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Biomarkers in croakers *Micropogonias furnieri* (Teleostei: Sciaenidae) from polluted and non-polluted areas from the Patos Lagoon estuary (Southern Brazil): Evidences of genotoxic and immunological effects

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Abstract

Biomarkers of exposure and effect of pollutants were analyzed in croakers *Micropogonias furnieri* (Teleostei: Sciaenidae) captured in winter and summer in a polluted and in a non-polluted site at the Patos Lagoon estuary (Southern Brazil). Catalase and glutathione *S*-transferase activities (exposure biomarkers) and lipid peroxidation (effect biomarker) were analyzed in liver samples. Other two effect biomarkers were also studied: blood cells DNA damage (through comet assay and micronucleus test) and respiratory burst measurements. In a broad view, results point to an important seasonal variation of the biochemical biomarkers analyzed. However, data obtained clearly indicate that croakers collected in winter at the polluted site were subjected to a level of clastogenic agents sufficient to generate irreversible genetic damages (mutations) and impair the fish immune system.

Keywords: Aquatic pollution; Biomarkers; Croaker; DNA damage; Estuaries; Immune responses

1. Introduction

Estuaries are important sinks of pollutants derived from anthropogenic activities. Fish inhabiting these areas have been proposed as sentinels for pollution monitoring through assessment of sensitive biomarkers. Biomarkers can be defined as a change in biological response, ranging from molecular through behavioral changes, which can be related to exposure or effects of environmental contaminants (Depledge et al., 1995). Exposure biomarkers can be defined as any xenobiotic, its metabolite or the interaction product between the xenobiotic and a molecule or cell that is measured in organisms or in subunits of them

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(Depledge et al., 1995; López-Barea and Pueyo, 1998). Effect biomarkers represent any biochemical, physiological, behavioral (or other) alteration that can modify the well-being of an organism. Several molecular and cellular components in different fish species have been used as exposure and effect biomarkers, including biochemical, immunological and genetic parameters (van der Oost et al., 2003).

In the present study, the croaker *Micropogonias furnieri* (Teleostei: Sciaenidae) was used as sentinel of aquatic pollution, since this species is estuarine-dependent, being restricted to the Patos Lagoon estuary (Southern Brazil) during its juvenile phase (up to 16 cm total length) (Gonçalves et al., 1999). Fish larger than 16 cm perform a reproductive migration out of the estuary. After this, they occasionally use the estuary for feeding. The Patos Lagoon estuary is characterized by semi-enclosed bays of shallow waters. Some of these bays have been reported to be highly polluted, such as the "Mangueira Inlet" (Almeida et al., 1993). Baumgarten and Niencheski (1998) reported that sediment collected in this region is enriched with copper, lead and zinc. Also, previous studies from Geracitano et al. (2004a,b) showed that the estuarine polychaeta *Laeonereis acuta* from the "Mangueira Inlet" had higher antioxidant enzyme activity than that collected at a reference site, the "Justino Inlet". Other non-polluted areas are also found in the Patos Lagoon estuary. An example is the "Arraial Inlet", which is part of the Atlantic Forest Biosphere Reserve (Marcuzzo et al., 1998), and is considered a reference site.

The aim of the present study was to investigate if aquatic pollutants present in the "Mangueira Inlet" are generating biological responses by comparing croakers from this site with those collected in the "Arrail Inlet", a non-polluted site. To accomplish this objective, exposure and effect biomarker responses were measured in tissue samples from croakers collected in these two regions, in winter and summer. This study is part of a large-scale project conducted in several estuaries along the Brazilian coast in order to evaluate and detect pollution concerns in coastal and estuarine areas (www.mileniodomar.org.br).

