



## Contaminant concentrations, biochemical and hematological biomarkers in blood of West Indian manatees *Trichechus manatus* from Brazil

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### ABSTRACT

The West Indian manatee *Trichechus manatus* is threatened with extinction in Brazil, and this study focused on nondestructive blood samples analyzed for metals, polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs), as well as biochemical and hematological biomarkers. Studied manatees were kept at Projeto Peixe-Boi headquarters in Pernambuco State, and at two natural areas in estuaries where they are released to the wild. Manatees kept at the natural estuary in Paraíba State have blood concentrations of Al, Pb, Cd, Sn that are 11, 7, 8 and 23 times greater, respectively, than the concentrations found in blood of animals from the same species in Florida, USA. An inhibition of butyrylcholinesterase in manatees kept at the two reintroduction sites in Alagoas and Paraíba States indicated possible exposure of the animals to cholinesterase inhibitor insecticides. PCBs and OCPs were not detected. Results from this study will help delineate conservation efforts in the region.

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### 1. Introduction

Mammal species belonging to the order Sirenia have relatively long lives, low reproductive rates and are distributed across over 90 countries. However, the five living species including the dugong and the manatees have the lowest number of individuals among all orders of mammals (Reynolds and Odell, 1991). The West Indian manatee *Trichechus manatus* is an herbivorous animal that has been tentatively divided into two subspecies, the Florida manatee (*Trichechus manatus latirostris*), found in the coasts and rivers of Florida, and the Antillean or Caribbean manatee (*Trichechus manatus manatus*) (Domning and Hayek, 1986), found in the Greater Antilles, eastern Mexico, Central America, and in the North and Northeast of Brazil in a discontinuous distribution (Lefebvre et al., 2001). This subdivision has been questioned, although genetic differences between

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populations are present (Vianna et al., 2006). We will refer to the species that is the subject of this study in Brazil as *Trichechus manatus*, classified as “Vulnerable” (IUCN, 2010). It is the aquatic mammal most threatened with extinction in Brazil (Brasil, 2003) with an estimated population of approximately 500 (Luna et al., 2008).

Problems that have contributed to this situation include the accidental capture of manatees in fishing nets (Lima, 1997), loss of habitat used for breeding and parental care, as well as stranded calves (Parente et al., 2004), collision by boats (Borges et al., 2007), and some etiological agents such as *Salmonella panama* bacteria (Vergara-Parente et al., 2003).

In order to bring populations of manatees in Brazil out of the endangered status and to sustainable levels, Projeto Peixe-Boi, a national conservation and management program was established in 1980. One of its crucial conservation initiatives involves the rescue of orphaned pups. The rescued specimens are sent to a rehabilitation Unit located in the Island of Itamaracá, state of Pernambuco, where the animals receive veterinary care and are fed until an average age of 6 years. At this age, these animals are translocated to two distinct natural rehabilitation bases in the states of Alagoas and Paraíba, where they are reintroduced to their natural habitat and finally released, usually with radio tracking equipment.

Around these manatee reintroduction areas human activities related to sugar cane plantation, shrimp cultivation and lack of

adequate public sanitation impose environmental challenges to the manatees. A large and varied amount of agrochemicals are used in the sugar cane industry in the region, including herbicides such as ametryn and glyphosate, and insecticides such as caborfuran, a carbamate used to control nematodes of sugar cane with a significant potential to leach into aquatic environments (Plese, 2005). Exposure of birds and mammals to this group of contaminants showed certain disturbances in their behavior such as changes in level of activity, aggression, foraging, learning and memory (Grue et al., 1997). In addition to organic contaminants, metals like mercury can cause immunosuppression in marine mammals (Bennett et al., 2001), while other elements, such as cadmium, nickel, lead and tin have been found in significant concentrations in marine mammals, and may be an important risk factor related to death from infectious diseases (Das et al., 2003; Kannan et al., 2006).

Biomarkers are measures of biological effect of chemical compounds including biochemical, physiological, histological, morphological and behavioral endpoints (Depledge, 1993; Walker et al., 2005). Nondestructive biomarkers are those that can be assessed in wild animals using tissue samples collected without causing significant harm to the animals, a key necessity in the case of the Brazilian manatee. Studies using non-destructive biomarkers in marine mammals are scarce due to the fact that few specimens are kept in captivity and to the difficult access to fresh carcasses, as well as the limitations in assessing the animals in the wild. Studies that used this approach include pinnipeds (Fossi et al., 1997, 2006, 2008; Nyman et al., 2003; Tanabe, 2002; Troisi and Mason, 1997) and cetaceans (Fossi et al., 2010; Teramitsu et al., 2000). Among the animal tissues that can be used to evaluate both the accumulation of chemical contaminants as well as different types of nondestructive biomarkers, blood is considered the best option (Fossi et al., 1993).

Biochemical biomarkers like the inhibition of esterases such as the enzyme butyrylcholinesterase have been used as indicators of exposure to organophosphates and carbamates in biomonitoring programs of pesticide exposure in vertebrates (Sánchez-Hernández et al., 2004; Thompson and Walker, 1993).

