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Assessing environmental pollution in birds: a new methodological approach for interpreting bioaccumulation of trace elements in feather shafts using geochemical sediment data

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Abstract

1. Environmental trace element composition can have an important impact on ecosystem and population health as well individual fitness. Therefore carefully assessing bioaccumulation of trace elements is central to studies investigating the ecological impact of pollution. Colonial birds are important bioindicators since non-invasive sampling can easily be achieved through sampling of chick feathers, which controls for some confounding factors of variability (age and environmental heterogeneity). However an additional confounding factor, external contamination (ExCo), which remains even after washing feathers, has frequently been overlooked in the literature.
2. We developed a new method to reliably interpret bioaccumulation of 10 trace elements (As, Cd, Cr, Cu, Hg, Ni, Pb, Se, Sn, and Zn) in feathers using chicks of a colonial species: the Greater Flamingo, *Phoenicopterus roseus*. First, only shafts were used to remove ExCo retained in vanes. Second, we applied a thorough washing procedure. Third, we applied a new analytical method to control for ExCo, which assumes that ExCo is mainly due to adhered sediment particles and that the relative concentration of each trace element will be similar to sediment geochemical composition of sampling sites. We validated this new methodology by comparing trace element composition and particle composition (by scanning electron microscopy and mass-spectrometry) of washed and unwashed feathers.
3. The washing procedure removed < 99% of K indicating that most of the ExCo from salt was removed. Scanning electron microscopy and mass-spectrometry revealed that some sediment particles remained after washing, especially clays which are likely to severely bias bioaccumulation interpretation. We successfully controlled for ExCo by calculating the ratio of ExCo due to sediment using the geochemical fingerprint of sediment samples. Our methodology leads to conservative estimates of bioaccumulation for As, Cd, Cr, Cu, Hg, Ni, Pb, Se, Sn, and Zn.

4. We have validated a new more reliable method of analysing trace element concentrations in feathers, which effectively controls for ExCo, if geochemical sediment data can be meaningfully compared to ExCo of feathers. We have demonstrated that overlooking ExCo leads to potentially erroneous conclusions and we urge that the method applied in this study be considered in future studies.

Introduction

Most metals and trace elements are omnipresent in the environment as a consequence of natural processes and anthropogenic activities. Some of them play an essential role in biological processes (e.g. metabolism, neuronal functions). However, other elements (e.g. mercury, lead, cadmium, arsenic, etc.; Kabata-Pendias & Pendias 2001) may also exert detrimental, toxic effects on species if they accumulate in the food chain (Amaral *et al.* 2006) which will negatively affect fitness and life history traits of plants and animals, as well as cause diseases in wildlife and humans (Nriagu 1989; Järup 2003).

During the last centuries, the anthropogenic exposure level of trace elements has hugely increased after the industrialization era, especially in wetlands, which, in many cases, act as geochemical endpoints and tend to accumulate pollution (Reddy & DeLaune 2008). The total concentration of metals in soil and sediments persists for a long time because they do not undergo microbial degradation (Kirpichtchikova *et al.* 2006). It has been demonstrated that metals from anthropogenic inputs are often weakly associated to the finest fraction of the top layers of sediment and organic matter (e.g. Salomons & Förstner 1984; Palanques *et al.* 1995; Migani *et al.* 2015) and consequently tend to be much more bioavailable and bioaccumulable than the same elements of natural origin (Bryan *et al.* 1979; Di Giuseppe *et al.* 2014). Monitoring environmental metal contamination and investigating how organisms are affected by the excess of trace element intake or, more generally, the alteration of the natural geochemical profile is of central importance in evolutionary ecology and human and wildlife health. A prerequisite for such monitoring is to develop reliable methods to correctly measure metal exposure, intake, and bioaccumulation.

For several decades, birds have proven to be valuable biomonitors for various types of pollutants, including metals (Furness & Greenwood 1993). Ecotoxicological studies in the last three decades have frequently used feathers in order to assess metal accumulation in birds and feather analysis has proven to be a very informative tool to unravel various physiological, ecological and toxicological processes inherent to individuals and populations (Burger 1993; Smith *et al.* 2003; Tsipoura *et al.* 2008). An important advantage of feathers with respect to blood metal concentration is that feathers are relatively easy to collect, preserve, and transport and sampling is virtually harmless to birds (Burger 1993). Moreover, metal accumulation in feathers generally represents a longer-term contamination process, while levels in blood represent a recent contamination directly associated with feeding (Carvalho *et al.* 2013). Since concentration levels in feathers reflects the body accumulation during the entire time of feather development, potential age biases can be circumvented by restricting the analyses to chick feathers. However, external contamination (ExCo) has always challenged researchers and has often been overlooked (but see: Hahn *et al.* 1993; Fasola *et al.* 1998; Ek *et al.* 2004; Hollamby *et al.* 2006; Valladares *et al.* 2010; Borghesi *et al.* 2016). ExCo is defined as the part of the concentration that is not attributable to bioaccumulation in the keratin structure (i.e. metals stored during feather growth as an effect of internal bioaccumulation and metabolic processes, hereafter referred to as bioaccumulation for brevity). ExCo is normally attributed to atmospheric dust, water, or deposition of contaminants on feathers during preening (Dmowski 1999; Dauwe *et al.* 2002; Jaspers *et al.* 2004). However, a recent study on the Greater Flamingo, *Phoenicopterus roseus*, pointed out the major importance of sediment particles in complicating the interpretation of analytical results (Borghesi *et al.* 2016). Most of the previous field studies have tried to remove ExCo through washing, however to date no washing procedure is completely effective in ensuring the total removal of ExCo from feathers (Cardiel *et al.* 2011; Espín *et al.* 2014). Furthermore, so far no studies have tried to quantify the magnitude of ExCo and to consequently validate the bioaccumulation data of trace elements. To continue to use feathers as indicators of bioaccumulation of trace elements, it is important to improve the methodology by reducing the relevance of ExCo, and at the same time, find new methods for estimating more accurate data about bioaccumulated concentrations.

In our study, we adopted five measures for that purpose: 1) we used only shafts, because feathers deprived of vanes capture dirt less efficiently (Cardiel *et al.* 2011); 2) we sampled chicks, which avoids variability due to age; furthermore chicks have sediment particles of proven origin entangled in their plumage; 3) we used local geochemical information from sediments collected around nesting islets, in order to compare the local geochemical fingerprint to the element ratios in feathers (Borghesi *et al.* 2016); 4) we chose an extensive set of elements (14), including some of which are supposed to have little or no bioaccumulation and are useful to check for ExCo in the investigated sites as they are indicators of clays (i.e. Al and La) and other fine fractions of the sediment such as oxides and hydroxides (i.e. Fe), and salt (i.e. K). The comparison between sediment and feather concentrations has been performed by adopting a new method capable of estimating the relative importance of ExCo for each element and to correct the analytical result for ExCo. The aim of this study is to validate this new method.

In order to achieve this goal, we used the Greater Flamingo as a model species. The ecology and biology of this species are well known due to long term studies (Johnson & Cézilly 2007), a major advantage for an ecotoxicological study. The Greater Flamingo has a large breeding range including many important Mediterranean wetlands (Balkız *et al.* 2007), feeds mainly on small benthonic invertebrates by filtering sediments of brackish wetlands and saltpans. During feeding it can ingest a considerable quantity of sediments from which the organic matter contained therein is digested as a component of diet (Jenkin 1957). Their particular feeding behaviour leads flamingos to be directly exposed to polluted sediments. In addition, flamingos feed their chicks with a liquid secreted from the upper digestive tract, rich in proteins, fat, carotenoids, blood cells, and, as a consequence, with part of the pollutants previously bioaccumulated and metabolized (Lang 1963;

Fisher 1972). All of these reasons make greater flamingo chicks a good choice among birds as an environmental indicator of the effect of trace element accumulation in Mediterranean wetlands (Borghesi *et al.* 2011, 2016). However, from the age of 3 weeks old, chicks form a large crèche in the muddy and brackish wetland near the vicinity of the breeding islet (Johnson & Cézilly 2007), leading to high exposure to local environmental elements. Therefore, as highlighted by Borghesi *et al.* (2016), ExCo can dominate trace element concentration of Greater Flamingo chick feathers.

