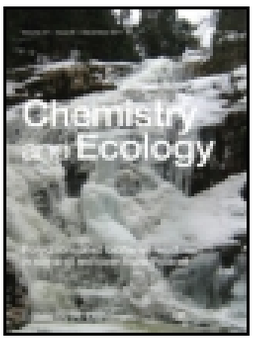

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Mineral and essential element measurements in dolphin bones using two analytical approaches

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ABSTRACT

We explored the potential of using energy-dispersive X-ray analysis (EDX), a non-destructive technique, to assess elemental contents in dolphin bones. Specimens were deposited in museum collections, and prepared by different methodologies. Fifty eight Commerson's dolphins (*Cephalorhynchus c. commersonii*) chevron bones and 24 Franciscana dolphins (*Pontoporia blainvillei*) were analysed. The EDX allowed us to detect the following elements: Ca, P, Na, Mg, Fe, K, Zn, S, Cl and Al; and quantify their proportion (weight percent of element). Principal components analysis differentiates two groups according to the cleaning procedures applied, supporting that cleaning methods could influence the chemical integrity of bone. No significant age-dependent increase was found for elements analysed in species, and no significant differences were found between sex and physical maturity stages. Alternative assessment was made through atomic absorption spectrophotometry, providing quantitative information on the principal elements in bones (Ca, P, Mg, Na, Fe and Zn) and allowing comparisons with other studies. A standard protocol for bone cleaning and conditioning is needed to exclude any effect on the mineral integrity of calcified tissue. This would enable future comparative studies on the bone mineral matrix over time housed in natural history museums or other scientific collections.

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Elements; calcified tissue; skeleton; odontocetes; southwestern south Atlantic Ocean

Introduction

Natural history museum collections have become an important source of information, involving a variety of studies worldwide. As part of those collections, the cetaceans recovered have been derived from by-catch or stranding events. Carcasses are found in different

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stages of freshness or decomposition or even only the skeletons in various degrees of cleanness. Osteological studies provide biological information such as sex and growth of specimens.[1–3] Moreover, the calcified tissues, such as teeth or bones, offer an opportunity to estimate ages and physical maturity.[4–8] It has been suggested that bone matrix durability can remain essentially unchanged for centuries after death.[9] Knowledge concerning feeding habits and genetic diversity using teeth and bone of specimens were performed with this biological material.[10–12] Nevertheless, there are other important data regarding the chemical composition of hard tissue that is still limited for marine mammals.[13–19] Bones potentially provide powerful tools to monitor long-term pollution, but only if consistency of preparation is ensured. To guarantee accurate analysis, the museums should follow a rigorous procedure for the care and preservation of such calcified tissue.

In the southwestern South Atlantic Ocean, among other species, inhabit two small odontocetes the Commerson's dolphin (*Cephalorhynchus c. commersonii*) and the Franciscana dolphin (*Pontoporia blainvillei*); both affected by artisanal fishery nets due to their coastal distribution.[1,3,20,21] This is, in fact, one of the most important conservation threats small cetaceans face and the major reason why the Franciscana dolphins has been categorised as Vulnerable by the International Union for Conservation of Nature, IUCN.[22] Although the Commerson's dolphin has been categorised as Data Deficient since 1996 by the IUCN, further studies are significant for this species' status.

Quantification of macro and trace elements in soft tissue, such as kidney, liver and muscle, has been previously determined for both species.[23–30] However, these analyses in bone are poorly developed, only a few studies regarding elemental concentrations in marine mammal's calcified tissue can be found.[15,16,31] Analytical techniques include an atomic absorption spectrophotometer, inductively coupled plasma spectroscopy and others mass spectrometries, all of which involve time-consuming techniques and complete destruction of the sample. Conversely, scanning electron microscopy coupled with X-ray spectroscopy (SEM-EDX) includes easy sample preparation and, more importantly, undamaging analysis for the integrity of the collection pieces.[32,33]

In this study, we explored the potential of utilising an analytical and non-destructive technique, the SEM-EDX, to assess mineral contents in bone pieces of coastal dolphins archived in museum collections. The specific goals of this study were: (a) to characterise qualitatively the elemental composition of two species of dolphin bones by SEM-EDX, (b) to quantify the concentration of Ca, P, Mg, Zn, Fe and K in bones by atomic absorption spectrophotometry (AAS), (c) to assess differences between bone cleaning procedures and (d) to assess differences between the sex and age classes of species.

