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ASSESSMENT OF ESSENTIAL HEAVY METAL ACCUMULATION IN THE SKIN OF *EUPHLYCTIS CYANOPHLYCTIS* FROM THATTA DISTRICT, SINDH, PAKISTAN

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Abstract

Investigations were carried out to determine the concentrations of two essential heavy metals i.e. Mn and Zn in the skin of Euphlyctis cyanophlyctis from the urban area of Makli and the agricultural area of Babra village, Thatta District, Sindh along with the water samples. The study was conducted from December 2020 to November 2021. Higher concentrations of Mn and Zn were observed in water and skin samples of E. cyanophlyctis from agricultural area. Mn showed significant relationships between water and skin samples from urban area and agricultural area. This study assists in monitoring heavy metal concentration in the skin of Euphlyctis cyanophlyctis which can help in creating countermeasures to conserve local biodiversity.

Keywords: Skin, essential heavy metals, Manganese, Zinc, Common skittering frog, biomarker, Thatta, Sindh, Dicroglossidae, *Euphlyctis cyanophlyctis*, Pakistan.

Introduction

Amphibians serve as environmental bio-indicators as their skin is permeable and sensitive, making them an excellent bio-indicator of an ecosystem, but leaving them defenceless in the extreme environments. An ecosystem's health can be arbitrated through the amphibian population (Saber et al. 2017). Globally, the disappearance of amphibian variety might be regarded as an emergency. Climate change, habitat loss, widespread and excessive use of pesticides and fertilizers, which also include inorganic pollutants (Relyea et al. 2005; Brühl et al. 2011; Khattab et al. 2021), are only a few of the causes. The main sources of heavy metals in the environment are irregular and, sometimes, excessive use of pesticides and fertilizers, drainage of anti-knocking agents (used in automobiles, containing Mn) in the water bodies, improper disposal of wastes and urban runoff. According to Iqbal et al. (1998), Zaman and Ara (2000), and Pandey (2006), fertilizers, pesticides, hazardous emissions from cars, and industrial wastes are the main drivers of environmental pollution. (Iqbal, *et al*., 1998; Zaman and Ara, 2000; Pandey, 2006).

Euphlyctis cyanophlyctis is known as common skittering frog which belongs to family Dicroglossidae. It is one of the most abundant species of amphibians as distributed throughout the Thatta District, Sindh (Khan et al. 2010), however, their populations are declining due to anthropogenic activities. Heavy metal pollution is becoming a serious threat to the health of amphibian population when left unsupervised.

To run the body functions smoothly, essential heavy metals are necessary. Mn and Zn are essential heavy metals but their excess amount can harm the body of amphibians. In this study, the concentrations of two essential heavy metals; Manganese (Mn) and Zinc (Zn) were determined by using atomic absorption spectrometer in the skin of *E. cyanophlyctis*, along with the water samples from the study areas.

Various studies have been carried out to estimate the risk of heavy metal concentrations fresh water, fishes, reptiles, birds and mammals but only few studies exist in the case of anurans inhibiting a contaminated environment (Mahmood, et al. 2016; Qureshi, et al. 2015). As widely distributed species in the Thatta District, and since it is capable of accumulating heavy metals, *E. cyanophlyctis* was able to present the differences between the heavy metal concentration and the areas which were analysed, however, no work has been reported on the skin of *Euphlyctis cyanophlyctis*. This study provides the basis for the determination of heavy metals to monitor environmental pollution. Since the species are abundant in the region, it is recommended to study them thoroughly to ensure the residing biodiversity.

Materials and methods

Sampling area

Two habitats of *Euphlyctis cyanophlyctis* were selected from Thatta District. Study area 1 was chosen as urban area; Makli from TalukaThatta (24°44'52.0"N 67°54'25.4"E) while for Study area 2 Babra village was selected as agricultural area from TalukaMirpursakro (24°37'04.1"N 67°44'12.2"E) (Figure 1). Study areas were surveyed during the day time. The duration of this study was from December 2020 to November 2021 i.e., Northeast monsoon (December 2020 to February 2021), Pre monsoon (March 2021 to May 2021), Southwest monsoon (June 2020 to August 2021) and Post monsoon (September 2021 to November 2021).

