RESEARCH ARTICLE

Assessing pollution in marine protected areas: the role of a multi-biomarker and multi-organ approach

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Abstract Marine protected areas (MPAs) are vulnerable to many pressures, including pollution. However, environmental quality monitoring in these areas traditionally relies on only water chemistry and microbiological parameters. The goal of the current study was to investigate the role of a set of biomarkers in different target organs (liver, kidney, and gills) of fish in order to assess the environmental quality of an MPA (MTs, GPx, GST, GSH, DNA damage, LPO, AChE, and condition index). Chemical analyses were also performed on liver and muscle tissues to evaluate metal body burdens, and PAHs were identified in bile. A demersal fish (*Cathorops spixii*) that is widely consumed by the local population was used as bioindicator species, and the results were integrated using multivariate analysis. The use of the biomarker approach allowed for the identification of both seasonal and spatial variations in pollution sources around the Environmental Protected Area of Cananéia-Iguape-Peruíbe (APA-CIP). Higher metal body burdens associated with biological responses were found in the sites under the influence of urban areas during the dry season, and they were found in the sites under the influence of the Ribeira de Iguape River (RIR) during the rainy season. The liver was found to be more responsive in terms of its antioxidant responses, whereas gills were found to be more responsive to biomarkers of effect. These results show that this set of biomarker analyses in different organs of fish is a useful tool for assessing chemical pollution in an MPA.

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Abbreviations

AChE	Acetylcholinesterase
APA-CIP	Environmental Protected Area of Cananéia-Iguape-Peruíbe
D	Dry season
FA/PCA	Factor analysis (with principal component analysis as the extraction method)
GPx	Glutathione peroxidase
GSH	Non-protein reduced thiols
GST	Glutathione *S*-transferase
LPO	Lipid peroxidation
MPAs	Marine protected areas
MTs	Metallothionein-like protein
P	Partially dry season
PAHs	Polycyclic aromatic hydrocarbons

R   Rainy season
RIR   Ribeira de Iguape River

Introduction

Marine protected areas (MPAs) have been established to preserve the marine environment (Scherl et al. 2006). The rationale behind the MPA concept is that by reducing habitat loss and mortality due to anthropogenic pressures, individuals can survive longer and produce more offspring, and populations can grow. However, MPAs are usually planned, created, and managed following the same concepts and theories historically used for terrestrial protected areas, despite the substantial differences in ecosystem processes, historical perceptions, and regulatory frameworks between marine and terrestrial environments (Houde 2001). For example, marine populations, communities, and ecosystems are all connected to a broader landscape and seascape: holoplanktonic organisms, the early life stages of several species, diadromous fish, many forage fish species, and highly migratory species can all travel for long distances to settle, spawn, feed, or nurse (UNCLOS 1982; Carr et al. 2003).

Furthermore, water rarely respects man-made boundaries, and contaminants may be introduced into an MPA from adjacent areas (Palmer et al. 1996; Boersma and Parrish 1999; Pozo et al. 2009). It is widely accepted that MPAs are vulnerable to pollution as well as to fishing, habitat alteration, and climate change (Cicin-Sain and Belfiore 2005; Keller et al. 2009).

Most biological assessments carried out in MPAs consider only direct or indirect effects of fishing on the biota (refer to García Charton et al. 2000; Fraschetti et al. 2002 for reviews). Still, pollution studies in MPAs usually focus on measuring contaminant levels in environmental matrices such as water, sediments, and organisms (e.g., Michel et al. 2001; Conti and Cecchetti 2003; Chou et al. 2004; Pozo et al. 2009; Perra et al. 2011; King et al. 2013; García-Alvarez et al. 2014) rather than the effects of pollutants on the biota that is supposed to be protected (e.g., Terlizzi et al. 2004; Pinsino et al. 2008; Rodrigues et al. 2013; Araujo et al. 2013; Cruz et al. 2014).

The knowledge of the chemical concentrations in a given environment provides important information on the risks of pollution. However, chemical data alone is not capable of providing information on biological effects since the bioavailability and toxicity of chemicals in complex mixtures may be altered as a result of both contaminant synergies (Beyer et al. 2014) and interactions between contaminants and environmental conditions (Chapman and Wang 2001). Thus, the use of ecotoxicological tools (i.e., biological-based assessment tools) is imperative in order to directly assess the health of aquatic organisms within an MPA since these tools shift the focus of the assessment from the agents (contaminants) to the targets (biological/ecological responses).

Effects of pollution are particularly difficult to assess in MPAs since such areas are usually subjected to low to moderate levels of contamination and biological or ecological responses are therefore not as evident (Choueri et al. 2009). Under these unfavorable conditions, effects may not necessarily be lethal, but the conditions can deteriorate the health status of the biota and can affect populations in the long term. Sensitive biological responses are potentially suitable tools for monitoring the environmental quality of mildly contaminated MPAs.

Biochemical and cellular responses measured in organisms' tissues (i.e., biomarkers) can determine the health status of an individual and thus aid in the detection of the first signs of injury caused by pollutants. Biomarkers combined with metal body burdens of resident organisms have been widely used in aquatic pollution monitoring (Van der Oost et al. 2003; Au 2004; Giarratano et al. 2010; Duarte et al. 2011; Oliva et al. 2012, 2014; Barhoumi et al. 2014; Morachis-Valdez et al. 2015), though most biomarker-based environmental quality studies were performed exclusively in highly anthropized sites (Malins et al. 2006; Ramos-Gómez et al. 2011; Ben Ameur et al. 2012; Maranho et al. 2012; Pereira et al. 2014). We hypothesize that biomarkers are adequate for assessing the environmental quality of MPAs.

The evaluation of MPA performance is critical not only for the protection biodiversity itself but also because the failure of MPAs could erode public and political support for conservation (Mora and Sale 2011). The goal of the current study was to investigate the usefulness of a set of biomarkers (antioxidants responses, DNA damage, lipid peroxidation, metal body burdens in different organs, and total PAHs in bile) in the local fish in order to assess environmental quality of an MPA subjected to moderate levels of contamination. To achieve that, biological responses in a demersal fish (*Cathorops spixii*) that is widely consumed by the local population was used as a bioindicator of pollution.

Materials and methods

Study area

The Cananéia-Iguape-Peruíbe Environmental Protected Area (known as the APA-CIP) (24°40′S and 25°05′S) is an estuarine-lagoon ecosystem recognized by UNESCO as part of the Biosphere Reserve of the Atlantic Rainforest due to its relevance for environmental conservation. Since 2000, the region has been part of the global list of UNESCO's World Heritage Sites; in addition, the APA-CIP is considered an area of priority for future inclusion on the list of Brazilian wetlands

of international importance within the scope of the Ramsar Convention (Brazil 2012).

Two well-defined climate seasons dominate in this region, a drier winter and a rainier summer, with minimum precipitation rates occurring from July to August (monthly average of 95.3 mm) and maximum rates from January to March (monthly average of 266.9 mm). Monthly mean temperatures range from a maximum of 28 °C (February) to a minimum of 20 °C (July). Tides are semidiurnal, and mean tidal amplitude is 0.82 m (Cunha-Lignon et al. 2009).

The main freshwater contributor to the CIP estuary is the Ribeira de Iguape River (RIR). Approximately 70 % of the course of the river flows toward the lagoon waters through an artificial channel known locally as Valo Grande. The river basin is a metallogenic province with natural Pb and Zn deposits (Moraes 2004). Mining activities were performed in this area for many decades during the twentieth century, but the mines were closed down in the 1990s. Since then, high levels of metals (Pb, Zn, Cu, Cr) and arsenic (As) have been recorded in the river waters as well as in both bottom and suspended sediments (Eysink et al. 1988; Corsi and Landim 2003; Moraes 2004; Guimarães and Sígolo 2008). In the estuarine sediments, metals were found at only moderate levels (Mahiques et al. 2009) as defined by the international Sediment Quality Guidelines (Long et al. 1995; Environment Canada and *Ministère du Développement durable, de l'Environnement et des Parcs du Québec*, 2007).

Apart from its important natural features, the APA-CIP also encompasses three cities (Iguape, 30,259 inhabitants; Ilha Comprida, 9025 inhabitants; Cananéia, 12,601 inhabitants) (IBGE 2014) that lack proper sanitation infrastructure. Waste is discharged in rivers, in groundwater, or directly into the lagoon system since treatment is insufficient for local demand (Morais and Abessa 2014).

Fish collection and sample preparation

The madamango sea catfish (*Cathorops spixii*) is a demersal fish that lives in a wide salinity range and preys mainly upon zoobenthos (crustaceans and polychaetes in particular) and small fishes (Fishbase 2014). Adult individuals migrate from coastal zones to lower reaches of estuaries to spawn and the early juvenile development occurs in bays and estuaries (Araújo 1988). In addition to its ecological relevance, this species is of socioeconomic interest as it is widely consumed by the local population (Favaro et al. 2005).

