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Marsh Frog (*Pelophylax ridibundus*) as a Bioindicator to Assess Pollution in an Agricultural Area

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Authors' Contribution
TŞ designed and supervised the work. MÇK, EC and ŞA collected the samples and conducted the experimental work. HD helped the data analysis. FG and SÇ wrote the manuscript.

Key words
Agricultural pollution, Histopathology, Genotoxicity, *Pelophylax ridibundus*, Erzurum.

ABSTRACT

In order to determine the effects of environmental contamination, nuclear abnormalities of peripheral erythrocytes and lung, liver, and kidney histopathologies were evaluated in marsh frog (*Pelophylax ridibundus*) in agricultural areas. The most prominent histopathological alterations observed in lung tissue were noted as epithelial hyperplasia, thickness of alveolar septa, dilatations and congestions of blood capillaries, and melanomacrophage accumulation. An increase in the number and widening of melanomacrophage centers were also noted for liver, in parallel with hepatocyte degeneration and heterogenic vascular construction originating from congestion, deformation and dilatation. In kidney, degeneration, congestion, dilatation, and necrosis in proximal and distal tubules were recorded; moreover, expansions of Bowman's space, infiltrations of lymphocytes, and glomerulonephritis were observed. Also, the frequency of nuclear abnormalities in erythrocytes showed significant differences between the sites. Quantitative analyzes of histopathological and nuclear alterations indicated that the highest effects of pollutants in frogs were at the agricultural sites in June.

INTRODUCTION

Because of their little mobility, life cycle complexity, and skins with high water permeability, amphibians are very sensitive to environmental changes (Gonzalez-Mille *et al*., 2013). These unique properties can cause rapid and effective bioaccumulation of chemicals (Gurushankara *et al*., 2007) and make amphibians the most preferred bioindicator organisms to determine the effect of pollutants (Marquis *et al*., 2009). In amphibian specimens collected from agricultural areas, many malformations leading to population declines have been reported (Taylor *et al*., 2005). The causal link between the decrease of population and excessive use of pesticides and fertilizers were also reported more recently (Soloneski *et al*., 2016). The amphibian species globally suffer population decline, primarily due to anthropogenic pollution and increasing use of pesticides and synthetic fertilizers (Whittaker *et al*., 2013).

Pelophylax ridibundus Pallas, 1771, primarily known as *Rana ridibunda*, a widely distributed marsh frog species of Central Europe and Western Asia, is also found in Turkey (Baran *et al*., 2012). When compared to other aquatic vertebrates, *P. ridibundus* is a more useful bioindicator that can provide more information in environmental risk assessment due to spending its whole life in the watershed (Marques *et al*., 2009; Zhelev *et al*., 2013). The practical usefulness of *P. ridibundus* in bioindicative analyzes for the environmental assessment of agroecosystems was also described recently by Zhelev *et al*. (2018). Lung, liver and kidney are the most preferred organs for histopathological investigations due to their roles in absorption, distribution, biotransformation, detoxification and excretion of xenobiotics (Paunescu *et al*., 2012a). Several studies also showed that amphibians were sensitive organisms suitable for the detection of genotoxic chemicals (Mann, 2006). The micronucleus (MN) assay has been used as a measure of genotoxicity in amphibians and has shown potential in the in situ monitoring of water quality (Gauthier *et al*., 2004). Because MN assay measures chromosome loss and fracture, it is preferred in the evaluation of genotoxic damage (Fenech, 2000). Furthermore, some authors have interpreted that erythrocytic nuclear abnormalities (ENA) result from analogous damage (Serrano-Garcia and Montero-Montoya 2001; Marques *et al*., 2009). With MN, ENA such as notched, lobed and kidney-shaped nuclei can be counted to assess exposure to genotoxic pollutants (Guilherme *et al*., 2008; Marques *et al*., 2009).

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The current study was aimed to assess the water quality at three sites along Pulur Creek (Erzurum-Turkey) which receives agricultural effluents, using the frog *P. ridibundus* as a bioindicator in June and October 2014. The genotoxic effects and histological alterations of agriculture effluents were studied in peripheral blood samples, lung, liver and kidney of *P. ridibundus* collected from one control and two agricultural areas in the creek.

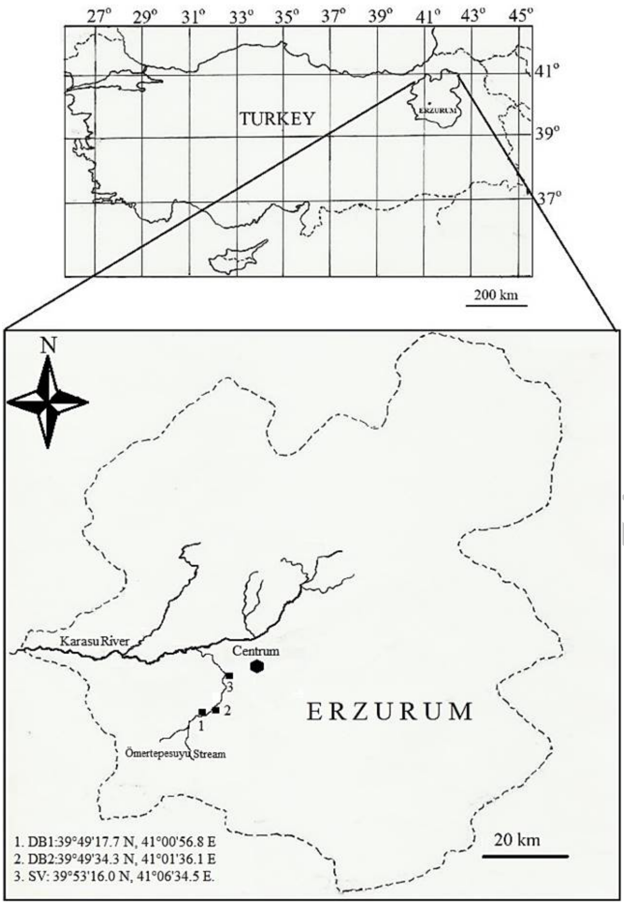

Fig. 1. The sampling stations.

