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Metal concentrations in common skittering frog (*Euphlyctis cyanophlyctis*) inhabiting Korang River, Islamabad, Pakistan

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The current study investigated metal concentrations (Zn, Cd, Pb, Mn and Fe) in the habitat and body tissues (blood, liver and kidney) of common skittering frog *Euphlyctis cyanophlyctis* along the Korang River, Islamabad, Pakistan. Samples were collected from three selected contaminated sites and a reference site at four different occasions from October 2010 to March 2012. All studied metals showed elevated levels in river water, as well as in blood, liver and kidneys of frogs from all contaminated sites. Specifically, Mn showed significantly higher levels in all analysed matrices from contaminated sites compared to the reference site. Histological sections of frog livers exposed to elevated metals showed abnormal hepatocytes, while their kidney sections showed discontinuous glomeruli with dead or ruptured cells exhibiting improper shapes with cytoplasmic depositions. This study indicates that frogs inhabiting Korang River are at potential risk of metal toxicity from the environment.

Key words: Dicroglossidae; histology; histopathology; metal bioaccumulation; pollution.

Concentraciones de metales en *Euphlyctis cyanophlyctis* del río Korang en Islamabad, Paquistán. Se investigaron concentraciones de varios metales (Zn, Cd, Pb, Mn and Fe) en el hábitat y en tejidos (sangre, hígado y riñones) de la rana *Euphlyctis cyanophlyctis* a lo largo del río Korang en Islamabad, Paquistán. Se tomaron muestras de tres sitios seleccionados como contaminados y de una localidad de referencia en cuatro ocasiones entre octubre de 2010 y marzo de 2012. Todos los metales analizados mostraron niveles elevados en el agua del río y en la sangre, hígado y riñones de las ranas procedentes de los sitios contaminados. Los niveles de Mn fueron significativamente mayores en lugares contaminados con relación a la localidad de referencia. Los cortes histológicos de hígados de ranas expuestas a metales mostraron hepatocitos anómalos, mientras que las secciones de los riñones presentaban glomérulos discontinuos y células muertas o rotas con formas anómalas y deposiciones citoplasmáticas. Este estudio sugiere que las ranas que habitan en el río Korang están en riesgo de sufrir intoxicaciones a causa de los metales presentes en el medio.

Key words: bioacumulación de metales; contaminación; Dicroglossidae; histología; histopatología.

Metal pollution has an important role in global biodiversity decline (Ficken & Byrne, 2013). In Pakistan, soils, air and water are heavily polluted with different

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metals including mercury (Hg), arsenic (As), lead (Pb), zinc (Zn), manganese (Mn), lithium (Li), cadmium (Cd) and chromium (Cr) (ABBAS *et al*., 2004). Several metals, including those that are essential for life, occur naturally in the water, but their levels must remain within acceptable ranges not to be toxic to aquatic organisms, as recommended by the environmental and health protection agencies (US EPA, 1993; WHO, 1998). Excess concentrations of several metals appear in *nullah* waters (i.e. water currents flowing at the bottom of steep valleys) from industrial effluents, posing serious threat to biodiversity dependent on such *nullah* waters. It is estimated that about 90 to 95% of all domestic sewage and 75% of all industrial effluents are discharged into surface waters without proper treatment (HINRICHSEN *et al.*, 1997; PANDEY, 2006) resulting in contamination of soil, water and air, and hence threatening human and biodiversity health (HAMIDULLAH *et al*., 1997; IQBAL *et al.*, 1998). Industrial effluents, organic wastes, refuse burnings, transport and solid sludge contain acids, alkalis, sodium chloride and metals (Cd and Cr), which are released in huge quantities into the *nullahs* and streams (ZAMAN & ARA, 2000; AGARWAL, 2002; TAHIR & NASEEM, 2007).