Fifteen specimens of *C. spixii* were collected at each sampling site using a bottom otter trawl (2-min trawling) and kept for the analyses (Fig. 1). Five animals were set aside for metal body burden analyses and ten specimens were set aside for biomarker analyses. Mean length of the animals from all sampling sites was 17.5 cm (±3.6), and mean weight was 76.3 g (±27.9). To account for seasonal variation, individuals were

collected during three seasons with different amounts of rainfall: (1) the partially dry season (P) (May 2012), (2) the dry season (D) (August 2012), and (3) the rainy season (R) (March 2013). The average rainfall during these seasons was 192, 111, and 390 mm, respectively (CEPAGRI 2014). In the first sampling campaign (during the partially dry season), four sampling stations distributed along the APA-CIP area (P2 to P5) were set up. In the subsequent campaigns, two additional sampling stations were included (P1 and P6) in order to enable a better understanding of the influence of important contaminants sources in APA-CIP. The scope of metal body burdens analyses in *C. spixii* was enlarged as well. Metal analyses were limited to axial muscle in the first sampling campaign, but liver was included in the subsequent campaigns (dry and rainy seasons). Before dissection, the collected specimens were kept in local water, under aeration until transportation to the laboratory. Before euthanized by spinal cord section, individuals were anesthetized with benzocaine in water, then weighed and measured. Fish gills, kidney, liver, bile, and axial muscle tissues were dissected, frozen, and stored at −80 °C until biochemical analyses. Axial muscle and liver tissues used in metal body burden analyses were stored in plastic vessels at −20 °C.

Condition factor

Fulton's condition factor was calculated according to the formula: $KF = (W/L^3 \times 100)$, where KF = Fulton condition factor, W = body weight in grams, and L = total body length in centimeters.

Biochemical determinations

Gills, kidney, liver, and muscle tissues were kept on ice and homogenized at 10 %w/v in Tris–HCl buffer (Tris 50 mM; EDTA 1 mM; DTT 1 mM; sucrose 50 mM; KCl 150 mM; PMSF 1 mM, pH 7.6). Homogenates were centrifuged at 10,000×g for 20 min at 4 °C, and in the case of the liver, gills, and kidney, aliquots of the supernatants were kept for the analyses of glutathione S-transferase (GST) and glutathione peroxidase (GPx) activities as well as for the quantification of non-protein reduced thiols (GSH), lipid peroxidation (LPO), and DNA strand breaks. In the case of the muscle tissue, supernatants were used for acetylcholinesterase (AChE) activity analyses. Liver, kidney, and gill tissue samples were also set aside for metallothionein-like protein (MTs) analysis, homogenized with 20 mM Tris–HCl buffer supplemented with 0.5 M sucrose, 0.01 % β-mercaptoethanol, and centrifuged at 15,000×g for 30 min at 4 °C.

GST (Keen et al. 1976) and GPx activities (Sies et al. 1979) were determined spectrophotometrically at 340 nm. GSH levels were measured spectrophotometrically at 415 nm (Sedlak and Lindsay 1968). AChE activity analysis was
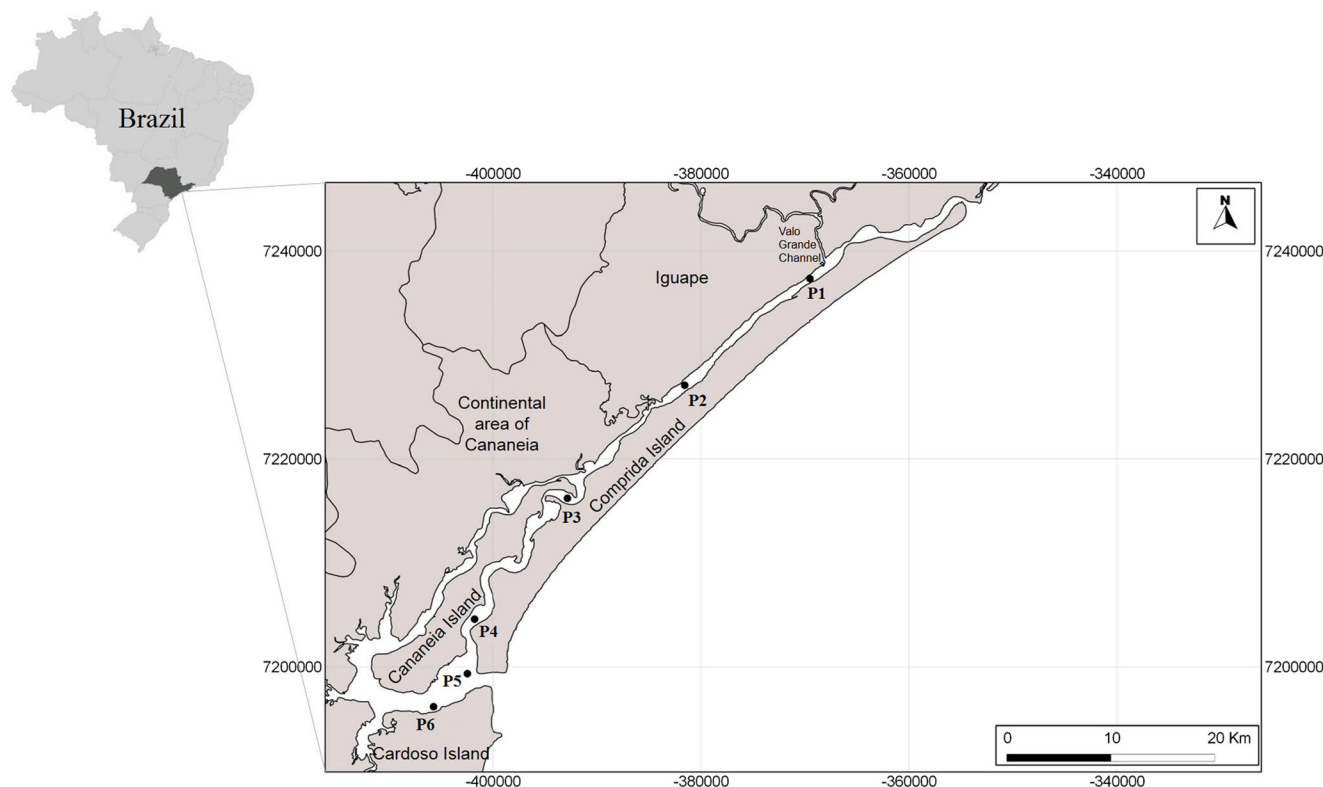
Fig. 1 Sampling stations located within the APA-CIP, Brazil

performed at 415 nm using the colorimetric method by Ellman et al. (1961). The concentration of metallothionein-like protein (MTs) was established based on cysteine residue titration of a partially purified MT extract obtained by acidic ethanol/chloroform fractionation of the tissue homogenate. In brief, after the homogenization, cold (−20 °C) absolute ethanol and chloroform were added to the supernatant in order to precipitate the high molecular weight proteins. Samples were then centrifuged at 6000×g for 10 min at 4 °C. This supernatant fraction was acidified with HCl to co-precipitate MT and improve recovery. Before acidification, samples were stored at −20 °C for 1 h. The samples were subsequently re-centrifuged at 6000×g for 10 min at 4 °C and the MTs pellet obtained was resuspended with an ethanol/chloroform/homogenizing buffer Tris–HCl 20 nM solution. Samples were re-centrifuged at 6000×g for 10 min at 4 °C. The MTs pellet obtained was resuspended in 0.25 M NaCl solution and then HCl/EDTA was added to remove metal cations still bound to the MT.

The MTs-like protein concentration was quantified using Ellman's reagent containing DTNB after a centrifugation of 3000×g for 5 min at room temperature. The absorbance was recorded at 412 nm (Viarengo et al. 1997).

Levels of lipid peroxidation (LPO) were determined by quantifying the concentration of 2-thiobarbituric acid reactive substrates (TBARS) through fluorescence (λ_{ex} 532 nm and λ_{em} 556 nm) (Wills 1987). DNA strand breaks were measured using an alkaline precipitation assay (Olive 1988; Gagne and

Blase 1995). The assay is based on the potassium dodecyl sulfate precipitation of protein-bound genomic DNA, which leaves protein-free DNA strand breaks in the supernatant. These DNA strands are quantified using fluorescence (λ_{ex} 360 nm and λ_{em} 450 nm) after staining with Hoechst dye. Standard solutions of salmon sperm DNA were used for calibration. Protein concentrations were determined spectrophotometrically at 595 nm (Bradford 1976), with BSA as the standard. All biomarkers analyses were performed in a microplate reader (Biotek-Synergy™ HT).

Chemical analyses

Metal body burden

Concentrations of As in muscle and liver tissues were determined using an atomic absorption spectrophotometer (Varian®, AA 240Z) equipped with a graphite furnace (AAS-GF) (Model, GTA 120). Metals were quantified using flame atomic absorption spectroscopy (FAAS) (Varian®, AA 240FS). All analyses were performed according to standard method 200.9 (USEPA 1994). Detection limits for As were 5.88 µg kg^{-1} and detection limits for metals (Cu, Mn, Zn, Cr, Co, Ni, Cd, Pb) were 0.034, 0.0697, 0.0525, 0.112, 0.146, 0.0623, 0.042, and 0.0602 mg kg^{-1}, respectively. Standard curves were prepared using reference material (Qhemis High Purity®). Recovery rates ranged from 80 to 120 % in all

investigations. Metal concentrations were expressed in milligrams per kilogram of dry weight.