Material and methods

Species and sample collection

The Commerson's dolphin occurs along the coast of Patagonia from Río Negro (40°30′S) to the Strait of Magellan, Cape Horn and the Malvinas (Falkland) Islands.[1] Based on geographic, morphological and genetic data, a separate subspecies was determined at the Kerguelen Islands.[34] The Franciscana dolphin also inhabits the coastal waters of southern South America, from Espírito Santo, Brazil (18°25′S, 30°42′W) to Chubut, Argentina (42°35′S, 64°48′W).[20]

The skull and postcranial skeletons of the Commerson's dolphins analysed are deposited in the RNP Goodall collection at the Museo Acatushun de Aves y Mamíferos Marinos Australes, Estancia Harberton, Tierra del Fuego, Argentina. These specimens were recovered from by-catch or stranding events along the coasts of Tierra del Fuego Island (Figure 1) throughout four decades (1975–2011). The specimens of Franciscana dolphins are deposited at the Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia', Buenos Aires, and specimens were also incidentally captured in fishing nets or stranded along the southern coast of the province of Buenos Aires (Figure 1) between years 2004 and 2011. All necropsies were made by standard methods of Norris [35] and Geraci and Lounsbury.[36] Sex was determined by external examination, which could be confirmed with most specimens via direct observation while the carcass was still fresh, or by DNA

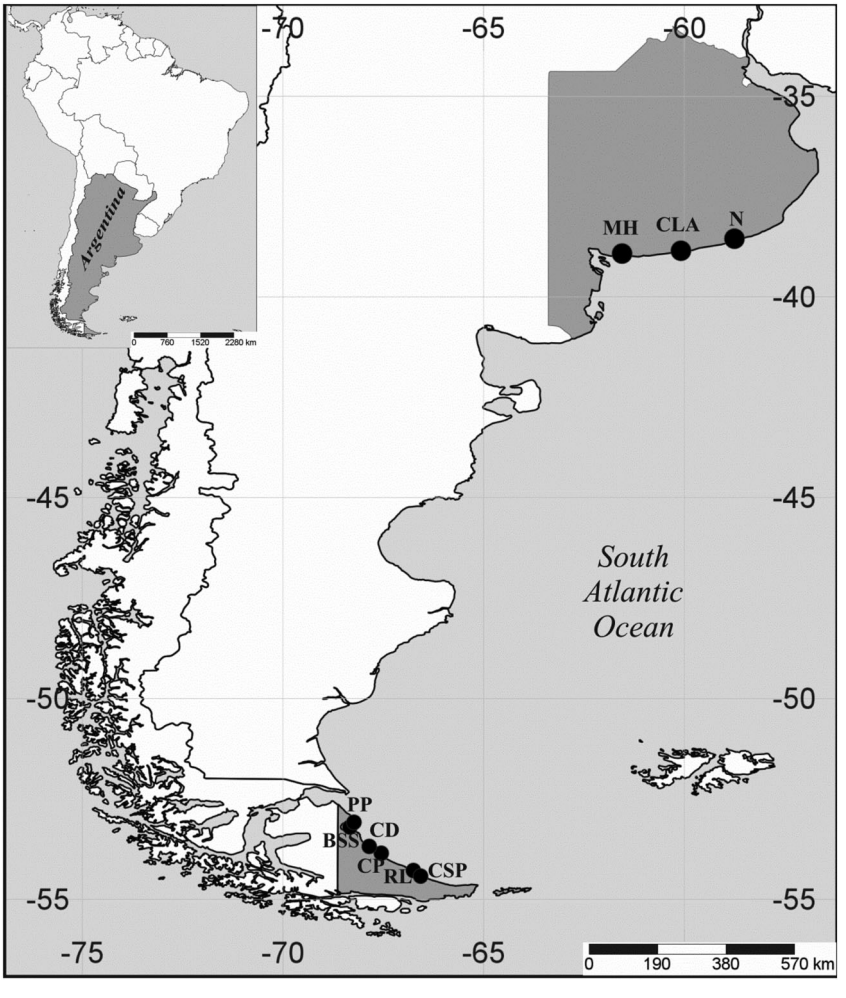

Figure 1. Location map of the analysed by-caught and/or stranded dolphins (*C. c. commersonii* and *P. blainvillei*) in coastal areas of Argentina, from north to south: N, Necochea; CLA, Claromecó; MH, Monte Hermoso; PP, Península el Páramo; BSS, Bahía San Sebastián; CD, Cabo Domingo; CP, Cabo Peñas; RL, Río Láinez and CSP, Cabo San Pablo.