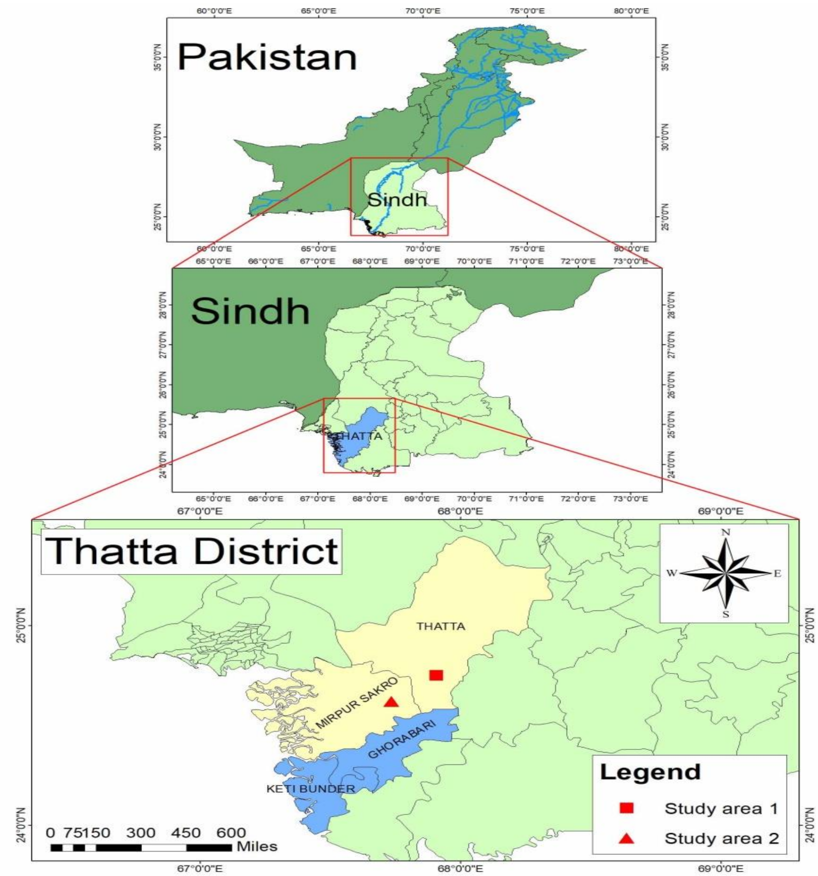

Figure 1: Map of Islamic Republic of Pakistan showing the study areas from Thatta District, Sindh.

Field sampling

Water samples and frogs, *E. cyanophlyctis* (Figure 2), were collected quarterly from two study areas (Makli and Babra village) of Thatta District. Frogs were caught with the help of net as a standard technique (Campbell and Christman 1982; Corn and Bury 1990; Heyer*et al*. 1994). Some were easily captured by hand picking method while they were basking on rocks. Two specimens were collected from each study area. Water samples were collected twice in a 100 ml beaker from either side of the habitat. The samples were stored in brown glass bottles for further analysis.

Figure 2: Adult *Euphlyctis cyanophlyctis* captured from agricultural study area of Thatta District, Sindh.

Sample digestion for metal determination

The collected water sample was initially filtered for the removal of soil particles and debris and for the detection of heavy metals in water, 100 ml of well filtered water sample were transferred to a beaker. 2 ml of concentrated HNO_3 and 5 ml of concentrated HCl were also added in the beaker. The beaker was then covered with watch glass and placed on hotplate (LabTechDaihanLabtech Co. Ltd.) for heating at 90 to 95 ^oC. The samples were heated until the total volume was reduced to 15 to 20 ml. The beaker was then removed from hotplate and was set aside to cool. After cooling, the walls of the beaker were washed by deionized water. The solution was then transferred to a 100 ml volumetric flask and final volume was made up with distilled water. The sample was also filtered to remove silicate and insoluble materials which could clog the atomizer (Fransion, 1981).

