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# Determination of Nuclear Abnormalities in Peripheral Erythrocytes of the Frog *Pelophylax ridibundus* (Anura: Ranidae) sampled from Karasu River Basin (Turkey) for Pollution Impacts

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## A B S T R A C T

Karasu River, which is the only river in the Erzurum plain, is the source of the river Euphrates (Eastern Anatolia of Turkey). The river is in a serious environmental situation as a result of pollution by agricultural and industrial sewage and domestic discharges. The present study aims to evaluate genotoxic effects of toxic metals in marsh frog, *Pelophylax ridibundus* collected from two contaminated wetlands environments of the Karasu River Basin, in comparison with frogs from one non-polluted reference site. Heavy metal concentrations in surface water of the river were determined. Genotoxicity assays such as micronucleus (*MN*) and other nuclear abnormalities (*NA*) were carried out on the frogs studied. *MN* and *NA* (kidney-shaped nucleus, notched nucleus and lobed nucleus) were assessed in peripheral blood erythrocytes of the frog. A significant elevation in *MN* and *NA* frequencies was observed in frog collected from the polluted sites compared with those from the reference site. Results of the current study suggest that there is a close realtion between increasing of erythrocytic nuclear abnormalities and environmental contamination. High concentrations of heavy metals have a potential genotoxic effects, and the toxicity is possibly related to industrial, agricultural and domestic activities.

**Keywords**: *Pelophylax ridibundus*, heavy metals, polluted river water, genotoxicity

## A R T I C L E  I N F O

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### Karasu Nehri (Erzurum) Havzasında Yaşayan P*elophylax ridibundus* (Anura: Ranidae) Kurbağalarında Kirliliğin Neden Olduğu Periferal Eritrositik Çekirdek Anormalliklerinin Tespiti

**Öz:** Erzurum ovasının tek nehri olan Karasu Nehri, Fırat Nehri'nin kaynağını oluşturmaktadır. Nehirde tarımsal, endüstriyel ve evsel atıkların karışması sonucu ciddi bir çevresel kirlilik durumu söz konusudur. Bu çalışmanın amacı nehrin metallerle kirlenmiş ve kirlenmemiş bölgelerinde yaşayan ova kurbağalarındaki (*Pelophylax ridibundus*) genotoksik etkileri araştırmaktır. Kirliliği tespit etmek için nehrin yüzey suyu örneklerinde ağır metallerin konsantrasyonları ölçülmüştür. Kurbağalarda genotoksisite mikro çekirdek (*MÇ*) ve diğer çekirdek anormalliklerinin (*ÇA*) oluşumuna bakılarak tespit edilmiştir. *MÇ* ve böbrek şekilli çekirdek, çentikli çekirdek ve loplu çekirdek gibi diğer *ÇA* oluşumları kurbağaların eritrositleri kullanılarak belirlenmiştir. Kirlenmiş bölge kurbağalarının kanlarında *MÇ* ve *ÇA* frekansı temiz bölge kurbağalarına göre önemli ölçüde arttığı bulunmuştur. Bu artış ile nehrin kirlenmiş olma durumu arasında kuvvetli ilişkinin olduğu sonucuna varılmıştır. Yüksek ağır metal seviyesinin toksisiteye yol açtığı ve tespit edilen genotoksisitenin endüstriyel, tarımsal ve evsel aktivitelerle ilgili olabileceği kanaatine varılmıştır.

**Anahtar kelimeler:** *Pelophylax ridibundus*, ağır metaller, kirlenmiş nehir suyu, genotoksisite

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## Introduction

It is well known that organisms are repeatedly exposed to low doses of a variety of chemical pollutants of environmental or occupational origin. The environmental contaminants from both natural factors and human activities have received considerable study. In many ecosystems, human-induced changes have overwhelmed the natural biogeochemical discharges of various pollutants, such as trace elements. The degradation of the aquatic resources due to effluent discharges containing pollutant substances, some of which persistent, toxic or bioaccumulable, has coming to put in risk their natural function of life support and their utilization for the most variety of human activities. Biomonitoring of whole effluent has been used as an indicator of total effluent effects that may not be easily identified with specific chemical analysis (USEPA 2000; Şişman et al. 2008). So, the Direct Toxicity Assessment (*DTA*) approach uses the whole sample and monitors the direct effect on organisms accounting for unknown chemicals and for those of uncertain toxicity. *DTA* is important for different reasons, for example to identify, track, and monitor the source of toxicity in a stream or industrial process and to assist in the decision to discharge (Daniel et al. 2004; Dane and Şişman 2014). Also *DTA* has some degree of ecological relevance.

