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Zn, Co, Cr, As, and genotoxic effects in the ichthyofauna species from polluted and non-polluted/protected estuaries of the São Paulo coast, Brazil

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Abstract: The human impact on estuarine regions results in an increasing concentration of trace elements in the environment, exposing aquatic organisms. In this study, Zn, Co, Cr, and As were determined in the muscle tissue of some fish species from Santos-São Vicente (SSV), a recognized polluted region, and in the Cananeia estuary (CAN). Genotoxicity was also determined for the evaluation of the chronic effect on ichthyofauna, to enlarge the bank of environmental data regarding the effects of pollution, and to suggest other fish bioindicator species that may contribute to monitoring programs. The obtained data showed a similar profile of an accumulation of Co and Zn in *Micropogonias furnieri* and *Chaetodipterus faber*, where: Santos Bay > CAN > inner area of SSV. A low frequency of micronucleus was observed. However, in general, fish from the inner area of the SSV estuary presented more nuclear abnormalities than fish from Santos Bay and CAN. In the inner area of SSV, *Centropomus paralelus* and *Diapterus rhomneus* showed more changes than other fish species. It was possible to consider that the habits of each species, contributed to a different profile of trace element accumulation, followed by the chemical components in the organism offering different vulnerability.

Key words: Trace elements, nuclear abnormalities in erythrocytes, protected areas, south atlantic, subtropical estuaries.

INTRODUCTION

In general, chemical compounds present higher concentration in coastal waters, which decrease according to the distance from the river mouth, due to dilution and flocculation, because the increase of salinity favors the removal of the dissolved-to-particulate phase of the metals (Kennish 1986). Estuarine organisms are normally more exposed to higher concentrations of trace metals and metalloids than those from the oceanic area. The characteristic and activity of ionic forms of these contaminants, associated with the sensibility of the species, play an important role in the pollution effect on bioindicators.

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The Santos/São Vicente (SSV) and Cananeia (CAN) estuaries, located in the coastal region of Brazil, have been the subject of several environmental monitoring studies, such as approaches to biological processes, mechanisms of the input of organic and inorganic pollutants, and the use of bioindicators of environmental contamination (Hortellani et al. 2005, Azevedo et al. 2009, 2012a). For this purpose, these aquatic systems have special peculiarities because SSV shows a long history of strong anthropogenic action by industrial discharges, domestic sewage, harbor activities, and tourism. SSV also has a significant population that contributes to a large amount of domestic sewage, partially eliminated by the sewage effluent that contributes to the eutrophication processes of Santos Bay (Braga et al. 2000). The Cubatão City, located in the inner area of SSV, has an industrial zone with about 1,100 industries in different fields of chemistry. Therefore, inorganic contaminants such as Zn, Co, and Cr may be introduced in the water and sediments, and they are bioavailable for organisms in the production of various chemical manufactures such as the production of chlorine, soda, styrene, paper, green coke, steel, petroleum products, latex, polystyrene, and fertilizers (Lamparelli et al. 2001, Hortellani et al. 2005, Bicego et al. 2006, Azevedo et al. 2012c).

In contrast, the CAN estuary is an aquatic ecosystem subject to minimal anthropogenic influence and was recently included in the Ramsar list of wetlands of international importance (https://rsis.ramsar.org/ris/2310). However, the northern region of this important estuary receives freshwater and continental material input such as human agricultural waste developed in the Ribeira de Iguape region. On the other hand, the Cubatão Sea is subject to tidal influxes and riverine contributions because it is located in the middle of the CAN estuary. Therefore, it is important to know the tidal conditions and characterize the areas when the biological sample is taken, to understand the influence and dynamics of abiotic parameters concerning the bioavailability of trace elements.

In this context, the evaluation of the contamination of marine organisms by metals is necessary because the metals represent a risk to the biodiversity of the studied areas and to human health. In the last decade, *Cathorops spixii* have been used as bioindicators of contamination in SSV and CAN, since they accumulate and participate in the dynamics of essential and non-essential metals, such as Zn, Cu, Mn, Fe, Hg, Pb, Cd, and Ni (Azevedo et al. 2012a). Besides, *C. spixii* responds to biochemical, histopathological (Azevedo et al. 2013), and genotoxic effects by nuclear abnormalities in erythrocytes alterations (Azevedo et al. 2012b) and these studies showed greater damage to catfish from SSV than the CAN estuary.

When in excess, even essential elements for maintaining and balancing health become harmful. Metals, such as zinc (Zn), cobalt (Co), and chromium (Cr) are essential micronutrients that are required by the organism in low concentrations and can collimate in association with nutrients and other metal traces (Saito and Goepfert 2008).

Zn is a micronutrient, which, in trace levels, is essential to organisms. It acts as a catalytic and structural component of several enzymes related to the energy of metabolism, transduction, and translation, and beyond their involvement in biological processes, such as the stabilization of structural membranes and cell protection, as well as preventing lipid peroxidation and auxiliary in the antioxidant system of the cell. In individuals, zinc deficiencies induce depression, skin lesions, and infertility. Metabolically, Zn depletion may lead to failure and/or defects in mRNA synthesis and in the function and reduction of the growth hormone (IGF-I) concentration (Alloway 1993). On the other hand, zinc excess may lead to copper reduction in the organism, causing muscular pain, anorexia, intestinal bleeding, and brain anomalies (Shimma 1995). Musa et al. (2017) observed that, in fish, high concentrations of Zn tend to compromise the functioning of the gills.

Biologically, Co is considered a micronutrient as part of the constitution of vitamin B12 (cyanocobalamin), essential for proper functioning of the organism (Martens et al. 2002). In humans, the intake of cobalt in higher quantities than necessary can occur through foods such as fish. Cobalt excess leads to a similar response to the hypoxia, which may result in nausea, vomiting, and even serious heart problems, along with a high hepatotoxic and nephrotoxic potential (Lippi et al. 2005). Regarding Cr, the most important chemical forms to health are Cr^{3+}, essential for metabolism, and Cr^{6+}, which is potentially toxic, carcinogenic, and mutagenic (ATSDR 2009).

On the other hand, elements, such as arsenic (As), are toxic to species in some cases, even in low concentrations. The damage caused by these metals is extensive, including changes in biochemistry role, growth rate, reproductive phase, cell mutations, behavioral changes, and modifications in the community structure (Oana 2006). Symptoms of arsenic poisoning appear as conjunctivitis, melanose, depigmentation, and keratosis (wounds). Attention is required concerning the study of As and its limits in aquatic organisms; regarding human consumption, once in marine organisms, such as fish, the predominant arsenic compound is arsenobetaine, which is widely considered non-toxic and it is involved in the regulation of osmotic stress (Julshamn et al. 2012, Zhang et al. 2018).

Regarding aquatic organisms such as fish, the toxic effect of Co excess is extremely low when compared to other metals, with few studies relating to Co levels in marine organisms, such as fish (Furness and Rainbow 1990). Cr content in marine fish often show concentration values close to the detection limit of many analytical methods (Furness and Rainbow 1990). Unlike Zn, Co, and Cr, As does not has a micronutrient function. However, marine organisms easily capture this metalloid (Barra et al. 2000). In foods, the highest levels are found in fish and mollusks (USEPA 1997).