MATERIALS AND METHODS

Study area

It is known that agrochemicals are used in some areas in the Erzurum Plain (ESR, 2012 and 2016). Three stations (Fig. 1) were selected based on diversities of agricultural activities in the plain. The first one was the entry point of Pulur Creek which is one of the main tributaries of Karasu River, and regarded as the reference/control site (DB1) where no agricultural activities were carried out, and there were no urban or industrial zones. The second one (DB2) was the exit point of the creek at Dereboğazı Village which has moderate agricultural activities, such as the use of artificial fertilizers. The last station was located in the same creek, downstream of the Söğütlü Village (SV), where high agricultural activities were carried out in terms of widespread use of insecticides (ESR, 2012 and 2016). The last sites which are two of the central villages of Erzurum, receive only agricultural effluents.

Sampling species

Permission (No:55885869/1132, 25/09/2013) was obtained from relevant authority before the sampling. In each station, equal numbers (n=5, total=30) of *Pelophylax ridibundus* species were caught from their natural habitats in June and October 2014. Samples were not differentiated in terms of sex. The weights and lengths of all of the samples were measured (mean; 80.34 g and 10.26 cm-SVL).

Histopathology

All specimens were anaesthetized by diethyl ether, and dissected by splitting into a “T” shape. The lungs, liver, and kidneys were removed and fixed in Bouin’s fluid for 24 h and kept in buffer for 12 h. Following dehydration and clarification, the tissues were embedded in paraffin. 7 µm-thick cross sections were performed by microtome, stained by hematoxylin and eosin (H&E), examined and photographed. Quantitative evaluations were made on 10 slides belonging to one organ. Quantitative analysis of histological lesions was determined according to Histopathological Alterations Index (HAI) (Abdel-Moneim *et al*., 2012) based on the prevalence of lesions. Observed abnormalities were noted and classified in accordance with the stages of damage: Stage I (SI), the status of tissue that functions normally; Stage II (SII), the status of moderate impairment of tissue’s normal function; Stage III (SIII), the status of strong, irreversible damage.

The HAI value was calculated on each tissue of each frog using the following formula:

$$HAI = (1 \times SI) + (10 \times SII) + (100 \times SIII)$$

The average HAI of each station was also calculated and interpreted for each frog. HAI values at the range of 0-10 were accepted as an indicator of normal organ functions. Values at the range of 11-20 indicate slight damage to the organ, while 21-50 indicate moderate damage, and 51-100 indicate the existence of severe lesions. HAI values above 100 were accepted as the existence of irreversible damage (Poleksic and Mitrovic-Tutundzic, 1994).

Genotoxicity

Genotoxicity was tested using micronuclei (MN) and erythrocytic nuclear abnormalities (ENA) and was carried out in mature peripheral erythrocytes according to the procedures of Fenech (2000) and Carrasco *et al*. (1990). Blood samples were collected with a heparinized insulin syringe and smeared on clean microscope slides. Four blood smears were prepared from each frog. The smears were fixed with methanol for 10 min and stained with Giemsa for 30 min. One thousand cells from each slide were scored using blind scoring under a 1000x magnification in order to determine ENA and MN. Only cells with whole cellular and nuclear membranes were analysed. The nuclei which ovoid or elliptical shaped and diameter 1/3rd that of main nucleus and clearly detached from it, were accepted as MN. Four ENAs were considered: notched, binucleated, lobed and blebbed (Pollo *et al*., 2015). All of these features were considered as ENA. The results were expressed as MN and ENA mean frequency (‰) of the sum of all abnormalities observed (Guilherme *et al*., 2008).

Statistical analysis

The assessment of the results was made using the SPSS 20.0 software. The one-way analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) tests were used for comparison with the average data obtained from each site. The difference between the stations was evaluated in terms of significance at the level of $p<0.05$.

