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Chronic Exposure to Heavy Metals Induces Nuclear Abnormalities and Micronuclei in Erythrocytes of the Marsh Frog (Pelophylax ridibundus Pallas, 1771)

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Abstract. Amphibians have big potential as bioindicators based on their combined life cycle as aquatic and terrestrial form. They can play the role of prey or predator, making them a key element in toxic substances transfer between aquatic and terrestrial habitats. The nuclear abnormalities (NAs) in amphibians' erythrocytes in recent years have been used as a successful biomarker for anthropogenic pollution. The NAs including micronuclei in erythrocytes of the marsh frog (*P. ridibundus*) have been studied to assess the cytotoxic and genotoxic effect in heavy metal polluted area *in situ*. Here we assess the cyto- and genotoxic potential of the polluted waters (Chaya River) close to the lead-zinc smelter near Plovdiv (Bulgaria) situated in an area that has been contaminated with heavy metals for 60 years. Frogs from Strandzha Natural Park were used as a negative control. Peripheral blood smears have been dyed with acridine orange. NAs of the following types: notched nuclei, nuclear buds and blebbed nuclei have shown the highest frequency. There is no sexual dependence in the formation of different types of NAs. The significant differences (P≤0.0001) in the mean Total NAs (‰) in erythrocytes of marsh frogs from the polluted area compared to the total NAs from the background region "Strandzha" NP demonstrate the presence of *in vivo* active cytotoxic and genotoxic agents in the impacted area. The obtained results for NAs in erythrocytes of *P. ridibundus* are evidence for successful application of NAs as a biomarker in amphibians for the purpose of biomonitoring.

Key words: genotoxicity, nuclear abnormalities, micronuclei, heavy metals, *Pelophylax ridibundus*.

Introduction

Negative changes in the environment, because of daily anthropogenic activity, are a continuous and irreversible process. Increased anthropogenic pressure leads to changes in the biosphere, which can disrupt the fragile ecological balance, as well as cause several harmful effects on humans and the environment (Ellis, 2015). This requires the application of biological monitoring, which is carried out through a modern integrated approach, research and state assessment as well as forecast of changes in individual organisms, communities, and ecosystems (USEPA, 2000; Şişman et al., 2015).

Heavy metals and their compounds have extremely harmful effects on the biota

(Gall et al., 2015). Therefore, they have been identified as some of the most hazardous toxic substances (The Priority List of Hazardous Substances; ATSDR, 2011). The caused contamination is especially adverse since traces of metals and metalloids are not biodegradable. According to Camizuli et al. (2018) the trace metals from mining sites and metallurgical industry stay bioavailable. They persist in the environment and tend to constant accumulation in plants and animals. Therefore, the local contamination continues to be a serious environmental risk and is subject to many biomonitoring studies.

Stress caused by heavy metals leads to a cascade of biological responses in living organisms, which could be used as a biomarker (Amiard-Triquet et al., 2012). Nuclear abnormalities, including micronuclei, are biomarkers of genotoxicity and chromosomal instability. They have been successfully used as biomarkers in fish, amphibians, and mammals (Fenech et al., 2011; Pollo et al., 2015, 2016; Ivanova et al., 2016). The mechanisms of their occurrence have not been fully studied yet – some authors define them as cytotoxic biomarkers (notched and lobbed nuclei) (Rocha et al., 2011), while others link the appearance of certain NAs not only with the cytotoxic effect of the environment, but also with genotoxic one (nuclear buds) (Bolognesi & Hayashi, 2011). In all cases, their presence is associated with disorders of cell division, apoptosis and genotoxicity or mutagenicity. Therefore, many scientists who have studied the effects of various genotoxicants recommend the use of the NAs method in conjunction with the micronucleus test (Ferraro et al., 2004; Çavaş, 2008; Hoshina et al., 2008). Multiple NAs – including blebbed, notched, lobed nuclei, nuclear buds and binucleated cells – have all been applied as potential biomarkers of genotoxicity (Ayllon & Garcia-Vazquez, 2000; Çavaş & Ergene-Gözükara, 2005; da Silva Souza & Fontanetti, 2006; Muranli & Güner, 2011; Ruiz de Arcaute et al., 2016).

