Metal content in the liver, kidney, and feathers of Northern gannets, Morus bassanus, sampled on the Spanish coast

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(Article begins on next page)

Comparative study of the influence of age and gender on the metal content in liver, kidney and feathers of Northern gannets, *Morus bassanus,* from Spain

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Abstract

The value of birds as bioindicators for monitoring the environmental inorganic elements has been globally recognized. In this context, due to its well-known ecology and population stability, the Northern gannet (*Morus bassanus*) could be particularly useful. Dead Northern gannets (n = 30) were collected and samples from liver, kidney and feathers were taken, dried, mineralized and finally analyzed via ICP-MS. Metals and metalloids, namely As, Cd, Hg, Pb, Zn, associated with environmental pollution and toxicity on living organisms, were evaluated. The mean highest concentrations of As, Hg and Zn were found in liver (0.916, 7.026 and 89.81 mg/kg dry weight, respectively). For Cd, kidney showed the highest mean concentration (17.51 mg/kg dry weight), whereas for Pb this value corresponded to feathers (0.399 mg/kg dry weight). Significant differences were found between the age classes in terms of contaminant concentrations, with the adults exhibiting higher metal levels. This difference was significantly relevant for Pb and Hg, where the effect of age was observed for all the considered tissues. When considering the effect of gender, no significant differences were observed, in agreement with similar studies performed in other geographical regions. Finally, positive correlations between the concentrations of Hg and Pb in the feathers and in the liver ($r=0.688$, $p<0.001$ and $r=0.566$, $p<0.001$, respectively) were observed, as well as between feather and kidney concentrations ($r=0.685$, $p<0.001$) indicating the possibility to use feathers, a non-invasive biomonitoring tissue, for better understanding Hg and Pb exposure in seabirds.

Keywords

Bioindicators, birds, environment, metals, pollution, feather, liver, kidney

Introduction

It is well known that the use of bioindicators is an essential way for monitoring the quantity and the environmental consequences of inorganic elements. The value of birds for biomonitoring the environmental quality has been globally recognized as an important tool for the environmental management. Among the aims of monitoring using sentinel animals, we can include: assessing human health risks, identifying food chain contaminants, determining levels of environmental contamination and identifying adverse effects on the animals themselves (García-Fernández 2014).

During the past years, many species of pelagic seabirds have been considered as good bioindicators of metals in the marine environment, mainly because they can live for many years (the Northern gannet can live for more than 20 years) and feed at different distances from land. They also stand in a high trophic position and have a high resistance to toxic effects (Mendes et al. 2013; Pereira et al. 2009). All these facts can be of relevance to evaluate their bioaccumulation capacities too, since it's very likely for a top predator to consume preys containing high levels of contaminants (Champoux et al. 2015). Furthermore, the ecotoxicological assessment of seabirds is important because they are exposed to varying levels of contaminants and to different natural environmental stressors which may produce physiological deficiencies that lead to a worse resistance or change in life habits, such as reproduction behavior or other endocrine functions (Mendes et al. 2008).

Northern gannets (*Morus bassanus*) could be particularly considered for an appropriate biomonitoring, since their general ecology and population stability is well known. They usually feed far from the coast, in the open sea. Actually, since they are pelagic seabirds, they only depend from the marine environment: Northern gannets eat schooling fish and also surface schools of squid found at the surface of oceans or seas, up to 15 m deep. Prey fishes are from 2.5 to 30.5 cm in length. They often feed in association with predatory fish and cetaceans, such as bluefish (*Pomatus saltatrix*), white beaked-dolphins (*Lagenorhynchus albirostris*), and Atlantic white-sided dolphins (*Lagenorhyncus acutus*). Their size also helps them to withstand the punishing environmental conditions in the areas these fish are found: Northern gannets are indeed the largest seabirds in Northern Atlantic (Mowbray 2002). Unlike gulls, they do not have contacts with human waste or rubbish from the coastal towns, rendering these seabirds as very interesting for monitoring the ecotoxicological status of open sea areas (Montevecchi et al. 2012). Moreover, the gannets are migratory seabirds and migratory patterns vary with age class: in summer, they are found at high concentrations near breeding colonies at higher and northern latitudes. Adults begin to migrate North towards breeding colonies in February, sub-adults in March and immature birds in April. Adults arrive at breeding colonies in April to mid-May, while younger birds arrive later. In fall, Northern gannets begin their southward migration (Montevecchi et al. 2012). For these reasons the toxicological analysis needs to be put in context to the seasonal period and the territory in which the birds are found.