For the digestion of tissue samples, wet ashing was performed. 0.5 g of skin was placed in a 125 ml Erlenmeyer flask with 5 ml HNO_3 and 0.5 ml perchloric acid. It was covered with watch glass and was left in the laboratory at room temperature to digest for 24 hours. Then the samples were heated up to 100^{o}C on hotplate with a magnetic stirrer (LabTechDaihanLabtech Co. Ltd.) until all the tissue particles were completely dissolved and a clear solution was obtained. The beaker was then removed and left to cool down in a fuming hood. The solution was filtered and the final volume was made up to 100 ml by using deionized water (Williams, 1972).

Elemental detection and Data analysis

Atomic Absorption Spectrometer (Perkin Elmer A Analyst 700) (USA) with hollow cathode lamps at air acetylene flame was used to analyze the concentration of Mn and Zn in the samples.

The different concentrations of heavy metals in water samples and selected tissues of *Euphlyctis cyanophlyctis* were analyzed on a software IBM Statistical package for social sciences (SPSS Inc., version 25, USA) by using one-way ANOVA followed by post-hoc Tukey's test. Minitab 19 was also used for Linear Regression Analysis to determine the dependency and interdependency of heavy metals. Pearson's correlation was also calculated. The significance (P-value) was also calculated at significance level of 0.05 (5%) ($P > 0.05$ = non-significant and $P \leq 0.05$ = significant). Principal component analysis (PCA) was used to extract significant (eigenvalues >1.0) factors of variability in the obtained data.

Results and discussion

In the present study, the two essential metals; Mn and Zn were observed in skin of *E. cyanophlyctis* and its habitat. The concentration of both essential heavy metals were expressed in parts per million (ppm). Seasonal variations were evident in the concentration of both elements in water and skin of *E. cyanophlyctis* from urban and agricultural areas of Thatta. The occurrence of both metals were found to be highly significant with the exception of Zn in skin of *E. cyanophlyctis* which was non-significant from both study areas (Table 1).

Table 1: Seasonal variations in water of habitat and skin of *E. cyanophlyctis* from both study areas; Urban and Agricultural, Thatta District.

	Heavy metals	Study Areas	P- value
Water	Zn	Urban area	0.00
		Agricultural area	0.00
	Mn	Urban area	0.00
		Agricultural area	0.00
Skin	Zn	Urban area	0.47
		Agricultural area	0.15
	Mn	Urban area	0.00
		Agricultural area	0.00

Keejhar Lake supplies water to cities of Sindh for domestic and agricultural use through interconnected canal system (Abbasi, 2021; Mehmood et al., 2020; Nizamani, 2020). Lashari et al. (2012) reported the concentration of Mn and Zn in water of Keenjhar Lake which ranged from 0.0002 to 0.0067 mg/l and 0.0014 to 0.1043 mg/l, respectively. In the present findings, it was observed that the concentration of Mn and Zn exceeded the defined range of the actual source of the water. Results ANOVA, the distribution of the concentration of both elements in water: Zn>Mn for urban area and agricultural area (Table 2). To observe the correlation between the heavy metals, linear regression was applied. Results showed high positively significant relation between Mn and Zn in water of both study areas (Table 3). This is due to the use of fertilizers and pesticides in the agriculture fields and near the canals which supply water to urban areas (Shah, 2016). Moreover, the high levels of Mn and Zn in water were to be associated with the presence of heavy metals in animal feed. Another reason is due to repairing of cars and batteries near water bodies and disposal of unwanted chemicals from cars (for instance, antiknocking agent which contain Mn) which is a common practice in Thatta District (Khan et al., 2015; Joy et al., 2017; Ghanghro, 2020).