Amphibians are of a great importance for both terrestrial and aquatic communities. They are an extremely important link in food chains and are very sensitive to anthropogenic environmental changes (Alton & Franklin, 2017). In addition, among some aquatic and terrestrial communities, certain amphibian species are the most abundant vertebrates, reaching densities of up to 2,500 for aquatic and 40,000 individuals per hectare for terrestrial communities (Burton & Likens, 1975; Petranka & Murray, 2001). The significant biomass, combined with the typical big appetite of the amphibian larvae (Taylor et al., 1988), their high mass and energy conversion efficiency (Grayson et al., 2005), allows them to play an important role in the transfer of energy and nutrients through food chains (Beard et al., 1998).

Due to their specific characteristics, amphibians can be used as bioindicators to detect toxic waste in water, soil, or bottom sediments (Boone & Bridgs, 2003). Most of their gas exchange takes place through the skin, which leads to an easy absorption of substances that pollute their habitat. In addition, this group of animals cannot take long-distance movements, and this makes them intricately connected to the environment in which they live (Sievers et al., 2018). The sensitivity of anurans as zoomonitors of heavy metals contamination is present in various studies (Leontyeva et al., 1997; Lefcort et al., 1998; Şişman et al., 2015; Zhelev et al., 2015, 2020).

The marsh frog *P. ridibundus* is an unprotected anuran with the widest distribution in Bulgaria. It is a bioindicator of the long-term environmental impact of biological parameters and can be used to assess anthropogenic pollution (Corduk et al., 2018). The aim of the present study is to determine the cyto- and genotoxic effect *in situ* in marsh frog (*Pelophylax ridibundus* Pallas, 1771) from anthropogenically polluted area.

Material and Methods

Study area

The area of study covers two regions listed in the "National Biomonitoring Program of Bulgaria", one as impacted and the other as background (Peev & Gerasimov, 1999). The polluted region includes the area of the lead-zinc smelter (KCM AD) near the city of Plovdiv and the unpolluted – the Strandzha Natural Park (SNP).

The Marsh frogs were captured in a sampling site close to the lead-zinc smelter in the Thracian valley: the Chaya River (synonyms: Chepelare River, Assenitsa River) near the confluence with the Maritza River (42.1561° N, 24.8973° E, 162 m a. s. l.). The place of capture of the studied marsh frogs is near the tailings pond close to the outflow of industrial waters. Industrial pollution with SO_2, NO_2, Pb, Cd, Zn and other toxic substances has been registered. Micro-aggregates of lead, cadmium, and zinc are released into the atmosphere through air emissions (aerosols). They accumulate in soil, spreading over vegetation and aquatic areas. The degree of pollution and the nature of pollutants for the investigated period (2018) are included in the annual reports on KCM and are controlled by the Executive Environment Agency in the Republic of Bulgaria. The areas around the plant are agricultural ecosystems. Studies show that 80% of pollution occurs in the air (pollution torch), and the remaining 20% is due to polluted irrigation water. The physicochemical analysis of the surface waters at the place of sampling is of a highest importance. The data in present study originates from a physicochemical monitoring of the surface water done by the Basin Directorate for Water Management-East Aegean Sea, Region-Plovdiv, Ministry of the Environment and Waters. Table 1 presents information about studied site in Chaya river for a three-year period 2015–2017 (average annual values and lowest and highest measured values) as well as data collected during present study (April 2018). The main pollutants are heavy metals and metalloids (lead, cadmium, zinc, copper and arsenic) as well as nutrients (ammonium nitrogen, nitrate nitrogen, nitrite nitrogen, and total nitrogen).

