Marine Pollution Bulletin 153 (2020) 110975

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Baseline

Total and subcellular Ti distribution and detoxification processes in *Pontoporia blainvillei* and *Steno bredanensis* dolphins from Southeastern Brazil

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ARTICLE INFO

Keywords:
TiO_2 nanoparticles
Bioavailability
Titanium
Delphinids
Metalloproteins
Detoxification

ABSTRACT

Titanium (Ti), used in many dailyuse products, such as shampoos and sunscreen filters, in the form of TiO_2 nanoparticles (NPs), may elicit adverse marine biota effects. Marine mammal Ti data is scarce, and subcellular distribution and detoxification information is non-existent. Ti concentrations and metalloprotein detoxification in *Pontoporia blainvillei* and *Steno bredanensis* dolphins from Southeastern Brazil were assessed. Metallothionein (MT) concentrations were determined spectrophotometrically, total and subcellular Ti, by ICP-MS and detoxification, by HPLC-ICP-MS. Ti detoxification occurred through MT complexation. Statistical Ti-MT associations were observed in *S. bredanensis* liver, indicating TiO_2 NPs contamination, as Ti binds to MT only as NPs. MT-Ti correlations were observed for both the coastal (*P. blainvillei*) and offshore (*S. bredanensis*) dolphins, evidencing oceanic TiO_2 diffusion. Ti detoxification through binding to reduced glutathione occurred in both species. Thermostable subcellular fractions are a valuable tool for cetacean Ti detoxification assessments and should be applied to conservation efforts.

Titanium (Ti) has been infrequently assessed in marine environments, in part because it is generally considered to display low toxicity (Wise Sr. et al., 2011). However, its increasing use in the form of titanium dioxide nanoparticles (TiO_2 NPs) in foodstuffs, toothpastes, shampoos, cosmetics, sunscreens, deodorants, photocatalysts and drug delivery systems, among others (Weir et al., 2012), has recently elicited environmental concerns, and Ti has now been termed a contaminant of emerging concern (Sauvé and Desrosiers, 2014). However, its effects in this form are still not yet well understood.

TiO_2 NPs are usually present at the highest concentrations in all environmental compartments compared to other NPs, as they are produced worldwide at extremely high production volumes (Nam et al., 2014). Estimates indicate that up to 7.5 million tons of particulate TiO_2 are expected to enter the marine environment in the next decades (Farrokhpay et al., 2010; Owen and Depledge, 2005), as they are not removed from influent sewage by wastewater treatment plants (Kiser et al., 2009; Weir et al., 2012; Westerhoff et al., 2011). This, in turn, leads to their discharge in recipient water bodies, where they may interact with aquatic biota (Weir et al., 2012).

While their mechanisms of action are not yet fully understood, effective TiO_2 NP bioaccumulation, biomagnification and trophic transfer in aquatic biota have been previously reported (Asztemborska et al., 2018; Hosseini et al., 2015; Wang et al., 2017). In addition, deleterious effects have been observed, including oxidative stress (Skocaj et al., 2011; Sureda et al., 2018), characterized by an imbalance between radical oxygen species formation rates and intracellular antioxidant

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https://doi.org/10.1016/j.marpolbul.2020.110975
Received 3 January 2020; Received in revised form 29 January 2020; Accepted 10 February 2020
Available online 22 February 2020

