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Using an apex predator for large-scale monitoring of trace element contamination: Associations with environmental, anthropogenic and dietary proxies

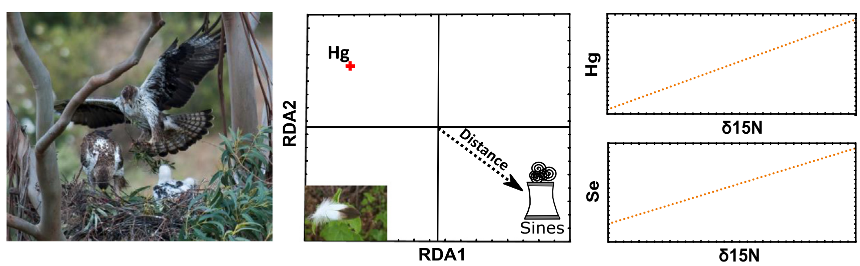
Alexander Badry [a,b,**], Luis Palma [d], Pedro Beja [d,e], Tomasz M. Ciesielski [a], Andreia Dias [d,f], Syverin Lierhagen [c], Bjørn Munro Jenssen [a], Nicolas Sturaro [g], Igor Eulaers [h], Veerle L.B. Jaspers [a,*]

[a] Department of Biology, Norwegian University of Science and Technology, Høgskoleringen 5, 7491 Trondheim, Norway
[b] Aquatic Ecology, University of Duisburg-Essen, Universitätsstr. 5, 45141 Essen, Germany
[c] Department of Chemistry, Norwegian University of Science and Technology, Høgskoleringen 5, 7491 Trondheim, Norway
[d] CIBIO/InBio, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Campus de Vairão, Vila do Conde, Rua Padre Armando Quintas 7, 4485-661 Vairão, Portugal
[e] CIBIO/InBio, Centro de Investigação em Biodiversidade e Recursos Genéticos, Instituto Superior de Agronomia, Universidade de Lisboa, 1349-017 Lisboa, Portugal
[f] Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals, Facultat de Biologia, Universitat de Barcelona, Diagonal 643, 08028 Barcelona, Catalonia, Spain
[g] Laboratory of Oceanology, FOCUS, University of Liège, B6C, 4000 Liège, Sart Tilman, Belgium
[h] Arctic Research Centre, Department of Bioscience, Aarhus University, Fredriksborgvej 399, 4000 Roskilde, Denmark

HIGHLIGHTS

- Trace elements and stable isotopes were assessed in Bonelli's eagle feathers
- Higher Hg exposure was found closer to the coal-fired power plant of Sines
- Hg concentrations were above a reported toxicity threshold for raptors around Sines
- $\delta^{15}N$ indicated biomagnification of Hg and Se in the terrestrial food web
- As, Pb, Cr, Cu & Zn levels were mostly low and unrelated to anthropogenic activity

GRAPHICAL ABSTRACT



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ABSTRACT

Understanding the levels and drivers of contamination in top predators is important for their conservation and eventual use as sentinels in environmental monitoring. Therefore, metals and trace elements were analyzed in feathers of Bonelli's eagles (*Aquila fasciata*) from southern Portugal in 2007–2013, where they are believed to be exposed to a wide range of contamination sources such as agricultural land uses, urban areas, active and abandoned mines and a coal-fired power plant. We focused on concentrations of aluminum (Al), arsenic (As), copper (Cu), chromium (Cr), mercury (Hg), lead (Pb), selenium (Se) and zinc (Zn), as these contaminants are potentially associated with those sources and are known to pose a risk for terrestrial vertebrates. Stable isotope values of nitrogen ($\delta^{15}N$: $^{15}N/^{14}N$), carbon ($\delta^{13}C$: $^{13}C/^{12}C$) and sulphur ($\delta^{34}S$: $^{34}S/^{32}S$) were used as dietary proxies to control for potential effects of prey composition on the contamination pattern. The spatial distribution of potential contamination sources was quantified using geographic information systems. Concentrations of Hg in the southern part of the study area were above a reported toxicity threshold for raptors, particularly in territories closer to a coal-fired power plant at Sines, showing that contamination persisted after a previous assessment conducted in the 1990s. Hg and Se levels were positively correlated with $\delta^{15}N$, which indicates biomagnification. Concentrations of As, Cr, Cu, Pb and Zn were generally low and unrelated to mining- or industrial activities, indicating low

* Correspondence to: V. Jaspers, Department of Biology, Norwegian University of Science and Technology, Høgskoleringen 5, 7491 Trondheim, Norway.
** Corresponding author.
E-mail addresses: a.badry@outlook.de (A. Badry), veerle.jaspers@ntnu.no (V.L.B. Jaspers).

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environmental background concentrations. Al was found at higher concentrations in the southernmost areas of Portugal, but this pattern might be related to external soil contamination on feathers. Overall, this study indicates that, among all elements studied, Hg seems to be the most important contaminant for Bonelli's eagles in southern Portugal, likely due to the power plant emissions and biomagnification of Hg in terrestrial food webs.

1. Introduction

Contamination with metals and trace elements like aluminum (Al), arsenic (As), cadmium (Cd), chromium (Cr), mercury (Hg), selenium (Se) and lead (Pb) can have a variety of adverse effects on wildlife and may be a cause of conservation concern for many species (Burger, 1993; Govind and Madhuri, 2014; Scheuhammer, 1987). Adverse effects may be particularly pronounced for toxic elements such as Hg, Se and As that bioaccumulate in wildlife and biomagnify through terrestrial and aquatic food webs, thereby potentially reaching high concentrations in apex predators (Barwick and Maher, 2003; Cristol et al., 2008; Palma et al., 2005). Therefore, monitoring and assessing the distribution of toxic elements in the environment is crucial to predict their potential effects, and informing risk management to prevent potential population declines of threatened species (Gall et al., 2015; Ortiz-Santaliestra et al., 2015; Palma et al., 2005).

Birds are considered useful bioindicators to achieve these goals, because of their long history in ecotoxicological research, their worldwide distribution and their well-known ecology and physiology (Jaspers et al., 2004). In this context, birds of prey are in general considered suitable sentinel species for environmental contamination, mainly because of their high position in food webs (Gómez-Ramírez et al., 2014). Furthermore, birds of prey often forage over relatively large territories and therefore may be particularly suited to indicate landscape scale contamination, as they integrate contamination sources over relatively large areas, which are used more or less exclusively by breeding pairs (Bosch et al., 2010; Fernández et al., 2009). In territorial species, the levels of contamination of each individual likely reflect the sources of contamination within its home range, as well as contamination that reaches the territory from external sources. Sources of potentially toxic elements from within the home range may be related to both, natural releases from eroding bedrock and human activities such as mining, waste disposal, agriculture or urban related emissions (Figueira et al., 2002; Gall et al., 2015). Finally, obtaining samples for estimating contamination in birds of prey is relatively straightforward, because like in other birds the feathers are known to be a reliable archive of contaminant exposure during the period of feather growth in several species (Burger, 1993; Jaspers et al., 2004; Palma et al., 2005). However, despite these advantages, only few studies have used terrestrial birds of prey to investigate the spatial distribution of multiple metals and trace elements in relation to anthropogenic land uses and other human activities.