Table 1. Ecological status of the studied site at the Chaya river for the period 2015–2017, based on the data contained in newsletters of the Basin Directorate of Water Management in the East Aegean Sea – Plovdiv, Ministry of the Environment and Waters. Physicochemical substances are presented with average annual values and the lowest and highest measured values for each year (for April 2018: recent data at the time of the study). *Legend:* Temperature (Temp), electrical-conductivity (EC), dissolved oxygen (DO), oxygenation (Ox), biological oxygen demand five days (BOD5), chemical oxygen demand (COD), calcium carbonate hardness (CCH), ammonium nitrogen (NH_4^+-N), nitrite nitrogen (NO_2^--N), nitrate nitrogen (NO_3^--N), total nitrogen (TN), orthophosphates (PO_4^{3}), total phosphorus, as P (TP), iron dissolved in water (Fe), lead (Pb), copper (Cu), zinc (Zn), cadmium (Cd), depending on the hardness classes of water, manganese (Mn), nickel (Ni), arsenic (As), mercury (Hg), aluminum (Al), not monitored (NM). Physicochemical data (water): * – The values deviating (< , >) Standards for high water quality according to Ordinance No. H-4 (Ordinance, 2012) on the characterization of surface waters in Bulgaria (State Gazette No.22 of 5.03.2013); ' – the values above: AAV (Average annual value) and '' – MPC (maximum permissible concentration) according to Ordinance No. 256 (Ordinance, 2010) for Standards on environmental quality for priority substances and for certain other pollutants (State Gazette No. 88 of 9.11.2010).

Parameters	Standards for high water quality	Polluted site (Chaya River)			
		2015	2016	2017	April 2018
Temp °C	-	13.2 (5.0–22.1)	15.7 (11.0–21.0)	14.5 (6.0–22.0)	10.0

pH units	-	7.5 (7.3–8.1)	7.7 (7.1–8.1)	8.0 (7.9–8.3)	8.03
EC µS/cm	700.0	477.2* (248.0–703.0)	648.7* (253.0–1096.0)	543.0* (250.0–996.0)	390.0*
DO mg/l	9.0–7.0	9.1 (7.2–11.9)	9.3 (6.7–11.3)	9.9 (7.5–12.5)	12.2
BOD_5 mg/l	<2.0	1.47 (0.50–2.0)	1.36 (0.50–3.3)	2.35 (0.9 –4.5)	2.0
COD mg/l	25.0	8.9 6.7–11.1	15.2 6.0–41.0	17.3 6.0–30.0	16.0
Ox %	100–105	88.2 (68.0–92.0)	90.2 (68.0–114.0)	93.5 (76.0–106.0)	114.0
CCH mg $CaCO_3$/l	-	181.7 (105.0–255.0)	218.5 (112.0–357.0)	193.6 (108.0–320.0)	143.0
NH_4^+-N mg/l	<0.10	0.15* (0.12–0.20)	0.21* (0.12–0.31)	0.35* (0.19–0.56)	0.21*
NO_2^--N mg/l	<0.03	0.04* (0.03–0.07)	0.03 (0.02–0.04)	0.05* (0.02–0.07)	0.03
NO_3^--N mg/l	<0.7	1.98* (0.49–4.0)	2.49* (0.47–4.1)	1.42* (0.75–3.2)	0.62
TN mg/l	<0.7	2.78* (1.4–3.9)	2.96* (1.5–4.0)	2.13* (1.3–3.6)	1.3*
PO_4^{3-} mg/l	<0.07	0.05 (0.04–0.06)	0.03 (0.02–0.05)	0.07 (0.02–0.12)	0.03
TP mg/l	<0.15	0.09 (0.06–0.17)	0.07 (0.05–0.12)	0.13 (0.05–0.21)	0.05
Fe µg/l	AAV: 100.0; MPC: not applicable	24.0 (20.0–32.0)	27.0 (20.0–41.0)	34.2 (20.0–69.0)	19.06
Pb µg/l	AAV (1.2); MPC (14.0)	3.24′ (1.12–8.0)	10.17′ (1.12–55.0)	3.87′ (1.55–9.4)	6.4′
Cu µg/l	AAV: 1.0 ($CaCO_3$ 0–50 mg/l); 6.0 ($CaCO_3$ 50–100 mg/l); 10.0 ($CaCO_3$ 100–250 mg/l); 22 ($CaCO_3$ > 250 mg/l); MPC: not applicable	2.31 (1.81–2.6)	2.55 (1.81–3.3)	6.95 (3.8–12.1)	12.2′
Zn µg/l	AAV: 8.0 ($CaCO_3$ 0–50 mg/l); 40.0 ($CaCO_3$ 50–100 mg/l); 75.0 ($CaCO_3$ 100–250 mg/l); 100.0 ($CaCO_3$ > 250 mg/l); MPC: not applicable	143.3′ (54.0–244.0)	651.0′ (186.4–1160.0)	64.0 (52.0–72.0)	57.0
Cd µg/l	AAV: ≤0.08 (class 1); 0.08 (class 2); 0.09 (class 3); 0.15 (class 4); 0.25 (class 5); MPC:≤0.45 (class 1); 0.45 (class 2); 0.6 (class 3); 0.9 (class 4); 1.5 (class 5)	1.92′,″ (0.63–4.2)	8.74′,″ (0.61–49.0)	1.96′,″ (0.18–3.0)	1.11′,″
Ni µg/l	AAV: 4.0; MPC: 34.0	0.61 (0.46–1.41)	0.70 (0.46–2.1)	1.49 (0.47–3.8)	0.67
As µg/l	AAV: 10.0; MPC: 25.0	2.72 (0.47–6.6)	4.89 (0.64–14.0)	5.11 (1.2–13.4)	0.46
Hg µg/l	AAV: not applicable; MPC: 0.07	<0.01	<0.01	<0.01	0.01
Al µg/l	AAV: 15.0; MPC: 10 (pH < 6.5); 25 (pH > 6.5)	5.53 (5.56–5.82)	10.76 (4.5–22.0)	15.6 (5.4–39.0)	1.45