2. Material and methods

Fish sampling was performed in August 2003 (winter) and February 2004 (summer) in two sites of the Patos Lagoon estuary (Southern Brazil). The first one is located close to the "Marinheiros Island" in a region called "Arraial Inlet" (32°02′S–52°12′W), which was considered the non-polluted site (reference site). The second site is located at the "Mangueira Inlet" (32°05′S–52°07′W), which was previously characterized as a polluted site (Fig. 1). Fish collection was performed for 15 min using a trawl-net. Fish between 10 and 20 cm were anaesthetized with benzocaine (50 ppt) and weighed. Blood samples were taken from the caudal artery with heparinized syringes with needles 27/7. Samples were immediately used for comet assay and micronucleus test, as described below. Liver, spleen and anterior kidney were immediately dissected. Livers were frozen in liquid nitrogen and then stored at −80 °C until enzyme assays and metallothionein-like proteins determination, as described below. Spleen and anterior kidney were collected, placed in 10 mL of Hank's balanced salt solution (HBSS: 200 mM NaCl, 1 mM KCl, 0.2 mM Na_2HPO_4, 0.08 mM KH_2PO_4, 1.2 mM Glucose, 100 U/mL Sodium Heparin, 100 U/mL Penicillin and 100 μg/mL Streptomycin; pH 7.4) and maintained on ice until leukocyte respiratory burst measurements, as described below.

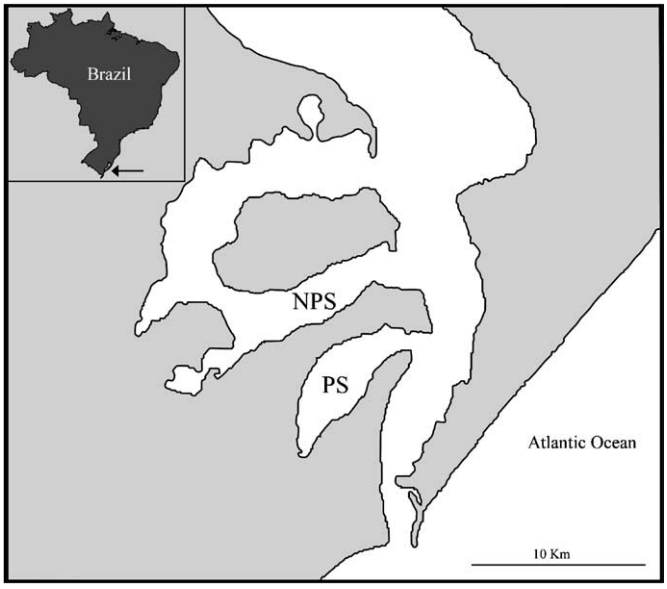

Fig. 1. Sites of *Micropogonias furnieri* (Teleostei: Scianidae) collection at the Patos Lagoon estuary (Southern Brazil). Non-polluted site (NPS) at "Arraial Inlet" (32°02′S–52°12′W) and polluted site (PS) at "Mangueira Inlet" (32°05′S–52°07′W).

Water chemistry parameters (salinity, pH, and temperature) were measured directly in the field.

2.1. Exposure biomarkers

To determine catalase (CAT) and glutathione-*S*-transferase (GST) activities, liver samples were processed according to previously established protocols with minor modifications (Livingstone, 1988). They were homogenized (1:5 w/v) in cold (4 °C) buffer solution containing 20 mM Tris–Base, 1 mM EDTA, 1 mM dithiothreitol (Sigma, St. Louis, MO), 500 mM sucrose, 150 mM KCl, 0.1 mM phenylmethylsulphonyl fluoride (PMFS, Sigma), with pH adjusted to 7.60. Homogenates were then centrifuged at $9000 \times g$, for 30 min at 4 °C. The supernatant of each sample was collected and stored at −20 °C, for no longer than a week, until analysis.

CAT activity was determined following the method described by Beutler (1975), which measure the rate of enzymatic decomposition of H_2O_2 (Merck, Darmstadt, Germany) as absorbance decrements at 240 nm. Enzyme activity was expressed in CAT units, where one unit is

the amount of enzyme needed to hydrolyze 1 μmol of H_2O_2/min/mg protein, at 30 °C and pH 8.00.

GST activity was assessed following the method described by Habig and Jakoby (1981). This method is based on the conjugation of 1 mM glutathione (Sigma) with 1 mM of 1-chloro-2,4-dinitro-benzene (CDNB; Sigma). Enzyme activity was measured as absorbance increments at 340 nm and was expressed in GST units, where one unit is the amount of enzyme necessary to conjugate 1 μmol de CDNB/min/mg protein, at 25 °C and pH 7.00.

