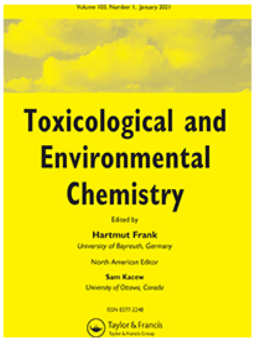

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Levels of zinc, cadmium, and lead in liver, kidney, and feathers of Atlantic puffins (*Fratercula arctica*) from Spain

Ignacio Barrales[a], David Hernández-Moreno[b], Luis Eusebio Fidalgo[c], Ana López-Beceiro[c], Salomé Martínez-Morcillo[a], Lourdes Sánchez-Montero[a], María Prado Míguez[a,d], Francisco Soler[a,e] and Marcos Pérez-López[a,d]

[a]Faculty of Veterinary Medicine, Toxicology Area, University of Extremadura, Cáceres, Spain; [b]Department of Environment and Agronomy, National Institute of Agricultural and Food Science and Technology, Madrid, Spain; [c]Faculty of Veterinary Medicine, Department of Veterinary Clinical Sciences, Universidade de Santiago de Compostela – Campus de Lugo, Lugo, Spain; [d]Institute of Biotechnology in Agriculture and Livestock (INBIO G + C), University of Extremadura, Cáceres, Spain; [e]IPROCAR Research Institute, University of Extremadura, Cáceres, Spain

ABSTRACT

Zinc, cadmium, and lead in livers, kidneys, and feathers of 48 young and adult Atlantic puffins found dead or dying off the coast of Galicia (Northwest Spain) were determined. The most abundant between the three elements was the essential metal zinc, with highest mean levels (173 ± 9 mg/kg dry weight) in livers. For the two non-essential metals, the highest mean levels of cadmium were found in kidneys (22.1 ± 1.0 mg/kg dry weight), and of lead in feathers (1.31 ± 0.10 mg/kg dry weight). For some birds, concentrations of zinc and cadmium exceeded established risk levels. The concentrations of the three metals were positively correlated in livers. In kidneys and livers, cadmium levels were correlated. With respect to age, the levels of the three metals in adults were higher than in young animals. Female birds showed significantly higher levels than males. The results are useful for establishing baseline data of the concentrations of the three metals for this species.

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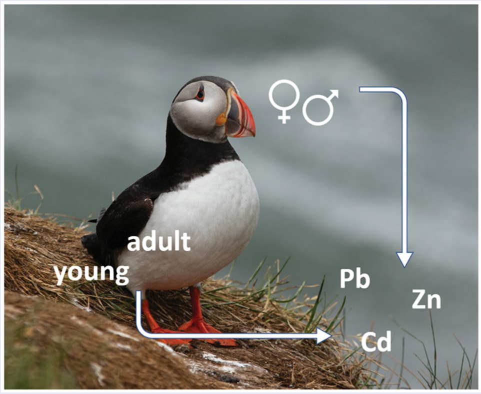
CONTACT David Hernández-Moreno hernandez.david@inia.es Department of Environment and Agronomy, National Institute of Agricultural and Food Science and Technology, Madrid 28040, Spain; Marcos Pérez-López marcospltoxi@gmail.com Toxicology Area, University of Extremadura, Cáceres 10003, Spain

GRAPHICAL ABSTRACT



Abbreviations: Cd: cadmium; dw: dry weight; HNO3: nitric acid; H2O2: hydrogen peroxide; ICP-MS: inductively coupled plasma mass spectrometry; LOD: limit of detection; Pb: lead; SE: standard error; Zn: zinc

Introduction

Pollution is a great problem affecting our planet, a consequence of the presence of chemical agents in some environmental compartments at concentrations that may threaten the health and well-being of living organisms (Hermoso et al. 2008). Especially Cd and Pb are relevant in this respect as they are potentially toxic at low levels. They occur naturally, but through human activities, they are mobilized to become present in some ecosystems at concentrations higher than at previously pristine conditions (He et al. 2020). Specifically, in the marine environment, some metals and pollutants are of concern which bioaccumulates in food chains to reach levels that may constitute health risks to top consumers including humans (Mansouri, Babaei, and Hoshyari 2012). Different anthropogenic sources such as uncontrolled disposal of domestic and industrial waste or direct or indirect industrial emissions contribute strongly, although natural processes are also involved in the release of some metals into the marine environment (Nardiello et al. 2019).