Among the different contaminants, metals are identified as natural occurring elements having atomic number (Z) greater than 20 and an elemental density greater than 5 g cm^{-3} (Ali and Khan 2018). The term is generally used for metals and metalloids associated with environmental pollution, toxicity and adverse effects on living organisms including humans. With the assumption that heaviness and toxicity are inter-related, heavy metals also include metalloids, such as arsenic, that are able to induce toxicity at low level of exposure. Heavy metals end up in marine environments as results of waste products, industrial activities and natural processes, too. By the way, most of the environmental contamination result from anthropogenic activities: mining and smelting operations, industrial production and use, domestic and agricultural use of metals and metal-containing compounds used for medical treatments. Environmental pollution can also be caused by metal corrosion, atmospheric deposition, soil erosion of metal ions and leaching of heavy metals, sediment re-suspension and metal evaporation from water sources to soil and ground water (Tchounwou et al. 2012).

After all these considerations, we chose to assess by ICP-MS some metals (As, Cd, Hg, Pb) in kidneys, livers and feathers of adult and juvenile Northern gannets. Age and sex were considered as parameters to be evaluated too, in order to assess the influence of them on the metal levels. One of the most relevant aims of the present study is to determine whether a correlation between the heavy metal content in some internal tissues (liver and kidney) and feathers exists, in order to evaluate the use of feathers as non-destructive samples, as some previous studies have indicated, for example, when determining mercury levels (Espín et al. 2012). This could be assumed since the vast majority of feather-based studies have been concerned with metal contamination, demonstrating that feathers can be used as an alternative to internal tissues. Feathers, actually, are connected to the bloodstream only for a certain period of time during their initial development and molt period. That is why birds deposit heavy metals in feathers during their formation. When they mature, vascular connections become atrophic and compound concentrations remain stable. Therefore, feathers can provide information on concentrations in the blood circulation at the time of their growth (García-Fernández 2014).

Materials and methods

In order to determine the metal levels in liver, kidney and feathers of Northern gannets, samples from dead animals were collected, dried, mineralized and analyzed.

Field procedure and sampling

Northern gannets, *Morus bassanus* (n = 30), were collected during the period 2015-2017 in the region of Galicia (North West of Spain). Collected animals were found dead or died after being injured and referred to the Wildlife Recovery Centers situated in the considered coastal area. For the study, a choice was made to use only those birds that had not been held at the Recovery Centre for more than 5 days before dying. The average period of stay in the Centre of the chosen birds, however, was about 2 days, and the diet provided during this period was supposed to be free of environmental contaminants. All dead specimens were immediately frozen and stored at -20ºC until samples were prepared for analysis. From each corpse, a portion of approximately 10 g of liver and kidney tissue was taken, placed individually in plastic bags, and stored at -20ºC. Similarly, body feathers were pulled out from the lower back (approximately 20 g) and stored in plastic bags. For each individual, different feathers were pooled to limit potential inter-feather differences.

During the necropsy, some parameters such as mass measurements (g), organ weights, bill development and physical condition were registered for collateral studies. Moreover, the age was determined based on the color plumage, as well as with the study of the bill development, size and appearance of the gonads. According to this data, the animals were classified as juveniles or adults (16/14). With respect to gender, birds were sexed during necropsies by visual gonad examination, determining the sexual organ development and divided accordingly (males/females, 15/15). After sampling, the remains were removed hygienically by incineration, under current European legislation.

Reagents

Nitric acid (69%) and hydrogen peroxide (30%) were purchased from TraceSELECT^{TM}, Fluka (Seelze, Germany). Multi-element Calibration Standard 3 solution was purchased from PerkinElmer Inc. (Shelton, CT). The certified sample of lyophilized bovine liver was provided by the Institute for Reference Materials and Measurements (IRMM).

