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Original Research

Impact of Industrial Pollution and Genotoxic Effects in Marsh Frog (*Pelophylax ridibundus* (Pallas, 1771)) Populations in Kosovo

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Abstract

Industrial pollution from anthropogenic activities is a critical global issue, significantly affecting biodiversity. Due to their unique life cycle, limited mobility, permeable skin, and dual habitat in aquatic and terrestrial environments, amphibians exhibit high sensitivity to environmental changes and serve as reliable bioindicators of pollution. This study assesses the impact of industrial pollution on *Pelophylax ridibundus* populations by examining erythrocyte nuclear morphology. Specimens were collected from four polluted industrial sites (Kishnica, Obiliq, Mitrovica, and Drenas) and one control site (Dragash) in Kosovo during the spring and autumn seasons of 2023. Blood samples were obtained via ventricular puncture, and smears were prepared following standard protocols, stained using the May-Grünwald-Giemsa method, and examined under a light microscope. The observed nuclear abnormalities included micronuclei, binuclei, vacuolated nuclei, and irregularly shaped nuclei. Significant variations were identified in the frequency of nuclear abnormalities between the studied locations and across seasons. The findings of this study highlight the practical utility of analyzing micronuclei and nuclear abnormalities in *P. ridibundus* as evaluative parameters for assessing the environmental health of ecosystems exposed to industrial pollution.

Keywords: bioindicators, nuclear abnormalities, environmental monitoring, industrial contamination, amphibian erythrocytes

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Introduction

Amphibian populations across the globe have experienced significant declines in both numbers and diversity since the 1960s, primarily due to the destruction of their natural habitats [1]. This alarming trend can be largely attributed to the escalating impact of anthropogenic activities on the environment, which has resulted in continuous alterations to the biosphere, an issue recognized as one of the most pressing global challenges of our time [2]. Among the various threats to amphibians, anthropogenic pollution, particularly stemming from agricultural and industrial practices, stands out as a critical concern [3]. Such pollution not only jeopardizes freshwater and marine ecosystems [4] but also poses significant risks to human health [5]. Addressing this multifaceted problem necessitates a comprehensive and integrated approach, employing modern methodologies for biological monitoring to assess and predict changes in natural ecosystems [6].

Environmental pollution in Kosovo presents a major threat to biodiversity, adversely affecting numerous ecosystems and species. From industrial emissions to agricultural runoff, a range of anthropogenic activities contribute to pollution in terrestrial and aquatic environments. The continuing reliance on coal-fired power plants, for instance, results in substantial emissions of particulate matter and heavy metals, adversely affecting both air and water quality. Agricultural practices further exacerbate the situation, as fertilizers and pesticides are commonly overused, leading to nutrient loading and contamination of water bodies. These environmental stressors not only threaten the health of numerous wildlife species, including amphibians, but also have broader implications for human health and ecosystem stability [7-10].

Heavy metals and their by-products represent a category of pollutants with profoundly detrimental effects on living organisms. The issue of heavy metal contamination in wetland ecosystems is a widespread environmental concern of global significance, primarily due to the persistence and bioaccumulation of these pollutants within food chains [11]. Unlike organic contaminants, heavy metals are non-biodegradable, leading to their accumulation in water, soil, sediments, and biota. The accumulation of heavy metals in organisms can result in a range of adverse effects, including cytogenotoxicity, immunotoxicity, hepatotoxicity, nephrotoxicity, and endocrine disruption [12-13].

Amphibians are particularly vulnerable to environmental pollutants compared to other vertebrate groups owing to several biological factors: their eggs lack protective shells, their life cycle is complex, and their skin is permeable [14-15]. Additionally, their limited mobility and intricate life cycles render amphibians among the most sensitive bioindicators of both aquatic and terrestrial pollution, as well as environmental changes [16-17]. Hematological assessments have long been utilized to investigate the physiology and health of amphibian species, particularly in natural habitats [18]. Non-invasive techniques, such as blood biomarker analysis, have proven effective in revealing the mutagenic effects of pollution. Cytogenetic biomarkers, including nuclear abnormalities in erythrocytes, are essential for evaluating genotoxicity [19-21]. Among these biomarkers, the micronucleus test is favored for its simplicity and reliability in detecting toxicant-induced clastogenic effects [22-24]. This method is capable of assessing the early impacts of prolonged exposure to xenobiotics in both field and laboratory settings [25]. In recent decades, the frequency of micronuclei has been employed to indicate cytogenetic damage across various animal groups, including amphibians [26-28].

Research on the impact of anthropogenic pollution on amphibians in Kosovo is still in its nascent stages, yet it has begun to garner attention due to the alarming decline of these species in the region [29]. One significant study by Kittner et al. [30] highlights the health risks associated with trace metals found in lignite coal, which is prevalent in Kosovo's energy sector. The study underscores that the pollutants released from coal-fired power plants, including heavy metals, contribute to environmental degradation and pose serious health risks to both humans and wildlife, potentially including amphibians [30]. This study's primary objective was to investigate industrial pollution's effects on frog species populations of *Pelophylax ridibundus* in different seasons, sexes, and industrial hotspots within Kosovo. Specifically, this research aims to evaluate the health status of frogs inhabiting these polluted areas and to ascertain whether the pollutants released from industrial activities pose a risk of genotoxic damage in two different seasons (spring and autumn) and both sexes. This study assesses genotoxic effects in frogs from industrial pollution hotspots in Kosovo, focusing on nuclear abnormalities in erythrocytes and heavy metal concentrations in environmental samples. Correlation analyses explore associations between metal exposure and genotoxic markers, providing baseline data for environmental health assessment.