Therefore, methods to estimate the presence and concentrations of potentially toxic elements in the relevant ecosystems are needed (Lodenius and Solonen 2013). Bioindicators are a useful tool to monitor the environmental consequences of pollutants. Seabirds participate in marine ecosystems and feed in different geographic areas, and as they are at the top of the trophic chain, their body burden reflects the extent and effects of bioaccumulation. Furthermore, they respond relatively quickly to the effects of pollution (Mallory et al. 2010; Zhang and Ma 2011).

Exposure of birds to some metals can result in reproductive disturbance, predisposition to disease, and behavioral changes (Zhang and Ma 2011). The concentrations of metals in birds' body tissues are a consequence of their levels in the diet and reflect the degree of environmental contamination (Castro et al. 2011). In most studies with seabirds, potentially toxic metals have been determined in internal organs such as the liver or kidney (Fenstad et al. 2017). Since birds may eliminate certain metals into their feathers, the latter might be useful for pollution monitoring (Mansouri, Babaei, and Hoshyari 2012). As reported before (Castro et al. 2011), metal accumulation depends upon several aspects including sex or age of the bird, but there are no clear patterns regarding elements or tissues.

Whether differences in the metrics of seabird monitoring are due to local or large-scale environmental disturbances remains questionable. This knowledge gap is due to the lack of baseline data regarding important factors of environmental conditions (Mallory et al. 2010). For example, the presence of metals in Atlantic puffin (*Fratercula arctica*) samples has been rarely studied. Like several other seabirds, the Atlantic puffin spends most of the year far from the mainland in the North Atlantic, North Sea, and even the Mediterranean and visits the coast only to breed. The diet consists mainly of fish, including crustaceans, mollusks, and some worms (Guilford et al. 2011).

For the present study, concentrations of Zn, Cd, and Pb were determined in the livers, kidneys, and feathers of Atlantic puffins by inductively coupled plasma mass spectrometry (ICP-MS), considering age and sex as possible factors that affect metal concentrations. Correlation between the levels of metals in different types of samples was estimated to establish the use of feathers for non-invasive sampling. The principal aim was to collect baseline data regarding Zn, Cd, and Pb levels in the Atlantic puffins.

Materials and methods

Sampling

Atlantic puffins, *F. arctica* ($n = 48$), which were found dead on the coast of Galicia (Northwest Spain), or had died at wildlife recovery centers within 5 days, were collected from 2015 to 2017. Of each animal, samples of liver and kidney (10 g each) were obtained and stored in individual plastic bags. Feathers (15–20 g) were pulled from each bird's chest and washed with tap water, distilled water, Milli-Q water, and acetone. Size and appearance of the gonads were recorded for classification according to age (16 young (less than 1-year-old), 32 adults). Sex was determined during necropsies by visual gonad examination and sexual organ development and divided accordingly (28 males, 20 females). All bodies and samples were stored at −20 °C until preparation for analysis.

Metal determination

The protocol was that of Nardiello et al. (2019). Nitric acid (69%) and hydrogen peroxide (30%) were obtained from Fluka (TraceSELECT™, Seelze, Germany). The certified freeze-dried bovine liver was provided by the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). All glass- and plastic-ware materials were washed with 2% HNO_3 prior to use.

Microwave digestion was carried out according to Fromant et al. (2016) and Morton, Tan, and Suvarna (2017). To 2 g biological sample, 6 mL of a mixture of HNO_3 (69%) and H_2O_2 (30%) (3:1, *v/v*) was added. Pre-digestion was run for 12 h at room temperature. Subsequently, for microwave digestion, the temperature was raised to 180 °C within 15 min and kept at this temperature for 5 min. After cooling to room temperature, the digest was diluted to 10 mL with deionized water and stored. Blank digestion was also carried out. Samples from identical specimens were oven-dried at 80 °C until constant weight.

