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DR-CALUX® BIOASSAY RESPONSE TO SCREEN PCDDS, PCDFS, AND DIOXIN-LIKE PCBS IN WHITEMOUTH CROAKERS (MICROPOGONIAS FURNIERI) FROM RIO DE JANEIRO STATE, BRAZIL

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Introduction

Polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are persistent organic pollutants which are of omnipresent in the global environment[1]. These substances cover a group of 210 congeners, among which 17 are of toxicological concern. PCDD and PCDF toxicity is related mainly to their capacity to interact with the cytosolic specific protein called the aryl hydrocarbon receptor (AhR). The binding to the AhR constitutes a first and necessary step to initiate the toxic and biochemical effects[2]. It is also known that twelve out of 209 polychlorinated biphenyls (PCBs) congeners have the same (AhR)-dependent mechanism of toxicity, due to a chlorine substitution and their planar geometry; these PCBs are known as "dioxin-like" PCBs (dl-PCBs)[3]. These substances, i.e., PCDD/Fs and dl-PCBs, also called dioxins and related compounds (DRCs), can be easily absorbed by biota. Due to their lipophilicity and metabolic resistance, they are concentrated and accumulated in fatty tissues and most DRCs tend to biomagnify up the food web. This last feature generates high DRC levels in organisms that occupy high trophic positions, including humans[2]. It is estimated that more than 90% of the human exposure to PCDD/Fs and dl-PCBs is due to consumption of foodstuff of animal origin, such as meat, milk and dairies, as well as fish and derived products[4, 5].

The accurate measurement of DRCs requires high standard analytical strategies, including the use of mass spectrometric analysis i.e. GC-HRMS[6]. The high cost of the analysis using a mass spectrometric technique can be an obstacle for large-scale monitoring of DRCs in food and environmental samples. In this context, the cell-based screening methods such as the chemically activated luciferase gene expression (CALUX) cell bioassay and, more specifically, the dioxin responsive-CALUX (DR-CALUX®), could be an alternative. This cell bioassay allows a higher sample throughput and is cheaper than the analysis using mass spectrometry[7]. DR-CALUX® and other similar cell-based assays are more and more widely used, since they are validated method for screening for DRCs in food and feed according to international standards such as Regulations (EU) n° 589/2014 and 278/2012. Therefore, it can be concluded the DR-CALUX® to be a valuable tool for improving the knowledge on environmental contamination by DRCs as well. This is especially important for newly industrialized nations, such as Brazil.

The aim of this work was to estimate the DRC contamination of estuarine environments under the influence of industrialized and highly urbanized areas of Rio de Janeiro State, Southeast Brazilian region, through analyses of samples from whitemouth croaker, Micropogonias furnieri (Desmarest, 1823) (Perciformes, Sciaenidae). This croaker is the most important target for fisheries among demersal fishes of the South-West Atlantic coast[8], Additionally, the whitemouth croaker occupies the second place among coastal fishes of commercial value in Brazil[9]. In order to achieve the abovementioned objective, we have employed the DR-CALUX® to screen samples from whitemouth croaker for DRCs using a quantification approach relative to the tetrachlorodibenzodioxin (TCDD) response, allowing the estimation of bioanalytical equivalents (BEQ).

Materials and methods

The Rio de Janeiro State coast is the most densely populated area of the Brazilian littoral[10]. In this region, at least two coastal systems are pointed as critical areas concerning environmental contamination, which are Guanabara Bay (GB) and Sepetiba Bay (SB) (Figure 1). The BG is under the direct influence of the approximately 11 million people living in its surroundings, which comprise 80% of the Rio de Janeiro state population[10]. The estuary receives sewage, industrial waste and, consequently, many contaminants

that are transported along its drainage basin[11]. The environmental impact of anthropogenic and industrial activities on SB is also well known. Its drainage basin is surrounded by approximately 1.2 million people and over 400 industries, including metallurgical, petrochemical and pyrometallurgical smelters[12].
The samples analysed in the present study were obtained from whitemouth croakers that were captured in GB (n=14) and SB (n=6). These estuaries are two important fishing areas in the Rio de Janeiro state (RJ), Southeast Brazilian region, despite being anthropogenically-disturbed areas along the Brazilian coast[13]. After dissection, samples were lyophilized and stored up to the moment of the analyses.
The DR-CALUX® was developed by Wageningen University[14] and is distributed by BioDetection Systems (BDS, NL). The analytical procedure was detailed elsewhere[7]. Briefly, freeze-dried muscles were rehydrated with 15 ml of water and 15 ml of isopropanol (Promochem) and their fat content was extracted by a shaken solvent extraction method using hexane/diethylether (97/3 v/v; Promochem/Sigma-Adrich). The resulting extracts were evaporated to constant weight for gravimetric fat determination. After this step, the samples were transferred to an open glass column filled with acid silica (20g 33% H_2SO_4 and 20g 20% H_2SO_4), covered with 1 g anhydrous sodium sulphate and eluted with 125 ml of hexane/diethylether (97/3 v/v). The eluates were concentrated avoiding dryness and transferred to vials, with the addition of 15µL of DMSO. 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 0.8% (v/v). After incubation, the medium was removed, and the cells were lysed. After the addition of luciferin (buffer containing 1% luciferin [Promega] and 0.5mM ATP [Roche Diagnostics Belgium]), the luciferase activity was determined using a luminometer (ORION II, Berthold Detection System, Pforzheim, Germany). BEQ 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). The Mann-Whitney U test and Spearman's (Rs) correlation test were used since data significantly deviated from a normal distribution (Shapiro-Wilk's W test). The level of significance was set at $p<0.05$.