Concerning food safety, Brazilian law (ANVISA 2013) established a maximum limit of 50 mg Kg^{-1}, 0.10 and 1.00 mg Kg^{-1} for Zn, Cr, and As, respectively. There is no maximum limit recommended by Brazilian Law (ANVISA 2013) for Co ingestion.

Arsenic is significant in terms of food safety because very high concentrations can be found in fish and other seafood. However, the most toxic form, inorganic arsenic, is only usually found in very low concentrations in fish (Julshamn et al. 2012, Hong et al. 2018, Zhang et al. 2018). Arsenobetaine, which is the predominating form of arsenic accumulating in seafood, is not considered toxic at all. This is very important, as it is essential to the evaluation of arsenic and food safety.

There is evidence that, even at low concentrations, trace metals can cause lethal damage to organisms (Ansari et al. 2004). Prolonged exposure of essential and non-essential metals may have chronic effects on sentinel organisms, such as modifications in genetic material. Therefore, the use of genotoxic biomarkers, such as nuclear abnormalities in erythrocytes (NAE) and more specifically the micronuclei (MN) assay are very interesting with regard to the biomonitoring of aquatic systems (Capela et al. 2016). Notably, MN are effective responses to clastogenic and aneugenic compounds in the aquatic environment and can specify latent risk to the biota (Guilherme et al. 2008, Hoshina et al. 2008).

This work aimed for the determination of the metals Zn, Co, Cr and the metalloid As in fish with ecological and commercial importance from two estuaries on the Brazilian coast (SSV and Cananeia) with different physicochemical water characteristics and anthropic influence. Ecological responses in these fish species were also observed by evaluation of the chronic effects using genotoxic biomarkers (NAE) to enlarge the environmental data bank of effects of pollution to suggest other fish bioindicator species that could contribute to monitoring programs.

MATERIALS AND METHODS

SAMPLING SITES

The sampling of water and fish was performed on August 26 and 27 in 2006 (during the winter), in two estuaries of the São Paulo coast, Southeastern Brazil. The first was Santos/São Vicente estuary (SSV) that had two regions for sampling: the inner area and Santos Bay. The second region was the Cananeia estuary (CAN) (Figure 1). The inner area of the SSV receives the input of waste water from industries in Cubatão city, including domestic discharge from illegal buildings, such as stilts located on the edge of the estuary and materials from freshwater influx due to continental and riverine contributions. On the other hand, Santos Bay is characterized by the presence of a submarine emissary and a system of six canals that receive domestic sewage from SSV cities. More depurative conditions are also evident due to the hydrodynamism on the Ocean. Samples were also performed in the CAN, in the most southern region of the São Paulo littoral, where human influence is minimal and rich vegetation, with an enormous area of mangrove, is preserved. In the CAN, the sampling was conducted in a more protected area, located in the inner area of the estuary, namely the Cubatão Sea, which contributes significantly to the marine and fluvial influx due to the regime of the tide.

PHYSICAL AND CHEMICAL DATA

Information concerning the tides for both estuaries was obtained from Brazil's Navy. The field expedition was done on board "Albacora", the research vessel of the Instituto Oceanográfico da Universidade de São Paulo. The local depth was obtained using the vessel's echosounders. All physicochemical parameters were measured only in the water at the bottom of each sampling site. For the water sample collection, a Hidrobios bottle (go-flo) was used, in polycarbonate, trapped in the boat's cable, and lowered into the water to the maximum depth at each collection station. Following the water collection, the temperature was immediately registered using a reversing thermometer coupled to the Hidrobios bottle. Three aliquots of about 200 mL of the water were separated for the physicochemical measurements. The salinity was determined using a hydrometer Beckman RS-10 and dissolved oxygen concentrations were determined using the Winkler method (Grasshoff et al. 1983). pH values were obtained using a potentiometer in the same way as Aminot and Chaussepied (1983).

FISH SAMPLING AND BIOMETRIC DATA

Fish were sampled using a bottom otter net trawl with 1.6" mesh wall and 1.2" mesh end and 11m of total length that was dragged for 10 minutes at 3 mn h^{-1} to avoid major impact on the local aquatic fauna. The fish species selection from the SSV was conducted according to the degree of socioeconomic importance and consumption, with authorization of ICMBio (Instituto Chico Mendes de Conservação da Biodiversidade - Sisbio process nº 18803). In the CAN estuary, the species selection followed the ecological importance to human consumption; the standard of species caught in the SSV was to promote comparisons in the metal data. After capture, fish were removed from the net, identified using Figueiredo and Menezes (1978) and a selection of the species was, finally, put into boxes with ice and taken to the laboratory.

In the laboratory, biometric measurements of the fish were taken, for instance, the total length (TL) and total weight (TW) and were then dissected to extract the epaxial muscle getting the fillet from both parts of the fish. To avoid contamination from other organs, dissection was conducted

