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Dietary proxies ($\delta 15$N, $\delta 13$C) as signature of metals and arsenic exposure in birds from aquatic and terrestrial food chains

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ABSTRACT

In this study, exposure to arsenic (As), lead (Pb), cadmium (Cd), copper (Cu) and zinc (Zn) was investigated in the blood, pectoral muscles and tail feathers of two terrestrial (spotted owlet; *Athena brama* and bank myna; *Acridotheres ginginianus*) and two aquatic (cattle egret; *Bubulcus ibis* and pond heron; *Ardeola grayii*) bird species inhabiting Pakistan. Food chain specimens, as well as the dietary proxies δ^{15}N and δ^{13}C, were also analyzed to validate potential trophic and dietary transfers of metals and As in birds. Zn was found to be the most prevalent metal in the tissues of birds followed by Pb, As, Cu, and Cd. The bioaccumulation of metals and As was higher in tail feathers reflecting the combined effect of both endogenous and exogenous contamination. Pectoral muscle and blood harbored lower levels of As and metals, indicating less recent exposure through diet. Aquatic birds feeding at higher trophic levels accumulated significantly higher concentrations of metals and As in their tissues ($P < 0.05$) and, therefore, may be at a greater risk of metal and As toxicity than terrestrial birds. Linear regression model depicts δ^{15}N as a strong predictor of metals and As levels in the tissues of both aquatic and terrestrial birds, followed by the δ^{13}C dietary proxy. All metals in aquatic species, except for Cd, as well as terrestrial species, except for Cu, exhibit bioaccumulative potential through the food chain (Trophic transfer factor: TTFs > 1) indicating potential harmful consequences for birds. Elevated concentrations of metals and As in tissues may cause harmful effects in birds potentially leading to declines in their populations.

1. Introduction

In response to growing human populations, recent developments in industry and technology has led to the deterioration of the natural environment which poses a serious threat to living organisms (Deng et al., 2007). Various natural processes and anthropogenic activities, such as mining, electroplating, drugs, pigments, dyes, plastics, batteries and agrochemical manufacturing, urban runoff, agricultural activities and dumping of industrial untreated effluents, increase organic and inorganic pollutants in the environment (Waseem et al., 2014; Farooqi et al., 2007). Major inland aquatic systems, such as lakes and rivers, along with their terrestrial adjacent areas are often contaminated with metals and metalloids (Fu et al., 2014). Metalloids and non-essential metals are inorganic contaminants which are notorious for their hazardous impacts on both quality and sustainability of ecosystems (Ackerman et al., 2016). These elements are persistent, ubiquitous and non-biodegradable with long half-lives (Burger et al., 2007). In recent years, toxic metals and metalloids have gained attention because of their toxicity in living tissues (Abbasi et al., 2015a, 2015b). Once these contaminants enter aquatic and terrestrial ecosystems, as a result of agricultural, industrial, urban and/or domestic sources, toxic metals and metalloids tend to bioaccumulate in living organisms with increasing concentrations at higher trophic levels (Burger et al., 2007). Birds are particularly prone to higher levels of toxic metal and metalloid contamination because, not only are they often positioned high on the food chain (Burger and Gochfeld, 2005), but they also cover large distances for food and reproduction and hence exhibit higher exposure (Shahbaz et al., 2013).

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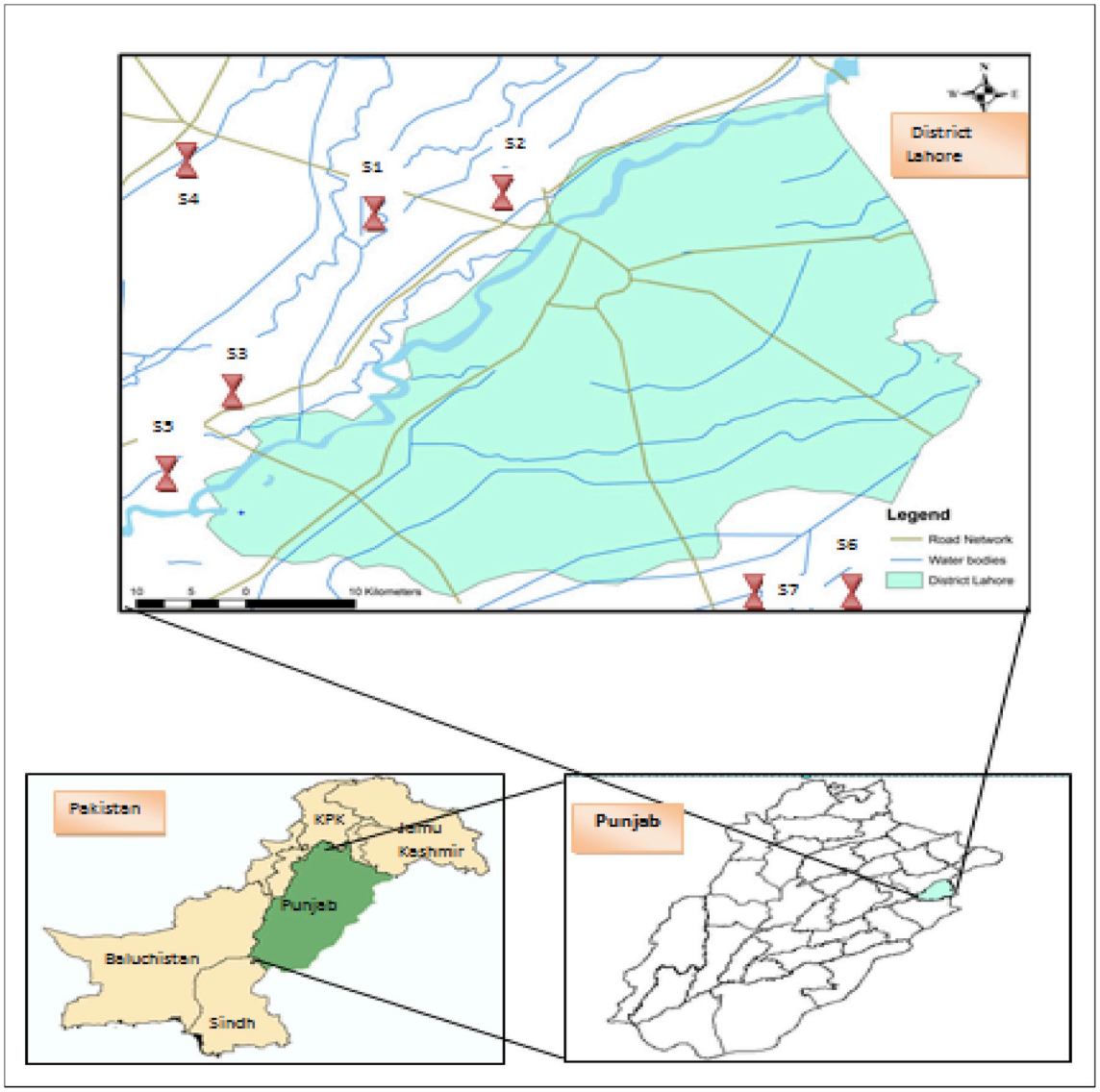

Fig. 1. Sampling sites showing sampling points from the outskirts of Lahore, Pakistan.