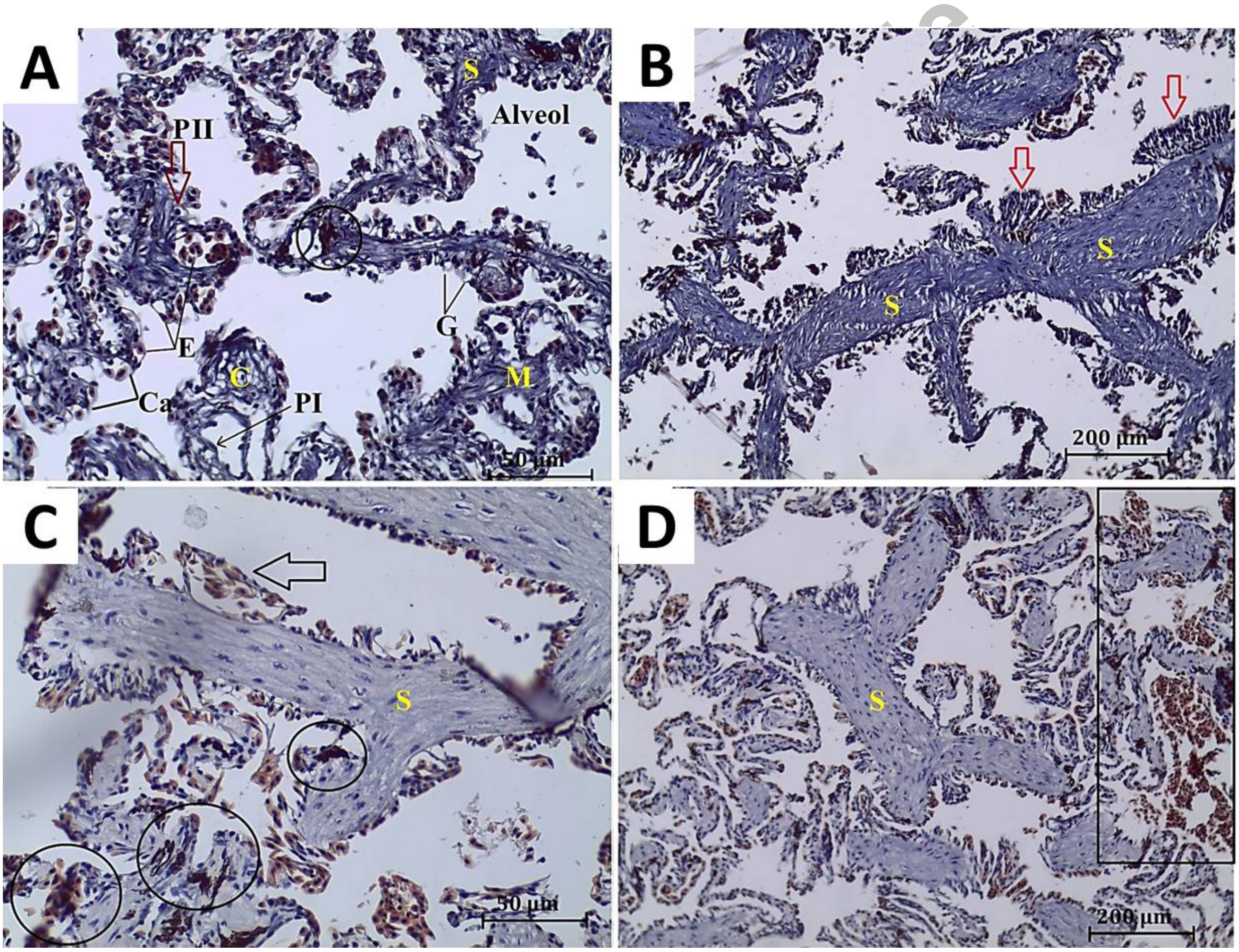

Fig. 2. **A,** normal alveolar construction of *P. ridibundus* collected from DB1. Septum (S), type I and II pneumocytes (PI, PII), erythrocytes (E), goblet cells (G), capillaries (Ca), alveol, melanomacrophage aggregate (encircled), connective (C) and muscle tissue (M). B, C and D, histopathological alterations of the lung of *P. ridibundus*: B: Samples of DB2; C, D: Samples of SV. **B,** thickness of alveolar septum and hyperplasia of alveolar epithelium (arrows). **C,** dilated blood capillaries (arrow) and melanomacrophage aggregation (encircled). **D,** congestion (squared), (H&E).

RESULTS

The frequencies of histological lesions and HAI values in the lung, liver, and kidney of *P. ridibundus* caught from the stations DB1, DB2 and SV are given in Table I. As seen, the frequencies of the histological abnormalities were very low at DB1, while they were increased slightly and significantly at DB2 and SV, respectively. In SV samples, the numbers of tissue lesions exhibited seasonal variations as more lesions were recorded for June 2014 when compared to October 2014 (Table II). It was observed that the difference was statistically significant.

Table I.- The frequencies and HAI values of the histopathological lesions of lung, liver and kidney tissues of the *P. ridibundus* collected from different stations.

Tissue / Lesion	HAI stage	DB1	DB2	SV
Lung				
Melanomacrophage aggregates	I	0+	+	++
Capillary dilatation	I	0	0+	+
Congestion	I	0	+	++
Hyperplasia of alveolar epithelium	II	0	+	++
Alveolar septum thickening	III	0+	+	++
Liver				
Melanomacrophage aggregates	I	+	++	+++
Non-homogenous parenchyma	I	+	+	++
Congestion of the central vein	I	+	+	+
Sinusoidal dilatation	I	+	++	++
Degeneration of the central vein	II	0	+	++
Vascular degeneration	II	0	+	+++
Kidney				
Glomerulonephritis	I	0	0+	+
Congestion	I	0	+	++
Tubular dilatation	I	0	0	+
Bowman's space expansion	I	0+	+	++
Lymphocyte infiltration	I	0+	+	++
Tubular degeneration	II	0	0+	+
Tubular necrosis	III	0	0	0+

0, absent; 0+, rare; +, low frequent; ++, frequent; +++, very frequent.

Lung histology and pathology

As seen in control specimens caught from DB1 (Fig. 2A), the alveolar septum that separates adjacent alveoli contains smooth muscle and connective tissue cells and fibers. Alveolar epithelia mainly consist of pneumocytes (I and II), typical goblet cells and neuroepithelial endocrine cells (the last cell could not be detected in Fig. 2A). Darkly stained, cubic or columnar pneumocytes have large and rounded nuclei. Goblet cells, located as individuals and in groups among the pneumocytes, can easily be identified with their apical cytoplasmic areas. Erythrocytes in the blood capillaries were also easily observed with their large, ovoid nuclei. Some slight damages such as thickness of alveolar septum and hyperplasia of alveolar epithelia were noted in DB2 specimens (Fig. 2B). In addition to thickness of septum, capillary dilatation and melanomacrophage accumulation (Fig. 2C), and congestion (Fig. 2D) were observed in SV specimens and indicated moderate damage. HAI values were calculated as 4.57±2.97 for DB1, 17.96±3.09 for DB2, and 42.43±3.20 for SV (Table III), in parallel with the histopathological findings.

Table II.- Seasonal variations of the total numbers of lesions observed in the tissues of SV samples (n=10).

Tissues	Total lesion in June 2014*	Total lesion in October 2014
Lung	276	258
Liver	363	328
Kidney	304	267
Total	943**	853

*The numbers showed the pathologies as a piece in tissue section, and the data in each organ and month were obtained from 50 slides belong to 5 frogs. **There was a statistical difference between the values.

Table III.- HAI values calculated for each tissue of the samples collected different stations (n=10).

Station	Lung	Liver	Kidney
DB1	4.57±2.97[a]	6.05±2.09[a]	1.40±0.30[a]
DB2	17.96±3.09[b]	45.35±15.09[b]	13.58±1.25[b]
SV	42.43±3.20[c]	87.88±22.08[c]	23.24±3.90[c]

Results are mean ±SE. Means with a different letter in the same column for each station are significantly different at p<0.05.

