Use of feathers to assess polychlorinated biphenyl and organochlorine pesticide exposure in top predatory bird species of Pakistan

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Abstract

Little is known about the levels of organochlorines (OCs) in predatory bird species from Asia or the factors governing their concentration. This study is the first report on concentration of polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) in predatory birds of Pakistan. The concentrations of PCBs and OCPs were investigated using tail feathers of ten different species of predatory birds. In addition, concentration differences among body, tail, primary and secondary feathers were investigated for six individuals of black kite (*Milvus migrans*). Ranges of concentrations were highest for dichlorodiphenyldichloroethylene (*p,p´*-DDE: 0.11-2163 *ng* g^{-1} dry wt.) followed by dichlorodiphenyltrichloroethane (*p,p´*-DDT: 0.36-345 ng g^{-1} dry wt.), hexachlorobenzene (HCB: 0.02-34 ng g^{-1} dry wt.), ∑PCBs (0.03-16 ng g^{-1} dry wt.) and *trans*-nonachlor (TN; 0.01-0.13 *ng* g^{-1} dry wt.). CB 118, 153, 138, and 180 along with *p,p´*-DDE were found as most prevalent compounds. ∑PCBs and ∑DDTs were significantly different among species (both $p<0.01$) and omnivorous, scavengers, carnivorous and piscivorous trophic guilds (all $p<0.03$). Whereas only ∑PCBs were significantly different ($p<0.01$) among different families of birds. Values of stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) differed significantly (all $p<0.01$) among species, families, trophic guilds as well as terrestrial and aquatic habitat but not between nocturnal and diurnal predators ($p=0.22$ for $\delta^{13}C$; $p=0.50$ for $\delta^{15}N$). Concentrations of ∑PCBs, ∑DDTs and *trans*-nonachlor, but not HCB ($p=0.86$), were significantly different among different feather types (all $p<0.01$). Trophic and taxonomic affiliation as well as dietary carbon sources ($\delta^{13}C$) for species were identified as the variables best explaining the observed variation in exposure to the studied compounds. The significance of contributing factors responsible for OC contamination differences in predatory birds should be further elucidated in future studies.

Key words: trophic guild, feathers, habitat, POPs, $\delta^{13}C$, $\delta^{15}N$

1. Introduction

During the past few decades, prolific discharge of legacy persistent organic pollutants (POPs) from industrial, urban and agricultural sources have remained a cause of many environmental concerns particularly related to their toxic effects in humans and wildlife (Letcher et al., 2010). These chemicals are persistent, bioaccumulative, toxic and travel large distances through long-range transport (Vorkamp and Rigét, 2014). Two major classes of POPs, i.e. polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) have been introduced after the industrial revolution in 1920 and are still widespread in the environment (Lohmann et al., 2007) despite being legally mitigated on a worldwide scale (UNEP, 2011). PCBs were used in a wide array of substances as a coolant or additives and escape into the environment during their usage, packaging and storage as well as through leaching from landfills (Covaci et al., 2006). On the other hand, OCPs are chlorine-containing organic pesticides which were predominately used as insecticides (Ali et al., 2014). Among a variety of compounds, dichlorodiphenyltrichloroethane (DDT) was the mostly heavily used pesticide after World War I and its production was banned from USA, Europe, China and Japan after 1972 when its toxic effects became established (Tanabe et al., 1998). Hexachlorobenzene (HCB) was found among the most prevalent OCPs because of its use as insecticide as well as an industrial by-product (Corsolini et al., 2006). Besides these, metabolites of chlordanes (CHLs) were also found as compounds of concern because of their exacerbated use as insecticides and their reported adverse effects upon wildlife (Letcher et al., 2010). These OCPs mainly get their way into the environment during their production, application and storage and are eventually dispersed through runoff and air currents (Guan et al., 2009).

Ever since the toxicological significance of POPs was suspected, predatory birds have been successfully used as sentinels to assess the levels of these compounds in the environment (et al., 1993; Dauwe et al., 2005; Jaspers et al., 2006). However, sampling of predatory birds often encounters various practical and ethical impediments. Use of non-destructive tissues, such as blood, feathers and preen oil, is usually recommended as a preferable choice in case of predatory birds. Among these, the use of feathers has become more and more applicable because it is less invasive, comes along with easy collection and storage, and provides a valuable assessment of internal body burdens of POPs (Jaspers et al., 2006). Some of the recent studies have also emphasized to evaluate different types of feathers i.e. body, tail, primary and secondary which could best represent the level of the studied compounds (Eulaers et al., 2014b; García-Fernández et al., 2013; Jaspers et al., 2011).

Levels of POPs in avian tissues are influenced by a multitude of biological, spatial and ecological factors (Eulaers et al., 2013; Lavoie et al., 2010). Trophic levels/feeding guilds and taxonomic affiliation of species, locational and dietary exposure well as individual condition factors such as gender, age and reproductive status may significantly influence the concentration of POPs in birds (Eulaers et al., 2013; Behrooz et al., 2009). In general, POP concentrations at higher trophic level species mainly stem from dietary intake, which can be quantified using ratios of stable nitrogen and carbon isotopes (SIs; Eulaers et al., 2014a). The ratio of heavier ^{15}N to lighter ^{14}N ($\delta^{15}N$) provides information about the trophic level of an individual because it enriches with each trophic level (Huang et al., 2013). The ratio of carbon SIs ($\delta^{13}C$: $^{13}C/^{12}C$) is used as an indicator for dietary origin because of the varying degree of depletion of ^{13}C stable isotopes in primary producers from different habitats (Boecklen et al., 2011; Jardine et al., 2006). Although the use of SIs has shown promising to investigate trophodynamics of POPs, it has had less focus in predatory birds, particularly those from the Asian continent.

Predatory birds of the southern Asian region are particularly exposed to a high magnitude of legacy POPs because of their historical and current use in this region (Ali et al., 2014). Levels of POPs have been documented in biotic as well as abiotic components of the environment from South Asia (Sarkar et al., 2008; Yadav et al., 2015), but predatory birds have received less attention (Abbasi et al., 2016). Contamination of birds with POPs has only been reported in eggs of little (*Egretta garzetta*) and cattle egret (*Bubulcus ibis*) from Pakistan (Khan et al., 2014; Malik et al., 2011; Sanpera et al., 2003). Seeing this scarcity of exposure data, the present study was designed to investigate the current concentration levels of different OC compounds using feathers of multiple predatory bird species of Pakistan. Further, we evaluated the importance of various factors governing interspecific variation of OC exposure including intraspecific variations through carbon and nitrogen SI values. Lastly, the suitability of different feather types to characterize OC exposure was evaluated by comparing body, tail, primary and secondary feathers from black kites (*Milvus migrans*).

2. Methodology

2.1. Sample collection

Feather samples (*N*=76) from ten different species of predatory birds were collected between June 2012 to September 2014 (Fig. 1). Species selected for this study included black kite (N=13), Eurasian sparrowhawk (*Accipiter nisus*, N=10), common kestrel (*Falco tinnunculus*, N=4), red-necked falcon (*Falco chicquera*, N=2), Indian vulture (*Gyps indicus*, N=9), white-rumped vulture (*Gyps bangalensis*, N=12), spotted owlet (*Athene brama*, N=10), little owl (*Athene noctua*, N=6), great cormorant (*Phalacrocorax carbo*, N=4) and grey heron (*Ardea cinerea*, N=6). Tail feathers were obtained from all these species. In addition, tail, body, primary and secondary feathers were collected from six individuals of black kite to investigate concentration differences among feather types. Sampling details of each site are summarized in Table S1 (supplementary information). Predatory birds were sampled mainly from different towns and cities and their outskirts in Punjab province, which is considered a hub of agricultural activities of the country. Samples of black kite and spotted owlet were also collected from two metropolitan cities, i.e. Lahore and Rawalpindi, with higher expected anthropogenic input than other sites. Samples of both the vulture's species were obtained from their isolated and remotely located colonies (S1&S2) at Nagar Parker, Sindh Province. Grey heron was the only species sampled from northern regions at Lulusar Lake (S13), which is a remote waterbody. Each species was sampled from one location except black kite, Eurasian sparrowhawk and spotted owlet, which were sampled from two different locations (Table S1). Black kites and spotted owlet were sampled around the outskirts of Lahore, which is a metropolitan city with extensive agricultural activities in its suburbs, and Islamabad, which is a relatively smaller city with very small scale agricultural activities in its premises. The third species, Eurasian sparrowhawk, was sampled from Mianwali and Khaniwal, which are both small cities with extensive agricultural lands around. Further, species are discussed under various categories based on their taxonomic affiliation (families; accipitridae, ardeidae, falconidae, phalacrocoracidae, strigidae), trophic guilds (Omnivorous, scavenger, carnivorous, piscivorous, habitats (terrestrial or aquatic) and feeding regimes (diurnal or nocturnal). All the samples used in this study were taken from birds captured in the framework of other studies. A special permit from CITES authorities in Pakistan was acquired for shipping and transport of the samples of the two critically endangered vulture species. After collection, feathers were kept in zipped plastic bags and stored at -20˚C until chemical analysis.