Similarly, indicators of oxidative stress enzymes such as catalase and glutathione S-transferase are often related to exposure of animals to organic chemical contaminants such as aromatic hydrocarbons (Gutteridge and Quinlan, 1993) and organochlorines like polychlorinated biphenyls (PCBs) (Ferne et al., 2005; Vega-López et al., 2007).

Hematological parameters are another group of non-destructive biomarkers that can provide clinical diagnosis of different diseases in mammals, including immune disorders, anemia, hydration status, among others (Harvey et al., 2009; REMANE-IBAMA, 2005; Vos et al., 2003).

Belanger and Wittnich (2008) report that 482 papers were published from 1954 to 2008 about Sirenians, and 163 of those focused on manatees. Most of the articles address issues related to habitat, food, abundance estimates and impacts of anthropogenic activities other than environmental contaminants. Stavros et al. (2008a) published the first paper to provide information on bioaccumulation of metals in West Indian manatees inhabiting Florida. However, we are unaware of studies regarding bioaccumulation of contaminants and their potential effects evaluated by non-destructive biomarkers in West Indian manatees from Brazil.

Manatees are usually prone to a relatively small bioaccumulation of metals due to their herbivorous nature (O'shea, 2003). However, as they inhabit coastal areas and have a long life, they are still subject to metal bioaccumulation processes (Belanger and Wittnich, 2008). Thus, further studies are required to generate more information on contaminant levels and their possible effects on this marine mammal. In addition, the potential chemical pollution and the decreasing population of the West Indian manatees along the northeastern Brazilian coast render as urgent such an

investigation, in order to improve our understanding of the effects of contaminant bioaccumulation to the health of these marine mammals, providing information for environmental decisions towards their conservation and welfare.

Therefore, the objectives of this study involved the evaluation of metals, PCBs and organochlorine pesticides concentrations, as well as biochemical and hematological biomarkers, in blood collected from West Indian manatees in northeastern Brazil.

## 2. Materials and methods

### 2.1. Study area, animal care and sampling

Samples of blood were collected from 16 West Indian manatees (*T. manatus*) distributed in three groups that were captive in Pernambuco, Paraíba and Alagoas states. The first group with eight animals was kept in oceanarium tanks in the headquarter facilities of Projeto Peixe-Boi, within "Centro Nacional de Pesquisa, Conservação e Manejo de Mamíferos Aquáticos", in Itamaracá Island, state of Pernambuco (PE). This group was composed of five females and three males with age between three and 6 years old, who had been in the projects' facilities since they were rescued, about 3 years before. They were in the rehabilitation period and were maintained in the oceanarium tanks, which consisted of two interconnected tanks with total volumes of 67.84 m<sup>3</sup> and 31.80 m<sup>3</sup>, respectively. The dimensions of the larger tank was 5.3 m long by 4 m wide by 3.2 m deep, and of the smaller tank was 5.3 m long by 4 m wide by 1.5 m deep. Water inside the tanks was pumped from the beach in front of the facility, and filtered through sand filters.

The two other groups of four animals were confined in corrals constructed in natural areas (estuaries) in the states of Paraíba (PB) and Alagoas (AL), and were composed of individuals at the end of the period of readjustment to their natural environments, approximately 6 months after their transfer from the headquarter facilities in Pernambuco. The perimeter fence of the corrals was made of wood in both locations. All manatees studied were part of the reintroduction program, and were subject to constant veterinary monitoring based on procedures outlined in (Lima et al., 2007). They exhibited similar age and health conditions. Group 2 was composed by three males and one female between 6 and 15 years old, all kept in a 2500 m<sup>2</sup> corral at Mamanguape River estuary, surrounded by extensive mangrove vegetation in the state of Paraíba, and. Group 3 was composed by one female and three males between 6 and 8 years old, which were kept in a 1050 m<sup>2</sup> corral at Tatuamunha River estuary, in the state of Alagoas. All animals were fed with sea-grass and vegetables through pipes submerged in the water, in an attempt to simulate the fixed vegetation they naturally feed on in the natural environment.

Blood samples from the 16 manatees were collected during routine clinical evaluation of the animals. This procedure was performed by venipuncture of the brachial vein located in the pectoral fin and collected blood was stored in two separate vacutainer tubes (Stavros et al., 2008a), one of them with heparin anticoagulant for samples for heavy metal analysis, and the other with EDTA anticoagulant, for biomarker samples. Each blood sample was shaken several times to mix well. Samples were immediately frozen in dry ice and then kept at -20 °C until heavy metal, organochlorine and PCBs analysis.

The samples for the biomarker analysis were centrifuged immediately after collection at 7000 rpm using a portable centrifuge (FANEN, São Paulo, SP). Plasma and red blood cells fractions were stored in 2 mL cryovials, and immediately frozen in liquid nitrogen, where they remained until analysis.

## 2.2. Metals analysis

The metals aluminum (Al), magnesium (Mg), iron (Fe), zinc (Zn), copper (Cu), cadmium (Cd), chromium (Cr), tin (Sn) and lead (Pb) were analyzed in whole blood samples by high resolution inductively coupled plasma mass spectrometry (HRICP-MS), using Element 1 with reverse geometry (Element 1, Finnigan MAT, Bremen), at Instituto de Pesquisas Energéticas Nucleares (IPEN). Calibration curve solutions were prepared for five different concentrations: 0.1, 0.5, 1, 2 and 5 ppb. Digestion of the samples consisted of adding 100  $\mu\text{L}$  of blood to 400  $\mu\text{L}$  of concentrated nitric acid suprapure in Falcon tubes, and the mixture was placed in a water bath on a hot plate at 40 °C for approximately 20 min. After digestion of the sample, 1 mL of Indium (In) 10 ppb was added as an internal standard, and the total volume was increased to 10 mL using 0.25% EDTA in deionized MilliQ water (18.2 M $\Omega$ . cm at 25 °C).