Methods

Sample collection

All of the feathers from flamingo chicks were collected between July and August 2014, during the ringing operations in three breeding colonies of the western Mediterranean: Aigues-Mortes (AIG), southern France (N 43° 33’, E 4° 11’); Fuente de Piedra (FDP), southern Spain (37° 06’N, 04° 45’W) and the heavily polluted Odiel marshes (ODI) (Guillén *et al.* 2011), southern Spain (37º 17′ N, 06º 55′ W) (Figure 1). All of the sampled birds were between 5 and 8 weeks old (Johnson & Cézilly 2007). Ten feathers were obtained by cutting the distal part from random individuals using stainless steel scissors. We selected the longest internal scapulars that were protected from aerial deposition (Borghesi *et al.* 2011, 2016). Feathers were kept in envelopes at room temperature until analysis. In addition for each sampling site we collected seven sediment samples of 200-500g within and on the reeve of the water body where the breeding islet was situated. Each of the 21 sediment samples was kept in plastic containers in dry room temperature conditions prior to analysis.

Sample preparation and analysis

We chose to analyse 14 elements in both sediments and feathers. Ten elements were chosen because of environmental concern: As, Cd, Cr, Cu, Hg, Ni, Pb, Se, Sn, and Zn (ATSDR 1994; Hamasaki *et al.* 1995; Hamilton 2004; Cempel & Nikel 2006; Stern 2010; Tchounwou *et al.* 2012; Walters *et al.*

2014; Herrmann *et al.* 2016). Aluminium, Fe, K and La were chosen as indicators of clay and the finest fraction of sediment (Leeder 1982).

a. *Sediments*

Digestion and trace analysis of sediment samples was carried out by ACME Labs, Vancouver (Canada). Samples were digested with a modified *aqua regia* solution of equal parts concentrated in HCl, HNO_3 and DI-H_2O for one hour in a heating block within a hot water bath. Digestion of sediments was done using a modified *aqua regia* solution from ACME labs in order to compare sediment element concentration to element concentration obtained by nitric-chloridric acid digestion of organic material. Indeed the acidic solution of both methods should have a similar dissolving effect on samples (whether sediment or biological). The modified *aqua regia* solution was chosen since it is even more similar to the solution used for feather dissolution than the stronger original *aqua regia* solution (3HCl:1HNO_3). This sediment digestion method has previously been successfully used when analysing feathers in previous studies (Borghesi *et al.* 2016). Each sample volume was equalised with diluted HCl. The concentrations of 64 chemical elements (Ag, Al, As, Au, B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Dy, Er, Eu, Fe, Ga, Gd, Ge, Hf, Hg, Ho, In, K, La, Li, Lu, Mg, Mn, Mo, Na, Nb, Nd, Ni, P, Pd, Pr, Pt, Rb, Re, S, Sb, Sc, Se, Sm, Sn, Sr, Ta, Tb, Te, Th, Ti, Tl, Tm, U, V, W, Y, Yb, Zn, Zr) were determined by Inductively coupled plasma mass spectrometry (ICP-MS). To evaluate the analysis quality, an Internal Reference Material (IRM), named DS10, with a composition similar to our sediment samples, was used. Only the 14 trace elements analysed in feathers are considered in this study (Al, As, Cd, Cr, Cu, Fe, Hg, K, La, Ni, Pb, Se, Sn, and Zn). As for feathers, concentrations in sediments are expressed in mg/Kg.

b. *Feathers*

Vanes were manually separated from shafts by keeping fingers of one hand on the feather tip and then detaching each vane by pulling from the top to bottom with the other hand. Subsequently, the 2 mm distal portion (which still had some tiny barbs) was cut off. This method allowed us to obtain rachides completely deprived of barbs and the cuticle connecting barbs to the shaft. From each specimen, five rachides were prepared, in order to reduce variability between feathers and obtain enough feather weight per sample.

Thirty-nine samples in AIG and 40 samples in FDP and ODI (in total 119 samples, corresponding to 595 rachides) were thoroughly washed through three steps by sequentially using acetone, Triton X(™) detergent, and deionized water. During each step, washing sonication was performed for 20 minutes. After washing, feathers were dried in a dry box at room temperature. From this point forward we now refer to the latter feather samples as “washed feathers”.

In order to test the effect of washing on feather trace element composition, we duplicated 10 individuals from each site (30 of the 119 individuals in total). For this treatment, five rachides from each individual (a total of 150 rachides) of similar weight were directly sent to the digestion process described below (i.e. no washing procedure was performed prior to digestion). From this point forward we now refer to the latter feather samples as “unwashed feathers”.

All samples (approximately 0.100 g from each sample) were digested and analysed at the Trace Element Analysis Core Laboratory of Dartmouth College, Hanover, NH, USA. Digestion was carried out in 0.5 ml acid mixture (9:1 HNO_3:HCl) and then diluted to a final volume of 10 ml with deionized water in polypropylene tubes. Digestion was performed with open polypropylene vessels in a microwave digester reaching a temperature of 105°C (Beck et al. 2013). Similar digestion methods have previously been used to analyse feathers (Latta et al. 2015) and toenails (Amaral et al. 2012; Davis et al. 2014; Punshon et al. 2015; Freeman et al. 2015). Total concentration of 14 trace elements (Al, As, Cd, Cr, Cu, Fe, Hg, K, La, Ni, Pb, Se, Sn, and Zn) were measured by Agilent 8800 ICP-MS. QA/QC was evaluated by adding to the batches: blanks (frequency: one every 25 samples); six samples of oyster, tomato, and hair Standard Certified Materials (2 of each type). Matrix duplicates and matrix duplicate spikes were also digested and analyzed (frequency: 1 every 21 and 19 samples, respectively), and fortified blanks added in batches (frequency: 1 every 50 samples). Additional QC consisted of reporting calibration checks and blanks (see Supplementary Materials Tables S1-S8). The average recovery of the separate digestions from the National Institute of Standards and Technology (NIST 1566b, 1573a, NIES #13), for As, Cd, Cu, Fe, Pb, Se, and Zn was around 100%, for K was 117.5% (SD 11.5%), and, for Hg, La and Ni was around 80% (see Supplementary Materials Table S4). For Al and Cr recovery averaged around 30% presumably because these metals were in a form that is not solubilized by the open vessel acid digestion used here (Beck et al. 2013). Tin analyzed in the feather samples was not certified in the NIST standards. Since QA/QC of all trace elements were very good and hair was the most comparable certified reference material (CRM) with feathers, Al and Hg concentrations were corrected using the hair CRM whereas Cr, K, La, and Ni for the other available CRMs (tomato and oyster), since no reference values for these metals were available for hair. Concentrations in feathers are expressed in mg/Kg dry weight (dw). Method detection limits (MDLs) were calculated as three times the standard deviation of the average value of the 6 calibration blanks, and based on a sample weight of 100 mg. Limits of quantification (LOQs) have also been calculated as 3 times the MDL (see Supplementary Materials Table S8).

Examination of feather with Scanning Electron Microscopes (SEM)

In order to make morphological observations of external particles and possibly infer the nature of external contaminants, shaft segments 1 cm long have been scanned with a Jeol JSM-5400 Multi-Purpose Digital SEM equipped with WDS and EDS Systems at University of Bologna, Department of Biological, Geological, and Environmental Sciences (BiGeA). Six feather shafts were selected from each site (18 feather shafts in total), and prepared for SEM without any washing treatment (i.e. the same treatment as unwashed feathers). In addition, 3 feather segments from ODI were scanned after a thorough cleaning procedure with tap water, a commercial detergent, and acetone (i.e. substantially the same treatment as washed feathers but without sonication).

Correction of element concentrations in feathers for environmental contamination

Feathers, even washed, retain a certain quantity of sediment (see results). For the sake of argument, if we assume that all bioaccumulation is masked by ExCo, analytical results from chick feathers should tend to represent the geochemical characteristics of local sediments instead of the actual assimilation and accumulation in keratin structure of trace elements. If so, the relative abundances of elements in sediment and feathers should be similar. In contrast, if elements are mostly bioaccumulated then they should be in a higher concentration than expected if the chemical fingerprint of feathers is only determined by ExCo. Using the 14 elements analysed in this study, and investigating the ratios between concentration in feathers and sediment, we can check which elements in feathers are clearly enriched with respect to expected ExCo concentrations.