2.2. Quantification of PCBs and OCPs

The procedure for cleanup and extraction of POPs was adapted from previous described methods (Dauwe et al., 2005; Jaspers et al., 2006). Feathers were thoroughly washed with deionized water to remove exogenous dust particles and other unwanted depositions. After washing, feathers were covered with standard laboratory paper and dried overnight at ambient temperature. Dried feathers were cut into pieces of ~1 mm, weighed and transferred to analytical glass recipients. Initially, feather samples were spiked with the internal standard CB 143 (50µL of 200 pg μL^{-1}) and incubated overnight at 45˚C in HCl (4M) and hexane:dichloromethane (4:1; v:v). From the incubated mixture, analytes were liquid-liquid extracted using hexane:dichloromethane (4:1; v:v). Cleanup of the resulting extract was performed on acidified silica (800 mg; 44% H_2SO_4) topped with anhydrous Na_2SO_4 (400 mg), and analytes were eluted with hexane:dichloromethane (4:1; v:v). Finally, the cleaned-up extracts were concentrated using a gentle flow of Nitrogen gas, reconstituted in 80 µL *iso*-octane, and transferred to injection vials. The whole process of clean-up and extraction was performed at the Bird ecotoxicology laboratory, Norwegian University of Science and Technology (Trondheim, Norway), whereas the concentrations of PCBs and OCPs were quantified at the Toxicological Center, University of Antwerp (Wilrijk, Belgium).

The concentrations of PCBs and OCPs were quantified using a mass spectrometer (Agilent MS 5973, Palo Alto, CA, USA) operated in electron-capture negative ionization mode to a gas chromatograph (Agilent GC 6890, Palo Alto, CA, USA). A total of 19 PCB congeners (CB 105, 118, 146, 153, 138, 187, 183, 128, 174, 177, 171, 156, 180, 170, 199, 196/203, 194, 206, 209), HCB, *trans*-nonachlor (TN), *cis*-nonachlor (CN), oxychlordane (OXC), and DDTs, i.e. *p,p´*-DDE, *p,p´*-DDT, were measured. In all samples, only high chlorinated PCBs were measured. However, in a few samples (those with the highest concentrations of PCBs), we have attempted to measure lower-chlorinated PCB congeners. Yet, detection limits were higher and the lower chlorinated PCB congeners measured (CB28, CB52, CB95 etc.) were below the limit of quantification (<LOQ) and are thus not reported. Concentrations of analytes were expressed as ng g^{-1} dry weight (dw). Internal standards were purchased from Accustandard (New Haven, CT, USA), while pesticide-grade solvents (Merck, Darmstadt, Germany) were used throughout the entire process. Mean recoveries of internal standards were 52%±13 for PCBs in all samples. The same internal standard (CB 143) was used for other OCPs. For quality assurance, in each

batch of 10 samples, a procedural blank was prepared and analyzed. LOQs for different analytes were set at 3*SD of the procedural blank values. When analytes were not detected in blanks, the LOQ was calculated using a 10:1 signal to noise ratio.

2.3. Stable isotopes measurement

Composition of stable nitrogen and carbon isotopes was measured at the Center for Permafrost (University of Copenhagen, Denmark). We adapted the previously reported procedure by Eulaers et al. (2014a) for the measurement of SIs in feathers. Briefly, a representative homogenized subsample of 0.5 to 2.0 mg was wrapped into a tin combustion cup, and the ratios for stable carbon and nitrogen isotopes were measured by continuous flow using an elemental analyzer (CE 1110, Thermo Electron, Milan, Italy) coupled to a mass spectrometer (Finnigan MAT Delta PLUS, Thermo Scientific, Bremen, Germany). The ratios of SIs were expressed as

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

with X representing the C or N SIs and R representing their corresponding ratios ($^{13}C/^{12}C$, $^{15}N/^{14}N$) in the sample or standard. References samples (Atropin) were included for the positive evaluation of analytical performance. The instrument was calibrated by employing pure gases of CO_2 and N_2 against the certified reference material of sucrose and $(NH_4)_2SO_4$ provided by the International Atomic Energy Agency (IAEA, Vienna, Austria). The SI ratios were calculated against the international standards Vienna PeeDee Belemnite (vPDB) and atmospheric N_2 (AIR) respectively. Analytical precision was maintained at 0.1‰ SD.

2.4. Statistical analysis

All the statistical computations were performed using SPSS (IBM 20) and R (version 3.2.3). Firstly, screening of the data was performed as suggested by Zuur et al. (2010) to avoid common statistical errors. Data was log_{10} transformed after testing for normality using Q-Q plots and Shapiro-Wilk's tests (all p<0.05). Only those compounds which were detected above the limit of quantification (>LOQ) in at least 50% of the samples of a species were treated for further statistical analysis. Missing values for these compounds were substituted with the proportion of detected samples*LOQ. The *null*-hypothesis was rejected at α=0.05. Firstly, differences of PCB and OCP concentrations among species, families, omnivorous, scavengers, carnivorous and piscivorous trophic guilds, habitats (aquatic/terrestrial), feeding regime (diurnal or nocturnal) as well their associations to dietary proxies ($\delta^{13}C$ and $\delta^{15}N$) were tested through analysis of variance (ANOVA). Subsequent post-hoc Tukey's tests for honest significant differences (HSD) were used for multiple comparisons. Further, above-mentioned variables were evaluated for their capacity to explain the observed variation in levels of PCBs and OCPs using Akaike's Information Criteria (AICc) as discussed previously (Johnson and Omland, 2004). A separate AIC-based selection was run for each compound to evaluate the factors best governing the observed variation in PCB and OCPs concentrations. Separate AIC based model was run for each compound based on of the fact that they have different physicochemical properties and may be influenced differently by different factors. Associations of PCBs and OCPs with dietary proxies ($\delta^{13}C$, $\delta^{15}N$) were tested through linear regression. A separate ANOVA was performed to determine the variation in concentrations among different feather types sampled from black kite.