Total protein content in homogenates was measured using a commercial kit (Doles Reagentes Ltda., Goiânia, Brazil), which is based on the Biuret protein assay. Both enzymatic and protein content determinations were performed at least in duplicate.

Metallothionein-like proteins (MT) were determined employing the method described by Viarengo et al. (1997). Livers were homogenized in a cold buffer solution containing sucrose (500 mM), Tris–HCl (20 mM), PMFS (0.5 mM) and β-mercaptoethanol (0.01%) as reducing agent. The pH was adjusted to 8.60. MT content was estimated spectrophotometrically (412 nm) using 5,50-dithiobis(2-nitrobenzoic acid) (DTNB 0.43 mM; from Sigma). Different glutathione (GSH) concentrations ranging from 0 to 500 μM were employed as standards. MT concentration was expressed in terms of GSH equivalents (μmol GSH/g of wet tissue).

2.2. Effect biomarkers

Lipid peroxidation (LPO) was determined in liver samples, according to Hermes-Lima et al. (1995). The method is based on the oxidation of Fe^{2+} by lipid hydroperoxides (FOX reactive substances) at acid pH in the presence of the Fe^{3+}-complexing dye, xylenol orange (Sigma). Samples were homogenized (1:15 w/v) in 100% cold (4 °C) methanol. The homogenate was then centrifuged at $1000 \times g$, for 10 min at 4 °C. The supernatant was collected and used for LPO determination (580 nm). Cumene hydroperoxide (CHP; Sigma) was employed as standard.

DNA damage was assessed through two tests: comet assay that detect DNA double or single strand breaks which can be repaired; and micronucleus test that detect irreversible genetic damage. Comet assay was performed as described by Singh et al. (1988) and Tice et al. (2000) with some modifications. Fully frosted microscope slides were coated with 300 μL 0.65% normal melting point agarose (NMPA; Gibco BRL) in TAE buffer (40 mM Tris–Acetate, 1 mM EDTA). Croaker blood samples were diluted (5:1000 v/v) in phosphate buffer saline (PBS) and 20 μL of cell suspension were mixed with 150 μl of 0.65% low melting point agarose (LMPA; Gibco BRL) prepared with Kenny's salt solution (400 mM NaCl, 9 mM KCl, 0.7 mM K_2HPO_4, 2 mM $NaHCO_3$, pH 7.5) at 30 °C (Steinert, 1995). Slides were immersed in freshly made lysing solution (10% DMSO, 1% Triton X-100, 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium sarcosinate; pH 10) overnight at 4 °C. To allow DNA to unwind, slides were placed in an electrophoresis buffer (10 N NaOH, 200 mM EDTA; pH between 12 and 13) for 15 min. Electrophoresis was carried out at 25 V and 300 mA for 20 min. After electrophoresis, slides were washed three times with neutralizing solution (0.4 M Tris, pH 7.5) and stained with 70 μL of ethidium bromide (20 μg/mL). The presence of comets was examined using a Zeiss axioplan fluorescent microscope (400X). DNA migration was visually determined in 100 cells per croaker. Comets were classified into five different groups: 0 for intact cells; 1, 2 and 3 for intermediary levels of breaks; and 4 for maximum damage. Results were expressed as scores, where 0 represents absence of damage and 400 indicates the highest damage registered in the 100 cells analyzed.

Micronucleus test was performed as described by Hooftman and de Raat (1982). A drop of croaker blood was smeared on microscope slides and air-dried. After fixation with methanol for 10 min, slides were stained with 5% Giemsa (Merck) in phosphate buffer (60 mM KH_2 PO_4 and 60 mM Na_2HPO; pH 6.8) for 20 min, washed with distilled water and air-dried. The relative frequency of micronucleated cells was evaluated under light microscope (1000*x* magnification) by scoring an average of 2000 mononucleated erythrocytes per slide.