The background region is located in south-eastern Bulgaria and is part of the largest protected area from Strandzha Nature Park. The whole territory of the SNP is included in the network of EU areas of nature protection Natura 2000. There are no important local sources of industrial pollution (Peev & Gerasimov, 1999). No exceedance of the heavy metals soil concentrations has been found in any of the heavy metal monitoring stations in the SNP as well as in the whole surrounding region for the investigated period (Annual report for the activity of RIEW Burgas). The sampling site of the Veleka river was near the confluence with the Black Sea (N42°03′40′′, E27°57′56′′).

Material

Forty animals from KCM area and 39 animals from the SNP, adults (average snout-vent length 83.16 ± 17.91 cm) and sexually mature (Bannikov et al., 1977), were randomly caught. The field research was conducted in the period April - June 2018. Following the Sutherland's (2000) methodology, 1-kilometer-long and 4-meters-wide stretches of shores have been passed. The animals were captured alive at night, blinded by artificial light, and then transported in buckets full of water to the laboratory where the entire laboratory analysis was performed. All individuals (61 males and 18 females) were identified by sex based on secondary sexual characteristics: the presence of "marital corns" on the first finger and resonator bubbles in the corners of the mouth of the males.

The animal handling and laboratory methodology was approved by the Ethics Board for Experimental Animals at the Faculty of Biology at the University of Plovdiv.

Methods

Haematological analyses were done in laboratory conditions, 1 day after the capture (actually less than 24 h after the catch). In order to prepare blood smears, blood was isolated by cardiac ventricular puncture after the anesthesia of frogs. The blood was spread in a thin layer on a glass slide. A minimum of 2 blood smears were prepared from each individual and dried for about 24 hours at a room temperature. The dried slides were fixed for 10 min in absolute methanol (Merk) and stored in a dark and dry place until stained. All prepared blood smears were stained with the fluorescent dye acridine orange (AO) according to Hayashi et al. (1983). AO was prepared first as a 0.1% aqueous solution and then 0.24 mM AO was dissolved in 1/15 M Sörensen phosphate buffer (pH 6.8). Fixed cells were stained with this solution for 3 minutes at room temperature. The slides were rinsed in the buffer three times each 1 to 3 minutes. The samples were studied immediately after AO staining by a Leica DM 1000 fluorescence microscope equipped with a special filter (I3), with a lens 100 × below immersion.