Several studies report a wide range of toxicological impacts of metals and metalloids in birds such as decreased reproductive success, genotoxicity, nervous and endocrine disorders, immunity, behavioral and physiological abnormalities (Bauerova et al., 2017; Abbasi et al., 2015a; Eeva and Lehikoinen, 2015; Geens et al., 2010). It has also been established that birds are among the best bioindicators to predict environmental health in both aquatic and terrestrial habitats (Abbasi et al., 2015a,b; Dauwe et al., 2004). The trophic level and dietary sources of birds are usually traced through stable isotopic (SIs) ratios of nitrogen and carbon respectively. The ratio of heavier ^{15}N to lighter ^{14}N ($\delta^{15}N$) illustrate the trophic level of an individual because it is enriched with every succeeding trophic level (Huang, 2016). The ratio of carbon SIs ($\delta^{13}C$: $^{13}C/^{12}C$) reflects the dietary origins of the carbon because of the degree of depletion of ^{13}C stable isotopes, which differ in primary producers from varying habitats (Boecklen et al., 2011). The monitoring of aquatic and terrestrial birds represents a powerful tool for the assessment of the levels and effects of arsenic and toxic metal contamination in aquatic and terrestrial environments (Pablo Seco Pon et al., 2011). Feathers represent a potentially valuable indicator of exposure as they accumulate toxic metals in proportion to their levels in the blood during the period of feather growth (Sadeghi et al., 2019; Einoder et al., 2018). Past literature suggests that toxic metals are also deposited externally on the feathers and cannot be removed completely through the washing process (Jaspers et al., 2019; Borghesi et al., 2016). As a result, this can inflate the amount of these contaminants detected in feathers (Jaspers et al., 2019; Borghesi et al., 2016). Hence concentrations of metals in feathers are considered to originate both from endogenous and exogenous sources. Blood, on the other hand, reflects the recent dietary exposure of contaminants in birds (Burger et al., 2007). Pectoral muscles could also harbor toxic metals and As signatures and may have a stronger correlation with feather concentrations than other internal organs.

Among metalloids, Arsenic (As) is recognized as a major threat to the environment in many countries including Pakistan. Various reported studies document exceeded levels of As in many areas of Pakistan mainly in Punjab (Waseem et al., 2014; Sánchez-Virosta et al., 2015). Higher levels of As in birds can lead to an inability to coordinate muscle movements as well as weakness in the wings and legs. This leads to difficulty standing, walking and flying, as well as adverse effects on the nervous and immune system that can cause an impairment in reproduction (Misztal-Szkudlińska et al., 2011; Sánchez-Virosta et al., 2015). Pb and Cd are of great concern in Pakistan and are found above the safe limits in surface waters and other environmental compartments (Azizullah et al., 2011). Birds exhibit reduced reproduction, decreased

bodyweight and coordination, paralysis of the wings and legs and blindness when exposed. In addition, mortality due to Pb and Cd poisoning has caused negative effects on reproduction, increased bone damage and serious health risks to wild birds (Sadeghi et al., 2019; Kim and Oh, 2013). In many studies metal toxicity in living organisms has been found to be associated with the higher concentrations of Cu and Zn (Bauerova et al., 2017; Abbasi et al., 2015a; Geens et al., 2010). Exposure of birds to As and toxic metals depends on their chemical forms, which determines their bioavailability along food chains (Dauwe et al., 2004; Beyer et al., 2004). So far, very few studies have reported the trophic transfer of metals and metalloids in birds. In addition, a comparison between aquatic and terrestrial food chains of birds has rarely been shown. Therefore, we have designed this study to investigate the levels of As, Pb, Cd, Cu and Zn in the tail feathers, pectoral muscles and blood of four bird species from a terrestrial and an aquatic habitat. Comparisons among tissues of birds and habitats were also been drawn. Food chain samples were analyzed to correlate the concentration in tissues of birds with their diet and dietary proxies of $\delta^{15}N$ and $\delta^{13}C$ were analyzed to validate the potential trophic and dietary transfer of metals and As in birds.

2. Materials and methods

2.1. Study area

Sampling was carried out from February to March 2017 from the outskirts (Manga mandi and Muridke) of a metropolitan city Lahore, Pakistan (Fig. 1). A total of 42 birds from four species were captured using mist nets. Two aquatic species i.e. Cattle egret; *Bubulcus ibis* (n = 10) and pond heron; *Ardeola grayii* (n = 10) were collected from Muridke (S1–S3). Two terrestrial species i.e. Spotted owlet; *Athena brama* (n = 6) and Bank myna; *Acridotheres ginginianus* (n = 16) were obtained from Manga mandi (S4–S7). Approximately 2–3 ml of blood was sampled from each bird and taken into a micro hematocrit tube after puncturing the brachial veins using 26G needles fitted with 5 ml syringe. Tail feathers and approximately 5 g of pectoral muscle was sampled from each bird after dissection. The birds used in this study were collected by WWF Pakistan in collaboration with multiple research projects, including this project. Food chain samples of each species were taken in triplicates to minimize any statistical bias. Fish (*C. puntata*), frog (*H. tigerinus*), aquatic insects (*L. americanus*) and plant leaves (*P. hysterophorus*) were also collected as prey items for the cattle egret and pond heron. Lizard (*H. flaviviridis*). Moths (*S. luridata*) were collected as prey items for the spotted owlet and white mulberry (*M. alba*) was collected as a common fruit consumed by the bank myna. All the samples were stored in zipped-locked bags and blood was stored in vials before they were transported to Environmental biology and Ecotoxicology laboratory, Quaid-i-Azam University, Islamabad for further analysis.

