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# Essential and non-essential heavy metals in skin and muscle tissues of franciscana dolphins (*Pontoporia blainvillei*) from the southern Argentina coast

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Franciscana dolphin, *Pontoporia blainvillei*, is a small and ‘vulnerable’ cetacean, which exhibits coastal marine habits according to the IUCN (2008). The aim of this work was to determine the presence of essential (Zn and Cu) and non-essential (Cd, Pb, Cr and Ni) heavy metals in skin ($n = 33$) and muscle ($n = 36$) tissues of franciscana dolphins from southern Buenos Aires, establishing the influence of biological parameters on the accumulation of these pollutants. Histological standardised methods were used to determine both age and sexual maturity of the dolphins. Heavy metal concentrations were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Zn was 8-fold higher than Ni in the skin. Zn was significantly affected by the age of the specimens and might be influenced by the state of body condition. Ni showed a behaviour pattern similar to that of non-essential heavy metals. The relationship between Ni and Zn concentration determined in skin and muscle might provide a tool for studying the general condition of endangered marine mammal species such as franciscana dolphins.

**Keywords:** Argentina; biological parameters; heavy metals; marine mammals; multivariate analysis

## 1. Introduction

Marine mammals integrate and reflect ecological variation across large spatial and long temporal scales; they are prime sentinels of marine ecosystem change and represent the last sink for persistent pollutants in the environment.[1] As a high-level predator of the marine food chain many marine mammals (such as odontocetes and pinnipeds) tend to bioaccumulate high concentrations of anthropogenic contaminants, such as organochlorine contaminants and heavy metals.[2–4]

Franciscana dolphin, *Pontoporia blainvillei*, is an endemic species that inhabits coastal waters of the western South Atlantic Ocean, from Itaúnas (18°25′S-30°42′W), Brazil,[5] to Golfo Nuevo (42°35′S-64°48′W) Argentina.[6] As a result of its coastal marine habitat, this species is extremely vulnerable to be by-caught by artisanal fishing nets all around its geographical distribution [6,7] and to accumulate pollutants within its tissues as numerous works have demonstrated.[8–14]

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The information that currently exists, about heavy metals concentrations in skin or muscle in *P. blainvillei,* is based on few dolphins from Argentina [2] and Brazil.[15] In this sense, the aim of this work was to determine the presence of heavy metals (Cd, Pb, Zn, Cu, Cr and Ni) in the epithelial and muscle tissues of dolphins from southern Buenos Aires, and thus establishing the influence of biological parameters (length, weight, age, sexual maturity stage and body condition) on the assimilation of these pollutants.

## 2. Materials and methods

Thirty three skin samples (14 females and 19 males) and thirty six muscle samples (15 females and 21 males) from franciscana dolphins by-caught in gillnet and shrimper nets from 2001 to 2010 were analysed. The studied area includes four localities of the southern coast of Buenos Aires, Argentina: Necochea (N, 38°37′ S, 58°50′ O), Claromecó (C, 38°51′ S, 60°04′ O) and Monte Hermoso (MH, 38°59′ S, 61°15′ O) (Figure 1).

The skin and muscle samples from carcasses were removed after dissection using stainless steel tools. The samples were kept inside plastic bags, and stored at −20°C until their analysis in the laboratory. Total length, weight and sex were recorded for each dolphin in situ.

To determine essential (Zn and Cu) and non-essential (Cd, Pb, Cr and Ni) heavy metals concentration ($\mu g\ g^{-1}$ wet weight) inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin-Elmer Optima 2100 DV) was used. The stratum corneum was removed from the skin samples to minimise external contamination during the chemical analysis. Tissue samples were weighted (0.60 ± 0.01 g) and placed into acid-washed Pyrex tubes. Then, 1 mL of perchloric acid and 3 mL of nitric acid (to a maximum of 10 mL) were poured to each tube. Subsequently, the