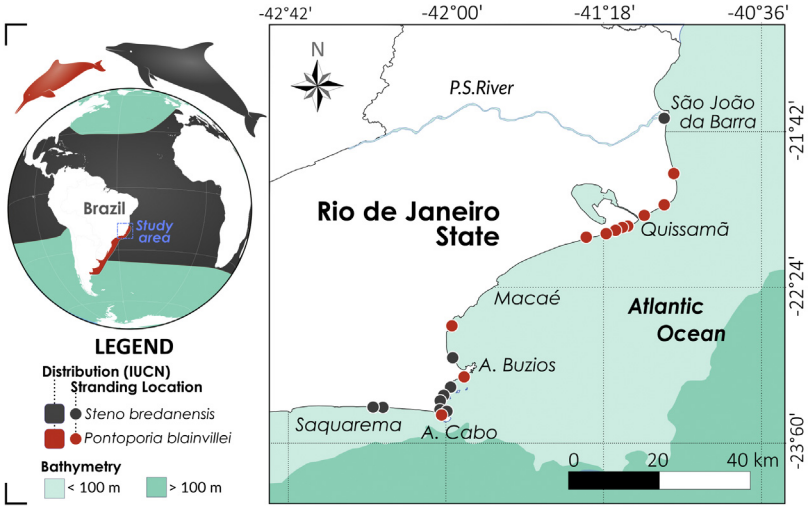

Fig. 1. *Pontoporia blainvillei* and *Steno bredanensis* sampling sites in Rio de Janeiro, Southeastern Brazil.

defenses (Halliwell and Gutteridge, 2015). This results in several negative effects, including interactions with lipids, proteins or nucleic acids, resulting in cellular and/or genetic damages (Costa et al., 2002; Ghezzi and Bonetto, 2003; Levine et al., 1990). Ti adsorption and transfer throughout the food chain have also been reported (Engates and Shipley, 2011). Thus, this contaminant may pose significant risks to both environmental and human health and has been classified by the International Agency for Research on Cancer as "possibly carcinogenic to humans" (Group 2B) (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2010).

Because of this, environmental monitoring assessments are paramount to evaluate environmental health and possible effects on the marine biota. In this regard, many marine mammals are considered excellent sentinel species concerning environmental contamination, as they are long-lived top predators, exposed to pollutants through food web bioaccumulation and biomagnification processes (Bossart, 2011; Gulland and Hall, 2007) However, Ti content data for aquatic organisms is scarce in general and almost no assessments concerning marine mammals are available (Frenzilli et al., 2014).

Conversely, determining total metal content is not the most adequate way to indicate metal bioavailability and possible adverse effects as, after entry in the organism, metals undergo internal subcellular compartmentalization, altering metal bioavailability (Marijić and Raspor, 2006; Wallace and Luoma, 2003). Thus, metals present in soluble cellular fractions are bioavailable and may lead to deleterious effects, while insoluble metals are inert and, thus, biologically unavailable (Dragun et al., 2015; Wallace et al., 2003; Wallace and Luoma, 2003). Assessments regarding metal subcellular distribution, however, have been rarely carried out in marine mammals, and data regarding subcellular Ti contents in these animals are represented by only one recent study published by our research group for *Steno bredanensis* (Monteiro et al., 2019). In addition, biochemical effects should also be determined, such as assessments on oxidative stress endpoints, i.e. reduced glutathione, an important anti-oxidant and metal-detoxifying tripeptide (Adamis et al., 2004; Forman et al., 2009; Ropero et al., 2016), and detoxification, i.e. metallothionein induction, where MT is a heat-stable cytosolic metalloprotein that displays the ability to bind to toxic and excess essential metals for excretion from the body and oxidant-scavenging properties (Das et al., 2000; Ruttkay-Nedecky et al., 2013). In addition, it also plays a role in the transport of certain essential elements, such as Cu and Zn and has the ability to act as a radical oxygen species scavenger when the first intracellular line of defense against oxidative stress (namely, reduced glutathione, GSH) is overwhelmed (Kagi, 1979; Kumari et al., 1998).