Heavy metals are commonly found as pollutants from industrial waste. In contrast to organic materials, they cannot be degraded and accumulate in water, soil, bottom sediments, and living organisms (Outridge and Noller 1991). Most heavy metals released into the environment find their way into water bodies as a result of direct input, atmospheric deposition, and erosion (Veena et al. 1997). As a consequence, aquatic organisms are exposed to higher levels of heavy metals, which accumulate in several tissues and so enter the food chain (Kalay and Canlı 2000; Altındağ and Yiğit 2005; Yazıcı and Şişman 2014).

During the last few years, many amphibian populations have been decreasing dramatically, and extinction caused by man-made changes in the environment (Care and Bryant 1995), has occurred in a few species. Frogs are more vulnerable than other vertebrates to environmental contaminants because frog eggs are not protected by semi-impervious shells (Duellman and Trueb 1986), they have both aquatic and terrestrial life stages and a semi-permeable skin (McDiarmid and Mitchell 2000). *Pelophylax ridibundus* is a marsh frog widely distributed in Europe and west Asia, with a wide distribution in Turkey. This kind of organism has shown to be a good bioindicator of aquatic contamination when compared to other aquatic vertebrates (Selvi et al. 2003; Marques et al. 2008) mainly because of the highly permeable skin that readily absorbs substances from the environment (Mitchell et al. 2005). Such vulnerability has also been responsible for the decline in amphibian populations worldwide (Stuart et al. 2004).

The micronucleus test is an easy and useful test for the in vitro and in vivo genotoxicity in frog living in contaminated water. The micronucleus number has been in use as an indicator of cytogenetic damage in molluscs, fish, amphibious animals and also in humans for more than 25 years (Hayashi et al. 1998; Rodriguez et al. 2003; Şişman and Türkez 2010; Türkez 2011).

Karasu River-510 km long is poured out Keban Barrage in Elazığ. Domestic and industrial waste, garbage, and fertilizers reach the river at different points. People living in the vicinity of the river use it for irrigation and fishing, so it is of great importance to establish the degree of damage to commercial fish species because of pollutants that might be present in the river waters. In the present study, we report the levels of heavy metals and physicochemical parameters of water pollutants and their genotoxic effects in frogs from the river.

## Materials and Methods

The main study area is Karasu Basin, which is the headwater of the Euphrates River. The basin is located in the Eastern Anatolia region of Turkey (Erzurum). The water source is snowmelt and rain. The river runs through several locations, such as Erzurum, Ilıca, and Aşkale. The human populations and distances to the sampling area from those locations are 250,000 and 60 km, 14,000 and 50 km, and 15,000 and 5 km, respectively. Some domestic and industrial wastes, garbage, and fertilizers reach the river at different points. At the upper of the river, various industrial activities are conducted, including textile dyeing and there are cement and sugar factories. The study sites were roughly selected on the basis of potential pollution or free contaminant, and the presence relatively large population of frogs. Sites were located in three regions in Karasu River: Serçeme site (S), Dumlu site (D) and Aşkale site (A). All located inside the city provinces of Erzurum, and at site S on the Serçeme stream, away from urban area, as a reference site. Site D on the Dumlu stream is relatively free contaminants. Site A at near the Aşkale shows strong anthropogenic influence, receiving domestic, agricultural and industrial effluents. The site is 58 km outside the city of Erzurum (Figure 1).