By investigating sediment element concentration, we are able to infer what the predicted concentration of feather elements would be if ExCo was 100% (predicted external contamination; PExCo) for each element. Here a reference element which indicates ExCo needs to be carefully chosen. The reference should be an element that: 1) is analytically reliable, 2) that is dominant in the source of ExCo (in our case soil and sediment) and 3) that is either negligibly or not bioaccumulated (i.e. concentrations are dominated by ExCo). A previous study (Cardiel *et al.* 2011) has suggested that Al is a good indicator of ExCo because it is known to be scarcely metabolized by birds (Beyer *et al.* 1999) and it is a main component of clays and hydroxides (Moore & Reynolds 1989). However, we found that Al is extracted in smaller concentrations by the acid digestion step than most of the other elements (see above). As a consequence a certain amount of ExCo of elements will be overlooked when using Al as the geochemical reference even if corrected using the CRM and, analytically, Al is not a sufficiently reliable element to be used as a reference element. In contrast Fe is well recovered by the methods applied in this study (see Supplementary Materials Tables S1-S8 for QA/QC results), it is reported to be only negligibly bioaccumulated in shafts of seabirds (Howell *et al.* 2012) and it represents a wider gamma of compounds in sediments than Al and La (Reddy & DeLaune 2008). Finally, it is important to note that a small amount of Fe maybe bioaccumulated, which means that we are actually using a conservative approach and may be slightly overestimating ExCo. For sound biological interpretation the latter is highly preferable than ignoring ExCo and reporting highly inflated bioaccumulated values. However we found a strong correlation between Al, Fe and La concentration in washed feathers, further suggesting that ExCo dominates bioaccumulation for these elements (see Supplementary Materials Figure S1-S4) (Borghesi *et al.* 2016). For all of the aforementioned reasons we chose to infer feather PExCo using Fe. We calculated the PExCo of feathers as:

$$PExCo_i = \frac{x_i y_j}{z_j}$$

where x_i is the concentration of Fe in the feather sample i and y_j is the concentration of the element studied in sediment at the breeding colony j and z_j is concentration of Fe in sediment at breeding colony j. From PExCo, we can deduce the proportion of element concentration found in feather that is due to ExCo (external contamination factor; ExCoF):

$$ExCoF_i = \frac{PExCo_i}{w_i}$$

where w_i is the element concentration of interest of the feather sample i. Using these two simple equations we estimated, for each feather, the proportion of ExCo for each element within each breeding colony site. For pedagogical reasons, we also applied the above equations to median feather concentrations for each breeding site and intervals which encompass 95% of the data (i.e. x_i and w_i are median values or 95% intervals of each element for each breeding site instead of for each individual feather). For each feather, we were then able to correct element concentration for ExCo by using the following formula:

$$corrected\ w_i = w_i - (ExCoF_i * w_i)$$

Statistics

All statistics were carried out in R version 3.2.4 (R Core Team 2016). To investigate the effect of washing of feathers on element concentrations we applied a paired Wilcoxon-Pratt signed-rank test

(Pratt 1959) between element concentrations for feathers that were not washed and for feathers that were washed (n = 30). We calculated r as a measure of effect size which is the z-value divided by the square root of the sample size (in our case 30; Pallant 2007). An r value between 0.1-0.3 is considered as small, a value between 0.3-0.5 to be medium and finally any value above 0.5 is considered as large. Median differences between washed feathers and unwashed feathers as well as associated 95% confidence intervals were also reported.

To investigate the effect of correcting ExCo on element concentration of feather shaft we calculated the mean difference in feather concentration between raw element concentration of feather shaft and element concentration of feather shaft corrected for ExCo (n = 119) and the associated Cohen's D (Cohen 1988) (note that applying a paired Wilcoxon-Pratt signed-rank test here always yielded a significant result since ExCo correction always reduces concentration of elements, however this does not allow us to assess whether ExCo correction had a negligible or strong effect). Since many element concentrations were not normally distributed we calculated 95% confidence intervals by bootstrapping (1000 bootstraps) as recommend by Nakagawa and Cuthill (2007) using the boot package implemented in R (Canty & Ripley 2015). A Cohen's D of below 0.2 is considered as negligible, between 0.2-0.5 small, between 0.5-0.8 medium and larger than 0.8 as large (Nakagawa & Cuthill 2007). We therefore considered that ExCo correction to have an appreciable effect on element concentration when Cohen's D was equal to or greater than 0.2.

Results

The effect of washing feathers

The washing procedure significantly reduced trace element concentration for 12 of the 14 elements analysed in feathers: Al, As, Cd, Cr, Cu, Fe, K, La, Ni, Pb, Se, and Zn (Figure 2). The effect was strong (r > 0.500) for Al, As, Cu, Cd, Cr, Fe, K, La, Ni, and Zn. A medium effect (r > 0.300) was observed for Se and Pb. For Sn and Hg washing did not significantly reduce trace element composition (Figure 2).

Examination of feathers with SEM

SEM examination of 18 unwashed shaft segments of 1 cm revealed a large diversity of particles which densely covered the feathers. A quantitative count of external particles was not possible due to their abundance and complexity. A large number of particles (>200) were found, most of them predominantly composed of sulphur (S) associated with other elements. We concluded that these particles were probably mostly from organic matter derived from feathers, which were discarded from further analysis. Of the remaining particles, one to six putative external contaminants per segment were thoroughly examined for their dimension, shape and chemical composition (for the most abundant elements only according to instrumental limitations). This resulted in a total of 66 lithic particles analysed for their element composition by SEM.

The analysed particles tended to range from less than 1 to 30 µm in all segments, although on rare occasions, they measured up to 100 µm. Particles appeared as amorphous terrigenous aggregations (Figure 3a), definite solid crystals (Figure 3b), piles of stacked sheets (Figure 3c), electrostatically adhered soft objects, or a combination of the aforementioned.

By observing the spectrum, a classification of the geological nature of each x-rayed particle has been provided. As shown in Table 1, a variety of Na and Mg salts emerged as the most abundant components of particles in all sites. In salts, K was detectable only in AIG samples. Occasionally, Ca was appreciably present in FDP salts. In all sites, clay particles were often associated with salt particles.

Aluminium was a common element in clays in all sites, but the composition of other elements changed according to sites. Potassium was detected in clay particles investigated in AIG, whereas clays from FDP and ODI showed heterogeneous composition, being either calcic, sodic, or potassic. Noticeably, Mg was detectable in clays only in ODI samples (7 out 8), which were very variable in their overall composition and in some cases particularly rich in Fe, Ti, Cr and potentially many other metals.

Hydroxides were present in particles from all sites, but were not very frequent. They appeared as Al-hydroxides, Mn was detectable in one particle from FDP. Minerals such as quartz, mica, chlorite, muscovite and gypsum were occasionally found in ODI samples, while Ca-carbonates were found in FDP. Four particles (2 in FDP and 2 in ODI samples) were apparently composed uniquely of Al. This may be due to the use of metallic tools, such as scissors and tweezers. A few lithic particles containing Cl or Ca remained undetermined.

In addition to the 18 unwashed shafts, three different shaft segments from ODI (the site where external contaminants are more likely to be rich in trace elements) were analysed by SEM, which were submitted to a washing procedure with water and detergents and then rinsed under running water. Much less lithic particles were visibly found, but some scattered particles were still present. In general, they were less frequent, smaller and seemed less complex in shape. At least 5 lithic objects were found and have been classified as sodium chloride crystals (2), carbonatic mineral (2), metallic aluminum (1).

