USING TOP PREDATORS AND DR-CALUX TO SCREEN COASTAL ENVIRONMENTS FROM THREE DIFFERENT BRAZILIAN REGIONS FOR DIOXINS AND RELATED COMPOUNDS

Dorneles PR[1]*, Lailson-Brito J[2], Bisi TL[2], Domit C[3], Barbosa LA[4], Meirelles ACO[5], Carvalho VL[5], Malm O[1], Azevedo AF[2], Das K[6], Scippo ML[7]

[1]Radioisotope Laboratory, Biophysics Institute, Federal University of Rio de Janeiro (UFRJ), Brazil; [2]Aquatic Mammal and Bioindicator Laboratory (MAQUA), School of Oceanography, Rio de Janeiro State University (UERJ), Brazil; [3]Laboratory of Ecology and Conservation, Centre of Marine Studies (CEM), Federal University of Paraná (UFPR), Curitiba, Brazil; [4]ORCA Institute, Vila Velha - ES, Brazil; ; [5]Association for Research and Conservation of Aquatic Ecosystems (AQUASIS), Caucaia - CE, Brazil; [6]Laboratory for Oceanology, MARE Centre, University of Liege (ULg), Belgium; [7]Analysis of Foodstuffs of Animal Origin, Centre for Analytical Research and Technology (CART), University of Liege (ULg), Belgium

Introduction

The hotspots of chemical pollution in Brazil are related to the heterogeneity in the distribution of urban centres and industries, as well as to peculiarities of land use at the country[1-9]. Brazil is a newly industrialized nation, with few governmental restriction laws over production and use of endocrine disrupting chemicals (EDCs)[1]. Aquatic systems have been considered ideal final sinks for persistent and bioaccumulative toxicants (PBTs), such as metals and organohalogen compounds (OHCs), which turns coastal environments of urbanized and industrialized areas into PBT-contaminated bodies of water. Due to some features that include chemical stability and affinity for proteins or lipids, some PBTs are efficiently bioaccumulated and end up undergoing biomagnification, a concentration increase up the trophic chain[3-9]. Therefore, predator nektonic organisms are critical groups and may present high PBT concentrations[3-9]. In Brazil, the predator species of greatest ecotoxicological concern is the Guiana dolphin (*Sotalia guianensis*), since this small marine mammal inhabits shallow waters and is often found year-round in bays and estuaries. Residency and habitat fidelity are patterns described to this species along its distribution[10, 11].

Some studies have evaluated the exposure of nektonic organisms to toxic metals[5] and organohalogen compounds[3, 4, 6-9, 12] in Brazilian environments. However, knowledge on dioxins and related compounds (DRCs), such as polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), as well as on non-ortho and mono-ortho polychlorinated biphenyls (dioxin-like PCBs) is still scarce in the country[6]. The reason for the paucity of data on DRCs is partially related to the high cost of the analyses. In this context, it is worth mentioning the existence of the chemically activated luciferase gene expression (CALUX) cell bioassay and, more specifically, the dioxin responsive-CALUX (DR-CALUX®). The DR-CALUX® is an increasingly used bioanalytical tool for the screening and relative quantification of dioxin related compounds (DRCs), such as PCDDs and PCDFs[13].

The aim of this work was to evaluate the DRC contamination of coastal marine environments under the influence of highly urbanized and industrialized areas of Brazil. In order to achieve this goal, we have employed the DR-CALUX® to screen samples from Guiana dolphins for DRCs using the quantitative approach.

Materials and methods

Liver samples were obtained by different marine mammal research groups from four Brazilian states, including the North-eastern [Ceará (CE) state], the South-eastern [Espírito Santo (ES) and Rio de Janeiro (RJ) states] and the Southern [Paraná (PR) state] regions of the country (Figure 1). They were collected through the necropsy of 28 (five individuals from CE, eight from ES, seven from RJ and eight from PR) adult male Guiana dolphins that had been incidentally captured in fishing operations or found stranded dead on the beaches. The carcasses were classified as early decomposition stage[14]. After dissection, liver samples were stored in individual aluminium foil and kept frozen (-20^{o} C) until being dried at 50-55 ◦C (72 h) for the analyses.

