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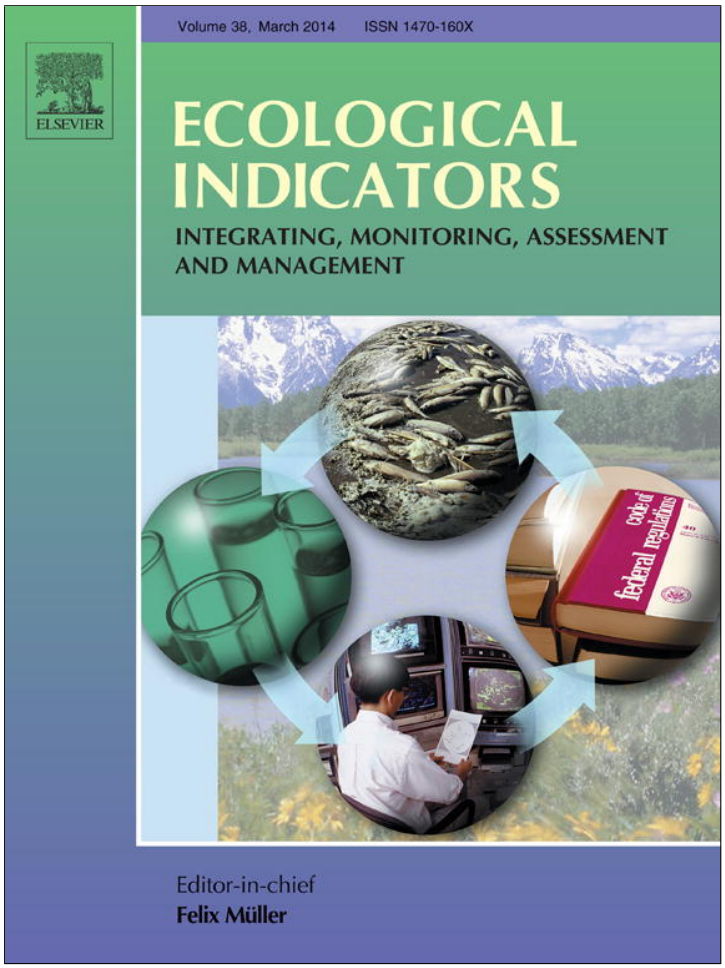

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Deleterious effects of wastewater on the health status of fish: A field caging study

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ABSTRACT

We carried out a caging field experiment to assess the potential effects of an untreated sewage effluent on the health status of *Prochilodus lineatus*. We analyzed multiple biomarker responses, which included morphological indices, biochemical and hematological parameters as well as oxidative stress markers. In addition, we investigated the energetic demand of that exposure. Our findings showed that fish caged at the effluent showed a differential physiologic profile, suggesting a strong impact on fish health. Particularly, mortality, monocytosis, transaminase increase, antioxidant enzyme activation, lipid oxidative damage in several tissues and hepatic and muscle glycogen depletion were observed. According to multivariate analysis, oxidative stress markers and metabolic parameters were key biomarkers to contribute in separating fish caged at effluent site from those caged at upstream and downstream sites. So, these biomarkers allied to a caging strategy are recommended for future environmental monitoring assessments.

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1. Introduction

Sewage effluents represent an important point source of water pollution since their discharges consisting of complex mixture of chemicals. Chemicals like metals, polycyclic aromatic hydrocarbons, pesticides, organotins, volatile organic compounds, chlorobenzenes, phthalates and alkylphenols have been reported in wastewater, as well as certain pharmaceuticals and hormones (Abessa et al., 2005; Gasperi et al., 2008; Bolong et al., 2009; Metcalfe et al., 2010).

Although chemical analyses are able to measure many of these compounds qualitatively and quantitatively, it is not viable to quantify all the pollutants that are potentially present in the sewage water. Furthermore, chemical analyses alone do not reveal the impact of chemical pollution on the aquatic environment because of potential synergistic/antagonistic effects of complex mixtures of chemical pollutants (Kerambrun et al., 2011). In this context, alternative monitoring methods involving biomarkers have been developed in order to provide a reliable assessment of the environmental quality (van der Oost et al., 1996). However, there is no single biomarker that can give a complete diagnosis of the effects of effluent exposure on organisms. Consequently, the use of a battery of complementary biomarkers is recommended to gain an understanding of how an organism responds to the total pollution load in an area (Lavado et al., 2006; Cazenave et al., 2009).