Several dolphin species occur in southeastern Brazil, such as the critically endangered *Pontoporia blainvillei* and data-deficient *Steno bredanensis*. The Franciscana dolphin *P. blainvillei* is a coastal species distributed throughout South America, namely Argentina, Uruguay and south-southeastern Brazil and presents a decreasing population trend, with continuing decline of mature individuals, thus classified by the IUCN as vulnerable (Zerbini et al., 2018). This species is non-migrant and experiences several threats, including entanglement in fishing gear, impacts resulting from oil and gas drilling, shipping lanes, aquatic resource harvesting and human recreational activities, pollution and climate change and severe weather, leading to habitat shifts and alterations (Zerbini et al., 2018). Franciscana dolphins usually feeds on shallow-water fish and crustaceans, feeding regularly on shrimp when young and tending to eat higher amounts of small teleost fish (up to about 10 cm) and squid when adult (Walley et al., 1995). The rough-toothed dolphin *S. bredanensis*, on the other hand, displays a still poorly understood distribution and population, inhabiting the Pacific, Atlantic, and Indian Oceans, as well as the Mediterranean, mainly in deep offshore waters beyond the continental shelf, although they are known to inhabit coastal waters in Brazil (Cardoso et al., 2019; Gannier and West, 2005). Threats to this species include habitat degradation, chemical and noise pollution, and entanglement in fishing gear (Walley et al., 1995). This species feeds mainly on squid, octopuses and large teleost fish (Zerbini et al., 2018). No data concerning Ti toxicokinetics are available for either species.

In this context, total Ti contents and subcellular Ti distribution and metalloprotein detoxification were assessed in *Pontoporia blainvillei* and *Steno bredanensis* individuals from southeastern Brazil, in order to assess total and intracellular and, thus, bioavailable Ti contamination. Metallothionein (MT) concentrations were also evaluated to infer detoxification via this biochemical pathway.

Carcass samplings were authorized by the Brazilian Ministry of the Environment (Licenses no. 19665-1, no. 32550-1 and no. 32550-2). Cetacean individuals were found beached between 2005 and 2012 (Fig. 1) in Southeastern Brazil. Carcasses were mostly classified as in decomposition stages 2–3, valid for toxicology assessments (Geraci and Lounsbury, 1993). The tissue samples were removed from the carcasses, placed on ice and transported immediately to the laboratory for processing. For *P. blainvillei*, 8 muscles, 5 livers and 1 kidney sample were analyzed, and for *S. bredanensis*, 10 muscles and 8 livers. Small sample sizes are usual in this type of ecotoxicological assessment, due to the inherent difficulty of studying these animals (Kajiwara et al., 2001; Lavandier et al., 2016; Lemos et al., 2013). Total body length, maturity stage and sex were determined for each specimen, when possible. *P.*

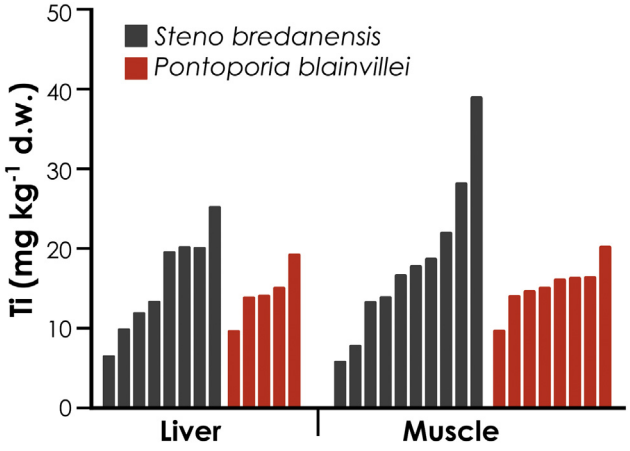

Fig. 2. Total Ti concentrations in *Steno bredanensis* and *Pontoporia blainvillei* liver and muscle tissue. Data are displayed as mg kg^{-1} d.w.

blainvillei individuals were all adults (> 115 cm for males and > 130 cm for females) (Ramos et al., 2000), except for one non-sexed juvenile (86 cm), while *S. bredanensis* specimens were all adult or subadult (> 240 cm) (Siciliano et al., 2007).