The ANOVA reveals the concentration of both elements, distributed in the skin was in the following order: Mn>Zn for urban area and agricultural area (Table 2). Mn is an essential heavy metal required in the body as cofactors for enzymes and in various metabolic processes (Chang and Cockerham, 1994). Correia et al. (2014) determined the concentration of heavy metals in the skin of *Leptodactylusocellatus*. The concentration of Mn was found in very low concentration in skin as compared to the sediments. In the present findings, the concentration of Mn was also low in skin of *E. cyanophlyctis* than the water samples from agricultural area. Whereas, the concentration of Mn was high in skin of *E. cyanophlyctis* than the water samples from urban area. This could be due to the drainage and improper disposal of acid in the aquatic environment at urban areas (Winger et al., 1990). Zn is another nutritionally important essential heavy metal, required by body to run metabolic processes (Abdulla and Chmielnicka, 1989). Correia et al. (2014) determined the concentration of heavy metals in the skin of *Leptodactylusocellatus*. The concentration of Zn was found relatively high in skin. In the present findings, higher concentrations of Zn were detected in water samples from the agricultural area than the urban area but even lower concentrations were observed in the skin of *E. cyanophlyctis*. No traces of Zn were observed in the skin of *E. cyanophlyctis* from urban area in Southwest monsoon.

Table 2: Results of ANOVA for essential heavy metals; Mn and Zn in water and skin of *E. cyanophlyctis* obtained from the two study areas of Thatta.

		Study Areas	N	Mean (ppm)	S. D. (±)	P-value	F-value	R-sq	Comparison
Mn	Skin	Urban area	8	0.0578	0.0338	0.561	0.35	2.47%	S2 > S1
		Agricultural area	8	0.0743	0.0709				
	Water	Urban area	8	0.0378	0.0349	0.105	3.01	17.69%	S2 > S1
		Agricultural area	8	0.0721	0.0437				
Zn	Skin	Urban area	8	0.0218	0.0322	0.198	1.83	11.53%	S2 > S1
		Agricultural area	8	0.0554	0.0624				
	Water	Urban area	8	0.1362	0.0388	0.002	13.82	49.68%	S2> S1
		Agricultural area	8	0.532	0.299				

S1 = Urban area; S2 = Agricultural area

Noble (1925) explained the semi-permeability of skin which is used for cutaneous respiration by anurans. This makes them vulnerable, being exposed to the pollutants in aquatic environment (Quaranta et al., 2009). Correia et al. (2014) observed the not significant correlation between the heavy metals in sediments and in the skin of *Leptodactylusocellatus*. To observe the correlation between the heavy metals, linear regression was applied. Results showed high negatively non-significant relation between Mn and Zn in skin of *E. cyanophlyctis* from both study areas. This indicated that the concentration of essential heavy metals, present in habitat, varied inversely with the skin of *E. cyanophlyctis* (Table 3).

Table 3: Linear regression analysis between Mn and Zn in water and skin of *E. cyanophlyctis* from Urban and Agricultural area during the year (2020-2021)

		Study Areas	P-value	F-value	R-sq	regression equation
Zn vsMn	Water	Urban area	0.00	2187.23	99.73%	Zn = 0.04037 + 6.823 Mn
		Agricultural area	0.002	27.7	82.20%	Zn = 0.09820 + 1.007 Mn
	Skin	Urban area	0.103	3.70	38.16%	Zn = 0.09584 - 0.5443 Mn
		Agricultural area	0.627	0.26	4.19%	Zn = 0.03310 - 0.1953 Mn

On the basis of statistical analysis, the average concentration of Mn and Zn from urban and agricultural area showed higher concentration in the ambient environment than skin. In both study areas, for the concentration of Mn, significantly positive linear regression was observed between water and skin which indicated that the concentration of Mn increases in skin with an increase in the water of the habitat; urban area (P=0.001) and agricultural area (P=0.008). Hence, the skin of *E. cyanophlyctis* can be considered as a good pollution biomarker for Mn pollution. However, the concentration of Zn was found to be non-significant with negative linear regression for both study areas which indicated that the concentration of Zn increases in skin with a decrease in water from both study areas; urban area (P=0.352) and agricultural area (P=0.456). Hence, the skin of *E. cyanophlyctis* cannot be considered as a good pollution biomarker for Zn pollution (Table 4).

Table 4: Linear regression analysis of Mn and Zn between water and skin of *E. cyanophlyctis* from Urban and Agricultural area of Thatta District.