Determination of metal levels

A microwave assisted acid digestion procedure adapted from Fromant et al. (2016) and Morton et al. (2017) was carried out to obtain metal contents. Two grams of each sample were weighed into Teflon PTFE flasks and 6 ml of a freshly prepared mixture of concentrated HNO_3 (69%) and H_2O_2 (30%) (3:1, v/v) were added. The flasks were closed and left to predigest for 12 hours at room temperature. The vessels were then sealed, and microwave digested (15 min with constantly increasing temperature up to 180 °C, and finally 5 min at this maximal temperature). Once the digestion was completed, the vessels were allowed to cool to room temperature, and the digest was diluted to 10 ml with deionized water. A blank digest was carried out in the same way. All sample solutions were clear. In order to avoid losses of volatile elements, a second set of identical samples from the same individuals was ovendried at 80 ºC till constant weight in order to calculate the percentage of humidity in each sample (average humidity of 74.02, 68.22 and 20.85% in kidney, liver and feathers, respectively). The accuracy of the microwave digestion method was checked by standard reference material (BCR® certified reference materials - ref. 185R, Community Bureau of Reference, EU). Four replicates were done on NIST SRM 1577b Bovine liver to check the accuracy, and the results were in good agreement with the certified material, with a mean recovery rate of 85-102%. It must be indicated that, in order to remove external contamination from the surface of the feathers, a washing process was performed prior to analytical determination, subsequently using tap water, distilled water, Milli-Q water and acetone (Jaspers et al. 2004).

A platform collision cell inductively coupled plasma mass spectrometer ICP-MS 7900 equipped with an integrated autosampler (Agilent Tech) was used for element detection. For an optimal nebulization of the sample, a Peltier-cooled (2ºC) cyclonic chamber (Elemental Scientific, Omaha, NE, USA) and a low-flow (0.25 mL/min) Meinhard® concentric nebulizer (LGC, London, UK) was employed. Both the collision gas and the argon for the plasma have a purity of 99.999% and have been supplied by Praxair (Madrid, Spain).

Each day, the ICP-MS was calibrated to obtain the highest values of intensity indicated by the ratios CeO/Ce < 2.5 %, Ce^{++}/Ce < 3 % and background (220) < 1 cps. The instrumental detection limits were 0.005 mg/kg for all the elements. Calibrating solutions were prepared daily from a 10 mg/L Multi-element Calibration Standard 3 solution (PerkinElmer, Inc., Shelton, CT). The same certified sample of lyophilized bovine liver previously indicated was used for quality control of the analytical procedure. The values obtained for these elements were consistent with certified reference values, and the recovery yields varied between 89% for Cd and 107% for As. The limit of detection (LOD) and of quantification (LOQ) were determined according to the ICH-Q2 guideline on method validation (ICH 2005), after analysing repeated blanks with the same procedure used for the samples and determining the standard deviation. The final values of both parameters were calculated taking into account the samples dilution factor and the weight and were in all cases lower than 0.003 and 0.009 mg/kg for LOD and LOQ, respectively. The coefficients of variation for replicate samples (n=5) were determined to be lower than 5.3%. All samples were run in batches that included analytical blanks.

Metal concentrations are expressed as mg/kg dry weight (dw), since dry values are considered to be more reliable and consistent compared to wet weight values (ww) (Adrian and Stevens 1979).

Table 1 Operation conditions for the ICP-MS

Potency RF (W)	1550
Plasma Mode	General purpose
Omega Bias (V)	-120
Omega lens (V)	9.3
Extract 2 (V)	-245
Deflect Lens (V)	1.0
Energy discrimination (V)	5
Collision gas (ml/min)	5
Cell Entrance (V)	-40
Cell Exit (V)	-60

Statistical analysis

Data were analyzed using statistical software Prism 6 (version 6.02) for MacOS (GraphPad Software Inc., La Jolla, CA, USA). The concentrations of metals were presented as mean values ± SEM, median and range. Shapiro-Wilk normality test was performed to determine whether the data were normally distributed. Due to the non-normal distribution of the data, a non-parametric Kruskal-Wallis test was used to determine the influence of the considered tissue on the metal concentrations. Similarly, a Mann Whitney *U-test* was used to assess the influence of gender and age. Moreover, a Spearman test was performed to determine the correlations among metal levels. The level of statistical significance was set as $p < 0.05$. A value of 50% of the limit of detection (LOD) was assigned to samples with metal concentrations below LOD. These values were included in the data-set for statistical testing, a technique that minimizes nominal type I error rates (Clarke, 1998).