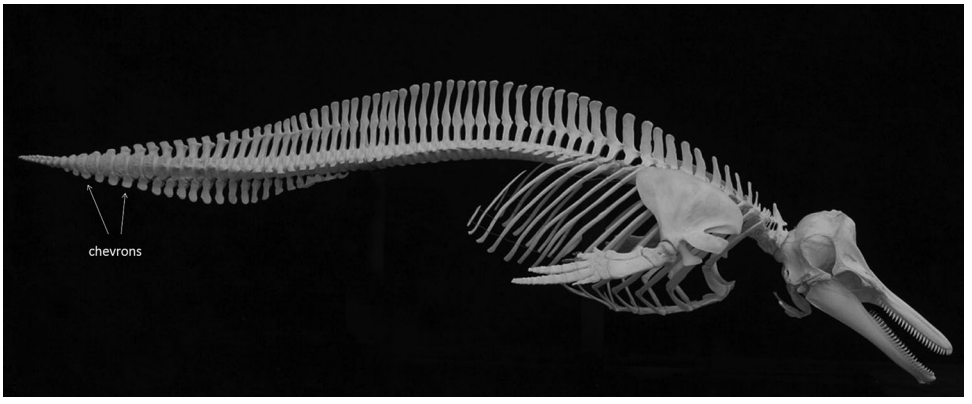

Figure 2. Skeleton of dolphin indicating the position of the chevron bones analysed (adapted from http://commons.wikimedia.org).

analysis, and in the case of the Commerson's dolphin by pelvic bone morphology. [1,3,10,37] Chevron bones were sampled from both species. These bones are paired ventral intervertebral ossifications found in the caudal region of cetaceans (Figure 2), [38] among other mammals. The age of animals was estimated by counting the number of growth layer groups (GLGs, Perrin and Myrick [39]) in dentine for the Commerson's dolphin,[4,5,8] and in the dentine and cement for the Franciscana dolphin.[21] It is assumed for these species that 1 GLG represents a one-year period. The animals used in this study ranged from 0 to 17 years old for the Commerson's dolphins and 0–13 for the Franciscana dolphins (Table 1). For the Commerson's dolphin, the largest animal in our sample (male, 13 year) was 148 cm in length and the shortest animal (male neonate) was 72.2 cm. Whereas for the Franciscana dolphin the largest animal (female, 13 year) was 144 cm in length and the shortest animal was a female neonate with 63 cm (Table 1). All specimens of both species were examined and classified for the physical maturity as follows: Class 0, foetus or neonate in which the neural spine was still unfused to the body of the cervical vertebrae; Class 1, juvenile, with neural spines fused but no epiphyseal fusion; Class 2, subadult, with some epiphyses fused to their vertebral body, starting at head and tail; and Class 3, physically mature individuals with all vertebral epiphyses fused.[1]

Bone cleaning procedures

A total of 81 bones sampled from 57 specimens of Commerson's dolphins (23 females, 31 males and 3 unknown sex) and 24 Franciscana dolphins (12 females and 12 males) were studied. According to their cleaning procedures, the material was classified as fairly recently prepared museum bones (PMB), fresh bones (FB) and dermestids cleaning bones (DCB). Thus, implying three forms of cleaning methods of the bones pieces: (a) ordinary procedure, namely PMB, including a period of soaking in fresh water followed by a warming period in stainless steel pans with soap powder (without boiling) and finally the remains of adhered muscle and ligament were removed by using a scalpel or titanium knives and soft brushes, (b) standard procedure, or FB, here the adhering

Table 1. Biological parameters of dolphins analysed (*C. c. commersonii* and *P. blainvillei*).

		Age (GLGs)				Total body length (cm)			
Species	Sex	Mean	SD	Range	*n*	Mean	SD	Range	*n*
Commerson's dolphin *C. c. commersonii*	Female	5.28	4.23	0.5–14	23	126,83	14.58	95.0–145.5	23
	Male	6.62	4.86	0–15	31	126.57	13.21	72.2–148.0	30
	Unknown	9.43	7.87	1.3–17	3	132.15	4.74	128.8–135.5	2
Franciscana dolphin *P. blainvillei*	Female	2.92	2.47	0–8	12	123.32	25.99	63.0–149.0	12
	Male	3.83	3.51	0–13	12	118.56	14.9	87.7–141.5	12

Note: *n*, number of specimens; SD, standard deviation.