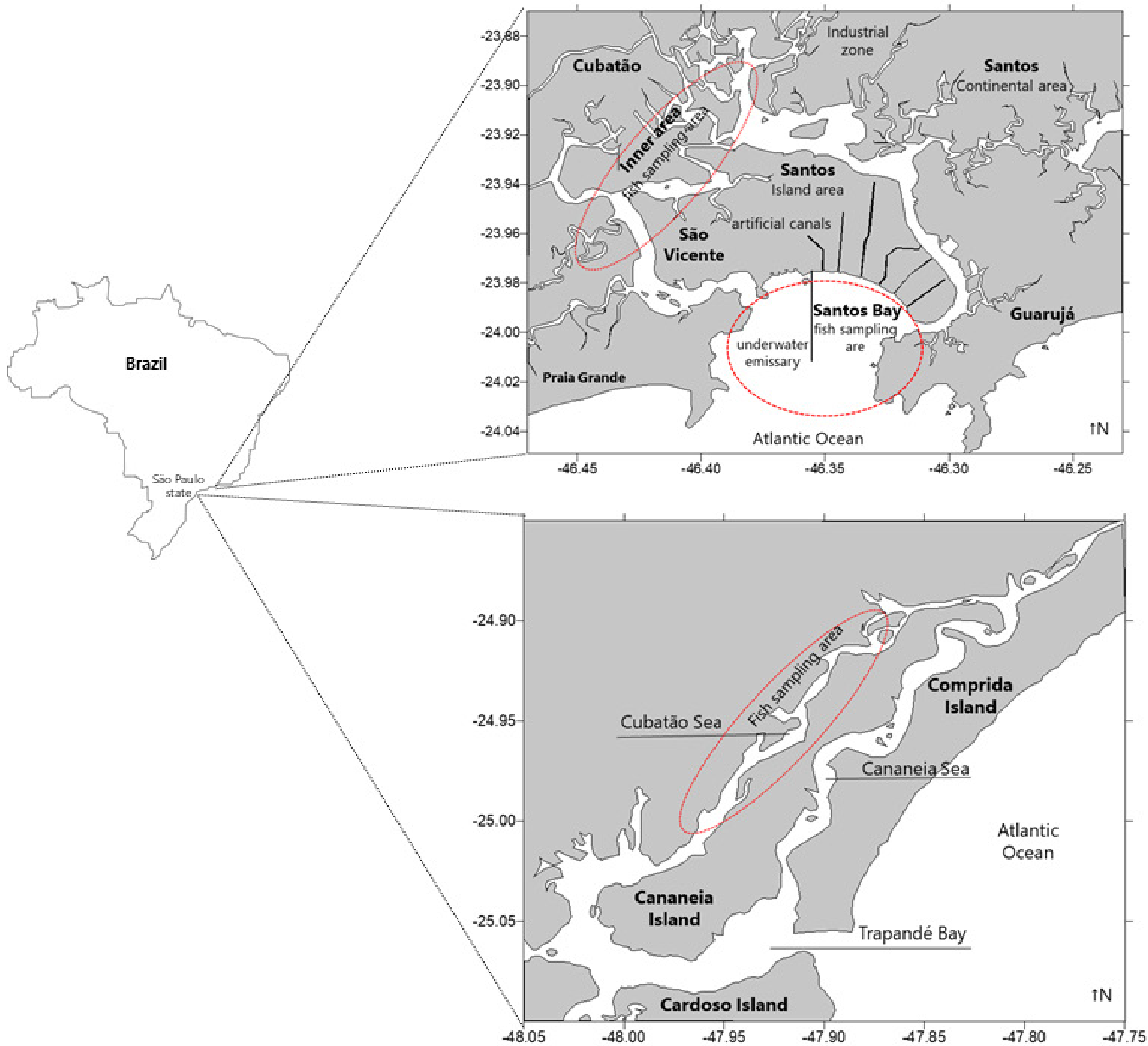

Figure 1 - Sampling sites showing the areas of fish collection in Santos/São Vicente (SSV) and Cananeia estuary, São Paulo, Brazil.

using a surgical scalpel and the obtained material was immediately washed with milli-q water. The muscle tissues were conditioned at -20°C until the analysis of trace elements was conducted. From the 10 species, 59 individual sampled were taken from the SSV and from four species, 27 specimens were taken from the CAN estuary.

TRACE ELEMENTS DETERMINATION BY INAA

The fish samples were dried for 24h using a lyophilizer Liobras-L101 and homogenized before irradiation to determine some metals and metalloids using Instrumental Neutron Activation Analysis (INAA) (Larizzatti et al. 2001).

Circa of 0.2 mg of each individual muscle tissue and reference materials (DORM-2 – dogfish muscle tissue, NRCC and Peach Leaves – SRM 1571, NIST) were accurately weighed and sealed in pre-cleaned polyethylene bags, for irradiation. These materials were irradiated for eight hours, under a thermal neutron flux of 1 to $5x10^{12}$ cm^{-2} s^{-1} in the IEA-R1 nuclear research reactor at the Nuclear and Energy Research Institute (IPEN-São Paulo). The identification of the radioisotopes formed was conducted using the half-life and energy of the gamma-ray. ^{76}As was determined in the first count, after seven days of decay, and the radioisotopes ^{60}Co, ^{57}Cr, and ^{95}Zn were determined during the second count, after 20 days of decay. Gamma-spectrometry was performed using a Canberra gamma X hyperpure Ge detector (EG&G ORTEC) and associated electronics, with a resolution of 0.88 keV and 1.90 keV for ^{57}Cr, ^{60}Co, ^{95}Zn and ^{76}As, respectively. The concentrations of the elements were determined by comparative method, using the programs VISPECT and ESPECTRO, to identify the gamma-ray peaks and calculate the concentrations, respectively.

The data from the reference materials DORM-2 and Peach Leaves showed a relative standard deviation (RSD) ranging of 1.7 to 7.2% and relative error (RE) ranging from 2.0 to 14.8%, indicating the precision and accuracy of the INAA for these elements, respectively.

NUCLEAR ABNORMALITIES IN ERYTHROCYTES (NAE)

Immediately after removal of the fish from the net, while they were still alive, blood was collected from the caudal artery using a fine needle syringe with heparine. Shortly thereafter, a drop of fish blood was smeared onto a microscope slide and air-dried, while on board. On landing, after fixation with methanol for 10 min, the slides were stained with Giemsa 10% for 30 min. The counting of NAE was performed in the prepared slides using microscopy under immersion light with an optical magnification of 1000 times, scoring an average of 2000 mature mononucleated erythrocytes, as described by Carrasco et al. (1990). The NAE identified and analyzed in this work were classified as notched, lobed, blebed, vacuolizated, and micronuclei.

RESULTS

PHYSICOCHEMICAL DATA

Data concerning the abiotic water parameters measured in three sites of the SSV and in the Cubatão Sea (CS) in the CAN estuary are shown in Table I. The sampling at all stations occurred in syzygy flood tides in both estuaries, except for station 1 in the SSV, close to the industrial zone. The characteristics of the inner area, and of Santos Bay in the SSV estuary, increased in salinity towards the bay (station 3). In addition, the lowest salinity value was obtained in the inner area surrounded by mangroves and urban occupation such as stilts (station 2).

In the SSV estuary, the temperature of the deepest water was 21.96ºC at station 1, with a maximum value of 23.50ºC observed at station 2, which was a shallow region and surrounded by mangroves. Higher values of pH and dissolved oxygen (DO) were found near the ocean, in Santos Bay (station 3), due to the major influence of ocean waters on the hydrodynamic function. In general, the obtained pH followed the conceptual standard considering that a pH values of about 8 is characteristic of ocean regions with a decreasing tendency towards the interior of the system, as observed for stations 3, 2, and 1, respectively.

In the CAN estuary, the sampling performed on the Cubatão Sea showed similar abiotic characteristics regarding to the winter period. DO values higher than 3 mL L^{-1} are indicative of a healthy aquatic system. Lower values of salinity and pH demonstrate major continental influence, since the Cubatão Sea is located in the inner area of the CAN estuary.

BIOLOGICAL ASPECTS

A total of 10 fish species were sampled in the SSV estuary and four species were in the CAN estuary, so they were collected in this estuarine system. In

TABLE I
Physicochemical parameters from the water bottom of different sites in the both estuaries. CS = Cubatão sea.

	Sites	Tidal	Depth (m)	T (ºC)	Salinity	pH	DO (mL L^{-1})
SSV	1	Syzygy Ebb	7.6	21.96	28.07	7.96	3.00
	2	Syzygy Flood	5.0	23.50	24.94	7.69	3.20
	3	Syzygy Flood	7.0	22.60	33.18	8.62	6.12
CAN	CS	Syzygy Flood	7.3	22.05	26.87	7.95	4.61

* DO = Dissolved Oxygen; 1 and 2: inner area of SSV; 3: Santos Bay in Santos/São Vicente (SSV) estuary; CAN: Cananeia estuary.

general, all species have commercial importance to both regions and, in fact, are ecologically very important. Furthermore, all collected species show a benthic feeding habit.

The individuals of all analyzed species from both estuaries were more homogeneously biological because the mean values of the total length and total weight demonstrated low standard deviation (Table II). Concerning to the species in both estuaries, the *Micropogonias furnieri* from the two sites of the SSV (inner area = 218±29 mm; Santos Bay = 238±43 mm) were larger than the fish from the CAN estuary (TL=139±17 mm). The same pattern was observed in *Chaetodipterus faber* because fish from Santos Bay (TL=214±37 mm) and the inner area (TL=170±8 mm) in SSV estuary were longer than the individuals from the CAN estuary (TL=84±6 mm). *Achirus lineatus* and *Cynoscion* sp. were sampled only in the inner area and in Santos Bay of the SSV and CAN estuary. In fact, these species were also greater in the SSV ($TL_{A.\ lineatus}$=132±11 mm; $TL_{Cynoscion\ sp.}$=265±77 mm) than in the CAN estuary ($TL_{A.\ lineatus}$=107±6 mm; $TL_{Cynoscion\ sp.}$=206±20 mm). Sexual differences were not considered due to the low sample size.