Contaminant levels in birds of prey are affected by the composition of their diet, and this may confound the identification of contamination sources (Palma et al., 2005; Ruus et al., 2002). For instance, Bonelli's eagles (*Aquila fasciata*) feeding mainly on herbivores tend to have lower concentrations of Hg than those feeding more on insectivorous and omnivorous birds (e.g. corvids), irrespective of environmental contamination (Palma et al., 2005). Because of this, there is a need to control for the effects of diet on contamination exposure, which may be done directly through diet studies (Palma et al., 2005), but also indirectly through stable isotope-based investigations of the effect of diet on contaminant exposure (Eulaers et al., 2013; Eulaers et al., 2014; Kelly, 2000). For instance, the stable isotopes of nitrogen ($\delta^{15}N$), carbon ($\delta^{13}C$) and sulphur ($\delta^{34}S$) in tissue samples have been shown to provide useful proxies for the dietary plasticity of the studied species, such as the trophic position at which a given predator is feeding (Eulaers et al., 2013; Resano-Mayor et al., 2014), or the use of primarily aquatic or terrestrial food webs by a predator. Nitrogen stable isotopes are useful to estimate the species trophic position since consumers are typically enriched in the heavier isotope (^{15}N) by ~2.0 to 3.4‰ compared with the food they consume (Post, 2002; Vanderklift and Ponsard, 2003). The analysis of the stable carbon isotope ($\delta^{13}C$) can be used to determine the habitat origin (i.e. terrestrial vs. aquatic) of the respective prey since different photosynthesis mechanisms in aquatic and terrestrial plants (e.g. C3 vs. C4) result in different isotopic carbon patterns (Kelly, 2000). Additionally, the stable sulphur isotope ($\delta^{34}S$) can be used to discriminate between marine and terrestrial foraging habitats (Resano-Mayor et al., 2014; Resano et al., 2011).

In the present study, we analyzed 56 chemical elements (Table SI-1) in feathers of Bonelli's eagle collected in 80 territories in southern Portugal, where a previous study has found relatively high Hg contamination in feathers of this species, possibly related to emissions from a coal-burning power plant at Sines (Palma et al., 2005). That study also found strong dietary effects on contamination, with lower Hg concentration in feathers collected in territories where diet was dominated by herbivores such as rabbits (*Oryctolagus cuniculus*), red-legged partridges (*Alectoris rufa*) and pigeons (*Columba livia*), and higher concentrations where insectivorous and omnivorous birds such as corvids accounted for a large proportion of the diet (Palma et al., 2005). However, this study examined only Hg, although other contaminants may be important in the region as well. For instance, both active and abandoned mines may represent a major source of trace elemental pollution (Ferreira da Silva et al., 2004; Freitas et al., 2004), as As, copper (Cu), Pb and Zn have been found in high concentrations in soils in the southeast of Portugal (Freitas et al., 2004). To address these issues, the present study aims to: (i) assess the elemental concentrations in relation to thresholds considered potentially harmful to Bonelli's eagles; (ii) investigate to what extent the trophic position of the eagles influenced the elemental concentrations in feathers; and (iii) model the spatial variation in elemental concentrations in feathers in relation to the spatial distribution of potential contamination sources. Results were used to evaluate how contamination resulting from human land use and activities can potentially impact Bonelli's eagles and other top predators and also to evaluate the merit of wide-ranging predators for monitoring environmental contamination at large spatial scales.

2. Methods

2.1. Study area

The study was carried out in southern Portugal within an area of about 4×10^4 km^2 (Fig. 1), where there is a dense tree-nesting Bonelli's eagle population that has been increasing since the early 1990s (Dias et al., 2017). Human density is low throughout much of the area, with most population concentrated along the coast and in urban centers in the hinterland. The main potential sources of contamination include the petrochemical industrial complex of Sines (Fig. 1), whose coal-fired power plant was previously shown to affect Bonelli's eagles (Palma et al., 2005). There are also active and inactive mines, urban areas and intensive agricultural areas (Freitas et al., 2004; Freitas et al., 1999), as well as some major landfills (Fig. SI-1 and Table SI-2).