2.2. Analysis of metals (EPA method 3050B)

Metals such as Pb, Cd, Cu, and Zn were analyzed using the polarography voltammeter technique (Computrace 797, Swiss-made). This method, which is described by Dolan et al. (2017), was followed with modifications. To remove any exterior material, tail feathers were first washed with deionized water before they were dried and rinsed with acetone alternated with deionized water. For digestion, EPA method 3050B was followed as a standard protocol. Feather samples were first dried in an oven for 1 h at 80 °C. To avoid metal contamination, tail feathers were then cut into pieces using plastic scissor before they were ground into a powder and weighed. Approximately 2.5–3 g were taken for digestion. The grounded feathers were first treated with 4 ml HNO3 (70% Merk) and heated to 120 °C for 24 h. This was followed by the addition of 2 ml of hydrogen peroxide (H_2O_2) before the solution was heated to 110 °C for 24 h. This produced white fumes and the solution became light in color. After digestion, samples were filtered using Whatman No. 42 filter paper, before the volume was raised to 25 ml with distilled water. It was then poured into an amber glass bottle for quantification of metals and As. Blanks were prepared after every 10th sample to measure the precision of the instrument. The certified reference material DOLT-3 (dogfish liver, National Research Council of Canada, 11 Ottawa, CA) was used to validate the method. The standard recoveries ranged between 83% and 97% which is an acceptable limit. Detailed analytical procedures are mentioned in the Supporting Information (S.I 1.1). All other samples, except for blood, water, and soil, were digested using the same method as described for the feathers. Weighed blood samples (1 ml) were digested on a hotplate with 2.5 ml mixture of sulfuric acid (95% H_2SO_4), perchloric acid (70% $HClO_4$), and nitric acid (70% HNO_3) in a ratio of 1:8:8 at 160 °C for 15–20 min. Soil samples were weighed to 0.5 g before 3 ml of concentrated HNO_3 was added. The samples were then slowly heated on the hotplate. Approximately 4 ml of $HClO_4$ was added and the mixtures were heated until white fumes were produced. Water samples were weighed to approximately 0.5 ml before 3 ml HNO_3 was added to acidify the water samples for metal analysis. Water samples were then filtered and their volumes was raised to 25 ml with distilled water. For each element analysis, the limit of detection (LOD) was calculated by conducting three repeated blanks analysis. The standard deviation of the blanks was then multiplied by three to generate the LODs. The LOD was 0.007μg/L for As, 0.021μg/L for Pb, 0.0009μg/L for Cd, 0.09μg/L for Cu and 0.3 μg/L for Zn respectively. All the concentrations were blank corrected and expressed in μg/g dw for tail feathers and soil, μg/g ww for pectoral muscle and other tissues and μg/ml for blood and water samples.

2.3. Analysis of arsenic (As)

Arsenic was analyzed using Atomic Fluorescence Spectrometer (3200, Al). The method employed for As analysis was developed by Sankararamakrishnan and Mishra (2018) with modifications. The initial sample preparation for As was the same for metals. Before the concentration of As was determination for each sample, 3 ml of concentrated HCl was added to each 25 ml sample to increase its acidity (10%) and 2 ml of 10% ascorbic acid and 50% KI was added to reduce As (V) to AS (III). Blanks and sample tubes were subsequently analyzed by a hydride generator. Argon gas was used as a carrier for the transportation of samples from hydride generator to the atomizer. The detailed analytical procedure is given in supporting information (S.I 1.2).

2.4. Organic analysis of stable nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) isotopes

Stable isotopes were analyzed following the procedure adopted by Rogers (2003) with modifications. For carbon ($\delta^{13}C$) isotope analysis, organic carbon in tail feathers was converted to CO_2 using a sample preparation system. Approximately 20–30 mg of moisture-free powdered tail feathers were placed in a porcelain boat (8 cm × 1 cm x 0.8 cm) and introduced into the quartz portion of the vacuum system. After preliminary evacuation (10^{-2}torr), high purity oxygen was added into the combustion part of the line at slightly less than 1 atm pressure. The sample boat was externally heated with a gas burner (~600 °C). The evolved CO/CO_2 was then circulated in the line with the help of a magnetically driven circulation pump. During this circulation, CO_2 gas and moisture were condensed in a trap held at liquid nitrogen temperature (−196 °C). The CO gas was converted to CO_2 while passing over the heated copper gauze (~900 °C) in the presence of oxygen. After 5 min of circulation, all CO was converted to CO_2 and collected into the CO_2 trap. The temperature of this trap was then raised to −80 °C with the help of liquid nitrogen-freon mixture in order to transfer the CO_2 in a suitable sample collector for mass spectrometric analysis.

In order to prepare the tail samples for measurements of stable

nitrogen ($\delta^{15}N$) isotope, 2–5 ml of liquid sample (pink color solution as obtained after Kjeldahl Digestion of total nitrogen) was prepared containing approximately 8–20 mg of NH_4^+-N. This was carefully dispensed in one leg of a Rittenberg Y-Tube. To the other leg of the Rittenberg Y-Tube, 5–12 ml of NaOBr reagent was dispensed (without coming in contact with the sample in the other wing of Y-tube). Five tubes containing samples were prepared each time. Each tube was connected to the vacuum line for degassing to a pressure less than 10^{-2}mb via the external pump. Finally, the evacuated Y-tube containing the reagent and the sample was close to the external pump and the contents were mixed by turning the Y-Tube to generate N_2 gas. The Y-Tube was then opened to the mass spectrometer inlet line bearing a liquid nitrogen trap to condense vapors and the dry N_2 gas was analyzed for ^{15}N–^{14}N ratios. These ratios were converted to $\delta^{15}N$ per ml values against the atmospheric nitrogen standard i.e. ‰ Air-N_2. The analytical precision of 0.1‰ SD for $\delta^{13}C$ and $\delta^{15}N$ were obtained with the routine analysis. The ratio of stable isotope is expressed as

$$X(‰) = \left(\frac{R_{sample}}{R_{standard}} - 1\right)$$

Where X represents $\delta^{13}C$ or $\delta^{15}N$ and R_{sample} show ratio of $^{13}C/^{12}C$ or $^{15}N/^{14}N$. ^{13}C $R_{standard}$ based on PeeDee Belemnite and ^{15}N on atmospheric N_2 (Cui et al., 2011).

2.5. Statistical analysis

Statistical software SPSS (IBM 20 version), Minitab (trial version) and Excel (window 10) were used for statistical computations. To avoid error in the statistical procedures, data screening was performed as recommended by Zuur et al. (2010). For all statistical procedures, the null hypothesis was rejected at 5% ($\alpha = 0.05$) and significance was set at ($p < 0.05$). The distribution was found to be normal after checking the acceptable range of skewness using Kolmogorov–Smirnov test (KS-test) (Sadeghi et al., 2019). Descriptive statistics were calculated for all the variables. Linear regression was applied to investigate the relationship between the concentrations of metals and the dietary proxies of $\delta^{13}C$ or $\delta^{15}N$. The A $\delta^{13}C$ vs $\delta^{15}N$ layout is drawn to illustrate the location of species with respect to its trophic level. Pearson correlation was also applied to investigate the associations between metals and tissues. Slopes were also drawn between log-transformed concentrations of each metals and As with $\delta^{15}N$ to elucidate the association of contaminants with the trophic level of the species. Variation in biomagnification potential of arsenic and metals was explained using modified equations developed by Murillo-Cisneros et al. (2019). Bioaccumulation factor (BAF) was calculated with respect to the route of exposure to characterize the aquatic and terrestrial toxicity suggested by DeForest et al., (2017).

$$\text{Bioaccumulation factor (BAF)} = \frac{\text{Concentrations in tissues of birds}}{\text{Concentration in water and soil}}$$

$$\text{Trophic transfer factor (TTF)} = \frac{\text{Concentration in bird}}{\text{Concentration in Prey}}$$

