): 25625. doi: 10.1038/srep25625

Guilherme S, Valega M, Pereira ME, Santos MA, Pacheco 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. doi: 10.1016/j.ecoenv.2007.08.016

Guo R, Zhang W, Ai S, Ren L, Zhang Y (2017). Fluctuating asymmetry rather than oxidative stress in Bufo raddei can be an accurate indicator of environmental pollution induced by heavy metals. Environmental Monitoring and Assessment 189 (6): 189-293. doi: 10.1007/s10661-017-5991-6

Häder DP, Banaszak AT, Villafañe VE, Narvarte MA, González RA et al. (2020). Anthropogenic pollution of aquatic ecosystems: Emerging problems with global implications. Science of the Total Environment 713: 136586. doi: 10.1016/j.scitotenv.2020.136586

Houlahan JE, Findlay CS, Schmidt BR, Meyers AH, Kuzmin SL (2000). Quantitative evidence for global amphibian population declines. Nature 404 (6779): 752-755. doi: 10.1038/35008052

Jha AN (2008). Ecotoxicological applications and significance of the comet assay. Mutagenesis 23 (3): 207-221. doi: 10.1093/mutage/gen014

Kuru M (editor) (2020). Vertebrates. 12nd ed. Ankara, Turkey: Palme Press (in Turkish).

Lajmanovich RC, Cabagna-Zenklusen MC, Attademo AM, Junges CM, Peltzer PM et al. (2014). Induction of micronuclei and nuclear abnormalities in tadpoles of the common toad (Rhinella arenarum) treated with the herbicides Liberty® and glufosinate-ammonium. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 769: 7-12. doi: 10.1016/j.mrgentox.2014.04.009

MacDonald DD, Ingersoll CG, Berger TA (2000). Development and evaluation of consensus-based sediment quality guidelines for freshwater ecosystems. Archives of Environmental Contamination and Toxicology 39 (1): 20-31. doi: 10.1007/ s002440010075.

Masindi V, Muedi KL (2018). Environmental contamination by heavy metals. In: Saleh HED, Aglan RF (editors). Heavy Metals. 1st ed. London, UK: IntechOpen, pp. 115-132.

Mohammadi Z, Khajeh A, Ghorbani F, Kami HG (2015). A biosystematic study of new records of the marsh frog Pelophylax ridibundus (Pallas, 1771) (Amphibia: Ranidae) from the southeast of Iran. Journal of Asia-Pacific Biodiversity 8 (2): 178-182. doi: 10.1016/j.japb.2015.04.001

Moiseenko TI, Morgunov BA, Gashkina NA, Megorskiy VV, Pesiakova AA (2018). Ecosystem and human health assessment in relation to aquatic environment pollution by heavy metals: case study of the Murmansk region, northwest of the Kola Peninsula, Russia. Environmental Research Letters 13 (6): 065005. doi: 10.1088/1748-9326/aab5d2

Møller AP, Swaddle JP (2002). Asymmetry, Developmental Stability and Evolution. 2nd ed. Oxford, UK: Oxford University Press.

Montalvão MF, Da Silva Castro AL, De Lima Rodrigues AS, De Oliveira Mendes B, Malafaia G (2018). Impacts of tannery effluent on development and morphological characters in a neotropical tadpole. Science of the Total Environment 610: 1595-1606. doi: 10.1016/j.scitotenv.2017.06.134

Özgül CN, Kurtul D, Çiğdem G (2020). Haematological and genotoxicological research on Pelophylax ridibundus and Bufotes variabilis living around the Çan (Çanakkale, Turkey). Turkish Journal of Bioscience and Collections 4 (2): 105-111. doi: 10.26650/tjbc.20200011

Özdemir ZK (2014). Investigation of the effects of domestic and industrial pollution in Kocabaş Stream (Çanakkale) on fresh water mullet (Leuciscus cephalus L., 1758) using biomarkers. Ms, Çanakkale Onsekiz Mart University, Çanakkale, Turkey (in Turkish).