Polycyclic aromatic hydrocarbons in bile

The metabolites of polycyclic aromatic hydrocarbons (PAHs) in the bile of *C. spixii* were quantified via fixed-wavelength fluorescence in the spectrofluorometer (Sunrise-Tecan) at wavelengths of 288/330, 334/376, 364/406, and 380/422 nm ($\lambda_{ex}/\lambda_{em}$), which correspond respectively to naphthalene type (two rings), pyrene type (four rings), benzo[*a*]pyrene-type (five rings), and benzo[*ghi*]perylene type (six rings) (Aas et al. 2000; Oliveira Ribeiro et al. 2005). PAH concentrations were determined through a comparison with a standard curve for each group of rings. The results were expressed as units of PAH milligrams per protein.

Data treatment

Statistical analyses

First, biomarker data and total PAH contents in bile were tested for normality (Kolmogorov-Smirnov's test) and homoscedasticity (Bartlett's method). The statistical differences of mean values of each data series ($n=10$) that met analysis of variance (ANOVA) assumptions were tested through ANOVA followed by post hoc Tukey's test. Non-parametric statistical tests (Kruskal-Wallis test, with Dunn's multiple comparisons as post-test) were used to compare data series that violated ANOVA assumptions. The significance level was set at $p=0.05$.

The biological data was integrated by two different methods. In the first one, only biological responses data were integrated through the calculation of an index, i.e., the Biomarker Response Index (BRI) (Hagger et al. 2008). Shortly, biomarker data (GST, GSH, GPX, MTs, LPO, DNA damage, and AChE) were ranked numerically to represent varying degrees of severity from normal reference responses. Since there is no baseline biomarker data for *C. spixii*, the responses obtained in fish from the sampling site showing the least effects and bioaccumulation was deemed the reference condition. The index ranges from numeral ranks of one (severe alterations) to four (no or slight alterations). The biomarkers were also weighted according to their potential of impact on the health of the organisms (i.e., biomarkers of effects weighted as two, while biomarkers of exposure weighted as one). The BRI was calculated as the following (Hagger et al. 2008):

$$Biomarker\ response\ index\ (\text{BRI}) = \sum (\text{biomarker}_1\ rank \times \text{biomarker}_1\ weighting) + (\text{biomarker}_2\ rank \times \text{biomarker}_2\ weighting)_n$$
$$\times \left[\sum (\text{biomarker}_1\ weighting) + (\text{biomarker}_2\ weighting)_n \right]^{-1}$$

The second integration method aimed to highlight associations among the variables measured in this study (both biomarker responses and bioaccumulation levels) during each of the three seasons (partially dry, dry, and rainy). Factor analysis with principal component analysis as the extraction method (FA/PCA) was used. Associations between the different biomarkers (GST, GSH, GPX, MTs, LPO, DNA damage, and AChE), metal loads (Cu, Mn, Zn, Cr, Co, Ni, Cd, Pb) and As loads in liver and muscle tissues, and total PAH metabolites in bile were assessed. The variables that failed to present significant variation among sampling stations were removed from the original datasets. The variables were autoscaled (standardized) so as to be treated with equal importance. The selected variables to be interpreted were those associated with the factors with a loading ≥0.50, a value which is more conservative than the loading cut-off recommended by Tabachnic and Fidell (1996). The relevance of the observed associations to each of the six sampling stations (cases) was estimated by calculating the factor score from each case for the centroid of all cases for the original data. All the statistical and multivariate analyses were performed using STATISTICA 12 software (StatSoft Inc. USA).

Results

The responses of the biomarkers of exposure in the liver and gills of *C. spixii* specimens collected along the APA-CIP revealed both seasonal and spatial variations (Fig. 2a–l). During the periods of lower rainfall (dry, partially dry), biomarkers of exposure (MT, GST, GPX, and GSH) presented significantly higher values among the fish collected at the estuarine sections influenced by the city of Cananéia (P4, P5, and P6); during the rainy season, these responses are more pronounced in fish sampled from the region under the influence of the RIR (P1, P2, P3). Biomarkers of effect (LPO, DNA damage, and AChE) found in *C. spixii* liver tissue also followed these seasonal and spatial variations (Fig. 3a–g). In addition, liver genotoxicity responses were higher in specimens sampled during the dry and partially dry seasons in the stations under the influence of Cananéia (P4, P5, P6); during the rainy season, LPO levels in the liver were higher ($p < 0.05$, ANOVA). AChE activity was inhibited, particularly in the stations under the influence of the RIR ($p < 0.05$, ANOVA). Responses of biomarkers found in
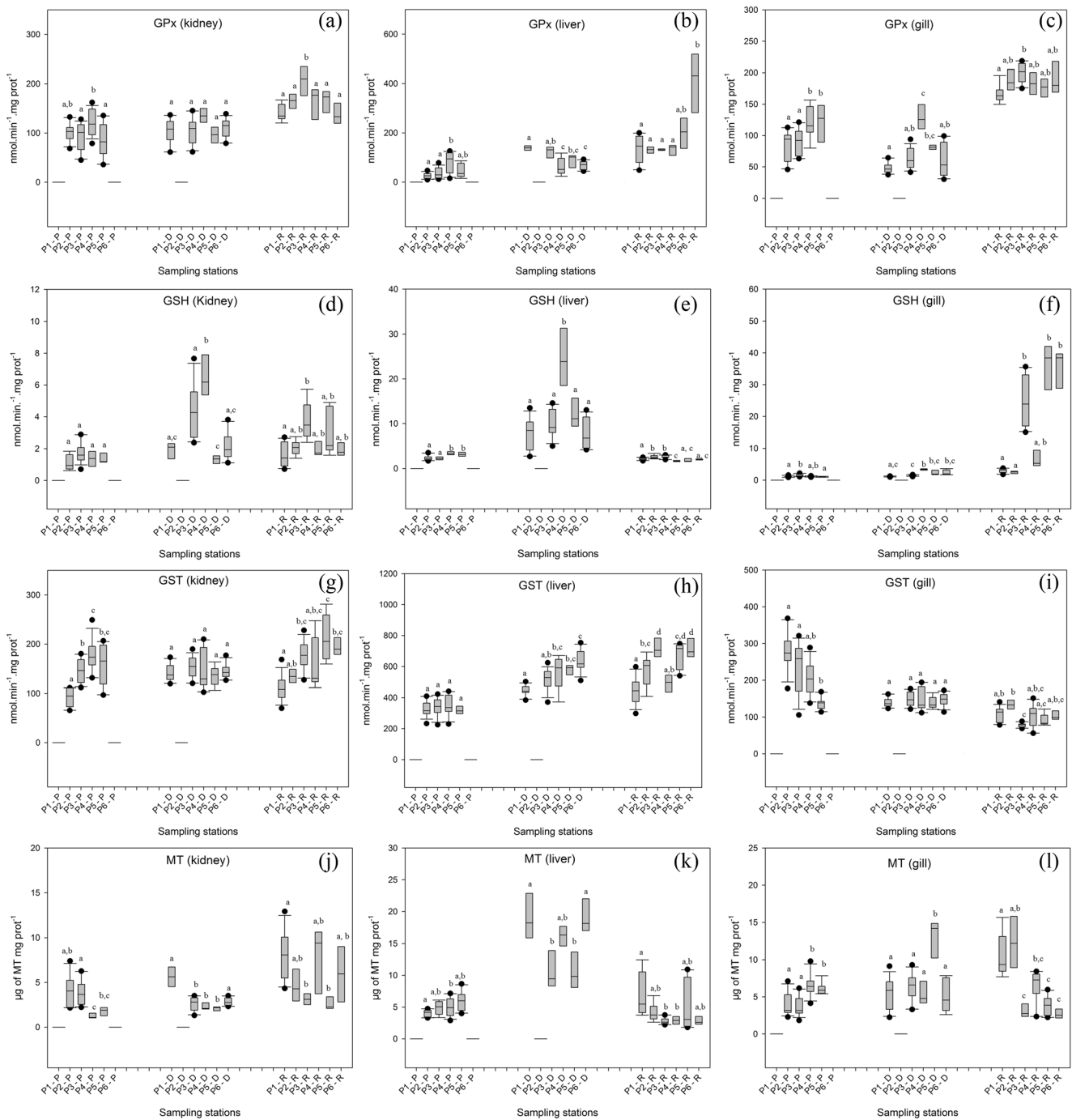
Fig. 2 a–l Responses of biomarkers of exposure in kidney, liver, and gill tissues from *Cathorops spixii* from the APA-CIP collected during the partially dry season (*P*), the dry season (*D*), and the rainy season (*R*). Sites with a *line* instead of a *boxplot* mean that no organisms were sampled. Data are presente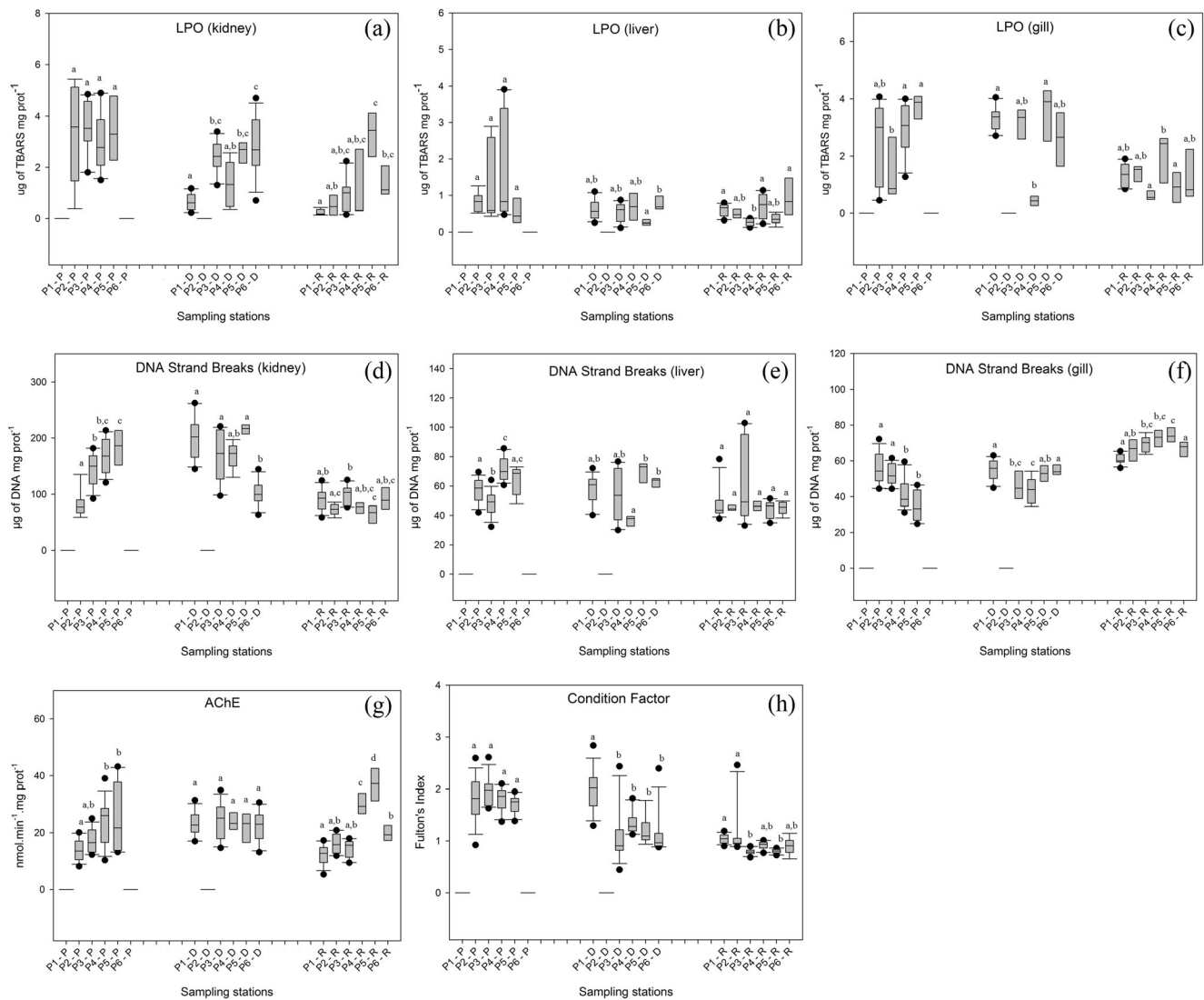d as *boxes* with boundaries that indicate 25th and 75th percentiles; a *line* within the *box* marks the median value; *whiskers* below and above the *box* indicate 10th and 90th percentiles; the outliers are presented with *black dots*. The use of different *letters* above the data indicates significant differences during the same season (*p*=0.05)