Samples were freeze-dried, homogenized in an anti-oxidant Tris-HCl 20 mmol L^{-1} buffer at pH 8.6 containing 0.01% beta-mercaptoethanol as a reducing agent and 0.5 mmol L^{-1} phenyl-methyl-sulphonyl-fluoride as a protease inhibitor and submitted to a sequential extraction, followed by heat-treatment (Erk et al., 2002), which has also been applied to mammalian blood (Kizek et al., 2001; Petrlova et al., 2006) and dolphin (Decataldo et al., 2004; Monteiro et al., 2019) samples. Briefly, a first centrifugation step at 20,000 ×*g* was carried out for 1 h at 4 °C in order to precipitate insoluble metals and perform a preliminary supernatant purification. The resulting insoluble fraction (ISF) contains insoluble, and thus, biologically unavailable metals, poorly associated to the main metal detoxifying protein, MT. Thus, MT is poorly involved in the detoxification of ISF metals (Decataldo et al., 2004). The partially purified supernatant fraction contains bioavailable thermo-labile metals (TLF) which may be detoxified by enzymes (when fresh tissues are analyzed, not freeze-dried) and other labile biomolecules (Decataldo et al., 2004). The TLF was then heated at 70 °C in order to denature most proteins followed by a second centrifugation for 30 min in the same conditions to further purify the samples and obtain a fraction containing bioavailable heat-stable metals and proteins, including MT (thermo-stable fraction, TSF). After processing, all samples were kept at −80 °C if not analyzed at once.

MT contents were determined in both species by UV–Vis spectrophotometry. The heat-treated supernatants were incubated for 30 min in the dark in a 0.2 mol L^{-1} phosphate buffer at pH 8.0 containing 1 mol L^{-1} HCl, 4 mmol L^{-1} EDTA, 2 mol L^{-1} NaCl and 0.43 mmol L^{-1} 5,5-dithiobis-2-nitrobenzoic acid (Ellman, 1959). Absorbances were then determined at 412 nm and MT contents were calculated by using GSH as an external standard, which displays a stoichiometric relationship of 20:1 to MT (Linde and Garcia-Vazquez, 2006).

Total Ti contents were determined in crude aliquots of the three tissues without undergoing any subcellular fractionation treatment. Briefly, about 200 mg of each sample were acid-digested in bidistilled HNO_3 for approximately 4 h at 100 °C in closed polypropylene vessels. After cooling, samples were made up with ultra-pure water and analyzed on a NexIon 300X ICP-MS using ^{103}Rh as an internal standard. Ti subcellular distribution was determined in the three fractions by inductively coupled plasma mass spectrometry (ICP-MS), following the same sample preparation as for total Ti content, using aliquots (~200 μL) of each fraction. A certified reference material (DORM-4) was used to validate sample preparation methodology.

Ti detoxification by metalloproteins in the TSF was characterized by Size Exclusion Chromatography on a high-performance liquid chromatograph coupled to the aforementioned ICP-MS spectrometer (SEC-HPLC-ICP-MS), according to previous studies using the same technique (Gonzalez-Fernández et al., 2011; Lavradas et al., 2016). The same amount of total protein (40 μg) of each sample was injected, to enable comparisons regarding peak intensity and, thus, Ti contamination. Column calibration was performed using serum Bovine Albumin (BSA) (67 kDa, determination of Zn), MT-I (7 kDa Cd determination) and GSH (0.3 kDa Cu determination).

Intra- and inter-species differences between tissues means were evaluated by a one-way ANOVA followed by Tukey's post-hoc test. Pearson correlation coefficients (r) between animal total lengths and Total Ti concentrations as well as for all variables in the three subcellular fractions were calculated, following a linear regression analysis (r^2). Correlations presenting $p \leq 0.05$ were considered indicative of statistical significance. Variable data were log-transformed prior to the correlation analysis. Statistical analyses were carried out using the Statistica (Statsoft) and Prism (GraphPad Software) statistical data software packages.