For respiratory burst measurements, head–kidney and spleen leukocytes were isolated following procedures described by Chung and Secombes (1988). Head–kidneys and spleens were pushed through a 100 μm nylon mesh with HBSS. The cell suspension obtained was centrifuged at $400 \times g$, for 10 min. The pellet was resuspended in 2 mL of HBSS and centrifuged again at $600 \times g$ for 30 min in a 34–51% Percoll® gradient. Isolated leucocytes were then collected, resuspended in 5 mL of HBSS and centrifuged at $400 \times g$ for 10 min. The pellet was resuspended in 2 mL of Roswell Park Memorial Institute-1640 (RPMI-1640, Gibco), supplemented with penicillin (140 IU/mL), streptomycin (58 μg/mL), gentamicin (10 μg/mL), sodium bicarbonate (0.75 mg/mL), HEPES solution 1 M (0.02 mL/mL), heparin sodium (50 IU/mL) and heat-inactivated (37 °C for 30 min) fetal bovine serum (0.01 mL/mL). Leucocytes were counted in a Neubauer hemocytometer and the cell suspension was adjusted to 10×10^6 cells/mL. Cell viability was greater than 95%, as determined by the Trypan blue exclusion test. The phagocyte oxidative burst assay (nitroblue tetrazolium reduction) was performed according procedures described by Rice et al. (1996). In a microplate, 100 μL of cell suspension, 30 μL of fetal bovine serum, 140 μL of a nitroblue tetrazolium (NBT) solution (179 μg/mL) made with phosphate buffered saline solution (PBS) and 30 μL of phorbol myristate acetate (10^{-5} M) were added for the stimulated assay. For the non-stimulated assay, phorbol myristate acetate was replaced by PBS. Each sample was analyzed in triplicate. The microplate was then incubated for 30 min at 25 °C in a wet chamber with occasional shaking and centrifuged at $200 \times g$ for 10 min. Pellets obtained were resuspended in 250 μL of

PBS after centrifugation at $200 \times g$ for 10 min, and fixed with 100 μL of 100% methanol for 1 min. In each well, 150 μL of 70% methanol were added and the microplate was centrifuged at $400 \times g$ for 5 min. Supernatants were removed and the microplate was air-dried overnight at 25 °C. Pellets were resuspended in 20 μL of a 0.1% Triton X 100 solution. After 30 min, they were solubilized in 140 mL of a 2 M KOH solution, and 120 mL of dimethylsulfoxide (DMSO) were added and mixed by pipetting. The optical density was then measured in a spectrophotometer at 630 nm.

2.3. Statistical analysis

Data were analyzed through two-way ANCOVA (factors: season and sampling site; covariate: weight) followed by post-hoc mean comparisons test (Tukey's test). Analysis assumptions (normality and variance homogeneity) were previously checked (Zar, 1984). Significance level adopted was 95%. Results were expressed as mean ± standard error.

3. Results

Water chemistry (temperature, pH, salinity and dissolved oxygen) measured at the moment of fish collection is shown in Table 1. Values for all parameters measured were similar in the two sites for both seasons and had no relationship with any biomarker data.

Morphometric (length and weight) parameters of collected fish are listed in Table 2. At the non-polluted site, winter croakers were bigger ($P < 0.05$) than summer ones. They were also bigger than that from the polluted site in both seasons. However, in all cases weight (covariate) did not influence the statistical analysis, being verified a parallelism in the relationship between weight and both factors (season and sampling site).

Table 1
Environmental parameters registered in winter 2003 and summer 2004 at the polluted and non-polluted sites of the Patos Lagoon estuary (Southern Brazil)

Site	Season	Temperature (°C)	Salinity	DO (mg/L)	*n*
Non-polluted	Winter	13 ± 0	0.2 ± 0.1	9.2 ± 0.2	4
Non-polluted	Summer	24.3 ± 0.3	11.4 ± 3.8	8.5 ± 0.1	4
Polluted	Winter	9.5 ± 0.3	1.8 ± 0.2	10.1 ± 0.3	4
Polluted	Summer	24.3 ± 0.5	6.2 ± 1.7	7.6 ± 0.4	4

DO: dissolved oxygen; *n*: number of measurements. Values are expressed as means ± 1 standard error.