Using geochemical data to assess the importance of ExCo on shaft trace element concentration

We found strong variation between elements of the importance of ExCo on trace element concentration in feathers. For Cu, Hg, Se, and Zn we found a median ExCoF lower than 0.5% in all the investigated sites and 95% of the data (95% interval; hereon referred to as 95%Iter) ranged between 0.3% and 0.7% indicating that Cu, Hg, Se, Zn are clearly bioaccumulated in feathers and dominate ExCo (Table 2). In contrast, for Al, K, and La median ExCoF were much higher than 100% (Table 2) suggesting that ExCo dominates any bioaccumulation for these elements. The ExCo was less clear cut for the other elements (As, Cd, Cr, Ni, Pb, and Sn; Table 2). Among these elements, Sn seems to be mostly bioaccumulated, with little variation between sites and median ExCoF ranging between 2-5% (Table 2). For As, Cd, Cr, Ni, and Pb, the ExCoF was more variable between the sampling sites. Arsenic had ExCoF of 14% (95%Iter = 3-43%) in AIG, ExCoF of 38%

(95%Iter = 18-87%) in FDP, and only a ExCoF of 3% (95%Iter = 2-8%) in ODI (Table 2). There was a lower variation of the effect of ExCo for Pb which has a ExCoF of 13% (95%Iter = 6-20%) in AIG, ExCoF of 22% (95%Iter = 14-37%) in FDP and a ExCoF of 10% (95%Iter = 5-15%) in ODI (Table 2). For Cd, Cr, and Ni, there was strong variation of the effect of ExCo on trace element concentrations within site, although there was little variation between sites (Table 2). For Cd, we calculated a median ExCoF of 21%, 32% and 35% in ODI, AIG and FDP respectively (Table 2). For Ni, median ExCoF ranged between 26-47% (AIG>FDP>ODI), with 95%Iter within site ranging between 13-78%, 10-91%, 9-39% in AIG, FDP, and ODI, respectively (Table 2). For Cr, AIG and ODI had a median ExCoF of 20% and 22% respectively (95%Iter=5-92% and 4-53% respectively), while FDP had the highest ExCo for this element (median ExCoF=31%, 95%Iter = 7-78%; Table 2).

Using geochemical data to correct for external contamination of feathers

Correcting each individual sample mirrored median ExCoF results (Figure 4). Prior to ExCoF correction Al, La and K could erroneously be interpreted as bioaccumulated (Figure 4). However, ExCoF correction revealed that actually Al, La and K concentrations in feather is likely to be almost entirely due to external contamination and bioaccumulation is either highly unlikely or below instrumental detection limits (Figure 4). Of the remaining elements ExCoF had an appreciable effect (Cohen's D > 0.200) on element concentration for Ni and Pb (Figure 4). However, ExCoF correction had a negligible effect (Figure 4; Cohen's D < 0.200) on the concentration of bioaccumulation for As, Cd, Cr, Cu, Hg, Se, Sn and Zn (Figure 4).

Discussion

Our results show that our novel methodological approach efficiently dealt with external contamination found in feather shafts and significantly changed interpretation of feather element concentration. We sampled chicks which allowed us to control for the effect of age on bioaccumulation and the shorter time of exposure to external environmental agents than adults

(Burger 1993). Prior to analysis, we took two methodological measures to minimise ExCo and unreliable biological interpretations. First, unlike most studies in feathers, in this study we removed the vanes in order to limit the tendency of feathers to entangle dirt among barbs (Cardiel *et al.* 2011). Furthermore, vane and shaft sequester metals differently, (Bortolotti 2010; Howell *et al.* 2012) which may confuse biological interpretation if analysed together. Indeed, high resolution images from X-ray fluorescence microscopy of shearwater chick breast feathers revealed a different distribution of As, Br, Ca, Fe, and Zn among the calamus, shaft and vane (Howell et al. 2012). The latter study was preliminary and did not give a physiological explanation of such a finding but pointed out that elements can be mostly concentrated in the calamus (Ca), shaft (As, Br, and Zn), or vane (Fe). In addition, most of the mass of a feather is the shaft for a given section of a feather and this may consequently affect the concentration, according to Bortolotti (2010), which advocates two mechanisms related to bioaccumulation in feathers depending on each trace element: mass-dependent and time-dependent accumulation. Scanning electron microscopy on our samples highlighted that a huge quantity of lithic particles and salt crystals are trapped in unwashed feathers, even when deprived of vanes. Therefore, our second measure was to wash shafts, combining the most common methods applied in the literature to date (Ansara-Ross *et al.* 2013; Costa *et al.* 2013; Carvalho *et al.* 2013; Rubio *et al.* 2016) with a prolonged ultrasonic bath treatment (Weyers *et al.* 1988). SEM observation also revealed that some ExCo remained in washed feathers and that ExCo cannot be ignored prior to data analysis. We successfully controlled for the remaining ExCo by calculating the ratio of ExCo due to sediment using the geochemical fingerprint of sediment samples. Our methodology allowed us to have conservative estimates of 10 bioaccumulated elements (As, Cd, Cr, Cu, Hg, Ni, Pb, Se, Sn, and Zn).

The effect of washing

After washing, more than 99% of K was removed (the concentration of K went from 116-809 mg/kg in unwashed shafts to 0.103-5.051 mg/kg in washed shafts), a much higher percentage than any of the other elements analysed in this study. Since K is a dominant element in salt, we can conclude that the washing effect was near complete in removing salt, which is likely to be a dominant residue in coastal bird feathers. We note however, that despite the effectiveness of washing, some K remained (0.103 - 5.051 mg/kg), suggesting that either some residual ExCo remained (rare small salt crystals were observed even in washed shafts by SEM), or some bioaccumulation or both.

The washing treatment of feathers also significantly reduced the concentration of 11 of the remaining 13 elements, with only Sn and Hg not significantly reduced. For Sn either trace element concentration from environmental contaminants was negligible relative to the concentration from bioaccumulation, or the efficiency of the washing procedure was lower than for the other trace elements. Like most elements, very little is known about the characteristics of Hg ExCo, however feather concentration of Hg is considered to be a good indicator of bioaccumulation irrespective of washing procedures (Jaspers *et al.* 2004; Pedro *et al.* 2015). Previous studies have shown that Hg levels in feathers are highly correlated with Hg concentration in the diet (Lewis & Furness 1991, 1993; Hahn *et al.* 1993; Monteiro & Furness 1995) and in internal tissues (Thompson *et al.* 1991) even when potential ExCo is ignored. Furthermore, Hg concentration in feathers is stable over time under various experimental environmental treatments suggesting that ExCo has little effect on this element (Appelquist *et al.* 1984). Our study is therefore consistent with the literature that ExCo of Hg is irrelevant regardless of the washing treatment.

Observing shafts by SEM demonstrated that unwashed feathers are very rich in lithic particles and are likely to be the main contributors of ExCo in feathers. Most lithic particles are salt crystals, clays and other fine residuals which can be removed in part by washing (Font *et al.* 2007). In fact, SEM observations on some ODI washed samples revealed that since these lithic particles are electrostatic and very small (typically 1-30 μm) some ExCo remain, even after the thorough washing treatment. ExCo of lithic particles from salt crystals is essentially made of Na, Mg, and K chlorides and Ca and Mg carbonates. However, we believe that any remaining ExCo by salt is likely to have a negligible effect on metal concentration because K is hundreds of times more concentrated in salt than Cu, Cr, and Zn (1,800-3,900 mg/Kg of K, 0-1.2 mg/Kg of Cu, 12-14 mg/Kg of Cr, and 7.4-7.5 mg/Kg of Zn in two collected and analysed samples of salt from Aigues-Mortes water; see dryad data: "Dryad hyperlink if accepted"). In contrast, terrigenous particles, such as clays, hydroxides and organic matter contain higher concentrations of metals, and the presence of a few of these lithic particles in a feather sample is sufficient to mask bioaccumulation for some elements (Borghesi *et al.* 2016). Therefore, further analytical methods are necessary to soundly interpret feather data.

Assessing the importance of ExCo on shaft trace element concentration

We found strong variation between elements on the importance of ExCo on trace element concentration in feathers. On the one hand, ExCo had a negligible effect on trace element concentrations for some elements (median ExCoF less than 0.5% for Cu, Hg, Se, and Zn; and around 5% for Sn), while on the other hand, ExCo dominated element concentrations for Al, K, and La (median ExCoF more than 100%). The latter is consistent with the hypothesis that residual ExCo after washing is essentially made of clays. Indeed, K is incorporated in the structure of certain clay minerals such as illite, and commonly adsorbed on the surfaces of many others (Salminen 2005), and clays have the capability of adsorbing rare earth elements (REEs) released/dissolved during weathering, with La being one of the most abundant REEs (Moldoveanu & Papangelakis 2012). Aluminum, K, and La concentrations are therefore good signals of residual ExCo and should only be used as controls of ExCo in trace element studies in feathers. Regarding the remaining five elements (As, Cd, Cr, Ni, and Pb), ExCo had a more nuanced effect on trace element concentration (depending on the element and the site), and the use of these elements in feathers to infer bioaccumulation needs some ExCoF corrections in order to avoid inflated interpretation of bioaccumulated concentrations.