Materials and Methods

Study Area

This study was conducted in four industrial regions and one control site in Kosovo. The industrial sites Kishnica (S-1), Obiliq (S-2), Mitrovica (S-3), and Drenas (S-4) were selected for their significant industrial activities and pollution levels, while Dragash-Brezne (S-5) served as a pristine, uncontaminated control site (Fig. 1).

Industrial regions are directly affected by pollutants from nearby facilities, including heavy metals, ash by-products from coal combustion, phenols, and other chemicals:

– S-1 (Kishnica) and S-3 (Mitrovica) are impacted by lead (Pb) and zinc (Zn) processing and mining activities.
– S-2 (Obiliq) is affected by lignite extraction and coal combustion.
– S-4 (Drenas) is influenced by the processing of iron (Fe) and nickel (Ni).
– S-5 (Dragash–Brezne) represents a clean, natural ecosystem.

These sites were chosen to represent a gradient of environmental conditions, ranging from heavily industrialized and polluted regions to a pristine natural habitat. This approach enables a comprehensive assessment of the impact of industrial pollutants on local ecosystems.

Water Samples and Analysis

Water samples were collected from the natural habitats of frogs. Five samples were taken from each locality at different points and stored in plastic bottles for laboratory analysis. The samples were filtered, standardized using Zeman's method, and analyzed for metal concentrations using an ICPE Shimadzu. The analysis was performed with a Plasma Atomic Emission Spectrometer, Shimadzu, ICPE-9820, with ppb detection levels. To assess the health of frog habitats and determine the maximum permissible levels of heavy metals in water and soil, the analysis followed the Kosovo Standard (2009), EU standards, and German standards for water quality.

Soil Samples

Soil samples were taken from frog habitats near industrial sites. Five samples were collected per locality using a hand probe at a 15 cm depth, following Kluge & Wessolek [31] and ISO11466. Samples were sealed, dried at 105ºC for 48 hours, and treated with a mixture of 69% HNO_3 and HClcc in a 2:6 ratio. The mixture was processed in a microwave at 200ºC for 45 minutes, filtered, standardized to 50 ml with distilled water, and analyzed for metal concentrations using a Plasma Atomic Emission Spectrometer, Shimadzu, ICPE-9820.

Animal Samples

Fifteen marsh frogs for each locality (*Pelophylax ridibundus*; 8 ♂♂ and 7 ♀♀) were collected during May and October 2023, totaling 150 individuals. Frogs were

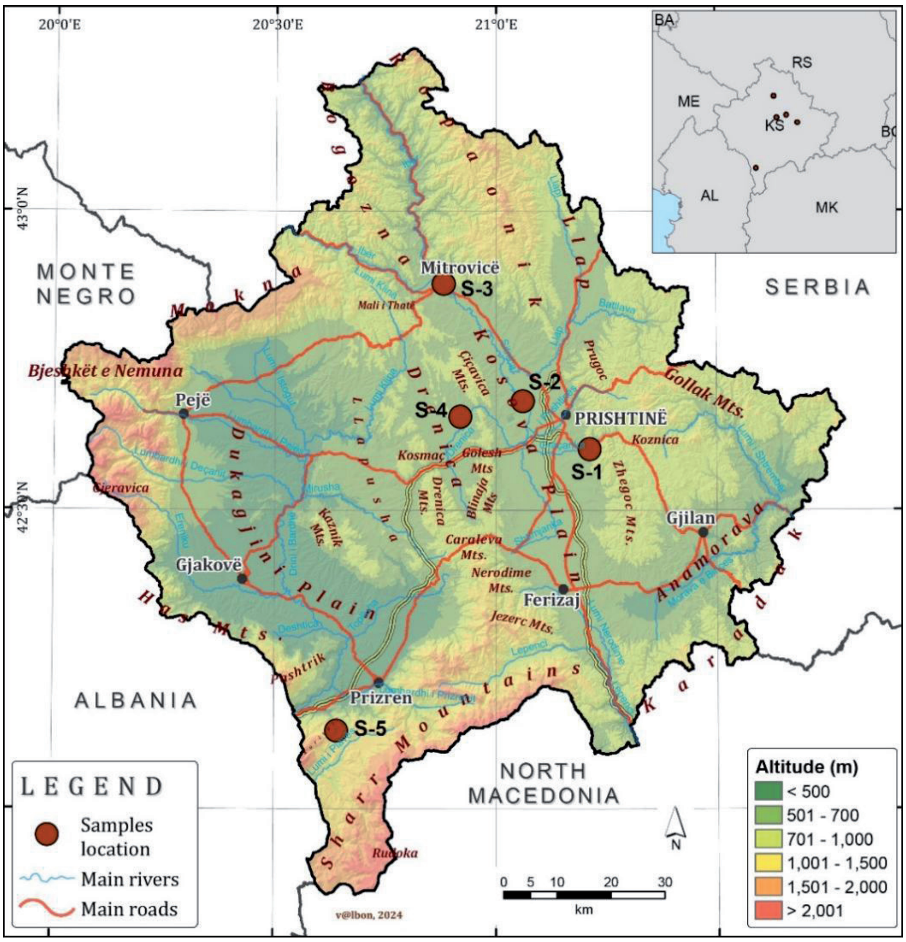

Fig. 1. Map of sampling stations in Kosovo.