Results

Element concentrations

The main statistical parameters corresponding to the Zn, As, Cd, Hg and Pb levels found in organs (liver and kidney) and feathers of Northern gannet are reported in the table below (Table 2). Since the distributions of the heavy metal levels included in this study were skewed, not only the means ± standard errors are shown, but also the ranges and coefficients of variation (CV), as well as the number of samples below the level of detection. In this sense, while Zn, As and Hg could be detected in all the samples, Cd was no detected in 23% (7/30) of the feather samples. Similarly, Pb was under the limit of detection in 10% (3/30) and 13% (4/30) of feather and liver samples, respectively. This percentage of undetected samples markedly raised when considering Pb concentration in kidney (37%, 11/30).

The highest concentrations of all the considered elements were found in liver, except for Cd, which was found in kidney, and Pb in feathers. Pooling all samples, Zn presented the highest values in all tissues. Moreover, levels of this essential element did only statistically differ between liver and feathers ($p<0.05$), with little variability between kidney and liver. When considering the toxic elements As and Pb, their levels also varied among tissues, with feather concentrations being statistically different when compared to both liver and kidney ($p<0.001$ in all cases, except for Pb in liver and feathers, with $p<0.05$). However, the relevance of this result when considering Pb in kidney samples is relative, according to the high number of samples below the detection limit for this tissue, as previously observed in table 1.

With respect to Cd, even if it accumulated mostly in kidney, while its concentrations in feathers were relatively low, there were statistical differences among all the three studied organs ($p<0.001$ for all tissue combinations). At last, for Hg, only statistical differences were observed when liver levels were compared with the other two considered tissues, kidney ($p<0.001$) and feathers ($p<0.05$), whereas Hg levels did not statistically differ between kidney and feathers.

Table 2 Main statistical parameters corresponding to the Zn, As, Cd, Hg and Pb concentrations, expressed in mg/kg of dry weight, in Northern gannet (n=30) liver, kidney and feathers

	Element	Mean ± SEM[a]	Range (CV[b])	<LOD
Liver	Zn	89.81 ± 4.205	58.95 - 157.3 (25.64)	0
	As	0.916± 0.1180	0.240 - 3.090 (70.56)	0
	Cd	4.530 ± 0.4507	1.950 - 14.35 (54.49)	0
	Hg	7.026 ± 0.8674	0.951 - 18.32 (67.62)	0
	Pb	0.210 ± 0.0268	<LOD - 0.625 (69.91)	4
Kidney	Zn	78.65 ± 3.5891	45.25 - 129.7 (25.04)	0
	As	0.646 ± 0.0894	0.195 - 2.810 (75.78)	0
	Cd	17.51 ± 3.2890	2.850 - 85.25 (102.9)	0
	Hg	2.760 ± 0.4654	0.350 - 10.91 (92.34)	0
	Pb	0.138 ± 0.0178	<LOD - 0.421 (71.07)	11
Feathers	Zn	72.75 ± 3.8931	19.28 - 114.1 (29.31)	0
	As	0.307 ± 0.0245	0.112 - 0.685 (43.85)	0
	Cd	0.211 ± 0.0373	<LOD - 1.090 (97.06)	7
	Hg	4.161 ± 0.7140	0.354 - 13.42 (94.05)	0
	Pb	0.399 ± 0.0480	<LOD - 0.952 (65.95)	3

[a]SEM: standard error of mean, [b]CV: coefficient of variation (%).

<LOD: number of samples below the limit of detection.

Age and gender influence

When analyzing the effect of age, it must be noted that the data of the analysis of metal concentrations in liver, kidney and feathers of Northern gannets showed relevant and sometimes statistically significant differences between the age classes (adult *vs* immature) in terms of contaminant concentrations, with the adults exhibiting higher metal levels compared to the immature animals. Regarding the influence of sex on metal levels, a different situation could be observed, with no specific patterns on metal accumulation, and no statistically significant differences found comparing the concentrations in the same organs of males and females. More specifically, Zn concentration (Figure 1) in the feathers was significantly higher in adults compared to immature animals (medians 86.07 and 60.01 mg/kg dw, respectively; $p<0.0001$) whereas, regarding the two organ levels, no significant differences were found between the two age classes. When considering the influence of gender, no statistically differences between males and females were observed for any of the considered tissues, even if mean values were slightly higher in females.

As levels (Figure 2) in adults were significantly higher in the organs ($p<0.0001$) as well as in the feathers ($p<0.01$), compared with those of the immature subjects. With respect to the effect of gender, and as previously described for Zn, no statistically significant differences were associated to this factor.