TRACE ELEMENT CONCENTRATIONS

Metal concentration in fish species sampled in the SSV and CAN estuaries is shown in Table II. In general, the highest mean concentrations of Co, Zn, and As were obtained in fish from the Santos Bay-SSV (Co - $_{M.\ furnieri}$=52.79 µg Kg^{-1}; $_{P.\ brasiliensis}$=30.69 µg Kg^{-1}; $_{S.\ rastiffer}$ =25.03 µg Kg^{-1}; $_{Csp.}$=17.57 µg Kg^{-1}; $_{C.\ faber}$= 40.06 µg Kg^{-1} / Zn - $_{M.\ furnieri}$=46.93 mg Kg^{-1}; $_{P.\ brasiliensis}$=11.75 mg Kg^{-1}; $_{S.\ rastiffer}$=23.12 mg Kg^{-1}; $_{Csp.}$ =19.38 mg Kg^{-1}; $_{C.\ faber}$= 35.29 mg Kg^{-1} / As - $_{M.\ furnieri}$=34.07 mg Kg^{-1}; $_{P.\ brasiliensis}$=10.03 mg Kg^{-1}; $_{S.\ rastiffer}$=2.13 mg Kg^{-1}; $_{Csp.}$=3.10 mg Kg^{-1}; $_{C.\ faber}$=4.21 mg Kg^{-1}). The lowest mean levels of Co were observed on fish species from the inner area of SSV ($_{M.\ furnieri}$=9.43 µg Kg^{-1}; $_{C.\ paralelus}$=2.69 µg Kg^{-1}; $_{B.\ ronchus}$=5.82 µg Kg^{-1}; $_{A.\ lineatus}$=4.89 µg Kg^{-1}; $_{C.\ faber}$=7.62 µg Kg^{-1} ; $_{D.\ rhombeus}$= 6.33 µg Kg^{-1} ; $_{E.\ brasiliensis}$=3.96 µg Kg^{-1}). Furthermore, no significant differences were observed among the mean levels of Zn in the fish species from the inner area of SSV ($_{M.\ furnieri}$=3.32 mg Kg^{-1}; $_{C.\ paralelus}$=3.26 mg Kg^{-1}; $_{B.\ ronchus}$=5.03 mg Kg^{-1}; $_{A.\ lineatus}$=6.89 mg Kg^{-1}; $_{C.\ faber}$=3.16 mg Kg^{-1} ; $_{D.\ rhombeus}$=6.33 mg Kg^{-1} ; $_{E.\ brasiliensis}$=4.96 mg Kg^{-1}) and the fish species from the unpolluted estuary (Cananeia) ($_{M.\ furnieri}$=4.86 mg Kg^{-1}; $_{A.\ lineatus}$=15.18 mg Kg^{-1}; $_{C.\ faber}$=5.02 mg Kg^{-1} ; $_{Csp.}$=4.23 mg Kg^{-1}). Arsenic contents were lowest in fish species from Cananeia estuary ($_{M.\ furnieri}$=0.55

TABLE II

Total Length (TL - mm) and Total Weight (TW - g) and metals contents (in wet weight) in fish species from the inner area and Santos Bay of the Santos/São Vicente (SSV) and Cananeia estuaries. Data are showed as mean ± standard deviation and minimum and maximum values.

	species	*n*	TL	TW	Co (µg Kg^{-1})	Zn (mg Kg^{-1})	Cr (mg Kg^{-1})	As (mg Kg^{-1})
Inner area	*Micropogonias furnieri*	7	218±29	115±52	9.43 (6.65-13.51)	3.32 (2.91-3.62)	0.06 (<DL-0.06)	0.89 (0.35-1.83)
	Centropomus paralelus	9	346±173	629±1004	2.69 (0.81-4.65)	3.26 (2.48-4.19)	0.17 (<DL-0.17)	0.47 (<DL-0.64)
	Bairdiella ronchus	3	175±4	65±5	5.82 (4.44-7.47)	5.03 (4.04-5.99)	<DL	0.30 (<DL-0.33)
	Achirus lineatus	6	132±11	51±13	4.89 (3.39-6.28)	6.89 (4.78-9.99)	<DL	0.60 (0.44-0.67)
	Chaetodipterus faber	2	170±8	166±20	7.62 (7.47-7.77)	3.16 (2.70-3.62)	0.15 (<DL-0.15)	0.56 (0.56-0.57)
	Diapterus rhombeus	2	170±15	73±12	6.33 (5.98-6.61)	6.33 (6.21-6.50)	<DL	0.63 (0.57-0.68)
	Eugerres brasiliensis	5	227±37	160±78	3.96 (3.06-5.74)	4.96 (3.71-5.59)	<DL	0.89 (0.39-1.25)
Santos Bay	*Micropogonias furnieri*	6	238±43	159±108	52.79 (32.44-75.83)	46.93 (14.78-113.71)	0.16 (<DL-0.82)	34.07 (20.93-64.86)
	Paralonchurus brasiliensis	3	194±37	76±42	30.69 (27.59-32.95)	11.75 (0.79-19.05)	<DL	10.03 (9.44-10.82)
	Steliffer rastiffer	9	188±37	91±43	25.03 (19.24-21.36)	23.12 (0.85-19.14)	0.09 (<DL-0.80)	2.13 (1.05-3.68)
	Cynoscion sp.	2	265±77	155±137	17.57 (10.30-24.83)	19.38 (18.20-20.56)	0.06 (<DL-0.11)	3.10 (1.22-4.98)
	Chaetodipterus faber	5	214±37	351±125	40.06 (25.69-78.48)	35.29 (17.43-96.81)	0.41 (0.09-0.77)	4.21 (1.63-7.49)
Cananeia estuary (CAN) – Cubatão sea	*Micropogonias furnieri*	4	139±17	26±12	13.90 (11.40-16.31)	4.86 (4.10-5.46)	<DL	0.55 (0.16-0.90)
	Achirus lineatus	8	107±6	25±5	10.91 (4.59-23.65)	5.18 (4.59-5.76)	0.02 (0.02-0.04)	1.02 (0.64-1.27)
	Chaetodipterus faber	11	84±6	23±4	14.39 (11.15-16.05)	5.02 (2.06-6.78)	<DL	2.11 (0.08-3.05)
	Cynoscion sp.	4	206±20	90±29	4.07 (3.09-6.23)	4.23 (4.30-4.53)	0.04 (0.02-0.06)	0.09 (0.05-0.17)

n = number of samples; DL = detection limit.

mg Kg^{-1}; $_{A. lineatus}$=1.02 mg Kg^{-1}; $_{C. faber}$=2.11 mg Kg^{-1} ; $_{Csp.}$=0.09 mg Kg^{-1}) than fish species from the inner area of SSV ($_{M. furnieri}$=0.89 mg Kg^{-1}; $_{C. paralelus}$ =0.47 mg Kg^{-1}; $_{B. ronchus}$=0.30 mg Kg^{-1}; $_{A. lineatus}$=0.60 mg Kg^{-1}; $_{C. faber}$=0.56 mg Kg^{-1} ; $_{D. rhombeus}$= 0.63 mg Kg^{-1} ; $_{E. brasiliensis}$=0.89 mg Kg^{-1}).