muscle and ligament were carefully removed from the bone samples using titanium knives, and then the surface was washed with double-distilled water; and finally (c) biological procedure, where bones are cleaned using dermestid beetles (*Dermestidae*) in the dermestary of the Mammalogy Collection at the Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia' after each necropsy of the Franciscana dolphin specimens, namely DCB ($n = 9$). The PMB procedure was used in both species (Commerson's dolphins – PMB, $n = 49$ and Franciscana dolphins – PMB, $n = 15$), although the second cleaning procedures were only applied for the Commerson's dolphin specimens (FB, $n = 8$). All bone pieces were stored in plastic bags or mounted on cards tagged with their data, in a designated cupboard at the museums. Prior to the analytical procedures, all chevron bones were conditioned as follows: each one was placed in a plastic vial, covered with a cleaning solution consisting of 9:1 parts (absolute ethanol-to-hydrogen peroxide), and agitated in an ultrasonic cleaner for 3–5 min to remove surface contamination.[40] It was then rinsed with ultrapure distilled water in an ultrasonic cleaner for 15 min, dried overnight and then at 80°C for 12 h. Each bone was mounted on a stub (cylindrical aluminium-slide) for SEM.

Energy-dispersive X-rays (EDX)

Quali- and semi-quantitative characterisation of bone samples were carried out using SEM-EDX at the Laboratorio de Caracterización de Materiales, Centro Atómico Bariloche, Comisión Nacional Energía Atómica (CAB-CNEA). Ca, P, Mg, Na, Zn and Fe were measured by EDX and expressed in weight percent of the element (wt%). The microanalysis was carried out using a Philips 515 coupled to a Thermo Electron Vantage 9900 EDX system. Three spectra were taken from each sample using an accelerating voltage of 20 kV, a probe current of 1.27 nA and a working distance of 500–1000 µm. The measurement of samples was made with magnification to around 300 times. Complementary images were made using backscattered electrons. The microanalysis technique through the SEM-EDX has no destructive impact on the material being analysed and has highly focused and discrete sample areas.

The EDX fitted to a SEM measures the energy of emitted X-rays from specific elements by using a lithium-drifted silicon detector cooled by liquid nitrogen. The relative elemental concentration (at three points on the surface of the bone) was calculated using standardless analysis of the EDX spectra. Peak fitting was made using a digital top-hat filter to remove the background from the spectra before fitting the spectrum with a reference

spectrum and using the Phi-Rho-Z correction method. Each sample measurement was taken with the live time set at 200 s for triplicates (corresponding to the three spectra). A detector measures and records the X-rays; the intensity is converted to elemental proportions. Software EDAX was used for the classification of the elements. The analytical error for most elements is anticipated to be approximately between 0.05% and 1%.

Atomic absorption spectrophotometry

In order to determine the concentration of elements, a quantitative technique was used. The concentration of Ca, P, Mg, Zn, Fe and K was determined in an additional set of 16 chevron bones from 7 female and 9 male specimens of Commerson's dolphins using an atomic absorption spectrophotometer (AAnalyst 300 – Perkin Elmer) (Table 2). Bone samples were pulverised, homogenised and dried overnight at 80°C and then left to cool in desiccators. Aliquots of about 1000 mg from each sample were weighed and then digested in a microwave oven (Millestone Ethos D) with a mixture of nitric and perchloric acids at 80°C.[41] After cooling, the solution obtained was transferred to a 50-mL plastic tube and made up to 30 mL with Milli-Q water (18.2 MΩ cm^{-1}; ±20°C) and the modifier solution ($NH_4H_2PO_4$) according to Pereira et al.[41] The Merck certified standard solutions were analysed together with the samples in order to evaluate the analytical quality control. All analyses were made in duplicate. The detection limits of the method (mg kg^{-1}) for elements were – Ca: 0.1; P: 0.1; Mg: 0.01; K: 0.01; Zn: 0.01 and Fe: 0.1. Comparisons were made assuming 20% of moisture loss for bone tissue.[42] We compared our concentrations with other similar bone type (skeletal vertebras) results, since no other studies including chevron bones were found among the literature.

Table 2. Mineral and essential elements in bones of dolphins (*C. c. commersonii* and *P. blainvillei*) according to cleaning procedures.