3. Results and discussion

3.1. Metals profiles in aquatic and terrestrial food chain

Concentration profiles of As, Pb, Cd, Cu and Zn in tail feathers, pectoral muscles and blood of two aquatic bird species (cattle egret, pond heron) and two terrestrial (spotted owlet, bank myna) bird species are presented in Table 1. Fish, frog, aquatic insects and plants were collected as prey items for cattle egret and pond heron. The concentrations of metals and As were investigated in water as well as in whole bodies of fish, frog, aquatic insects, and leaves of aquatic plant and is summarized in Table S2. All the studied metals and As were detected in all tissues of birds (Table 1), which were found comparable or relatively higher than previously reported studies from Pakistan (Abbasi et al., 2015a,2015b: Abdullah et al., 2015: Shahbaz et al., 2013: Malik and Zeb, 2009: Movalli, 2000). In the aquatic food chain, the highest mean concentrations in samples (μg/g ww) were 251 of Cu in insects (*L. americanus*), 184.33 and 3.96 of Zn and As in frogs and 15.85 and 12.33 μg/g ww of Pb and Cd within the leaves of aquatic plants (*P. hysterophorus*) (Table S2). The lowest concentrations (μg/ml) of Zn 1.9, Cu 0.97, Pb 0.64, As 0.37 and Cd 0.01 were recorded in water samples. In birds of the aquatic habitat, the highest concentrations (μg/g dw) of Pb 31.62 and As 16.35 were found in the tail feathers of the pond heron. The highest concentrations of Zn 279.5 and Cu 8.06 were found in the tail feathers of the cattle egret, which also harbored the highest Cd (μg/g ww) 1.05 levels within its pectoral muscles. Similarly, the lowest concentrations (μg/ml) of Zn 7.26, Cu 0.69 and As 4.36, within aquatic birds, was observed in the blood of the pond heron while the cattle egret harbored the lowest Pb 1.93 and Cd 0.19 within its blood. For the terrestrial food chain samples, the highest concentrations (μg/g ww) of Zn 344.2 were observed in the lizard (*H. flaviviridis*), Pb 41.5 and Cd 33.83 were observed in the moth (*S. luridata*) (Table S2). The highest concentrations of As 26.7 and Cu (μg/g dw) 28.5 were detected in the soil (Table S2). The lowest concentrations (μg/g ww) of Zn 23.47, Cu 6.73, Pb 0.64 and Cd 0.015 were observed in the leaves of the white mulberry (*M. alba*) and As 0.54 was recorded in the lizard. In terrestrial birds, we found the highest concentrations (μg/g dw) of Pb 19.88, As 8.96, Cd 1.06 and Zn 142.7 in the tail feathers of the spotted owlet and Cu (μg/g ww) 6.89 was observed in the pectoral muscles of the spotted owlet (Table 1). However, the lowest concentrations (μg/ml) of Pb 2.57, As 3.12, Cd 0.15, Cu 1.13 and Zn 21.92 were recorded in blood of the bank myna. The concentrations of essential and non-essential elements in tissues of birds and their respective food chain are discussed below.

3.1.1. Essential elements

Essential elements such as Cu and Zn play important roles in the functioning of the body. However, constant exposure at elevated concentrations can distress the body's physiology (Lucia et al., 2010). In the present study, Zn was found to be a predominant essential element in birds tissues. Further, the concentrations of Zn and Cu observed in this study were comparably higher than other studies reported worldwide (Teraoka et al., 2018; Zarrintab and Mirzaei, 2018; Mikoni et al., 2017; Dolan et al., 2017; Finger et al., 2016; Kim and Oh, 2013; Binkowski et al., 2013; Jayakumar and Muralidharan, 2011; Lucia et al., 2010; Nam and Lee, 2009; Beyer et al., 2004). This is because Zn is essential in the maintenance homeostasis in birds (Dolan et al., 2017). Zn and Cu play important roles in the regulation of certain biochemical reactions in living organism (Hashmi et al., 2013; Nam and Lee, 2009). The highest concentrations of Zn were found in the tail feathers of the cattle egret and the lowest concentrations of Zn were found in the blood of the bank myna. Aquatic species tend to accumulate more Zn when compared to terrestrial species. The deposition of Zn on feathers through exogenous sources may also elevate the Zn levels observed in tail feathers (Jaspers et al., 2019). Earlier studies documented the bioaccumulation of Zn at faster rates in several aquatic birds (Einoder et al., 2018; Dolan et al., 2017). Further, Zn is an essential metal for feather growth (Philpot et al., 2019) and birds regulate Zn effectively within a wide range of Zn exposure. Zn concentrations differ widely among bird species due to differences in immune responses, nutritional requirements and the chemical composition of their melanin (Chatelain et al., 2016). There are no reported incidences of Zn toxicity below 400 μg/g in bird tissues (Philpot et al., 2019). The behaviour of Cu was found to be different in aquatic and terrestrial species. The high concentrations of Cu observed in the tail feathers of aquatic bird species and in the pectoral muscles of terrestrial species may reflect the differences in bioavailability between habitats and regulatory mechanism of species (Abbasi et al., 2015b). The overall pattern of essential metals in the

Table 1
Levels (mean ± SD, min-max) of targeted metals and As in tail feathers (μg/g dry weight), pectoral muscles, blood (μg/g wet weight) and levels of $\delta^{15}N$ and $\delta^{13}C$ (mean ± SD (min-max) ‰) in tail feathers of selected birds from Pakistan.