Liver histology and pathology

The liver of DB1 specimens (Fig. 3A) was parenchymatous in appearance with central vein, hepatocytes and sinusoids. Hepatocytes were polygonal in shape, with their centrally located, one or two prominent nuclei. A few clusters of melanomacrophages were also observed. In addition to sinusoidal dilatation, degeneration and congestion of central and portal veins (Fig. 3B, C) and melanomacrophage aggregation were noted as the prominent alterations for the livers of DB2 specimens (Fig. 3C). In SV specimens, vascular degenerations (Fig. 3D), non-homogenous appearance of parenchyma (Fig. 3E), sinusoidal dilatations and increasing melanomacrophage aggregation (Fig. 3F) were observed.

Histological findings were in accordance with HAI values determined as 6.05±2.09 for DB1, 45.35±15.09 for DB2, and 87.88±22.08 for SV (Table III). Consequently, the functions of the liver of DB1 could be accepted as normal. However, the functions seemed to be moderately and severely damaged in DB2 and SV, respectively.

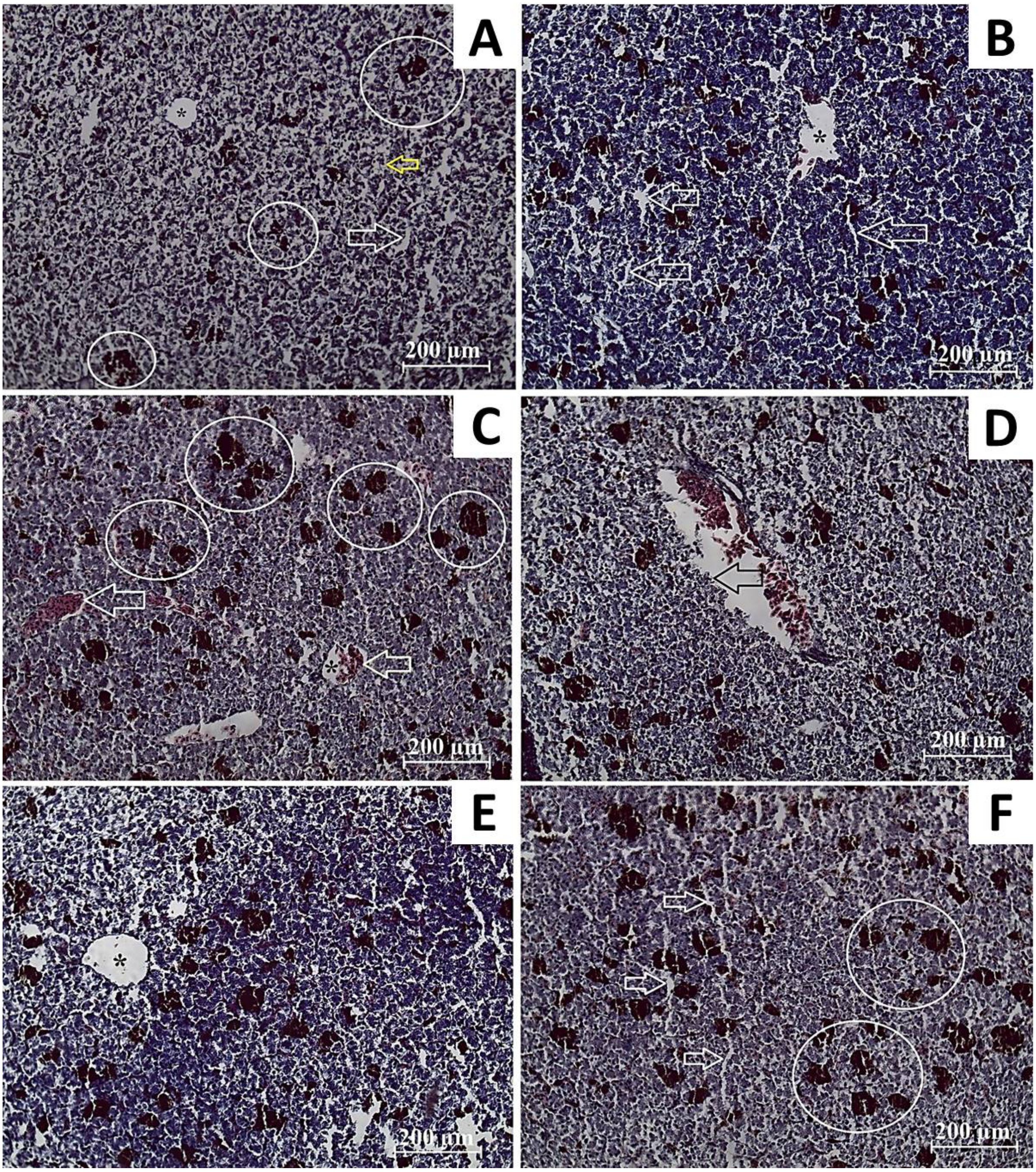

Fig. 3. Histological structures and alterations of the liver of *P. ridibundus*. A; samples of DB1, B, C; samples of DB2: **A,** sinusoids (white arrow), central vein (asterisk), hepatocytes (yellow arrow) and melanomacrophage aggregation (encircled). **B,** degeneration of central vein (asterisk) and sinusoidal dilatation (arrows). **C,** increasing melanomacrophage aggregation (encircled), congestions of the central (asterisk) and portal vein (arrows). D, E, F samples of SV. **D,** degeneration of vascular epithelium (arrow). **E,** non-homogenous parenchyma. **F,** sinusoidal dilatations (arrows) and increasing melanomacrophage aggregation (encircled), (H&E).