In this way, the use of biological markers in transplanted organisms is an integrative tool that measures the toxic effect of an effluent mixture as a whole. The development of caging field experiments are useful in effluent monitoring as they permit to assess the habitat quality in the outfall discharge area and the impact of contamination on biota. Furthermore, caging strategies are one of the techniques that integrate true ambient conditions over the chemical exposure and allow an interpretation of the exposure effects to complex mixtures (de la Torre et al., 2000). Thus, active biomonitoring using cages offer several advantages: the precise knowledge of the place and the precise duration of exposure, and the selection of a representative species and its particular developmental stage and genetic background (constancy of the test organism) (Oikari, 2006; Wepener, 2008). So, results from different sites are validly comparable.

In the present caging experiment the chosen species was *Prochilodus lineatus*, a neotropical fish representative of the water body of the region, with ecological relevance and economic importance. Besides, previous studies have demonstrated that *P. lineatus* gives a rapid response to an early exposure of various pollutants

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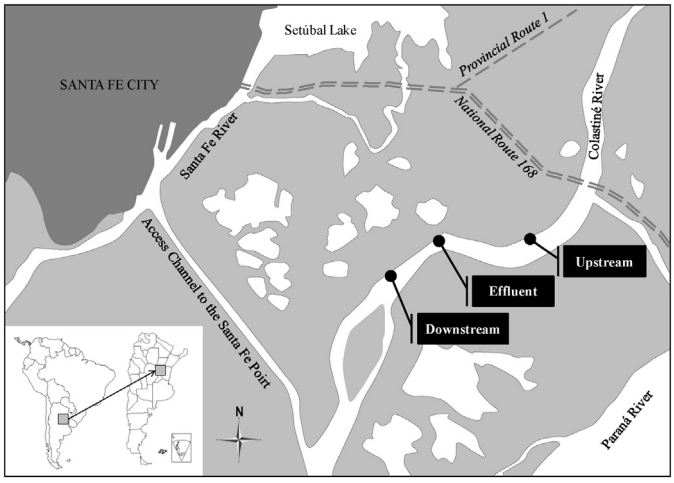

Fig. 1. Map showing study area and location of caging sites on the Colastiné River, Santa Fe, Argentina.

in both laboratory and field studies (Parma et al., 2007; Camargo and Martinez, 2006; Langiano and Martinez, 2008; Simonato et al., 2008; Cazenave et al., 2009; Lombardi et al., 2010; Bacchetta et al., 2011a; Paulino et al., 2012a,b; Troncoso et al., 2012; among others).

Effects of wastewater have been evaluated on individual analysis of serum chemistry (Bernet et al., 2001), hematology (Maceda-Veiga et al., 2010, 2013), oxidative stress (Almroth et al., 2008), some reproductive parameters (Akande et al., 2010; Galus et al., 2013), histopathology and infectious agents (Escher et al., 1999; Bernet et al., 2000; Fontaínhas-Fernandes et al., 2008). However, to obtain a holistic and integrative overview of how untreated sewage effluent exposure affects fish health we carried out a field caging experiment to analyze multiple biomarker responses, which included morphological indices, biochemical and hematological parameters as well as oxidative stress markers. Additionally, we investigated the energetic cost of that exposure.

2. Materials and methods

2.1. Study area and exposure sites

Colastine River, the main tributary of the Middle Parana River, is 35 km long, has a mean depth of 11 m, and its discharge at high water is about 2800 $m^3\ s^{-1}$ (Iriondo, 1975). This river supplies drinking water to Santa Fe city (more than 525,000 inhabitants). On the other hand, untreated domestic wastewater is discharged directly into the same watercourse.

In order to assess the effects of this wastewater effluent, fish were caged in the Colastiné River at the following three sites: at a reference site, located 2 km upstream from the sewage effluent (site upstream) (a natural area without having any known industrial and domestic sewage); at immediately (0.2 km; site effluent) and 2 km downstream (site downstream) from the sewage effluent site (Fig. 1). The experiment was carried out during the wet season in May 2011 (average monthly water level of 4.51 m).

2.2. Test organisms and experimental caging

Juveniles *P. lineatus* (three-month-old; $n = 60$) were obtained from a local hatchery at one week before the caging experiment, and held in two 500 L-tanks. Then, fish were transported from the laboratory to the exposure sites (in boat for <1 h) in large plastic bags (100 L) of oxygenated water.