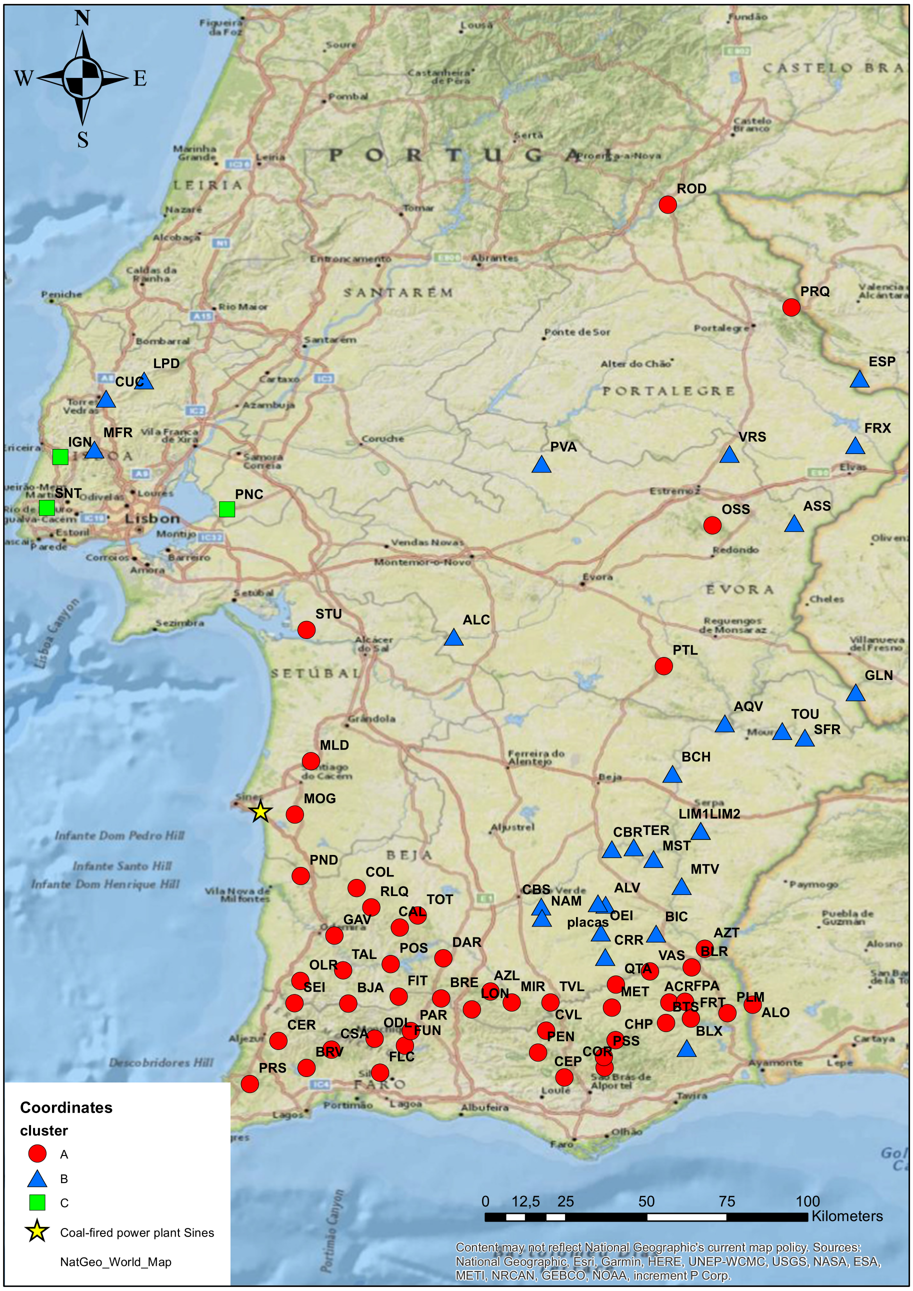

Fig. 1. Locations of the 80 Bonelli's eagle territories sampled in southern Portugal, categorized in three groups according to the dominant habitats (A = the southwestern uplands, B = the peneplain in the inland, C = urban dominated territories around Lisbon). Groups were formed using a cluster analysis based on environmental variables within a 10 km radius circle around nest sites. The star shows the location of a coal-burning power plant that was a main source of Hg contamination identified in previous studies.

2.2. Sampling procedure

Feather samples were collected between 2007 and 2013 from 80 Bonelli's eagle territories, corresponding to 83% of the 96 breeding pairs confirmed in 2013 (Palma et al., 2013). Feathers were collected around nest sites and adult roosts, where thorough searches were made for adult molt feathers (mostly ventral body feathers, Table SI-3). Searches were carried out during the nestling and post-nestling periods, mainly between May and September, with a small number in October (Table SI-4), in order to concentrate efforts when molt feathers were most likely to be found, while at the same time reducing risk of disturbance in the early breeding season. Different territories were sampled in different years, because some pairs did not breed every year, while others only settled in later study years of the expanding population (Dias et al., 2017). Furthermore, some of the nests were located in remote places, which were difficult to access every year. We believe that this is unlikely to have significantly affected the results, because land uses and contamination sources did not change much during the study period (Dias et al., 2017). In addition, a previous study showed a remarkable stability in diet composition within each territory during extended periods (Palma et al., 2006).

2.3. Elemental analysis

All feathers were washed sequentially with purified water (Milli-Q®), acetone (Sigma-Aldrich, ≥99.5%, GC grade) and 0.64 M nitric acid (Ultra-Pure grade) to remove external contamination as much as possible (Dolan et al., 2017). Subsequently, feathers were dried (Termaks

Series TS8000) in polypropylene vials covered by filter paper at 50 °C for 24 h. After determining dry weight, feathers were transferred into polyethylene ultraclave vials (PFA vessels, 18 mL) and 2 mL of nitric acid was added (50% v,v, Ultra-Pure grade, obtained by distillation with Milestone SubPur, Sorisole, BG, Italy) for a subsequent digestion in a high-pressure microwave system (Milestone UltraClave, EMLS, Leutkirch, Germany) with a maximum temperature of 245 °C at 110 bar for 2.5 h. After digestion, the samples were diluted with purified water to a final volume of 15 mL. Next, element concentrations were determined using a high resolution inductively coupled plasma mass spectrometry (HR-ICP-MS, Thermo Finnigan model Element 2 instrument, Bremen, Germany). Method detection limits (MDLs) were calculated using instrumental detection limits (IDL) as well as detection limits based on three times standard deviation of the blanks, and the higher value was selected as MDL. IDLs were estimated by the analysis of solutions containing a decreasing, low concentration of the respective element. The concentration which resulted in a relative standard deviation of 25% ($n = 3$ scans) was selected as IDL. Human hair (GBW09101b) was used as certified reference material to check for accuracy of the analysis. The concentrations found were within 95–117% of the certified values, except for Al with a recovery of 140%. The concentrations were not corrected for recovery.

2.4. Stable isotope analysis

The stable isotope ratios of carbon (C), nitrogen (N) and sulphur (S) of feathers were determined at the Laboratory of Oceanology of the University of Liège, using an isotope ratio mass spectrometer (Isoprime 100, Isoprime, UK) coupled in continuous flow to an elemental analyzer (vario MICRO cube, Elementar, Germany). Isotopic ratios were conventionally expressed as δ values in ‰ relative to the vPDB (Vienna Pee Dee Belemnite) for C, atmospheric N_2 for N and CDT (Canon Diablo Troilite) for S (Coplen, 2011). Certified reference materials from the International Atomic Energy Agency (IAEA, Vienna, Austria) used were sucrose (IAEA-C6, $\delta^{13}C = -10.8 \pm 0.5‰$; mean ± SD), ammonium sulfate (IAEA-N1, $\delta^{15}N = 0.4 \pm 0.3‰$; mean ± SD) and silver sulfide (IAEA-S1, $\delta^{34}S = -0.3 \pm 0.3‰$; mean ± SD). Hundreds of replicate assays of internal laboratory standards (powder of sulfanilic acid) indicate measurement errors (SD) of ±0.2‰ for $\delta^{13}C$, ±0.3‰ for $\delta^{15}N$ and ± 0.3‰ for $\delta^{34}S$. Samples from 15 territories could not be analyzed for their stable isotope composition because there was insufficient sample material available.

2.5. Environmental variables

Eight environmental variables describing land use compositions, human occupation, and distances to potential sources of contamination (Table SI-5) were used to model factors affecting regional distribution of elements. Variables were quantified within a buffer zone of 10-km radius (i.e. 314 km^2) around the nesting site of each territory, corresponding to areas presumably used by foraging eagles. Because there was no data on the size of the actual home range, the buffer zone was based on the average home range estimated from satellite telemetry data from 10 adult individuals from 10 contiguous territories (*L. Palma* Unpublished Data). The area occupied by each land use type ("habitat typology") within the buffer zone was estimated from the Corine Land Cover 2006 (Caetano et al., 2009; EEA, 2007), with land cover classes aggregated in five main categories (adapted from Kosztra et al., 2017): forest and natural vegetation; agriculture; pastures; water bodies; and artificial structures. Artificial structures refer mainly to industrial facilities and urban fabrics but also include other kinds of artificial structures such as airports (Table SI-6). We estimated human population density using a 100-m resolution population density grid of the European Union, which is based on the Eurostat 2001 population data disaggregated with Corine Land Cover 2000 (Gallego et al., 2011). We estimated road density and the density of buildings using OpenStreetMap data (Geofabrik, 2017). Regarding the potential point sources of contamination, we considered the industrial complex at Sines (Palma et al., 2005), the spatial distribution of landfills as well as active mines and abandoned mines (Matos and Rosa, 2001). We then computed the distance from the center of each territory to the nearest potential point source of each type. All variables were extracted using ArcGIS 10.6.0.8321 software by Esri (ESRI, 2018).