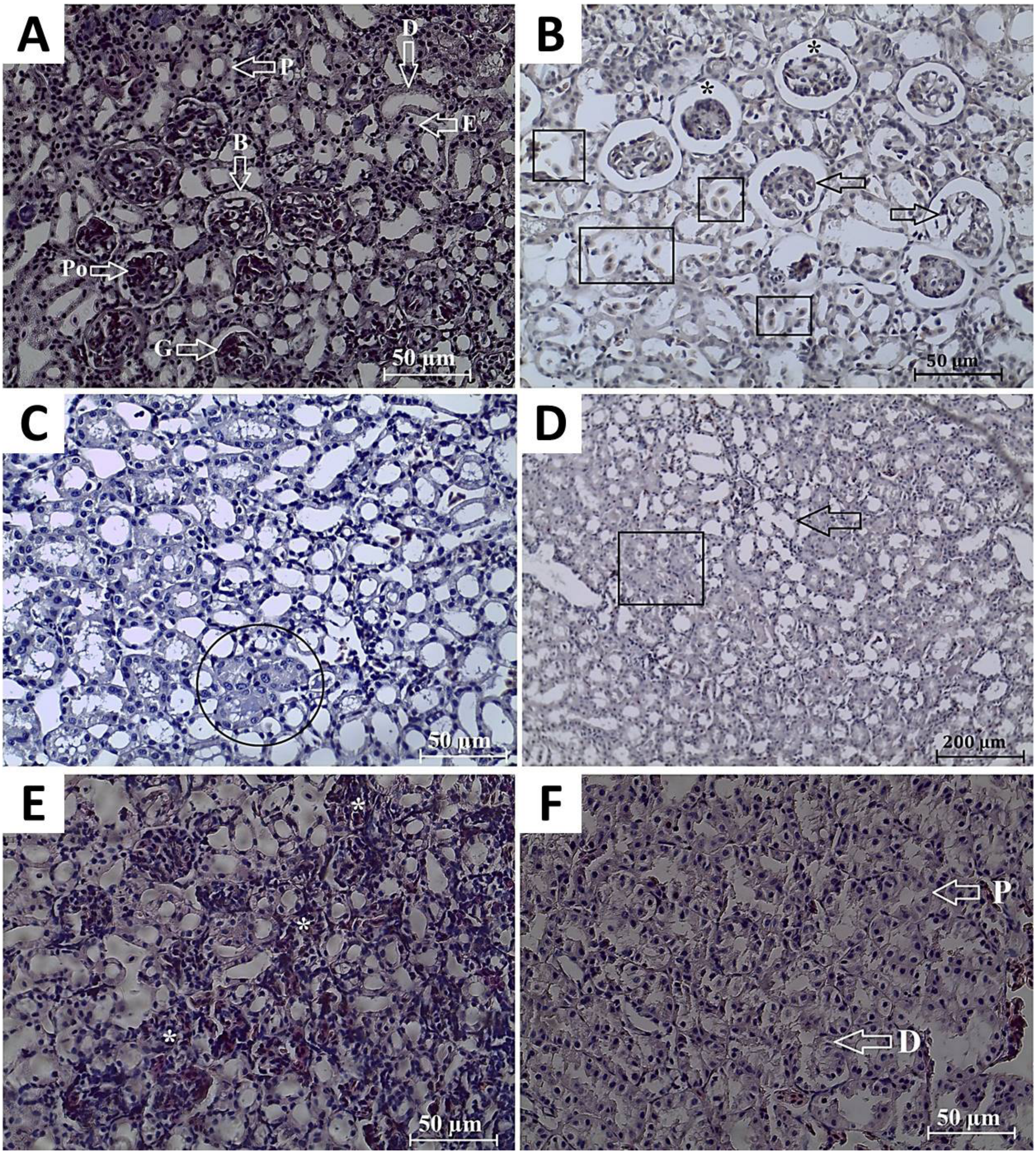

Fig. 4. **A,** normal histological properties of the kidneys of *P. ridibundus* from DB1: renal corpuscles with glomerulus (G) and Bowman's space (B); podocyte (Po), erythrocytes (E), proximal (P) and distal tubules (D). B, C and D; histological alterations of the specimens of DB2. **B,** glomerulonephritis (black arrows), congestion in renal parenchyma (squared), and expansion of Bowman's space (asterisks). **C,** tubular necrosis (encircled). **D,** tubular dilatation (arrow), tubule lumens as eosinophilic in appereance (squared). E, F; Histological changes in the kidney of SV specimens. **E,** lymphocytes infiltration (asterisks). **F,** degeneration of proximal (P) and distal (D) tubules, (H&E).

Kidney histology and pathology

It was observed that the renal parenchyma of the specimens of DB1 (Fig. 4A) was normal in structure with renal tubules and with their corpuscles surrounded by a Bowman's capsule. The inner visceral layer of Bowman's capsule consisted of clearly identified podocytes. Proximal tubules were observed to be round or spherical in shape, while distal ones were ovoid or ellipsoid. The most prominent alterations observed in the kidneys of DB2 were noted as glomerulonephritis, in addition to congestion in renal parenchyma and expansion of Bowman's space (Fig. 4B). Tubular necrosis (Fig. 4C) and dilatation (Fig. 4D) were also observed, and the lumens of some tubules were noted to be eosinophilic in appearance. In SV samples, the most prominent alterations were observed as lymphocytes infiltration (Fig. 4E) and tubular degenerations (Fig. 4F). The HAI values, as an indicator of normal kidney function, were calculated as 1.40 ± 0.30 for DB1; 13.58 ± 1.25 for DB2, and 23.24 ± 3.90 for SV (Table III). Based on both histopathological and statistical data, the kidneys of DB1 frogs were assumed to be normal. The function of the kidneys of DB2 was accepted to be slightly damaged, while moderately damages were noted for SV samples. The results obtained from all these findings may be summarized as that the pathological effects were determined in all frog tissues. Pathological effects in SV frogs were found to be more than other areas.

Genotoxic abnormalities

Some variations were observed in the normal erythrocyte shape in the frog samples. The different types of ENAs are presented in Figure 5. The kidney-shaped, notched, lobed nuclei and MN abnormalities were identified. Table IV shows the frequencies of ENA in the frogs from the sampling sites. The frequencies were determined in the following order: notched nuclei> lobed nuclei> kidney-shaped nuclei>MN. There was a significant increase in the ENA considered at the DB2 and SV sites compared to those at the reference site (DB1). ENAs were found exclusively in June in the agricultural sites.