Documented effects of pollutants on amphibians range from lethal to sublethal effects including decreased growth and development, as well as increased frequency of developmental abnormalities, susceptibility to diseases and behavioural alterations (e.g. BRIDGES, 1999; ORTIZ *et al*., 2004; RELYEA, 2005; GRIFFIS-KYLE, 2007; KARRAKER *et al*., 2008; SHINN *et al*., 2008; SNODGRASS *et al*., 2008; RELYEA & JONES, 2009). Anuran amphibians are particularly susceptible to the uptake of metals because they have highly permeable skin, which allows the rapid absorption of metal ions. Additionally, due to the larval microphagous feeding habit of most species, tadpoles frequently ingest sediment in which metals can accumulate (HOPKINS & ROWE, 2010). Additionally, due to the larval microphagous feeding habit of most species, tadpoles frequently ingest sediment in which heavy metals can accumulate (HOPKINS & ROWE, 2010). Some previously published studies have reported that over one third of the world's amphibians are facing extinction, and that almost half of extant species are experiencing widespread population declines (STUART *et al*., 2004), and one of the major causes widely implicated in population declines of amphibians is the contamination of freshwater systems with metals derived from industrial and agricultural sources (GREIG *et al*., 2010; HOPKINS & ROWE, 2010). Different researchers have attempted to document impacts of metal accumulations under laboratory (HERKOVITS & HELGUERO, 1998; JAMES & LITTLE, 2003) and field conditions (DEMICHELIS *et al*., 2001; FLYAKS & BORKIN, 2004; FENOGLIO *et al*., 2006), while some other studies have reported deformities, delayed metamorphosis, decreased flight response and altered interactions with predators in amphibians after exposure to metals in the water (CHEN *et al*., 2009; JAMES & SEMLITSCH, 2011; ZOCCHE *et al*., 2013).

In Pakistan, concentrations of various contaminants including heavy metals in ground and surface water and soil (YOUSAFZAI & SHAKOORI, 2008; SHAH *et al*., 2009; GHUMMAN, 2011; GILANI *et al*., 2013),

and in bird feathers (MOVALLI, 2000; BONCOMPAGNI *et al*., 2003; BOSTAN *et al*., 2007) have been assessed, which indicates that metal pollution is an environmental problem in the country. However, in the case of amphibians, although a few studies (KHAN *et al*., 2003, 2007; KHAN, 2004) have reported decreases in enzymatic activities in frogs after exposure to pesticides, there is a dearth of research pertaining to effects of metals on these animals. The present paper reports a biomonitoring study designed to estimate the concentrations of various metals in habitats (water), and blood samples of common skittering frogs (*Euphlyctis cyanophlyctis*) inhabiting Korang River (Islamabad, Pakistan) and to investigate the related histological changes in frogs' body tissues (i.e. liver and kidney).

MATERIALS AND METHODS

Study area

The Korang River originates from Murree, upstream the Rawal Lake in Islamabad. The outflow from the lake runs approximately 20 km before discharging in the Sowan River in Rawalpindi (Fig. 1). This area belongs to the Potohar plateau and has an altitude range from 502 to 609 m. The river water receives sewage and other wastes from the adjoining human dwellings on either side. In addition, there are two small marble factories that also discharge their waste directly into the river water.

Three sampling sites were selected along the river (Fig. 1) for collection of frog specimens and water samples. The sampling site I (33º41′27′′N, 73º07′54′′E) was located near the spillways of Rawal Lake, with a relatively clean water. Sampling sites II and III were situated downstream, near Burma (33º38′31′′N, 73º07′94′′E) and Highway Bridges (33º35′17′′N, 73º08′20′′E), respectively, both sites showing visible signs of water pollution such as a dark water colour and a foul smell. The land distance between the selected sampling sites was approximately five kilometres.

The vegetation along the Korang River is of riparian type, consisting mainly of *Cynodon dactylon*, *Euphorbia helioscopia*, *Malvastrum coromandelianum*, *Stellaria media*, *Melilotus indica*, *Coronopus didymus* and *Cannabis sativa* (AHMAD *et al*., 2014).

Sample collection

Six adult frogs and six water samples from each sampling site were collected on four occasions in October 2010, February 2011, December 2011 and March 2012 (total N = 24) using pitfall traps as standard technique for frog capture (CAMPBELL & CHRISTMAN, 1982; CORN & BURY, 1990; HEYER *et al*., 1994) and also by hand picking and using dip nets in the water. For using as control samples in the current study, six samples of water and six frogs were collected from the campus of the PMAS Arid Agriculture University, Rawalpindi, where a couple of temporary, pollution-free water ponds are found, especially in the rainy season.