Fig. 2. Fieldwork - frogs captured at the sampling sites.

captured at night with artificial lights, transported in water-filled buckets, and processed in the laboratory. Weight, length, and secondary sexual characteristics were recorded for identification (Fig. 2).

Blood Samples

Hematological analyses were prepared immediately after the frogs were brought to the laboratory. Blood was drawn from the ventricular apex using heparinized syringes, with five smears prepared per frog. The smear preparations were dried at room temperature, fixed, and stained with the May-Grünwald-Giemsa method [32]. The slides were rinsed in distilled water three times for 1 to 3 minutes; Canadian balsam was added to the slides and covered with a cover glass. Approximately 2000-3000 erythrocytes for each preparation were examined for nuclear abnormalities (NAs), including micronuclei (MN), binuclei (BN), vacuolated nuclei (VN), and irregularly shaped nuclei (ISN), following Carrasco et al. [33] and Fenech [34]. Nuclei with small formations of nuclear material separated from the central nucleus were designated as micronuclei (MN); nuclei divided into two parts were designated as binuclei (BN); nuclei with the presence of holes in the nucleus were designated as vacuolated nuclei (VN); and nuclei with larger shapes and a number of anomalies were designated as irregularly shaped nuclei (ISN). Slides were analyzed with a Motic microscope with a 100x lens below immersion. Images were captured with a Motic camera and processed using ImageJ software [35].

Statistical Analyses

Statistical analyses were conducted using Statistica 7 software. ANOVA with Newman-Keuls post-hoc tests evaluated the effects of locality, sex, and season on the parameters investigated. The average frequency of nuclear abnormalities in 2000-3000 labeled erythrocytes, expressed per mile, was calculated for each individual using the following formula:

$$Frequency\ of\ NAs‰ = \frac{Number\ of\ cells\ containing\ NA}{Total\ number\ of\ labeled\ cells}\ x\ 1000$$

Spearman correlation analysis identified relationships between metal concentrations in water, soil, and frog blood parameters. Spearman's rank correlation coefficient (rs) was calculated using the formula:

$$r_s = 1 - \frac{6\sum d^2}{n(n^2-1)}$$

where: d represents the difference between the ranks of each pair of observations, and n is the number of observations. The significance of the correlation coefficients was determined using a p-value threshold of <0.05, indicating statistical significance.

Results and Discussion

Water Chemical Analysis

Multiple water samples were read with a Schizmandu-type ICPL with ppb detection levels. The results of the chemical analysis for heavy metal concentrations in water are presented in Table 1. Based on the findings, the water samples from the investigated localities were found to be contaminated with Pb and Zn, while other elements such as Cd, Ni, Co, Mo, Cr, Cu, and As exhibited very low concentrations, all below the detection limit in both the spring and autumn seasons.

- Lead (Pb): The concentration of Pb exceeded the allowed critical limit only in the Kishnica locality during the autumn season. The concentration remained within the permitted limits in other localities in both seasons.
- Zinc (Zn): Zinc concentration exceeded the allowed critical limit only in the Drenas locality during the autumn season. In all other localities, the concentration remained within the permissible limits.

Table 1. Mean metal concentrations[1] of water samples collected from the sampling sites in two different seasons (spring-autumn).

Locality	S – 1	S – 2	S – 3	S – 4	S – 5
Season	S – A	S – A	*S – A*	*S – A*	*S – A*
Metals					
Pb (mg/L^{-1})	0,030 – 0.502	0.104 – 0.036	0.09 – 0.32	0.04 – 0.04	0.003 – 0.043
Zn (mg/L^{-1})	0.5 – 7.85	4.38 – 0.12	15.4 – 21.5	106.1 – 225.8	0.2 – 0.11
Ni (mg/L^{-1})	nld - nld	nld - nld	nld - nld	nld - nld	nld -nld
Cd (mg/L^{-1})	nld - nld	nld - 22.2	nld - nld	nld - 35.5	nld - nld
Co (mg/L^{-1})	nld - nld	nld - nld	nld - nld	nld - nld	nld - nld
As (mg/L^{-1})	nld - nld	nld - nld	nld - nld	nld - nld	nld - nld
Mo (mg/L^{-1})	nld - nld	nld - nld	nld - nld	nld - nld	nld - nld
Cr (mg/L^{-1})	nld - nld	nld - nld	nld - nld	nld - nld	nld - nld
Cu (mg/L^{-1})	nld - nld	nld - nld	nld - nld	nld - nld	nld - nld

Results are presented as mean values. Localities are indexed with numbers (S-1, S-2, etc.), and seasons are marked as Spring and Autumn. “nld” indicates “not limit detected”.

– Cadmium (Cd): In the autumn season, the Cd values in both Obiliq and Drenas were below the allowed critical limit (see Table 1).

Soil Chemical Analysis

The analysis of soil samples revealed contamination with several heavy metals, including Pb, Zn, Ni, Cd, Co, Cr, Cu, and As (Table 2).

– Lead (Pb): The concentration of Pb exceeded the allowed critical limit in the soil samples from Kishnica and Drenas during the autumn season and in Obiliq during the spring season.