Table 2
Morphometric data of croakers (*Micropogonias furnieri*) collected in winter 2003 and summer 2004 in the polluted and non-polluted sites of the Patos Lagoon estuary (Southern Brazil)

Site	Season	Length (cm)	Weight (g)	*n*
Non-polluted	Winter	15.9 ± 0.7 a	42.0 ± 7.4 a	10
Non-polluted	Summer	13.7 ± 0.2 b	22.1 ± 1.3 b	10
Polluted	Winter	13.3 ± 0.5 b	22.5 ± 3.0 b	10
Polluted	Summer	13.9 ± 0.5 b	24.3 ± 1.9 b	10

Different letters indicate significant differences ($P < 0.05$) between mean values for fish collected in the different sites and seasons. *n*: number of croakers collected.

3.1. Exposure biomarkers

3.1.1. Enzyme (CAT and GST) activities and metallothionein-like proteins (MT) concentration

Summer croakers from the non-polluted site showed significantly ($P < 0.05$) higher CAT (90.8 ± 13.2 U CAT) and GST (0.65 ± 0.09 U GST) activities than those collected in winter at the same site (CAT = 40.0 ± 4.5 U CAT; GST = 0.38 ± 0.04 U GST). Summer croakers from the polluted site also showed significantly ($P < 0.05$) higher CAT (130.0 ± 12.0 U CAT) and GST (0.45 ± 0.06 U GST) activities than those collected in winter at the same site (CAT = 40.5 ± 4.0 U CAT; GST = 0.31 ± 0.02 U GST). In each season, no significant differences ($P > 0.05$) in CAT (Fig. 2a) and GST (Fig. 2b) activities were found

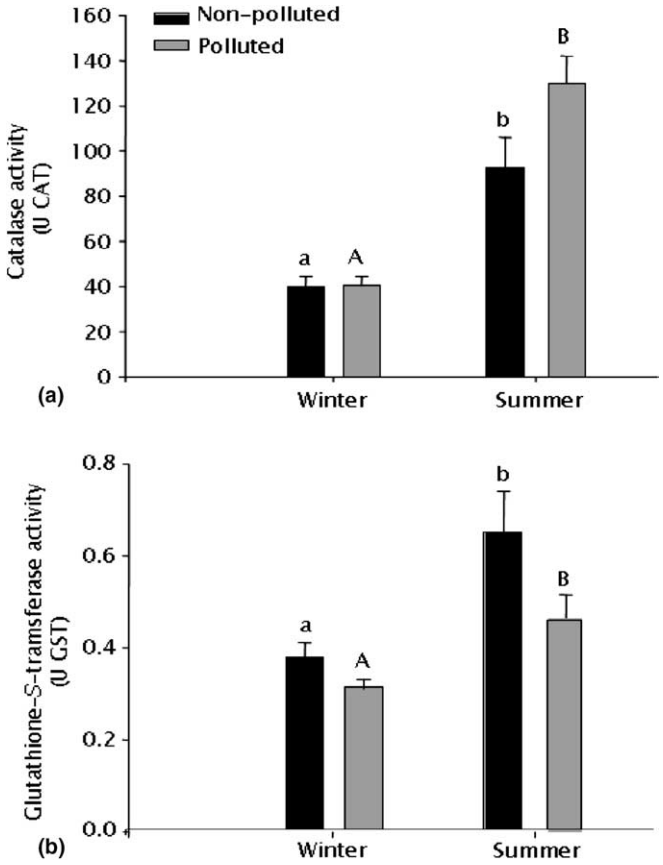

Fig. 2. Catalase (a) and glutathione-*S*-transferase (b) activity in croakers (*Micropogonias furnieri*) collected at the non-polluted and polluted sites in winter and summer. Different letters indicate seasonal significant differences ($P < 0.05$) for croakers from the non-polluted (lower case letters) and polluted (capital letters) sites. Significant differences ($P < 0.05$) between croakers from the non-polluted and the polluted site in the same season are indicated by an asterisk. Data are expressed as mean + 1 standard error.

between croakers from the polluted and non-polluted site. Also, no seasonal variations and significant differences ($P > 0.05$) were observed in the MT concentration of croakers from the non-polluted site (winter $= 2.90 \pm 0.47$ and summer $= 2.02 \pm 0.60$ μmol GSH/g of wet tissue) and the polluted site (winter $= 3.13 \pm 0.47$ and 2.53 ± 0.37 μmol GSH/g of wet tissue).