Approvals were obtained from the Atatürk University Local Ethics Commission (Number: B.30.2.ATA.0.23.85-99, Date: 06/09/2011) and the Turkish Ministry of Environment and Forest (B.18.0.DMP.0.02-510.02-48951, Date: 30/05/2011), and the rules of the commission were followed during the investigations. The frogs (both sexes) were captured in June and July 2012 from the surrounding areas of the sites. Fifteen (8♀, 7♂), fourteen (6♀, 8♂) and fifteen (7♀, 8♂) adult *P. ridibundus* were collected from the site S, D and A, respectively. The frogs were carried to the laboratory live in plastic aquaria in 2-3 cm of water obtained from the sites.

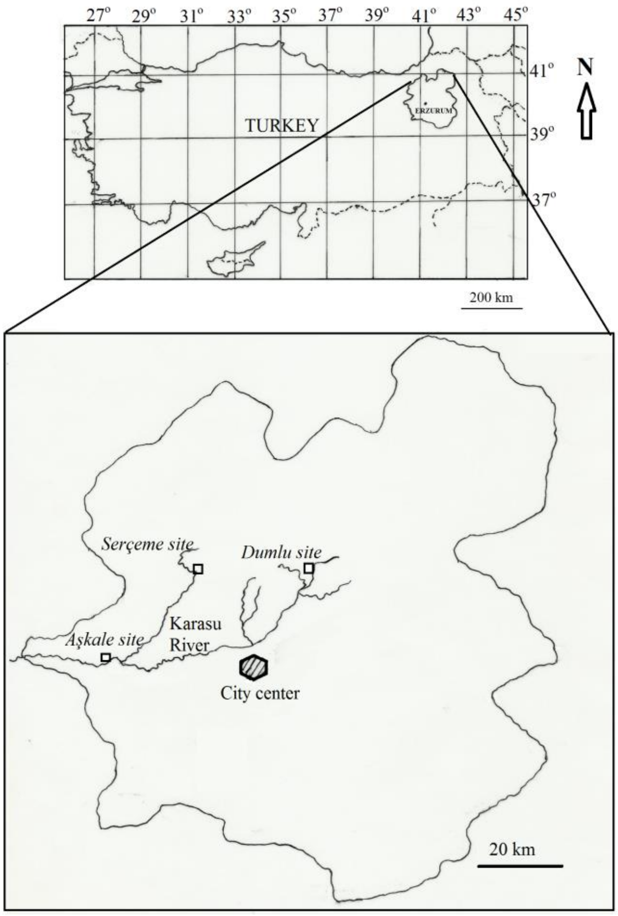


**Figure 1**. Map of Erzurum city and Karasu River showing the three sampling sites.

Water samples were collected from a depth of 0.5 m below the surface into clean 1-L polyethylene bottles by means of a Nansen Sampler. The water temperature, electrical conductivity, dissolved oxygen, and pH were measured in situ. Then, 1 ml of 0.5% $HNO_3$ was added to acidify the water samples. The samples preservation and analyses were made following the American Public Health Association and the American Water Works Association standard methods (APHA-AWWA-WPCF 1998). For the metal analysis, the water samples were acidified with nitric acid. A 100 $cm^3$ aliquot of the sample was digested with $HN0_3$ in a beaker at 120 ºC until a clear solution was obtained **(**Abulude 2005). All the sample were then stored at 40 ºC prior to analysis. The samples were analyzed by using a Perkin Elmer model 306 atomic absorption spectrophometer (APHA-AWWA-WPCF 1998).

In order to evaluate genotoxic effects, the erythrocytic nuclear abnormalities (*ENA*) was performed in mature peripheral erythrocytes according to previously described by Carrasco et al. (1990). For such aim, four blood smears were prepared from each animal. Blood smears were fixed with methanol for 10 min and stained with Giemsa for 30 min. A thousand erythrocytes were scored on every slide under a 1000× magnification in order to determine erythrocyte nuclear abnormalities. The scored nuclear alterations were divided in the following categories: kidney shaped, notched, micronucleus (*MN*) and lobed. The one-way analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) tests were performed to test statistical significant differences for *ENA* between frogs from the sampling sites by using SPSS Software (version SPSS 15.0). Statistical decisions were made with a significance level of $p$<0.05.