2.6. Selection of priority metals and trace elements

The present study focuses on eight elements and the selection is partly based on the Substance Priority List which prioritizes substances based on toxicity and potential for human exposure (ATSDR, 2017). The numbers in brackets indicate the position of the respective elements within the list: As (1) < Pb (2) < Hg (3) < Cr (17) (ATSDR, 2017). In addition, Zn (75) and Cu (118) were included as common mining related pollutants in the sampling region (Table SI-2). Se (146) and Al (183) were also investigated due to their potential toxicity in birds (Ohlendorf et al., 1989; Scheuhammer, 1987). Se is particularly important given its potential impact on Hg toxicity (Spiller, 2018). Therefore, the Hg/Se molar ratio was also calculated. An assessment of Cd (7) was not conducted because >50% of the samples had Cd concentrations below the detection limit.

2.7. Data analysis

Results below the detection limits for elements which have at least 50% detects were replaced with values estimated based on robust regression-on-order statistics (ROS) using the R package NADA (Helsel, 2005). Elements which were detected in <50% of the samples were excluded from further analysis. Environmental variables showing skewed distributions were transformed to approach normality and reduce the influence of extreme values. A $\log_{10}(x + 1)$ transformation was applied to latitude, altitude [m], pastures [m^2/km^2], salt and fresh water [m^2/km^2], artificial structures [m^2/km^2], human populations [pop/km^2], roads [km/km^2] and buildings [nr/km^2] as well as for all metals and trace elements [$\mu g\ g^{-1}$]. Stable isotope values and longitude were not transformed since their distribution approached normality. Prior to statistical analysis environmental and anthropogenic variables were scaled by subtracting the mean and dividing by the standard deviation to enhance comparability of effect sizes across variables measured in different scales.

To investigate whether the Bonelli's eagle territories belonged to different habitat typologies and whether these were associated with different contamination levels, a cluster analysis was performed based on environmental variables using the between-group linkage method in SPSS with squared Euclidean distance interval (Scott and Clarke, 2000). A generalized linear model with Gaussian distribution and identity link was performed to detect significant differences ($p < 0.05$) in trace element concentrations and stable isotope values among the clusters identified. We then investigated the main gradients in contaminant concentrations, by using principal component analysis (PCA) with the R package "FactoMineR" (Lê et al., 2008). To visualize the gradients spatially, we mapped the scores of each territory in the two main PC axes (eigenvalues >1) using ArcMap software (ESRI, 2018).

A partial redundancy analysis (RDA) was performed to investigate how variation in multiple metal and trace element contamination was related to environmental variables. To account for potential effects of dietary variations, a second RDA was performed including isotope values as covariables, thereby excluding territories without isotope data. The overall significance of the models was investigated using analysis of variance with 100,000 permutations. The significance of each constraining variable was investigated using the function "ordistep" with 100,000 permutations in the R package vegan (Oksanen et al., 2007). Variables significant at $P < 0.10$ were retained for subsequent model building.

Thereafter, a stepwise selection was performed to reduce the model and to assess the significance of each variable.

3. Results

3.1. Habitat typologies

The cluster analysis identified three main habitat typologies of Bonelli's eagle territories (Fig. SI-2), with a marked spatial pattern separating (A) the southwestern uplands, (B) the inland peneplain, and (C) urban dominated territories around Lisbon (Fig. 1). Habitat A aggregated territories located mainly in the hilly areas of south and southwest Portugal, with low human density and landscapes dominated by forested areas. Habitat B consisted mostly of territories located in the peneplain, as well as some to the north of Lisbon, encompassing areas with a larger representation of agricultural land uses. Finally, there was a habitat type including only three territories among those north of Lisbon (C), in areas with higher human population densities, and with more roads and built-up areas.

3.2. Variation of trace elements and stable isotopes among habitats

The concentrations of trace elements and stable isotope values per habitat cluster, as well as the overall values are given in Table 1. A list of the concentrations for elements that exceeded 50% detects and were not listed in Table 1 is given in Table SI-1.

For Al, Cr and Pb, the feather concentrations were significantly lower in habitat B compared to habitat C ($p \leq 0.05$). The concentration of Cr in habitat A was also significantly lower than in habitat C ($p \leq 0.05$). Zn concentrations were significantly lower in habitat A compared to habitat B, in contrast with the results obtained for Al and Pb. For As, Cu, Hg and Se, there were no significant differences among the three habitat typologies. The strongest correlation between elements was found for Al and Cr ($r = 0.99$, $p < 0.001$, Table SI-7). The $\delta^{15}N$ and $\delta^{34}S$ values were significantly higher in habitat C than habitat A ($p \leq 0.05$) whereas no significant differences were identified for $\delta^{13}C$.

3.3. Relation between stable isotopes and trace elements

The correlation matrix showing all significant correlations among trace elements and stable isotope values is given in Table SI-7. Both Hg ($p < 0.01$, $r^2 = 0.21$, Fig. 2A) and Se ($p < 0.01$, $r^2 = 0.19$, Fig. 2B) were positively related to $\delta^{15}N$. Additionally, there were significant positive associations between $\delta^{13}C$ and $\delta^{15}N$ ($r = 0.28$, $p < 0.05$) and between $\delta^{13}C$ and $\delta^{34}S$ ($r = 0.57$, $p < 0.001$, Table SI-7).

3.4. Spatial contamination trends and drivers

The PCA identified a dominant contamination gradient (PC1) that was mainly related to Al concentrations and accounted for 55.2% of the overall variation in contaminant concentrations (Fig. SI-3). This gradient did not show a marked spatial pattern, suggesting instead the presence of diffuse multiple point sources. The second gradient (PC2) accounted for 32.7% of variation in contaminant concentrations and was mainly related to the variation in Hg (0.31) and Hg/Se molar ratio (0.22), with higher values at several territories scattered through the study area (Fig. SI-4).

The first RDA model, including all 80 territories ($p < 0.01$) revealed significant effects of anthropogenic variables on the spatial gradients of metals and trace elements concentrations. The variables contributing significantly to these effects were the distance to the Sines power plant ($p < 0.01$), distance to active mines ($p < 0.01$), longitude ($p = 0.01$), artificial structures ($p = 0.02$) and a weak effect also of latitude ($p = 0.1$; Fig. 3). The first RDA axis was mainly related to variations in concentration of Hg and Hg/Se molar ratio, which were both positively associated with higher cover of artificial structures, smaller distances to the Sines power plant, and larger distances to active mines (Fig. 3). The second axis was mainly related to variation in Al concentrations, which were negatively related to distance to the Sines power plant and to longitude (Fig. 3).