The contents in the *M. furnieri* and *C. faber* species from the two sites of the SSV and CAN estuary showed a similar profile of the accumulation of the essential metals Co and Zn, i.e., Santos Bay > Cananeia > inner area of SSV. Concerning Cr and As, a similar accumulation profile was observed, as Santos Bay > inner area of SSV > Cananeia. In relation to As, an ambiguous pattern was observed with Santos Bay > inner area of SSV> Cananeia in *M. furnieri* and inner area of SSV> Santos Bay > Cananeia in *C. faber*. Regarding *Cynoscion* sp., all metal contents (Co, Zn, Cr and As) presented higher concentrations in fish from Santos Bay than those from the CAN estuary. Finally, a comparison between *A. lineatus* from the inner area of the SSV and the CAN estuary showed the highest concentrations of Co, Cr, and As in fish from the CAN estuary. However, the inverse profile was observed for Zn contents.

Specific evaluation of the metal contents in species from the SSV estuary showed higher mean metal concentrations in *M. furnieri* (Co=52.79 µg Kg^{-1}; Zn=46.93 mg Kg^{-1}; As=34.07 mg Kg^{-1}) and the lowest in *Cynoscion* sp. (Co=17.57 µg Kg^{-1}; Zn=19.38 mg Kg^{-1}; Cr=0.06 mg Kg^{-1}; As=3.10 mg Kg^{-1}) and *Steliffer rastiffer* (Co=25.03 µg Kg^{-1}; Zn=23.12 mg Kg^{-1}; Cr=0.09 mg Kg^{-1}; As=2.13 mg Kg^{-1}) from Santos Bay. One exception was observed with respect to Cr levels, with low mean value in *M. furnieri* (Cr=0.16 mg Kg^{-1}) and high mean contents in *C. faber* (0.41 mg Kg^{-1}). Low Zn and Cr concentrations were also observed in *Paralonchurus brasiliensis* (Zn=11.75 mg Kg^{-1}; Cr=<DL). Regarding metal contents in fish species from the inner area of the SSV estuary, a major mean values of Co and As was observed in *M. furnieri* (Co=9.43 µg Kg^{-1}; As=0.89 mg Kg^{-1}). With the exception of the mean value of As in *Eugerres brasilensis*, which was equal to the *M. furnieri*, an absence of significant differences were observed in the concentration of metals in other collected species from the inner area of the SSV estuary (*C. paralelus*, *B. ronchus*, *A. lineatus*, *C. faber*, *D. rhombeus*, and *E. brasiliensis*).

CHRONIC RESPONSES BY NUCLEAR ABNORMALITIES IN ERYTHROCYTES (NAE)

Normal fish erythrocytes and nuclear abnormalities, such as micronucleus, lobed, notched, vacuolizated, and blebed observed in fish from the SSV and CAN estuaries are shown in Figure 2. The frequency of the total NAE in fish species from the inner area and Santos Bay in the SSV and CAN estuary are also exhibited in Figure 2. In general, fish from the inner area of the SSV estuary had more NAE than fish from Santos Bay and the CAN estuary. In the inner area of SSV, *C. paralelus* and *D. rhomneus* presented more alterations than other fish species. Regarding Santos Bay, NAE ranged from 1.6 to 3.1, with little difference among the species, apart from *P. brasiliensis*, for which the lowest NAE frequency was observed. A similar profile was observed in fish species from the CAN estuary, where NAE ranged from 0.5 to 2.2, with the lowest frequency in *M. furnieri.* Micronucleus were observed only in fish species from the SSV estuary, but in low frequency for fish from both sites, the inner area and Santos Bay (Figure 3).

Lobed (L) and notched (N) nuclei were more frequent than other NAE. Regarding fish species from both estuaries, a similar frequency of N and L was found in *M. furnieri* from the inner area and Santos Bay in the SSV estuary. On the other hand, *C. faber* sampled in Santos Bay showed more N and L nuclei than individuals from the inner area of SSV estuary, but with a similar frequency of these NAE concerning *C. faber* from Santos Bay and the CAN estuary.

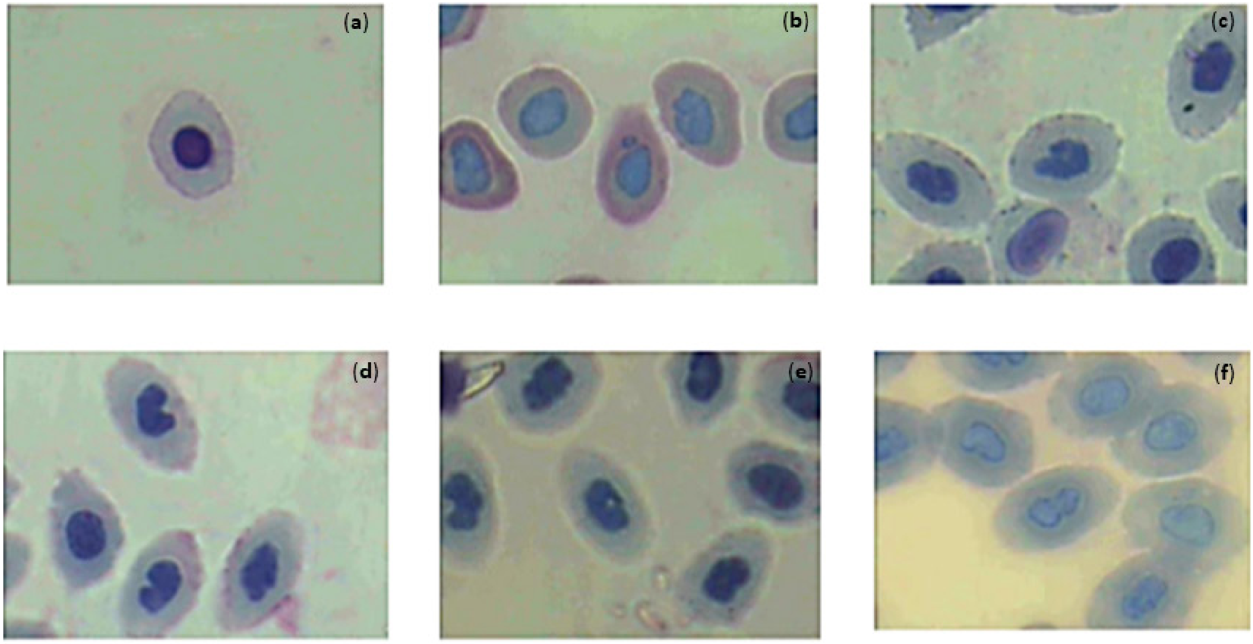

Figure 2 - Fish erythrocytes indicating the nuclear abnormalities as: (**a**) Normal; (**b**) Micronuclei; (**c**) Lobed; (**d**) Notched; (**e**) Vacuolizated; (**f**) Blebed. 1000-fold amplification.

INTEGRATIVE APPROACH OF THE DATA

Table III shows the correlation matrix of biometric variables, such as length and weight, trace elements (Co, Zn, Cr, As), and genotoxic biomarkers, such as NAE and micronucleus in fish species from the SSV. Because there were no significant differences concerning these variables on the fish species from the CAN estuary, these data were not presented. Regarding variables in fish species from the SSV, positive and significant correlation was found between length (TL) W *versus* weight (TW) (r_s=0.664, *p*=0.027), Zn *versus* Co (r_s=0.755, *p*=0.005), Cr *versus* TW (r_s=0.788, *p*=0.002), As *versus* Co (r_s=0.844, *p*=0.001), and As *versus* Zn (r_s=0.781, *p*=0.003).