kidney tissue generally followed a pattern of response based on the sample's proximity to the two main sources of contamination (the RIR and Cananéia).

Fulton's condition factor results (Fig. 3h) showed that specimens from P1 (during both the dry season and the rainy season) and P2 (during the rainy season) were more well nourished ($p<0.05$, ANOVA). No spatial differences were observed in the partially dry season ($p>0.05$, ANOVA).

Biological responses were consistent with As and metal concentrations in liver and muscle tissues of *C. spixii* (Fig. 4a–d). In general, As, Mn, Zn, Cd, and Pb concentrations in muscle were higher in stations closer to the city

Fig. 3 a–h Responses of biomarkers of effects in all analyzed tissues and condition factor of *Cathorops spixii* from the APA-CIP collected during the partially dry season (*P*), the dry season (*D*), and the rainy season (*R*). Sites with a *line* instead of a *boxplot* mean that no organisms were sampled. Data are presented as *boxes* with boundaries that indicate 25th and 75th percentiles; a *line* within the *box* marks the median value; *whiskers* below and above the *box* indicate 10th and 90th percentiles; the outliers are presented with *black dots*. The use of different *letters* above the data indicates significant differences during the same season (*p*=0.05)

(P4, P5, and P6) during the dry season and decreased during the rainy season. The opposite was found in analyses of Co and Ni levels in specimens from the station closest to the city (P4): levels were higher during the rainy season. A similar pattern was found in the analyses of metals in liver tissue: in general, metal concentrations in the dry season tended to be elevated in stations closer to the outskirts of the city (P5 and P6), although the station closest to the city (P4) showed higher levels of As, Cu, Co, and Cd during the rainy season compared to the dry season. In analyses of muscle and liver tissues from specimens collected closer to the RIR, higher metal concentrations (Mn, Zn, Co, Cr, Ni) were found during the rainy season than during the dry season (Fig. 4a–d). Exceptions were only Cd in muscle, and

Cu and As in the liver, of which higher concentrations were found during the dry season.

Levels of cadmium (annual means in sampling stations ranging from <0.11 to 1.78 mg kg^{-1} w.w.), lead (from 3.80 to 6.94 mg kg^{-1} w.w.), and total arsenic (from 0.0004 to 1.45 mg kg^{-1} w.w.) in the muscle were, in some instances, higher than the levels set by Mercosul (2011) and the Brazilian Sanitary Vigilance Authority (ANVISA 2013) (Brazil 2012) (both established a range from 0.05 to 0.3 mg kg^{-1} w.w. for Cd, 0.3 mg kg^{-1} w.w. for Pb, and 1.0 mg kg^{-1} w.w. for total As) as well as FAO/WHO (2014) (1.0, 2.0, and 1.0 mg kg^{-1} w.w. for Cd, Pb, and total As, respectively) and EC (2002, 2006) (Cd=0.1 to 0.30 mg kg^{-1} w.w.; Pb=0.30 mg kg^{-1} w.w.).

Fig. 4 a–d Metal body burdens
(muscle and liver) from
Cathorops spixii sampled in the
APA-CIP during both the dry
season and the rainy season. Data
were transformed (square root)
for better visualization
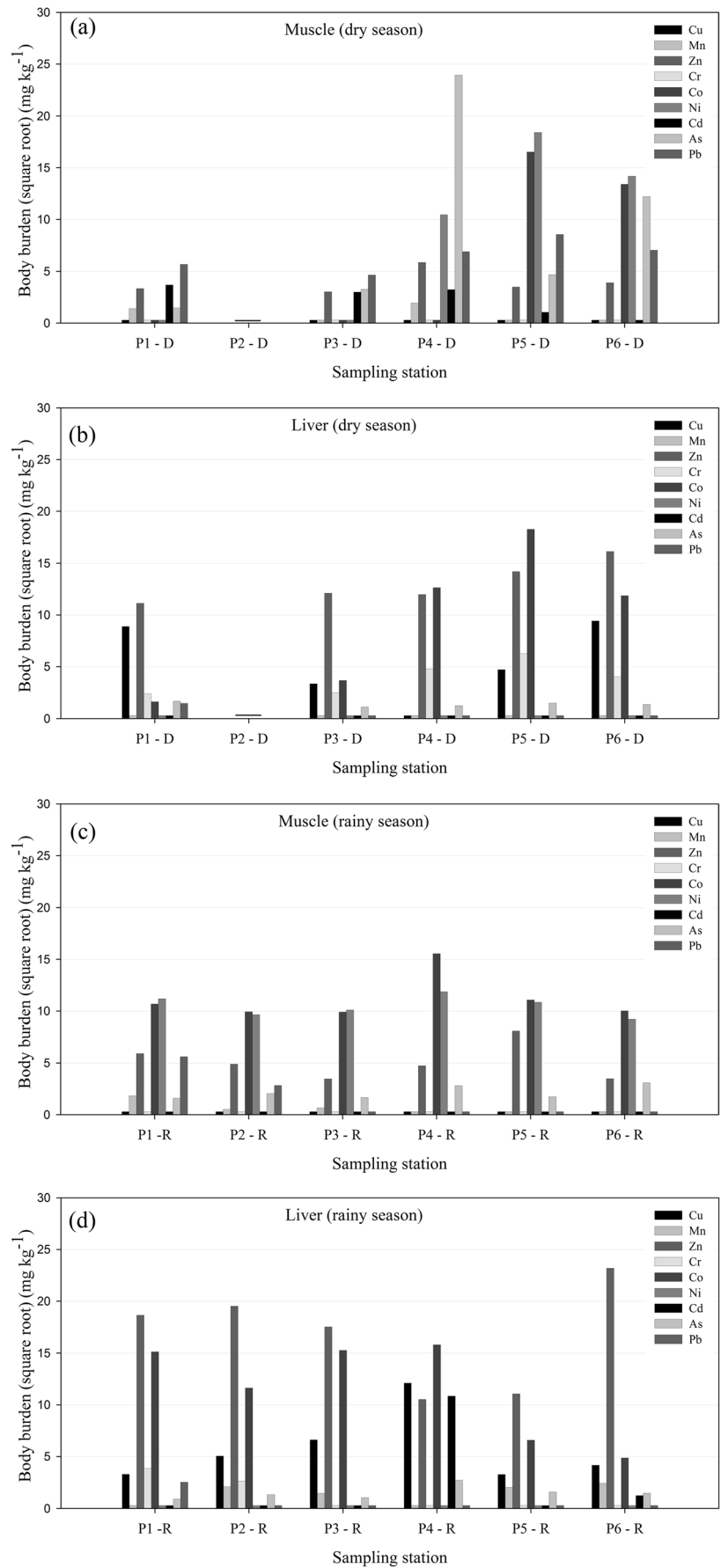
Total PAH metabolites in bile also showed a seasonal trend, with higher concentrations reported during the rainy season ($p<0.05$, ANOVA) (Fig. 5) for stations under the influence of both the river and the city. During the dry season, there was an increase in total PAH metabolites in bile in specimens from P3 and P4 compared to the other sampling stations, whereas during the rainy season, specimens from P1 and P4 presented higher levels.