– Zinc (Zn): Zinc concentrations were elevated in the soil samples from Kishnica and Obiliq in both seasons but did not exceed the allowed critical limit (Table 2).

– Cadmium (Cd): Cadmium levels were particularly high in the soil samples from Kishnica in the autumn and Drenas in both seasons, surpassing the allowed critical limits.

– Nickel (Ni): The concentration of Ni exceeded the allowed critical limit only in Kishnica during the autumn season and in Obiliq across both seasons.

– Chromium (Cr), Cobalt (Co), and Arsenic (As): The concentrations of these metals were found to be high across all investigated localities. However,

Table 2. Mean metal contents1 of sediment samples collected from the sampling sites in two different seasons (spring-autumn).

Locality	S – 1	S – 2	S – 3	S – 4	S – 5
Season	S – A	S – A	S – A	S – A	S – A
Metals					
Pb (mg/kg-1)	35.4 – 113.8	70.3 – 1.68	2.51 – 2.75	37.5 – 65.4	0.13 – 1.34
Zn (mg/kg-1)	146.6 – 232.3	103.9 – 109.0	8.97 – 33.7	65.5 – 73.6	5.5 – 6.8
Ni (mg/kg-1)	15.8 – 72.3	90.3 – 70.1	12.0 – 49.0	13.4 – 16.4	1.76 – 3.6
Cd (mg/kg-1)	0.45 – 5.28	nld – 1.6	nld – 1.49	9.2 – 10.4	nld - nld
Co (mg/kg-1)	793.6 – 1411.6	571.1 – 855.3	946.3 – 569.3	1035.5 – 1812.6	nld – 190.1
As (mg/kg-1)	461.7 - 520.9	3232.3 - 1228.4	973.0 - 1393.3	1900 - 395.5	nld - 224.2
Mo (mg/kg-1)	nld – 316.6	nld - nld	nld - nld	nld - nld	nld - nld
Cr (mg/kg-1)	2558.3 – 4337.4	4910.0 - 4150.0	1306.0 - 1512.7	1803.9 - 6893.6	129.8 - 234.0
Cu (mg/kg-1)	nld – nld	nld – nld	nld - nld	nld - nld	nld - nld

[1]Results are presented as mean values. Localities are indexed with numbers (S-1, S-2, etc.), and seasons are marked as Spring and Autumn. “nld” indicates “not limit detected.”

only in Kishnica during the autumn season did the concentration of these metals exceed the allowed critical limit for soil samples (Table 2).

Cytohematological Analysis

Various erythrocyte nuclear abnormalities were observed using an optical microscope, including MN, BN, VN, and ISN.

Tables 3, 4, 5, and 6 present the study's results on the frequency of MN and other NAs in frog populations from different localities. The findings reveal significant differences in the investigated parameters between localities and sexes, as well as notable seasonal variations.

Statistical analysis of the univariate test of significance ANOVA/MANOVA showed that the natural factors locality and season alone (or their interactions) have an impact on the frequency of MN, BN, and ISN; while for VN, in addition to the impact of locality and season alone or in interaction, the interaction between season and sex also impacts the frequency of VN.

MN showed the lowest values for this parameter in the control site, in both seasons and for both sexes. In spring, these values are statistically different from those obtained in males from Drenas and females from Drenas and Mitrovica. The statistical difference is stronger and more noticeable in the autumn, though, and practically every site differs from the other for both sexes (Table 3; Fig. 3a).

Regarding BN in spring, only one statistical difference was observed between males from the control site and Mitrovica, while in autumn, the low values for this parameter observed in Drenas and Dragash differ significantly from the other sites in both sexes (Table 4; Fig. 3b).

With the exception of Mitrovica in males and Obiliq in females, the low VN values observed in the control site during the spring differ significantly from those found in the polluted sites. Furthermore, males from Mitrovica showed significantly different values from those from Obiliq and Kishnica, while females from Drenas differed significantly from all other sites. In autumn, the highest values were recorded in both sexes from Drenas, which are significantly different from all other sites (Table 5; Fig. 3c).

Kishnica had the highest ISN values among males in the spring, which was significantly different from Obiliq's and Dragash's values. In the spring, females from Obiliq showed a significant difference from those from Kishnica, while the lowest values found in Dragash were significantly different from all polluted sites except Obiliq. In the autumn, males from Dragash, Drenas, and Mitrovica, as well as females from Dragash and Drenas, varied greatly from one another and from the other sites (Table 6; Fig. 3d).

In general, all the parameters studied showed seasonal significance. The frequency of MN, BN, and ISN was lower in spring compared to autumn, with the exception of VN, which showed a higher frequency of changes in spring. Significant seasonal differences in the frequency of MN were recorded in the localities of Kishnica, Obiliq, and Drenas for both sexes and female frogs from Mitrovica (Table 3; Fig. 3a), while for BN, only the locality of Kishnica showed significant seasonal differences for both sexes (Table 4; Fig. 3b). Regarding VN, significant seasonal differences were observed for males from Kishnica and Obiliq and females from Mitrovica (Table 5; Fig. 3c). Furthermore, ISN showed significant seasonal differences for both sexes in the localities of Drenas, Mitrovica, and Obiliq (Table 6; Fig. 3d).