Frogs were taken to the laboratory, anaesthetized using chloroform and sacrificed to collect blood by cardiac puncture and body tissue (i.e. liver and kidney). All animal handling and sacrifice was carried out in accordance with the guidelines provided by the Ethics Committee of the University (PMAS-AAUR/2646) and were ap-

proved by such Committee. In addition, it is pertinent to mention that the species used in the present study has no associated conservation issues.

All solutions were prepared with analytical reagent grade chemicals and distilled water. All plastic and glassware were cleaned by soaking in 2 M HNO_3 for 48 h, and rinsed five times with distilled water, and then five times with deionized water prior to use.

Estimation of metal concentrations

We estimated concentrations of Zn, Cd, Pb, Mn and Fe in the water, blood and tissue samples using atomic absorption spectrophotometry (AASP, FRANSION, 1981). To digest the tissues for atomic absorption spectrophotometry, we placed 0.5 g of tissue in 5 ml nitric acid (69%) and 0.5 ml

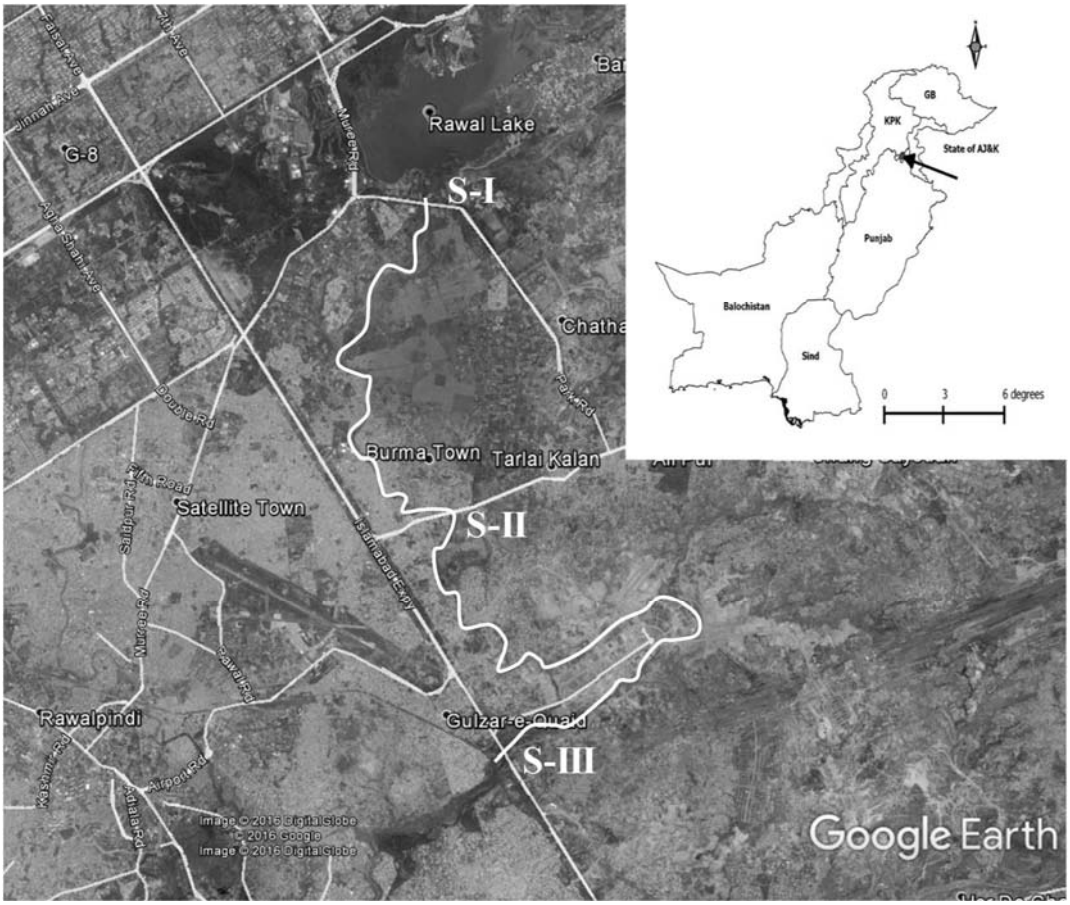

Figure 1: Location of the study area within Pakistan (black arrow in the right top map) and location of the three study sites (S-I, S-II, S-III) along the Korang River (white line) outflowing from Rawal Lake (image downloaded and modified from Google Earth, and published according to the guidelines established by the copyright owner; see Google Permissions at http://www.google.com/permissions/geoguidelines.html).