For quantitative analysis, an ICP-MS equipped with an autosampler (7900, Agilent Technologies, Santa Clara, CA, USA) was used. A Peltier cooled (2 °C) cyclone chamber (Elemental Scientific, Omaha, NE, USA) and a low flow Meinhard® concentric nebulizer (0.25 mL/min) (LGC, London, UK) were used for optimal nebulization of the sample. All gases, with a purity of 99.999%, were supplied by Praxair (Madrid, Spain). Daily calibration was performed, with calibration solutions prepared daily from a 10 mg/L multi-element calibration standard solution (PerkinElmer, Shelton, CT, USA). Instrumental detection limits were 0.005 mg/kg for all three elements, and all sample lots included analytical blanks. The working conditions for the ICP-MS were: RF power 1550 W, plasma mode for general purpose, omega bias −120 V, omega lens 9.3 V; deflect lens 1.0 V; energy discrimination 5 V, collision gas 5 mL/min, cell entrance −40 V, and cell exit −60 V.

Standard reference material (BCR® certified reference materials, ref. 185R, Community Bureau of Reference, EU) was used to verify the accuracy of the digestion method, and the obtained results were adequate. For all three metals, the limits of detection (LOD) were 0.003 mg/kg, and the limits of quantification (LOQ) 0.009 mg/kg, determined according to the ICH (2005) guideline on method validation [13]

Table 1. Main statistical parameters of Zn, Cd, and Pb concentrations (mg/kg dw), in liver, kidney, and feathers (total $n = 48$) of Atlantic puffin.

	Element	Mean ± SEM	Median	Range	$n <$ LOD
Liver	Zn	172.6 ± 9.4	163.4	84.0–303	0
	Cd	13.7 ± 0.9	12.6	2.20–24.75	0
	Pb	0.43 ± 0.04	0.46	<0.003–0.85	5
Kidney	Zn	124.1 ± 6.9	117.0	53.8–209	0
	Cd	22.1 ± 1.0	23.0	7.0–35.0	0
	Pb	0.19 ± 0.011	0.192	<0.003–0.35	7
Feathers	Zn	62.2 ± 2.6	62.0	27.3–94.6	0
	Cd	0.35 ± 0.04	0.290	<0.003–0.81	7
	Pb	1.31 ± 0.10	1.48	<0.003–2.32	1

SEM: standard error; $n <$ LOD: number of samples below the limit of detection.

analyzing blank samples. Precision was calculated by fortifying samples at three different levels (five replicates per level) in the validation and determining the coefficients of variation for the replicates as <5.3%. Specificity was performed by analyzing negative samples ($n = 15$) and verifying the absence of interferences. Linearity was performed analysing standard concentrations from 0.1 to 100 µg/L (six concentrations – five replicates per concentration) and blank matrices (liver) fortified with ten defined (five replicates) concentrations of Zn (1–200 mg/kg), Cd (0.01–50 mg/kg), and Pb (0.003–2 mg/kg). Accuracy was evaluated as a recovery percentage (Pb – 84%, Cd – 92%, and Zn – 102%).

Metal concentrations were expressed in mg/kg dry weight (dw), more reliable and consistent than wet weight (ww) values (Adrian and Stevens 1979).

Statistical analysis

GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis. Final concentrations were expressed in dw as mean ± standard error (SE), median, and range. Data normality was assessed by the Shapiro–Wilk test. Given that the data was not normally distributed, the non-parametric Kruskal–Wallis test was used to analyze the influence of the tissue on the element concentrations. The influence of sex and age was assessed by the Mann–Whitney *U* test. Correlations among metal levels were by Spearman's test. The level of statistical significance was established as $p < 0.05$. For statistical tests, a value of 50% LOD was assigned to samples with concentrations below LOD.