After accounting for isotopic ratios as proxies of dietary differences, the second RDA produced broadly similar results (Fig. 4). The $\delta^{15}N$ ($p < 0.01$) was the only significant contributing isotope. Thus. $\delta^{13}C$ ($p = 0.84$) and $\delta^{34}S$ ($p = 0.48$) were excluded from further analysis. The RDA model controlling for variation in $\delta^{15}N$ was significant (p 0.01), and it underlined the significant effects of the distance to the Sines power plant ($p = 0.02$), distance to active mines ($p = 0.02$), and

Table 1
Mean concentrations (±SD, range; μg g^{-1} dw) of metal and trace elements and stable isotope values (‰) in adult molt feathers from 80 territories of Bonelli's eagle (*Aquila fasciata)* in southern Portugal. For each metal and trace element we present the mean per each of three territory clusters reflecting habitat typologies (Habitat A, B and C – see Fig. 1), the range of observed concentration in brackets as well as the overall mean. Significance testing was done using a generalized linear model with identity link on $\log_{10}$ transformed data. Significant differences are indicated in bold, and different letters annotate significant differences between the clusters A, B and C ($p \leq 0.05$).

	Habitat A	Habitat B	Habitat C	Overall
Al	312.75 ± 489.22 (21.36–2941.81)	**180.81 ± 217.35[C]** (19.55–931.76)	**820.11 ± 1005.46[B]** (196.29–1980.01)	285.93 ± 450.37 (19.55–2941.81)
As	0.07 ± 0.09 (0.02–0.65)	0.11 ± 0.14 (0.02–0.73)	0.15 ± 0.13 (0.05–0.3)	0.09 ± 0.12 (0.02–0.73)
Cu	4.77 ± 2.55 (2.4–19.21)	5.09 ± 1.64 (3.15–10.09)	4.85 ± 2.69 (2.79–7.89)	4.88 ± 2.26 (2.4–19.21)
Cr	**0.34 ± 0.44[C]** (0.04–2.61)	**0.23 ± 0.21[C]** (0.01–0.89)	**0.83 ± 0.89[AB]** (0.25–1.86)	0.32 ± 0.40 (0.01–2.61)
Hg	2.58 ± 2.76 (0.06–11.18)	2.18 ± 3.94 (0.06–17.78)	0.42 ± 0.27 (0.21–0.73)	2.36 ± 3.18 (0.06–17.78)
Pb	0.17 ± 0.19 (0.02–0.87)	**0.14 ± 0.12[C]** (0.03–0.47)	**0.40 ± 0.39[B]** (0.15–0.85)	0.17 ± 0.18 (0.02–0.87)
Se	0.80 ± 0.30 (0.35–2.31)	0.70 ± 0.26 (0.39–1.59)	0.83 ± 0.38 (0.51–1.25)	0.76 ± 0.29 (0.35–2.31)
Zn	**12.61 ± 6.81[B]** (5.06–43.80)	**14.95 ± 5.92[A]** (6.37–32.78)	12.15 ± 6.71 (7.94–19.89)	13.40 ± 6.53 (5.06–43.8)
$\delta^{15}N$	**8.1 ± 1.1[C]** (5.9–11.1)	8.3 ± 1.3 (6.6–12.0)	**12.5 ± 0.0[A]** (12.5–12.5)	8.3 ± 1.3 (5.9–12.5)
$\delta^{13}C$	−23.1 ± 1.9 (−27.8–(−19.2))	−23.0 ± 1.3 (−26.0–(−20.6))	−24.8 ± 0.0 (−24.8–(−24.8))	−23.1 ± 1.7 (−27.8–(−19.2))
$\delta^{34}S$	**6.9 ± 1.5[C]** (2.6–10.2)	6.9 ± 1.3 (4.0–9.0)	**3.9 ± 0.0[A]** (3.9–3.9)	6.9 ± 1.5 (2.6–10.2)

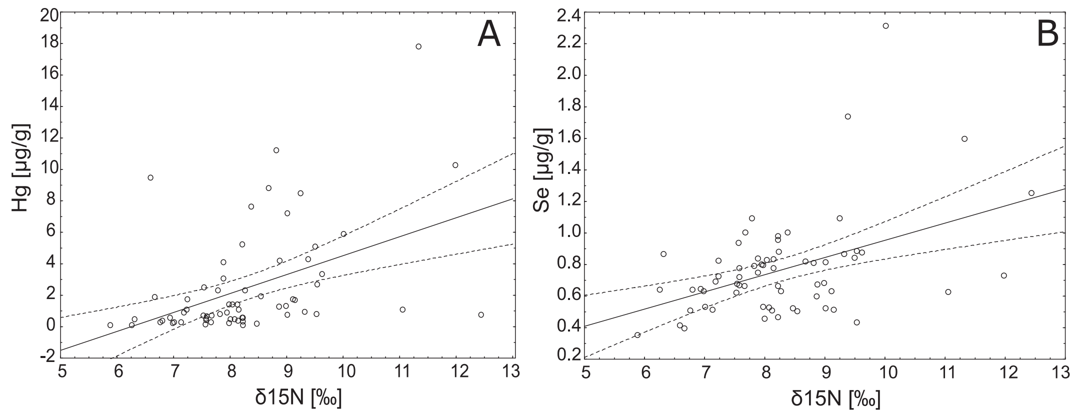

Fig. 2. Relationship inferred from a linear regression analysis between the concentrations of Hg (A), Se (B) and $\delta^{15}N$ values, using data from 66 Bonelli's eagle territories sampled in southern Portugal. A: $y = -7.52 + 1.20*x$; $p < 0.01$, $r^2 = 0.21$; B: $y = -0.14 + 0.11*x$; $p < 0.01$, $r^2 = 0.19$. Each panel presents the linear trend line and the corresponding 95% confidence intervals.

longitude ($p = 0.04$), and weaker effects of artificial structures ($p = 0.07$) and latitude ($p = 0.09$), on elemental feather concentrations (Fig. 4). The relationships observed were the same as for the first RDA without accounting for dietary differences.