The only significant difference between sexes was noticed for the frequency of VN between males and females from Obiliq in spring and Mitrovica in both seasons (Table 5; Fig. 3c).

Table 3. MN[1] (‰) from investigated localities. The data are shown for both sexes separately in two sampling seasons: spring and autumn.

MN (‰)				
Season	Spring		Autumn	
Sex	Male	Female	Male	Female
Locality				
Obiliq[a]	2,70 (0,8)*[abcde]	2,19 (0,6)*[abce]	9,73 (2,5)*[a]	7,85 (1,4)*[ac]
Kishnica[b]	3,72 (1,3)*[abcde]	3,46 (2,6)*[abcde]	15,44 (2,1)*[b]	14,45 (1,7)*[b]
Mitrovica[c]	3,36 (1,2)[abcde]	4,04 (1,5)*[abcd]	6,41 (1,9)[c]	7,68 (1,0)*[ac]
Drenas[d]	5,87 (0,9)*[abcd]	5,46 (1,6)*[bcd]	9,15 (2,7)*[acd]	11,23 (2,4)*[d]
Dragash[e]	0,79 (0,8)[abce]	0,44 (0,3)[abe]	0,71 (0,4)[e]	0,79 (0,2)[e]

[1]Values are expressed as mean (standard deviation). Each locality was marked with a corresponding lowercase letter. Different lowercase letters in the same columns represent differences between sampling sites, i.e., if the letters are present at all sites, then there are no significant differences between them, while when a letter is missing from any of the sites, then that site is significantly different from the others. Stars represent differences between frogs of the same sex between different seasons within the sampling site, according to ANOVA, followed by the Newman-Keuls test.

Table 4. BN[1] (‰) from investigated localities. The data are shown for both sexes separately in two sampling seasons: spring and autumn.

BN (‰)				
Season	Spring		Autumn	
Sex	male	female	male	female
Locality				
Obiliq[a]	3,09 (1,2)[abcde]	2,26 (0,5)[abcde]	4,45 (2,3)[ac]	4,53 (2,0)[abc]
Kishnica[b]	1,55 (0,8)*[abcde]	1,58 (0,5)*[abcde]	7,38 (2,2)*[bc]	5,38 (0,9)*[abc]
Mitrovica[c]	3,91 (1,6)[abcd]	2,93 (0,7)[abcde]	6,41 (2,0)[abc]	5,58 (4,2)[abc]
Drenas[d]	3,36 (0,6)[abcde]	3,16 (1,6)[abcde]	1,11 (0,6)[de]	1,03 (0,4)[de]
Dragash[e]	0,45 (0,9)[abde]	0,09 (0,1)[abcde]	0,55 (0,3)[de]	0,43 (0,2)[de]

[1]Values are expressed as mean (standard deviation). Each locality was marked with a corresponding lowercase letter. Different lowercase letters in the same columns represent differences between sampling sites, i.e., if the letters are present at all sites, then there are no significant differences between them, while when a letter is missing from any of the sites, then that site is significantly different from the others. Stars represent differences between frogs of the same sex between different seasons within the sampling site, according to ANOVA, followed by the Newman-Keuls test.

Table 5. VN[1] (‰) from investigated localities. The data are shown for both sexes separately in two sampling seasons: spring and autumn.

VN (‰)				
Season	Spring		Autumn	
Sex	male	female	male	female
Locality				
Obiliq[a]	70,11 (45,4)[A*abd]	29,08 (19,4)[Babce]	5,28 (1,5)*[abce]	6,94 (0,8)[abce]
Kishnica[b]	68,81 (17,3)*[abd]	47,85 (11,9)[abc]	24,11 (12,3)*[abcde]	28,26 (17,5)[abce]
Mitrovica[c]	32,12 (19,0)[Acde]	60,05 (11,8)[B*abc]	10,27 (2,0)[Aabce]	11,59 (4,73)[B*abce]
Drenas[d]	57,29 (13,1)[abcd]	103,14 (43,5)[d]	53,26 (31,6)[bd]	95,49 (8,9)[d]
Dragash[e]	0,72 (0,5)[ce]	0,71 (0,3)[ae]	6,87 (1,2)[abce]	6,43 (0,8)[abce]

[1]Values are expressed as mean (standard deviation). Each locality was marked with a corresponding lowercase letter. Different lowercase letters in the same columns represent differences between sampling sites, i.e., if the letters are present at all sites, then there is no significance between them, while when a letter is missing from any of the sites, then that site is significantly different from the others. Stars represent differences between frogs of the same sex between different seasons within the sampling site, while capital letters represent differences between sexes of a site in the same season, according to ANOVA, followed by the Newman-Keuls test.

The curves in Fig. 3 (a-d) represent the analysis of micronuclei (MN), binucleated cells (BN), vacuolated nuclei (VN), and irregularly shaped nuclei (ISN) frequencies from samples collected at different sites (Obiliq, Kishnica, Mitrovica, Drenas, and Dragash) during spring and autumn, categorized by gender (male and female). Mean values were calculated, and 95% confidence intervals were added as error bars.

Correlation Analysis

Statistical analysis of linear correlations was conducted to examine the relationships between the mean values of the blood parameters and the mean values of metal concentrations in water and metal content in soil. Correlation analysis was performed based on localities and seasons. The data, including correlation coefficients and significance levels, are presented in Table 7.