## Results

The water quality of the sampling sites was evaluated by comparing the results of physicochemical parameters (Table 1). Qualitatively, the Site D and A water samples had comparatively significantly higher levels of assayed ions, metals than samples collected from Site S water (reference site). The temperature and pH of the samples were approximately same.

The mean concentrations of the heavy metals in water samples at the selected sites together with Turkish Standardized Institute (*TSE*) (Anonymous 2005) *TSE* 266 limits are presented in Table 2. In water samples, according to the analysis results, the following findings were obtained for the concentration ranges of metals: Cd 0.05-7.1 µg/L, Al 69.2-287.8 µg/L, As 2.2-12.5 µg/L, Pb 3.05-11.3 µg/L, Mn 23.4-85.35 µg/L. Chemical analysis of surface water samples indicated that water from the downstream of the river (Site A) had higher concentrations of some heavy metals (Cd, Al, As, Pb and Mn) than sites from the upper river (Site D and S). While the heavy metal concentrations recorded in water of Site S and D were low compared to *TSE* 266 limits, Site A metals were high.

Table 3 shows the frequencies of *MN* and *NA* and their mean values with standard deviation in *P. ridibundus* from three sampling sites. Frogs collected from Site K and Site D showed a significant increase in *MN* frequency compared with the frog sampled at the reference site, Site S. Generally, the genotoxic effects of heavy metals studied were represented by the formation of *MN* and *NA* in the order: Site A < Site D < Site S. The most frequent nuclear aberration was lobed nuclei. Other nuclear abnormalities (*NA*) are also illustrated in Table 3. There was highly significant increase in the *NA* considered at Site A and Site D compared with those at the Site S.

Different erythrocytic nuclear abnormalities (*NAs*) recorded in frogs collected from the polluted sites (Site K and D) are presented Figure 2. The lobed nuclei (Figure 2A), notched nuclei (Figure 2B), micronuclei (Figure 2C) and kidney shaped nuclei (Figure 2D) abnormalities were identified.

Least square estimation analysis showed strongly significant positive correlation ($p<0.01$) between total heavy metal concentrations with *NAs* frequencies since the correlation values were 0.7-1.0 (Table 4).

**Table 1**. The physicochemical analysis of water samples obtained in the sampling sites.

| Physicochemical parameters | Site S[a] | Site D[b] | Site A[c] |
|---|---|---|---|
| Temperature (°C) | 25.2±8.5 | 25.2±8.3 | 25.8±8.4 |
| pH | 6.80±0.07 | 7.15±0.07 | 7.18±0.06 |
| Conductivity (S/m) | 1561±1.22 | 1650±1.25 | 1671±10.25 |
| DO (mg/L) | 7.48±0.44 | 5.8±0.53 | 5.6±0.52 |
| $NH_4$ (mg/L) | 0.26±0.20 | 0.55±0.44 | 0.57±0.01 |
| $NO_2$ (mg/L) | 0.04±0.017 | 0.07±0.06 | 1.12±0.37 |
| $NO_3$ (mg/L) | 0.05±0.008 | 0.04±0.046 | 0.03±0.004 |
| $K^+$ (µg/L) | 4.10±0.02 | 6.70±0.45 | 7.90±0.55 |
| $Na^+$ (µg/L) | 27.73±1.43 | 46.12±1.25 | 57.46±2.15 |
| $Ca^{2+}$ (µg/L) | 26.50±0.02 | 37.10±0.01 | 62.99±0.60 |
| $Mg^{2+}$ (µg/L) | 13.22±1.02 | 13.71±0.92 | 26.17±1.29 |

[a] Serçeme Stream, [b] Dumlu Stream, [c] Karasu River at Cement Factory

**Table 2.** Mean metal concentrations of surface water samples collected from the sampling sites as compared to *TSE* permissible limits (*TSE* 266).