Figure 3 shows the Principal Component Analysis (PCA) for trace element concentrations (Co, Zn, Cr, As), NAE, and micronucleus and biological variables such as length and weight in fish species from the three sites (SSV inner area and Santos Bay and the CAN estuary). PCA was applied to organisms sampled in SSV; PC1 explained 40% of the variance and 36% was explained by PC2. Two groups were clearly separated, representing Cr, Zn, As, and Co in *C. faber* and *M. furnieri* collected in Santos Bay (PC1); and TW, ANE and MN in *Cynoscion* sp. from the inner area of SSV (PC2). Concerning PCA and these variables in fish species from the CAN estuary, two weak groups were formed in PC1, representing As in *C. faber* and *A. lineatus*, and TL in *Cynoscion* sp. The variances attributed to PC1 and PC2 were 70% and 18%, respectively.

DISCUSSION

Due to the localization of the sampling sites, freshwater influx, and tidal changes, an increase in salinity was found at the inner area towards the bay. Temperatures equal to, or lower than, 23.50ºC are characteristic of winter in the region, as shown by other authors (Braga et al. 2000). Due to the strong influence of circulation and primary marine production, the DO contents were higher at station 3 than in the inner area of the SSV estuary, where circulation is more restricted.

TABLE III
Correlation Spearman coefficient (rs) of biological parameters (total length – TL - and total weight - TW), cobalt (Co), zinc (Zn), chromium (Cr), arsenic (As), and genotoxic biomarkers (NAE and MN) in fish species from the inner area and Santos Bay in Santos/São Vicente estuary (SSV). Analyzed samples = 59.

		TL	TW	Co	Zn	Cr	As	ΣANEs	MN
TL	rs	1							
TW	rs	**0.634***	1						
	p	0.027							
Co	rs	0.108	0.063	1					
	p	0.737	0.846						
Zn	rs	0.109	-0.154	**0.755****	1				
	p	0.737	0.633	0.005					
Cr	rs	0.455	**0.788****	0.374	0.174	1			
	p	0.137	0.002	0.231	0.588				
As	rs	0.354	0.130	**0.844****	**0.781****	0.213	1		
	p	0.258	0.688	0.001	0.003	0.507			
ΣANEs	rs	-0.046	0.0140	-0.343	-0.238	0.131	-0.392	1	
	p	0.888	0.966	0.276	0.457	0.685	0.207		
MN		0.125	-0.067	-0.396	-0.113	0.095	-0.455	0.263	1
	p	0.698	0.837	0.202	0.727	0.768	0.137	0.409	

**Correlation is significant at the 0.01 level; *Correlation is significant at the 0.05 level; NAE: nuclear abnormalities in erythrocytes; MN: micronucleus.

Oxygen consumption related to organic matter (OM) decomposition is another important aspect and this OM concentration is higher close to the region with freshwater influx, than in the ocean. DO levels around 3 mL L^{-1}, as observed in the waters of the inner areas of the system (stations 1 and 2), draw attention once hypoxia can reach these waters, causing disorders in the biota. However, environmental data such as DO in Santos Bay and in the CAN estuary are indicative of more depurative conditions; the highest element concentrations found in fish from these sites may reflect a punctual source of contamination by these elements. Therefore, it deserves the attention of environmental managers and should be subject to monitoring over time. Furthermore, and perhaps the most important in this case, the toxicity of inorganic elements, such as metals and metalloids in the aquatic system, is high in low salinity and high temperature (Garrison 2016). Consequently, changes in the abiotic parameters of the aquatic system can modify bioavailability and the effect of inorganic elements on the biota. The highest traces of metals and arsenic contents were found in fish species from the most saline areas (Santos Bay and the CAN estuary). The profile of high levels of mercury in catfish from the most saline area in the CAN estuary was observed by Pecoraro et al. (2018). In fact, changes in salinity may influence the solubility of trace elements in the environment

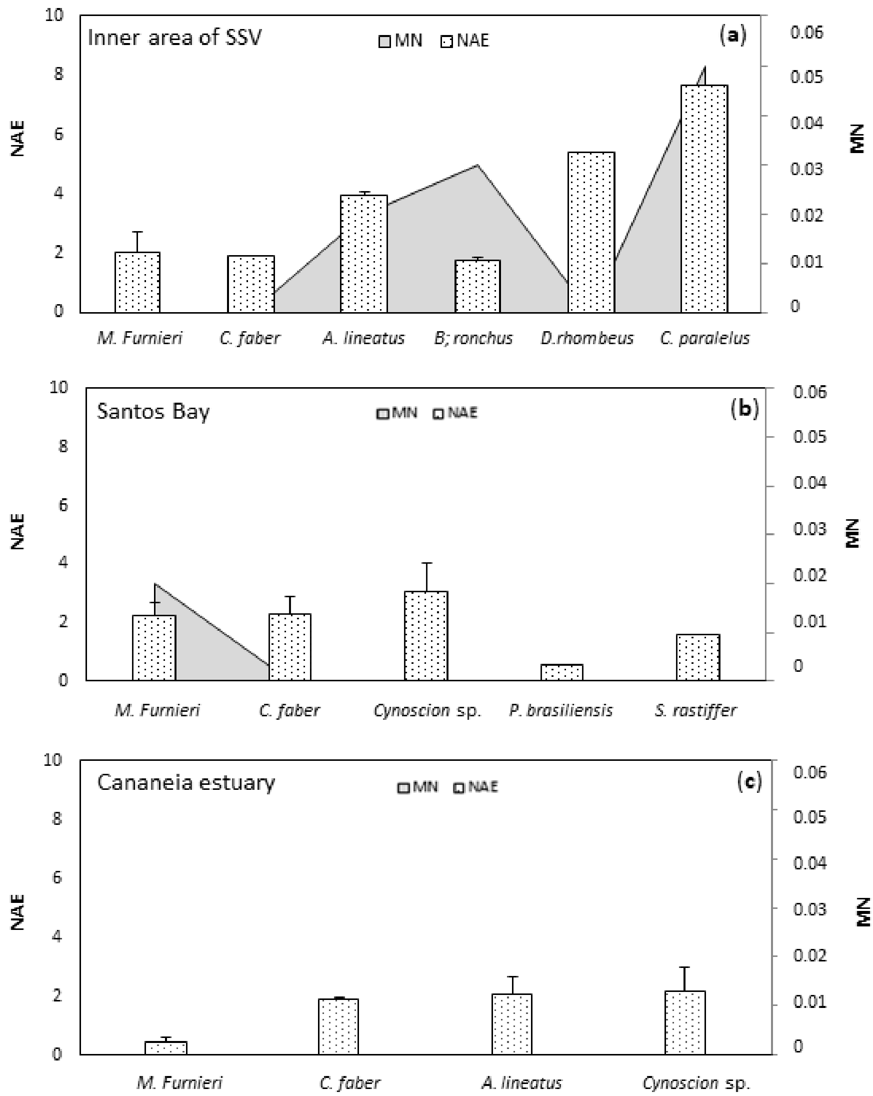

Figure 3 - Frequency of micronucleus (MN) and total of nuclear abnormalities in erythrocytes (NAE) of some fish species from the inner area (**a**) of the Bay (**b**) of the SSV estuary and from the Cananeia estuarine system (**c**).

and consequentially their bioavailability to aquatic organisms (Kennish 1986). Thus, environmental conditions, such as high salinity associated with punctual source, mainly the SSV, of trace elements can explain the major concentration of elements found in fish species in this study.