	Aquatic: Cattle egret (*Bubulcus ibis.*), [Ardiadae][f] D [Piscivorous][T] R n = 10[a], n = 10[b]			Aquatic: Pond heron (*Ardeola grayii*), [Ardiadae][f] D [Piscivorous][T] R n = 10[a], n = 10[b]			Terrestrial: Spotted owlet (*Athena brama*), [Strigidae][f] D [Carnivorous][T] R n = 6[a], n = 6[b]			Terrestrial: Bank myna (*Acridotheres gingi.*), [Sturnidae][f] D [Omnivorous][T] R n = 16[a], n = 10[b]		
Metals and Arsenic	Tail feathers	Pectoral muscles	Blood	Tail feathers	Pectoral muscles	Blood	Tail feathers	Pectoral muscles	Blood	Tail feathers	Pectoral muscles	Blood
As	16.30 ± 0.63 (15.44–17.71)	14.45 ± 1.9 (10.27–17.4)	4.38 ± 0.88 (2.28–5.35)	16.35 ± 2.46 (13.84–22.44)	15.98 ± 2.15 (11.18–18.21)	4.36 ± 0.53 (3.48–5.11)	8.96 ± 0.68 (8–9.95)	4.29 ± 1.76 (2.29–6.54)	4.29 ± 0.58 (3.57–5.1)	6.97 ± 2.04 (2.67–10.54)	6.02 ± 2.23 (2.61–9.71)	3.12 ± 1.26 (1.16–4.73)
Pb	25.14 ± 5.9 (16.53–36)	15.07 ± 3.87 (8.17–20.95)	1.93 ± 0.39 (1.47–2.55)	31.62 ± 9.80 (14.2–49.92)	20.95 ± 4.87 (15.99–30.19)	4.02 ± 2.03 (1.99–7.86)	19.88 ± 3.63 (14.55–25.58)	17.1 ± 2.70 (14.19–20.13)	5.25 ± 1.46 (2.9–6.96)	15.07 ± 4.23 (5.35–21.30)	13.79 ± 3.83 (3.4–23)	2.57 ± 1.33 (0.025–4.2)
Cd	1.05 ± 0.04 (1.01–1.13)	0.62 ± 0.11 (0.48–0.79)	0.19 ± 0.12 (0.05–0.35)	0.98 ± 0.23 (0.82–1.14)	0.74 ± 0.47 (0.15–1.53)	0.26 ± 0.13 (.025–0.46)	1.21 ± 0.06 (1.14–1.29)	1.06 ± 0.39 (0.63–1.57)	0.3 ± 0.11 (0.19–0.45)	0.76 ± 0.23 (0.61–1.6)	0.09 ± 0.03 (0.03–0.15)	0.15 ± 0.12 (0.015–0.3)
Cu	4.66 ± 2.60 (0.35–9.86)	8.06 ± 0.44 (7.45–8.8)	7.06 ± 1.14 (5.06–8.77)	2.62 ± 0.53 (2.05–3.18)	3.2 ± 0.30 (2.91–4.03)	0.69 ± 0.31 (0.27–1.17)	6.44 ± 0.53 (5.77–7.09)	6.89 ± 1.41 (5.25–8.53)	1.71 ± 0.54 (0.74–2.29)	1.40 ± 0.59 (0.4–1.95)	2.86 ± 0.49 (2.14–4.07)	1.13 ± 0.44 (0.54–2.35)
Zn	279.5 ± 17.3 (252.6–315)	119.5 ± 23.4 (87.85–169.5)	15.60 ± 9.7 (8.72–22.49)	122.1 ± 47.5 (36.03–224.3)	119.5 ± 37.4 (65.3–166.02)	7.26 ± 1.72 (4.86–9.53)	142.71 ± 16.46 (120.4–165.3)	98.27 ± 58.6 (6.24–190.5)	28.38 ± 6.6 (18.5–36.7)	169.9 ± 75.3 (71.5–311.1)	140.49 ± 52.6 (29.3–273.35)	21.92 ± 4.3 (9.7–28.65)
$\delta^{15}N$	9.2 ± 0.91 (7.7–10.74)			8.49 ± 0.27 (8.04–8.84)			7.6 ± 0.40 (6.85–7.92)			6.82 ± 0.32 (6.34–7.44)		
$\delta^{13}C$	-19.63 ± -0.42 (-20 – -19.01)			-24.16 ± -0.48 (-24.2 – -23.4)			-19.5 ± -1.71 (-21.7 – -17.4)			-19.8 ± -0.24 (-20.1 – -19.4)		

[f T] family and trophic guild is given within [ ], D: diurnal, R: resident.
[a] represented no. of tail feathers, pectoral muscles and blood samples for metals and arsenic measurements.
[b] showed sample no. of tail feathers for stable isotope analysis.

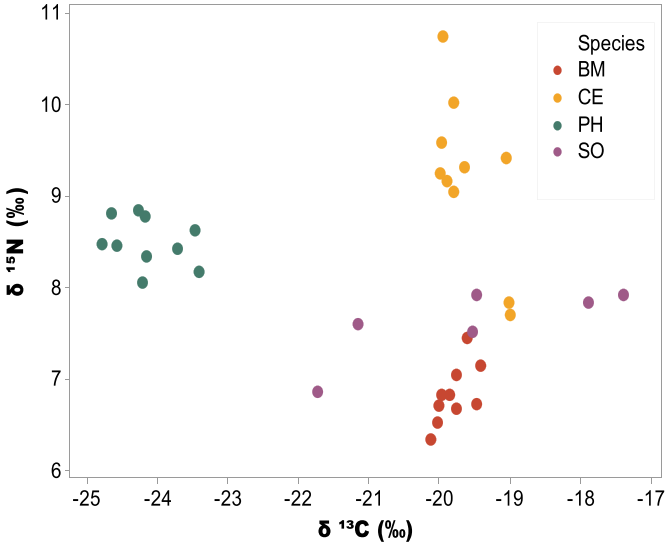

Fig. 2. Distribution of species on δ ^{13}C vs δ ^{15}N layout indicating differences in trophic level and habitat. BM = Bank Myna, CE = Cattle egret, PH = Pond heron and SO = Spotted owlet.

tissues of birds suggests that, like other metals, Zn and Cu are recorded in relatively higher concentrations at higher trophic level. However, higher concentrations of these metals in feathers corroborate the phenomenon of potential exogenous contamination. .

3.1.2. Non-essential elements

In this study, three non-essential elements, As, Pb and Cd, were investigate in the tissues of birds. These non-essential elements cannot be detoxified and/or regulated properly once they enter living tissues. As is a toxic metalloid that readily bioaccumulates in living tissues (Costa et al., 2013; Sánchez-Virosta et al., 2015). In this study, As concentrations were recovered in the tail feathers of species from both habitats indicating a combined effect of exogenous and endogenous contamination. However, As concentrations were found to be alarmingly higher in the samples collected in this study than in tissues reported from multiple bird species in other studies (Finger et al., 2016; Fu et al., 2014; Cui et al., 2011; Lucia et al., 2010; Tsipoura et al., 2008; Deng et al., 2007; Dauwe et al., 2005). The increasing concentration pattern of As through the food chain suggests that it has biomagnification potential both in aquatic and terrestrial habitats. The biomagnification potential of As has been documented earlier in the form of lipid-soluble arsenic in the tissues of birds (Sánchez-Virosta et al., 2015; Furtado et al., 2019; Picone et al., 2019). Higher concentrations of Pb was also observed in the tail feathers of both aquatic and terrestrial species, which further indicates the contribution of external deposition in addition to internal sources by blood. Pb preferentially bioaccumulates in feathers and bones and measuring Pb exposure is potentially useful for the monitoring of anthropogenic contamination due to its role in altering the regulation of metabolism in living organisms. In this current study, Pb concentrations were well above the level which can cause lethal or sublethal effects in the birds (Malik and Zeb, 2009). Comparatively, Cd levels were not as elevated in birds, however, their concentrations were found higher in plant leaves and moths in aquatic and terrestrial food chains respectively. The reason for low Cd levels can be attributed to its less accumulative nature in tissues and is, hence, excreted rapidly (Lucia et al., 2010). In general, concentration of Pb and Cd were either comparable or higher than those reported in birds from different parts of the USA, Europe, Asia and elsewhere (Tsipoura et al., 2017; Fu et al., 2014; Jayakumar and Muralidharan, 2011; Tsipoura et al., 2011; Lucia et al., 2010; Nam and Lee, 2009; Burger et al., 2009; Pérez-López pez et al., 2008; Rattner et al., 2008). The concentration trends of metals and As within tissues was established in order of tail feathers > pectoral muscles > blood. The greater concentration of metals and As in tail feathers appears to be associated with potential external contamination (Jaspers et al., 2019). In addition, the birds were collected form the same locations with similar exposure and similar molting seasons and, hence, the concentrations in feathers may be due to the combined effect of endogenous and exogenous exposure. Concentrations of As and metals in blood depicts recent exposure through diet (Abbasi et al., 2017). The relatively lower concentration patterns of metals and As in blood suggests that there is less exposure through the diet. Pectoral muscles, on the other hand, reflect exposure that occurred in the recent past through the diet which bioaccumulates differentially among tissues (Kim and Oh, 2013). The correlation analysis reveals that concentrations of metals and As are weakly associated between tissues (Table S3), suggesting that the bioaccumulation, detoxification, excretion and regulation of As and metals may occur through different mechanisms in different tissues.