Table 1: Metal Concentrations (Mean ± SE) in experimental river water, and blood, liver and kidney samples of frogs (*Euphlyctis cyanophlyctis*) inhabiting Korang River, Islamabad, Pakistan. Asterisks identify groups that were significantly different from reference site according to the Least Significant Difference post-hoc tests ($P < 0.05$). LoD: limit of detection.

Sample	Metal	Reference site	Korang River sites		
			Site I	Site II	Site III
Water (μg/ml)	Zn	0.32 ± 0.03	0.15 ± 0.09	0.17 ± 0.09	0.11± 0.02*
	Cd	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.08± 0.02
	Pb	0.02 ± 0.01	0.97 ± 0.35*	1.44 ± 0.66*	1.41± 0.61*
	Mn	0.06 ± 0.10	0.04 ± 0.02	0.09 ± 0.04	0.19± 0.08*
	Fe	0.19 ± 0.05	0.13 ± 0.03	0.26 ± 0.11*	0.36± 0.14*
Blood (μg/ml)	Zn	2.40 ± 0.69	0.58 ± 0.18	0.92 ± 0.54	0.46 ± 0.16
	Cd	0.02 ± 0.00	0.02 ± 0.00	< LoD	0.12 ± 0.02*
	Pb	< LoD	< LoD	0.09 ± 0.02	0.003 ± 0.00
	Mn	0.06 ± 0.02	0.65 ± 0.17*	0.37 ± 0.10*	0.55 ± 0.10*
	Fe	1.90 ± 0.50	2.30 ± 0.98*	3.10 ± 1.04*	2.50 ± 1.71*
Liver (μg/g)	Zn	1.53 ± 0.75	2.69 ±1.02	2.14 ± 0.64	1.99 ± 0.83
	Cd	0.06 ± 0.01	0.12 ± 0.00	0.05 ± 0.00	0.16 ± 0.01*
	Pb	1.37 ± 0.41	1.26 ± 0.04	2.71 ± 0.16	1.58 ± 0.20
	Mn	0.19 ± 0.03	0.56 ± 0.07*	0.42 ± 0.13*	0.44 ± 0.12*
	Fe	2.80 ± 0.8	3.40 ± 0.14	3.80 ± 0.97	2.9 ± 0.84
Kidney (μg/g)	Zn	1.45 ± 0.70	2.46 ± 0.06*	1.54 ± 0.87	1.72 ± 0.08
	Cd	0.02 ± 0.00	0.15 ± 0.00	0.08 ± 0.00	0.09 ± 0.01
	Pb	0.60 ± 0.17	0.92 ± 0.10	0.88 ± 0.09	0.98 ± 0.14
	Mn	0.14 ± 0.02	0.59 ± 0.12*	0.46 ± 0.04*	0.50 ± 0.12*
	Fe	3.41± 0.59	4.53 ± 0.13	4.87 ± 0.62	3.99 ± 0.58

perchloric acid and left them overnight in the laboratory at room temperature for digestion. Next morning, these samples were heated for complete digestion by using hot plate with magnetic stirrer (HB 502 Bibby) for 20 minutes. Temperature range for heating was 30 ºC to 100ºC. The samples were then filtered and diluted up to 10 ml final volume. Determination of metal concentrations was conducted in an air-acetylene flame atomic absorption spectrophotometer GBC 932 Plus (GBC Scientific Equipment, Braeside, Victoria, Australia) (FRANSION, 1981). The water and blood samples were processed and prepared using the same procedure as described above for animal tissues for metal estimation through AASP. Standard dilutions of each metal (Zn, Cd, Pb, Mn and Fe) were used during analyses to quantify metal concentration in the samples, but no data on recovery were retrieved.

Histological analysis

The collected liver and kidney tissues of frogs were fixed in 10% formalin solu-

tion and processed for standard histological studies. Tissues were embedded in paraffin wax and then tissue sections were cut (5 to 7 μm thick) using a rotary Microtome Shandon® Finesse® 325 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The sections were stained with haematoxylin and eosin (BANCROFT & STEVENS, 1990; RIDDELL, 1996) and analysed directly under a light microscope Olympus BX50 (Olympus, Tokyo, Japan). Although the tissues were cut randomly, an effort was made to get sections from both the periphery and the central part of each organ. At least 20 slides from each organ were prepared for histological analysis.