Correcting for the effect ExCo on shaft trace element concentration

By calculating an ExCoF for each individual sample we were able to correct concentration values for ExCo for each sample by subtracting from the element concentration the proportion of element concentration that was estimated to be due to ExCo. Of the ten elements of environmental concern analysed in this study (As, Cd, Cr, Cu, Hg, Ni, Pb, Se, Sn and Zn), ExCoF correction for Ni and Pb did appreciably change mean concentrations (Figure 4). This may have important consequences when investigating the relationship between element concentration and other variables (such as body condition or fitness traits), including differences in bioaccumulation between sites which is beyond the scope of this study.

Trace element concentration in sediment at ODI was higher for 13 out of 14 elements (the exception was Se which was similar in ODI and FDP, and lower in AIG). These results are consistent with the extensive literature which demonstrates that ODI is one of the most polluted estuarine areas in the world (Guillén *et al.* 2011). However, following careful consideration of ExCo, appreciably higher trace element concentrations in feathers in ODI were only found for As and Pb. The latter suggests that there are important differences in how chicks metabolise each element during feather development and that not all trace elements in feathers are reliable environmental bioindicators. For example, Al, La and K were negligibly bioaccumulated and therefore poor bioindicators, whilst the other analysed trace elements were bioaccumulated to some extent and may be good bioindicators. However, there is strong indication that the bioaccumulation rate in feathers is not the same for all elements (e.g. the level of Cu, Hg, Se, Sn and Zn in feather shafts, while high in all samples, appears to be relatively independent of environmental levels, whilst As, Cd, Cr, Ni and Pb levels seem to be more heterogeneous between individuals and sites). A detailed interpretation of bioaccumulation and differences between sites is beyond the scope of this article.

In conclusion, as pointed out by previous studies, without careful consideration of ExCo, conclusions about the validity of the concentration of element bioaccumulation in feathers are unreliable. We have developed a new more reliable method of analysing trace element concentrations in feather shafts which effectively controls for ExCo. While Fe was used as the reference element to infer ExCo in feathers in this study, a different reference may be used in other studies depending on sampled species and environmental characteristics, in other words, the predicted main source of external contamination and the pollutants which are the object of the research. We also note that while our study focused on feathers, a similar strategy can easily be applied to other non-invasive organic samples when residual soil/sediment particles may bias interpretation of bioaccumulation (for example when assessing trace elements in plants, invertebrates, feaces and hair samples of vertebrates). Many studies continue to overlook ExCo leading to potentially erroneous conclusions and we urge that methods applied in this study be considered in future studies investigating bioaccumulation of trace elements in organic samples in contact with the external environment.

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Data accessibility

All the data used in this study is available in the public database Dryad: "Dryad hyperlink if accepted"

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Figure 1: Map showing the location of the three breeding colonies sampled for greater flamingo chick feathers and sediment. Sample sizes of washed feather shafts are in brackets.

Figure 2: Boxplot of paired unwashed and washed shaft feathers for the 14 elements investigated (*n* = 30). Median difference between washed and unwashed feathers, 95% confidence intervals, the z-value and p-value of the paired Wilcoxon-Pratt signed-rank test and the effect size *r* are shown within the boxplots of each element. Elements with a * were plotted on the log scale (but were not log transformed prior to analysis).

Figure 3: Electron microscopy pictures of three typical examples of three types of particles found in unwashed feather shafts: a. amorphous terrigenous aggregations; b. definite solid crystals; and, c. piles of stacked sheets

Figure 4: Boxplot of paired shaft feathers not corrected for ExCo and corrected for ExCo for the 14 elements investigated (*n* = 119). Mean difference and Cohen's D between not corrected and corrected concentrations and associated 95% confidence intervals (calculated by bootstrap, *n* = 1000) are shown within the boxplots of each element. Elements with a * were plotted on the log scale (but were not log transformed prior to analysis).

Table 1: Summary of element composition for each particle analysed by SEM showing the number of particles with a certain element composition and its geological interpretation within each site: Aigues-Mortes (AIG), Fuente de Piedra (FDP) and Odiel marshes (ODI).

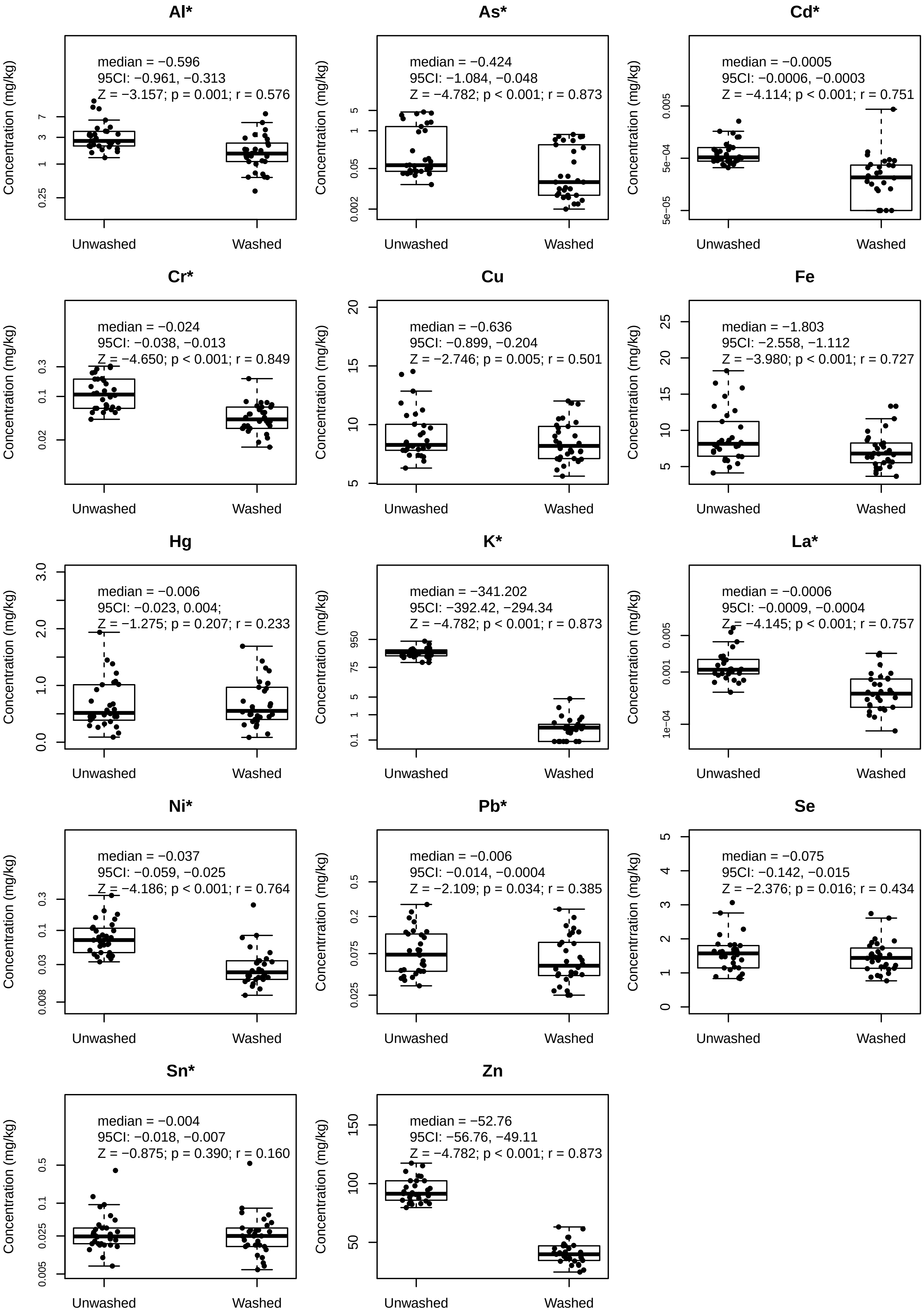
Site	Number of particles	Element composition	Geological interpretation
AIG	5	Na, Mg, Cl	Salt crystals
	2	Na, Cl	Salt crystals
	2	Mg, Cl	Salt crystals
	2	Na, K, Cl	Salt crystals
	1	N	Organic matter
	1	K, Cl	Salt crystals
	1	Al, O	Al hydroxide
	1	Al	Aluminum
	1	Al, Si, K, Ca	Clay
	1	Al, Si, K	Clay
	1	Mg, Cl, Al	Salt and Al hydroxide/oxide
	1	Si	Quartz
FDP	3	Na, Mg, Cl	Salt crystal
	2	Cl	Chloride
	1	Mg, Na, Ca, Cl	Salt crystals
	1	Mg, Cl	Salt crystals
	1	Na, Cl	Salt crystal
	1	Mg, Cl, Al, Si, Ca, Fe	Salt and clay
	1	K, Ca, Mg, Cl, Al, Si	Salt and clay
	1	Mg, Cl, Al, O	Salt and Al oxide/hydroxide
	1	Mg, Cl, Al	Salt crystal and aluminum
	1	Na, Mg, Cl, Al	Salt crystal and aluminum
	1	Na, Cl, Ca, C, O	Salt and carbonate
	1	Na, Al, Si	Clay
	1	Ca, C, O, Mn	Carbonate and Mn oxide hydroxide
ODI	9	Na, Cl	Salt crystals
	4	Mg, Cl	Salt crystal
	2	Na, Mg, Cl	Salt crystal
	2	Mg, Al, K, Ti, Fe, Si	Clay (mica)
	2	Si	Quartz
	2	Ca, S, O	Gypsum
	2	Al	Aluminum
	1	Mg, Al, Fe, Si	Clay (phyllosilicates)
	1	Na, Mg, Al, K, Cl, Si	Clay (phyllosilicates)
	1	Na, Mg, Al, K, Fe, Si	Clay (phyllosilicates)
	1	Al, K, Fe, Si	Clay (phyllosilicates)
	1	Al, Fe, Cr, Ca, Al, Si	Clay
	1	Mg, Al, Si	Clay
	1	Cl	Chloride
	1	Ca	Calcium oxide