The DR-CALUX® was developed by Wageningen University and is distributed by BioDetection System (BDS, NL). The analytical procedure was detailed elsewhere[13]. Briefly, the fat from liver samples has been extracted by liquid/solid extraction using hexane/diethylether/isopropanol (87.3/2.7/10). DR-CALUX® analysis was performed by exposing the cells (rat hepatoma H4IIE cell line stably transformed with an AhR-controlled luciferase reporter gene), in triplicate, in 96 wells plates, during 24 h to sample extracts or to standard TCDD solutions in DMSO diluted in culture medium (α-MEM, Invitrogen) containing 10% (v/v) of foetal calf serum

(FCS, Invitrogen). The final concentration of DMSO in culture medium was 0.4% (v/v). After cell lysis and substrate addition (buffer containing 1% luciferin [Promega] and 0.5mM ATP [Roche Diagnostics Belgium]), luminescence was measured using a luminometer Orion II (Berthold Detection System, Germany). DR-CALUX® concentrations were calculated from a standard calibration curve, ranging from 0 (blank DMS0) to 20 pg TCDD per well, and established in triplicate on each 96 wells plate. Dose response curves were fitted using a user-defined curve fit (Slide Write Plus v. 6.1, Advanced Graphics Software, USA).
Regarding the statistical treatment, depending on data normality (Shapiro-Wilk's W test), parametric [Student's t-test and Pearson's (r) correlation test] or non-parametric tests [Mann–Whitney U test and Spearman's (Rs) correlation test] were used.