			EDX – elements (wt%)					
Species	Cleaning method	Statistics	Ca	P	Mg	Na	Fe	Zn
Commerson's dolphin	PMB	Mean	51.57	32.40	3.64	3.63	1.93	0.84
C. c. commersonii		Median	52.84	32.91	3.27	3.47	1.26	0.75
		SD	4.15	3.11	1.45	1.14	1.77	0.63
		Range	40.63–58.59	17.37–38.09	0.98–7.66	1.68–6.63	0.25–7.64	0.25–3.39
		n	49	49	49	49	49	49
	FB	Mean	47.68	40.10	2.65	5.17	0.93	ND
		Median	48.30	39.99	2.51	5.43	0.74	ND
		SD	5.07	1.52	0.73	2.02	0.51	
		Range	39.47–55.28	37.88–42.58	1.64–3.79	2.18–7.84	0.53–1.98	ND
		n	8	8	8	8	8	8
Franciscana dolphin	PMB	Mean	50.58	33.34	2.80	3.56	1.28	0.44
P. blainvillei		Median	51.05	33.90	3.11	3.07	0.76	0.25
		SD	4.33	2.68	1.27	1.15	1.64	0.26
		Range	44.20–56.03	29.37–38.34	0.90–5.15	2.11–5.41	0.49–7.13	0.25–0.84
		n	15	15	15	15	15	15
	DCB	Mean	49.54	36.78	3.47	4.43	1.53	0.64
		Median	50.60	35.77	3.51	4.24	0.93	0.69
		SD	2.35	3.19	1.02	1.34	1.30	0.30
		Range	45.07–52.13	33.30–40.97	1.93–5.17	2.90–6.76	0.49–4.05	0.25–1.17
		n	9	9	9	9	9	9

Note: PMB, fairly recently prepared museum bones; FB, fresh bones; DCB, dermestids cleaning bones; *n*, number of specimens; SD, standard deviation.

Statistical analyses

The element concentrations of bones (wt% and μg g^{-1}; in each analytical case) are presented as a mean ± standard deviation (SD) and median values are also provided. The coefficient of variation (CV%, the ratio of SD to the mean) reflects the variability of each element among species. Data were tested for normal distribution by Kolmogorov–Smirnov's test and homoscedasticity was checked by Levene's test. Principal component analysis (PCA) was used on elemental measurement and species to outline general data. In order to verify the existing relationship between Principal components (PC's) scores (resulting for the grouped PCA) and biological parameters (age and total body length (TBL)), simple linear regressions were performed. One-way ANOVA followed by the *post hoc* Tukey test/ Fisher's least significant difference (LSD) test have been applied to evaluate differences in sex and physical maturity. The Mann–Whitney *U* test or the Kruskal–Wallis test was used when the statistical assumptions were not gathered. The samples below the analytical error and/or detection limits (EDX = 0.05% and AAS = 0.005–0.05 mg kg^{-1}) were given a value of half the detection limit for statistical analyses. For statistical analysis, only those elements with detectable concentrations above 40% of the whole sample were considered. The level of statistical significance was set at $p < .05$. Analyses were performed using InfoStat (7).

Results

By using the EDX, a total of 10 elements with the following abundance were detected in bones of both species under study: Ca > P > Na ≈ Mg > Fe > Zn, among others (Al, Si, S and Cl). Measurements of Ca, P, Mg, Na, Fe, Zn and K in the bones by two analytical approaches EDX and AAS are presented in Tables 2 and 3, respectively. Due elements such as Al, Si, S and Cl are not commonly in calcified tissue were not considered in the statistical analysis. Nevertheless, Al and Si were found in 84–98% of the bone pieces in both species (Commerson's dolphins: Al: 1.49 ± 0.92; Si: 1.92 ± 1.53; Franciscana dolphins: Al: 2.18 ± 1.91; Si: 3.66 ± 3.59). Cl and S contents in bone were detected up to 50% in the Commerson's dolphin samples (Cl: 1.26 ± 1.20; S: 2.92 ± 2.93) and also in the Franciscana bones (Cl: 0.35 ± 0.08; S: 1.02 ± 0.39). Those elements presented a high coefficient of variation in both species (Commerson's dolphins: 62–100%; Franciscana dolphins; 39–98%).

Energy-dispersive x-rays

Ca and P measurements were the highest among macro-elements in both species, whereas Zn and Fe had the lowest values (Table 2). Mean Ca content in PMB was the highest, whereas mean P and Na contents were higher in FB followed by DCB. Mg mean contents were comparable among all types of bones. Zn was not detectable in FB and about 22% of the PMB had detectable concentrations (0.84 ± 0.63; Table 2) of the Commerson's dolphin. In bone samples of Franciscana dolphin, this element had detectable concentrations (DCB: 0.64 ± 0.29, PMB: 0.44 ± 0.26; Table 2) and higher variability was observed in both types of bone cleaning procedure.

Throughout multivariate analysis, the PCA formed three significant axes which comprise 71% of the variance in the bone samples (cophenetic correlation = 0.96, Figure 3).

Table 3. Review of mineral and essential elements in bones of small cetaceans.