Nuclear abnormalities (NAs), including micronuclei, have been reported according to the criteria of Carrasco et al. (1990), Fenech (2000) and Furnus et al. (2014). Nuclei with a substantial notch into the nucleus were noted as notched nuclei (NotchN); those with larger evaginations (lobe), including those with several lobes, are lobbed (LobeN); the nuclei with relatively small evaginations of the nuclear membrane and contained euchromatin are reported as blebbed (BlebN); those that have relatively small formation connected to the nucleus by a stalk of nucleoplasmic are considered to be nuclei with nuclear buds (NBud); "Eight" shape nuclei (EN) were distinguished according to Furnus et al. (2014) and represented a constriction resembling the shape of the digit eight; kidney shaped nuclei (KN) were reported also; cells in which, in addition to the main nucleus, there is a less isolated nucleus with a round or oval shape, which is not larger than 1/5 of the main nucleus, focuses and fluoresces with the same color as the main nucleus, we consider as erythrocytes with micronucleus (MN). Binucleated cells (BN) were defined as cells with two nuclei of approximately equal sizes. Bridge-like formation (NBr) between

two daughter erythrocytes were described according to Anbumani & Mohankumar (2011).

The average frequency of nuclear abnormalities per 2000 scored erythrocytes (polychromatic and normochromatic), expressed in per mille, was calculated for each individual with the following formula:

$$NAs\ Frequency\ ‰ = \frac{Number\ of\ cells\ containing\ NAs}{Total\ number\ of\ cells\ scored} \times 1000$$

Photomicrographs taken with the Leica Application Suite were processed with the ImageJ program (Abràmoff, 2004) with the addition of the "Cell Counter" plugin.

Statistical methods

The data were first tested for both normal distribution (D'Agostino and Pearson omnibus normality test) and homogeneity of variance (Levene, F-test). The MN and NAs data was not normally distributed and therefore the non-parametric Mann-Whitney test was used. For all tests, the level of significance was set at $P \leq 0.05$. All calculations were performed with the software Prism, version 4.02 (GraphPad Software, San Diego, CA, USA).

Results and Discussion

Mature erythrocytes of *P. ridibundus* have an oval shape with a centrally located nucleus (Fig. 1A). The nucleus is oval, clearly structured and has a well-defined boundary, which facilitates the identification of fragments in the cytoplasm. Fluorescence microscopy showed different types of nuclear abnormalities – notched nuclei (NotchN), blebbed nuclei (BlebN), nuclear buds (NBud), lobbed nuclei (LobeN), kidney shape nuclei (KN) and eight-shaped nuclei (EN), cells with MN, binuclear cells, mitotic erythrocytes (erythroblasts) (Fig. 1I). The frequency of the first three nuclear abnormalities – NotchN, BlebN and NBud, is many times higher than the other abnormalities. Therefore, all other abnormalities, except micronuclei, were pooled in "Other NAs".

The non-parametric Mann-Whitney test proved the absence of statistically significant differences between sexes in all scored NAs: BlebN ($P = 0.3157$; $U = 1773$), NotchN ($P= 0.6956$; $U = 1896$), NBud ($P = 0.0553$; $U = 1584$); Other NAs ($P = 0.7973$; $U = 1924$); MN ($P = 0.5573$; $U = 1909$) and Total NAs ($P = 0.4574$; $U = 1824$). For the subsequent analyses, we combined males and females per site. The lack of sex dependence in the frequency of NAs in *P. ridibundus* is not surprising, as similar biomonitoring studies also did not report statistical differences between sexes in detection of NAs in erythrocytes of anurans (Pollo et al., 2015; Pollo et al., 2016).

Significant differences in NAs frequency among impact and background sites were found (Fig. 2) with the lowest frequency recorded for SNP.

The highest values of the average frequency of Total NAs at KCM area can be associated with the effect of polymetallic pollution in this area. The obtained average value for Total NAs (49.14 ± 36.59) differs significantly ($P \leq 0.0001$) from the established value in the background region SNP (9.75 ± 13.94) (Fig. 2 F). It is also higher than that recorded by Şişman et al. (2015) in *P. ridibundus* in Turkey. The highest recorded value by the authors for Total NAs (‰) is 11.07 ± 4.06, however only four types of anomalies were reported – LobeN, NotchN, MN and KN. The authors also prove a direct correlation between the frequency of NAs and the concentration of heavy metals in surface waters.