MT concentrations ranged from 7255 to 14,285 μmol g^{-1} for liver (means and standard deviation of 10,930 ± 2610 μmol g^{-1}) and 4425 and 15,621 μmol g^{-1} for muscle (means and standard deviation of 12,189 ± 3794 μmol g^{-1}) for *P. blainvillei*. The MT value for kidney species was of 13,242 μmol g^{-1} (only one sample was analyzed). For *S. bredanensis*, MT concentrations ranged between 5056 and 10,357 μmol g^{-1} for liver (means and standard deviation of 7187.19 ± 1825.30 μmol g^{-1}) and 5490 and 9899 μmol g^{-1} for muscle (means and standard deviation of 7425.02 ± 1435.49 μmol g^{-1}). No significant differences ($p < 0.05$) were noted between MT concentrations in the three assessed tissues of both species. MT levels in muscle were unexpectedly similar to liver concentrations, as liver is the most metabolically active organ, followed by kidneys and, as such, should display the highest MT concentrations. Thus, a certain amount of metal accumulation seems to be occurring in muscle, as this tissue presents low excretion capacity, leading to cellular contaminant accumulation (Dural et al., 2007). This seems to indicate high environmental Ti exposure, possibly overwhelming primary metal detoxification and excretion routes applied by both cetaceans in liver.

Total Ti concentrations are displayed in Fig. 2 for each assessed individual, expressed as μg g^{-1} dry weight (d.w.). The only kidney sample is not shown (18.2 μg g^{-1} d.w.). No significant differences were observed when comparing the means of each species for each analyzed tissue ($p > 0.05$). For *S. bredanensis* Ti means and standard deviations were as follows: 16.20 ± 6.33 μg g^{-1} d.w. in liver and 18.67 ± 9.71 μg g^{-1} d.w. in muscle. For *P. blainvillei*, 14.74 ± 3.41 μg g^{-1} d.w. in liver and 15.70 ± 2.91 μg g^{-1} d.w. in muscle. No statistically significant correlations were observed between total Ti concentrations and animal length (age).

Although both assessed species present different diets and habitats, no significant inter-species differences between total Ti for either liver or muscle were observed. This may be due to the high bioavailability of TiO_2 NPs throughout the study area, as effective bioaccumulation, biomagnification and trophic transfer of these compounds in aquatic food webs has been demonstrated previously (Asztemborska et al., 2018). Therefore, we postulate that different trophic web components throughout the coast of Rio de Janeiro, such as different prey items for both dolphin species, are exposed to and efficiently bioaccumulate TiO_2 NPs. Unfortunately, however, no studies regarding titanium in species predated by dolphins in the study area are available. This hampers discussions in this regard but indicates the novelty and importance of the present study. Future assessments in this regard will be carried out by our research group in order to shed further light on Ti bioaccumulation and potential biomagnification processes.

Most studies indicate relatively low Ti levels in samples obtained from different cetaceans, such as sperm whales (*Physeter macrocephalus*), where skin levels ranged from 0.4 to 119.2 μg g^{-1} dry weight