		Elements									
Species	*n*	Ca	P	Mg	K	Na	Fe	Zn	Concentration	Analytical methods	References
C. c. commersonii	16 (7 *f*, 9 *m*)	0.11 ± 0.013	na	0.004 ± 0.008	137 ± 63.7	na	391 ± 301	921 ± 414	µg g^{-1} DW	AAS	This study
C. c. commersonii	58 (23 *f*, 30 *m*, 2 *unknown*)	50.19 ± 3.69	34.63 ± 3.29	3.05 ± 1.20	nd	3.88 ± 1.27	0.52 ± 0.28	1.37 ± 1.50	wt%	EDX	This study
P. blainvillei	24 (12 *f*, 12 *m*)	51.03 ± 4.45	33.47 ± 3.97	3.51 ± 1.41	nd	3.84 ± 1.38	0.76 ± 0.62	1.79 ± 1.69	wt%	EDX	This study
Stenella coeruleoalba	1 (*m*)	na	na	na	na	na	40.7–401	429–487	µg g^{-1} WW	AAS	[13]
Stenella coeruleoalba	3 (Ft)	73.3–89.3	na	0.69–0.80	1.86–2.49	na	na	na	mg g^{-1} WW	AAS	[14]
	4 immature + mat.	159–189		2.09–2.54	1.28–1.90						
Stenella coeruleoalba	13 (Ft)	na	na	na	na	na	85.4	82.2	µg g^{-1} WW	AAS	[15]
	11, *m* calves						70.9	323			
	6, *m* immature						79.8	305			
	5 *f*, 5 *m* mature						101–129	382–409			
Phocoenoides dalli	1 Ft	na	na	na	na	na	170	220	µg g^{-1} WW	AAS	[16]
	1 *f*						281	na			
	1 *m*						na	296			

Note: *f*, female; *m*, male; Ft., foetuses; na, not analysed; nd, not detected.

PC1 represents a contrast of Na versus Zn – Ca (loadings: Zn = 0.60, Ca = 0.77, Na = −0.68); PC2 represents a gradient of Mg and Fe (loadings: Mg = 0.52, Fe = 0.69) in contrast with P (loading: P = −0.63) and PC3 represents a gradient of Zn (loading: Zn = 0.50). No differences were found in PC1, PC2 and PC3 between the two species (PC1–PC2 ANOVA, *Post hoc* Tukey $F = 1.81–2.85$, $p \geq .01$; PC3 Mann–Whitney U test: $U = 640$, $p = .74$). Due to the lack of differences between species, data were pooled together; and there statistical differences were found in PC1 and PC2 among cleaning procedures (PMB vs. FB and DCB) with the exception of PC3 for which those differences were not verified. This outcome can be verified by noting the distribution pattern; PMB and DCB data are distributed to more negative values on PC1, whereas PMB are dispersed more on the same component, a similar distribution can be observed for PC2. A simple linear regression was performed for the three PC's between the age and the total body length, yet no relationship was verified for the PC's (linear regression: age PC1 $R = .08$, $F = 0.38$, $p = .54$; PC2 $R = .24$, $F = 3.79$, $p = .06$; PC3 $R = .14$, $F = 1.25$, $p = .25$; TBL PC1 $R = .03$, $F = 0.07$, $p = .79$; PC2 $R = .14$, $F = 1.26$, $p = .27$; PC3 $R = 1.15$, $F = 1.48$, $p = .23$). No significant differences were obtained for the three PC's related with sex (ANOVA *post hoc* LSD Fisher, PC 1 $F = 1.76$, d.f. = 1, $p = .19$; PC2 $F = 1.92$, d.f. = 1, $p = .17$; PC3 $F = 0.86$, d.f. = 1, $p = .36$), as well as with physical maturity stages (ANOVA *post hoc* LSD Fisher, PC 1 $F = 1.56$, d.f. = 1, $p = .21$; PC2 $F = 1.90$, d.f. = 1, $p = .14$; PC3 $F = 0.20$, d.f. = 1, $p = .89$).

Atomic absorption spectrophotometry

The presence of Ca, P and K contents was highest in the bone, followed by Mg, Zn and Fe (Table 2). A positive relationship was found for Ca with respect to the P, Fe and K contents (Spearman's rank test Ca–P: $R = .81$, $p < .0001$; Ca–Fe: $R = .65$, $p = .006$; Ca–K: $R = .61$, $p = .01$); additionally, P had a similar relationship with Zn (Spearman's rank test P–Zn: $R = .58$, $p = .02$) and Mg had a negative relationship with K (Spearman's rank test $R = -.54$, $p = .03$). The level of these elements had no relationship with the age of the dolphins (Spearman's rank test). No significant differences were found between sexes for each element (Mann–Whitney U test $p > .05$).