Quality assurance methods included blanks, duplicates and analysis of the certified reference material seronorm TM trace element whole blood level III every 10 samples. Recovery rates ranged from 86.3% to 97%. Method detection limits (in  $\mu\text{g g}^{-1}$  wet weight) for the elements under the experimental procedures used were Fe (0.00014), Mg (0.00042), Zn (0.00032), Cu (0.00020), Cr (0.00006), Cd (0.00001), Sn (0.00020), Pb (0.00024) and Al (0.00044).

## 2.3. Organochlorine pesticides and polychlorinated biphenyls analysis

Organochlorine pesticides analyzed in this study were DDTs ( $\alpha$ , $\beta$ -DDT,  $p,p'$ -DDT,  $\alpha,p'$ -DDD,  $p,p'$ -DDD,  $\alpha,p'$ -DDE, and  $p,p'$ -DDE), CHLs ( $\alpha$ - and  $\gamma$ - chlordane, oxychlordane, heptachlor, and heptachlor epoxide), HCHs ( $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -HCH), Drins (aldrin, dieldrin, isodrin, and endrin), HCB, and Mirex. Polychlorinated biphenyls (PCBs) investigated included the following congeners: 8, 18, 28, 31, 33, 44, 49, 52, 56/60, 66, 70, 74, 87, 95, 97, 99, 101, 105, 110, 114, 118, 123, 126, 128, 132, 138, 141, 149, 151, 153, 156, 157, 158, 167, 170, 174, 177, 180, 183, 187, 189, 194, 195, 199, 203, 206, 209. Method detection limits for organochlorine pesticides and polychlorinated biphenyls were 0.10  $\text{ng g}^{-1}$  wet weight.

The analytical procedure for analysis of PCBs and organochlorine pesticides in blood was based on (Grimalt et al., 2010). An aliquot of one gram of blood was extracted 3 times with 3 mL of *n*-hexane and dichloromethane 50% (v/v). Before extraction 100 ng of the standards 2,2',4,5',6-pentachlorobiphenyl (PCB 103); 2,2',3,3',4,5,5',6-octachlorobiphenyl (PCB 198) were added to all samples, blanks and standard reference material (SRM) as surrogate PCBs. SRM 1958 (Organic Contaminants in Fortified Human Serum) was obtained from the National Institute of Standards and Technology (NIST, USA).

Organochlorine compounds were eluted in partially deactivated alumina column chromatography with 10 mL of a 1:1 mixture of *n*-hexane and methylene chloride. The eluate was concentrated to 100  $\mu\text{L}$  and the internal standard for chlorinated pesticides 2,4,5,6-tetrachlorometaxylene (TCMX) was added with final concentration of 100  $\text{ng } \mu\text{L}^{-1}$ . An aliquot of the final extract was injected in a gas chromatograph (Agilent Technologies, model 6890) equipped with an electron capture detector (GC-ECD) for organochlorine pesticide analysis. The PCBs were analyzed by gas chromatography coupled with mass spectrometry (GC/MS) (Agilent Technologies, model 6890/5973 N) in a selected ion mode (SIM).

The identification of pesticides and PCBs was made by comparison of sample retention times with Accustandard of USA reference standards. The quantification was made by ratios between surrogates and compounds of interest, based on the analytical curves built with at least 5 different concentrations for each group of compounds. The compounds analyzed by GC/MS were also identified

through the quantitation ion ( $m/z$ ) and its respective ions of confirmation.

## 2.4. Enzymatic biomarkers

Butyrylcholinesterase (BChE) activity was evaluated in plasma, and catalase (CAT) and glutathione S-transferase (GST) activities were measured in erythrocytes. Total protein was measured according to (Peterson, 1977), using bovine serum albumin standard. Hemoglobin concentration was analyzed using a Standard Kit for hemoglobin (Bioclin, Belo Horizonte, Brazil), and readings were made at 540 nm.

Butyrylcholinesterase activity was conducted according to (Worek et al., 1999), using 50  $\mu\text{L}$  of plasma and 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), and readings were made in duplicates at 412 nm using a UV/vis spectrophotometer (Pharmacia Biotech, Ultrospec 3000).

Catalase activity (CAT) was determined according to Beutler (1975), based on hydrogen peroxide decomposition. We used 5  $\mu\text{L}$  of samples diluted in 995  $\mu\text{L}$  of buffer, and absorbance readings were performed at 240 nm, and activity was expressed in  $\mu\text{mol min}^{-1} \text{mg hemoglobin}^{-1}$ . Glutathione S-transferase (GST) activity was determined according to the method described by Keen et al. (1976), by complexation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CNDB). Absorbance was read at 340 nm and activity was expressed in  $\text{nmol min}^{-1} \text{mg hemoglobin}^{-1}$ .