| Metals | *TSE* 266* | Site S | Site D | Site A |
|---|---|---|---|---|
| Cd (µg/L) | 5.0 | <0.05 | 6.6 | 7.10[a] |
| Al (µg/L) | 200 | 69.2 | 192.1 | 287.80[a] |
| As (µg/L) | 10 | 2.2 | 10.4 | 12.50[a] |
| Pb (µg/L) | 10 | 3.05 | 6.25 | 11.30[a] |
| Cu (mg/L) | 2 | <0.1 | 0.13 | 0.15 |
| Mn (µg/L) | 50 | 23.4 | 29.2 | 85.35[a] |
| Cr (µg/L) | 50 | 16.3 | 26.7 | 45.80 |

*Anonymous (2005)

[a] Values are higher than the recommended Anonymous (2005) drinking water quality guidelines

**Table 3.** Nuclear abnormalities in peripheral blood erythrocytes of *P. ridibundus* from the sampling sites ($\overline{X}$±SEM).

| Nuclear abnormality | Site S | Site D | Site A |
|---|---|---|---|
| Lobed (L) | 1.24 ±0.09* | 5.29±1.39* | 7.04±1.11* |
| Notched (N) | 0.32±0.07* | 1.02±0.32* | 1.78±0.34* |
| Kidney shaped (KS) | 0.00±0.00 | 0.12±0.01* | 0.29±0.01* |
| Micronuclei (MN)** | 0.00±0.00* | 1.70±0.04* | 1.96±1.34* |
| Total (L+N+MN+KS) | 1.56±0.22* | 8.13±1.49* | 11.07±4.06* |

* Represents a statistical significant difference at the level of 0.05 between the stations.

** *MN* rates were presented as *MN* frequency/1000 cells for each sitation.

**Table 4.** Relationship between *NAs* frequencies and total heavy metal concentrations of *P. ridibundus* caught from the study sites.

| Sites | Total heavy metal concentrations (µg/L) | Total *NAs* |
|---|---|---|
| Site S | 114.3 | 1.56 ± 0.22 |
| Site D | 271.38 | 8.13 ± 1.49 |
| Site K | 450.0 | 11.07 ± 4.06 |
| *R*-value | | 0.956** |

Data are represented mean±SEM, **Highly significant difference ($p<0.01$).

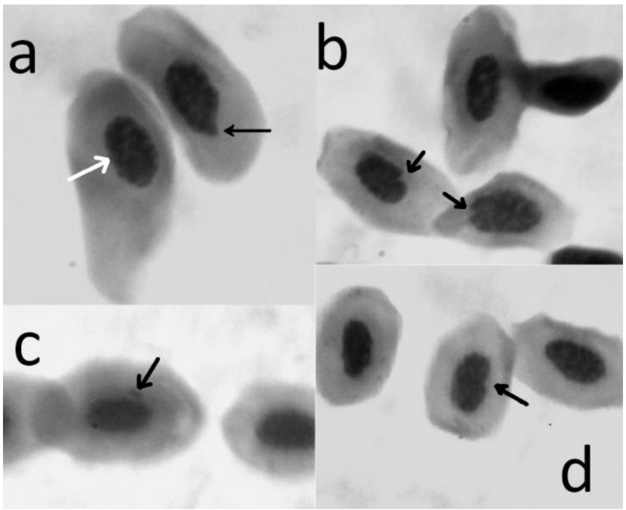


**Figure 2.** Representative nuclear aberrations of erythrocytes recorded in *P. ridibundus*. Normal (white arrow) and lobed nucleated erythrocyte (black arrow) (a), notched nucleus (black arrow) (b), micro-nucleated erythrocyte (black arrow) (c), kidney-shaped nucleus (black arrow) (d).