Discussion

The EDX technique offered qualitative information on the elements present on the bone samples surface and permitted us to quantify its proportion in the preserved museum specimens. Macro-elements such as Ca, P, Na and Mg were measured in all bone samples of the two species analysed, where Ca and P were the highest. Contents of such mineral components (Ca and P) of dolphin bone are consistent with findings reported for calcified tissue such as bone and teeth of mammals.[32,42] It is well known that the main components of hydroxyapatite, Ca and P, constitute the mineral bone matrix and contribute to approximately 65% of the wet weight of bone.[9,43,44] As in other studies, no differences were found between sex of the species.[14] We found that elemental contents were comparable among the physical maturity of specimens analysed here by EDX. Honda et al. [14] report for stripped dolphins an age-trend of Ca concentrations in the calcified tissue, with an increase during the foetal and weaning stages of individuals which thereafter remained constant. The foetal period is characterised by a

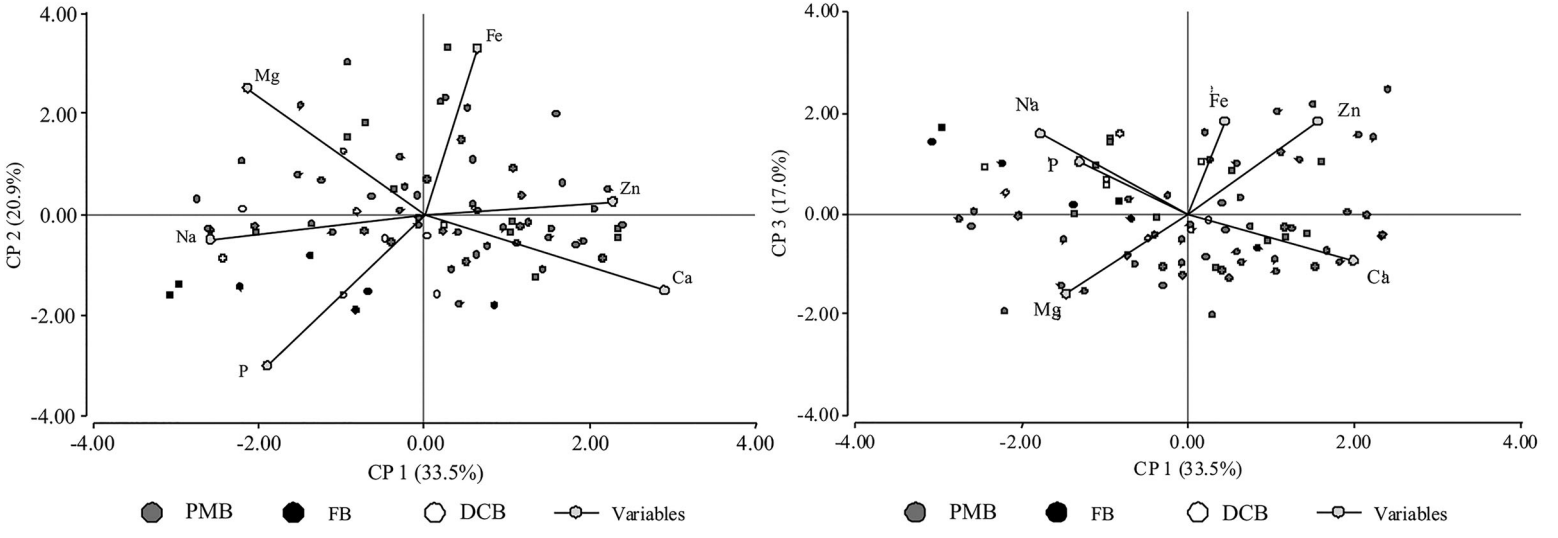

Figure 3. Byplot obtained by the PCA based on Fe, Ca, P, Na, Zn and Mg contents (wt%) in bones of dolphins (*C. c. commersonii* and *P. blainvillei*).

high metabolic rate, elevated development and growth, and high amounts of nutrients, such as Ca, are involved in such processes.[14] Information concerning the distribution characteristics of diverse elements in bones is useful for physiological and ecological aspects. Further studies are likely to provide new insights into the nature of Ca and other minerals in bone of these species.