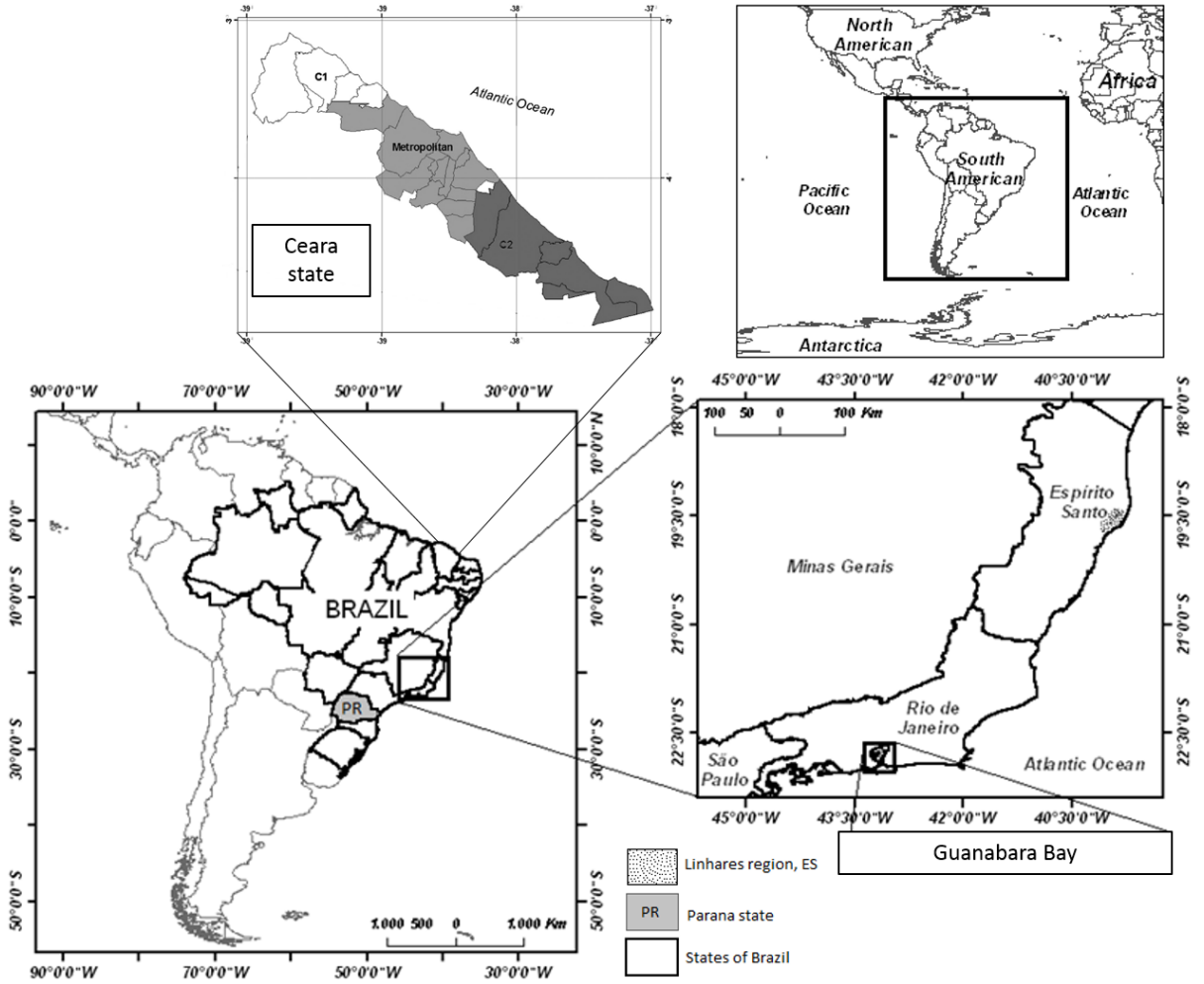

Fig. 1. Brazilian map highlighting the Paraná state (in grey) and amplifying Rio de Janeiro (RJ), Espírito Santo (ES) and Ceará states. Linhares region (dotted area), in ES state, and Guanabara Bay, in RJ state, are additionally highlighted.

Results and discussion

Extracts of samples from six dolphins, two from CE, three from ES and one from RJ state, exhibited cytotoxicity. Therefore, these six samples have not rendered results for this study. Bioanalytical equivalent (BEQ) concentrations [dioxins (pg BEQ / g lipid)], in liver of Guiana dolphins from North-eastern, South-eastern and Southern Brazilian regions, are presented in Table 1.
Since crude data on BEQ concentrations [dioxins (pg BEQ / g lipid)] in liver samples from North Sea harbour porpoises (*Phocoena phocoena*) were available[15], they were statistically compared with the data generated by the present study. Significantly higher concentrations were found for Guiana dolphins from Brazilian waters ($p=0.00007$), demonstrating the high exposure of coastal top marine predators to DRCs in Brazil.
Regarding the geographical differences in the Guiana dolphin exposure, levels (pg BEQ / g l.w.) were significantly higher in individuals from the South-eastern (ES and RJ states grouped) than in those from the

Southern (PR state) Brazilian region (p=0.01). The same pattern was found when the statistical comparison was performed between ES and PR states, *i.e.*, levels were significantly higher in the SE region (p=0.003). However, there was no significant difference when comparison was carried out between RJ and PR states. Higher levels were expected to occur in SE Brazil, as it is the most industrialized region of the country, as well as the region that generally renders the highest PBT concentrations in the Brazilian littoral[1, 3-9]. When data from the two areas of SE Brazil (ES and RJ states) were compared, no significant difference was found. This later finding alone demonstrates the high exposure of Guiana dolphins from ES state, since the sample set of RJ state is entirely composed of Guiana dolphins from Guanabara Bay (GB). GB not only constitutes the most dramatic example of man-made degradation of the Brazilian coast[3, 4, 8], but it is also an important hotspot of environmental contamination by polychlorinated biphenyls[6].

Table 1. BEQ [dioxins (pg BEQ / g lipid)] concentrations [Mean (Median) ± SD; Min - Max] in liver of male Guiana dolphin (*Sotalia guianensis*) from North-eastern [Ceará (CE) state], the South-eastern [Espírito Santo (ES) and Rio de Janeiro (RJ) states] and the Southern [Parana (PR) state] regions

	North-eastern region	South-eastern region		Southern region
	CE state (*n*=3)	ES state (*n*=5)	RJ state (*n*=6)	PR state (*n*=8)
BEQ concentrations [dioxins (pg BEQ / g lipid)]	8.63 (8.68) ±5.56 3.05 - 14.2	10.1 (11.2) ±3.82 4.54 - 14.8	8.57 (7.71) ±5.7 1.94 - 15.6	4.32 (3.89) ±1.64 2.56 - 6.88