(Wise Sr. et al., 2011) in a global assessment, while kidney and liver ranged from 0.3 to 1.2 μg g^{-1} dry weight, determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) in individuals found on the southern North Sea (Belgian and The Netherlands) (Holsbeek et al., 1999), common dolphins (*Delphinus delphis*) from the French Atlantic coast, below 0.15 μg g^{-1} dry weight up to 0.22–0.83 μg g^{-1} dry weight in muscle, liver and kidneys, also determined by ICP-AES (Holsbeek et al., 1998) and Commerson's dolphins (*Cephalorhynchus c. commersonii*), from the southwestern South Atlantic Ocean (Argentina), where all samples were below the relatively high limits of detection of the Instrumental Neutron Activation equipment (100–150 μg g^{-1} dry weight in liver, 100–250 μg g^{-1} dry weight in kidney and 40–90 μg g^{-1} dry weight in muscle samples) (Cáceres-Saez et al., 2013). Thus, the levels observed herein are one order of magnitude higher than those noted for several species worldwide (excluding comparisons to the study by Cáceres-Saez et al. (2013), due to the high limits of detection of their study, and skin levels observed for sperm whales, as we analyzed internal organs), indicating significantly high environmental contamination by this element in both species in southeastern Brazil. This is probably due to the fact that TiO_2 production has increased exponentially in the last decades (Farrokhpay et al., 2010; Owen and Depledge, 2005) and current wastewater treatment plants are not able to remove this contaminant during treatment (Kiser et al., 2009; Weir et al., 2012; Westerhoff et al., 2011). In addition, sewage and water treatment is still very limited in the state (Fistarol et al., 2015), with only about 78% of permanent and regular Rio de Janeiro households connected to the sewage system (IBGE, 2000), while slum households, which house a very large population living irregularly are not connected to the sewage system. Thus, huge volumes of untreated effluents are discharged daily into the Rio de Janeiro coastal environment (Fistarol et al., 2015).

Regarding Ti concentrations in the subcellular fractions, this paper expands on the data reported in Monteiro et al. (2019) for only a few *S. bredanensis* individuals. In the present study, significant differences were observed between the assessed species. Ti-metalloprotein detoxification was observed in liver and muscle, both in TLF and TSF for both species. Percentage Ti data for the three subcellular fractions are displayed in Fig. 3. As only one kidney was analyzed, it is not displayed in the graph.

For *P. blainvillei*, a significant difference ($p < 0.05$) between ISF and TSF was observed in liver (mean percentages of 76% and 21%, respectively, with the TLF displaying a means of about 45%). Intracellular Ti distribution displays a very heterogeneous profile in this species, with varying percentage concentrations in the ISF. All the analyzed individuals showed a certain amount of detoxification in the TLF and TSF, indicating protein detoxification by MT and other proteins. Ti presented higher percentage concentration in both muscle TLF and TSF (mean percentages of approximately 11% and 20%, respectively, while the ISf displayed a means of about 57%). This corroborates the MT data regarding seemingly low efficiency of primary metal detoxification and excretion routes.

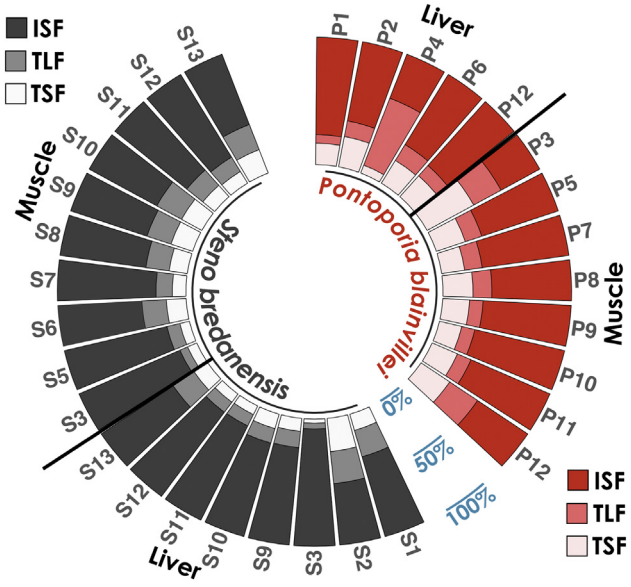

Fig. 3. Percentage Ti data for *Pontoporia blainvillei* and *Steno bredanensis* muscle and liver insoluble (ISF), thermo-labile (TLF) and thermo-stable (TSF) fractions.

For *S. bredanensis*, a significant difference ($p < 0.05$) between ISF and TSF was also observed for Ti concentrations in liver (mean percentages of 77% and 10%, respectively). In muscle, a significant difference was noted between ISF and TLF (mean percentages of 63% and 14%, respectively), as well as between ISF and TSF (mean percentages of 63% and 10%, respectively). Thus, although both tissues performed some level of Ti detoxification, a very high accumulation of this analyte in ISF is still observed.