3. Results and Discussion

3.1. Variation in OC concentrations and profiles

To the best of our knowledge, so far PCBs and OCPs have never been quantified in feathers of predatory birds from Pakistan. The measured concentrations for different compounds of PCBs and OCPs are summarized in table S2. Out of the 19 PCBs congeners targeted, 14 congeners, i.e. CB 105, 118, 146, 153, 138, 187, 183, 128, 156, 180, 170, 199, 196/203, 194 were detected above LOQ in ≥50% samples for minimum one to maximum all the species. Among the studied compounds CB 153, HCB, *p,p´*-DDE and *p,p´*-DDT were detected in all the studied species whereas all other compounds were variably detected (Figure S1). Compounds such as CB 171, 174, 177, 206, 209, as well as OXC and CN were not detected above LOQ in ≥50% of the samples in any species (Figure S1), hence not further discussed. In general, the trend ∑DDTs>HCB>PCBs>TN was depicted in tail feathers of predatory birds from Pakistan. The concentration ranges (minimum-maximum) recorded in this study were 0.11-2163 $ng\ g^{-1}$ dry wt. for DDTs, 0.02-34 $ng\ g^{-1}$ dry wt. for HCB and 0.03-16 $ng\ g^{-1}$ dry wt. for ∑PCBs and 0.01-0.13 $ng\ g^{-1}$ dry wt. for TN respectively. Previously, screening of OCs has only been carried out in eggs of little egret (Sanpera et al., 2003) and cattle egret (Khan et al., 2014; Malik et al., 2011) from Pakistan. In those studies, reported concentrations of OCs were higher probably due to the higher lipid content in egg. In recent global literature, PCBs and OCPs in predatory birds have been mostly reported in egg, muscle, liver, kidney and other non-keratinous tissues (Chen et al., 2009; Jaspers et al., 2006; Kocagöz et al., 2014; Lavoie et al., 2010; Peng et al., 2015; Sun et al., 2014; Zhang et al., 2011) whereas only few studies are available for comparison of PCBs and OCPs in feathers. Compared to findings of the present study, ∑DDTs were found comparable whereas ∑PCBs and HCB were approximately 5 to >50 fold higher in feathers from different predatory bird species from south-west of Iran (Behrooz et al., 2009). Similarly, feather concentrations of ∑DDTs and HCB were found comparable to our findings, whereas ∑PCBs levels were relatively higher in different waterbird species from the Caspian Sea coast, Northern Iran (Rajaei et al., 2011). Regarding the European scenario, compared to our study Jaspers et al., (2007) reported relatively higher ∑PCBs, comparable HCBs and lower ∑DDTs levels in tail feathers of multiple predatory species from Belgium. Further, Jaspers et al., (2009) reported a comparable level of *p,p´*-DDE (1.07-139 $ng\ g^{-1}$ dry wt.), relatively lower level of *p´p´*-DDT (0.38-11.8 $ng\ g^{-1}$ dry wt.) and fairly high range of ∑PCBs (2.92-236 $ng\ g^{-1}$ dry wt.) in tail feathers of common magpie (*Pica pica*) from Belgium. Similarly, concentrations of *p,p´*-DDE, HCB and TN but not ∑PCBs of this study were found comparable or slightly higher than reported in tail feathers of white-tailed eagle (*Haliaeetus albicilla*) from western Greenland (Jaspers et al., 2011).

The contribution of the detected compounds is illustrated in figure 2a, whereas profiles for PCBs and DDTs are shown in figure S2a,b. Among the detected compounds, *p,p´*-DDE was found as the predominant compound in predatory birds of the current study followed by *p,p´*-DDT and congeners of PCBs, HCB and TN respectively, which is in line with previous studies (Chen et al., 2009; Rajaei et al., 2011). Among PCBs, CB 118, 153, 138, 180, 170 and 194 were observed as more prevalent congeners in tail feathers (figure S1). In the present study, PCB congeners with six (*hexa*-CBs) and seven chlorines (*hepta*-CBs) dominated in terrestrial species whereas those containing five chlorines (*penta*-CBs) were more prevalent in aquatic species (Figure S2a), which is in agreement with previous findings (Yu et al., 2014; Jaspers et al., 2007). Previously, Abbasi et al., (2016) reported that *p,p´*-DDE has been unanimously detected as predominant metabolite of DDTs in Asian studies on birds. In contrast, *p,p´*-DDE has been found as a predominant compound in feathers of European predatory birds (Jaspers et al., 2007; Eulaers et al., 2013). In general, the elevated level of DDTs in this study corresponds to their wide scale use as pesticide in Pakistan (Ali et al., 2014). Similar to our findings, CB 153, 180, 138 have been reported as predominant congeners in feathers of predatory birds from different parts of the world (Chen et al., 2009; Behrooz et al., 2009, Jaspers et al., 2007, 2011). Earlier, Dauwe et al. (2005) suggested that elevated levels of lower-chlorinated PCB congeners in feathers may be associated with differential elimination and distribution mechanisms.

3.2. Intraspecific variation

We aimed at elucidating intraspecific variation in OC exposure through linear regression of concentrations versus the dietary proxies (SI values) and also by plotting the individuals of species on a δ^{13}C/ δ^{15}N biplot (Figure 3a). The distribution of species in the δ^{13}C/ δ^{15}N biplot reflects within and among species variations based on the differences in values of dietary proxies (figure 3a). δ^{13}C values reflect dietary separation of carbon sources, whereas values of δ^{15}N are used as a proxy for their position at trophic food chain (Yu et al., 2011). Aquatic species i.e. grey heron and great cormorant, in the present study were found to be feeding at a higher trophic position compared to terrestrial birds (p<0.01), which is in line with previous studies (Hong et al., 2014; Jaspers et al., 2007). However, relatively scattered distribution (figure 3a) of the individuals of aquatic birds depicted their wide dietary flexibility. Earlier, based on stable isotope characterization, Sørmo et al., (2011) found that the diet of coastal herring gull (*Larus argentatus*) was influenced by terrestrial sources. Moreover, Morkūnė et al., (2011) reported that great cormorant switched its diet at various stages of life whereas grey herons showed consistent dietary habits throughout their life span. We suspect that higher trophic positions on δ^{13}C/ δ^{15}N layout and relatively scattered distribution of aquatic birds in our study were because of their more specialized dietary habits as well as varying exposure when compared to terrestrial species. In contrast, individuals of Indian vulture and white-rumped vultures were found with a relatively clustered distribution in the δ^{13}C /δ^{15}N biplot which might be associated with more specialized dietary habits (Yu et al., 2011). We sampled these two vultures from their isolated remote colonies where they mostly consumed the locally available carrions, which restricts their choice for diverse food sources. Interestingly, Indian vultures were observed to be feeding at a relatively higher trophic level as compared to white-rumped vultures suggesting differences in dietary habits of these two species. Similarly, a scattered distribution of black kites in the *SI* biplot reflects the availability of diverse food choices for birds dwelling in human proximity and close to urban environments. Earlier, Barón et al., (2014) observed black kite as a versatile feeder ranging from human refusals, small insects, invertebrates, up to small mammals, frogs and snakes in urban and township areas. The results confirm our assumption of exploitation of diverse feeding sources by black kites. Similarly, Eurasian sparrowhawk, red-necked falcon and common kestrel were also found with relatively wide ranging distributions in the δ^{13}C /δ^{15}N biplot indicating flexibility in food choices for these species as well (Chen et al., 2009; Elliot at al., 2009, Luzardo et al., 2014). Among two owl species, spotted owlet residing in urban and suburban localities depicted a relatively more scattered distribution in the δ^{13}C /δ^{15}N biplot suggesting its dietary flexibility compared to tight clustering of little owlet. Certain overlap among terrestrial species but not aquatic species is obvious from the δ^{13}C/δ^{15}N biplot (figure 3a) suggesting their potential sympatric distribution (Zhang et al., 2011) as well as shared feeding sources (Elliott et al., 2009). Distribution of individuals birds of different predatory species on δ^{13}C/δ^{15}N layout suggest that OC bioaccumulation is considerably influenced by habitat and dietary exposure in addition to different other factors. Regression analysis revealed a weak and non-significant (except few) but positive association between dietary proxies and concentration of compounds analyzed (R^2 ranged between 0.01 to 0.99). For δ^{15}N, regression was significant in black kite (for PCBs; R^2=0.44 and HCB; R^2=0.42, both p<0.01) and great cormorant (for DDTs; R^2=0.42, p=0.02). Conversely, for δ^{13}C values regression was only significant in Eurasian sparrowhawk (for PCBs, p<0.03; and DDTs p<0.04). The regressions between dietary proxies (δ^{13}C, δ^{15}N) and OCs concentrations were found non-significant (p>0.05) for other studied species.