In Brazilian states for which the area of origin of dolphin carcasses comprises a long coastal extension, which is the case of CE and ES states, the sample set may not be composed of individuals from the same ecological population[10, 11]. This makes further analyses necessary for both states. Regarding the ES state, the dolphin that provided the lowest level (8.45 pg BEQ / g lipid) was the only individual originating from an area regarded as a non-urbanized region, the Anchieta city[16]. The three highest concentrations among the ES dolphins, ranging from 11.2 to 14.8 pg BEQ / g lipid, were found in individuals from the Linhares city region, an area under the influence of the paper and pulp industry[17], which is an industrial process well-known to produce dioxins and dioxin-like chemicals as unwanted contaminants. The CE state area considered in the present study was divided here and in previous investigations[12] in three regions, comprising C1, Metropolitan (M) and C2 regions (Figure 1). As the name suggests, the M region corresponds to a highly industrialized and urbanized area, while the C1 and C2 regions present human population densities that are around 10% of that found in the M region[12]. In addition, the most important economic activities for C1 and C2 CE state regions are agriculture, fishing and tourism[12]. In this context, it is important to mention that the highest concentration among the CE dolphins (14.2 pg BEQ / g lipid) was found in the individual originated from the M region, an area that harbours a reduced Guiana dolphin population, assessed to be around 40 individuals[11]. The small number of useful samples from the CE (*n*=3) prevented statistical comparison comprising this Brazilian state. However, the concentration ranges in CE state (3.05 to 14.2) and SE region (1.94 to 15.6) do not suggest a significant difference (Table 1). This apparent absence of difference between North-eastern (CE state) and South-eastern (ES and RJ states) regions may be a result of a combination of two factors: (1) the small sampling number (*n*=3) from CE state and (2) the presence of an individual from a highly impacted area of this state (M region).

In which concerns possible correlations between BEQ concentrations [dioxins (pg BEQ / g lipid)] and total length of dolphins, no significant correlations were found for ES or PR states. A significant negative correlation was observed between BEQ concentrations and the month of stranding (p=0.003), suggesting a temporal variation [from December 2002 (month 1) to August 2009 (month 81)] in Guiana dolphin exposure in the ES state. Furthermore, this pattern was still the same when dolphins from Linhares region were exclusively evaluated. Therefore, this temporal trend may have hampered the existence of a length-related correlation. The same holds for dolphins from PR state, since only PR dolphins that died in 2008 and 2009 were analysed and the highest BEQ concentration found among dolphins that died in 2009 (3.94 pg BEQ / g lipid) was still lower than the lowest level (4.51 pg BEQ / g lipid) found among dolphins that died in 2008. Differently from the pattern found for PR and ES dolphins, a significant negative correlation was found between BEQ concentrations [dioxins (pg BEQ / g lipid)] and total length of Guiana dolphins from RJ state. BEQ values result from the concentrations of the different DRCs as well as from the ability of each compound to induce the Ah-receptor mediated response. Therefore, this finding may be a consequence of chemically-induced developmental disruption. The latter hypothesis is based on the fact that inhibition of growth and development is among the effects attributed to exposure to DRCs[6]. On the other hand, an increasing efficiency of the detoxifying activity

with the growth of the animal may be a plausible explanation as well[6]. Other important aspect to be considered while evaluating pollutant bioaccumulation through the life of dolphins is that the length is not the best proxy for age in mammals. The aging process may continue for decades after the growing of the animal has ceased.
Further investigations comprising a much wider sampling are required before strong conclusions can be reached. The necessity of a wider sampling is reinforced when the site fidelity[10] exhibited by the Guiana dolphin is taken into account. The large concentration range observed in CE state constitute an important example of this requirement. It is important to highlight that BEQ concentrations [dioxins (pg BEQ / g lipid)] in liver of dolphins from North-eastern, South-eastern and Southern Brazilian regions seem to mirror the expected environmental contamination by DRCs. Considering the difficulties associated with sampling marine mammals, including legal and ethical issues, a similar approach using a high trophic level fish species is strongly recommended. In this context, it is important to mention that the whitemouth croaker (*Micropogonias furnieri*) would be an interesting sentinel species, as this scianid fish not only is a crucial prey species for Guiana dolphins but also occupies (the adult whitemouth croakers) high trophic positions in Brazilian coastal bays[2].