Results and discussion
Biological data and bioanalytical equivalent (BEQ) concentrations (pg BEQ / g fat) in whitemouth croaker muscle samples are summarized in Table 1. Previous studies have reported the occurrence of two whitemouth croaker populations along the southeastern and southern Brazilian coast (population I: 23° - 29°S; population II: 29° - 33°S)[8, 15]. Based on these geographical data, the whitemouth croakers from GB and SB, in the state of Rio de Janeiro, should be included in the same population (I). The maturation size (L_{50}) of whitemouth croaker is 275 mm; and the average length of the species in adjacent coastal areas is estimated to be 510 mm[8, 16]. In this context, only adult specimens were used in this study. The fishes caught in SB were all smaller than 510 mm, but the samples from GB were divided into two groups (group I ≤ 510mm; group II > 510mm).
Significant negative correlation was found between BEQ concentrations (pg BEQ / g fat) and the total length ($p=0.03$, $Rs= -0.5$). The whitemouth croaker has a wide geographical distribution in the Western Atlantic, this species is an estuarine-dependent fish, which presents movement patterns during their life cycle. These movements display habitat shifts with the adult fish feeding in coastal waters and moving into estuaries to spawn, which suggests that the juvenile fishes reside in estuaries for several years before moving out to coastal waters to recruit into the adult stock[17]. In this context, a significant negative correlation between BEQ concentrations and total length might be indicating a reduction on the DRC bioaccumulation rate by the oldest individuals, since the lengthier whitemouth croakers feed out of the estuaries, i.e., out of the hotspot contamination area. Another possible explanation would be an increased efficiency of the detoxifying activity with the animal growth as it has been hypothesised for other marine vertebrates[18, 19].
Regarding the geographical differences in whitemouth croaker exposure, levels (pg BEQ / g fat) were significantly higher in individuals from SB than in those from all specimens (n=14) from GB ($p = 0.007$). The same pattern was found when the statistical comparison was performed between SB and GB group II, i.e., levels were significantly higher in the whitemouth croakers from SB ($p= 0.007$). However, there was no significant difference when the comparison was carried out between SB and GB group I ($p = 0.08$). Some characteristics of each body of water may influence DRC bioavailability. GB presents

significant loads of suspended material and high biological productivity. In this case, DRCs could be complexed and bound to the particulate suspended material, reducing their residence time in the water column and bioavailability[13, 20].

To the best of our knowledge, there is no study using cell bioassays to report DRC concentrations in fish samples from South Atlantic. However, a recent investigation was performed focusing on BEQ concentrations in liver samples from male Guiana dolphins (Sotalia guianensis) from three regions (Northeastern, Southeastern and Southern) of Brazil. The higher levels were found in the Southeastern region, where the GB and SB are localized. As abovementioned this region shows the highest DRC concentrations in the Brazilian coast[13, 18, 19, 21, 22]. Thus, it is important to mention that juvenile whitemouth croakers could be used as sentinel species, reflecting the DRC levels from the GB and SB. However, further studies on whitemouth croaker ecology are required for investigating if migrating towards the continental shelf waters outside estuaries is a general pattern repeated in all bays along the distribution of the species.

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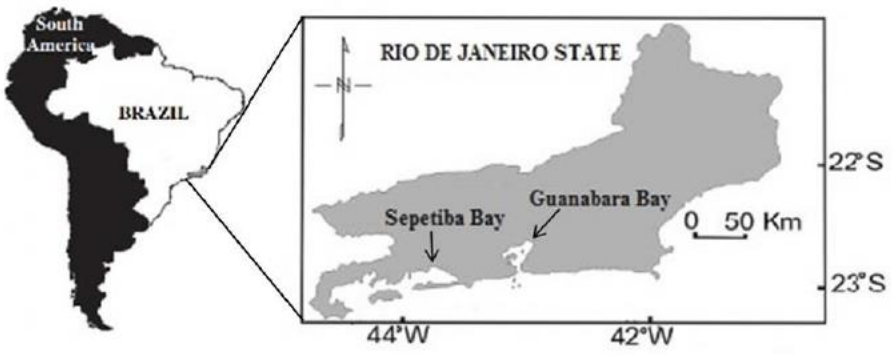

Figure 1: Brazilian map is stressing the state of Rio de Janeiro (gray). The map of Rio de Janeiro state is amplified and shows Guanabara and Sepetiba Bays.

Table 1: Biological data and BEQ [dioxins (pg BEQ / g fat)] muscular concentrations (Mean ± SD; Min – Max) of whitemouth croakers (*Micropogonias furnieri*) from Rio de Janeiro state, Brazil.

Sampling	Length (mm)	% fat	BEQ conc. (pg BEQ / g fat)
Sepetiba	470 ± 16	2.3 ± 0.7	5.6 ± 2
(n = 6)	450 – 490	1.2 – 3.1	2.5 – 8.3
Guanabara Bay			
(group I; n = 5)	486 ± 21	3.1 ± 1	3 ± 2.1
	460 - 510	2.2 – 4.7	0.9 – 6.4
(group II; n = 9)	612 ± 71	4 ± 2.1	2.1 ± 1.1
	520 - 750	1.3 - 8	0.3 – 4.3
(Total n = 14)	567 ± 85	3.7 ± 1.8	2.4 ± 1.5
	460 - 750	1.3 - 8	0.3 – 6.4
TOTAL (n = 20)	540 ± 83	3.3 ± 1.7	3.4 ± 2.2
	450 - 750	1.2 - 8	0.3 – 8.3