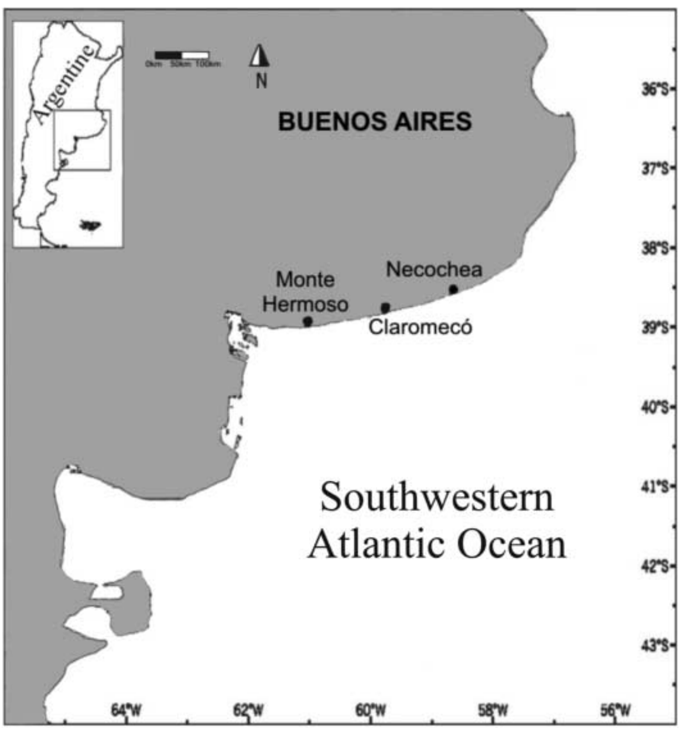


Figure 1. Sampling localities along the Southern coast of Buenos Aires.

tubes were placed in a glycerin heating bath at controlled temperature (110 ± 10°C) until complete mineralisation. Nitric acid (0.7%) was poured to the residue up 10 mL into centrifuge tubes after cooling.[16]

Certified reference materials (CRM; Mussel tissue flour, R.M. No 6, NIES, Japan) were used for the analytical quality control. The detection limit of the method (MDL) ($\mu g \cdot g^{-1}$) and recovery percentages (%) for all heavy metals are: Cu: 1.63–100.6; Zn: 0.26–93.56; Ni 0.21–73.12; Cr: 1.03–80.95; Cd: 1.90–113.17; Pb: 5.34–97.80. All of the relative SDs of the replicate samples are <25%.

Detailed descriptions of the analytical method to ascertain the age of the dolphins were previously described in [17]; therefore we present brief descriptions for analytical method here. Teeth were extracted from each specimen and then fixed in a 10% formaldehyde solution. Subsequently, they were decalcified in commercial acid mix RDO® and sectioned in a cryostat at −21°C. Sections of 25 μm were stained with Mayer's Haematoxylin and mounted in slides with glycerine. Age was determined by counting growth-layer groups (GLGs). Only central well-contrasted layers were used for GLG readings in both dentine and cement. Although no direct validation exists for GLGs, indirect evidence supports that one GLG represents one year of age.[17] The dolphins were divided into three different groups of age: group 1 ($> 0- \leq 1$ years old), group 2 ($\geq 2- \leq 4$ years old) and group 3 ($\geq 4$ years old).

Ovaries and testis were separated from the remained reproductive tract and fixed in 10% formalin solution. A subsample of each testis and ovaries was removed and examined by using standards histological preparations. Sexual maturity groups (Immature and Mature) were determined by macro and microscopically examination.[18–20] Mammary glands were examined for the presence of milk to determine whether a female was lactating. The reproductive tract was carefully inspected looking for the presence of a foetus.

For each dolphin, fat index (BW) was established in order to take heed of their general body condition. This index resulted from the contribution in percentage of the total fat weight to the total body mass. For those specimens whose value of total fat weight was not available, it was estimated through a linear regression between total fat (BW) and axillary girth (CA): $\mathrm{LogBW} = (\mathrm{LogCA} - 0.79)/0.27$ ($R^2 = 0.86, n = 19$).

Heavy metals concentrations were log-transformed. Parametric and nonparametric tests were used to compare different groups: Kolmogorov–Smirnov and Levene tests were used to assume the normality and homogeneity of the data.

Principal component analysis (PCA) was used on heavy metals concentration, biological parameters (standard length and age) and blubber weight (BW) to outline the general structure of the dolphins analysed. ANOVA followed by post-hoc Tukey ANOVA comparison tests have been used to compare the data between the different groups of age. When the necessary assumptions to realise ANOVA were not gathered, Kruskall–Wallis was used followed by multiple comparisons based on the Kruskall–Wallis rank sums to test for pairwise differences among groups of age. Mann–Whitney U test was carrying out to compare differences among sexes and maturity groups when variances were not homogenous. Spearman coefficient was used to test correlations between those values. Generalised lineal model (GLM) was used to evaluate jointly effect of the biological parameters on the heavy metals concentrations in both tissues analysed assuming a log link functions assuming a log link function. The explanatory variables were: age (year), sex, maturity stage (M/I), standard length (L, cm), total weigh (kg). The selection of the best subset of variables was based on the Akaike Information Criterion (*AIC*), those with the lowest *AIC* value are the most explicative. Those models with less than two units of difference between their AIC value and the lowest one might be considered equal with high empirical support.[21] All the analyses were performed with the software Statistica 7.0. For all tests performed the level of statistical significance was set at $p \leq 0.05$ and the data presented as mean ± SD.