Due to intense industrial activity in the inner area of the SSV and the disposal of domestic waste by the submarine emissary in Santos Bay, the SSV has been biomonitored since 2004. This biomonitoring considers the bioaccumulation pattern of toxic and non-toxic metals, such as Pb, Cd, Zn, Fe, Mn, Cu, and Hg (Azevedo et al. 2009, 2012a, 2012c), as well as the expression of biochemical, histopathological, and genotoxic biomarkers in the bioindicator species *Cathrops spixii* (Azevedo et al. 2012b, 2013) and shows major damage in catfish from the SSV, along with higher levels of metals. Thereby, the investigation of other fish species with potential use as bioindicators of contamination in estuarine environments is very important and may be used as an additional tool in

TABLE IV

Zn, Cr, Co and As mean concentration ± standard deviation (mg Kg^{-1}) in muscle tissue of fish species with demersal habitat from different sampling polluted and non-polluted areas on estuarine and coastal areas on the world. NA: not analyzed. QL: quantification limit.

Species	Region	Environmental Characteristic	Zn	Cr	Co	As	Reference
Platichthys flesus (Flounder)	Western Scheldt estuary, Netherlands	Polluted in the upstream river site by domestic, industrial and agricultural waste water discharges	21±4	QL	0.03±0.01	32±1	Maulvault et al. (2015)
E. vipera (Weever)	Dalio River estuary, Southern Lioning Province, China	Industrial waste water	30	0.21	NA	3.66	Radomyski et al. (2018)
Achirus lineatus (Lined sole)	Santos Bay, São Paulo, Brazil	Domestic waste water discharge by submarine emissary on the Bay, but with accentuated hydrodynamic by the Ocean	35.08	3.27	NA	10.37	Rocha et al. (2014)
Trinectes Paulistanus (Slippen sole)	Santos Bay, São Paulo, Brazil	Domestic waste water discharge by submarine emissary on the Bay, but with accentuated hydrodynamic by the Ocean	26±2	0.22±0.14	0.095±0.01	5.1±1.4	Rocha et al. (2014)
Cathorops spixii (Catfish)	Inner area of Santos/São Vicente estuary, São Paulo, Brazil	Industrial waste water	11.57	NA	NA	NA	Azevedo et al. (2012c)
Cathorops spixii (Catfish)	Southern area of the Cananeia estuary, São Paulo, Brazil	Low human influence and accentuated hydrodynamic by the ocean	10.16	NA	NA	NA	Azevedo et al. (2012c)

biomonitoring programs as a way to take advantage of all species collected by the net during sampling, such as that in the SSV and CAN estuaries, in the São Paulo State, Brazil.

Elements such as Cr, Co, As, and Zn can potentially become toxic to biota and humans, therefore, they were selected in this study. The elements levels determined in this study have been compared to others obtained for fish species with a similar habitat (demersal) and submitted to different anthropogenic influences around the world (Table IV). The low Cr values found in this study are in accordance with the data obtained for other fish species submitted to industrial and domestic polluted and non-polluted areas and, therefore, it is not a great problem for the fish species from the SSV and the CAN estuary. On the other hand, Co and As concentrations were higher than those observed in other studies (Table IV), mainly in fish species from Santos Bay (SSV). Elements such as Cr, Co, and As can be toxic to organisms due to their high affinity with sulfur (S) and nitrogen (N) ligands, which interfere in the metabolism with the lost protein binding ability (Luoma and Rainbow 2008). The Zn content was lower in fish species from the CAN estuary and the inner area of SVV than in fish species from estuarine and coastal polluted sites around of the word (Table IV). In fact, the obtained Zn values in fish from the Santos Bay (SSV) area were in accordance with those obtained from other polluted sites. Thereby, differences in the observed concentrations profile of metals accumulation obtained in this study may have a consequence on ecological strategies such as feeding habits, but not necessarily on their habitat, metabolism, or the levels of these elements in the environment (SSV and the CAN).

Concentrations of the analyzed trace metals in all fish species from both estuaries were also compared with the maximum limit established by Brazilian Health Legislation (ANVISA 2013). Several samples showed concentrations below the detection limit (DL) for Cr determined by INAA. In fact, low Cr concentrations are expected, since the maximum limit established to human consumption of this metal is 0.10 $\mu g\ g^{-1}$ (ANVISA 2013). Therefore, low Cr levels observed in all fish species from CAN and the two sites of the SSV estuary indicate more depurates and/or a reduction in anthropogenic influence in these systems and, finally, can reflect the significant effectiveness of the actions of monitoring and environmental surveillance, mainly in the Santos region. Additionally, concentrations of Zn found in all fishes were below the established limit (ANVISA 2013).

Regarding Co contents in fish from both regions, no values above the maximum limit for human consumption were found. Although Co is considered an essential micronutrient, high concentrations of Co can be harmful to organisms (Furness and Rainbow 1990). Thus, further studies would be required to determine the maximum tolerated concentrations in fish to maintain the biochemistry integrity of these organisms.

Although the high arsenic contents observed in *C. faber* from the CAN estuary and all fish species from Santos Bay in SSV were above 1.0 $\mu g\ g^{-1}$, which is the maximum tolerated limit for human consumption, established by Brazilian law (ANVISA 2013), these levels should be carefully considered. The small amounts of arsenic found in fish are usually inorganic, the most toxic form. Arsenobetaine is the organic and main form of arsenic in fish tissue. Concerning food safety, it is important to consider that this form is non-toxic and non-metabolized by human organisms when contaminated fish are consumed. Levels in a human are independent of abiotic aspects of the aquatic system such as salinity (Zhang et al. 2018, Julshaman et al. 2012).

The total arsenic content found in fish species from areas with the highest salinity (e.g., Santos Bay and the CAN estuary) may be associated with salinity. Despite the fact that not all chemical species are toxic, it is important to consider the influence of salinity under Arsenic chemical speciation, once the profile of arsenic transfer related to salinity is already reported in the literature for other aquatic organisms (Zhang et al. 2018, Hong et al. 2018, Kalantzi et al. 2017) and may be explained by the osmoregulation of aquatic organisms exposed to differential haline conditions. In the presence of salts from marine waters, many elements are incorporated into cells after being transported from the water. Consequently, in less saline waters, the metals are less (or not) available for the organism. More studies are recommended concerning the biogeochemical cycle in the aquatic environment and the speciation of arsenic to offer more information about the uptake and distribution of different forms in the organism.