NotchN, BlebN and NBud frequency increased significantly in KCM in respect to SNP ($P \leq 0.0001$). NotchN occurs in the impact regions with the highest frequency, followed by NBud and BlebN. This indicates that a cytotoxic effect is observed in *P. ridibundus* erythrocytes in the impacted region. Although the origin of notched nuclei remains unclear and has not been fully studied, their occurrence in fish and amphibian erythrocytes has been associated with the presence of cyto- and genotoxic agents

(Pollo et al., 2015). Increased frequencies of notched nuclei have been observed in other studies of amphibians inhabiting contaminated areas (Pollo et al., 2015; 2016; Şişman et al. 2015; Raghunath et al., 2017; Corduk et al., 2018). Erythrocytes of individuals of *P. ridibundus* inhabiting a river with pollution of anthropogenic origin (Şişman et al., 2015) have an increased but lower average frequency of NotchN, ‰ (1.78 ± 0.34) compared to our impact region (21.70 ± 15.24). On the other hand, an anomaly of another type (BlebN) prevails with the highest frequency, which shows the potential of different pollutants or their combined impact to predominate the different types of NAs. According to Fenech et al. (2011) NBuds are associated with DNA amplification and repair processes and may be associated with unnecessary chromosomes in aneuploid cells. Shimizu et al. (1998, 2000) used *in vitro* experiments with mammalian cells to show that amplified DNA is selectively localized at specific sites on the periphery of the nucleus and is eliminated by nuclear buds during the S phase of the cell cycle. Amplified DNA can be eliminated from chromosomes by recombination of homologous regions of amplified sequences forming minicircles of centric regions called "double minutes". NBuds are characterized by the same morphology as MN, except that they are connected to the nucleus by a narrower or wider column of nucleoplasmic material, depending on the stage of the process. Bolognesi et al. (2006) reported a strong association between the induction of MN and NBuds in fish erythrocytes. The mean frequency of NBuds (‰) obtained in the present study is the highest in KCM area (Fig.2.B), which may be related to the recorded long-term heavy metal contamination in this region. For example, the observed values (15.32 ± 11.06) are ten times higher than those published by Pollo et al. (2015) for *Rhinella arenarum* inhabiting an artificial water basin in urban conditions (1.14 ± 1.27).