Statistical procedures

Data were checked for their normal distribution, and then analyses of the variance (ANOVAs) were run to compare metal levels among study sites. Least significant difference post-hoc tests were conducted for significant ANOVAs to check which specific sites showed differences with regards to controls.

RESULTS

Metal concentrations in water and tissues

Among all studied metals, Pb, Mn and Fe were the ones showing elevated concentrations in water samples of the river as compared to the control site (Pb: $F_{3,36}$ = 2.909, P = 0.048; Mn: $F_{3,40}$ = 3.797, P = 0.017; Fe: $F_{3,40}$ = 3.794, P = 0.017). Among the river sites, Pb showed significantly high concentrations at the three sites, while levels of Mn were elevated at site III and levels of Fe at sites II and III (Table 1). On the other hand, Zn concentration in river waters was lower than that at the reference site ($F_{3,60}$ = 8.442, P < 0.001), with a significant difference observed between reference and site II levels of this element (Table 1).

Blood samples of *E. cyanophlyctis* from the Korang river showed higher concentrations of Cd, Mn and Fe than frogs from control site (Table 1). In the cases of Mn and Fe, elevated concentrations compared to control were detected at the three river sites, while increased blood levels of Cd were observed in frogs from river site II. In addition, although no comparative test was possible in the case of Pb because of the levels below detection limit at the reference site, detectable concentrations of this element were found at sites II and III. With regards to liver and kidney samples, metal concentrations followed a similar pattern than in blood, with the exception of kidney Zn concentrations, which were higher at river site I than at the reference site, contrarily to the trend observed for blood levels of this element (Table 1). In general, liver and kidney were less sensitive than blood as indicator of inter-site differences. Only Mn in both organs was systematically increased at all river sites compared to control, whereas liver Cd at site III was also higher than at reference site. On the contrary, the elevated Fe levels observed in blood samples from river frogs were not confirmed with the analyses of liver and kidneys (Table 1).

Histological observations

When observed under light microscopy, histological sections of livers of *E. cyanophlyctis* collected from the reference site showed normal continuous hepatocytes with centrally placed nuclei; these cells presented normal shapes with no cytoplas-