Peltzer PM, Lajmanovich RC, Attademo AM, Junges CM, Cabagna-Zenklusen MC et al. (2013). Effect of exposure to contaminated pond sediments on survival, development, and enzyme and blood biomarkers inveined treefrog(Trachycephalus typhonius) tadpoles. Ecotoxicology and Environmental Safety 98: 142-151. doi: 10.1016/j.ecoenv.2013.09.010

Peskova TY, Zhukova TI (2007). The usage of amphibians for bio-indication of water pollution. Science Kuban 2: 22-25.

Pollo FE, Bionda CL, Salinas ZA, Salas NE, Martino AL (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. doi: 10.1007/s10661-015-4802-1

Pollo FE, Grenat PR, Otero MA, Salas NE, Martino AL (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. doi: 10.1016/j.ecoenv.2016.08.003

Pollo FE, Grenat PR, Otero MA, Babini S, Salas NE et al. (2019). Evaluation in situ of genotoxic and cytotoxic response in the diploid/polyploid complex Odontophrynus (Anura: Odontophrynidae) inhabiting agroecosystems. Chemosphere 216: 306-312. doi: 10.1016/j.chemosphere

Pyle GG, Rajotte JW, Couture P (2005). Effects of industrial metals on wild fish populations along a metal contamination gradient. Ecotoxicology and Environmental Safety 61 (3): 287-312. doi: 10.1016/j.ecoenv.2004.09.003

Qureshi IZ, Kashif Z, Hashmi MZ, Su X, Malik RN et al. (2015). Assessment of heavy metals and metalloids in tissues of two frog species: Rana tigrina and Euphlyctis cyanophlyctis from industrial city Sialkot, Pakistan. Environmental Science and Pollution Research 22 (18): 14157-14168. doi: 10.1007/s11356-015-4454-2

Saquib Q, Al-Khedhairy AA, Siddiqui MA, Abou-Tarboush FM, Azam A et al. (2012). Titanium dioxide nanoparticles induced cytotoxicity, oxidative stress and DNA damage in human amnion epithelial (WISH) cells. Toxicology in Vitro 26 (2): 351-361. doi: 10.1016/j.tiv.2011.12.011

Shaapera U, Nnamonu LA, Eneji IS (2013). Assessment of heavy metals in Rana esculenta organs from River Guma, Benue State Nigeria. American Journal of Analytical Chemistry 4 (9): 496-500. doi: 10.4236/ajac.2013.49063

Sönmez, AY, Haşıloğlu S, Hisar O, Aras Mehan HN, Kaya H (2013). Fuzzy logic evaluation of water quality classification for heavy metal pollution in Karasu Stream, Turkey. Ekoloji 22 (87): 43-50 (in Turkish). doi: 10.5053/ekoloji.2013.876

Spirina EV (2009). Morpho-physiological adaptations in Rana ridibunda Pall. under the influence of pollution. Bulletin of the Altai State Agrarian University 12 (62): 64-68.

SWQR (2015). Turkey's Ministry of Forestry and Water Affairs Surface Water Quality Regulations. Official Gazette, No: 29327 (in Turkish). Ankara, Turkey: Official Gazette of the Republic of Turkey.

Şişman T, Aşkın H, Turkez H, Ozkan H, Incekara U et al. (2015). Determination of nuclear abnormalities in peripheral erythrocytes of the frog Pelophylax ridibundus (Anura: Ranidae) sampled from Karasu River Basin (Turkey) for pollution impacts. Journal of Limnology and Freshwater Fisheries Research 1 (2): 75-81.