The BRI values of each site at each season are presented in Fig. 6. Although the final classification was "no or slight alteration from normal responses" for most sites (following classification provided by Hagger et al. 2008) (exceptions were P1 and P4 during rainy season, both showing "moderate alteration"), the values show a trend of presenting more severe responses at sites closer to P1 and P4 at all seasons.

Three different datasets were used to perform FA/PCA: samples from the (1) partially dry, (2) dry, and (3) rainy seasons. Total explained variance of each FA/PCA was 100 % for the partially dry season dataset, 87.9 % for the dry season dataset, and 93.4 % for the rainy season dataset.

Three factors were extracted from the original dataset of the partially dry season. The first factor (F1, which explained 62.2 % of the variance) (Table 1) reflected metals (Cd and As) found in muscle and associated with MTs in the gills and liver tissues, several antioxidant responses in different organs, and DNA damage in the kidneys. Factor score analysis showed that these associations were more relevant (i.e., presented higher scores) in specimens from stations under the influence of the urban area (P4 and P5) (Table 4). Also in F1, another group of associated variables (with negative values) included Pb in muscles, PAH metabolites in bile, MT levels in kidneys, genotoxicity in gills, and neurotoxicity

(AChE inhibition). This group of variables was found to have the highest factor score for P2, which is the site that is under the greatest influence of the RIR. The second factor (F2, 25.1 % of explained variance) related, in one group, metals in muscle (Pb and Zn) and antioxidant response in liver and kidney tissues (GPx) (with the highest factor score estimated for P4), and, in another group, levels of Mn were found to be related to MTs in the liver (highest score for P5). The third factor (F3, 12.7 % of explained variance) associated As, Mn, and Zn levels in muscle tissues, MTs, oxidative damage, and antioxidant response in the gills, in addition to an indication of antioxidant activity and genotoxicity in the liver. This factor shows the highest score for P5, followed by P2 (Table 4). Another group of variables associated with F3 included antioxidants and genotoxic responses in the gills, without, however, any relationship with the contaminants quantified in organisms. These associations are relevant for P3.

Three factors were extracted from the original dry season dataset (Table 2). In one group, F1 (37.8 % of explained variance) represented metal levels in muscles associated with antioxidant responses (GSH in the kidney, liver and gills, and GPx in the gills) (with highest score for P4). F1 also associated another group of variables: DNA damage and LPO in the gills, DNA damage in liver tissue, and levels of As and Cu in the liver (highest score for P1, followed by P6). F2 (35.0 %) (Table 2) associated metal levels in the liver (Cr, Co) and muscle (Pb, Ni, and Co) with increased MTs in gills and genotoxicity in liver and kidney tissues. These associations are highly relevant only in P5 (Table 4). Another group of variables showed a relationship between levels of PAH metabolites in bile and damage in liver cells (genotoxicity and lipid peroxidation) (Table 2). The estimated scores of this

Fig. 5 Total PAH metabolites in the bile of *Cathorops spixii* sampled in the APA-CIP during the partially dry season (*P*), the dry season (*D*), and the rainy season (*R*). Sites with a *line* instead of a *boxplot* means that no organisms were sampled. Data are presented as *boxes* with boundaries that indicate 25th and 75th percentiles; a *line* within the *box* marks the median value. The use of different *letters* above the data indicates significant differences during the same season (*p*=0.05)
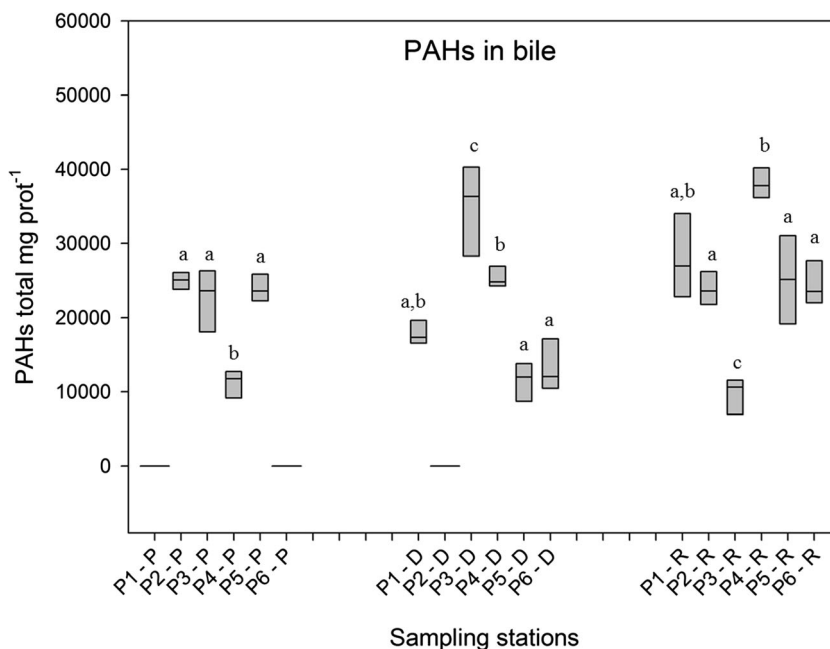
Fig. 6 Biomarker response index estimation for *Cathorops spixii* sampled in the APA-CIP during the partially dry season (*P*), the dry season (*D*), and the rainy season (*R*). Sites with a *line* instead of a *bar* mean that no organisms were sampled. Higher ranks mean lesser difference to baseline conditions
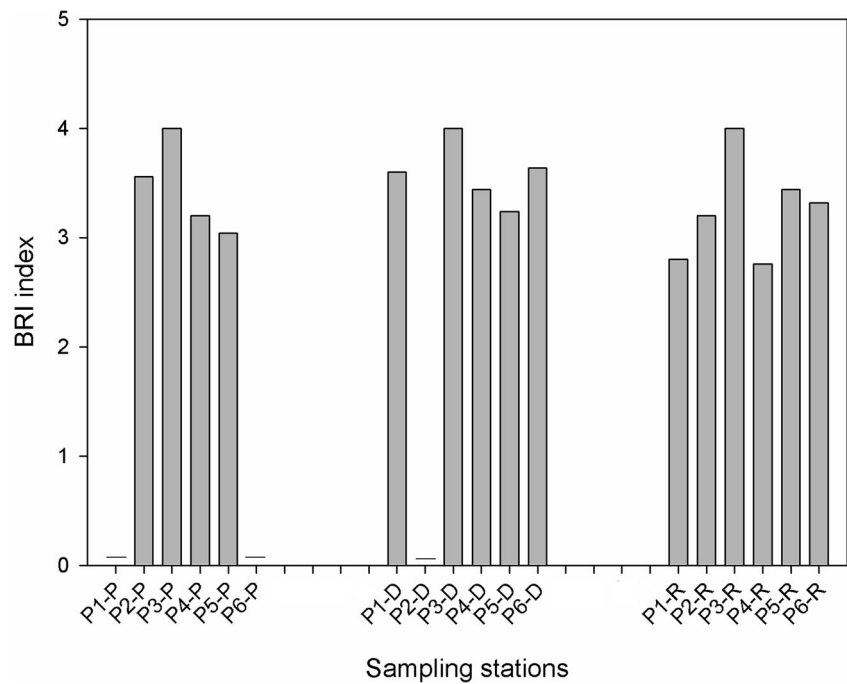
Table 1 Loadings of partially dry season after varimax rotation for the three factors obtained in the PCA[a] (the variance of the principal factors is given in percentage of the total variance in the original data matrix)