Locational differences of POPs accumulation were also done for three species for which samples were available for comparison between sites. We have only three species, i.e. black kite, Eurasian sparrowhawk and spotted owlet, for comparison between two different locations because all other species were sampled from only one site (table S1). For black kite, significant differences for δ^{13}C (p=0.03), δ^{15}N (p=0.02) and HCB (p=0.02) were observed between sites. ∑DDTs at Lahore and ∑PCBs at Rawalpindi were found slightly higher although not significantly different (p>0.05) between sites which is possibly associated with significant differences for the values of dietary proxies. None of the compounds nor stable isotope values differed significantly (p>0.05) between sites for spotted owlet or Eurasian sparrowhawk indicating similar exposure to pollutants at both sites. The above results for stable isotopes suggest that black kite, being a more urban dwelling species, may switch its feeding choices (Barón et al., 2014) based on availability at different sites hence reflect differential exposure to OCs. Conversely, spotted owlet and Eurasian sparrowhawk, which remain consistent between sites due to lower availability of choices at suburban to forested sites hence reflect similar exposure to OCs.

3.3. Interspecific differences

In the present study, highest median concentrations (minimum-maximum) of ∑PCBs at 7.9 *ng* g^{-1} dry wt. (0.4-15.4 *ng* g^{-1} dry wt.) in red-necked falcon, ∑DDTs at 195.5 *ng* g^{-1} dry wt. (7.1-1022.2 *ng* g^{-1} dry wt.) in common kestrel and HCB in Eurasian sparrowhawk at 0.7 *ng* g^{-1} dry wt. (0.1-34.4 *ng* g^{-1} dry wt.) were recorded. All three species of the current study are terrestrial predators that mainly feed upon small birds, rats, mouse, frogs, snakes and invertebrates with some flexibility in their dietary choices (Behrooz et al., 2009). Further, these species mainly reside in suburbs and agricultural lands around cities where they can get their prey easily. Relatively higher concentrations of OCPs in common kestrel and Eurasian sparrowhawk in particular is possibly attributed to their higher dietary exposure to agricultural used pesticides (Behrooz et al., 2009). This was further corroborated through the significant regression between origin of dietary carbon (δ^{13}C) and PCBs (R^2=0.45, p=0.03) as well as with DDTs (R^2=0.41; p=0.04) in Eurasian sparrowhawk. Regression was not significant for common kestrel possibly because of limited movement and exposure of this species (Jaspers et al., 2007). However, relatively higher concentrations of PCBs in red-necked falcon must be considered with caution because of the low sample size. Moreover, it is observed that in winter Eurasian sparrowhawk and red-necked falcon move towards towns and cities from the agricultural lands to overcome winter harshness, which increases their exposure to the urban sources of OCs (Chen et al., 2009). Based on their utility and disposal, PCBs in particular and HCB up to some extent are originating from urban sources and hence bioaccumulate in the tissues of top predators during their winter feeding exposure at temporary stopover sites. To the best of our knowledge, the exposure to PCBs and OCPs in vultures has never been studied. In the present study, we sampled both vulture species from their remotely located colonies from Sindh province (figure 1, table S1), where the exposure to urban as well as agricultural chemicals is expected to be minimal, corresponding to the lower concentrations of PCBs and OCPs in vultures of the current study. In contrast, the concentrations of PCBs and OCPs were found relatively higher in black kite and spotted owlet because of higher exposure of these urban/suburban dwelling species to both agriculture and urban sources of OCs (Barón et al., 2014). The concentrations of ∑DDTs but not ∑PCBs were found relatively higher in aquatic birds of our study suggesting that the surplus use of OCPs and environmental leaching of PCBs is more bioavailable in water reservoirs than terrestrial food chain (Rajaei et al., 2011). Unexpectedly, we have observed relatively high concentrations of ∑DDTs in grey herons, which were sampled from a relatively high altitude pristine location (figure 1, table S1), suggesting that these compounds may also move towards high laying areas through long range transport from their origin (Wania and Mackay 1996).

Previously, interspecific variation in contaminant levels was attributed to the combined influence of several biological, ecological and spatial factors (Eulaers et al., 2013, 2014; Peng et al., 2015). In the present study, we have evaluated the importance of various factors, such as trophic level, taxonomic affiliation, habitat and feeding regime, as drivers of OC exposure, and have investigated how values of $\delta^{13}C$ and $\delta^{15}N$ may specifically serve as dietary tracers that govern difference in OC concentrations among species. Concentrations of compounds such as ∑PCBs and ∑DDTs, but not HCB (p=0.08), differed significantly among species (p<0.01 for both) as well as trophic guilds (all p<0.03). Multiple comparison (Tukey HSD) test revealed that more specialized predators such as Eurasian sparrowhawk and red-necked falcon differed significantly from omnivorous and piscivorous birds (Table 1). Among families, only ∑PCBs (p<0.01), but not ∑DDTs (p<0.88) nor HCB (p<0.82), were significantly different. The bioaccumulation trend of PCBs in falconidae family was found significantly different from all other families. Conversely, the corresponding differences between habitats and feeding regimes were non-significant for ∑PCBs (p<0.27; p<0.91), ∑DDTs (p<0.45; p<0.62) and HCB (p<0.45; p<0.62) respectively (Table 1). Values for $\delta^{13}C$ and $\delta^{15}N$ are found significantly different (p<0.01) for all the above mentioned variables, except for feeding regime (both p=0.22). Further, associations between dietary habits of species and contaminants concentrations were obtained by regressing the $\log_{10}$ normalized concentrations for ∑PCBs, ∑DDTs and HCB with $\delta^{15}N$ values (figure 3b, c, d). Regression slops for species depicted that association of log normalized ∑PCBs and ∑DDTs with $\delta^{15}N$ values were stronger when compared to HCBs. This suggest that different OC compounds vary in their potential of bioaccumulation in birds. This bioaccumulation differences could possibly be attributed to differences of exposure and physicochemical properties of compounds (Behrooz et al., 2009). However, species feeding at different trophic level ($\delta^{15}N$ values) depicted similar trends for each ∑PCBs, ∑DDTs and HCBs accumulation except for few species. In case of common kestrel, regression slop was positive for ∑PCBs while it is relatively straight or negative for ∑DDTs and HCB. Alternatively, regression slop for ∑PCBs was negative for Indian vulture followed by positive and straight lines for ∑DDTs and HCB respectively. Regression slops for all other species somehow depicted similar bioaccumulation trend for ∑PCBs, ∑DDTs and HCB with varying degree of positive or negative trends. Although there is no clear differences, as depicted by slops that bioaccumulation of PCBs and OCPs vary between terrestrial and aquatic species, however the bioaccumulation trend (slops) for grey heron but not greater cormorant were somehow similar to most of the terrestrial species. This differences between two aquatic species could be attributed to sufficient terrestrial exposure (Ito et al., 2013) and potential influence of terrestrial feeding sources in grey heron (Sørmo et al., 2011) compared to great cormorant which strictly rely on aquatic food sources. We have also evaluated the importance of different variables in explaining the magnitude of exposure of PCBs and OCPs in feathers of predatory birds through their respective AIC values (table 3). In different models which are separately run for each of the compounds, variables with the lowest AIC value (shown bold in table 3) best explain the observed variation in concentrations of the different compounds. The models suggested that the concentrations of ∑PCBs, *p,p´*-DDE, *p,p´*-DDT and ∑DDTs are best explained by the variable species. But we have run separate models for each compounds assuming that physicochemical differences of these compounds may varyingly influenced by factors governing their bioaccumulation. Interestingly, most of the PCB congeners are best explained either by trophic guild, δ^{13}C values or taxonomic affiliation of species. Concentration differences of lower-chlorinated compounds were found to be more governed by trophic guilds. Earlier, Behrooz et al., (2009) suggested that the interspecific variations in OC concentrations are mainly due to differences in feeding habits of the species. Very few congeners, i.e. CB 128 and 187, were best predicted by habitat or feeding regime differences. For future studies, we recommend to further elucidate the factors best predicting the bioaccumulation of OCs by investigating large sample size for each species.