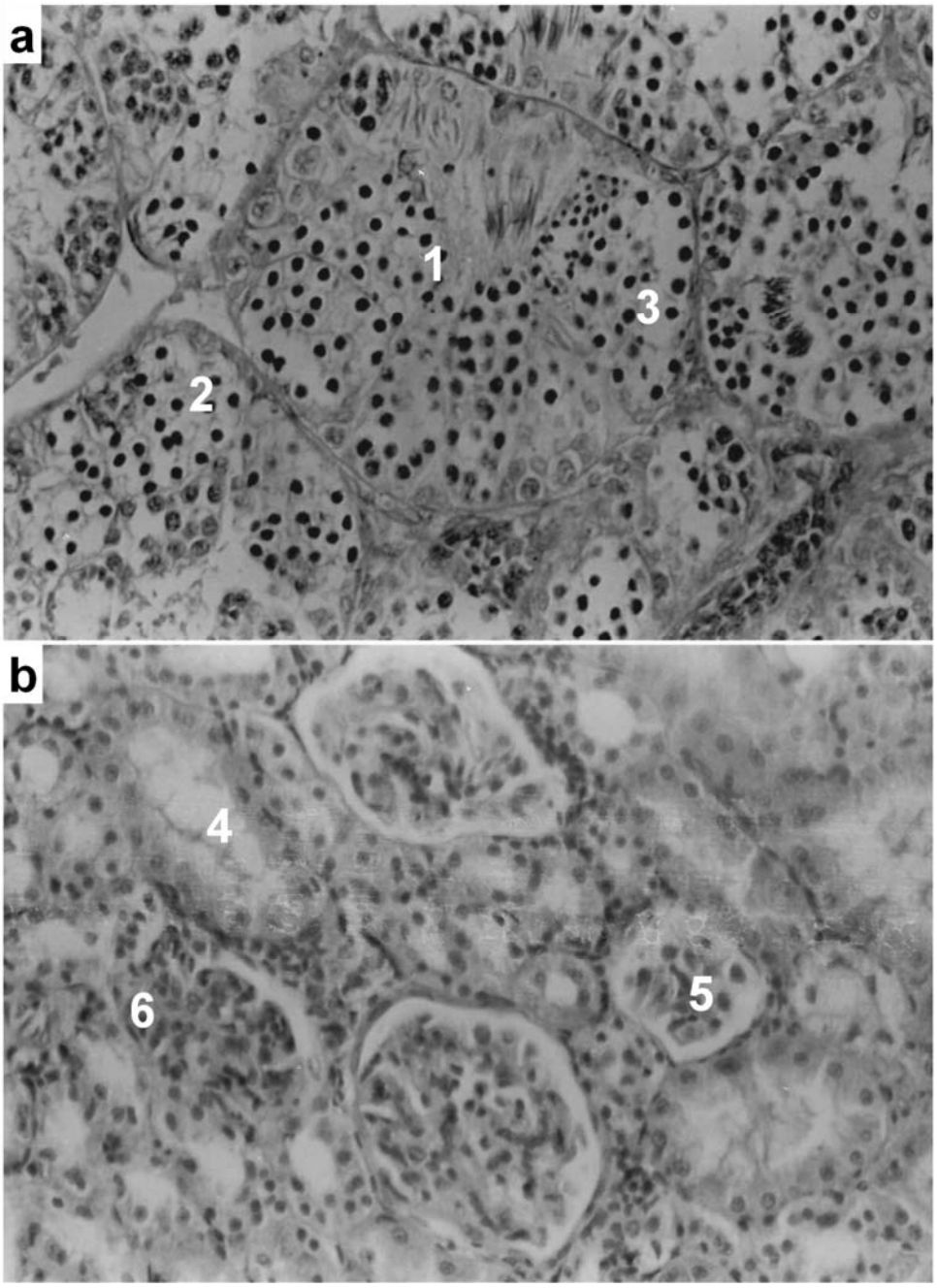

Figure 2: Sections of frog (*Euphlyctis cyanophlyctis*) livers. (a) Control liver (10X) showing continuous hepatocytes (1), centrally placed nuclei (2), and cells with proper shapes (3) with no cytoplasmic vacuolization. (b) Liver of a frog (10X) captured from river site II showing spacing among cells (4), irregularly placed nuclei (5) with cytoplasmic vacuolization, and cells having irregular shapes (6).