## 2.5. Hematological biomarkers

Whole blood samples were preserved in 4 mL vacutainer tubes with EDTA as anti-coagulant, and stored at  $-20$  °C until analysis. After thawing, each blood sample was shaken several times to mix well. Analyses of red blood cells (RBC), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and leukocytes (Le) were performed at the same day of sampling using an automatic blood cell counter (Celm<sup>®</sup> Analyzer CC 550, São Paulo, Brazil). Differential analysis was done using blood films stained with a fast panoptic stain kit. The slides were read by optical microscopy at 40 $\times$  and 100 $\times$  magnification.

## 2.6. Statistical analysis

Statistical analysis was performed using Sigmaplot software (Sigmaplot version 11.0, Jandel Scientific, Erkrath, Germany) at an alpha of 5%. Normality was checked by Kolmogorov–Smirnov test and homoscedasticity by Levene median test. If the data were normal and homoscedastic, we used one-way ANOVA to compare means for metals, enzymatic and hematological biomarkers from different treatment groups, which were the three different regions. If statistically significant differences were detected, Bonferroni corrected Tukey multiple comparison tests were used for parametric analysis. In case data failed normality or homoscedasticity a non-parametric Kruskal–Wallis ANOVA was used, followed by Dunn's multiple comparison tests.

## 3. Results

### 3.1. Metals

All analyzed metals were detected in the manatees' blood samples. Essential metals were detected in higher concentrations in animals from the 3 regions including Fe, ranging from 169.4 to 341.8  $\mu\text{g g}^{-1}$  (minimum and maximum concentrations in all

**Table 1**

Trace element concentrations in blood ( $\mu\text{g g}^{-1}$  wet weight) of West Indian manatee *Trichechus manatus* (mean  $\pm$  SD; max. and min.) in the states of Pernambuco, Alagoas and Paraíba, Brazil.

| Element | Pernambuco (n = 8)               |               | Alagoas (n = 4)                  |               | Paraíba (n = 4)                  |               |
|---------|----------------------------------|---------------|----------------------------------|---------------|----------------------------------|---------------|
|         | Mean $\pm$ SD                    | Max. – min.   | Mean $\pm$ SD                    | Max. – min.   | Mean $\pm$ SD                    | Max. – min.   |
| Fe      | 235.3 <sup>a</sup> $\pm$ 60.2    | 341.8–172.0   | 216.4 <sup>a</sup> $\pm$ 14.1    | 241.0–207.8   | 229.0 <sup>a</sup> $\pm$ 45.6    | 278.8–169.4   |
| Mg      | 36.3 <sup>a</sup> $\pm$ 4.9      | 42.0–27.1     | 40.3 <sup>a</sup> $\pm$ 5.9      | 48.3–34.1     | 36.2 <sup>a</sup> $\pm$ 3.9      | 41.9–32.9     |
| Zn      | 10.1 <sup>a</sup> $\pm$ 2.5      | 14.9–7.6      | 9.5 <sup>a</sup> $\pm$ 1.6       | 12.2–7.9      | 11.0 <sup>a</sup> $\pm$ 2.2      | 13.3–9.0      |
| Cu      | 0.90 <sup>a</sup> $\pm$ 0.49     | 1.80–0.39     | 0.70 <sup>a</sup> $\pm$ 0.08     | 0.79–0.57     | 0.92 <sup>a</sup> $\pm$ 0.36     | 1.46–0.69     |
| Cr      | 0.010 <sup>a</sup> $\pm$ 0.003   | 0.015–0.006   | 0.007 <sup>a</sup> $\pm$ 0.003   | 0.010–0.004   | 0.009 <sup>a</sup> $\pm$ 0.002   | 0.013–0.007   |
| Al      | 0.27 <sup>a</sup> $\pm$ 0.11     | 0.41–0.11     | 0.19 <sup>a</sup> $\pm$ 0.07     | 0.29–0.12     | 0.37 <sup>b</sup> $\pm$ 0.11     | 0.48–0.27     |
| Pb      | 0.050 <sup>a</sup> $\pm$ 0.020   | 0.094–0.029   | 0.043 <sup>a</sup> $\pm$ 0.007   | 0.049–0.032   | 0.100 <sup>b</sup> $\pm$ 0.066   | 0.192–0.052   |
| Cd      | 0.0017 <sup>a</sup> $\pm$ 0.0011 | 0.0042–0.0007 | 0.0019 <sup>a</sup> $\pm$ 0.0010 | 0.0035–0.0011 | 0.0082 <sup>a</sup> $\pm$ 0.0145 | 0.0300–0.0007 |
| Sn      | 0.019 <sup>a</sup> $\pm$ 0.001   | 0.020–0.017   | 0.018 <sup>a</sup> $\pm$ 0.000   | 0.018–0.017   | 0.049 <sup>b</sup> $\pm$ 0.056   | 0.133–0.018   |

<sup>a,b</sup>Different letters indicate significant differences among groups (Anova followed by Tukey,  $p < 0.05$ ).