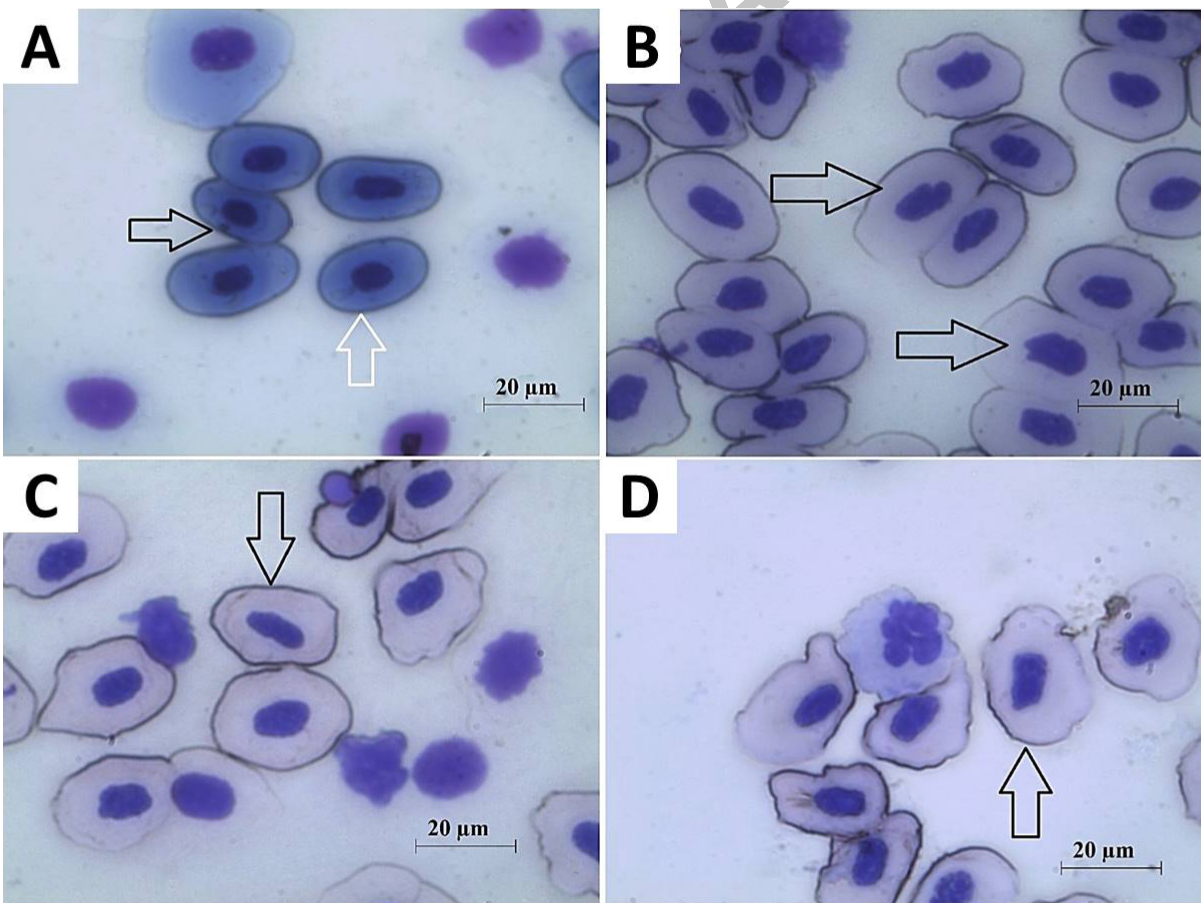

Fig. 5. The ENAs recorded in *P. ridibundus:* **A,** Normal (white arrow) and micronucleus (black arrow); **B,** notched nucleus (black arrows); **C,** kidney-shaped nucleus (black arrow); **D,** lobed nucleus (black arrow), Giemsa.

Table IV.- ENAs in peripheral blood erythrocytes of *P. ridibundus* from the different sites and months.

Season	Site	Micro nucleus (MN)	Lobed nucleus (L)	Notched nucleus (N)	Kidney-shaped nucleus (K)	Total (MN+L+N+K)
June	DB1	0.07±0.01	0.02±0.00	0.05±0.01	0.02±0.01	0.16±0.03[a]
	DB2	0.50±0.02	0.60±0.04	2.10±0.50	0.90±0.08	4.10±0.60[b]
	SV	0.80±0.08	1.90±0.70	6.40±1.20	1.70±0.40	10.80±2.40[c]
October	DB1	0.05±0.01	0.01±0.00	0.06±0.02	0.01±0.00	0.13±0.03[a]
	DB2	0.30±0.06	0.50±0.06	1.20±0.20	0.50±0.10	2.50±0.40[b]
	SV	0.50±0.04	1.50±0.07	5.20±0.80	1.20±0.20	8.40±1.10[c]

Results are mean ±SE. Means with a different letters in the same column for each station are significantly different at $p<0.05$.

DISCUSSION

There is no doubt that extensive use of agricultural chemicals including fertilizers and pesticides is one of the main reasons of environmental pollution. It was investigated in this study whether agricultural contamination of Erzurum Plain affected the marsh frogs. One of the indicators of agricultural pollution in the plain is the analysis by the provincial directorate of environment and city planning. According to the water analyzes in 2012 and 2016 by official Erzurum provincial directorate of environment and urbanism, 37.85 mg/L and 115.17 mg/L nitrate levels were reported in groundwater with nonpoint sources of nitrogen pollution in the plain, and 32.08 tons fertilizers (N, P and K in total) and 31.51 tons pesticides (insecticides, herbicides and fungicides) were used in Erzurum Plain (ESR, 2012, 2016). In the same report, it was noticed that the Pulur Creek water, where we collected the frogs, was classified a third-class irrigation water, and one of the causes of pollution in the creek was shown as the use of pesticides (ESR, 2012). On the other hand, the Turkish Official Water Quality Control Regulation accepted that third-class irrigation water was polluted water and that the water could not be used for cultivation without refinement (Official Gazette, 2004). ESR (2016) also reported that the pesticides and fertilizers used in the agricultural areas of Erzurum caused soil and water contamination. The data clearly showed that there was an agricultural pollution in the plain. Based only on the results of the present study, we have to note that it is impossible to identify the various, probably agonistic or antagonistic effects of different fertilizers and pesticides that used in the sampling areas. Thus, all of the comparisons mentioned below must only be evaluated on the axis of agricultural pollution-marsh frogs.