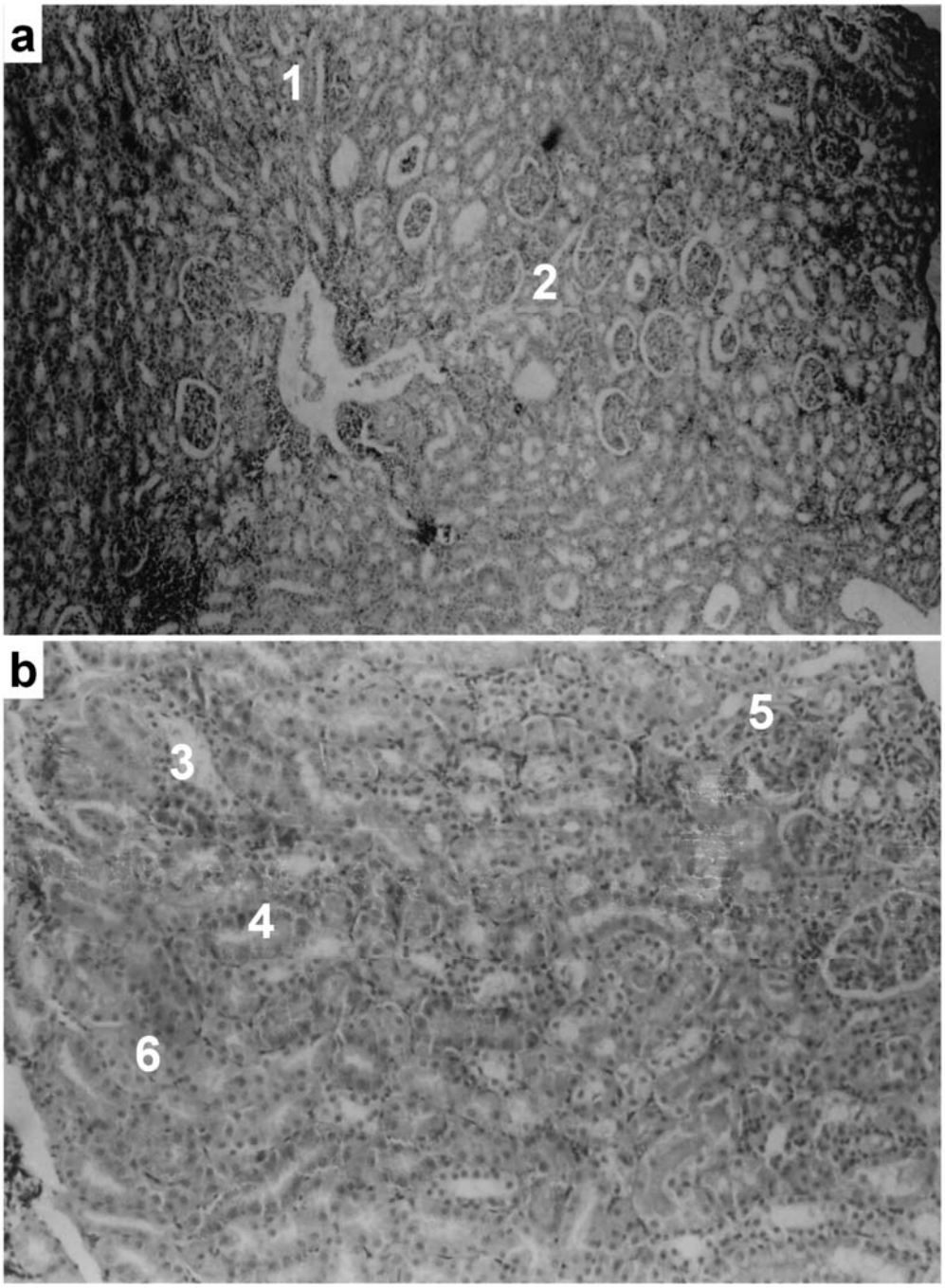

Figure 3: Sections of frog (*Euphlyctis cyanophlyctis*) kidneys. (a) Control kidney (5X) showing continuous glomeruli (1) and regularly shaped cells (2). (b) Kidney of a frog (5X) captured from site III showing non-continuous glomeruli (3), dead and ruptured cells (4), some depositions in the cells (5) and irregularly shaped cells (6).

mic vacuolization (Fig. 2a). On the contrary, liver sections of frogs collected from river site II showed irregularly shaped hepatocytes (Fig. 2b). Likewise, histological sections of kidney tissues of frogs collected from sampling site III showed how these kidneys presented discontinuous glomeruli with dead or ruptured cells; kidney cells exhibited improper shapes with cytoplasmic depositions (Fig. 3).

Discussion

Environmental pollution may result from human activities such as oil spilling or disposal of industrial wastes. It is one of the most important ecological problems affecting developing countries from southern Asia, such as Nepal, India, Bangladesh and Pakistan (Karn & Harada, 2001), where water pollution is especially severe and critical near urban stretches due to the huge amounts of pollutants discharged by urban activities. In these areas, about 90 to 95% of all domestic sewage and 75% of all industrial effluents are discharged into surface waters without any proper treatment (Hinrichsen *et al.*, 1997; Pandey, 2006), resulting in contamination of soil, water and air, and the consequent threat for human, animal and plant health (Hamidullah *et al.*, 1997; Iqbal *et al.*, 1998).

Human activities are also behind the increase of metal concentrations to toxic levels in natural aquatic environments; for instance, Owe *et al.* (1982) demonstrated that urban runoff contributes significantly to the pollution of rivers with Cr. Similarly, La Zerte (1984) showed that high levels of aluminium (Al) might be the result of acid precipitation, which enhances the mobilisation of Al from the soil. The main sources of environmental pollution in Pakistan include sewage water, city refuse, fertilisers, pesticides, automobiles, industrial effluents and leather tanning (Tahir *et al.*, 1998; Aftab *et al.*, 2000). Huge amounts of solid sludge and effluent water containing acids, alkalis, sodium chloride and heavy metals, mainly Cd and Cr, are released daily into the *nullahs* and streams. This wastes directly flow into rivers and eventually into the ocean (Zaman & Ara, 2000; Tahir & Naseem, 2007). Metals are released in the environment mostly through industrial effluents, organic wastes, refuse burnings, as well as transport the power generation activities.