Factors	F1	F2	F3
% variance	62.21	25.10	12.69
MTs kidney	**−0.831**	−0.365	−0.419
GPx kidney	−0.154	**0.969**	−0.196
GST kidney	**0.988**	0.155	0.022
DNA damage kidney	**0.974**	−0.224	0.033
MTs liver	**0.837**	**−0.528**	0.143
GPx liver	0.498	**0.837**	0.226
GSH liver	**0.770**	0.285	**0.571**
DNA damage liver	0.440	0.168	**0.882**
MTs gill	**0.754**	0.278	**0.594**
GPx gill	**0.667**	−0.022	**0.745**
GSH gill	−0.127	0.144	**−0.981**
GST gill	**−0.781**	0.286	**−0.555**
LPO gill	0.118	−0.060	**0.991**
DNA damage gill	**−0.831**	0.061	**−0.553**
AChE	**−0.858**	−0.477	−0.189
PAHS in bile	**−0.500**	**−0.868**	−0.048
Mn muscle	0.310	**−0.708**	**0.634**
Zn muscle	0.027	**0.817**	**0.576**
Cd muscle	**0.919**	−0.197	0.341
As muscle	**0.782**	0.305	**0.543**
Pb muscle	**−0.564**	**0.766**	−0.309

[a] Only variables with loadings 0.5 (in bold text) were considered components of the factors

factor are especially high for stations P3 and P6 (Table 4). F3 (15.0 %) revealed strong associations between levels of some metals in the tissues (Zn in the liver, Ni and Co in muscles), oxidative damage in kidney cells, and a decreased condition factor. These responses were particularly relevant for specimens from P6 (Table 4). In other associations with F3, other contaminants in tissues (Pb and As in liver tissues and Cd in muscle tissues) were linked to MTs and genotoxic damage in the kidney, and these associations were relevant for specimens from P1.

Four factors were extracted by the FA/PCA applied to the rainy season dataset (Table 3). F1 (36.9 % of explained variance) reflected the association of metal burdens in the tissues (Pb in muscle and liver tissues, Mn in muscle tissues, and Cr in liver tissues), MTs in the liver and gills, and an indication of antioxidant response in gills (GST) and of neurotoxicity (AChE inhibition). This group of variables was found to have higher scores for the sampling stations under higher influence of the RIR (P1 and P2) (Table 4). Another group of variables was also related to F1: Mn in the liver (associated with decreased condition factor), the presence of DNA damage in the gills, and antioxidant responses in different organs (GSH in the gills and kidneys, GST in the liver and kidneys). These associations are relevant to P3, P4 (moderately), P5, and P6 (Table 4). F2 (27.1 %) reflects the associations between Cd, Co, Cu, and As levels in the liver, Ni and Co in muscle tissues, MTs in kidney tissues, lipid peroxidation in the gills, and PAH metabolites in bile (Table 3). Only P4 (the sampling station under highest influence of the urban area) was found to have a high score for this group of variables

Table 2 Loadings (correlation coefficients) of dry season after varimax rotation for the three factors obtained in the PCA[a] (the variance of the principal factors is given in percentage of the total variance in the original data matrix)

Factors	F1	F2	F3
% variance	37.782	35.024	15.048
MTs kidney	**−0.528**	−0.326	**−0.736**
GSH kidney	**0.868**	−0.429	−0.057
LPO kidney	−0.204	0.309	**0.833**
DNA damage kidney	0.075	**0.610**	**−0.730**
MTs liver	0.035	**−0.782**	−0.025
GPx liver	**−0.691**	0.013	**−0.573**
GSH liver	**0.985**	0.086	−0.119
GST liver	0.352	0.294	**0.881**
LPO liver	0.296	**−0.924**	0.131
DNA damage liver	**−0.842**	**0.519**	0.143
MTs gills	−0.181	**0.933**	−0.043
GPX gills	**0.960**	0.271	0.057
GSH gills	**0.911**	0.096	0.292
LPO gills	**−0.952**	0.257	−0.124
DNA damage gills	**−0.897**	0.136	−0.036
Condition factor	0.123	0.080	**0.876**
PAHS in bile	0.370	**−0.626**	−0.233
Mn muscle	**0.757**	−0.195	−0.490
Zn muscle	**0.923**	−0.069	0.078
Co muscle	−0.341	**0.748**	**0.541**
Ni muscle	−0.023	**0.815**	**0.528**
Cd muscle	0.285	−0.457	**−0.835**
As muscle	**0.920**	−0.150	0.146
Pb muscle	0.109	**0.861**	0.335
Cu liver	**−0.727**	−0.262	0.030
Zn liver	−0.327	0.203	**0.865**
Cr liver	0.280	**0.901**	0.307
Co liver	0.204	**0.879**	0.402
As liver	**−0.501**	0.318	**−0.571**
Pb liver	−0.396	−0.192	**−0.830**

[a] Only variables with loadings 0.5 (in bold text) were considered components of the factors

Table 3 Loadings (correlation coefficients) of rainy season after varimax rotation for the three factors obtained in the PCA[a] (the variance of the principal factors is given in percentage of the total variance in the original data matrix)

Factors	F1	F2	F3	F4
% variance	36.873	27.110	15.656	13.754
MTs kidney	0.432	**−0.663**	**0.523**	−0.106
GPx kidney	−0.492	−0.183	**−0.830**	−0.186
GSH kidney	**−0.503**	0.220	**−0.724**	−0.415
GST kidney	**−0.807**	**0.501**	−0.088	0.275
LPO kidney	−0.499	0.402	−0.269	**0.719**
DNA damage kidney	0.089	0.306	−0.033	**−0.734**
MTs liver	**0.991**	0.067	−0.009	0.118
GPx liver	−0.406	0.445	**0.756**	0.061
GSH liver	0.208	0.375	−0.319	**−0.805**
GST liver	**−0.783**	**0.589**	−0.178	0.018
LPO liver	0.213	−0.291	**0.920**	−0.102
MTs gill	**0.704**	−0.296	0.050	−0.076
GPX gill	−0.295	−0.052	−0.387	**−0.854**
GSH gill	**−0.692**	**0.568**	0.078	0.314
GST gill	**0.579**	−0.276	0.431	−0.187
LPO gill	0.243	**−0.911**	0.110	0.169
DNA damage gill	**−0.860**	−0.461	−0.077	0.194
AChE	**0.534**	0.329	0.050	**−0.777**
Condition factor	**−0.818**	0.230	**−0.510**	0.086
PAHS in bile	0.206	**−0.671**	**0.534**	0.464
Mn muscle	**0.925**	0.124	−0.069	−0.015
Zn muscle	0.129	0.128	−0.291	**0.932**
Co muscle	−0.118	**−0.952**	0.122	0.220
Ni muscle	0.294	**−0.756**	−0.235	0.445
As muscle	−0.446	−0.281	**0.835**	−0.136
Pb muscle	**0.984**	0.117	0.025	0.014
Cu liver	−0.245	**−0.957**	0.033	−0.128
Mn liver	**−0.523**	**0.677**	0.331	0.036
Zn liver	0.131	**0.620**	**0.531**	**−0.563**
Cr liver	**0.989**	0.128	0.028	−0.041
Co liver	0.424	**−0.632**	−0.431	−0.425
Cd liver	−0.168	**−0.966**	0.166	0.054
As liver	−0,329	**−0,890**	0,237	0,209
Pb liver	**0.919**	0.095	0.020	0.083

[a] Only variables with loadings 0.5 (in bold text) were considered components of the factors

(Table 4). Another group of variables associated with F2 included Mn and Zn and antioxidant responses (GST in liver and kidney tissues, and GSH in the gills); high scores were estimated mainly for stations under urban influence (P5 and P6), but also in the case of P3, which is under the influence of RIR. Factor 3 (15.7 %) revealed the association of Zn in the liver and As in muscle tissues, MTs in the kidneys, an indication of antioxidant response (GPx) and oxidative damage (LPO) in the liver, and PAH metabolites in bile. Higher F3 scores were estimated for P4 and P6. F4 (13.8 %) loadings showed an association of Zn in muscle and LPO in kidney, and, in another group, Zn in the liver

was found to be associated with antioxidant responses in the liver (GSH), with antioxidant responses in the gills (GPx), and with genotoxicity in kidney and neurotoxicity (AChE inhibition). Estimation of F4 scores showed that high levels of Zn in muscle associated with oxidative damage in the kidney are relevant for specimens from P5, while Zn in the liver associated with antioxidant responses and damage are relevant for specimens from P2 and P3.

Table 4 Factor scores estimated for each of the sampling stations in partially dry (P), dry (D), and rainy (R) seasons evaluated in the APA-CIP to the centroid of all cases for the original data

Station	F1	F2	F3	F4
Partially dry season				
P2P	−1.444	−0.001	0.406	–
P3P	0.106	−0.280	−1.469	–
P4P	0.602	1.341	0.297	–
P5P	0.735	−1.059	0.765	–
Dry season				
P1D	−0.709	−0.343	−1.484	–
P3D	−0.175	−0.714	0.115	–
P4D	1.739	−0.170	−0.099	–
P5D	−0.229	1.752	0.143	–
P6D	−0.625	−0.524	1.324	–
Rainy season				
P1R	1.876	0.195	0.040	0.169
P2R	0.370	0.152	0.039	−0.550
P3R	−0.603	0.296	−1.376	−1.183
P4R	−0.332	−1.977	0.316	0.115
P5R	−0.579	0.506	−0.633	1.775
P6R	−0.732	0.829	1.614	−0.326

Discussion

The current study shows that rainfall seasonality plays an important role in the estuarine pollution of the APA-CIP in terms of the risk of exposure for the biota. The evidence includes the association of metal burdens in muscle and liver tissues as well as the association of PAH levels in the bile of *C. spixii* with the biomarker responses in target tissues. The spatial distribution of biomarker responses and pollutants that bioaccumulated in fish tissues changed throughout the year, a result which indicates that levels and/or bioavailability of these contaminants change according to the season. Although the climate in the region is classified as tropical rainforest with high humidity throughout the year, the present study shows that the differences in rainfall between seasons are enough to influence the environmental quality of the APA-CIP.