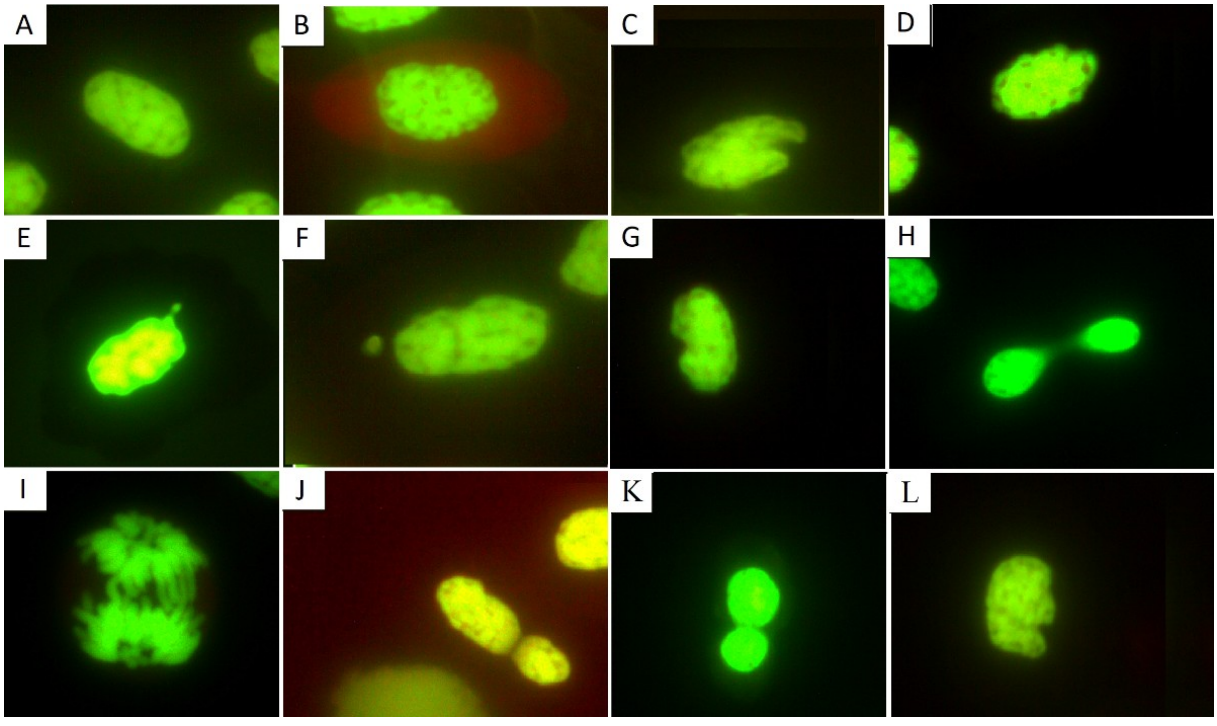

Fig. 1. Different type of NAs in erythrocytes of *P. ridibundus*: A) normal normochromatic erythrocyte without abnormalities; B) normal polychromatic erythrocyte; C) notched nucleus – NotchN; D) blebbed nucleus – BlebN; E) nuclear bud – NBud; F) micronucleus – MN; G) kidney shape nucleus – KN; H) nucleoplasmatic bridge - NBr; I) mitotic erythrocytes; J) binucleated erythrocyte – BN; K) "eight" shape nucleus – EN; L) lobed nucleus – LobeN.