Metal pollution in river waters may result in toxic effects on various tissues of animals inhabiting the polluted water site, affecting various functions like general metabolism, hormonal control, growth and reproduction. One of the most vulnerable groups of animals in this respect is amphibians, and especially frogs, since they totally depend upon water for their reproduction and have very permeable skins (Quaranta *et al.*, 2009). Previous studies have described the occurrence of various deformities following exposure of frogs and their larvae to metals (Nebeker *et al.*, 1994).

The results of the present study showed high concentrations of Pb, Mn and Fe in river water samples, which could be explained by the discharge of domestic sewage from human dwellings located on both sides of the river. Accordingly, elevated Mn levels were found in blood, liver and kidneys of frogs from river sites compared to control. This suggests that frogs did uptake these metals from their envi-

ronment (river water) and accumulate them body tissues. However, levels of Pb, although found to be elevated in tissues of animals from the contaminated sites in the river, did not show significant increases when compared to control animals. Fe, as well as Cd and Zn, were also found to be at higher concentrations in at least one of the analysed tissues (i.e. blood, liver and kidney) of frogs from the river as compared to animals from the reference site.

Metal accumulation by frogs has been previously reported in several studies. For example, livers of *Pelophylax ridibundus* contained high amounts of copper, cobalt, molybdenum, Cr and Cd while carcasses of these animals showed high levels of Al, Mn, nickel, strontium and barium (LOUMBOURDIS & WRAY, 1998). Similarly, BLAUSTEIN *et al.* (2003) showed that metals like Zn, Pb, Cd and Mn might adversely affect amphibian populations because of accumulation in different body tissues, which ultimately caused abnormal functioning of these tissue cells. Although in the current study we did not investigate the physiological functioning of the animals, the frequent increase of analysed metals in liver and kidneys of frogs from the Korang River, considering control animals as background reference, supports the necessity of conducting further studies to assess potential physiological malfunctions associated with metal accumulation by these frogs.

Some cellular abnormalities were noticeable in the histological sections of body tissues of frogs from the river sites, suggesting that metal uptake could interfere with cellular morphology and physiology. For example, stained sections of liver of *E. cyanophlyctis* from site II revealed irregularly shaped hepatocytes, and those of kidneys from sampling site III showed discontinuous glomeruli having dead or ruptured cells, as well as cells with abnormal shapes and cytoplasmic depositions, as compared to reference tissues. These findings would indicate that metals might alter the levels of ceratin biochemical parameters and cause tissue damage in frogs. Earlier studies conducted on these aspects reported that livers of frogs exposed to Cd had excessive bile secretion and dilatation of sinusoids (IKECHUKWU & AJEH, 2011). Similarly, individuals of *Euphlyctis hexadactylus* exposed to metals (Cd, Cu, Zn and Pb) exhibited marked changes in tissue development, severe bile secretion, haemorrhages and sinusoidal dilatations of liver, as well as distortion of alveolar sacs in the lungs and damaged Bowman's capsules in the kidney (JAYAWARDENA *et al.*, 2013). The potential of toxic effects of metal exposure in *E. cyanophlyctis* from the Korang River was also supported by the frequent observation of physical and behavioural abnormalities (e.g. asymmetry in limb length and skin coloration, or sluggish behaviour) displayed by frogs captured from this area.

Conclusion

From the results of the current study, we can conclude that Korang River waters exhibit levels of some metals higher than normal. Aquatic vertebrates, such as frogs, inhabiting this area can take up these metals from the environment, absorb them to the blood and accumulate them in liver and kidneys. The accumulation of these metals at high concentrations in the frog

body tissues proved toxic for various body functions, leading to histopathological responses in the affected organs. Thus, whereas the common skittering frog is potentially at high risk of metal toxicity at Korang River in the vicinity of Islamabad, further studies are suggested to assess and establish the toxic effects of metal accumulation in these frogs in order to elucidate whether frog survival can be threatened by metal pollution in the study area.

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