Correlation findings:

- MN showed a strong positive correlation with As, Co, Cd, Cr, Pb, and Zn content in sediment.
- BN was positively correlated with Pb and Ni content in sediment.
- ISN was positively correlated with As, Co, Cd, and Pb content in sediment.

These results indicate that higher concentrations of specific heavy metals in sediment are associated with increased frequencies of nuclear abnormalities in the erythrocytes of *Pelophylax ridibundus*.

The analysis of the results obtained from this study indicates the presence of micronuclei (MN) and other nuclear abnormalities (NAs) in erythrocytes of the marsh frog (*Pelophylax ridibundus*) from Kosovo (Fig. 4).

Table 6. ISN[1] (‰) from investigated localities. The data are shown for both sexes separately in two sampling seasons: spring and autumn.

ISN (‰)				
Season	Spring		Autumn	
Sex	male	female	male	female
Locality				
Obiliq[a]	58,10 (14,0)*[acde]	52,19 (6,3)*[acde]	136,24 (31,0)*[ab]	144,09 (8,3)*[abc]
Kishnica[b]	123,64 (15,8)[bcd]	113,32 (21,9)[bcd]	140,91 (26,6)[ab]	148,20 (20,6)[abc]
Mitrovica[c]	93,77 (26,9)*[abcde]	96,77 (8,5)*[abcd]	194,51 (42,0)*[c]	194,63 (0,9)*[abc]
Drenas[d]	116,76 (14,5)*[abcde]	102,23 (53,11)*[abcd]	278,01 (30,7)*[d]	290,33 (10,7)*[d]
Dragash[e]	55,98 (74,7)[acde]	33,34 (1,61)[ae]	46,84 (3,3)[e]	47,64 (3,2)[e]

[1]Values are expressed as mean (standard deviation). Each locality was marked with a corresponding lowercase letter. Different lowercase letters in the same columns represent differences between sampling sites, i.e., if the letters are present at all sites, then there is no significance between them, while when a letter is missing from any of the sites, then that site is significantly different from the others. Stars represent differences between frogs of the same sex between different seasons within the sampling site, according to ANOVA, followed by the Newman-Keuls test.

These findings align with the significant heavy metal contamination in the studied areas, particularly from industrial pollution. The chemical analysis of water and soil samples revealed elevated levels of heavy metals such as Pb, Ni, Zn, Cd, Cr, Mn, and Co, sometimes exceeding the critical limits for water quality [36-38].

Notably, elevated concentrations of Pb were found in Kishnica and Drenas during autumn and Obiliq during spring, which is consistent with heavy industrial activities in these regions. Similar seasonal variations in Zn concentrations were observed, with peak levels in Drenas and Kishnica, corroborating findings by Zhelev

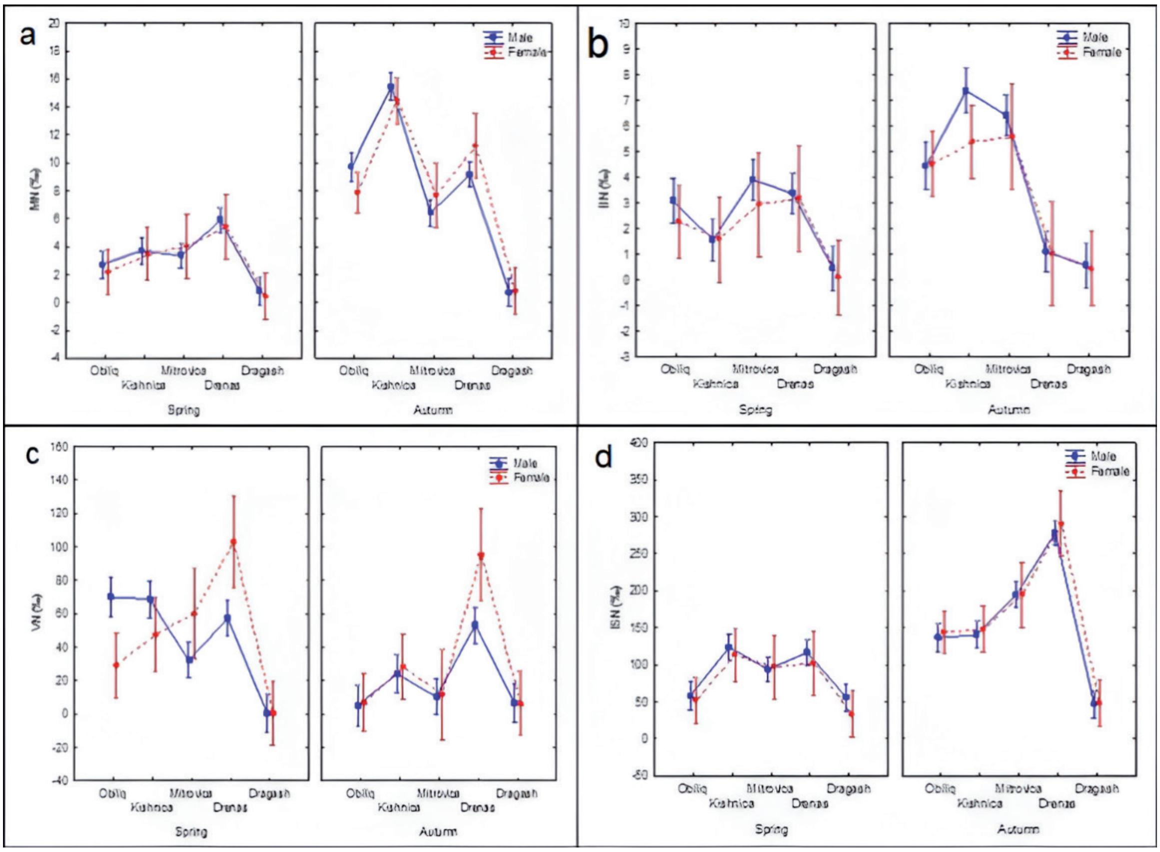

Fig. 3. Comparative analysis of frequencies a) micronuclei – MN; b) binuclei – BN; c) vacuolated nuclei – VN; d) irregularly shaped nuclei – ISN, from polluted and referent sites.