To understand the biological damage caused by mutagenic agents in polluted water, the frequency of nuclear abnormalities in erythrocytes (NAE) and micronucleus testing were employed. Although NAE are non-specific indicators of mutagenic damage, they can provide an additional tool for understanding the genotoxic effect, since it provides an indication of mutation above the normal frequency and can, therefore, be indicative of xenobiotic exposure (Azevedo et al. 2012b, Capela et al. 2016). The fish species from the CAN estuary and most fish species from the SSV estuary without micronuclei cells, are in accordance with the results presented by Azevedo et al. (2012b), which

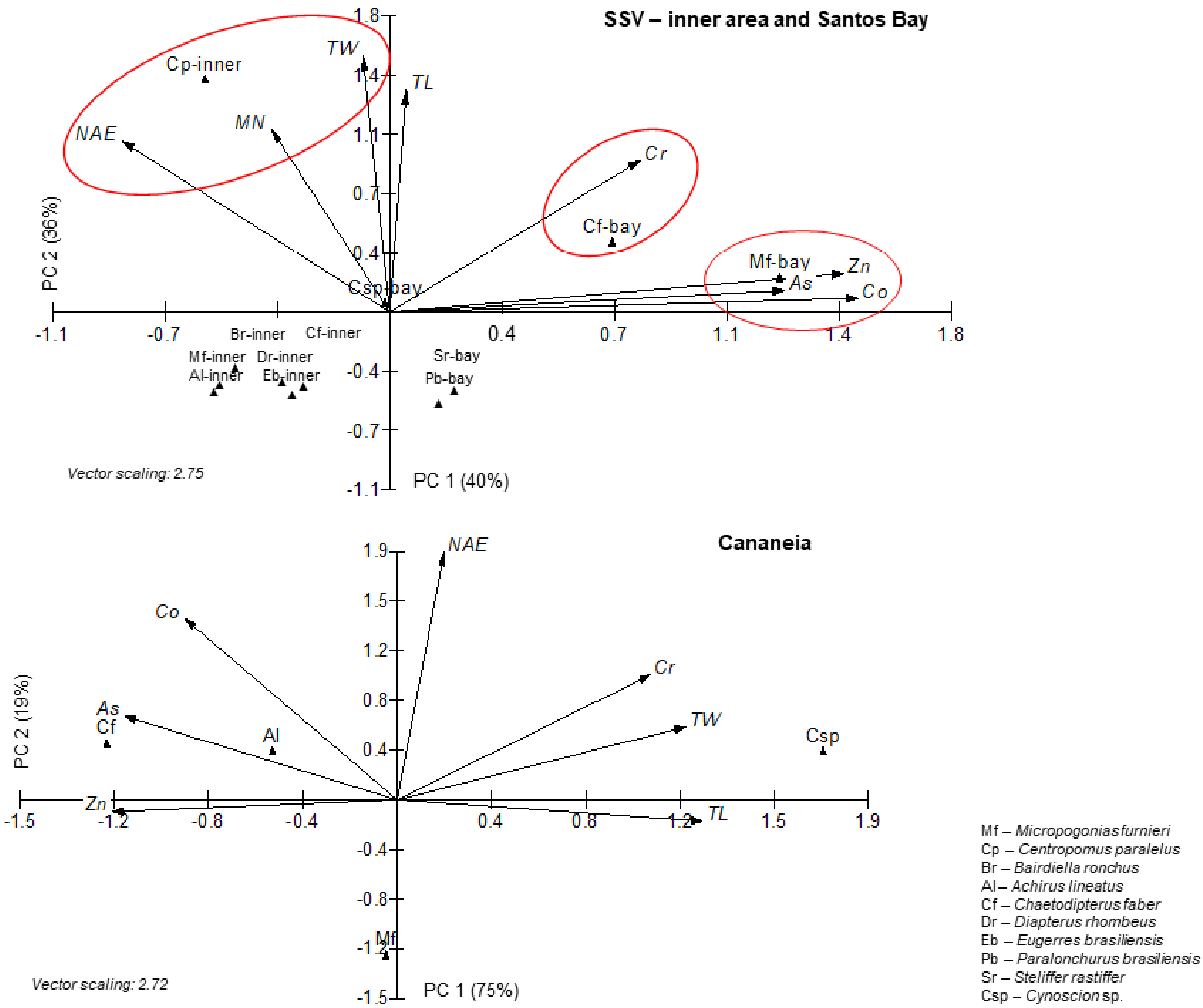

Figure 4 - Multivariate approach by Principal Components Analyses (PCA) of biological variables like total length (TL) and total weight (TW), genotoxic biomarkers as nuclear abnormalities in erythrocytes (ANEs) and micronucleus (MN) and metals and semi-metals (Zn, Co, Cr, As) in different fish species from two sites of the SSV and Cananeia estuaries.

analyzed nuclear abnormalities in erythrocytes of *C. spixii* from different sites of SSV and Cananeia estuaries in 2005/2006, providing evidence of the absence of clastogenic and aneugenic effects. Some authors consider that, in aquatic systems without anthropogenic pollution, the frequency of nuclear abnormalities in erythrocytes (NAE) range from 3 to 20‰ (Capela et al. 2016). Although the frequency of NAE found in this work was higher in fish such as *C. paralelus* (about 8‰) and *D. rhombeus* (about 6‰) from the inner area of the SSV than the observed in the other fish species, these data are lower than the observed in *C. spixii* from the same site (above 20‰). Therefore, non-significant genotoxic effects were detected in fish species from the CAN estuary, indicating the absence of clastogenic and aneugenic effects on these fish. Data from the Spearman correlation coefficient and PCA also evidence these aspects once no significant correlation was observed among the NAE, micronucleus, and the levels of the analyzed elements. Regarding fish species from the SSV, clastogenic and/or aneugenic effects may be related to *A. lineatus, B. ronchus,* and *C. paralelus*, from the inner area of SVV and in *M. furnieri* from Santos Bay (SSV). These data indicate a chronic effect on these fish and while they are not associated with the metal contents analyzed in this study (Table III, Figure 4), they can be a consequence of long exposure to other inorganic and organic chemical compounds, mainly in the inner area of SSV.

Much of the variation found is probably random, because the number of samples is rather low per species and area. Thereby, an effort is recommended to increase the sample number and perform a monitoring program with the species shown in this study to evaluate their potential use as a bioindicator species of contamination for the SSV and CAN estuaries. It is also important to consider that once all fish species analyzed in this study are demersal, the contents of the elements may be an input of their benthic feeding habits because the highest concentrations and bioavailability of the elements are found in the sediment. In fact, the integrative approach by PCA of the analyzed metals indicates the association of high Zn, As, and Co levels in *M. furnieri* from Santos Bay (SSV). The weight had an evidence association only of NAE and micronucleus in *C.* sp. from the inner area of SSV, suggesting the importance of biological variables when the chronic exposure was considered.

Finally, the trace elements analyzed in this study showed differences concerning the fish species from both estuaries. In fact, with these findings, it was possible to distinguish between the polluted and non-polluted/protected estuaries of the São Paulo coast submitted to differential anthropic influences. Environmental pollution was differently expressed in each species depending on the variability of abiotic conditions, such as salinity. The concept of environmental sustainability is reinforced by the maximum utilization of the species collected in a net trawl for quality assessment purposes.

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AUTHOR CONTRIBUTIONS

Elisabete de Santis Braga contributed to the financial support with the projects, organization and responsible for the oceanographic expedition,the hydrochemical strategy, results discussion of the manuscript. Juliana de Souza Azevedo contributed to the fish sampling strategy, organization and writing of the manuscript, statistical analysis and results discussion. Leonardo Kuniyoshi contributed to the hydrochemical, blood and muscle tissues analysis. Déborah Inês Teixeira Fávaro contributed to the trace elements strategy and analysis and results discussion.

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