3.2. Effect biomarkers

3.2.1. Lipid peroxidation (LPO) content

Summer croakers from the non-polluted site showed significantly ($P < 0.05$) higher LPO content (3325.8 ± 438.0 nmol CHP/g tissue) than those collected in winter at the same site (981.6 ± 188.2 nmol CHP/g tissue). Croakers from the polluted site showed no significant ($P > 0.05$) difference in LPO content in summer (1250.5 ± 486.4 nmol CHP/g tissue) and winter (1124.0 ± 157.9 nmol CHP/g tissue). Significant difference between the two sites was only found in summer, when croakers from the non-polluted site showed higher ($P < 0.05$) LPO content than those from the polluted site (Fig. 3a).

3.2.2. Respiratory burst measurements

Nitroblue tetrazolium reduction was higher in croakers from the non-polluted site than in those from the polluted site. In both seasons, optical density was higher ($P < 0.05$) in croakers from the non-polluted site (winter $= 0.22 \pm 0.07$; summer $= 0.20 \pm 0.04$) than in those from the polluted site (winter $= 0.09 \pm 0.04$; summer $= 0.05 \pm 0.02$). No significant ($P > 0.05$) seasonal difference was observed in each sample site (Fig. 3b).

3.2.3. DNA damage

In winter, both biomarkers (comet assay, CA, Fig. 3c; and micronucleus test, MN, Fig. 3d) showed higher ($P < 0.05$) levels of DNA damage in croakers from the polluted site (CA $= 101.67 \pm 7.17$; MN $= 0.87 \pm 0.21$) than in those from the non-polluted site (CA $= 38.00 \pm 5.83$; MN $= 0.18 \pm 0.04$). In summer, there was no significant difference ($P > 0.05$) between croakers from the polluted site (CA $= 99.4 \pm 17.65$; MN $= 0.32 \pm 0.05$) and the non-polluted site (CA $= 57.71 \pm 11.85$; MN $= 0.26 \pm 0.06$), although CA results showed a tendency of higher values ($P = 0.052$) in croakers from the polluted site.

4. Discussion

Several field studies with different fish species have used the response of antioxidant and biotransformation enzymes as exposure biomarkers (van der Oost et al., 2003). In the present study, exposure biomarkers (CAT and GST) showed similar patterns in croakers from both polluted and non-polluted sites, but a clear seasonal