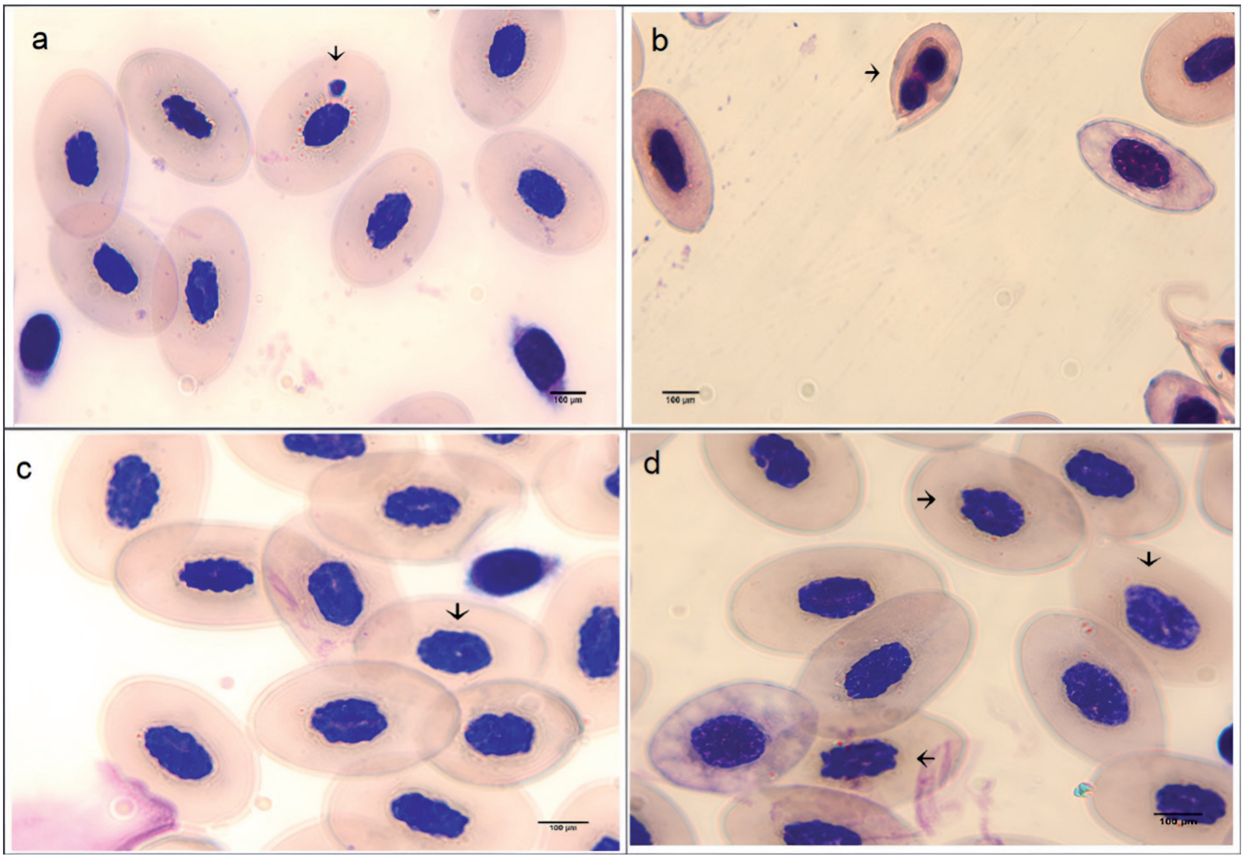

Fig. 4. Light micrograph showing different types of NAs in erythrocytes of marsh frog *Pelophylax ridibundus*: a) micronuclei (MN); b) binuclei (BN); c) vacuolated nuclei (VN); d) irregularly shaped nuclei (ISN).

Table 7. Results from correlation analyses[1] between blood parameters in marsh frog *Pelophylax ridibundus* vs. metal analyses from the water and the sediment.