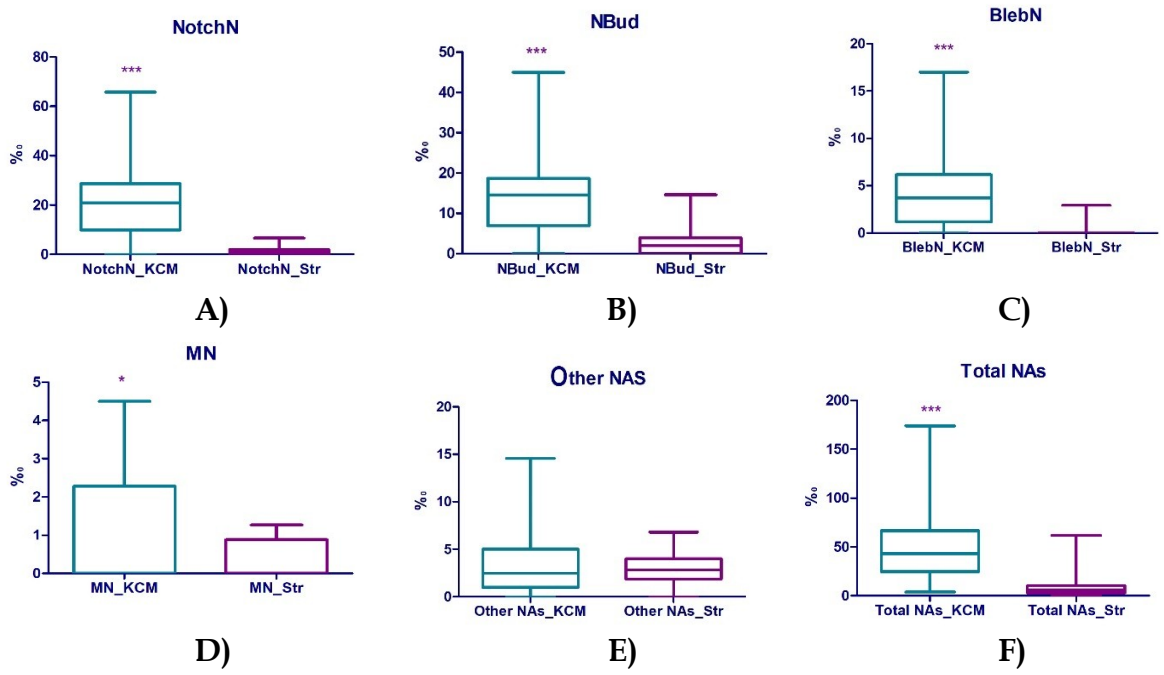

Fig. 2. Comparative analysis of frequencies (‰) of different types of NAs in polluted (KCM, Plovdiv) and background region (Strandzha Nature Park): A) notched nuclei B) nuclear buds; C) blebbed nuclei; D) micronuclei; E) Other NAs - lobbed nuclei, kidney shape nuclei and eight-shaped nuclei, binuclear cells, mitotic erythrocytes (erythroblasts); F) Total NAs. Bottom and top of the box represent 25 and 75% percentile values, respectively, with median values within the box. Error bars indicate minimum and maximum values (*** $P \leq 0.0001$, ** $P \leq 0.01$, * $P \leq 0.05$, compared to negative control).

Rare abnormalities reported in single cells of individuals were pooled in Other NAs. No statistically significant differences were found concerning Other NAs between the investigated sites ($P = 0.83$, $U = 367$) (Fig.2 E). The low values of the mean frequencies of Other NAs in the impact and background regions indicate that their values are probably close to the normal values for the species. In the impact region there is greater individual variability than in the background region, which proves the susceptibility of *P. ridibundus* as a zoomonitor species. This variability is probably due to the presence of genotoxic agents in the affected areas, which induce the appearance of rarer abnormalities, such as nucleoplasmic bridges and mitotic erythrocytes, which we included in the general sample.

The induction of NAs and micronuclei in our study is not surprising concerning the heavy metal pollution in the investigated area. Other authors (Zhelev et al., 2020) report also that *P. ridibundus* individuals inhabiting the same site in Chaya River have severely deteriorated general health status, suppressed hemopoiesis and a weakened immunity due to the high levels of toxicants of anthropogenic origin (human industrial activity) combined with nitrate fractions and heavy metals. The levels of cadmium and lead in the liver and muscles of frogs were significantly higher than those in frogs from reference site. In addition, our study proves that chronic exposure to heavy metals in polluted area causes clear cyto- and genotoxic effects on amphibians red blood cells *in situ*.

BlebN as well as LobeN are considered precursors of MN (Shimizu et al., 1998; Anbumani & Mohankumar, 2012) associated with a mutagenic effect. Their increased frequency depends on the dose of exposure

and can be explained by the cellular mechanism for dealing with excess chromatin, in which the secreted genetic material is incorporated into micronuclei and can be expelled from the cell as a "double minute" (Shimizu et al., 1998). It is hypothesized that accurate initiation of the Breakage-Fusion-Bridge cycle to separate entangled and attached chromosomes is associated with gene amplification and may lead to the formation of LobeN or BlebN, NBuds, nucleoplasmic bridges, and MN during isolation of amplificated nuclear DNA (Shimizu et al., 1998, 2000; Fenech et al., 2011). In this sense, the nuclear abnormalities registered in the present study in the impact region demonstrate genetic instability in the studied individuals, caused by the presence of cyto- and genotoxic agents in the environment.

Conclusions

The obtained results reveal the greater induction of NAs in erythrocytes of march frog, *P. ridibundus* inhabiting polluted waters (Chaya river) close to the lead-zinc smelter near Plovdiv (Bulgaria). NAs of the following types: notched nuclei, nuclear buds and blebbed nuclei have shown highest frequency. The NAs frequency and micronucleus frequency are significantly higher in the impact area than those from the control region (Veleka river, Strandzha Nature Park). Our findings demonstrate a clear cyto- and genotoxic effect, which shows that the anurans in the polluted region are vulnerable to polymetallic contamination. Our study also confirmed that the assessment of NAs in addition to the MN test determinese the potential cyto- and genotoxic effect of heavy metals on amphibians red blood cells *in situ*.

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