During the dry season, biomarker responses and metal burdens in muscle and liver tissues were generally higher in the sections of the APA-CIP around the city of Cananéia. In addition, *C. spixii* from areas closer to the RIR also were found to possess increased molecular damages in lipids and DNA. Previous studies in coastal areas subjected to seasonal rainfall variation reported that, though the amount of contaminants may decrease with lesser continental inputs during the dry season, the concentration of contaminants in the aquatic system may increase if freshwater inflow is diminished (Sainz et al. 2004; Fianko et al. 2007; Costas et al. 2011).

During the rainy season, biomarker responses and metal burdens in liver and muscle tissues from fishes are more evident in fish from sampling stations under the highest influence of the RIR. The BRI also showed that the alterations are greater during the rainy season compared to the other seasons. Increased rainfall during the rainy season leads to a higher contribution of the RIR to the APA-CIP, which may remobilize metals from the sediment and/or carry metals from land-based sources into the estuary. Previous studies have shown the major role played by rainstorms in the release of contaminants from surface soils into the RIR and its tributaries within the RIR basin (Corsi and Landim 2003; Cunha et al. 2007; Costa et al. 2009; Abessa et al. 2014).

In general, the levels of metals (Zn, Cu, Zn, Cd, and Pb) in the muscle and liver tissues found in the current study were higher than the levels previously reported for *C. spixii* collected in a sampling site that corresponds to the site P3 of current research (Azevedo et al. 2012). Exceptions were Cu in muscle, which was not detected in both studies, and Zn in liver, which was higher in the previous study (from 100.15 to 850.77 mg kg^{-1}) (Azevedo et al. 2012).

Although the RIR basin is a natural metal-rich geological area, evidence suggests that the increased levels of metals in fish tissues observed in this study were due to human activities in the river basin. High levels of metals (Pb, Zn, Cu, Cr, As) recorded in river waters, bottom sediments, and suspended sediments were largely linked to mining activities in the river headwaters (Eysink et al. 1988; Corsi and Landim 2003; Moraes 2004; Guimarães and Sígolo 2008). In the estuary, metals in sediments have been generally found only at moderate levels (Mahiques et al. 2009; Azevedo et al. 2011; Cruz et al. 2014); however, these values have increased substantially since the opening of the Valo Grande channel and even more so after industrial mining activities began (Mahiques et al. 2009). These reports show that metals in the APA-CIP are largely from anthropogenic sources.

Our results suggest the influence of rainfall seasonality on the bioaccumulation of metals and bioavailability of PAHs, though some physiological disturbances may also indicate that some changes are due to seasonal differences. Some DNA damages in fishes exposed to low temperatures may be explained by decreased protein synthesis, which results in less efficient DNA repair mechanisms (Benincá et al. 2012; Pellacani et al. 2006; Buschini et al. 2003). Although the austral winter presented lower water temperatures (21 °C) if compared to the rainy season (28 °C), this difference could not explain the effects on *C. spixii* described in the current study. In fact, it is more reasonable to assign DNA damages to pollutant exposure, as demonstrated above, than to suggest that lower temperatures alone could give rise to these effects on DNA.

Data integration using FA/PCA revealed that metal concentrations in the body of *C. spixii* are associated with antioxidant

responses, oxidative damage, neurotoxicity, and genotoxicity. Zn, As, and Mn followed by Pb, Co, and Cd were the contaminants that were most frequently associated with biomarker responses in *C. spixii*. Other metals, including Ni, Cu, and Cr, showed some association with biomarkers, though less frequently. In results that corroborate our study, a previous study showed that Pb and Cd levels in the muscle tissues of two catfish species (*C. spixii* and *Genidens genidens*) from the APA-CIP were comparable to a highly polluted estuary located within the Santos Estuarine System (Azevedo et al. 2012).

The induction of antioxidant enzymes present in different organs of *C. spixii* were associated with Zn, As, Mn, and Pb that had bioaccumulated in liver and muscle tissues, a finding which suggests both acute and chronic exposure. The relationship between metal exposure and antioxidant response has long been established (Livingstone 2001). Metals can stimulate the production of reactive oxygen species (ROS) through different pathways, such as NADPH oxidases, metal-catalyzed oxidation systems via Fenton reactions (Lushchak 2011; Sharma and Dietz 2009), or even the depletion of major antioxidants in the cell, particularly thiol-containing molecules (Atli and Canli 2010; Stohs et al. 2001). Consequently, increased ROS production triggers the cell defense mechanisms by increasing the activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), GST, and GPx, and also by increasing the expression of free-radical scavenger molecules (e.g., GSH). These responses can be used as biomarkers of a cellular pro-oxidant state.

This method has been validated for cases of waterborne exposure in both field and laboratory studies with fish, as reported by Nunes et al. (2014) in the gills of *Anguilla anguilla* exposed to Pb, Cu, and Cd; by Eroglu et al. (2014) in the liver of *Oreochromis niloticus* exposed to Cd, Cu, Cr, Pb, and Zn; and by Eyckmans et al. (2011), who described the induction of antioxidant responses in different freshwater fishes exposed to Cu. According to Fonseca et al. (2014), marine or estuarine fish species respond to waterborne or sediment exposure to metal, and metal was found to induce antioxidant responses in *Dicentrarchus labrax*, *Solea senegalensis*, and *Pomatoschistus microps*. Similar findings were reported Souza et al. (2013) in a study on *Centropomus paralellus* from two estuaries along the Brazilian coast.

In the current study, the induction of antioxidant mechanism in all of the tissues considered was most evident in the liver, followed by the gills and less frequently in the kidney. Previous studies on fishes also found that antioxidant responses in the liver are more responsive to pollution than in other organs, as described in a study on *Zacco platypus* after waterborne exposure to Cu (Kim et al. 2014), as well as in a study on *O. niloticus*, whose liver presented a higher sensitivity than its kidney (Atli and Canli 2010). The liver is a key organ in evaluating the effects of pollutants in fish (Bernet et al. 1999). Hepatocytes are known to exhibit a more efficient

antioxidant mechanism because the multiple functions increase the metabolism in the tissue and, consequently, function as a constant source of oxidant species (Gül et al. 2004; Avci et al. 2005).

In the gills of *C. spixii*, antioxidant activity was more responsive to metal bioaccumulation than it was in the kidney. Fish gills are areas of extensive epithelium that exhibit high permeability to ions per unit of surface area, and they are continuously ventilated to allow for the exchange of gases, ions, salts, and water with the environment (Hill et al. 2004). Therefore, this organ is in direct contact with contaminants present in the aquatic milieu, and it is the main organ for contaminant absorption (Sayeed et al. 2003). The current data show that antioxidant responses are associated with an increase of LPO in the gills, a finding which demonstrates the failure of these antioxidant responses to neutralize oxidative species. However, the prevalent pattern is one of increased gill damage (DNA damage and/or LPO) associated with decreased antioxidant activity in this organ, a result which clearly reflects the risk of exposure experienced by fishes living in the estuary around both the RIR and the diffuse and point sources located within the city of Cananéia. The susceptibility of the gills to pollutants, as described in the current study, means that there is a real risk of exposure for the local biota and an environmental health problem. Other studies have corroborated the same findings in the gills of other fish, including as *Solea solea* from the Tunisia coast (Jebali et al. 2013), *O. niloticus* from heavily contaminated sites in Egyptian lakes (Abdel-Moneim et al. 2012), and *A. anguilla* after exposure to metal-rich effluent from a bleached kraft pulp mill (Santos et al. 2004). The increased LPO in the gills suggests that the gills have a less developed antioxidant mechanism compared to the liver and the kidneys.

Current organ-specific responses support that organ-specific changes might be related to the exposure route as well the mode of entry and chemical uptake and bioaccumulation by the individuals (Ahmad et al. 2006). The results of FA/PCA suggested that the responses of antioxidant defenses and oxidative damage to metal exposure are different in the studied organs. In higher metal exposure (denoted by higher metal levels in *C. spixii* body), the liver showed increased antioxidant activity though less oxidative damage than the other organs; on the contrary, gill shows poorer association between antioxidant induction and metal loads in tissues, and a stronger association of metals and oxidative damage. In addition, antioxidant responses in gill in some instances (especially closer to contamination sources) are inhibited when oxidative damage is higher. Therefore, *C. spixii* describes that the liver shows more efficient antioxidant responses, and thus partially protects itself from oxidative damage, while the gills are more susceptible to oxidative damages. Meanwhile, the kidney did not represent a good target organ because fewer

associations of antioxidant responses, macromolecular damages, and metal body burdens were observed in FA/PCA. Although the current study showed that the biomarker responses in kidney of *C. spixii* were poorly associated to metals and As levels in tissues in this mildly contaminated estuarine area, it is known that fish kidney plays key homeostatic functions, and its antioxidant response is highly relevant to avoid a temporary or permanent derangement of homeostasis (Vieira et al. 2012).