## 3. Results

The concentration of Zn and Ni were higher than Pb, Cu, Cr and Cd in skin and muscle samples, only Zn and Ni were above MDL (Skin: Zn = 100%, Ni = 52%; Muscle: Zn = 100%, Ni = 83%). The total of Cd and Cr concentration values in skin samples were under the MDL. Meanwhile, a 99% and a 91% of Pb and Cu concentration values were under the MDL. Cd, Cr and Pb were always under the MDL as well as a 77% of Cu in muscle tissue.

Significant differences were obtained in Zn and Ni concentration between skin and muscle tissue samples (Mann-Whitney: Muscle vs Skin Zn, $p < 0.001$; Ni, $p = 0.04$, Table 1). A positive relationship between Zn and Ni concentration was found only for muscle tissue (Spearman: $r = 0.46, n = 34, p = 0.01$). Table 1 shows the percentage of variation (%CV) of Ni and Zn concentration for both tissues analysed. The Ni %CV were higher in skin and muscle than the same percentage of Zn.

The concentration of Zn was higher in group of age 1 (ANOVA-Tukey post-hoc test $F = 3.99$ $p = 0.03$; $118.39 \pm 42.64\,\mu g\,g^{-1}$) than in groups 2 and 3 ($86.70 \pm 43.29$ and $76.18 \pm 28.93\,\mu g\,g^{-1}$) in skin samples. The groups 2 and 3 show similar concentration of Zn in the same tissue. This result is consistent with the negative relationship observed between log Zn and age ($r^2 = 0.13$; $r = -0.36$; $p = 0.05$). In contrast, the relations between log Zn and length and weight were not significant, but they show a negative trend.

No significant differences were obtained in Ni and Zn concentration between sex, maturity and age groups in muscle samples. However, concentration of Zn and Ni were lower in the group of age 2 (Zn: $10.42 \pm 2.38$; Ni: $0.31 \pm 0.24\,\mu g\,g^{-1}$) than in groups 1 (Zn $= 12.28 \pm 3.65$; Ni $= 0.61 \pm 0.66\,\mu g\,g^{-1}$) and 3 (Zn $= 14.47 \pm 5.12$; Ni $= 1.03 \pm 1.24\,\mu g\,g^{-1}$) in this tissue. In addition, the relationship between both heavy metals and age were not statistically significant in muscle. In contrast, a significant association was observed between log Zn and length and weight in the muscle samples analysed (Spearman length: $r = -0.39$, $p = 0.02$; weight: $r_{sp} = -0.49$, $p = 0.003$; GLM length: AIC $= -44.77$ $p = 0.02$; weight: AIC $= -45.41$ $p = 0.01$).

Sexually mature males (BW skin: $24.16 \pm 7.91\,\mu g\,g^{-1}$, $n = 8$; muscle: $22.92 \pm 9.01\,\mu g\,g^{-1}$, $n = 6$) showed a lower body condition (BW) than sexually immature males (BW skin: $25,35 \pm 9,03\,\mu g\,g^{-1}$, $n = 11$; muscle: $27.09 \pm 5.84\,\mu g\,g^{-1}$, $n = 15$) and sexually immature (I) and mature (M) females in both tissues (skin I: $28.91 \pm 10.51\,\mu g\,g^{-1}$, $n = 10$, M: $27.38 \pm 10,15\,\mu g\,g^{-1}$, $n = 3$; muscle I: $27.98 \pm 10.28\,\mu g\,g^{-1}$, $n = 10$; M: $27.38 \pm 10.15\,\mu g\,g^{-1}$, $n = 3$), nevertheless that differences were no significant (see Table 2).

The PCA produced two significant axes, which jointly explained 68% and 71% of the variance in dolphins' data in skin and muscle, respectively (Figure 2). The first principal component (PC1) represents a gradient of growth (length, age and body condition) and Zn concentration (loadings: $L = 0.80, A = 0.82$, BW $= -0.71$, log Zn $= -0.66$) in skin samples. This analysis revealed that Zn is negatively related with the body condition of the dolphins. PC 2 represents a gradient of Ni

Table 1. Zn and Ni coefficient of variation (%CV) according the tissue analysed. Asterisks indicate significant differences $p \leq 0.05$.

| | Skin | | | | Muscle | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | $n$ | %CV | Mean | SD | $n$ | %CV |
| Zn | 97.9** | 43.3 | 33 | 44.2 | 12.1 | 3.8 | 34 | 31.6 |
| Ni | 0.6* | 0.9 | 21 | 155.6 | 0.6 | 0.8 | 35 | 124.5 |

SD: Standard deviation.
**Mann-Whitney: $p < 0.001$.
*Mann-Whitney: $p = 0.04$.