3.4. Variation based on feather type

To present date, very few studies have reported the differential accumulation pattern of OCs in different feather types of predatory birds. Earlier, Jaspers et al. (2011) found significant differences in levels of different organic contaminants among different feathers types of white-tailed eagles from Greenland. Similarly, Eulaers et al. (2014b) also detected varying accumulation trends of OCs in different feather types of barn owl (*Tyto alba*) from Belgium. Based on the assumption that OCs bioaccumulate differently among feathers types, we tested the concentrations of PCBs and OCPs as well as values of $\delta^{13}C$ and $\delta^{15}N$ among body, tail, primary and secondary feathers of six individuals of black kite. The concentration pattern of detected compounds in different types of feathers of black kite is shown in figure S5 (a-d) whereas detection frequencies are shown in figure S1b. Detection frequencies for body and tail feathers were similar, but higher than wing (primary and secondary) feathers. The concentration trend of OCs in different feathers types was also found as ∑DDTs>∑PCBs>HCB>TN. Test results for significant differences of OCs among different feather types are presented in table 2.

Analysis of variance revealed that concentrations of ∑PCBs, ∑DDTs, TN differed significantly (p<0.01 for all three compounds) among body, tail, primary and secondary feathers. Whereas differences were non-significant for HCB (p=0.86), $\delta^{13}C$ (p=0.65) and $\delta^{15}N$ (p=0.64) among feathers types. Among the different feather types, body feathers were found with highest mean concentrations of ∑PCBs, ∑DDTs, TN and $\delta^{13}C$ whereas HCB was highest in secondary feathers (table 2). Based on the higher detected concentrations and detection frequencies, we believe that body feathers could be used as a most useful tool for future biomonitoring studies in predatory birds as suggested earlier by Jaspers et al., (2011). However, we urge for a more elaborate investigation in the future with larger sample sizes to confirm this. Eulaers et al., (2014b) suggested that relatively elevated concentrations of OCs in tail and body compared to primary feathers are mainly associated with preening activity and moult pattern of the barn owl. Further, external contamination through preening has also been suggested to alter the level of OCs in feathers (Jaspers et al., 2008; 2013). The specific moulting pattern of different feathers types in black kite is currently unknown. However, we predict that a higher influence of preening activity on body and tail feathers of black kites because of their proximity to preen gland or beak (Eulaers et al., 2014b) may be associated with higher concentrations of OCs in these feather types. It has been suggested that moulting pattern and age (Jaspers et al., 2011) as well as length and growth of feathers (Bortolotti et al., 2010) are key factors to describe the variations in OCs levels among different types of feathers. Besides these, various confounding factors such as differences in moulting strategy (Jaeger et al., 2013) as well as feeding and migratory habits (García-Fernández et al., 2013) of species can influence the levels of OCs in feathers. Although we have not evaluated any of the above mentioned factors in this study because of lack of information during data collection, we suspect that a combined effect of just mentioned factors may be responsible for differences in bioaccumulation patterns of OCs. In future studies, we suggest to further elucidate the factors responsible for differential accumulation of OCs among various feather types.

4. Conclusions

The present study is the first to report the levels of PCBs and OCPs in predatory birds of Pakistan. Various contributing factors explaining the intra- and interspecific differences in PCBs and OCPs levels in predatory birds were also evaluated. We concluded that PCBs and OCPs could easily be quantified in predatory birds using feathers, particularly body feathers because of their high detectability. Significant differences in concentrations of PCBs and some OCPs among different feathers types emphasize the need for appropriate feather choice in future toxicological studies. Compared to PCBs, concentrations of OCPs, particularly DDTs, were found higher in predatory birds reflecting the large scale historical and current application of pesticides in Pakistan. In general, PCB levels reported in predatory birds of Pakistan are found lower than those of European studies whereas OCPs are relatively comparable. To get a broader picture, we urge future research to investigate the significance of contributing factors influencing the levels of OCs using multiple species of predatory birds from a wide geographical range.

Conflict of interest

The authors declare no conflicts with any person or organization.

Supplementary information

All the supporting material cited in this manuscript is available in the online supplementary files.

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Table 1: Pairwise comparisons for PCBs, DDTs, HCB (ng g^{-1} dry wt.) and stable isotope values (‰) in tail feathers among different groups of birds based on ANOVAs. Means are shown under each variable whereas differences through multiple comparisons are illustrated through different alphabetic characters. Groups with a single different letter differ significantly from the other. Sample sizes in each group are given in parentheses.

		Test for significance			Mean concentrations and Significance levels (alphabets) among/between groups (Tukey-HSD)									
Test variable	**Compounds/SIs**	**df**	**F**	**P**	**BK (10)**	**WRV (12)**	**IV (9)**	**ESH (10)**	**CK (4)**	**RNF (2)**	**GH (6)**	**GC (4)**	**SO (10)**	**LO (6)**
Species	**δ13C**	9,60	6.14	**<0.01**	-20.49 AB	-17.54 B	-17.53 B	-20.89 AB	-20.34 AB	-24.73 A	-24.00 A	-21.23 AB	-17.20 B	-20.64 AB
	δ15N	9,60	8.28	**<0.01**	9.01 AB	9.57 ABC	11.51 BCD	8.58 A	8.33 A	8.51 A	12.18 CD	13.60 D	10.67 ABC	9.97 ABC
	∑PCBs	9,66	2.55	**<0.01**	2.05 B	0.45 B	0.57 B	3.56 AB	5.20 AB	8.41 A	4.10 AB	2.46 AB	2.04 B	2.74 AB
	∑DDTs	9,66	2.61	**<0.01**	12.97 B	2.03 B	6.15 B	647.98 A	355.03 AB	43.4 B	55.69 B	13.15 B	132.49 B	25.97 B
	HCB	9,66	1.80	0.08	0.11	0.10	0.06	5.86	0.11	0.29	0.09	0.05	0.14	0.18
Families					**Accipitridae (44)**		**Ardeidae (6)**		**Falconidae (6)**		**Phalacrocoracidae (4)**			**Strigidae (16)**
	δ13C	4,65	5.1	**<0.01**	-19.17 B		-24.00 A		-21.81 AB		-21.30 AB			-18.76 B
	δ15N	4,65	9.19	**<0.01**	9.58 C		12.18 AB		8.39 C		13.60 A			10.35 BC
	∑PCBs	4,71	3.24	**<0.01**	1.55 B		4.10 AB		6.27 A		2.46 B			2.30 B
	∑DDTs	4,71	0.29	0.88	152.91		55.69		251.15		13.15			92.55
	HCB	4,71	0.37	0.82	1.41		0.09		0.17		0.05			0.15
Trophic guilds					**Omnivorous (13)**		**Scavengers (21)**		**Carnivorous (32)**		**Piscivorous (10)**			
	δ13C	3,66	9.95	**<0.01**	-20.49 AB		-17.53 C		-20.23 B		-23.08 A			
	δ15N	3,66	12.91	**<0.01**	9.01 B		10.4 B		9.26 B		12.65 A			
	∑PCBs	3,72	4.45	**<0.01**	2.05 AB		0.28 B		3.44 A		3.45 A			
	∑DDTs	3,72	2.92	**0.03**	12.97 B		3.80 B		295.86 A		38.67 B			
	HCB	3,72	1.09	0.35	0.11		0.09		1.94		0.08			
Habitat					**Terrestrial (66)**		**Aquatic (10)**							
	δ13C	1,68	12.66	**<0.01**	19.36		-23.08							
	δ15N	1,68	27.35	**<0.01**	9.61		12.66							
	∑PCBs	1,74	1.19	0.27	2.17		3.45							
	∑DDTs	1,74	0.56	0.45	147.22		38.68							
	HCB	1,74	0.37	0.54	1.00		0.08							
Feeding regime					**Diurnal (60)**		**Nocturnal (16)**							
	δ13C	1,68	1.5	0.22	-20.04		18.77							
	δ15N	1,68	0.44	0.50	9.93		10.35							
	∑PCBs	1,74	0.01	0.91	2.34		2.31							
	∑DDTs	1,74	0.24	0.62	143.70		92.55							
	HCB	1,74	0.58	0.44	1.07		0.16							

Table 2: Tests for strength of significance for PCBs and OCPs (ng g^{-1} dry wt.) and stable isotope residues (‰) among different feather types of 6 individuals of black kites from Pakistan. Means are shown under each feather types whereas different alphabetic characters are used to illustrate significant differences through multiple comparison (Tukey-HSD) test.