A significant positive correlation ($r = 0.71$; $p < 0.05$) was observed between MT and Ti in *S. bredanensis* liver TSF. This association seems to indicate TiO_2 exposure per se in the Brazilian marine environment and subsequent detoxification and excretion attempts, as this metalloprotein seems to induce and bind to mammalian MT only when in the form of nanoparticles (Lansdown, 2014; Sureda et al., 2018), seemingly corroborating Ti contamination in the form of NP. In addition, high oceanic diffusion of this contaminant also seems to be the case, either due to oceanic currents and/or differential prey distribution, as *S. bredanensis* lives in offshore waters further from the coast than *P. blainvillei* but presented the aforementioned MT-Ti correlations. The lack of correlations for *P. blainvillei* may be due to the lower sample number analyzed herein.

Only two studies are available in the literature regarding TiO_2 contamination and bioavailability effects in dolphins, both conducted in vitro. The first assessed the genotoxic potential of TiO_2 anatase and micro-sized rutile on bottlenose dolphin (*Tursiops truncatus*) leukocytes obtained from captive individuals (Bernardeschi et al., 2010). Three exposure times (4, 24 and 48 h) and three doses (20, 50 and 100 μg mL^{-1}) were tested. Genotoxicity was verified by the Comet assay, while cytotoxicity was detected by the Trypan blue exclusion method. The authors report that both forms of TiO_2 were genotoxic for bottlenose dolphin leukocytes, with a statistically significant increase of DNA fragmentation after exposure to 50 and 100 μg mL^{-1} for 24 and 48 h (Bernardeschi et al., 2010). The second study analyzed the genotoxic potential of the same compounds on bottlenose dolphin fibroblast and leukocyte cell lines (Frenzilli et al., 2014). Both cell lines were exposed for 4, 24, and 48 h to different TiO_2 concentrations (20, 50, 100, 150 μg mL^{-1}) and DNA damage was investigated by the Comet assay. Both TiO_2 anatase and micro-sized rutile induced DNA damage, and an ultrastructural investigation indicated that TiO_2 particles entered the cell and were compartmentalized within membrane-bound vesicles (Frenzilli et al., 2014).

Regarding the SEC-HPLC-ICP-MS analyses, all samples from both species presented evident high intensity Ti peaks, thus indicating contamination and bioavailability by this analyte in all individuals. Representative spectra for protein-bound Ti are displayed in Fig. 4.

Detoxification seems to be carried out by low molecular weight proteins. Ti retention time was of approximately 21 min for both species, the same retention time as the GSH standard. This indicates probable complexation between Ti and this tripeptide, as GSH is known to complex to other metals, such as Cu (Singh, 2001). However, as this is a contaminant of emerging concern, no records were found in the literature on the formation of the Ti-GSH complex, which, again, evidences the need for further studies to characterize proteins bound to this metal. Liver protein-bound Ti content in *P. blainvillei* was mostly higher than in *S. bredanensis*. This is probably due to the ingestion of prey contaminated with high levels of Ti, since shallow-water fish and