Acknowledgements
This work was part of a set of investigations included in the scientific mission of Dr. Dorneles (UFRJ Professor), as Visiting Professor at ULg, financed by FNRS (*Fonds de la Recherche Scientifique*). This work was supported by the Ministry of Education of Brazil-CAPES ("Ciências do Mar" - Proc. 23038.051661/2009-18), by a scientific cooperation established between CNPq (*Conselho Nacional de Desenvolvimento Científico e Tecnológico*) and FNRS (Proc. 490279/2013-9 CNPq), as well as by FAPERJ (*Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro,* CNE grant of Dr. Malm). Dr. Das is a F.R.S.-FNRS research associate. Dr. Azevedo and Dr. Lailson-Brito have research grants from CNPq (PQ-2) and FAPERJ (JCNE).

References:
1. Alonso MB, Eljarrat E, Gorga M, Secchi ER, Bassoi M, Barbosa LA, Bertozzi, CP, Marigo J, Cremer M, Domit C, Azevedo AF, Dorneles PR, Torres JPM, Lailson-Brito J, Malm O, Barceló D. (2012); Environ Pollut. 170: 152-60.
2. Bisi TL, Lepoint G, Azevedo AF, Dorneles PR, Flach L, Das K, Malm O, Lailson-Brito J. (2012); Ecol Indic. 18: 291-302.
3. Dorneles PR, Lailson-Brito J, Azevedo AF, Meyer, J.; Vidal, L. G.; Fragoso, A. B.; Torres JPM, Malm O, Blust R, Das K. (2008); *Environ Sci Technol*. 42(14): 5368-73.
4. Dorneles PR, Lailson-Brito J, Dirtu AC, Weijs L, Azevedo AF, Torres JPM, Malm O, Neels, H.; Blust R, Das K, Covaci A. (2010); *Environ Int.* 36(1): 60-7.
5. Dorneles PR, Lailson-Brito J, Fernandez MAS, Vidal LG, Barbosa LA, Azevedo AF, Fragoso ABL, Torres JPM, Malm O. (2008); *Environ Pollut*. 156(3): 1268-76.
6. Dorneles PR, Sanz P, Eppe G, Azevedo AF, Bertozzi CP, Martínez MA, Secchi ER, Barbosa LA, Cremer M, Alonso MB, Torres JPM, Lailson-Brito J, Malm O, Eljarrat E, Barceló D, Das K. (2013); *Sci Tot Environ*. 463-464: 309-18.
7. Lailson-Brito J, Dorneles PR, Azevedo-Silva CE, Azevedo AF, Vidal LG, Marigo J, Bertozzi CP, Zanelatto RC, Bisi TL, Malm O, Torres JPM. (2011); *Chemosphere*. 84(7): 882-7.
8. Lailson-Brito J, Dorneles PR, Azevedo-Silva CE, Azevedo AF, Vidal LG, Zanelatto RC, Lozinski CPC, Azeredo A, Fragoso ABL, Cunha HA, Torres JPM, Malm O. (2010); *Environ Pollut*. 158(5): 1800-8.
9. Lailson-Brito J, Dorneles PR, Azevedo-Silva CE, Azevedo AF, Bisi TL, Vidal LG, Legat LN, Azevedo AF, Torres JPM, Malm O. (2012); *Sci Tot Environ*. 433: 123-31.
10. Azevedo AF, Lailson-Brito J, Dorneles PR, van Sluys M, Cunha HA, Fragoso ABL (2009); *Mar Biodivers Rec*. 2: 1-5.
11. Meirelles, ACO (2013) Doctoral Dissertation, Universidade Federal do Ceara.
12. Santos-Neto EB, Azevedo-Silva CE, Bisi TL, Santos J, Meirelles ACO, Carvalho VL, Azevedo AF, Guimarães JE, Lailson-Brito J. (2014); *Sci Tot Environ*. 472: 194-203.
13. Scippo ML, Eppe G, De Pauw E, Maghuin-Rogister G. (2004) *Talanta*. 63(5): 1193-202.
14. Geraci JR. Lounsbury VJ, William FP, Bernd W, Thewissen JGM. (2009); Health. In Encyclopedia of Marine Mammals (2nd Edition), Academic Press: 546-53.
15. Das K, Holsbeek L, Jauniaux T, Bouquegneau J-M. (2005); Belgian Federal Science Policy Office: 74pp.
16. Carvalho VF. (2013) Master Thesis, Universidade Federal Rural de Pernambuco.
17. Nicolodi JL, Pettermann RM. (2011). *J Coast Res*. (sp. issue 64), 1372-79.