	Test for significance			Mean concentrations and Significance levels (alphabets) among feather types (Tukey-HSD)			
Compounds/SIs	**df**	**F**	**P**	**Body**	**Tail**	**Primary**	**Secondary**
δ13C	3,20	0.56	0.65	-20.24	-21.55	-20.97	-22.32
δ15N	3,20	0.57	0.64	9.17	10.16	8.68	9.22
∑PCBs	3,20	5.13	**<0.01**	4.21 B	2.57 AB	1.48 A	1.92 A
∑DDTs	3,20	13.57	**<0.01**	27.57 B	12.17 A	7.68 A	9.97 A
HCB	3,20	0.24	0.86	0.13	0.14	0.14	0.16
TN	3,20	7.37	**<0.01**	0.05 B	0.03 AB	0.019 A	0.016 A

Table 3. Evaluation of factors governing OCs concentration differences based on Akaike's information criteria (AIC). Variables with lowest AIC (shown in bold) best explained the concentration of the respective compound. Significant differences (ANOVA) of compounds among/between tested variables are shown with bold p values.

		Species	Families	Trophic guilds	Food chain	Feeding regime	$\delta^{13}C$	$\delta^{15}N$
CB-105	**AIC**	116.41	**114.42**	116.93	115.58	115.87	118.00	119.38
	F, (p)	2.16 (0.08)	2.78 (**0.04**)	3.99 (**0.02**)	8.17 (**0.00**)	3.74 (0.06)	NC*	NC
CB-118	**AIC**	204.85	203.05	**198.85**	202.92	213.28	210.78	212.81
	F, (p)	2.85 (**0.01**)	4.51 (**0.00**)	5.51 (**0.00**)	11.53 (**0.00**)	0.85 (0.35)	NC	NC
CB-146	**AIC**	132.21	128.37	127.41	125.65	124.94	**124.24**	125.88
	F, (p)	0.88 (0.51)	0.82 (0.51)	0.60 (0.55)	0.00 (0.99)	1.56 (0.21)	NC	NC
CB-153	**AIC**	226.48	240.69	**223.07**	245.41	251.33	249.54	250.66
	F, (p)	2.67 (**0.01**)	2.88 (**0.02**)	4.05 (**0.01**)	1.32 (0.25)	0.07 (0.79)	NC	NC
CB-138	**AIC**	209.73	214.53	**202.81**	212.32	215.32	214.25	215.42
	F, (p)	1.56 (0.15)	1.38 (0.25)	2.66 (**0.05**)	1.10 (0.29)	0.36 (0.55)	NC	NC
CB-187	**AIC**	114.26	108.47	108.06	**106.07**	107.79	107.69	107.69
	F, (p)	1.27 (0.29)	1.91 (0.14)	1.14 (0.32)	1.24 (0.27)	0.01 (0.89)	NC	NC
CB-183	**AIC**	84.34	**82.36**	82.79	83.89	84.09	84.07	84.05
	F, (p)	2.07 (0.10)	2.85 (**0.05**)	1.34 (0.27)	0.72 (0.40)	0.21 (0.64)	NC	NC
CB-128	**AIC**	154.67	149.27	148.62	147.42	**145.59**	146.78	148.07
	F, (p)	0.56 (0.77)	0.75 (0.56)	0.24 (0.78)	0.32 (0.57)	2.03 (0.15)	NC	NC
CB-156	**AIC**	87.59	86.16	87.16	86.22	86.81	**84.05**	85.09
	F, (p)	3.41 (**0.02**)	2.45 (0.08)	0.76 (0.47)	0.01 (0.90)	0.75 (0.39)	NC	NC
CB-180	**AIC**	218.61	215.15	**213.21**	216.7	216.9	214.6	216.82
	F, (p)	1.46 (0.18)	1.45 (0.22)	2.51 (0.06)	1.01 (0.31)	0.12 (0.72)	NC	NC
CB-170	**AIC**	202.26	196.43	196.79	197.19	197.07	**192.91**	196.50
	F, (p)	1.40 (0.21)	1.58 (0.18)	1.99 (0.12)	0.72 (0.39)	0.00 (0.95)	NC	NC
CB-199	**AIC**	66.40	64.51	**63.31**	**63.31**	66.57	66.66	66.66
	F, (p)	1.13 (0.36)	1.78 (0.19)	1.40 (0.25)	1.40 (0.25)	0.24 (0.62)	NC	NC
CB-196/203	**AIC**	139.60	135.90	136.15	134.23	134.57	**133.85**	134.52
	F, (p)	1.21 (0.32)	1.38 (0.26)	1.55 (0.22)	0.84 (0.36)	0.21 (0.64)	NC	NC
CB-194	**AIC**	149.63	**149.22**	150.14	153.12	153.52	150.73	153.69
	F, (p)	2.50 (**0.03**)	2.90 (**0.04**)	2.11 (0.10)	0.72 (0.40)	0.47 (0.49)	NC	NC
∑PCBs	**AIC**	**217.74**	273.26	238.12	279.91	283.48	282.77	282.57
	F, (p)	2.55 (**0.01**)	3.24 (**0.01**)	4.45 (**0.00**)	1.19 (0.27)	0.01 (0.91)	NC	NC
TN	**AIC**	71.03	71.03	**69.10**	75.06	74.02	75.90	80.23
	F, (p)	4.91 (**0.00**)	4.91 (**0.00**)	7.59 (**0.00**)	4.40 (**0.04**)	7.88 (**0.00**)	NC	NC
HCB	**AIC**	**207.83**	245.68	233.55	241.42	243.80	242.46	243.95
	F, (p)	1.80 (0.08)	0.37 (0.82)	1.09 (0.35)	0.37 (0.54)	0.58 (0.44)	NC	NC
p,p´-**DDE**	**AIC**	**272.43**	315.55	277.44	317.85	317.24	318.66	318.61
	F, (p)	2.60 (**0.01**)	0.21 (0.92)	2.71 (**0.05**)	0.45 (0.50)	0.26 (0.60)	NC	NC
p,p´-**DDT**	**AIC**	**268.20**	292.00	273.85	294.74	295.88	298.21	293.90
	F, (p)	2.67 (**0.01**)	1.51 (0.20)	3.15 (**0.03**)	1.10 (0.29)	0.10 (0.75)	NC	NC
∑DDTs	**AIC**	**263.33**	305.87	269.40	308.57	307.31	308.72	308.01
	F, (p)	2.61 (**0.01**)	0.29 (0.88)	2.92 (**0.03**)	0.56 (0.45)	0.24 (0.62)	NC	NC