	Heavy metal	P-value	F-value	R-sq	Regression equation
Urban Area	Mn	0.001	36.48	85.88%	skin = 0.006160 + 0.7163 water
	Zn	0.352	1.02	14.52%	skin = 0.04370 - 0.04111 water
Agricultural Area	Mn	0.008	15.27	71.80%	skin = 0.00940 + 1.719 water
	Zn	0.456	0.64	9.58%	skin = 0.1233 - 0.4983 water

Principal component analysis (PCA) was performed to evaluate the relationship of environment and heavy metal concentrations in water and skin of *E. cyanophlyctis*. On the basis of eigenvalue >1.0, the result visually shows that Mn in water and skin have large loadings on first component as PC1 was observed to be positively correlated for Mn in water and skin for both study areas, however, in PC1, Zn was found to be negatively correlated in the skin of *E. cyanophlyctis* from both study areas. PC2 indicated negative correlation for the concentration of Mn and Zn in water and skin from urban area, however, negative correlation was observed only in the case of Mn in water and skin from agricultural area (Table 5 and Figure 3).

Table 5: PCA of Mn and Zn in skin *E. cyanophlyctis* and water samples from Urban and Agricultural area of Thatta District.

Study Area	Variable	Heavy metal	PC1	PC2	PC3	PC4	PC5
Urban Area	Skin	Mn	0.334	-0.437	0.063	0.391	0.402
	Water	Mn	0.405	-0.239	-0.008	-0.050	0.097
	Skin	Zn	-0.263	-0.369	-0.889	0.033	-0.043
	Water	Zn	0.398	-0.273	-0.006	-0.066	0.134
Agricultural Area	Skin	Mn	0.383	0.256	-0.170	0.476	-0.665
	Water	Mn	0.386	0.188	-0.227	-0.740	-0.028
	Skin	Zn	-0.135	-0.664	0.332	-0.256	-0.592
	Water	Zn	0.427	-0.015	-0.121	-0.014	-0.124

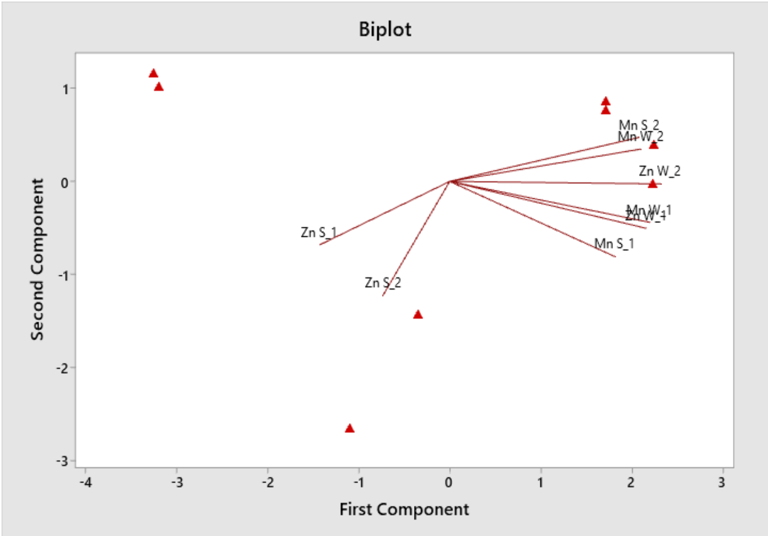

Figure 3: Multivariate analysis of data in the form of biplot, obtained from metal determination of Mn and Zn in skin and water of *Euphlyctis cyanophlyctis* collected from urban and agricultural areas of Thatta District.

Conclusion

Current study provided to monitor heavy metal contamination by using selected tissues of *E. cyanophlyctis* which can help in creating conservative countermeasures for local biodiversity for anuran species and other aquatic fauna which are susceptible to environmental pollution. The obtained results concluded that the skin of *E. cyanophlyctis* acts as a semi-permeable membrane for underwater cutaneous respiration, thus *E. cyanophlyctis* can be used as a good bio-indicator for our environment. In addition the information of elements obtained in the skin of *E. cyanophlyctis* is also important for considering it as a bio-monitoring agent for regulating the quality of the natural ecosystem.

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