Results and discussion

Element concentrations

The average Zn, Cd, and Pb concentrations found in the internal tissues (liver and kidney) and feathers of Atlantic puffins are shown in Table 1.

Given the variability observed in the results, the mean ± SE, median, range, and the number of samples below the LOD are presented. Zn was detected in all samples, while Cd was below the LOD in 14.6% ($n = 7$) of the analyzed feathers. Similarly, Pb was below the LOD in 10.4% ($n = 5$), 14.6% ($n = 7$), and 2.1% ($n = 1$) of the liver, kidney, and feather samples, respectively.

Overall, Zn was the most abundant of all three metals, with the highest concentration in the liver. The highest Cd concentration was in the kidney followed by the liver, and in both tissues, it was the second most abundant of the three elements. In feathers, Pb concentrations were highest, higher than that of the other hazardous, non-essential Cd – but lower than that of the essential Zn. The lowest mean Pb concentration was measured in the kidney. In general, the concentrations of Zn and Cd were higher in the liver and kidney than in feathers. Furthermore, each element differed in its concentrations between tissues, for all tissue combinations ($p < 0.05$). However, considering the number of samples below the LOD, the relevance of this result is relative, especially in the case of Pb – because there were samples of all types below the limit – and in the specific case of Cd in the kidney, since 7 samples were below the limit.

As an essential element, Zn is of great relevance given its role in different metabolic reactions; however, concentrations in the body above the requirements can be harmful. Liver samples of Atlantic puffins collected at various colonies of the Barents Sea revealed a Zn concentration range of 85–101 mg/kg, although the geographical area markedly influenced this value (Savinov, Gabrielsen, and Savinova 2003). There were strikingly higher mean Zn concentrations in liver samples from Galicia; this finding indicates the relevant role of this organ in metal detoxification and storage (Lucia et al. 2008). In fact, liver contents greater than 200 mg/kg are considered a risk for seabirds. Even though the mean concentration measured in this study was below this value, 33% of the samples were higher. In one bird Zn even reached 300 mg/kg (Honda et al. 1990). The measured levels are also higher than those previously reported for the same species sampled in the same area (Pérez-López et al. 2006). The areas sampled in this study were severely affected by the *Prestige* oil spill in 2002, when only 16 days after the sinking, Zn concentrations in the water were twice the normal level (Prego and Cobelo-García 2003). This fact might be related to the levels measured in animals from this area. However, the mean levels measured in feathers were lower than the risk limit of Zn toxicosis reflected in feathers (1200 mg/kg) (Solgi, Mirzaei-Rajeouni, and Zamani 2020). In feathers, metals are bound to the sulfur-containing protein keratin, which has a greater affinity for Zn than other metals. Hence, the Zn content in feathers is largely due to internal deposits (Lodenius and Solonen 2013). Moreover, a previous study undertaken in Galicia with Northern gannets reported Zn levels in liver (89.8 mg/kg dw) and kidney (78.7 mg/kg dw) that were lower than in the present study, while the feather level was higher (72.8 mg/kg dw) (Nardiello et al. 2019). In a study performed with five different bird species from the Bering Sea, the measured Zn levels were higher than in this study only for Tufted Puffin (liver: 156 mg/kg dw; kidney 153 mg/kg dw) and Northern fulmar's kidney (149 mg/kg dw) (Ishii et al. 2017).