Figure 3 shows the result corresponding to Cd concentrations. With regards to the age, no significant differences were observed on liver samples, whereas both kidney ($p<0.001$) and feathers ($p<0.01$) showed a clear influence of this endogenous factor on the final concentration. No effect of gender was observed for this heavy metal.

Hg concentrations (Figure 4) showed a similar distribution to that observed for As, with organs and feathers ($p<0.001$) being significantly lower in immature animals when compared to adults. No statistically significant effect of gender was observed for this heavy metal.

At last, Pb concentrations (Figure 5) in liver and feathers were significantly higher in adults ($p<0.001$) whereas no statistically significant difference was found with regards to the kidney level. As previously indicated for the other analyzed metals, no statistically relevant effect was observed associated to the influence of gender.

Correlation study

The correlation between the level of the same metal in the feathers and in the different internal organs was also evaluated. As could be observed in figure 6, a positive correlation between the concentration of Hg in the feathers and in the liver ($r=0.688$, $p<0.001$) was observed, as well as between feather and kidney concentrations ($r=0.685$, $p<0.001$). A positive correlation was also found between Pb concentration in feathers and liver ($r=0.566$, $p<0.001$). In a similar way, hepatic and renal Hg levels of great cormorants from Japan were positively correlated with levels in feathers (Nam et al. 2005), and similar results were observed in previous studies with, for example, herring gull (Hutton 1981) and brown pelican (Ohlendorf et al. 1985). Although the kinetics of Hg and Pb in feathers is complex and not fully understood, the significant positive relationship between feathers and internal organs suggests that the feathers may be used as a non-invasive biomonitoring tissue for better understanding Hg and Pb exposure in seabirds (Mendes et al. 2008). Element burdens in feathers express exposure and accumulation during the inter-molt period, thus they are more representative of long-term rather than acute exposure (Espejo et al. 2018). Understanding specific molt patterns, migration routes, and overwintering areas of Northern gannet will further elucidate the patterns and potential sources of heavy metal concentrations in feathers observed in the present study. However, since metals may be deposited from atmosphere onto feather surfaces, as well as incorporated into growing feathers from blood, the use of feathers in environmental biomonitoring must take into account that these hard tissues may be responding to a combination of these two processes (Furness and Camphuysen 1997), and an adequate cleaning of the samples (as developed in the present study) must be considered. Moreover, the special characteristics of feathers have to be taken in account: once feather growth is complete, the feather no longer exchanges blood with soft tissues. Therefore, given that there is a seasonal cycle of some element accumulation and elimination in the soft tissues relative to feather molt, for biomonitoring purposes it would be better to consider cautiously the use of feather element concentrations to predict metal concentrations in soft tissues (Nam et al. 2005).

Discussion

Few data on metal contamination in seabirds in the Atlantic coast of the Iberian Peninsula are reported, especially in relation to *Morus bassanus* (Mendes et al., 2008).

Samples of this species collected from the Portuguese coast (Mendes et al. 2008) showed markedly higher values of Zn in liver, kidney and feathers (means of 147.09, 111.58 and 178.40 mg/kg dw, respectively). Moreover, when a similar study was developed in the same Portuguese area but some years later (Mendes et al. 2013), Zn concentrations in gannets were always within the range of the previously reported values. When compared to other data reported in the literature, kidney and feather Zn levels quantified in the present study were in a similar range to those found in seabirds from the same Northern Hemisphere (30.2-183 and 42.9-189.2 µg/g, for kidney and feathers, respectively) (Kim et al. 1998; Lucia et al. 2010). However, the results obtained in the present work for hepatic Zn levels were markedly lower than those quantified in this seabird species (range of 14.92-541 µg/g) or even in penguins from the Southern Hemisphere (values ranging between 72 and 330.34 µg/g) (Espejo et al. 2018). It must be considered that despite being an essential element, an excessive concentration of Zn can generate some negative effects. In fact, 200 µ/g of hepatic Zn is considered the threshold value of physiological relevance in seabird species (Honda et al. 1990), but none of the considered animals exceeded this concentration.