4. Discussion

4.1. Contaminant concentrations

The concentrations of Hg detected in Bonelli's eagles from southern Portugal were particularly high and may potentially represent a threat to this species. In fact, 22.5% of the territories had concentrations in feathers above 4.1 $\mu g\ g^{-1}$ Hg, which was considered a threshold for potentially harmful effects in Bonelli's eagles (Palma et al., 2005). This threshold was found to correspond to a concentration in eggs of about 1.0 $\mu g\ g^{-1}$ (Palma et al., 2005), which may be associated with embryo malformations (Heinz and Hoffman, 2003), and thus contribute to negative effects at the population level (Scheuhammer et al., 2015). It should be noted, however, that a study of a top predator feeding on aquatic food webs, the bald eagle (*Haliaeetus leucocephalus*), reported no apparent reproduction impairment at feather Hg concentrations of 40 $\mu g\ g^{-1}$ (Weech et al., 2006). However, different biomagnification patterns of Hg in the terrestrial and aquatic environment as well as significant interspecific differences in sensitivity to Hg are expected (Scheuhammer et al., 2015; Wolfe et al., 1998). Therefore, continued monitoring and ecotoxicological analysis of Hg contamination in Bonelli's eagles would be strongly recommended. This is particularly important because in the south-western part of the study area the breeding pairs exceeding the critical threshold increased from only 2 out of 21 (9.5%) (Palma et al., 2005) to 14 out of 50 (28%) (habitat A, this study).

In contrast to toxic Hg, the overall concentration of Se in the Bonelli's eagle feathers detected in our study ($0.76 \pm 0.29\ \mu g\ g^{-1}$) was low, compared to background concentrations in bird feathers that normally range between 1 and 4 $\mu g\ g^{-1}$ (Ohlendorf and Heinz, 2011). Therefore, toxic effects to the eagles as well as a widespread Se contamination throughout the area seem unlikely (Mehdi et al., 2013; Ohlendorf

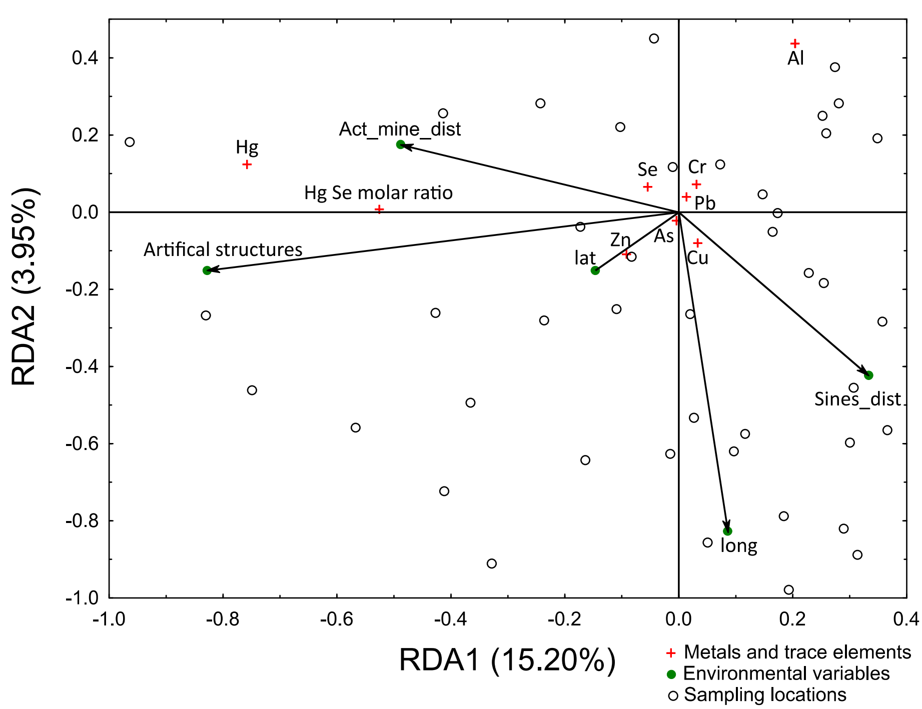

Fig. 3. Triplot of a Redundancy Analysis (RDA) showing the effects of environmental variables on the gradients in elemental concentrations recorded in molt feathers from 80 Bonelli's eagle territories in southern Portugal. Red crosses represent the eight metals and trace elements analyzed, black circles are the territories sampled, and vectors represent the effects of environmental variables. Act_mine_dist = distance to active mines; Artificial structures = proportional cover by fabrics and other artificial structures; lat = latitude; long = longitude; Sines_dist = distance to the coal-fired power plant of Sines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

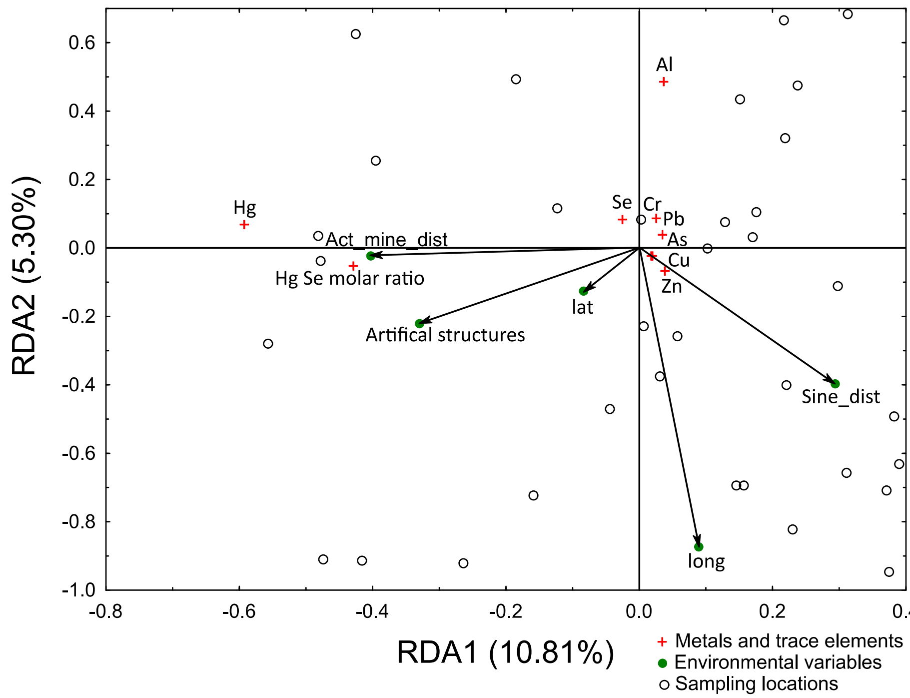

Fig. 4. Triplot of a Redundancy Analysis (RDA) showing the effects of environmental variables, after correcting for variation of $\delta^{15}N$, on the gradients in elemental concentrations recorded in molt feathers from 65 Bonelli's eagle territories in southern Portugal. Red crosses represent the eight metals and trace elements analyzed, black circles are the territories sampled, and vectors represent the effects of environmental variables. Act_mine_dist = distance to active mines; Artificial structures = proportional cover by anthropogenic and industrial structures; lat = latitude; long = longitude; Sines_dist = distance to the coal-burning power plant of Sines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 1986; Ohlendorf et al., 1989). However, Se has a protective function against the adverse effects of Hg, with a Hg/Se molar ratio above 1:1 being associated with increasing Hg toxicity (Berry and Ralston, 2008; Spiller, 2018). This is a cause of concern in our area, particularly in habitats A and B, where 21 out of 50 (42%) and 7 out of 28 (25%) territories, respectively, had Hg/se molar ratios >1. These results provide further support for the potential adverse effects of Hg contamination, especially in correspondence with generally low concentrations of Se.