Table 2. Zn and Ni concentration ($\mu g\, g^{-1}$ wet weight) in muscle and skin tissues according to sex and maturity stage of *Pontoporia blainvillei* survey.

| | | Zn | | | | Ni | | | |
|---|---|---|---|---|---|---|---|---|---|
| Tissue | Sex/Maturity | Mean | SD | Range | *n* | Mean | SD | Range | *N* |
| Muscle | Female | 11.6 | 3.3 | 8.3–19.3 | 14 | 0.7 | 0.9 | 0.1–3.4 | 15 |
| | I | 11.9 | 3.3 | 8.5–19.3 | 10 | 0.8 | 1.0 | 0.1–3.4 | 11 |
| | M | 10.6 | 3.3 | 8.3–15.4 | 4 | 0.4 | 0.5 | 0.1–1.1 | 4 |
| | Male | 12.5 | 4.2 | 7.2–22.3 | 20 | 0.5 | 0.6 | 0.1–1.9 | 20 |
| | I | 12.4 | 3.9 | 9.1–21.7 | 14 | 0.5 | 0.6 | 0.1–1.9 | 14 |
| | M | 12.8 | 5.3 | 7.2–22.3 | 6 | 0.5 | 0.7 | 0.1–1.9 | 6 |
| | Total | 12.1 | 3.8 | 7.2–22.3 | 34 | 0.6 | 0.8 | 0.1–3.4 | 35 |
| Skin | Female | 115.3 | 48.1 | 47.0–241.8 | 14 | 0.3 | 0.5 | 0.1–1.7 | 10 |
| | I | 115.0 | 53.4 | 47.0–241.8 | 11 | 0.2 | 0.1 | 0.1–0.4 | 8 |
| | M | 116.2 | 27.3 | 88.2–142.9 | 3 | 0.9 | 1.1 | 0.1–1.7 | 2 |
| | Male | 85.1 | 35.4 | 32.8–155.2 | 19 | 0.8 | 1.1 | 0.1–13.2 | 11 |
| | I | 84.4 | 38.5 | 32.8–155.2 | 11 | 0.6 | 0.8 | 0.1–1.8 | 4 |
| | M | 86.1 | 33.4 | 46.2–143.5 | 8 | 0.9 | 1.2 | 0.1–13.2 | 7 |
| | Total | 97.9 | 43.3 | 32.8–241.8 | 33 | 0.5 | 0.8 | 0.1–3.2 | 21 |

Note: I: sexually immature dolphins. M: sexually mature dolphins. SD: standard deviation.

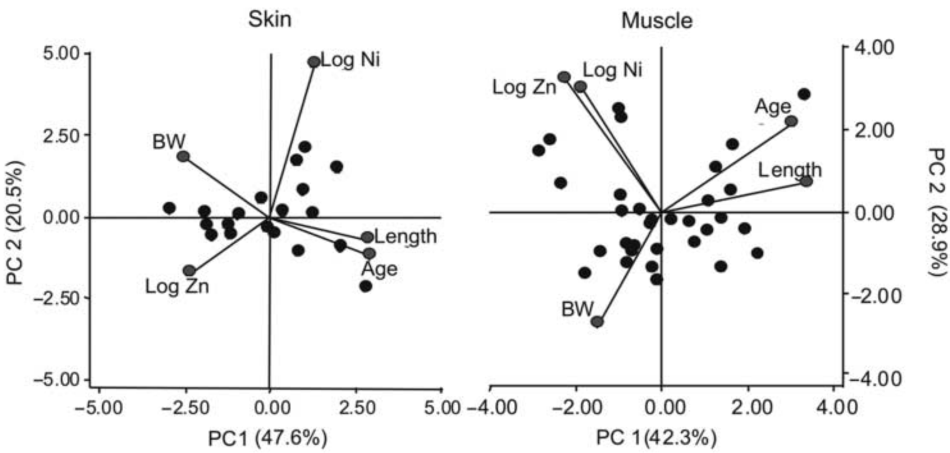


Figure 2. Byplot graph obtained by principal component analysis (PCA) based on Zn and Ni concentration ($\mu g\, g^{-1}$ wet weight) and biological parameters (age, length and body condition) of franciscana dolphins analysed. The black points represent the cases (or dolphins) and the variables are represented by vectors.

concentration (loading 0.86) in the same tissue (Figure 2). The same analysis (PCA) show that the PC1 represent a gradient of growth (length and age) and Zn concentration (loading: $L = 0.88$ and $A = 0.78$, Log Zn $= -0.58$) and the PC2 a contrast between heavy metals (Zn and Ni) and body condition (loadings: Zn $= 0.69$, Ni $= 0.64$, BW $= -0.58$) for muscle samples (Figure 2). It is worth pointing out that this relationship between body condition (BW) and heavy metals (Zn and Ni) was no found by univariate test: Spearman and GLM ($p > 0.05$ and 0.86) in both tissues.