In the present study, uncommon elements such as Al, Si and Cl were detected in bones of the specimens, and also S in a few samples, it is feasible that some of them may represent contamination during the previous cleaning process of the bone pieces. As mentioned above, these four elements were not considered within the statistical analysis. Aluminium was detected in 93% of samples analysed; this element is a main constituent of the stub for SEM and their presence could be related to the background of equipment. The presence of Si may give evidence of common airborne-dust particles deposited in the porous surface of bone that have not been sufficiently removed during the conditioning of bones prior to our chemical analysis. Several PMB bones under study were stored for longer than 25 years in the Museum's collection and the presence of Si accounts for about 53%, while any of FB exhibited this element was measured by EDX. In addition, elements such as Cl and S were found in approximately 50% of the whole sample analysed. Cl measurements ranged between 0.19% and 6.57%, whereas S ranged between 0.41% and 11.65%. Particularly, Cl was not found in the FB, and the presence of this element in other types of bones could be attributable to the cleaning method applied to some pieces subjected to bone-whitening for the admission to the scientific collection. Sulphur is an abundant element in the earth's crust and is found in large quantities combined by different ways in the vicinity of volcanic areas, mines and thermal waters.[45] Further studies related to the presence of both elements (S and Si) in the bones are needed through a quantitative analytical technique.

According to the multivariate analysis (PCA), two different groups were established in correspondence with the cleaning methods applied to the bones (PMB vs. FB and DCB) (Figure 3), which probably suggests that there may be an interference with the chemical integrity of calcified tissue. It is important to point out that Zn and Fe contents in the two dolphin species were not accurately determined; both of them showed a higher percentage of co-variation and scarce values measured within the relative error of the technique (0.05%). Uncertainties could arise because values of elemental measurements of bones are derived from a qualitative and semi-quantitative analytical technique, such as the EDX. Previous studies have reported trace elements such as Pb, Cd and Hg present in bones and teeth of odontocete and pinniped species, relating their occurrence to environmental influences and contamination.[13–16,46,47] These types of calcified tissue have been found to provide an appropriate archive for monitoring pollution over time and during the life history of an individual.[15,31,48] Although none of these elements were detected in our analysis by the SEM-EDX. Earlier studies reported that most of these elements occurred in very low concentrations,[15,16,32] lower than the detection limits of our analytical instruments (~0.05–1%) which means that they could have been present in the samples analysed, but not detected. Literature regarding the analysis of heavy metals and other elements in marine mammals is extensive; however, the knowledge about mineral and essential elements in calcified tissue [13,16] is limited, mostly due to inherent difficulties of performing studies using cetaceans and to the paucity to obtain appropriate samples for this kind of analysis. Here, we presented the mean values

obtained for bones of Commerson's dolphins with that of other small cetacean (Table 3) through AAS determinations. We found that mean concentrations of Ca, Mg and K in PMB of Commerson's dolphins are comparable or within the range of those reported for the vertebra of striped dolphins.[13] The concentration of Fe in bones of Commerson's dolphins was up to two orders of magnitude lower than that reported for vertebral bones of Dall's porpoises.[16] It should be noted that the data set analysed in our study is low, particularly was limited by the number of one of the dolphin species cleaned by 'standard procedure' (FB) in comparison with PMB and DCB, and, by the fact that most of them were young animals. Additional studies are necessary to verify this pattern. In this sense, researches should also include other species and a larger number of individuals.

Several years of research may be necessary to improve the method's accuracy; also, we consider that bone already represents a valuable tool to aid the knowledge of the dolphin's life history and their environmental studies. To our knowledge, this is the first study focused on the effect of cleaning procedures upon the chemical composition of bones stored in museum collections. These results show the need to improve a protocol for cleaning procedures of bones, avoiding possible interference with their original composition, and that permits further chemical analysis of such biological material. In spite of this, we suggest to follow a standard protocol for treatment and conservation of the bone material that will be subsequently analysed. During dolphin necropsies, selected bones as well as other soft tissues must be carefully excised from the skeleton using surgical tools. Remains of adhered muscle and ligament should be removed by using scalpels or titanium knives and soft brushes. All bone or pieces should be stored in labelled plastic bags at −20° C until analysis. If such proposed methods are considered and taken into account when performing chemical analysis, this may enable further comparisons of calcified tissue over time for a particular species from different scientific collections and/or museums.

Conclusion

The SEM-EDX allows a qualitative description of dolphin bones, taking into account that it is a non-destructive technique, offering the possibility of preservation of museum bone pieces. We recommend a main protocol and standard procedure for preserving this biological material, which does not affect the mineral component matrix, enabling further studies and inter-specific comparisons among diverse species over time.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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