Possible sources of exposure to increased Cd in the marine environment include food sources and naturally increased levels in upwelling areas (Summers et al. 2014). Consequently, seabirds from marine environments may be exposed to relatively high concentrations of Cd, which tends to accumulate in tissues (Burger 2008). In this study, the kidney represented the main organ for Cd accumulation, followed by the liver. These data illustrate the importance of the kidneys in the detoxification process of this metal (Lucia et al. 2010). This assumption is in agreement with the results of previous studies with birds (Lucia et al. 2008; Nardiello et al. 2019). In Atlantic puffins from the Barents Sea, Cd concentrations in the liver ranged from 2.6 to 9.8 mg/kg dw (Savinov, Gabrielsen, and Savinova 2003), slightly lower than in the present study. Beyer et al. (2004) compared Cd levels in waterfold from a Tri-State mining district (2.1 ± 3.1 mg k/g dw in the liver and 17 ± 30 mg k/g dw in the kidney) vs a reference site (0.92 ± 0.59 mg k/g dw in the liver and 7.4 ± 4.6 mg k/g dw in the kidney), being in both cases lower than the mean values from the present study. In Atlantic gannets from the coast of Portugal, the Cd concentration in the kidney (35.2 mg k/g dw) of adult birds was higher than our values, even though the Cd concentrations in liver (0.75 mg/kg dw) and feathers (0.12 mg/kg dw) were lower (Mendes et al. 2008). Previous studies with animals – of the same and different species – from the same region revealed markedly lower liver levels than our samples (Pérez-López et al. 2006; Nardiello et al. 2019). Moreover, the liver/kidney Cd concentration ratio is also informative: a ratio less than 1 indicates a low-level chronic exposure situation, while a ratio greater than 1 indicates an acute exposure to relatively high Cd doses (Ek et al. 2004). In this study, the liver-to-kidney ratio was 0.62, a value that suggests Cd accumulation in Atlantic puffin was due to chronic low-level exposure. Some authors suggest that seabirds have adapted to Cd-rich ecosystems (Summers et al. 2014). The presence of Cd, an abundant pollutant in the environment due to its industrial use, can affect the health of the birds and cause kidney toxicity, disrupt calcium homeostasis, thinning of eggshells, testicular damage, and loss of appetite (Burger and Gochfeld 2000; Lucia et al. 2010). On the other hand, Cd deposits in feathers are predominantly internal, rather than externally attached particles (Ek et al. 2004), and it is hypothesized that the feather concentration that corresponds with adverse health effects would be approximately 0.0001–2.0 mg/kg dw for terns (Burger and Gochfeld 2000). The average concentration obtained in the feathers exceeds this range. As no adverse effects of Cd have been documented in wild seabirds, it has been suggested that the threshold level for these species may be greater than for other birds (Burger and Gochfeld 2000).

In aquatic birds from the southwest Atlantic coast of France, Pb concentrations varied among tissues, with higher contents in the kidney and liver than in feathers (Lucia et al. 2010). By contrast, in the present study, the concentrations in feathers of Atlantic puffins are higher than in kidney and liver. These results agree with those for Northern gannets from the same area, but the Pb levels determined in the liver, kidney, and feathers (0.21 mg/kg dw, 0.14 mg/kg dw, and 0.40 mg/kg dw, respectively) of the latter species were markedly lower (Nardiello et al. 2019). Normal and non-risk concentrations for seabirds are considered to be at 0.5–5 mg/kg dw in the liver and 1–10 mg/kg dw in the kidney (Kehrig et al. 2015). Hence, for the present study, the Pb levels in the liver and kidney are not considered toxicologically relevant. Indeed, concentrations in the same order of magnitude were found in a reference site when waterfolds tissues were analyzed (Beyer et al. 2004). In birds, high Pb levels impair the growth and survival of chicks, cause hemolytic anemia, adversely affect reproduction and cause behavior problems (Lucia et al. 2010) In a study carried out with three waterfowl species from Northern Iran, Pb levels in feathers of Common coot (1.64 µg/g dw) were higher than those of the present study (Solgi, Mirzaei-Rajeouni, and Zamani 2020). Given the frequent high values of Pb in feathers, molting might be an effective mechanism of elimination (Lucia et al. 2010). In the present study, the mean Pb values in the feathers did not reach a level (4 mg/kg dw) assumed to elicit adverse effects. However, the atmospheric deposition of this metal in the feathers can constitute an important route of contamination (Jaspers et al. 2004). Thus, this type of sample may not reflect the actual content of the internal tissues because external contamination cannot be entirely removed by washing procedures (Mendes et al. 2008).