A similar situation in the present work was observed for As when compared to the mentioned studies developed on Northern gannets from the central coast of Portugal (Mendes et al. 2008; Mendes et al. 2013), with higher concentrations in liver, kidney and feathers (means of 1.66, 2.044 and 0.827 mg/kg dw, respectively). The concentrations of As in liver were in a quite similar range than those found in other seabirds from the Northern Hemisphere (0.22-5.62 µg/g) (Jerez et al. 2013; Lucia et al. 2010). However, these hepatic levels were markedly lower than those quantified in Antarctic prion (*Pachyptila desolata*), where mean values of 2.7 µg/g were quantified (Fromant et al. 2016). In general, the levels of As reported in feathers, blood, and organs of Northern gannets are below 3 µg/g, the limit considered normal and with no toxicological effect in living organisms (Jerez et al. 2013).

In birds, the accumulation of Cd can have adverse effects on health, such as renal and testicular damage, reduced feed intake and growth rate, thinning eggshells, decreased egg laying, or alterations in the behavior of the bird, among others (Burger 2008; Espejo et al. 2018). However, seabirds seem to be less vulnerable to the exposure to high levels of this heavy metal. In general, mean Cd concentrations in feathers and kidney in the present study were lower or similar than those found in seabirds of the Northern hemisphere (0.04-1.28 µg/g for feathers; 0.90-44.4 for kidney) (Kim et al. 1998; Malinga et al. 2010; Mansouri et al. 2012; Orlowski et al. 2007). It is interesting to note that mean Cd concentrations in feathers and liver of Northern gannets from Portugal (0.097 and 2.3 mg/kg dw respectively) were lower than those obtained in the present study, whereas kidney concentration was in the same range (17.53 mg/kg dw) (Mendes et al. 2008), a remarkable level that indicates the relevance of this organ in the general toxicokinetic of Cd. Moreover, it must be noted that a higher Cd concentration in kidney than in liver, as observed in the present study, usually indicates chronic exposure to low Cd levels (Scheuhammer 1987). The present data corroborated other studies conducted on penguins, in which seabird kidney is the soft tissue where total Cd is mainly concentrated, and only trace amounts are found in feathers (Kehrig et al. 2015; Vega et al. 2010).

As expected, liver presented the highest Hg concentrations, associated to its important role in Hg detoxification and storage (Kim et al. 1998), followed by feathers, where a large proportion of the Hg body burden can be excreted in the plumage during molt (Fromant et al. 2016). It is interesting to note that Hg concentrations in feathers in the range of 9-20 mg/kg can decrease reproductive success in some piscivorous birds (Bond et al. 2015; Espejo et al. 2018). Although the mean Hg concentration reported in Gannet feathers was below that known to cause adverse health and reproductive effects in birds, some animals showed values up to 13 mg/kg, indicating a possible toxic effect on these wild populations. Moreover, mean Hg concentrations in feathers of Northern gannet were slightly lower than those found in different species of seagulls and terns from Northern Hemisphere (0.31-20.2 µg/g) (Zamani-Ahmadmahmoodi et al. 2014), whereas liver concentrations in seagulls were markedly higher (4.9-306 µg/g) (Kim et al. 1996). Notwithstanding, the detected levels in kidney were in the same range than those found in seabirds from this same Hemisphere (Zamani-Ahmadmahmoodi et al. 2014). When the same species was considered, mean concentrations of Hg in feathers and kidney were slightly lower (3.51 and 1.88 mg/kg dw) in animals sampled in Portugal (Mendes et al. 2008), this difference being especially relevant when considering liver samples (1.79 µg/g dw). However, a recent study performed in the same Portuguese area and on the same seabird species revealed higher hepatic Hg concentrations, thus corroborating the potential effect of Hg pollution (Mendes et al. 2013), associated to the role of diet as a major contamination pathway in seabirds (Kim et al. 1998). More specifically, while Hg emissions from anthropogenic sources have decreased in North America and Europe in the last decade, Hg emissions have increased globally in recent decades, notably in Asia, which can increase deposition in Europe from global sources (Champoux et al. 2015). This is highly relevant, as Hg is one of the most toxic elements for seabirds (Savinov et al. 2003).