## Discussion

In the present study, analysis of water samples revealed elevated levels of Cd, Al, As, Pb and Mn in the downstream portion of the Karasu River (Table 2). The high levels of heavy metals in Karasu River could be attributed to the agricultural and industrial sewage drainage-water. There are several factories located in the study area which may contribute to the elevated amounts of heavy metals detected in the downstream portion of the river. The factory effluents may contain Pb, Ni, Cr, Cu, Cd (Anonymous 2004; Manzoor et al. 2006). Also, domestic effluents can contain elevated amounts of heavy metals such as Cd, Cu, Cr, Pb, and Zn (Aonhgusa and Gray 2002). The levels of Cd, Al, As, Pb and Mn in Site A are higher than the reference site (Site S). Heavy metals are generally found in water, foodstuffs, or as precipitates. Uptake of metals from contaminated water or food products depends on environmental conditions, the subject's metabolism, degree of contamination, salinity, temperature, and interactions between different chemical species (Gayer 1991; Canlı and Atlı 2003). Some authors reported that heavy metal toxicity is affected by temperature, dissolved oxygen, pH, and metal concentration and that increases of pH generally decreased the solubility of many heavy metals (Hellawell 1988; Avila-Pérez et al. 1999). On the other hand, high pH and high concentrations of ammonia and nitrates are toxic to aquatic life (Chapman 1998). Ammonia levels were high in the Dumlu and Aşkale sites. Nitrite was found to be high only in the Site A, possibly because of cement plant in the vicinity.

Micronuclei occur as a short-term response to cytogenetic injury, disappearing from the organism within a few days (De Flora et al. 1993), thus being a useful technique for investigating freshwater ecosystems. In our study, the *ENA* assay showed the existence of a significantly higher value of nuclear abnormalities in erythrocytes of *P. ridibundus* from the Site D and A when compared to Site S animals. Even though the number of *MN* did not differ significantly between sites, the presence of other anomalies in the nuclei on Site A frogs can indicate genotoxicity. In the current study, a positive correlation was also found between nuclear abnormality frequencies and the heavy metal content of river water. It was reported that similar results were obtained from the studies examining the genotoxicity of water pollution. According to Aymak (2010), the *MN* frequency is higher in the region where the heavy metal pollution is particularly high out of two localities considered uncontaminated and contaminated. In another study, the micronucleus analysis was made on the erythrocytes of the *P. ridibundus* specimens collected around Vize (Kırklareli) and Ida Mountains (Yenice, Çanakkale), nuclear abnormalities were detected. As a results of the study, it was determined that there was a significant difference in the total number of nuclear abnormalities between both localities and according to the investigation Vize samples (contaminated area) have higher frequency of micronuclei (Gürkan et al. 2012).

The blood of amphibians is very plastic tissue. In fact, variations of several hematological parameters in response to natural changes in the environment have been widely described by researchers (Stansley and Roscoe 1996). It is important to note that the occurrence of nuclear morphological aberrations like several binucleated cells in *P. ridibundus*, as well as the general degenerative changes in erythrocytes during the tadpole stage studied corresponding to the period of intense hematopoietic with active cell division in circulation blood. Jaylet et al. (1986) first adapted the *MN* test to amphibians. Many *MN* tests on amphibians have used genotoxic agents (Zoll-Morreux and Ferrier 1999). It has been used as a measure of genotoxicity in amphibians (Compana et al. 2003) and has shown potential for in situ monitoring of water quality (Gauthier et al. 2004). *MN* derive from chromosomal fragments or whole chromosomes which are not incorporated in to main nucleus during cell division as a consequence of DNA fragmentation (clastogenic origin) or of alteration of the mitotic apparatus (an eugenic origin) (Norppa and Flack 2003).

By the time the Dumlu Stream reaches the Karasu River, the already contaminated waters are mixed with those from a canal carrying the wastes from municipal wastes in that zone. In addition, waste and sewage from sugar and cement plants are dumped into the river. However, the tributary Serçeme Stream is clean because there is no any municipals, villages and plants upper and near the stream. It should be mentioned that during the field study, we observed many nearly dead fish at the Cement Factory area (Site A).

To the best of our knowledge, this study is the first study dealing with genotoxicity of frogs around the Karasu River. The results here presented confirm that there is a problem with water pollution in the river. The genotoxic damage observed in *P. ridibundus* used in this study shows that pollution is affecting organisms living around the river and that this is seriously threatening an important agricultural and industrial region in Turkey. Urgent measures must be taken to correct this situation before it becomes a critical issue for the region.

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