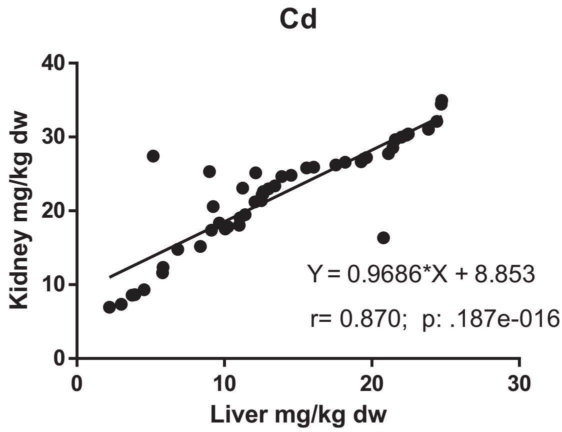


Figure 1. Positive significant correlation ($p < 0.001$) for Cd levels between kidney and liver. Data are expressed in terms of dry weight.

Correlation study

The correlations among the levels of the metals in/between tissues were also evaluated. In the liver, there were significant positive correlations between Zn and Cd ($r = 0.922$, $p < 0.001$); Zn and Pb ($r = 0.936$, $p < 0.001$), and Cd and Pb ($r = 0.967$, $p < 0.001$). These correlations might be related to the metal detoxification process, in which the liver plays a fundamental role, as evidenced by the high levels measured in the samples. The correlation between the liver Cd and Zn levels has been previously reported (Lucia et al. 2008; Malinga, Szefer, and Gabrielsen 2010). It may be due to the effect of metallothionein, a liver protein that binds Cd and Zn, on their metabolism (Van der Oost, Beyer, and Vermeulen 2003). In that case, high levels of one cause the accumulation of the other.

Moreover, Cd levels in liver and kidney were highly correlated ($r = 0.870$, $p < 0.001$) (Figure 1). This association is in agreement with that reported for the Glaucus gull of the Arctic (Malinga, Szefer, and Gabrielsen 2010). Unlike our results, significant correlations have been found in raptors for Cd levels between liver and feathers or between feathers and kidneys, as well as for Pb levels between liver and feathers (Castro et al. 2011). Furthermore, there has been a reported correlation between Pb levels in feathers and liver in Northern garnets (Nardiello et al. 2019). However, no correlations were found between metals levels in feather with respect to internal tissues for Atlantic puffins in the present study. The lack of concordance in the content of metals in feathers with the other tissues was expected: Once the feather is formed, the vascular irrigation is lost, so metals are no longer deposited. Consequently,

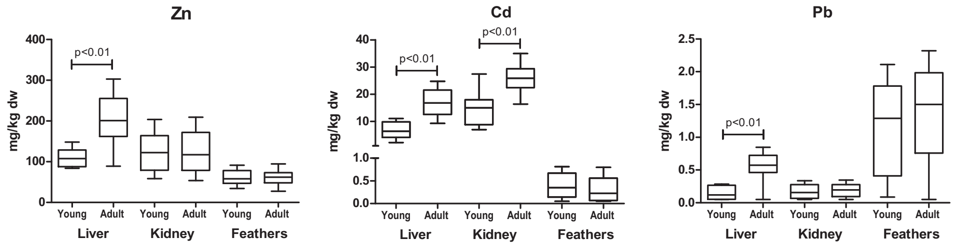


Figure 2. Zn, Cd, and Pb levels (mg/kg dw) in liver, kidney, and feathers of Atlantic puffin, according to age. Box plots represent median values and 25–75 percentile.

the levels measured in feathers correspond to periods prior to those of the sampling (Kojadinovic et al. 2007). The feather metal concentrations probably reflect the metal status in the animal of the year prior to the molt (Marques et al. 2011). Thus, although feather samples are a non-invasive method to measure metal concentrations, they may not the most appropriate samples to establish the actual status of metals in the organism.