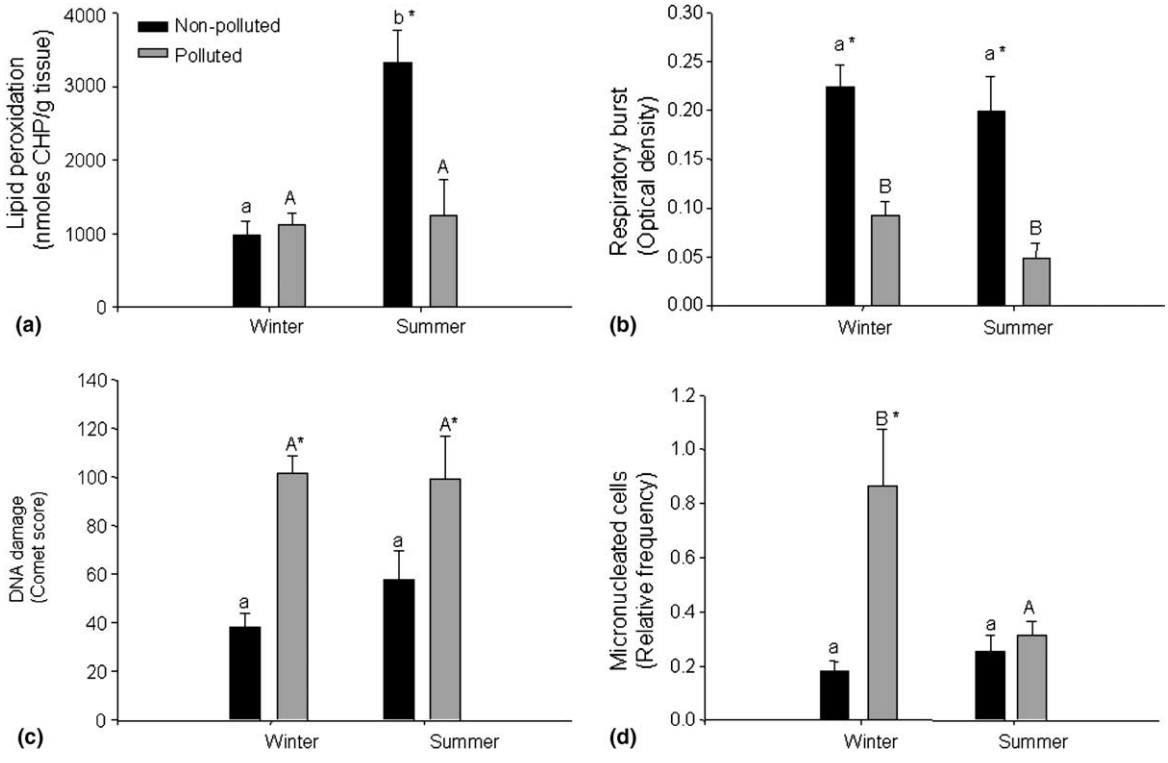

Fig. 3. Lipid peroxidation (a), respiratory burst (b), comet scores (c) relative frequency of micronucleated cells, (d) in croakers (*Micropogonias furnieri*) collected in the non-polluted and polluted sites in winter and summer. Different letters indicate seasonal significant differences ($P < 0.05$) for croakers from the non-polluted (lower case letters) and polluted (capital letters) sites. Significant differences ($P < 0.05$) between croakers from the non-polluted and the polluted site in the same season are indicated by an asterisk. Data are expressed as mean + 1 standard error.

variation was observed. Values were higher in the warmer season. Water chemistry parameters also showed an important seasonal variation, being similar in both studied sites (non-polluted and polluted). In a study with the fish *Geophagus brasiliensis* collected at a non-polluted site during spring and autumn, GST activity was also higher in the warmer period and CAT activity showed the same trend (Wilhelm Filho et al., 2001). Similar seasonal patterns of higher antioxidant and biotransformation enzymes activities in warmer seasons were also verified in other fish species (Ronisz et al., 1999). This response is probably related to the higher ambient temperature which can lead to an increase in oxygen consumption and therefore to an enhanced reactive oxygen species (ROS) generation. Seasonal adjustments in the antioxidant defense of thermoconformers, like most of fish and invertebrates, suggest that this mechanism is a common adaptation in these species (Wilhelm Filho et al., 2001).

The effect biomarkers (LPO content, DNA damage and respiratory burst measurement) showed different response patterns. LPO content in croakers from the non-polluted site showed a seasonal variation, being higher in summer. Polyunsaturated fatty acids (PUFA) from membrane phospholipids are the main target of ROS attack, leading to lipid peroxidation (Halliwell and Gutteridge, 1999). It is also known that membranes of cold-acclimated thermoconformers are enriched with PUFA, being more susceptible to suffer with oxidative stress (Parihar et al., 1996). On the other hand, croakers from the polluted site did not show any seasonal variation and had lower LPO values in summer than those from the non-polluted site, a priori, a non expected result. However, some authors have described the disruption of lipid metabolism due to PCBs exposure (Ferreira and Vale, 1998). Boutet et al. (2004) found an inhibition in the expression of the gene coding the enzyme Δ9 desaturase in oysters (*Crassostrea gigas*) exposed to hydrocarbons from 7 to 21 days. Desaturases are enzymes that catalyze the addition of double bonds in a fatty acyl chain, playing a critical role in PUFAs biosynthesis (Pereira et al., 2003). If pollutants are able to inactivate these enzymes, a possible interpretation to our results is that lipid peroxidation process could be reduced in croakers from the polluted site due to a lower content of PUFAs in the cell membranes of these fish. Thus, further studies analyzing the cell membrane fatty acid composition are needed to verify the lipid peroxidation potential of a specific tissue.