* NC (not calculated) are shown where ANOVA could not be quantified

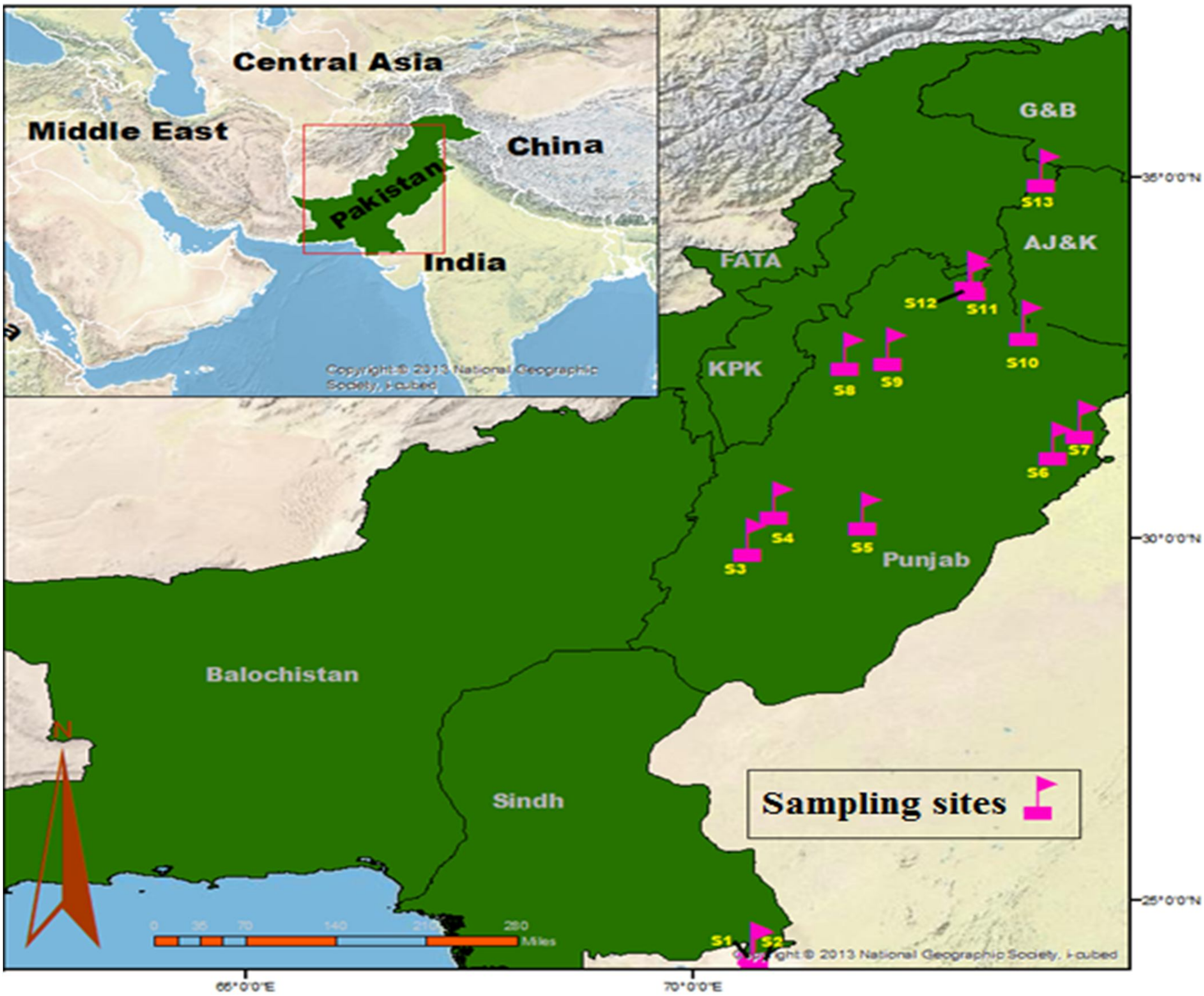


Figure 1. Map showing different sampling sites in Pakistan. The details of birds collected at different sampling sites are given in table S1 in the supplementary information.

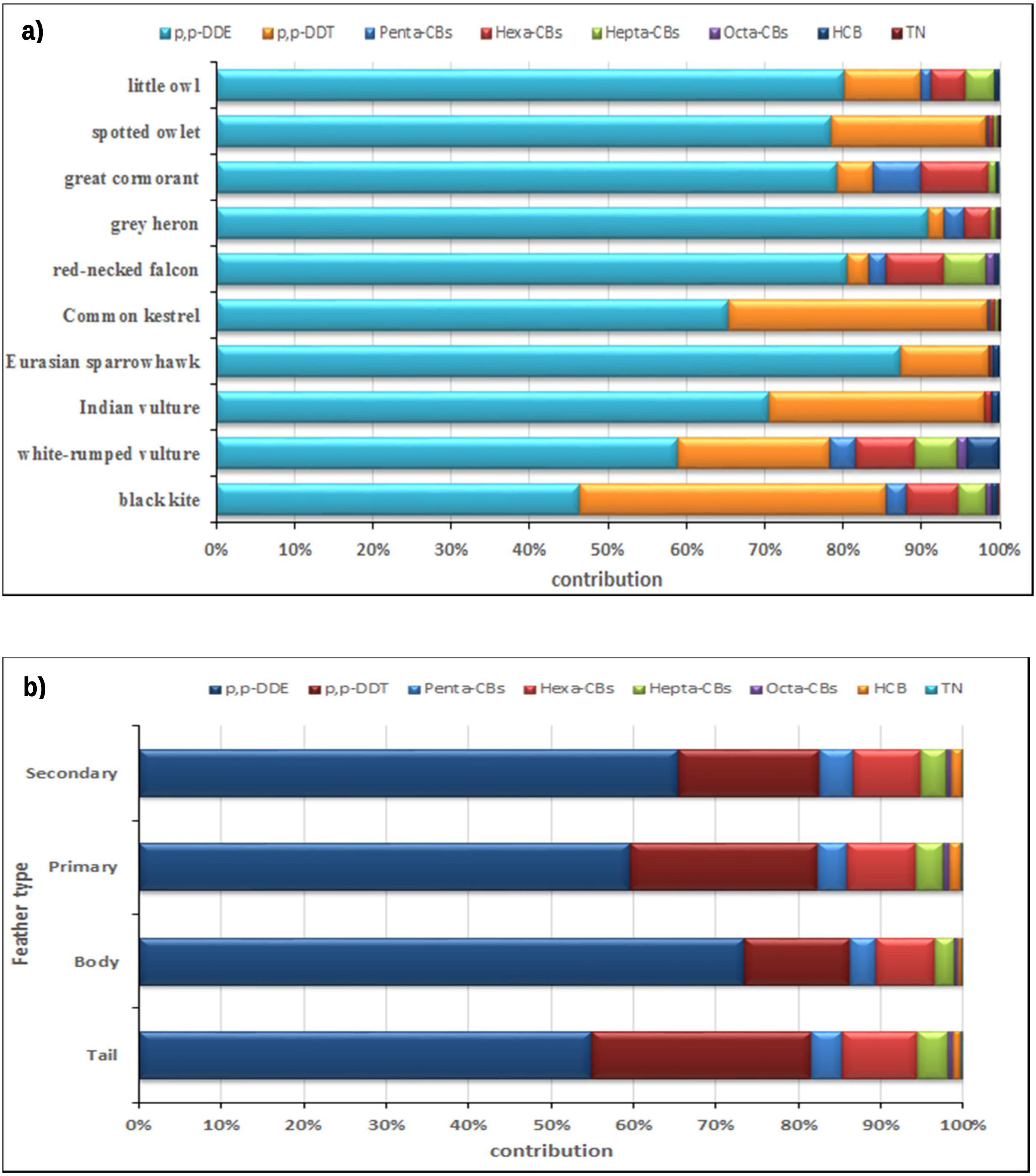
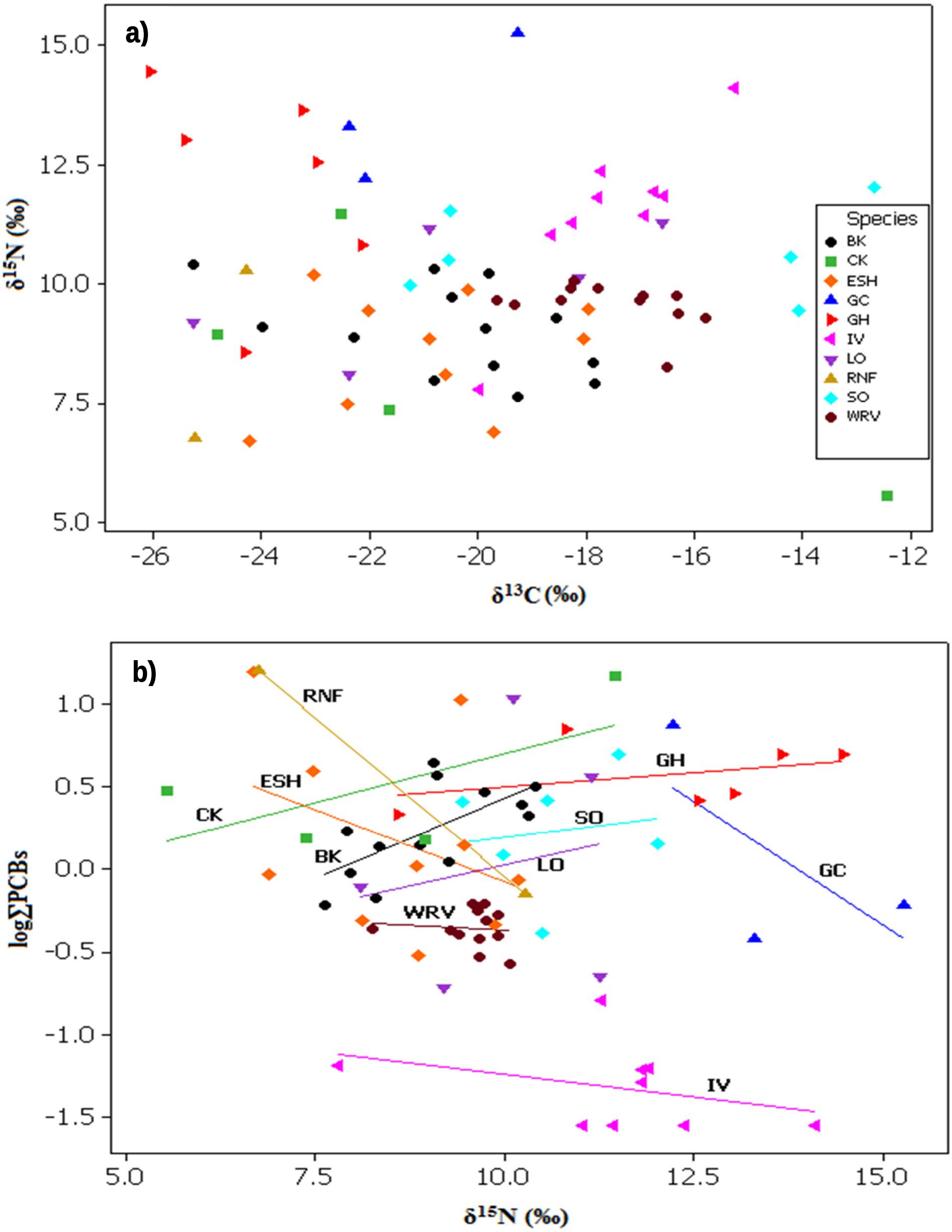