The overall concentrations of As (0.09 ± 0.12 μg g^{-1}) and Pb (0.17 ± 0.18 μg g^{-1}) were relatively low, compared for instance with a study reporting contamination in passerines close to mining areas in Mexico (As: 2.12 μg g^{-1}; Pb: 36.28 μg g^{-1}, Monzalvo-Santos et al., 2016). Concentrations of Pb were also much lower than those reported in Bonelli's eagles in Spain (0.82 ± 0.44 μg g-1), where the ingestions of lead particles from injured small game prey seem to be pervasive (Gil-Sánchez et al., 2018). This suggests that a similar problem does not occur in southern Portugal, probably due to the higher consumption of domestic pigeons and other non-game species by Bonelli's eagles in Portugal compared with those in Spain (Gil-Sánchez et al., 2018; Palma et al., 2006). Little is known about the toxicity threshold of As concentrations in feathers, whereas for Pb a threshold value of 4.0 μg g^{-1} in feathers has been suggested (Burger, 1993). Therefore, widespread contamination as well as toxic effects caused by As and Pb on the Bonelli's eagles of southern Portugal seems unlikely.

Concentrations of Cr in the present study (0.32 ± 0.40 μg g^{-1}) were generally lower than concentrations reported in lichens (1.53 to 32.3 μg g^{-1}) and mosses (0.04 to 107.26 μg g^{-1}) (Figueira et al., 2002; Freitas et al., 1999), thus suggesting that Cr is not biomagnified through the food web of Bonelli's eagles. However, although, concentrations seemed to be rather low, relating Cr concentrations in feathers to adverse effects needs further investigation since the relationship is still poorly understood (Burger et al., 2015). This is important because although Cr is an essential nutrient involved in various metabolic processes, its hexavalent form is considerably toxic to birds (Gilani and Marano, 1979; Sahin et al., 2004).

The average concentrations of Zn (13.40 ± 6.53 μg g^{-1}) and Cu (4.88 ± 2.26 μg g^{-1}) were also relatively low in the study area, possibly reflecting natural background concentrations. For instance, Zn concentrations were lower than those reported on feathers of sparrowhawks (*Accipiter nisus*) from Belgium (23.8 to 48.4 μg g^{-1}, Dauwe et al., 2003). The same study reported concentrations of Cu (3.1 to 6.6 μg g^{-1}, Dauwe et al., 2003) similar to those observed in southern Portugal, which were lower than concentrations in feathers of different terrestrial bird species from a polluted urban area in India with high industrial activity (Manjula et al., 2015). Overall, therefore, the low concentrations observed in southern Portugal are unlikely to be a cause of concern regarding the potential toxicity for birds described for either Zn (e.g., reduced fertility and hatchability, Palafox and Hoa, 1980) or Cu (e.g. oxidative damage and neurodegenerative disorder, Gaetke and Chow, 2003).

In contrast to all the previous elements except Hg, the average concentration of Al observed in our study was relatively high (285.93 ± 450.37 μg g^{-1}), and higher than that reported for instance in sparrowhawks from Belgium (35.0 μg g^{-1} to 49.5 μg g^{-1}, Dauwe et al., 2003). Reasons for this are unknown, but they may reflect natural sources because Al is a common earth crust metal, but they may also reflect contamination sources such as electrical, automobile and metal industry (Holm et al., 2002). However, the high values could also be caused by external contamination of feathers unrelated to ingestion by eagles, due to its high concentrations in sediments (up to 18,700 ± 1992 μg g^{-1}) and difficulty in removing external contamination by commonly applied washing procedures (Borghesi et al., 2016, 2017). Therefore, further investigations would be needed to disentangle possible pollution patterns from external feather contamination.

4.2. Evidence for dietary effects on elemental concentrations

The average $\delta^{13}C$ value found in the Bonelli's eagles feathers (−23.1 ± 1.7‰) corresponds to carbon fixing terrestrial C_3 plants as main base of the food web, and indicates a dominant contribution of terrestrial prey (Kelly, 2000). This agrees with a previous study on Bonelli's eagle diet in southern Portugal, which showed a 95% contribution of terrestrial prey (Palma et al., 2005, 2006). The values of $\delta^{34}S$ (6.9 ± 1.5‰) were significantly correlated with those of $\delta^{13}C$ (r = 0.57, P < 0.001; Table SI-7), and they were significantly higher around Lisbon (habitat C) compared to the southwestern uplands (habitat A). This was unexpected, because $\delta^{34}S$ tends to be depleted in urban areas, a fact that is possibly related to industrial discharges and H_2S production due to resulting anaerobic and iron rich conditions (Morrissey et al., 2013; Tucker et al., 1999). Higher $\delta^{34}S$ values near Lisbon may also reflect the consumption of marine prey such as gulls (Morrissey et al., 2013; Ramos et al., 2013), but further investigation would be required because of the low number of territories in this area. Neither $\delta^{13}C$ nor $\delta^{34}S$ significantly contributed to the variation of trace elements, possibly because they mainly discriminate diets based on aquatic vs. terrestrial food webs (Eulaers et al., 2013; Ramos et al., 2013), while Bonelli's eagles mostly feed on terrestrial prey (Eulaers et al., 2013; Palma et al., 2006; Ramos et al., 2013).

In contrast to $\delta^{13}C$ and $\delta^{34}S$, variation in the values of $\delta^{15}N$ was informative regarding spatial variations in the diet of Bonelli's eagle, possibly related to the prevalence of secondary consumers. In fact, $\delta^{15}N$ provides information on the trophic position of a predator, as consumers are typically enriched in ^{15}N by 2.0 to 3.4‰ relative to their prey (Kelly, 2000; Post, 2002; Vanderklift and Ponsard, 2003). The $\delta^{15}N$ values observed in southern Portugal (5.9–12.5‰) were generally higher than those reported for Bonelli's eagle nestlings in Spain (3.6–8.2‰), which confirms the higher intake in Portugal of insectivore and omnivorous birds such as corvids (Palma et al., 2005; Resano-Mayor et al., 2014). There was also variation in $\delta^{15}N$ within southern Portugal, which is in line with major variation across territories in the dietary prevalence of secondary consumers found in previous studies (Palma et al., 2005; Resano-Mayor et al., 2014). The values of $\delta^{15}N$ were strongly correlated with concentrations of Hg (r = 0.45, P < 0.001) and Se (r = 0.44; P < 0.001), which agrees with previous studies showing their bioaccumulation along the food chain (Palma et al., 2005; Schneider et al., 2015), and thus reaching the high values in top predators such as the Bonelli's eagle that often feed on secondary consumers (Palma et al., 2005)