In the present study, DNA damage was assessed through two tests: the comet assay (CA), which detects DNA strand breaks that can be repaired, and the micronucleus test (MN), which assess mutational events. It is known that breaks detected by comet assay can be transiently present when cells repair lesions via base or nucleotide excision. Thus, a high level of breaks in the comet assay may indicate either high damage or an efficient repair process (Collins et al., 1997). Our results suggest that DNA damage found through comet assay in croakers collected in winter at the polluted site was not efficiently repaired. This statement is based on the fact that micronucleus test indicated higher levels of mutations in these fish. Some of these mutations can be related to non-repaired breaks. In contrast, the tendency of higher DNA damage values in summer croakers did not lead to a higher micronucleus frequency, probably because breaks detected by comet assay in this season were of repairing nature. It seems that winter croakers were exposed to some contaminant or to a mixture of them that inhibited or exhausted their DNA repair mechanisms, leading to permanent damage, as showed by the micronuclei data. It should be stressed that the DNA damage determined through CA and MN was paralleled by a lack of CAT and GST induction in fish collected at the polluted site in winter. Catalase activity is essential to promote the degradation of H_2O_2, a precursor of hydroxyl radical, a reactive oxygen species that induces DNA damage (Halliwell and Gutteridge, 1999). In this context, the lack of CAT response in fish collected in winter at the polluted site should be unable to reduce the levels of hydroxyl radical promoters. The negative correlation between antioxidant defense competence and DNA damage was previously verified in other aquatic organisms such as *Mytilus galloprovincialis*, where individuals with lower total antioxidant capacity also showed lower DNA integrity (Frenzilli et al., 2001). Several in situ studies have been demonstrated the occurrence of higher DNA damage in organisms collected from polluted areas, both using the comet assay (Flammarion et al., 2002; Winter et al., 2004) and the micronucleus test (Minissi et al., 1996; Bombail et al., 2001), pointing to their utility in biomonitoring programs. However, few of the cited previous studies have analyzed the response in terms of DNA damage in different seasons, an important point to be considered among the several factors that can lead to augmented damage, as registered in the present study. Further research should consider the kinetics of DNA repair to analyze the effects of pollutants on this parameter.

Results from respiratory burst assay in croakers collected at the two sites of the Patos Lagoon estuary indicate that NBT reduction was higher in croakers from the non-polluted site than in those from the polluted site. Since previous studies reported suppression of phagocytic function by environmental contaminants (Lutz and Wasowicz, 2003), our results suggest that croakers phagocytes are being exposed to sublethal concentrations of environmental contaminants in the Patos Lagoon estuary, leading to manifestations of immunosuppression. These lower non-specific immune responses can lead to opportunistic diseases, such as viral infections and infestation with parasites, as previously determined in the fish *Ammodytes hexapterus* after hydrocarbon exposure (Moles and Wade, 2001).

Based on results presented here, it is not possible to point out a single chemical in the Patos Lagoon estuary that is causing the alterations observed in the present study. However, they suggest that synergistic effects from a com-

bination of chemicals can be affecting immune response and also causing DNA damage in croakers collected at the polluted site. The metabolism of several pollutants generates ROS that can attack any cellular components as DNA, fatty acids, carbohydrates and proteins, leading to serious damages to cellular macromolecules (Livingstone, 2001). Data showed here indicate that winter croakers from the polluted site were subjected to a level of pollutants enough to impair fish immunological activity and also to overwhelm the DNA repair mechanisms, generating irreversible genetic damages (mutations). Previous studies of Geracitano et al. (2004a,b) reported augmented antioxidant responses in the estuarine polychaeta *Laeonereis acuta* collected at polluted regions from the Patos Lagoon estuary, suggesting the existence of pollutants that can generate ROS. However, contrary to results reported by Geracitano et al. (2004a), no significant differences in liver MT levels were found in croakers from the non-polluted and polluted sites. Thus, higher rates of ROS production could be deleterious for croakers since no responses of any important antioxidant defense, such as catalase or GST activity, which helps to eliminate the oxidative by-products (Leaver and George, 1998), was observed in croakers collected in the polluted site. Furthermore, the fact that the polluted site is characterized by higher levels of metals like copper and arsenium (Seeliger and Costa, 1998; Mirlean et al., 2003) and hydrocarbons (Medeiros et al., 2005) that can generate ROS, gives support to our hypothesis.

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