The results regarding neurotoxicity showed that Mn and Pb in muscle tissues and Zn, Pb, and Cr in liver tissues are related with a decrease in AChE activity. Other researchers have highlighted the use of AChE activity in fish as a useful biomarker for studying the effect of toxic metals, such as neurotoxic chemicals, in the field (Payne et al. 1996; Fasulo et al. 2010). The current study found that Pb is most clearly associated with neurotoxicity. Pb is recognized as an environmentally toxic chemical that causes many biological consequences such as mutation, chromosome aberration, cancer, neurotoxicity, and birth defects (Hong et al. 2007; Nunes et al. 2014). Similar to the current study, previous studies have reported AChE inhibition in fishes exposed to Pb. The decrease in AChE activity has been attributed to the fact that metals can bind to the functional groups of the enzyme, an action which leads to enzyme activity inhibition and a consequent overstimulation of the acetylcholine (ACh) receptors (De Lima et al. 2013; Richetti et al. 2011). The physiological consequences include several adverse effects, such as movement impairment, respiratory failure (WHO/IPCS/INCHEM 1987), or even behavioral changes such as a loss of prey capture ability (Mager et al. 2010). Other results of ACh receptor overstimulation include an increase in peristaltic movement, muscle weakness, tremor, hypertension, and cardiovascular collapse (Boelsterli 2007).

The lowest levels of AChE activity were usually observed in fish collected in the areas around the RIR that contribute to metal exposure, including the area where mining activities were intense in the river headwaters for decades. The high quantity of metals reflects the fact that a large amount of contaminants have been introduced into the APA-CIP from land-based sources. Additionally, this hydrographical basin is an important agricultural area in Brazil, a factor which means that other contaminants could potentially affect AChE activity not measured in the present study. Two classes of pesticides in particular can affect AChE activity: organophosphorus compounds and carbamates, which are used as an insecticide and an herbicide, respectively (Payne et al. 1996). DDTs were also used in the region on a large scale until the early 1980s, and metabolites are still present (Yogui et al. 2003).

The increased levels of metallothionein-like proteins in different organs were associated with higher metal body burdens

in *C. spixii*. The metal scavenging role of MTs is well established, and the induction of their expression is a recognized biomarker of exposure to metals (Van der Oost et al. 2003; Viarengo et al. 2000; Stegeman et al. 1992). In the current study, the association between MT expression and metal bioaccumulation was more evident in the gills of specimens from sites closer to the city of Cananéia during dryer seasons and in those closer to the RIR during the rainy season. The data clearly show that the levels of MT expression are correlated with the levels of metal bioaccumulation, a result which corroborates the use of this biomarker in areas affected by metals. The higher metal body burdens in *C. spixii* associated with high levels of liver MTs in sites closer to the river during the rainy season may be explained by the release of metals that had accumulated in the sediment during the mining activities in the past. The explanation of the same result in specimens from sites closer to the city (P4 and P5) during the dry season could be the concentration of metals resulting from urban activities.

On the other hand, during the driest season, levels of metals in the organisms seem to decrease MT levels in the liver. The literature also reports decreased MT levels in fishes as a consequence of exposure to high metal concentrations (Carvalho et al. 2012; Romeo et al. 1997). This finding may be explained by an increased demand of cysteine residues for GSH synthesis during metal detoxification (Romeo et al. 1997).

In the gills of *C. spixii*, the strong evidence of high levels of MTs with metal bioaccumulation and lipid peroxidation suggests that this organ is an important target for metals in the APA-CIP. These findings show that metals are relevant contaminants in the estuary and that the activities along the river basin and in the cities located on the coast are important sources of pollutants. Therefore, from these results, and in light of a previous study showing high levels of metals (Pb, Zn, Cu, and Cr) in suspended sediments at the upper RIR as well as at its mouth (Guimarães and Sígolo 2008), it is possible to argue that metal-associated suspended particles may be an important uptake route of these contaminants for fishes in the protected area.

MTs behavior in both the liver and the gills, which is associated with metal body burdens, is in accordance with the other biomarkers in that it reflects two main sources of pollution in the APA-CIP: the city of Cananéia and the Ribeira do Iguape River. Additionally, the seasons also sometimes play an important role in the bioaccumulation of metals, depending on the site studied. The current findings corroborate the study by Cruz et al. (2014) in which the sediment quality of the APA-CIP was assessed using ecotoxicological tools. The study concluded that the contamination from both the RIR and urban areas (the city of Cananéia) are relevant sources of pollution in the APA-CIP.

Although metals may be the main class of contaminants affecting fish health in the APA-CIP, the presence of PAHs

in the bile of *C. spixii* indicates that fishes living in the estuary were exposed to PAHs. PAH metabolites in fish bile are useful for indicating chronic exposure to pyrogenic or petrogenic PAHs (Oliveira Ribeiro et al. 2005; Osório et al. 2014). The increase of LPO in the liver and gills and the genotoxicity in the gills were associated with high levels of PAHs in specimens from the estuary. However, these associations were more discreet than the associations between metals and damages in macromolecules. The increased levels of PAH metabolites in bile were found in individuals from the studied sites closer to the city of Cananéia (sites 4, 5, and 6). This finding strongly suggests that PAHs from urban sources, when combined with metals, lead to LPO increases and DNA damage in the liver of *C. spixii* specimens living in the APA-CIP. Sources of PAH in this area include nautical structures (marinas, decks, nautical garages, and small docks), ferry boat stations, a nautical gas station, municipal sewage discharges, and urban drainage (Cruz et al. 2014). PAHs are of concern in MPAs since they are potentially carcinogenic (Shailaja and D'Silva 2003) and may induce hepatic lesions, physiological disorders, and biochemical disorders in fish (Oliveira Ribeiro et al. 2005).

The BRI ranks showed that the greater alteration in biomarkers responses (i.e., smaller rank values) occurred in the sites P1 and P4, during the rainy season. During the partially dry and dry seasons, although there is a trend of increasing alteration in the sites near the RIR mouth and the Cananéia City, only minor differences from the reference site (P3) can be observed. It provided a classification of "no or slight alteration from normal responses" for almost all sites (following classification provided by Hagger et al. 2008). Thus, although the BRI is interesting to assist the interpretation of multiple biomarkers data for assessing environmental conditions, the index was not very sensitive and such situation may lead to a false-negative judgment.

The biological effects seen in fish from the estuary after natural exposure to a diversity of pollutants confirm the potential risk of exposure as described previously by Rodrigues et al. (2013) and Araujo et al. (2013). In the APA-CIP, the present study shows that, in addition to the pollution from RIR inputs, the city located within the Environmental Protected Area is another important pollution source that affects this important ecosystem.

Water chemistry and microbiological parameters are the water quality indicators recommended by the International Union for Conservation of Nature and Natural Resources (IUCN) for pollution assessment in MPAs (Pomeroy et al. 2005). Biological indicators are also recommended, but they are not specific to assess the health of fish exposed to contaminants. The results of the current study suggest that biomarker analyses in fish are useful tools for assessing effectiveness and for environmental quality monitoring of MPAs subjected to contamination pressure. This finding is important because they suggest that, even if contaminants in MPAs are at only moderate levels, they are still harmful to fishes in many instances. In these cases, shifting the assessment from agents (e.g., measuring contaminant levels) to targets (biota) can provide useful information for protective management.

It is important to evaluate sublethal responses combined with metal body burdens in tissues and PAHs in bile in order to assess how pollution affects the environment. This study adds to the knowledge on the effectiveness of MPA management. Such tools provide information for making sound decisions regarding environmental health status in vulnerable or valuable marine areas, and they support the development of MPAs and policies for them.

Conclusions

The current study shows that a set of biomarker analyses using different fish organs is a useful tool for assessing chemical pollution in an MPA. This is the first study on MPAs in which both biomarker responses and metal body burdens, or PAHs, in *C. spixii* reflected seasonal variation. During the dry season, antioxidant responses, LPO, genotoxicity, and MTs, combined with metal body burdens, clearly showed that fishes were affected in areas under the influence of the city of Cananéia (P4, P5, and P6); during the rainy season, the evidence is clearly present in sampling stations under the highest influence of the RIR (P1, P2, P3). In addition, the current study shows that PAHs from the city may have been causing LPO as well as DNA damages. The studies of AChE activity reinforce these data and include a new finding: lead may be the most relevant neurotoxicity-causing metal in *C. spixii*. Liver tissue was more responsive in terms of antioxidant responses, whereas the gills were found to be more responsive to biomarkers of effects. The BRI ranks showed that the greater alteration in biomarkers responses (i.e., smaller rank values) occurred in the sites P1 and P4, during the rainy season. However, BRI only showed minor alterations in almost all sites, which may lead to false-negative judgments. Finally, the use of sensitive biological tools to assess environmental quality provides useful information for the sound management of a valuable marine protected area.

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