**Table 2**

Enzymatic biomarkers (mean  $\pm$  SD) catalase activity (CAT: U mg hemoglobin<sup>-1</sup>), glutathione-S-transferase (GST: U mg hemoglobin<sup>-1</sup>) and butyrylcholinesterase (BChE:  $\mu\text{mol L}^{-1} \text{min}^{-1}$ ) quantified in blood of West Indian manatee *Trichechus manatus* in the regions of Pernambuco, Alagoas and Paraíba.

| Biomarkers | Pernambuco                       | Alagoas                          | Paraíba                          |
|------------|----------------------------------|----------------------------------|----------------------------------|
|            | Mean $\pm$ SD                    | Mean $\pm$ SD                    | Mean $\pm$ SD                    |
| CAT        | 75.5 <sup>a</sup> $\pm$ 20.6     | 56.7 <sup>a</sup> $\pm$ 17.9     | 85.1 <sup>a</sup> $\pm$ 9.6      |
| GST        | 25.7 <sup>a</sup> $\pm$ 4.9      | 26.2 <sup>a</sup> $\pm$ 4.6      | 25.3 <sup>a</sup> $\pm$ 6.1      |
| BChE       | 0.0016 <sup>a</sup> $\pm$ 0.0004 | 0.0010 <sup>b</sup> $\pm$ 0.0004 | 0.0009 <sup>b</sup> $\pm$ 0.0004 |

<sup>a,b</sup>Different letters indicate significant difference among groups (Anova followed by Tukey,  $p < 0.05$ ).

regions), Mg, ranging from 27.1 to 48.3  $\mu\text{g g}^{-1}$  and Zn, ranging from 7.6 to 14.9  $\mu\text{g g}^{-1}$ . No significant differences among mean blood concentrations from different regions were detected for Fe, (ANOVA I  $F_{2,16} = 0.238$ ,  $p = 0.792$ ), Mg (ANOVA I  $F_{2,16} = 1.161$ ,  $p = 0.342$ ) and Zn (ANOVA I  $F_{2,16} = 0.502$ ,  $p = 0.616$ ) (Table 1).

Other essential metals that were detected in a lower concentration range include Cu, ranging from 0.38 to 1.46  $\mu\text{g g}^{-1}$ , and Cr, ranging from 0.004 to 0.015  $\mu\text{g g}^{-1}$ . No significant differences among mean blood concentrations from different regions were detected for Cu (ANOVA I  $F_{2,16} = 0.528$ ,  $p = 0.601$ ) and Cr (ANOVA I  $F_{2,16} = 1.952$ ,  $p = 0.179$ ).

Among non essential metals, Cd ranged from 0.0007 to 0.0300  $\mu\text{g g}^{-1}$ , Sn ranged from 0.0017 to 0.133  $\mu\text{g g}^{-1}$ , Pb ranged from 0.032 to 0.192  $\mu\text{g g}^{-1}$ , and Al ranged from 0.112 to 0.489  $\mu\text{g g}^{-1}$ . No significant difference among mean blood concentrations from different regions were detected for Cd (Kruskal–Wallis,  $H = 0.982$ , 2 degrees of freedom,  $p = 0.610$ ), whereas a higher metal blood concentration in manatees from Paraíba was detected for Sn (Kruskal–Wallis,  $H = 8.1$ , 2 degrees of freedom,  $p = 0.017$ ; Dunn,  $p < 0.05$ ), Pb (ANOVA I,  $F_{2,16} = 4.17$ ,  $p = 0.038$ ; Tukey,  $p < 0.05$ ), and Al (ANOVA I,  $F_{2,16} = 3.87$ ,  $p = 0.046$ , Tukey,  $p < 0.05$ ).

**Table 3**

Hematologic biomarkers (mean  $\pm$  SD) of West Indian manatees *Trichechus manatus* in the regions of Alagoas, Paraíba and Pernambuco compared with Florida manatees: erythrocytes (Er), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and Leukocytes (Le).

|                      | Er                           | Hb                         | Ht                           | MCV                          | MCH                         | MCHC                        | Le                         |
|----------------------|------------------------------|----------------------------|------------------------------|------------------------------|-----------------------------|-----------------------------|----------------------------|
| Alagoas              | 2.25 <sup>a</sup> $\pm$ 0.09 | 9.4 <sup>a</sup> $\pm$ 0.4 | 28.2 <sup>a</sup> $\pm$ 1.26 | 126.2 <sup>a</sup> $\pm$ 4.9 | 38.7 <sup>a</sup> $\pm$ 5.0 | 32.5 <sup>a</sup> $\pm$ 0.6 | 4.9 <sup>a</sup> $\pm$ 1.0 |
| Paraíba              | 2.69 <sup>a</sup> $\pm$ 0.30 | 9.4 <sup>a</sup> $\pm$ 1.8 | 40.5 <sup>b</sup> $\pm$ 5.44 | 150.3 <sup>b</sup> $\pm$ 5.0 | 34.8 <sup>a</sup> $\pm$ 4.7 | 23.2 <sup>b</sup> $\pm$ 2.4 | 4.5 <sup>a</sup> $\pm$ 0.5 |
| Pernambuco           | 2.49 <sup>a</sup> $\pm$ 0.37 | 9.6 <sup>a</sup> $\pm$ 1.1 | 29.1 <sup>a</sup> $\pm$ 3.40 | 117.7 <sup>a</sup> $\pm$ 9.7 | 38.8 <sup>a</sup> $\pm$ 2.5 | 33.1 <sup>a</sup> $\pm$ 1.4 | 4.6 <sup>a</sup> $\pm$ 2.1 |
| Florida <sup>a</sup> | 2.81                         | 11.2                       | 16                           | 124                          | 40                          | 32                          | 6.6                        |

(Er) ( $\times 10^6 \mu\text{L}^{-1}$ ), (Hb) (g dL<sup>-1</sup>), (Ht) (%), (MCV) (fL), (MCH) (pg), (MCHC) (g dL<sup>-1</sup>) and (Le) ( $\times 10^3 \text{mL}^{-1}$ ).