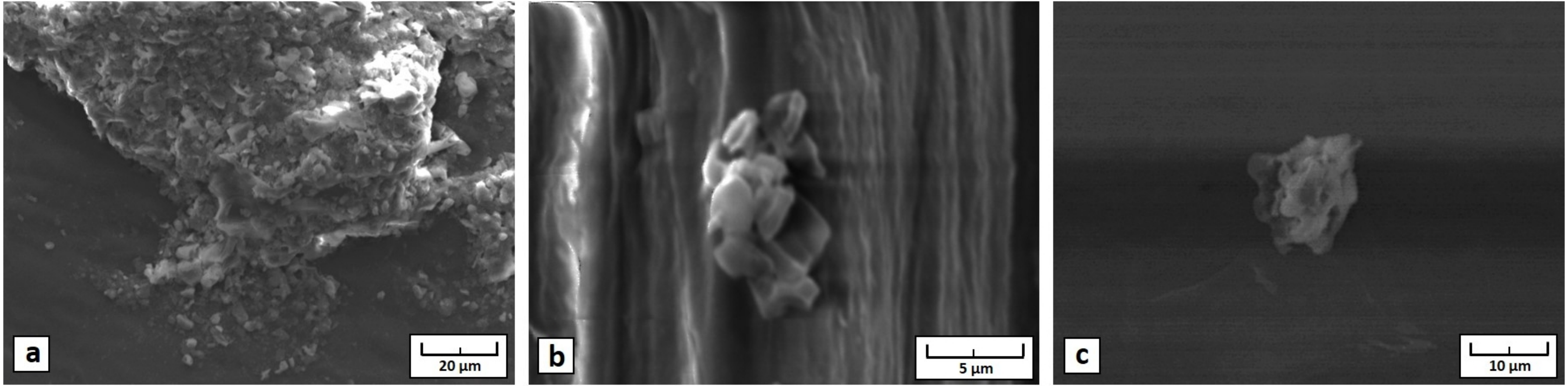
Table 2: Summary statistics for each samplings site showing median concentration of washed feathers prior to ExCo correction (Feather), median sediment concentration (Sediment), the predicted concentration if feather concentration is entirely due to external contamination (PExCo, see methods for calculation formula), the percentage of feather median concentration explained by external contamination (ExCo) and intervals which encompass 95% of the data (ExCoQ). Elements are ordered according to ExCo within each site. Iron (Fe) is highlithed in bold since this element was used as the reference for PExCo, ExCo and ExCoQ calculations and feather Fe concentration was assumed a priori to be 100% ExCo.

Elements	Feather (mg/kg)	Sediment (mg/kg)	PExCo (mg/kg)	ExCo (%)	ExCoQ (±95%Iter)
a. Aigues-Mortes (AIG; *n* = 29)					
Se	1.747	0.05	5.378E-05	0.003	0.002-0.007
Hg	0.539	0.12	1.291E-04	0.024	0.008-0.073
Cu	9.848	3.84	4.130E-03	0.042	0.032-0.051
Zn	43.312	19.7	2.119E-02	0.049	0.033-0.078
Sn	0.022	1.1	1.183E-03	5.286	1.844-12.998
Pb	0.061	7.22	7.766E-03	12.673	6.396-19.743
As	0.016	2.1	2.259E-03	14.050	2.657-42.743
Cr	0.047	8.6	9.250E-03	19.793	4.687-91.492
Cd	1.656E-04	0.05	5.378E-05	32.477	7.482-107.562
Ni	0.021	9.1	9.788E-03	47.151	13.257-78.014
Fe	**7.422**	**6900**	**7.422E+00**	**100**	**NA**
Al	0.938	3600	3.872E+00	412.619	179.506-1013.137
La	2.884E-04	2.9	3.119E-03	1081.612	335.137-2588.256
K	0.088	1000	1.076E+00	1227.041	116.867-1227.041
b. Fuente de Piedra (FDP; *n* = 30)					
Hg	0.623	0.021	1.365E-05	0.002	0.001-0.006
Se	1.597	0.6	3.901E-04	0.024	0.015-0.042
Zn	40.482	19.6	1.274E-02	0.031	0.024-0.058
Cu	7.225	13.74	8.933E-03	0.124	0.102-0.239
Sn	0.025	1.2	7.802E-04	3.106	0.113-10.958
Pb	0.041	13.76	8.946E-03	22.088	13.776-36.628
Cr	0.033	16	1.040E-02	31.348	6.599-77.545
Cd	1.289E-04	0.07	4.551E-05	35.317	6.611-91.019
Ni	0.023	13.4	8.712E-03	37.380	9.961-91.267
As	0.005	3.1	2.015E-03	37.959	17.532-87.451
Fe	**6.306**	**9700**	**6.306E+00**	**100**	**NA**
Al	1.410	13400	8.712E+00	618.075	214.011-1218.617
La	3.187E-04	4.6	2.991E-03	938.422	319.797-1856.099
K	0.247	4200	2.731E+00	1107.145	137.813-3114.956
c. Odiel (ODI; *n* = 30)					
Se	1.403	0.5	8.542E-05	0.006	0.004-0.009
Hg	0.398	0.168	2.870E-05	0.007	0.002-0.020
Zn	38.652	563.2	9.622E-02	0.249	0.157-0.382
Cu	8.509	247.1	4.222E-02	0.496	0.334-0.666
Sn	0.025	2.4	4.100E-04	1.627	0.076-4.419
As	0.499	91.2	1.558E-02	3.122	1.901-8.036
Pb	0.148	83.82	1.432E-02	9.666	5.003-15.170
Cd	1.933E-04	0.24	4.100E-05	21.216	6.641-82.005
Cr	0.035	45.3	7.739E-03	21.998	3.959-53.040
Ni	0.021	30.9	5.279E-03	25.735	9.184-38.706
Fe	**7.193**	**42100**	**7.193E+00**	**100**	**NA**
Al	1.460	23900	4.083E+00	279.655	65.607-599.191
La	5.760E-04	15	2.563E-03	444.940	107.003-1167.760
K	0.268	7000	1.196E+00	446.842	65.872-1364.259


Aigues-Mortes
(AIG; n=39)
Odiel marshes
(ODI; n=40)
Fuente de Piedra
(FDP; n=40)

The effect of washing feathers (duplicated samples only; n = 30)




a
20 µm
b
5 µm
c
10 µm

Trace elements of fearther (n = 119) before corrections (Not−corrected) and after (Corrected)