Şişman T, Keskin MÇ, Dane H, Adil Ş, Geyikoğlu F et al. (2021). Marsh frog (Pelophylax ridibundus) as a bioindicator to assess pollution in an agricultural area. Pakistan Journal of Zoology 53 (1): 337-349. doi: 10.17582/journal.pjz/20190103130130

Thammachoti P, Khonsue W, Kitana J, Varanusupakul P (2012). Morphometric and gravimetric parameters of the rise frog Fejervarya limnocharis living in areas with different agricultural activity. Journal of Environmental Protection 3 (10): 1403-1408. doi: 10.4236/jep.2012.310159

Toroğlu E, Toroğlu S, Alaeddinoğlu F (2006). Water pollution in the Aksu River (Kahramanmaraş). Coğrafi Bilimler Dergisi 4 (1): 93-103 (in Turkish).

Whittaker K, Koo MS, Wake DB, Vredenburg VT (2013). Global declines of amphibians. In: Levin SA (editor). Encyclopedia of Biodiversity. 2nd ed. Cambridge, MA, USA: Elsevier Incorporation (Academic Press), pp. 691-699.

Wojtaszek, J, Adamowicz A (2003). Haematology of the fire-bellied toad, Bombina bombina L. Comparative Clinical Pathology 12 (3): 129-134. doi: 10.1007/s00580-003-0482-2

Zakharov VM (2001). Ontogeny and population (the stability of development and population variability). Ecology 3: 177-191.

Zhelev ZM, Popgeorgiev GS, Angelov MV (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.

Zhelev Z, Arnaudov A, Boyadzhiev P (2014a). Colour polymorphism, sex ratio and age structure in the populations of Pelophylax ridibundus and Pseudepidalea viridis (Amphibia: Anura) from anthropogenically polluted biotopes in southern Bulgaria and their usage as bioindicators. Trakia Journal of Sciences 1: 1-12.

Zhelev ZM, Popgeorgiev GS, Georgieva ZK (2014b). Fluctuating asymmetry in the populations of Pelophylax ridibundus and Pseudepidalea viridis (Amphibia: Anura) in the region of the lead and zinc plant "Kardzhali" (South Bulgaria). Acta Zoologica Bulgarica 66 (1): 83-87.

Zhelev ZM, Popgeorgiev GS, Mehterov NH (2015a). Changes in the hepatosomatic index and condition factor in the populations of Pelophylax ridibundus (Amphibia: Ranidae) from anthropogenically polluted biotopes in Southern Bulgaria. Part II. Bulgarian Journal of Agricultural Science 21 (3): 534-539.

Zhelev ZM, Popgeorgiev GS, Arnaudov AD, Georgieva KN, Mehterov NH (2015b). Fluctuating asymmetry in Pelophylax ridibundus (Amphibia: Ranidae) as a response to anthropogenic pollution in South Bulgaria. Archives of Biological Sciences 67 (3): 1009-1023.

Zhelev ZM, Mehterov NH, Popgeorgiev GS (2016). Seasonal changes of basic erythrocyte-metric parameters in Pelophylax ridibundus (Amphibia: Ranidae) from anthropogenically polluted biotopes in Southern Bulgaria and their role as bioindicators. Ecotoxicology and Environmental Safety 124: 406-417. doi: 10.1016/j.ecoenv.2015.11.011

Zhelev ZM, Tsonev CV, Arnaudova DN (2017a). Health status of Pelophylax ridibundus (Pallas, 1771) (Amphibia: Ranidae) in a rice paddy ecosystem in southern Bulgaria: body condition factor and fluctuating asymmetry. Acta Zoologica Bulgarica 69 (Suppl. 8): 169-177.

Zhelev Z, Popgeorgiev G, Ivanov I, Boyadzhiev P (2017b). Changes o 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. doi: 10.1007/s11356-017-9364-z

Zhelev Z, Tsonev C, Georgieva K, Arnaudova D (2018). Health status of Pelophylax ridibundus (Amphibia: Ranidae) in a rice paddy ecosystem in Southern Bulgaria and its importance in assessing environmental state: haematological parameters. Environmental Science and Pollution Research 25 (8): 7884-7895. doi: 10.1007/s11356-017-1109-5