<sup>a,b</sup>Different letters indicate significant difference among groups (Anova followed by Bonferroni,  $p < 0.05$ ).

<sup>a</sup> Values for Florida manatees based on Harvey et al. (2009).

### 3.2. Organochlorine pesticides and polychlorinated biphenyls

All blood samples analyzed indicated concentrations below detection limits for all listed organochlorine pesticides and PCBs.

### 3.3. Enzymatic biomarkers

We detected an inhibition of approximately 40% in butyrylcholinesterase activity (BChE) in blood from manatees sampled in Alagoas and Paraíba, when compared to values obtained from animals kept in the headquarter facilities of the project, in Pernambuco (ANOVA I,  $F_{2,16} = 6690$ , Bonferroni  $t$ -test,  $p < 0.05$ ). Mean BChE activity was 0.0016  $\mu\text{mol L}^{-1} \text{min}^{-1}$  in animals from Pernambuco, and decreased to 0.0010 and 0.0009  $\mu\text{mol L}^{-1} \text{min}^{-1}$  in animals from Alagoas and Paraíba, respectively. Catalase (CAT) and glutathione S-transferase (GST) activities measured in erythrocytes did not differ among animals from the different regions (Table 2).

### 3.4. Hematological biomarkers

Counts of red blood cells, hemoglobin content, leukocytes and mean corpuscular hemoglobin did not differ between animals from the different regions, and averages were around  $2 \times 10^6$  erythrocytes  $\mu\text{L}^{-1}$ , 9 g hemoglobin dL<sup>-1</sup>,  $4.5 \times 10^3$  leukocytes  $\mu\text{L}^{-1}$ , and 35 pg, respectively. A higher hematocrit of 40.5% was detected in animals from Paraíba, when compared to a value of 29% in Pernambuco and Alagoas (ANOVA I,  $F_{2,15} = 15.4$ ,  $p < 0.001$ ). A higher mean corpuscular volume of 150.3 fL was detected in animals from Paraíba, when compared to a value below 130 fL in Pernambuco and Alagoas (ANOVA I,  $F_{2,15} = 22.5$ ,  $p < 0.001$ ). A smaller mean corpuscular hemoglobin concentration of 23.2 g dL<sup>-1</sup> was detected in animals from Paraíba, when compared to a value above 30 g dL<sup>-1</sup> in Pernambuco and Alagoas (ANOVA I,  $F_{2,15} = 59.25$ ,  $p < 0.001$ ) (Table 3).

**Table 4**  
Average metal concentrations in blood from Brazilian *Trichechus manatus* ( $\mu\text{g g}^{-1}$  wet weight) relative to metal concentrations in blood from Florida *Trichechus manatus*, Florida *Tursiops truncatus*, Wadden Sea *Phoca vitulina*, and Antarctica *Mirounga leonina*.

| Species | <i>Trichechus manatus</i>         |                    |                    |           |           | <i>Tursiops truncatus</i> |           |           | <i>Phoca vitulina</i> |           |           | <i>Mirounga Leonina</i> |           |           |
|---------|-----------------------------------|--------------------|--------------------|-----------|-----------|---------------------------|-----------|-----------|-----------------------|-----------|-----------|-------------------------|-----------|-----------|
|         | Region (A) Alagoas and Pernambuco | Region (B) Paraíba | Region (C) Florida | Ratio A/C | Ratio B/C | Region (D) Flórida        | Ratio A/D | Ratio B/D | Region (E) Wadden Sea | Ratio A/E | Ratio B/E | Region (F) Antarctica   | Ratio A/F | Ratio B/F |
| Fe      | 226                               | 229                | 388                | 0.6       | 0.6       | 458                       | 0.5       | 0.5       | 717.5                 | 0.3       | 0.3       | 700                     | 0.3       | 0.3       |
| Mg      | 38.3                              | 36.2               |                    |           |           |                           |           |           |                       |           |           |                         |           |           |
| Zn      | 9.8                               | 11.0               | 11.3               | 0.9       | 1.0       | 3.98                      | 2.5       | 2.8       | 3.5                   | 2.8       | 3.2       | 3.1                     | 3.1       | 3.5       |
| Cu      | 0.80                              | 0.92               | 0.77               | 1.0       | 1.2       | 0.76                      | 1.1       | 1.2       | 1.03                  | 0.8       | 1         | 1.04                    | 0.8       | 0.9       |
| Cr      | 0.009                             | 0.009              |                    |           |           |                           |           |           | 0.007                 | 1.3       | 1.0       | 0.007                   | 1.3       | 1.3       |
| Al      | 0.23                              | 0.37               | 0.03               | 6.9       | 11.2      | 0.03                      | 8.7       | 14.3      | 0.13                  | 1.7       | 1.5       |                         |           |           |
| Pb      | 0.047                             | 0.100              | 0.013              | 3.6       | 7.7       |                           |           |           | 0.004                 | 11.8      | 47.0      |                         |           |           |
| Cd      | 0.002                             | 0.008              | 0.001              | 2.00      | 8.20      |                           |           |           | 0.001                 | 1.4       | 4.1       | 0.004                   | 0.5       | 2.1       |
| Sn      | 0.018                             | 0.049              | 0.002              | 9.0       | 24.7      |                           |           |           | 0.0008                | 24.0      | 164.3     |                         |           |           |

Region (A) Alagoas and Pernambuco, this study.