As noted, the marsh frog *P. ridibundus* is a suitable indicator for environmental contamination (Zhelev *et al*., 2013, 2018). In a study, Sisman *et al*. (2015) showed the genotoxic effects of water pollution on *P. ridibundus* living in three different regions of the Karasu River, which is a single river of the Erzurum Plain, and reported that a significant elevation in ENAs was determined in the frogs collected from polluted areas. Sisman *et al*. (2016a) also investigated the histopathologic effects of pollution on *P. ridibundus* living around the same river and reported an increase in melanomacrophage clusters, non-homogeneous parenchyma, degeneration of hepatocyte, congestion, sinusoidal dilatation and fibrosis in the liver, while tubular degeneration, lymphocyte infiltration and fibrosis were detected in the kidney. In another study conducted on the frogs of the same sites, histopathological findings noted for lungs were found to be related to pollution (Sisman *et al*., 2016b). In the current study, there was a higher value of nuclear abnormalities in erythrocytes of *P. ridibundus* from the DB2 and SV, and different pathological effects were determined in the lung, liver and kidney of *P. ridibundus* samples collected from the areas. Although histopathological alterations were observed in all samples, minimal pathology was noted for DB1 specimens. Probably due to the extensive use of agricultural chemicals, more nuclear and histopathological alterations were observed in DB2, and they were observed the most in SV samples. Moreover, the alterations recorded for SV specimens exhibited seasonal variations, and the differences between June and October 2014 seemed to be originated from extensive local use of pesticides in early summer. By reducing the need for pesticides and via the disintegration of the chemicals used before, a decrease was noted in October. When taken together, our findings are in a great agreement with the reports of Sisman *et al*. (2015, 2016a, b) and indicate that the amphibian species in Erzurum were strikingly affected by agricultural pollutants that are currently flowing into and polluting the Karasu River.

According to some authors, the presence of ENA can indicate genotoxic effects of polluted water (Marques *et al*., 2009). MN and ENA are used to evaluate the genotoxic effects in aquatic organisms (Strunjak-Perovic *et al*., 2010), especially amphibian species (Babini *et al*., 2015). Previous studies showed that ENA frequencies were higher than MN frequencies. MN and ENA have been interpreted as analogous nuclear lesions (Serrano-Garcia and Montero-Montoya, 2001; Guilherme *et al*., 2008). In the current study, MN and ENA assays showed the existence of a significantly higher value of nuclear abnormalities in erythrocytes of *P. ridibundus* from DB2 and SV sites compared to DB1. The increase of ENA indicates that some pollutants are inducing genotoxic effects in the sites.

Considering the various contents of fertilizers and pesticides, the effects of heavy metals on different amphibian species have been widely investigated using both in vivo and in vitro techniques, and many researches were reported. However, only a few investigations were reported about the effects of pesticides and pesticide ingredients on amphibian lungs. In Hoplobatrachus occipitalis exposed to sublethal concentrations of cadmium, sinusoidal dilatations, pulmonary hemorrhage and the disruption of the parenchyma of the lungs were revealed (Ikechukwu and Ajeh, 2011). Although capillary dilatation was noted, pulmonary hemorrhage and disrupted parenchyma were not observed in the lung tissues of DB2 and SV samples of the present study. In another investigation, a fungicide/bactericide, Champion 50WP, was applied intraperitoneally to *P. ridibundus*, at two different temperatures for 3 weeks, and histopathological alterations observed in lungs were noted as loss of elasticity in the lung walls, enlargement of the blood vessels, excessive accumulation of erythrocytes around the pneumocytes, increase in the number of goblet cells, hyperplasia and inflamed areas in the pseudo-multilamellar cylindrical epithelium (Paunescu *et al*., 2011). In a similar study, Roundup® herbicide caused hyperplasia and enlargements of the nuclei of the respiratory epithelium, increasing number of goblet cells, and increasing edema and amount of melanin (Paunescu *et al*., 2012a). In our study, thickness of alveolar septum observed in DB2 and SV samples were evaluated as an indication for loss of elasticity. Although hyperplasia of alveolar epithelia, capillary dilatation and congestion noted for both of DB2 and SV samples were essentially similar to the results of Paunescu *et al*. (2011, 2012a), edema was not observed.

The main effects of environmental pollution on the liver of different amphibians were also investigated. In the livers of adult *R. esculenta* specimens collected at two sample rice fields, one heavily polluted and one relatively unpolluted, the prominent changes were noted in Kupffer cells along with melanomacrophages (Fenoglio *et al*., 2005). Similarly, increases in melanomacrophage areas, mild karyomegaly and polyploidy, solitary and focal accumulation of infiltrated neutrophils and lymphocytes, and progressive fibrosis were noted for *R. ridibunda* collected from a river classified as moderately to heavily polluted by heavy metals (Loumbourdis, 2007). Extremely high quantities of melanomacrophage centers and loss of structural architecture were also identified in the liver of *R. perezi* living close to a uranium mine (Marques *et al*., 2009). Not only the effects of heavy metals, but also the effects of common pesticides on the liver of different amphibian species were studied, especially under controlled conditions, and similar to the researches performed in laboratory conditions, dose and time-dependent results were recorded. Paunescu *et al*. (2010a) intraperitoneally applied an insecticide, Reldan 40EC (chlorpyrifos-methyl) to *R. ridibunda*. After 3 weeks, they noted an increase in Kupffer cells, darkening in parenchyma, karyomegaly and polyploidy. In both sexes of *R. ridibunda* treated intraperitoneally by a fungicide, Champion 50 WP, at one sublethal dose, similar results of increase in Kupfer cells, expansion in blood vessels, fibrosis, leukocyte infiltration, hepatocyte vacuolization, and necrosis were revealed after 3 weeks (Paunescu *et al*., 2010b). It was also reported that the Talstar 10EC insecticide led to vacuolization, nuclear pyknosis, fibrosis, sinusoidal dilatation and infiltration in the liver of *P. ridibundus* (Paunescu *et al*., 2012b). In *Bufotes variabilis* exposed to an insecticide, Carbaryl, at different doses for 96 h, hepatocyte vacuolization, increase in melanomacrophages, sinusoidal dilatations, hemorrhage and congestion were also observed (Cakici, 2015). When the extensive use of Carbaryl is considered, the results of the last-mentioned study can bring an explanation to why the livers of SV samples of the present study are mostly affected.