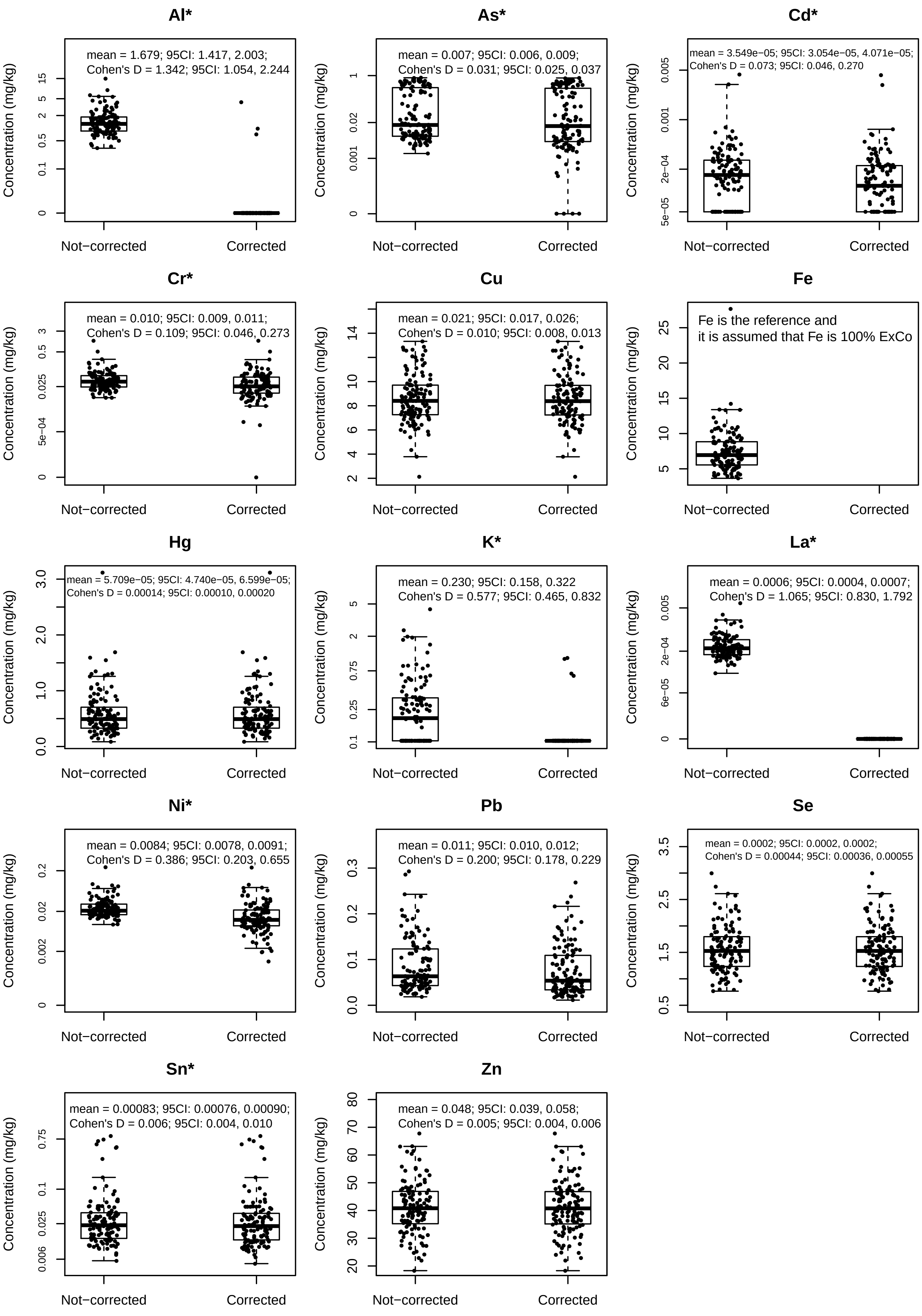


Figure S1: Pairwise Pearson correlation for each of the elements assumed to indicate ExCo (Al,Fe,K,LA) analysed in washed feathers in this study prior to ExCo correction (n=119). All correlations are significant except between Fe and K

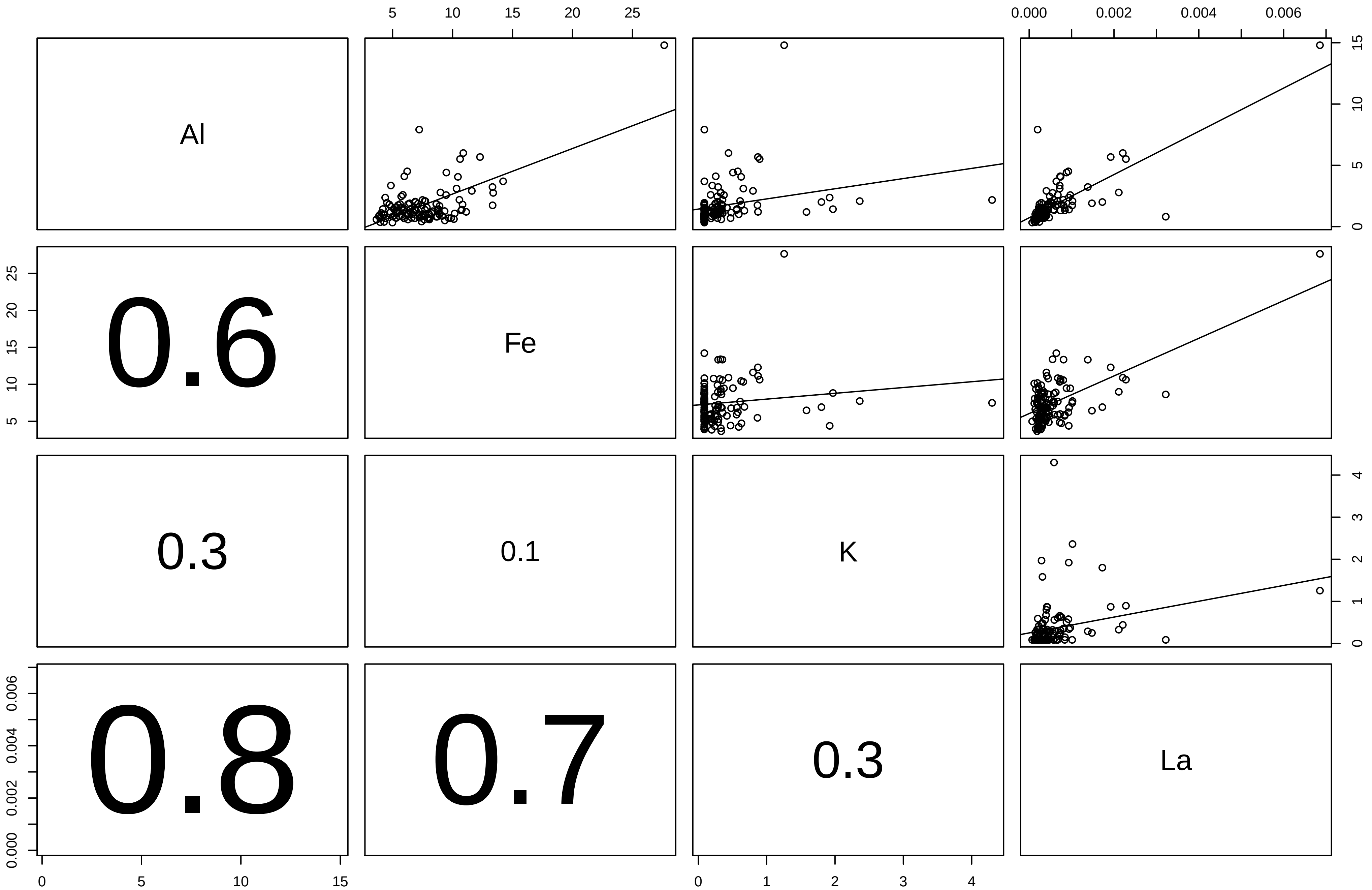


Figure S2: Pairwise Spearman correlation for each of the elements assumed to indicate ExCo (Al,Fe,K,LA) analysed in washed feathers in this study prior to ExCo correction (n=119) All correlations are significant except between Fe and K