Region (B) Paraíba, this study.

Region (C) Florida, USA, Stavros et al. (2008a).

Region (D) Florida, USA, Bryan et al. (2007) and Stavros et al. (2008b).

Region (E) Wadden Sea, Griesel et al. (2008) and Kakuschke et al. (2010).

Region (F) Antarctica, Baraj et al. (2001).

#### 4. Discussion

Our results indicate that West Indian manatees *T. manatus* from Paraíba have higher blood concentrations of the non essential metals Al, Pb, Cd, Sn, when compared to the animals from Pernambuco and Alagoas, as well as in comparison with the same species in Florida, USA (Stavros et al., 2008a). The magnitude of this difference was expressed by the ratio between metal blood concentrations in manatees used in this study and manatees in Florida, as well as the ratio between metal blood concentrations in manatees from this study and other marine mammals (e.g. bottlenose dolphins, common seals, pilot whales) in Table 4. Similarly, the animals from Paraíba have also higher blood concentrations of Al, Pb, Cd, and Sn when compared to other marine mammals in Table 4.

Blood concentrations of the essential metal iron in manatees from this study were lower than iron concentrations of other studied marine mammals (Table 4), ranging from 30% of blood iron measured in *Phoca vitulina* and *Mirounga Leonina* (ratio 0.3), to 50% of blood iron in *Tursiops truncatus*, and up to 60% of blood iron concentrations measured in *T. manatus* from Florida (Stavros et al., 2008a).

Blood concentrations of essential metals Zn and Cu were similar in animals from this study and those from Florida (Stavros et al., 2008a), with concentration ratios varying between 0.6 and 1.2. Cu and Cr concentrations were also similar when comparing manatees from Brazil with *T. truncatus* (Bryan et al., 2007; Stavros et al., 2008b), *P. vitulina* (Griesel et al., 2008; Kakuschke et al., 2010), and *M. Leonina* (Baraj et al., 2001), with ratios varying from 0.8 to 1.3 (Table 4). However, a 2- to 3-fold increase (ratios from 2.5 to 3.5) in Zn blood metal concentrations was detected when comparing manatees from Brazil with the other species mentioned above (Table 4).

The non essential metals Al, Sn, Pb e Cd were measured in higher concentrations in blood from Brazilian manatees, especially in individuals held in Paraíba. Blood Al in manatees from Brazil were 6- to 11-fold higher than blood Al in Florida manatees, and 14-fold higher than *T. truncatus*, but only 50% higher than blood Al in *P. vitulina* (Table 4). Al contamination can cause brain neurofibrillary degeneration including behavioral effects and oxidative stress in rabbits and rats (Rajasekaran, 2000; Savory et al., 2006; Xie et al., 1996), but to our knowledge no information regarding marine mammal's sensitivity to aluminum toxicity is available in the literature. Our results did not indicate any correlation between blood aluminum and any of the measured biomarkers.

Blood Pb concentrations in manatees from this study were 3- to 7-fold higher than concentrations in Florida manatees and 11- to 47-fold higher than concentrations in *P. vitulina* (Table 4). Food items offered to manatees studied included macroalgae *Gracilaria lemaneiformis* and *Hypnea musciformis*, species where high levels of Pb and Cd have been found in Itamaracá Island (Guedes, 2007), the region in Pernambuco State where the animals were kept before transfer to the reintroduction areas in Alagoas and Paraíba. It is possible that these algae were a potential source of Pb and Cd in their diet. The nervous system and bone marrow are critical targets for lead toxicity, and effects include anemia diagnosed as lowered blood hemoglobin, lower hematocrit or higher mean corpuscular volume from inhibition of  $\delta$ -aminolevulinic acid dehydratase (ALAD), an enzyme involved in haeme biosynthesis essential for hemoglobin production (Buekers et al., 2009).