3.2. Dietary proxies as signature of metals and As

We also aimed to identify the effects of trophic position and habitat on the bioaccumulation of metals and As in birds. Therefore, levels of δ ^{13}C and δ ^{15}N were analyzed in the tail feathers of four bird species belonging to terrestrial and aquatic habitats. As discussed earlier, the level of δ ^{15}N reflects the trophic position of birds as it increases 2–5% with each step of trophic level, while the δ ^{13}C value depicts the origin of dietary carbon, which provides information about foraging habitats (Yu et al., 2011). In the present study maximum δ ^{15}N levels were found in the cattle egret 10.74‰ and minimum levels were found in the bank myna 6.34‰ (Table 1). δ ^{13}C levels varied from −24.79 to −17.41‰ in pond herons and spotted owlets respectively (Table 1). We visualized interspecific differences by plotting values of dietary sources (δ ^{13}C) against trophic positions (δ ^{15}N) (Fig. 2). In the current study, aquatic species, i.e. cattle egret and pond heron, showed higher levels of δ^{15}N when compared to terrestrial birds, which is consistent with the previous studies (Abbasi et al., 2016; Hong et al., 2014). Among aquatic species, the cattle egret was found to be at a higher trophic position to the pond heron, which is mainly due to the inclusion of fish as a major portion in their diet (Abbasi et al., 2017; Barón et al., 2014; Jaspers et al., 2007). The pond heron, in contrast, mainly feeds on aquatic invertebrates (Abbasi et al., 2016). Spotted owlets and cattle egrets showed a relatively scattered distribution compared to the other bird species which reflects their dietary flexibility. In the δ^{15}N vs δ ^{13}C layout, the distribution of bank myna was found at similar levels to the spotted owlet, which reflects its relatively specialized diet (Fig. 3). We found significantly higher ($P < 0.05$) concentrations of metals and As in the tissues of aquatic birds with higher δ^{15}N levels than terrestrial birds. Higher concentrations of metals and As with larger δ^{15}N values suggests that metals and As tend to increase in the food chain with trophic levels, which is consistent with previous findings (Cui et al., 2011; Croteau et al., 2005). The variation in metals and As levels between aquatic and terrestrial species is also associated with differences in dietary carbon sources as δ^{13}C depletion is different between habitats (Bryan et al., 2012). Thus, relatively higher concentrations of metals and As found in aquatic birds when compared to terrestrial birds can also be partially explained by the differences in their primary food sources. This suggests that aquatic food chains with δ ^{13}C dietary sources are more contaminated than terrestrial food chains (Einoder et al., 2018; Bryan et al., 2012). The higher concentrations of metals and As in aquatic species feeding at higher trophic level suggests that these birds may be at a greater risk of toxic effects when compared to terrestrial birds. Linear regression model indicates that there is a relationship between metals, As and stable isotope proxies (δ ^{13}C/δ ^{15}N) in birds (Table 4). For birds inhabiting aquatic environments, δ ^{15}N showed a significantly positive association for Pb and Zn ($R^2 = 0.48$, $P < 0.03$ and $R^2 = 0.42$, $P < 0.05$ respectively) in the cattle egret.

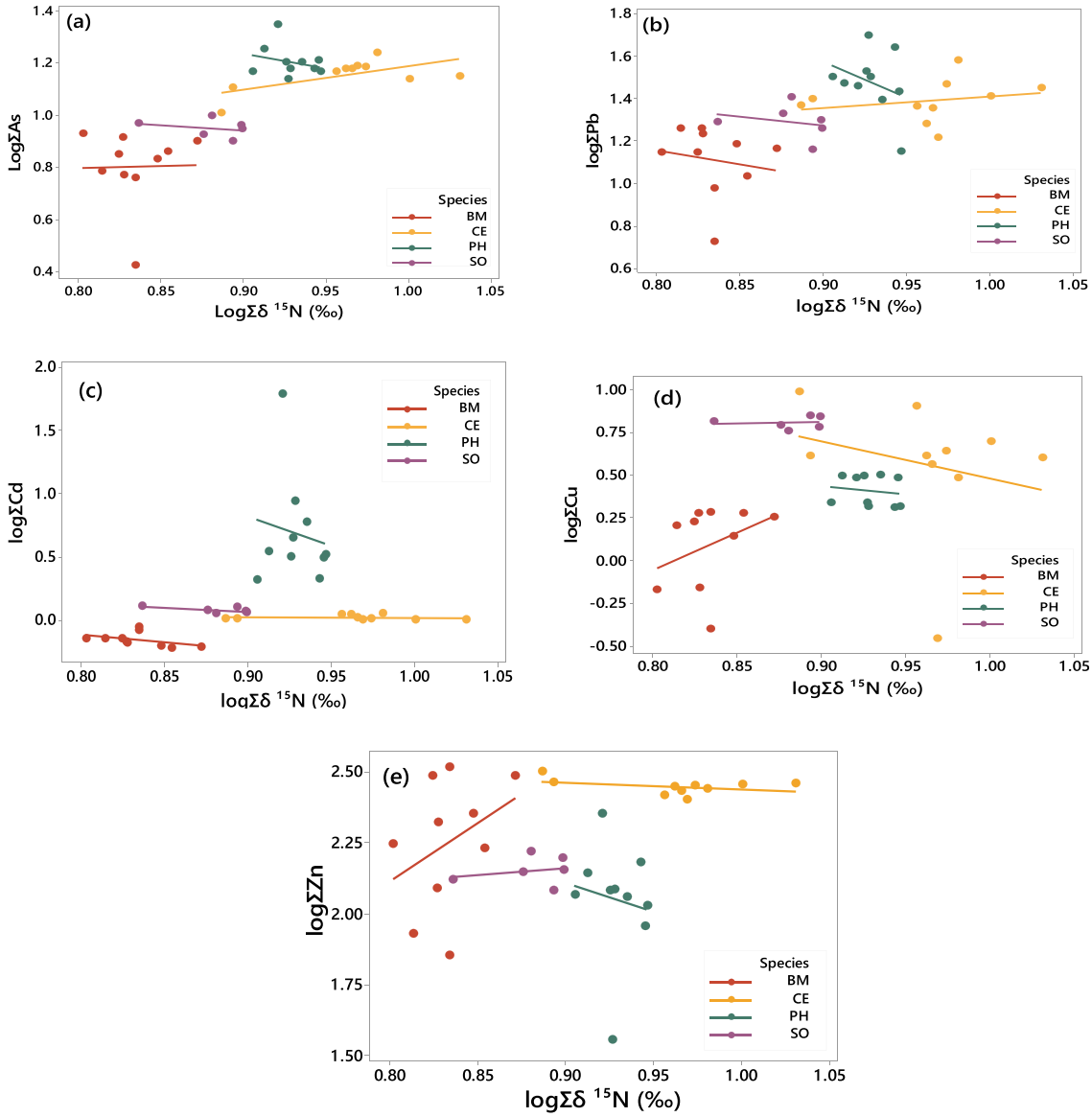

Fig. 3. Scatter plots indicating species specific regressions of $\log_{10}$ transformed concentrations of As (a), Pb (b), Cd (c), Cu (d) and Zn (e) with stable isotope log transformed values represented in tail feathers. BM = Bank Myna, CE = Cattle egret, PH = Pond heron and SO = Spotted owlet.