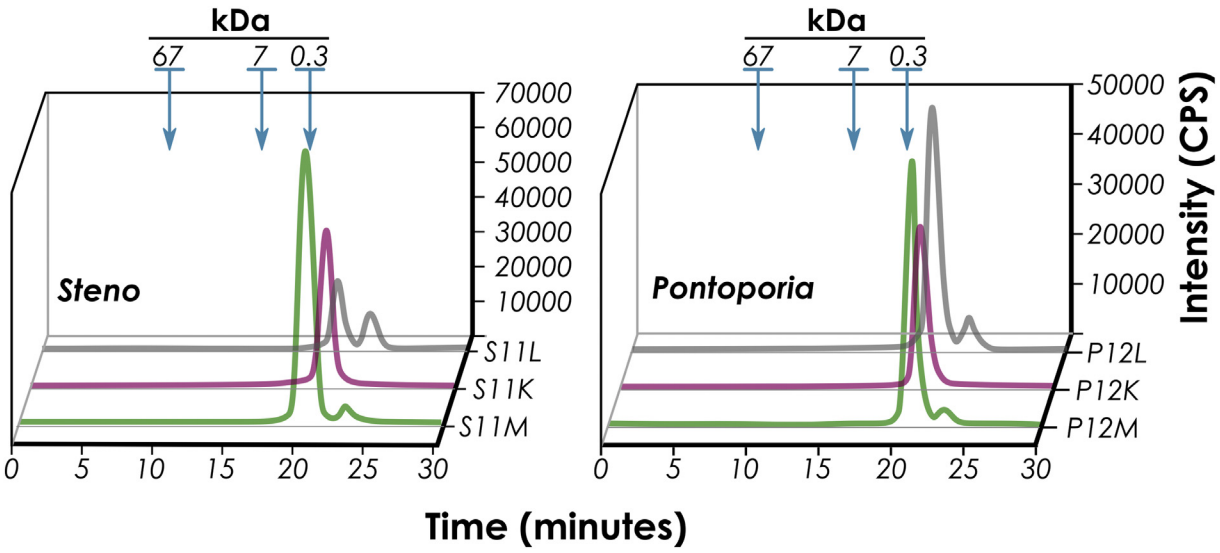

Fig. 4. Protein-bound Ti detected by SEC-HPLC-ICP-MS in *S. bredanensis* and *P. blainvillei* liver (gray line), kidney (pink line) and muscle (green line). The standard protein sizes contained are BSA (67 kDa), MT (7 kDa) and GSH (0.3 kDa). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

crustaceans are probably more exposed to Ti/TiO_2 NP contamination due to their proximity to the coast than offshore species, although baseline Ti data in these animals from southeastern Brazil are also scarce and no baseline Ti levels have been established in this area.

Differential subcellular distribution and thermo-stable metalloprotein detoxification profiles for both a costal and oceanic species were verified regarding Ti contamination which, associated to MT, seems to indicate source of TiO_2 nanoparticles in Brazilian marine waters and probable deleterious effects for these species. The TSF is proven herein as an interesting choice for the evaluation of the biochemical Ti detoxification pathway in the two assessed cetacean species. This report assessed Ti and its detoxification pathways in marine mammals, opening up a new field of possibilities regarding deleterious metal effect assessments and bioavailability studies, which may be applied to conservation efforts.

CRediT authorship contribution statement

Fernanda Monteiro: Investigation, Data curation, Formal analysis, Software, Writing - original draft, Writing - review & editing. **Leila S. Lemos:** Investigation, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Jailson Fulgêncio de Moura:** Investigation, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Rafael Christian Chávez Rocha:** Data curation, Formal analysis. **Isabel Moreira:** Data curation, Writing - original draft. **Ana Paula M. Di Beneditto:** Data curation, Writing - original draft, Writing - review & editing. **Helena A. Kehrig:** Data curation, Writing - original draft, Writing - review & editing. **Isabella C.A.C. Bordon:** Software, Writing - original draft, Writing - review & editing. **Salvatore Siciliano:** Supervision, Conceptualization, Funding acquisition, Project administration, Resources, Writing - original draft, Writing - review & editing. **Tatiana D. Saint'Pierre:** Supervision, Funding acquisition, Project administration, Resources, Writing - review & editing. **Rachel Ann Hauser-Davis:** Supervision, Conceptualization, Methodology, Funding acquisition, Project administration, Resources, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

FM would like to thank the National Council for Scientific and Technological Development (CNPq) for the Master's scholarshop and TDSP, for a productivity grant. APMDB acknowledges grants from CNPq and the Carlos Chagas Filho Foundation for Supporting Research in the State of Rio de Janeiro. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

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