As a common response to environmental chemicals, the loss of parenchymatic architecture by sinusoidal dilatations, expansion and congestion (Paunescu *et al*., 2010b; Cakici, 2015) were also noted in our study. Hepatocyte degenerations described with different terms have also been common in all reports. However, nuclear abnormalities such as karyomegaly, polyploidy (Paunescu *et al*., 2010a) and pyknosis (Paunescu *et al*., 2010b) were not evaluated, and hemorrhage (Cakici, 2015) was not observed in this study. All histopathological alterations noted for SV samples of *P. ridibundus* indicated that liver functions were disordered. The great differences of HAI values of DB1 (6.05±2.09) and SV (87.88±22.08) samples definitely confirm this conclusion.

The most prominent histological alteration, melanomacrophage aggregation observed in the liver of DB2 and SV specimens was also recognized as an effect of pollution. Melanomacrophagic aggregates are not only observed in liver, but also in spleen, lung and kidney (Loumbourdis and Vogiatzis, 2002), and may contain different types of pigments (melanin, lipofuscin, ceroid and hemosiderin/ferritin). Increased amounts of melanomacrophages may naturally occur due to aging, hibernating and infection; however, they may also appear in frogs living in polluted areas (Fenoglio *et al*., 2005). The main roles of the melanin content of the melanomacrophages are to protect the hepatocytes against reactive oxygen radicals and to reduce oxidative stress (Rozanowska *et al*., 1999). In addition, melanin plays some active roles, especially in the detoxification of metal ions and in many other biochemical reactions (McGraw, 2003). When taken together, increased and widened melanomacrophagic centers observed in DB2 and SV specimens may be accepted as a typical response to pollutants.

In parallel with the researches on the effects of chemicals on the livers of frogs, many investigations were performed on kidney histopathology and dysfunctions, which offer some great advantages for environmental monitoring. For example, several pathological and genotoxic damages such as enlargement of the renal tubules, tubular necrosis, and ENA were determined in the kidney of *R. perezi* living close to a uranium mine (Marques *et al*., 2009). To reveal the effects of heavy metals on frog kidney has been one of the main topics of several studies. Cadmium and zinc cause several kidney alterations (Förlin *et al*., 1986), and the kidney is assumed as one of the mostly damaged organs by the long-term effects of metals (Spry and Wiener, 1991). The nephrotoxic effects of lead nitrate on *R. ridibunda* were noted as vacuolization, infiltration, and damages to brush border and proximal tubule (Loumbourdis, 2003). In *R. ridibunda* treated with cadmium, apoptotic areas and hyaline spheres were observed in the kidneys after 30 days (Loumbourdis, 2005). Cadmium also caused lumen expansion and tubular necrosis in the kidney of *Rhinella arenarum* frogs after 15 days (Medina *et al*., (2016).

The effects of pesticides on frog kidney were also widely investigated. In *Euphlyctis heaxadactylus*, known as Indian green frog, exposed to Carbaryl, tubular dilatation, hemorrhage, increase in inflammatory cells, detachment of the tubular epithelium, glomerular atrophy and lymphocytic infiltration were identified (Renuka, 2007). Renal tissue abnormalities such as tubular cell degeneration, vacuolization, interstitial edema, karyomegaly, and glomerular degeneration were observed in *P. ridibundus* treated with Champion 50WP (Paunescu and Ponepal, 2011). Talstar 10EC insecticide also led to tubular necrosis expansion in Bowman's capsule, pyknotic nuclei in epithelial cells and edema in the kidneys of the same species (Paunescu *et al*., 2012b). Cakici (2015) reported renal tubular epithelial necrosis and karyolysis, glomerular shrinkage, hemorrhage and fibrosis in *B. variabilis* exposed to Carbaryl.

In the present study, the kidneys were slightly and moderately affected in DB2 and SV samples, respectively. In DB2 samples, congestion, expansion of Bowman's space, tubular necrosis and dilatation were noted as similar to the findings of Paunescu and Ponepal (2011), Paunescu *et al*. (2012b), Cakici (2015), and Medina *et al*. (2016). Eosinophilic appearance observed in some tubule lumens of DB2 samples may be strongly related to the hyaline spheres noted in *R. ridibunda* (Loumbourdis, 2005). Lymphocytes infiltration that was exhibited in SV samples were considered as a histological marker for inflammation. However, apoptotic areas (Loumbourdis, 2005) and the nuclear abnormalities recorded as karyomegaly (Paunescu and Ponepal, 2011), pyknosis (Paunescu *et al*., 2012b), and karyolyzis (Cakici, 2015) were detected neither DB2 nor SV frogs.

CONCLUSION

The lowest HAI was recorded in DB1, followed by the DB2 site with an intermediate rate and the SV site with the highest HAI. This highest HAI appeared probably because of the agricultural pollutants at the sites. Although other environmental factors that could be effective on health status were not studied, it seems that agricultural pollution is still one of the greatest threats for *P. ridibundus* living in the Erzurum Plain. Thus, more comprehensive studies are needed to protect amphibian species and populations, especially for Eastern Anatolia Wetlands close to farmlands. In order to protect human and environmental health, precautionary actions must be based on further investigations that will give more detailed and comparative results.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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