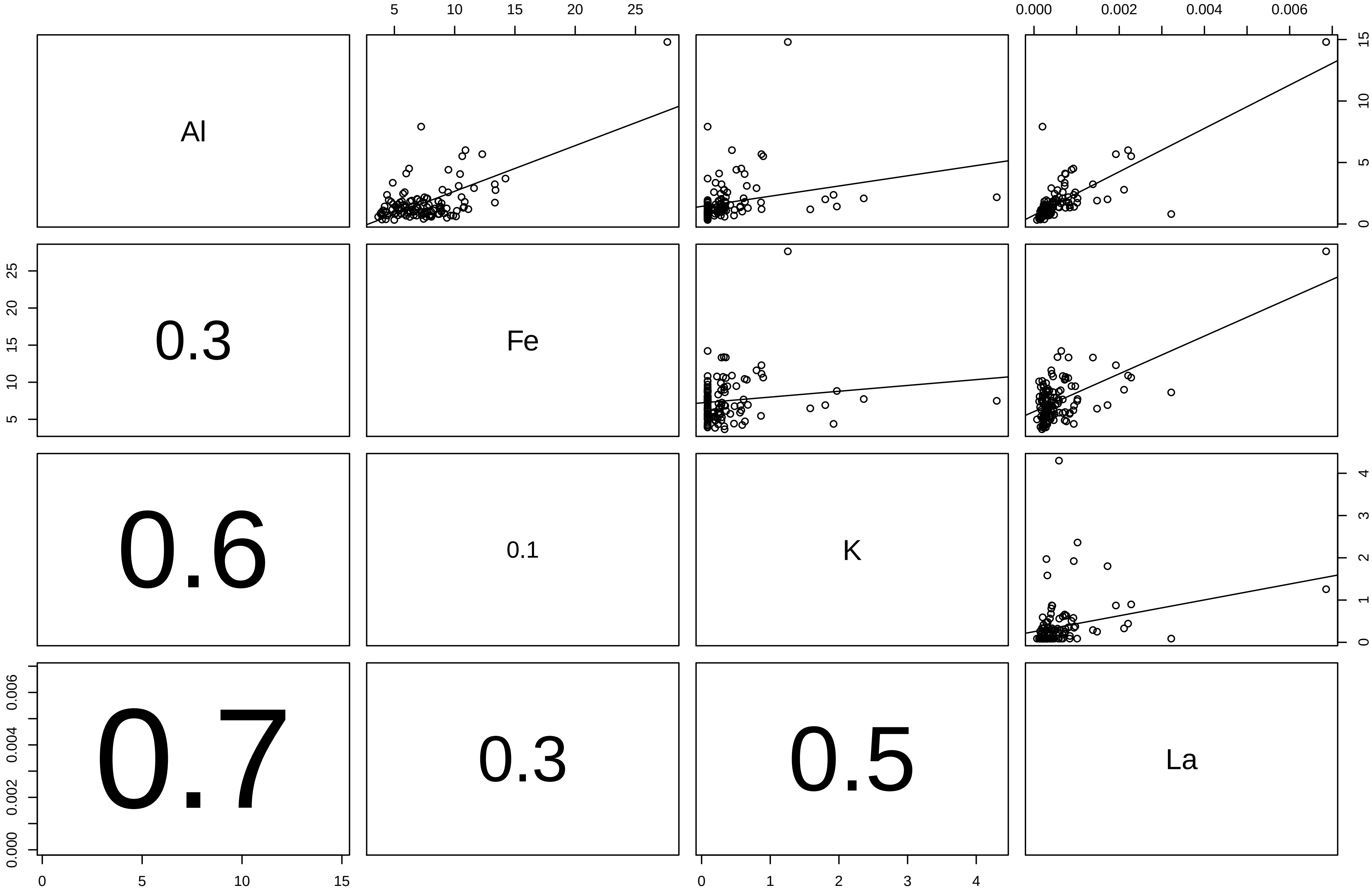


Figure S3: Pairwise Pearson correlation for each of the elements assumed to indicate ExCo (Al,Fe,K,LA) analysed in washed feathers in this study prior to ExCo correction (n=118). Without outlier with Fe = 27.64; All correlations are significant except between Fe and K

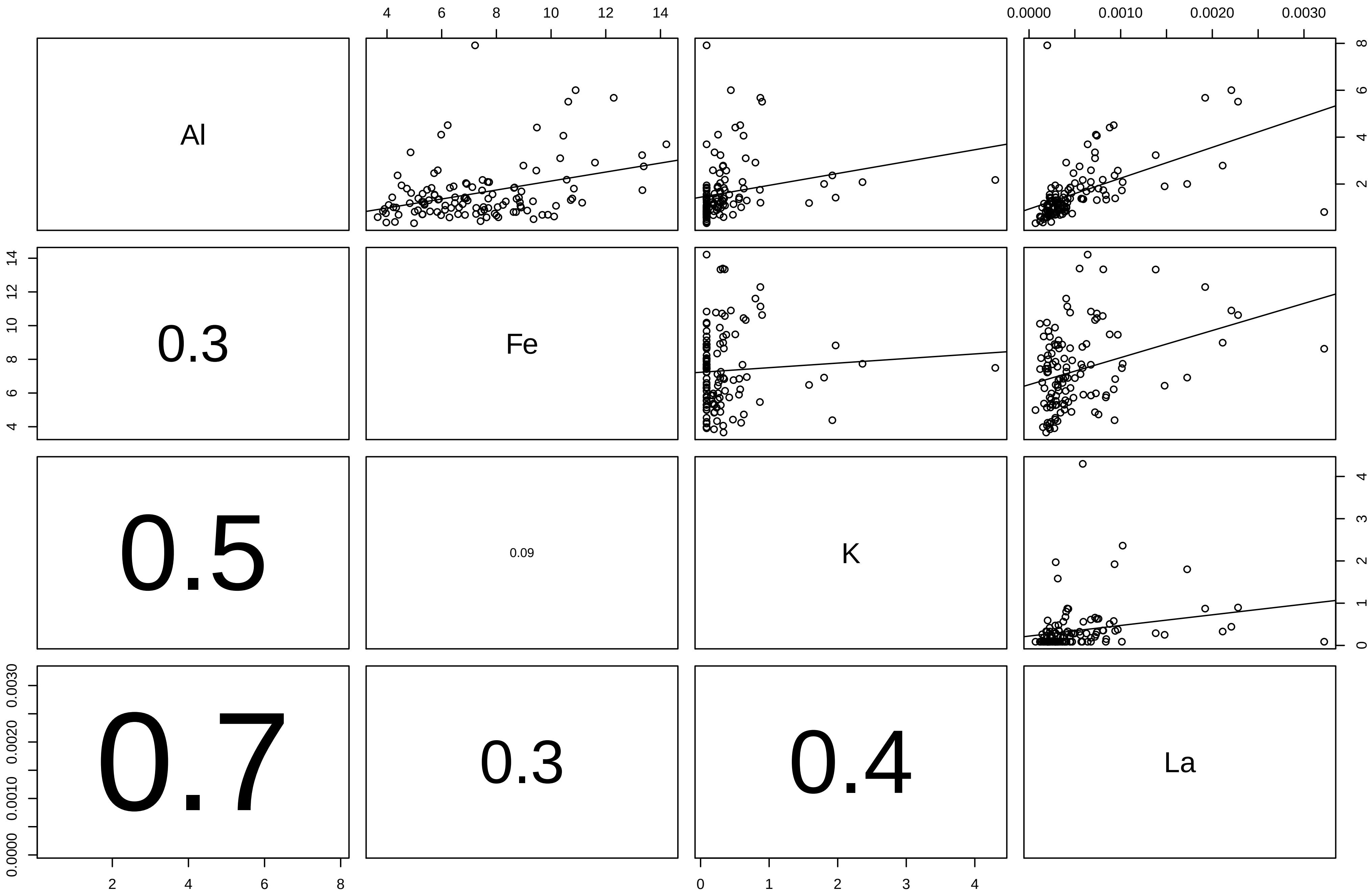


Figure S4: Pairwise Spearman correlation for each of the elements assumed to indicate ExCo (Al,Fe,K,LA)
analysed in washed feathers in this study prior to ExCo correction (n=118). Without outlier with Fe = 27.64; All correlations are significant except between Fe and K