## 4. Discussion

The heavy metals (Ni and Zn) concentration trends found are in agreement with previous results in different species of cetaceans [22–24] where the skin was labelled as a tissue that mainly accumulates Zn and Ni close to MDL. This study revealed statistically significant differences in the Zn and Ni concentration between the analysed tissues, which might suggest the existence of preference for deposition of each element according to chemical affinity and specific function of the tissue as well. In this sense we found that the concentration of Zn in skin was 8-fold higher than in muscle, 4-fold higher than in kidney and around 3-fold higher than in liver from *P. blainvillei* according to previous research.[11,12]

As was previously informed for different cetaceans' species there is an effect of age on Zn accumulation, but not of the sex [25] since the concentration of Zn was similar in males and females dolphins. The high levels of Zn may be associated with its protective function in healing skin wounds and against photo-oxidative damage from solar ultraviolets (e.g. tumour progression [23]). A large amount of Zn is also required for metalloenzymes, proliferation of cutaneous cells, and deposition of collagen at wound sites.[15,26]

Nickel has been shown to be essential for a wide variety of terrestrial organisms, many bacterial and plant species and for humans.[27] However, the essentiality of Ni to aquatic animals (invertebrate as well as vertebrate species) remains unknown.[22,27] Several studies revealed that the cost of chronic Ni acclimation was manifested in a reduced aerobic swimming capacity during strenuous exercise in fish species and may lead to metabolic unbalanced of Mg, Ca, and mainly of Zn.[27] Hyvärinen and Sipilä [28] found an association between Ni concentrations, contributed by the industrial activity in the area of study, and increased pup mortality in ringed seal, *Pusa hispida saimensis*.

Our results suggest that Ni may be behave as non-essential heavy metal in franciscana dolphins owing to two main outcomes: a) the absence of relationship between biological parameters (age, length and weight) and Ni concentration in muscle and skin; and b) the high percentage of variation coefficient (>155%) in both tissues. These variations in the concentration has been informed to be a characteristic feature of non-essential heavy metals in marine mammals and may reflect environmental exposure levels and food preferences of individuals. Essential heavy metals show lower %CV than the non-essential because the former are regulated by physiological processes that maintain homeostasis of the organism.[29] The relevance of this work resides in the incipient response to the presence of Ni in the species. This response was evident through the association between Ni and Zn –in muscle– and the inversed relation between Zn and Ni and body condition (BW) –in skin– (Figure 2). Further studies are likely to provide new insights into the nature of Ni in this species and other marine mammals.

We found that Zn was higher in muscle samples of franciscana dolphins from Rio de Janeiro ($27.00\,\mu g\,g^{-1}$) [15] and northern Buenos Aires [2] (t de Student: $t = 19.98$, $p < 0.001$) than in the dolphins from southern Buenos Aires coast ($12.13\,\mu g\,g^{-1}$). These results indicate that Zn concentrations may be lower in southernmost population of franciscana dolphins than in the other populations of Brazil, Uruguay and Argentina.

Concentration of several heavy metals (Cu, Zn, Pb, Zn Cd and Ni) above detection limits have been informed to occur in surface water and porewater from Bahía Blanca Estuary (cities and harbours associated etc. [30,31]). A probable source of Ni and Zn found in Monte Hermoso was related to the discharge of these pollutants into the streams or directly into the Bahía Blanca estuary by oil refineries, petrochemical industries, meat factories, leather plants, fish factories, textile plants, wool washing plants, silos and cereal mills that are located at the northern boundaries of the estuary.[30] Additional studies are necessary to obtain similar information about heavy metals sources in Necochea and Claromecó.

We found high concentration of Ni in Monte Hermoso and an association between this heavy metal and Zn with the body condition of the dolphins. We have also found that the 99% of beached dolphins were found in that locality. This point deserves further studies on the population franciscana dolphins from Monte Hermoso and Bahía Blanca in order to evaluate the general health status of this population. We also achieve to give novel information about the pattern and factors affecting of Ni and Zn distribution in *P. blainvillei*. Our outcomes strongly support the use of multivariate analysis for the evaluation of data obtained in the field.

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