4.3. Environmental and anthropogenic drivers of feather contamination

The present study clearly shows that Hg concentrations increase with decreasing distance to the Sines coal-fired power plant, and with increasing density of artificial structures. The importance of Sines power plant as a the main regional source of Hg contamination was underlined by the previous study of Palma et al. (2005) on Bonelli's eagles, but is also supported by a study of Hg contamination in lichens (Freitas et al., 1999). The observed Hg emission shows contamination leeward of the coal-fired power plant (predominant winds from the Northwest) into an area of otherwise low human impact (habitat A and B, Fig. 1), in contrast to other parts of the study area, namely the urbanised area of Lisbon and the associated industrial belt (habitat C, Fig. 1). For Hg no significant difference was found among the habitats, which emphasizes the fact that Hg emissions are not a result of varying large-scale environmental gradients but rather are a result of multiple point sources. The observed increase of Hg concentration with increasing distance to active mines might be an artefact caused by the emissions of the coal-fired power plant and the geographic distribution of active mines leeward of Sines. Interestingly, since Se increases with decreasing distance to Sines, the power plant of Sines may also be a source of Se contaminations, which agrees with a previous suggestion about the sources of Se in southern Portugal (Freitas et al., 1999). However, since Hg is associated with emissions from the coal-fired power plant, higher Se concentrations might also be the result of an increased binding to Hg within the Bonelli's eagles although the Hg/Se molar ratio indicates that this is insufficient.

Al, Cr and Pb showed the highest concentrations in the urban areas of habitat C, which indicates urban related sources for those elements. This is supported by elevated concentrations of Pb and Cr in lichens and mosses in the vicinity of the metropolitan area of Lisbon (Figueira et al., 2002; Freitas et al., 1999), broadly matching with our habitat C. However, other indicators of urbanization factors such as density of roads, buildings, and human population, did not significantly contribute to the observed variation of any of the investigated elements, suggesting rather spatially restricted emissions from such sources. As and Pb were not associated with distances to active or abandoned mines in the present study, which indicates rather low contamination at the landscape scale from mining activities. There was also no association between As and artificial structures, which have been previously suggested to influence As concentrations of Bonelli's eagles from Catalonia (Ortiz-Santaliestra et al., 2015). Interestingly, the CVL territory shows on average 10 to 20 times higher concentrations of Cr (2.61 μg g^{-1}) than the neighboring territories, which indicates a point pollution source. Leachates from the Sotavento landfill (Algarve) might be the cause for the observed pattern because CVL is the territory encompassing the landfill. These Cr concentrations are close to values found in the Tejo estuary (PNC territory, 1.86 μg g^{-1}), an industrial impacted area indicating that landfills may represent further emission sources within the study area. Even though the present study suggests background concentrations of Cr for most of the territories, distinctive point pollution sources like leachates from landfills may cause serious threats to wildlife in southern Portugal. However, external contamination such as physical contact with water polluted by dump leachates needs to be considered since we found high correlations between Al and As (r = 0.58, p < 0.001, Table SI-7), Pb (r = 0.82, p < 0.001; Table SI-7) and Cr (r = 0.99, p < 0.001, Table SI-7). An assessment for the spatial distribution of Al shows a negative association with latitude and longitude, meaning an increase of Al concentrations towards the southwest of Portugal. However, identifying a potential emission source is difficult due to the high abundance of Al in soil and sediments (Borghesi et al., 2016) and the fact that background concentrations of the respective elements have not been considered in the present study. Nonetheless, restricted point pollution sources might be related to leachates from landfills as previously discussed for Cr since the highest concentration of Al was again found for the territory CVL (2941.81 μg g^{-1}) that overlaps the Sotavento landfill.

Cu and Zn mines are considered to represent an important source of heap, soil and sediment contamination in southern Portugal (Table SI-2), though there are other potential contamination sources such as steel production, domestic waste and urban runoff (Davis et al., 2001; Roney, 2005). However, Cu and Zn showed relatively low concentrations in feathers, and were not associated with mining activities. This suggests that these elements, although present in the mine surroundings and neighboring streams (Table SI-2), do not disseminate into the eagles' home ranges. To detect such local Cu and Zn contamination, eagles do not seem to be the appropriate biomonitors, because of their wide foraging areas. The use of more local foraging birds, e.g. passerines, may therefore need to be considered (Jaspers et al., 2004; Monzalvo-Santos et al., 2016).

Altogether, the large variation observed for some of the elements within the study area was probably related to some point pollution sources and industrial activity (but not mining) rather than large-scale environmental gradients. However, it should be noted that besides anthropogenic pollution, biological factors, like the age and sex of the eagles, along with the age of the molted feather (Borghesi et al., 2016, 2017; Jaspers et al., 2004) are may be responsible for a large part of the variation in elemental concentrations that could not be explained by our current analysis. Indeed, our RDA analysis could only explain <20% of the variation through inclusion of environmental, anthropogenic and dietary proxies. Therefore, variations due to biological factors (age, sex, condition) are considered to be of high importance, but may be difficult to correct for when studying adult free-living birds of prey, especially when collecting molted feathers, with the exception of sex that can be determined by molecular methods. In addition, external contamination on the feathers may be of concern for several elements (Borghesi et al., 2016, 2017), although this is likely of minimal importance for Hg (Burger, 1993). An option to limit the influence of these confounding factors is the sampling of nestlings (Eulaers et al., 2013, 2014), which however brings other constraints at this geographical scale such as permits, work intensity and costs.

5. Conclusion

Our study shows that in the study area, especially in the southwest uplands, Hg often exceeds the threshold value in feathers for which biological impacts are reported in the literature, especially considering that more than a decade has elapsed since a previous study within in the area. Emissions from a coal-fired power plant and industrial activities seem to be the main drivers for Hg emissions in southern Portugal, which has shown to be biomagnified together with Se along the eagles' food web. On the other hand, pollution from mining activities was more difficult to assess as they were not clearly associated with any of the investigated metals and trace elements, possibly because residues from mines do not disseminate beyond their close vicinity. Therefore, assessing the pollution impact of mines calls for biomonitors other than eagles, for example passerine birds. Observed concentrations of As, Pb, Cr, Cu and Zn were relatively low, and a widespread contamination of these elements seems unlikely in the study region. Furthermore, our results indicated that investigating $\delta^{15}N$ to control for diet in contamination studies may be enough for species such as Bonelli's eagles that feed predominantly on terrestrial prey. The inclusion of biological factors such as molt sequence, age and sex of the birds might further improve the current biomonitoring approach by accounting for individual variation. Taken together, the present study demonstrates the potential of a novel large-scale biomonitoring approach, which is capable of identifying sources of metals and trace elements in a terrestrial apex predator by combining environmental, anthropogenic and dietary proxies.

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Conflict of interest

None declared.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.04.217.

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