Age and sex influence

Based on the measured metal levels in Atlantic puffin samples, there were some significant differences between age classes for metal accumulation. For all metals, adults presented higher levels compared with young animals (Figure 2). In the liver, there were higher concentrations in adults compared with young individuals for Zn (medians of 200 and 107 mg/kg dw, respectively; $p < 0.05$) and for Pb (medians of 0.57 and 0.12 mg/kg dw, respectively; $p < 0.05$). With regard to the age influence on Cd concentrations, adults showed significantly higher contents compared with young animals in the liver (medians of 6.3 and 16.8 mg/kg dw, respectively; $p < 0.05$) and kidney (medians of 25.9 and 15.0 mg/kg dw, respectively; $p < 0.05$). These results are consistent with previous studies on birds. For example, a study in Glaucous gulls reported higher Zn and Cd concentrations in the liver and Cd concentration in the kidney of adults (Malinga, Szefer, and Gabrielsen 2010). Another study with Greylag geese from France measured higher Cd liver and kidney contents in adults compared with young animals (Lucia et al. 2010). Higher metal content in adults may be explained by the long life of these elements, their capacity to bind to metallothionein, and their difficult excretion from living organisms (Mendes et al. 2008).

On the other hand, previous researchers have highlighted the importance of noting sex-specific effects when it is possible – even if the differences in metals levels by sex are not significant (Burger and

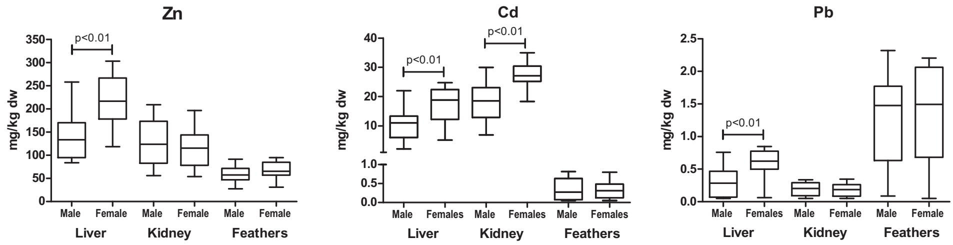


Figure 3. Zn, Cd, and Pb levels (mg/kg dw) in liver, kidney, and feathers of Atlantic puffin, according to sex. Box plots represent median values and 25–75 percentile.

Gochfeld 2009) – as it contributes to the collection of data. In this study, there were some significant differences between males and female Atlantic puffins in tissue metal concentrations, but in all cases, females showed higher levels (Figure 3).

There were differences between females and males for all liver metal contents: Zn (medians of 217 and 133 mg/kg dw for females and males, respectively; $p < 0.05$), Cd (medians of 18.8 and 11.0 mg/kg dw for females and males, respectively; $p < 0.05$), and Pb (medians of 0.62 and 0.28 mg/kg dw, respectively; $p < 0.05$). By contrast, in the kidney only Cd was different (medians of 27.1 and for females and 18.6 mg/kg dw for males; $p < 0.05$). There were no differences between the sexes for the feathers. These results differed from those obtained by previous researchers in seabirds, who did not find significant differences between sexes (Castro et al. 2011; Nardiello et al. 2019). Mansouri, Babaei, and Hoshyari (2012) found significant sex differences in the metal content of feathers of Western reef heron and Siberian gull. While females showed higher Zn and Pb contents, males presented higher Cd contents. Given that access to heavy metals is primarily through food, diet is thought to be related to the sex differences in eating different quantities of food diverse nature (origin) (Ishii et al. 2017).

Conclusions

The presence of essential and potentially toxic concentrations of metals can affect seabird species that live in contaminated environments. The results of this study highlight the need to carry out ecotoxicological studies to obtain data on specific species over a wider range. High metal levels could pose a risk to animal health, especially for Zn and Cd. However, there were no correlations between different tissues, except for Cd levels in the kidney and liver. Metal levels also manifested a cumulative effect on the body with age, and there were differences in tissue metal concentrations between sexes.

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Disclosure statement

The authors want to indicate that there is no potential conflict of interest.

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