Similarly δ ^{13}C exhibited significant regression for As, Cu and Zn (R^2 = 0.38, P = 0.05, R^2 = 0.62, P < 0.01, R^2 = 0.70, P < 0.01) in the cattle egret. Regression was not significant (P > 0.05) for δ ^{13}C and δ ^{15}N in the pond heron. For terrestrial species, δ ^{15}N depicted a significant positive relationship for Pb and Cu (R^2 = 0.63, P = 0.05 and R^2 = 0.66, P < 0.05 respectively) for the spotted owlet and for Cd (R^2 = 0.44, P < 0.05) in the bank myna. Values of δ^{13}C revealed a strong association for Pb (R^2 = 0.87, P < 0.01) in the spotted owlet and for As (R^2 = 0.42, P < 0.05) in the bank myna. The relationship between metals and trophic levels of birds was also analyzed by regressing $\log_{10}$ transformed concentrations for metals and arsenic with δ ^{15}N values (Fig. 3 a-e). The regression slopes indicate a weak and non-significant association between $\log_{10}$ transformed concentrations for metals and arsenic with δ ^{15}N values, which reflects a weak and heterogeneous trend of accumulation of metals and As with an increase in the food chain (Fig. 3 a-e). This weaker, and sometime negative, association could be attributed to 1) statistical biases in the results because of relatively smaller sample size and 2) collection bird samples from the similar locations with similar exposure and, hence, not showing any significant difference despite showing variation in trophic positions and 3) external contamination obscuring these relationships. The heterogeneous behaviour of metals and As suggests that their increasing bioaccumulation trend with trophic level synergistically depends on the species, habitat and possibly some physicochemical characteristics of metals.

3.3. Bioaccumulation and trophic transfer potential of metals and As

The bioaccumulation factor (BAF) and the trophic transfer factors (TTF) are used to elucidate the association of metal accumulation in birds and their prey as well as within soil and water. Bioaccumulation factors (BAF) reveal the concentration of metals and As in birds relative to their surrounding environment (Table S3). BAF was calculated based on the ratio between metals and As, within the tissues of birds, to the concentrations of metals and As within water and soil (Jakimska et al., 2011; DeForest et al., 2007; Li et al., 2007). BAFs were calculated to characterize the site-specific pollution rate and transfer capacity through the food chain (DeForest et al., 2007). Trophic transfer factors (TTFs), on the other hand, depict predator-prey relationships and the potential of trophic transfer of metals and As along food chains

(Murillo-Cisneros et al., 2019). It is broadly accepted that aquatic and terrestrial organisms attain chemical equilibrium with respect to the route of exposure or a particular media (Mountouris et al., 2002). In aquatic birds the highest BAF value was found for Zn (147) followed by Cd (81.2), Pb (49.4), As (44.2) and Cu (8.3) (Table S3) and was consistent with previous studies (Cui et al., 2011; Hsu et al., 2006). Results revealed that BAFs were found higher (BAF > 1) for aquatic birds and lower (BAF < 1) for terrestrial birds (Table S3). Information on BAF values provide knowledge of the relative ability of birds to adsorb toxic metals from their foraging sources (Hosseini Alhashemi et al., 2011; Hendozko et al., 2010; Fairbrother et al., 2007). Cattle egrets and pond herons were found to accumulate metals and As more efficiently from surrounding environment, which suggests a relatively higher dietary exposure through food items from aquatic sources (Hosseini Alhashemi et al., 2011). In contrast, all metals and As showed lower bioaccumulation factors (BAF < 1) in the spotted owlet and bank myna despite these elements showing significant accumulations in their bodies. This also suggests that additional external contamination from the surrounding environment may be occurring as well as species specific metal and As regulation along with other factors (Hsu et al., 2006). Based on tissue-specific predator-prey TTFs, results were comparable in both aquatic and terrestrial birds (Tables 2 and 3). Trophic transfer factors that are larger than 1 (TTF > 1) suggest that trophic transfer is likely, whereas a trophic transfer factor of less than 1 (TTF < 1) suggests that trophic transfer is unlikely to be occurring at particular step in the food chain (Jara-Marini et al., 2009). Among aquatic birds, Cu showed a maximum TTF value of 17.5 in the pectoral muscles of the cattle egret from fish followed by As 6.7 in the tail feathers of cattle egret and pond heron from aquatic insect. Pb showed a TTF of 5.4 in tail feathers of the pond heron from fish and Zn showed a TTF of 3.9 in the tail feathers of the cattle egret from fish. In general, As, Pb, Cu, and Zn showed a trophic transfer of larger than 1 in both aquatic bird species from their diet (fish, frog, and aquatic insect), which is consistent with the previous results. Cui et al. (2011) found comparable TTFs to this study for Pb, Cu, and Zn, and a relatively lower TTF values for As, in *Egretta garzetta* and *Chroicocephalus saundersi* from *Hypophthalmichthys molitrix*. Cd showed no trophic transfer trend (TTF < 1) owing to its low assimilation and high elimination rates that reduce its trophic transfer potential in aquatic food chains (Dutton and Fisher, 2011; Jara-Marini et al., 2009; Wang, 2002). Among terrestrial birds, As showed trophic transfer potential (range) in both the spotted owlet (8–16) and the bank myna (5–11) from their diet. Surprisingly, Cd (6–50) and Pb (4–23) showed significant trophic transfer potential in the bank myna (Reitan et al., 2013). Cu, however, exhibited no trophic transfer potential in terrestrial birds. Among avian tissues, we have observed higher trophic transfer trends in tail feathers followed by pectoral muscles and blood. This higher trophic transfer potential in tail feathers may be associated with potential external contamination factors which increase the concentrations of metals detected in feathers. Additionally, metal and As accumulation in bird tissues was strikingly affected by the type of prey items that was consumed as well as feeding behaviors and feeding tactics. In aquatic and terrestrial habitats, other studies showed less arsenic and metal bioaccumulation up the food chain than what is reported in this study. (Abbasi et al., 2015a, 2015b; Berglund et al., 2011; Cui et al., 2011). In summary, higher values of BMF and TTF reflect bioaccumulation and biomagnification of metals and As up the food chain. However, we have collected only a few representative food samples for each species which may cause bias in the results. Nevertheless, bioaccumulation and subsequent biomagnification of metals and As is affected by physicochemical nature of the contaminant as well as the detoxification potential of species.

Table 3
Trophic transfer factor (TTF) calculated for terrestrial birds species.