In those same studies developed in Portugal, mean Pb concentrations in feathers (2.617 mg/kg dw) were also markedly higher than those from the present study, whereas mean kidney concentrations (0.195 µg/g dw) were quite similar, and two times lower when considering liver samples (0.103 mg/kg dw). Similar to that observed in the present study, the highest concentration of Pb was quantified in feathers of great cormorants (*Phalacrocorax carbo*) from Japan (Nam et al. 2005), with concentrations up to 1.65 µg/g dw. The concentrations found in the present study for Pb in liver and kidney can be considered low, with no toxicological relevance. In fact, Pb concentrations ranging between 0.5-5.0 µg/g dw in liver and 1.0-10.0 µg/g dw in kidney can be considered as normal background levels for seabirds from uncontaminated areas (Kehrig et al. 2015; Scheuhammer 1987). Similarly, Pb concentrations of 4 µg/g dw in feathers are known to be the threshold level of toxicity (Burger and Gochfeld 2000a). A study conducted in South Korea (Kim and Oh 2014) found that high levels of Pb in liver (6.2 µg/g) could negatively affect both behavior and growth of gull chicks. However, Pb concentrations in Northern gannet from NW Spain are far below this threshold value. Influence of age on metal contents has been clearly demonstrated in some similar studies, even if studies are scarce (particularly in soft tissues). Significantly higher elemental levels were revealed in adult compared to juvenile seabirds from the Indian Ocean (Kojadinovic et al. 2007). Except for As and Pb, significant differences according to the age were also detected for Zn, Cd and Hg in Northern gannet from the Portugal coast (Mendes et al. 2008). Such age-dependent increments are a well-documented occurrence in many seabirds (Burger and Gochfeld 2000b; Mendes et al. 2008; Saeki et al. 2000), mainly in feathers (the only tissue where a significant correlation was observed for all the considered metals in the present study) even if birds have a molting system for metal removal (Mendes et al. 2008).

With respect to the gender, and in accordance with the data obtained in the present study, this endogenous factor did not seem to influence element levels of adult birds of each species with the exception of the hepatic Se (not considered in the present study), which was higher in male than in female tropicbirds, and in conclusion it was established that reproductive status, defined by the presence or absence of an incubation patch, did not significantly influence trace element levels among adult birds for all the considered species (Kojadinovic et al. 2007). In a similar way, gender difference for many essential and non-essential metals was not observed in seabird populations from Japan (Nam et al. 2005)

Conclusions

Exposure of migratory birds to contaminants is determined by their migration patterns, which can extend across the entire hemispheres between breeding and wintering areas, as for Northern gannets (Pérez-López et al. 2006). Moreover, and as previously indicated, the levels of elements may vary widely among different seabird species depending on the bird's feeding ecology, intensity and timing of exposure in foraging areas, as well as their physiological and biochemical characteristics (Savinov et al. 2003). With all those considerations the relevance of the present work is evident, as ecotoxicological studies need data on the same seabird species in a wide range of areas considering some different endogenous and exogenous factors, to help understanding the behavior and potential toxicity of metals in seabirds.

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Figure captions

Fig. 1 Zn levels (mg/kg of dry weight) in liver, kidney and feathers of *Morus bassanus*, according to age (I: immature, A: adult) and gender (M: males, F: females). Box plots represent median values and 25 to 75% percentile ranges. ***$p<0.001$, significance level.

Fig. 2 As levels (mg/kg of dry weight) in liver, kidney and feathers of *Morus bassanus*, according to age (I: immature, A: adult) and gender (M: males, F: females). Box plots represent median values and 25 to 75% percentile ranges. **$p<0.01$ and ***$p<0.001$, significance levels.

Fig. 3 Cd levels (mg/kg of dry weight) in liver, kidney and feathers of *Morus bassanus*, according to age (I: immature, A: adult) and gender (M: males, F: females). Box plots represent median values and 25 to 75% percentile ranges. **$p<0.01$ and ***$p<0.001$, significance levels.

Fig. 4 Hg levels (mg/kg of dry weight) in liver, kidney and feathers of *Morus bassanus*, according to age (I: immature, A: adult) and gender (M: males, F: females). Box plots represent median values and 25 to 75% percentile ranges. ***$p<0.001$, significance level.

Fig. 5 Pb levels (mg/kg of dry weight) in liver, kidney and feathers of *Morus bassanus*, according to age (I: immature, A: adult) and gender (M: males, F: females). Box plots represent median values and 25 to 75% percentile ranges. ***$p<0.01$, significance level.

Fig. 6 Positive significant correlations ($p<0.001$) in levels of Hg and Pb quantified between feathers and liver. Data are expressed in terms of dry weight.