Manatees kept in Paraíba had higher blood Pb concentrations as well as higher mean corpuscular volume and smaller mean corpuscular hemoglobin concentration when compared to animals from Alagoas and Pernambuco. The same effects were found in rats exposed to lead acetate (Jassim and Hassan, 2011), although in the lead exposed rats a decrease in hematocrit, hemoglobin concentration (Hb), and red blood cell count was also found. In our study, erythrocytes, blood iron content and hemoglobin concentration in manatees from Brazil were lower than values found in Florida manatees (Stavros et al., 2008a), although hematocrit in animals from Brazil was higher than in animals from Florida. An increase in hematocrit may indicate a dehydrated state of the animals (Costill and Fink, 1974). Overall, there are indications of blood alterations typical of lead exposure in manatees from Brazil. However, highest average blood lead concentrations of  $0.10 \mu\text{g g}^{-1}$  wet weight in manatees from Paraíba analyzed in this study are below the  $0.18 \mu\text{g g}^{-1}$  wet weight determined by Buekers et al. (2009) that would be protective for 95% of lead exposed wildlife, a limit based on lead toxicity data in wildlife focused on growth, reproduction, hematology and biochemical effects. It will be important to generate information on the biochemical biomarker inhibition of ALAD, which is an important marker of lead toxicity, to further investigate these possible toxicological effects of higher blood lead concentrations in manatees from Brazil.

Blood Cd concentrations in manatees from this study were 8-, 4- and 2-fold higher than concentrations in Florida manatee, *P. vitulina* and *M. leonina*, respectively, but only 0.47 and 0.001 of the concentrations found in whales *Globicephala melas* and *Physeter catodon*, respectively (Table 4). The highest blood cadmium

concentrations of  $0.017 \mu\text{g g}^{-1}$  in Faroe Islands *G. melas* are above the level for humans at which renal damage can be expected, which is  $0.010 \mu\text{g g}^{-1}$ , suggesting a tolerance of pilot whales to heavy metals as no anomalies likely to reveal a major toxic problem were found (Caurant and Amiard-Triquet, 1995). These high blood Cd concentrations are associated with their preference for squid as prey, which are considered the most significant source of cadmium for marine mammals (Nielsen et al., 2000). Blood Cd in manatees from Paraíba in this study is 20% below the minimum adverse-effect levels for humans, and we did not detect any other biomarker effect indicative of Cd toxicity. As blood Cd levels in these animals from Paraíba are 8 times higher than in animals from Florida, it is important to further investigate potential toxicological effects in these animals.

Blood Sn in manatees from Brazil were 9- to 24-fold higher than blood Sn in Florida manatees, and 24- to 164-fold higher than blood Sn in *P. vitulina* (Table 4). Butyl-tin organostatic compounds are immunotoxic, and lymphocytes proliferation in blood was suppressed in several species of marine mammals, at a concentration of  $0.077 \mu\text{g dibutyl-tin (DBT) g}^{-1}$  blood wet weight (equivalent to  $0.039 \mu\text{g Sn g}^{-1}$  blood wet weight in inorganic form), and at a concentration of  $0.089 \mu\text{g tributyl-tin (TBT) g}^{-1}$  blood wet weight (equivalent to  $0.036 \mu\text{g Sn g}^{-1}$  blood wet weight in inorganic form) (Nakata et al., 2002). If we consider that tin inorganic forms are not absorbed by the gastrointestinal tract (Hiles, 1974), and that tin exists predominantly in organic forms in marine mammals (Tanabe, 1999), we can suppose that total Sn measured in blood samples from the studied manatees were in organic form. The concentrations of tin measured in our study do not exceed the immunotoxic blood concentration of approximately  $0.03 \mu\text{g Sn g}^{-1}$  blood wet weight mentioned above, except for only one animal from Paraíba, whose tin blood concentration was  $0.133 \mu\text{g Sn g}^{-1}$ . Toxicological effects of organic tin exposure in experimental studies include cytotoxicity to bone marrow and red blood cells at relatively low exposure concentrations (Boyer, 1989). Hemoglobin concentration in manatees from Brazil were in the lower range of values found in Florida manatees (Harvey et al., 2009). It is worth investigating whether the higher Sn concentration in blood of manatees analyzed in this study could lead to immunotoxicity or red blood cell toxicity.

Activities of antioxidant enzymes catalase (CAT) and glutathione-S-transferase (GST) did not differ between animals from the different sites. This fact can be possibly explained because aquatic mammals can deal with nitrogen super saturation and tolerate hypoxia through morpho-physiological alterations, which increases their efficiency to cope with reactive oxygen species (Wilhelm Filho et al., 2002).

Butyrylcholinesterase (BChE) inhibition is the mechanism of carbamate and organophosphate toxicity, and measurement of BChE activity is a useful method to assess carbamate and organophosphate exposure. The significant inhibition of BChE in manatees at the two reintroduction sites in Alagoas and Paraíba relative to animals from Pernambuco indicates possible exposure of the animals to this class of insecticides, especially because there is sugar cane culture in those regions, where carbofuran is used.

The region where manatees were confined in estuarine corrals in the state of Paraíba (PB) is located at the city of Rio Tinto, close to the city of Cabedelo, where there is an industrial terminal and 47 industries, the majority of them localized in the coastal region, and operating with inadequate solid waste management programs. This is likely an important factor to explain the higher metal contamination in animals from Paraíba.

This study provided new information regarding blood contamination levels and its possible effects in manatees *T. manatus*. Such information is of interest to environmental agencies as it can be used to improve conservation and welfare efforts on West Indian

manatees. In addition, studies on marine mammal contamination by anthropogenic chemicals frequently take a bioaccumulation approach involving chemical analysis in different animal tissues exclusively. These results also highlight the importance of using both chemical measurements and effects based biomarkers to enhance our understanding of the potential detrimental effects of these contaminants on the conservation of marine mammals.

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