Variables	Sperman's RhO	p value
Sediment		
MN (‰) vs As (mg/kg^{-1})	+ 0.63	<0.05
MN (‰) vs Co (mg/kg^{-1})	+ 0.92	<0.001
MN (‰) vs Cd (mg/kg^{-1})	+ 0.86	<0.001
MN (‰) vs Cr (mg/kg^{-1})	+ 0.67	<0.05
MN (‰) vs Pb (mg/kg^{-1})	+ 0.95	<0.001
MN (‰) vs Zn (mg/kg^{-1})	+ 0.68	<0.05
BN (‰) vs Pb (mg/kg^{-1})	+ 0,63	<0.05
BN (‰) vs Ni (mg/kg^{-1})	+ 0,63	<0.05
ISN (‰) vs As (mg/kg^{-1})	+ 0.69	<0.05
ISN (‰) vs Co (mg/kg^{-1})	+ 0.87	<0.001
ISN (‰) vs Cd (mg/kg^{-1})	+ 0.80	<0.01
ISN (‰) vs Pb (mg/kg^{-1})	+ 0.79	<0.01

[1]Only significant correlations are presented

et al. [39-41] and Dönmez & Şişman et al. [42] regarding heavy metal bioaccumulation and erythrocyte size variations in *Pelophylax ridibundus* inhabiting polluted areas.

Cadmium (Cd) concentrations were notably high in water samples from Obiliq and Drenas during autumn, while soil samples from Kishnica and Drenas also exhibited elevated Cd levels. This aligns with previous research by Cruz-Santiago et al. [43] and Romanova et al. [44], indicating that Cd exposure is a major factor in genotoxic stress in amphibians. Similarly, Ni concentrations, although below detection limits in water, were significantly higher in soil samples, particularly in Kishnica and Obiliq, consistent with findings from previous studies [45-48]. The presence of Cr, Co, and As across all localities further confirms widespread contamination associated with industrial emissions, a phenomenon also reported by Alnoaimi et al. [49] and Patar et al. [50].

A clear seasonal relationship was observed between metal concentrations and nuclear abnormalities in erythrocytes of *Pelophylax ridibundus*. The increase in heavy metal levels during autumn corresponded with significantly higher MN, BN, and ISN values, with Kishnica being the most affected site, followed by Obiliq and Drenas. This pattern is consistent with the findings of Jayawardena et al. [51], who reported that seasonal variations in industrial pollutants influence genotoxic responses in amphibians. Interestingly, VN values were higher in spring, particularly in males from Kishnica and Obiliq, highlighting potential sex-specific responses to contamination, although no significant overall difference was observed between sexes, as previously noted by Pollo et al. [52] and Zhelev et al. [39-41].

The observed nuclear abnormalities in *Pelophylax ridibundus* agree with previous studies demonstrating the genotoxic effects of industrial pollutants on amphibian populations. Increased frequencies of MN and BN cells have been directly linked to exposure to heavy metals, particularly Cd, as highlighted in research by Özgül et al. [53] and Corredor-Santamaría et al. [54]. Furthermore, Chew et al. [55] emphasized the influence of water quality on amphibian health, reinforcing the necessity for ongoing monitoring of contaminated sites.

It is crucial to consider additional environmental stressors that contribute to nuclear abnormalities, including temperature fluctuations, oxygen availability, and food sources, as discussed by Huang and Peng [56]. Moreover, agricultural pollutants and municipal wastewater discharges likely exacerbate the genotoxic effects observed in this study, as pesticide contamination has also been linked to increased nuclear anomalies [57].

This research highlights that the studied areas are under ongoing pressure from industrial pollution. Persistent contamination could threaten local wildlife without effective mitigation strategies, potentially resulting in species decline or extinction. The study emphasizes the urgent need for environmental policies focused on reducing heavy metal emissions and improving habitat conditions for amphibian populations. Nuclear abnormalities in *Pelophylax ridibundus* from industrially polluted sites in Kosovo highlight the genotoxic impact of heavy metals and the genetic instability they induce. These findings align with previous studies emphasizing the ecological risks of industrial pollution and reinforce the importance of regular biomonitoring to detect early environmental health risks and mitigate potential long-term ecological consequences [58-60].

Conclusion

This study successfully achieved its goal of elucidating the impact of industrial pollution on the frequency of nuclear abnormalities (NAs) in the erythrocytes of *Pelophylax ridibundus*, filling a critical gap in the understanding of amphibian health in polluted aquatic environments in Kosovo. The findings revealed a significant increase in specific NAs, including binucleated and vacuolated and irregularly shaped nuclei, as well as micronuclei, in regions exposed to industrial pollutants compared to the control site. These results underscore the role of *Pelophylax ridibundus* as an effective bioindicator species, highlighting the particular sensitivity of amphibians to chemical pollutants.

Moreover, this study adds to the growing body of literature demonstrating heavy metal's cytotoxic and genotoxic effects on amphibian erythrocytes, reinforcing the significant risks these contaminants pose to amphibian populations. The observed increase in nuclear abnormalities serves as a clear indicator of industrial pollution's detrimental effects on amphibian health, aligning with previous research documenting similar impacts on various aquatic species [61-62].

The implications of this research extend beyond immediate findings, opening avenues for further studies on the expanded use of amphibians in biomonitoring programs. Such initiatives could deepen our understanding of the long-term consequences of industrial contamination on ecosystem health and species conservation. This study emphasizes the necessity for comprehensive monitoring of amphibian populations in polluted environments, as these organisms are not only integral to their ecosystems but also serve as critical indicators of environmental quality. Future research should explore the cumulative effects of multiple pollutants and potential synergistic interactions that may exacerbate genotoxic damage.

Ultimately, this study not only confirms the adverse effects of industrial pollution on *Pelophylax ridibundus* but also underscores the urgent need for continued monitoring and conservation efforts to mitigate the escalating threats posed by environmental contaminants.

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Conflict of Interest

The authors declare no conflict of interest.

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