Figure 2: Contribution profile (percentage) of PCBs and OCPs in (a) tail feathers of predatory birds and (b) different feather types of black kite from Pakistan.


a)
15.0
12.5
10.0
7.5
5.0
δ15N (‰)
-26
-24
-22
-20
-18
-16
-14
-12
δ13C (‰)
Species
BK
CK
ESH
GC
GH
IV
LO
RNF
SO
WRV
b)
RNF
CK
ESH
GH
SO
BK
LO
GC
WRV
IV
1.0
0.5
0.0
-0.5
-1.0
-1.5
logΣPCBs
5.0
7.5
10.0
12.5
15.0
δ15N (‰)

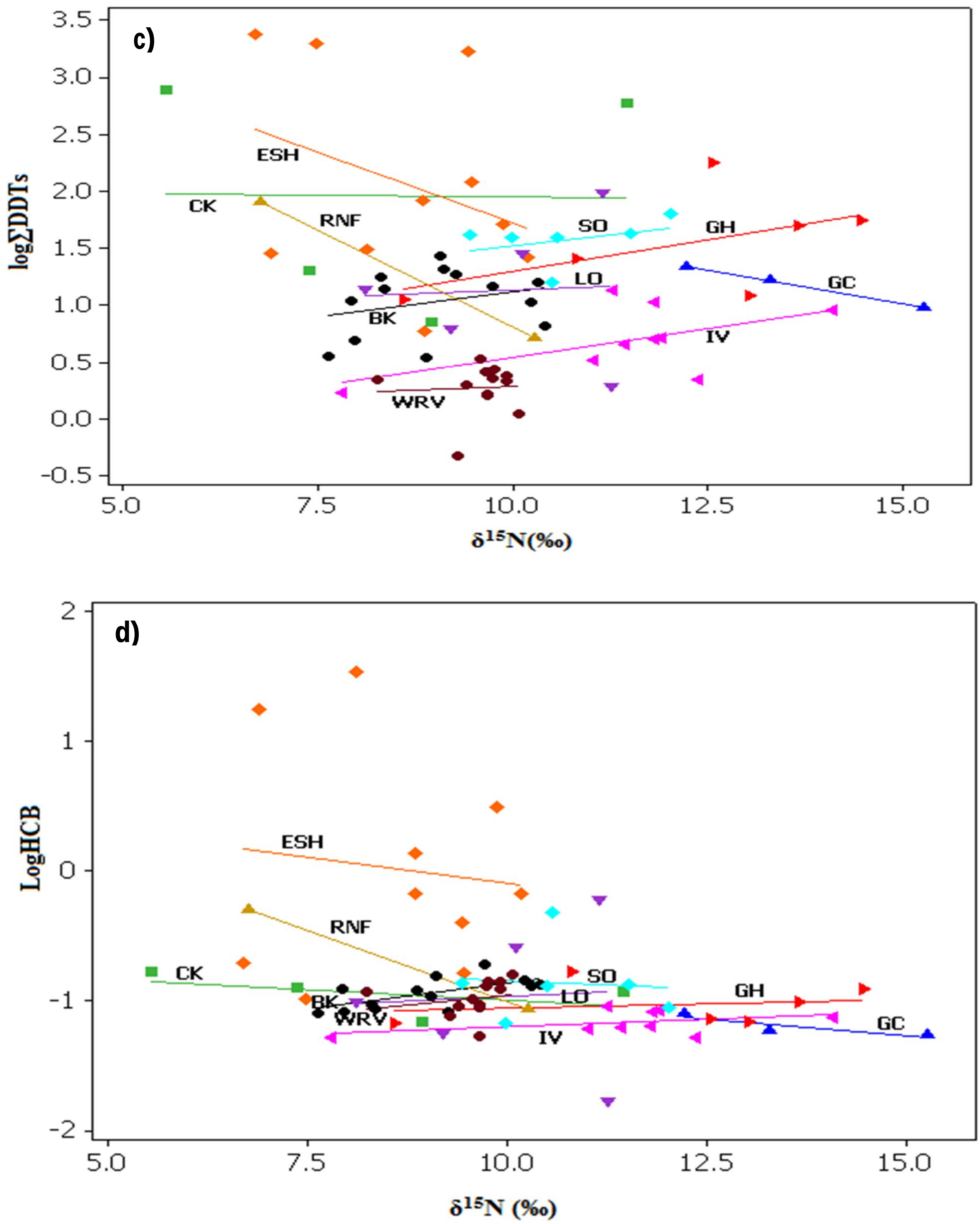


Figure 3. Scatter plots indicating interspecific differences in δ^{13}C and δ^{15}N values (a), and species-specific regressions of $\log_{10}$- transformed concentrations of ∑PCBs (b), ∑DDTs (c) and HCB (d) on stable isotope values. Species abbreviations are as follows: BK=black kite, WRV=white-rumped vulture, IV=Indian vulture, ESH=Eurasian sparrowhawk, GH=grey heron, RNF=red-necked falcon, CK=common kestrel, GC=great cormorant; SO=spotted owlet, LO=little owl