Trophic relation										
Prey-Predator (tissues)	Spotted owlet					Bank myna				
Lizard	As	Pb	Cd	Cu	Zn	As	Pb	Cd	Cu	Zn
H. flaviviridis -Tail feathers	**16**	1	**2.4**	0.3	0.4	–	–	–	–	–
H. flaviviridis -Pec.Muscle	**8**	0.8	**2**	0.3	0.3	–	–	–	–	–
H. flaviviridis-Blood	**8**	0.3	0.6	0.1	0.1	–	–	–	–	–
Moth										
S. luridata -Tail feathers	**4.5**	0.5	0.03	0.3	**2.2**	–	–	–	–	–
S. luridata -Pec.Muscle	**2**	0.4	0.03	0.3	**1.5**	–	–	–	–	–
S. luridata -Blood	**2**	0.1	0.01	0.1	0.4	–	–	–	–	–
White mulberry						–	–	–	–	–
M. alba -Tail feathers	–	–	–	–	–	**11**	**23**	**50**	0.2	**7**
M. alba -Pec.Muscle	–	–	–	–	–	**9.7**	**21**	**6**	0.4	**6**
M. alba -Blood	–	–	–	–	–	**5**	**4**	**10**	0.2	0.9

4. Conclusion

We concluded that exposure of metals and As remains an important environmental concern for both aquatic and terrestrial species. Despite environmental regulatory restrictions, increasing trends of metals and As continues to pose serious health risks to humans and wildlife. Elevated levels of metals and As in tail feathers is evidently associated with potential external contamination whereas lower contaminant levels in blood reflects less recent exposure through diet. Accumulation of metals and As in birds is mainly associated with level of dietary proxies ($\delta 15$N, $\delta 13$C) as well as habitat and dietary flexibility. Birds feeding at higher trophic levels are at high risk of contamination due to the significant potential of certain metals and As to bioaccumulate through the food chain. Terrestrial species, feeding at relatively lower trophic levels and with less dietary flexibility, accumulate lower levels of metals when compared to aquatic species. Nevertheless, increasing exposure of

Table 2
Trophic transfer factor (TTF) calculated for aquatic birds species.

Trophic relation										
Prey-Predator (tissues)	Pond heron					Cattle egret				
Fish	As	Pb	Cd	Cu	Zn	As	Pb	Cd	Cu	Zn
C. punctata -Tail feathers	**5.3**	**5.4**	0.3	**1.8**	**1.7**	**5.2**	**4.3**	0.8	**3.2**	**3.9**
C. punctata -Pec.Muscle	**5**	**3.6**	0.5	**6.9**	**1.7**	**4.6**	**2.6**	0.4	**17.5**	**1.7**
C. punctata-Blood	**1.4**	0.7	0.2	**1.5**	0.1	**1.4**	0.3	0.1	**15.3**	0.2
Frog										
H. tigerinus -Tail feathers	**4**	**2**	0.2	0.1	0.6	**4**	**1.6**	0.7	0.2	**1.5**
H. tigerinus -Pec.Muscle	**4**	**1.3**	0.5	0.1	0.6	**3.6**	0.9	0.4	0.4	0.6
H. tigerinus -Blood	1	0.3	0.2	0.03	0.04	1	0.1	0.1	0.3	0.1
Aquatic Insect										
L. americanus -Tail feathers	**6.7**	**4.4**	0.4	0.01	0.7	**6.7**	**3.5**	0.1	0.02	**1.5**
L. americanus -Pec.Muscle	**6.6**	**2.9**	0.1	0.01	0.6	**6**	**2.1**	0.1	0.03	0.6
L. americanus -Blood	**1.8**	0.5	0.04	0.003	0.04	**1.8**	0.3	0.03	0.03	0.08

Table 4
Relationship between metals and As concentrations and stable isotope signatures (δ ^{15}N, δ ^{13}C) using linear regression models.

				Tail Feathers-Isotopic proxies					Pectoral muscles-Isotopic proxies					Blood-Isotopic proxies				
				As	Pb	Cd	Cu	Zn	As	Pb	Cd	Cu	Zn	As	Pb	Cd	Cu	Zn
Aquatic Habitat	**CE**	δ ^{15}N (‰)	R^2	0.343	0.072	0.007	0.193	0.157	0.28	**0.488**	0.206	0.028	0.23	0.125	0.019	0.283	0.175	**0.425**
			p	0.075	0.455	0.812	0.205	0.257	0.116	**0.025***	0.187	0.646	0.16	0.316	0.703	0.114	0.229	**0.041***
		δ ^{13}C (‰)	R^2	**0.386**	0.003	0.128	0.127	0.31	0.12	0.364	0.263	0.035	0.6	0.035	0.018	0.057	**0.63**	**0.703**
			p	**0.05***	0.882	0.309	0.313	0.095	0.328	0.065	0.13	0.607	0.497	0.607	0.711	0.505	**0.006***	**0.001***
	PH	δ ^{15}N (‰)	R^2	0.073	0.044	0.042	0.022	0.042	0.23	0.071	0.27	0.123	0.002	0.035	0.006	0.1	0.153	0.003
			p	0.45	0.56	0.569	0.68	0.568	0.161	0.455	0.18	0.321	0.912	0.606	0.838	0.373	0.263	0.882
			p	0.406	0.545	0.917	0.057	0.412	0.339	0.228	0.665	0.175	0.88	0.97	0.727	0.474	0.687	0.785
Terrestrial Habitat	**SO**	δ ^{15}N (‰)	R^2	0.09	0.05	0.37	0.023	0.059	0.196	**0.636**	0.017	0.002	0.011	0.027	0.229	0.035	**0.66**	0.225
			p	0.563	0.669	0.2	0.773	0.642	0.38	**0.05***	0.807	0.93	0.842	0.756	0.337	0.724	**0.048***	0.341
		δ ^{13}C (‰)	R^2	0.506	0.454	0.03	0.479	0.115	0.013	**0.869**	0.14	0.022	0.329	0.052	0.243	0.066	0.425	0.142
			p	0.113	0.142	0.743	0.128	0.511	0.832	**0.007***	0.464	0.778	0.234	0.664	0.32	0.624	0.161	0.462
	BM	δ ^{15}N (‰)	R^2	0.001	0.041	0.175	0.16	0.143	0.069	0.015	**0.442**	0.015	0.048	0.161	0	0.016	0.083	0.009
			p	0.927	0.573	0.229	0.252	0.281	0.462	0.733	**0.036***	0.732	0.543	0.25	0.984	0.73	0.419	0.796
		δ ^{13}C (‰)	R^2	0.004	0	0.286	0.054	0.168	0.025	0.007	0.328	0.029	0.002	**0.423**	0.025	0.167	0.088	0.105
			p	0.869	0.961	0.111	0.517	0.239	0.664	0.812	0.083	0.635	0.911	**0.042***	0.661	0.242	0.04	0.36

metals and As is remains a major threat to an already declining avifauna, particularly in the developing world.

Declaration of competing interest

The authors declare no conflict of interest at personal and/or organizational level.

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Appendix A. Supplementary data

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