Once at the field sites, fish were selected randomly and they were placed into the cages. We used polyethylene cages ($0.60\ m \times 0.30\ m \times 0.36\ m$, 65-$dm^3$), perforated with many holes to allow water circulation through the cage. Cages were completely immersed (depth ≤ 1.5 m) near the sediment and they were firmly anchored at the sites to prevent their displacement. At each site, two cages (separated by approximately 3 m) were paced for 96 h (10 individuals per cage).

During the field exposure, water quality was evaluated three times (at 0, 48 and 96 h) at each site exposure. We recorded in situ water temperature, dissolved oxygen, conductivity, pH and transparency. Additionally, 2 L of water samples were taken for each station and transported to the laboratory at 4 °C in clean plastic bottles. The following parameters were measured according to standard procedures (APHA and AWWA, 1998): chemical oxygen demand, nitrates, nitrites, ammonia, total phosphorus, calcium, magnesium, and hardness. Besides, water samples for bacteriological analyses were kept in sterilized recipients and then total and fecal coliforms were determined by the More Probable Number (MPN) method.

Following the exposure period, fish were retrieved and they were rapidly transported (in river water with aeration) back to the laboratory for sample processing.

2.3. Biomarkers

Prior to blood sampling and dissection, fish were anaesthetized in benzocaine as described by Parma de Croux (1990). Body weight (g) and total length (cm) were recorded for each individual. Blood was collected immediately from the caudal vessel, and plasma separated via centrifugation (at $1409 \times g$ for 10 min). The brain, liver, kidney, gill, intestine and muscle were dissected and quickly frozen in liquid nitrogen and subsequently stored at −80 °C until biochemical determinations. Before freezing, the wet weight of the liver was determined.

2.3.1. Condition indexes

Condition factor (CF) was calculated according to Goede and Barton (1990): $CF = BW/L^3 \times 100$, with BW = body weight (g), L = total length (cm). The liver somatic index (LSI) was calculated as: $LSI = LW/BW \times 100$, with LW = liver weight (g).

2.3.2. Hematology

Red blood cells (RBC) counts were performed with a Neubauer chamber. Hematocrit (Ht) values were determined by the micromethod using capillary tubes and centrifuged at $1409 \times g$ for 10 min. Hemoglobin concentration (Hb) was measured by the cyanomethaemoglobin method at 546 nm (Houston, 1990). Mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were calculated from primary indexes (Cazenave et al., 2005).

A drop of freshly collected blood was smeared on clean slides to estimate the total white blood cells (WBC) counts and for determination of leukocyte frequency according to Tavares-Dias and de Moraes (2007).

2.3.3. Transaminases and alkaline phosphatase

Liver and kidney enzyme extracts were prepared according to Bacchetta et al. (2011b). Aspartate aminotransferase (AST) (L-Aspartate-2-oxaloglutarate aminotransferase) and alanine aminotransferase (ALT) (L-Alanine-2-oxaloglutarate aminotransferase) activities were measured spectrophotometrically at 505 nm following the protocol described by Reitman and Frankel (1957). Alkaline phosphatase (ALP) (Orthophosphoric monoester phosphohydrolase) activity was determined colorimetrically using a commercial kit. Each sample was measured by triplicate and the

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enzymatic activity was calculated in terms of protein content (Bradford, 1976).

2.3.4. Oxidative stress

Oxidative stress in different tissues was assessed both by antioxidant enzyme activities and lipid peroxidation levels.

For enzyme extracts preparation, tissues were homogenized in an ice-cold 0.1 M sodium phosphate buffer, pH 6.5 containing 20% (v/v) glycerol, 1 mM EDTA and 1.4 mM dithioerythritol. The homogenates were centrifuged at 20,000 × *g* (4 °C) for 30 min, and the supernatant was collected and stored at −80 °C for enzyme measurement.

Enzyme activities were assayed spectrophotometrically. The activity of soluble glutathione *S*-transferase (GST) was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, according to Habig et al. (1974). Glutathione reductase activity (GR) was assayed according to Tanaka et al. (1994). The activity of glutathione peroxidase (GPx) was determined according to Drotar et al. (1985), using H_2O_2 as substrate. Catalase activity (CAT) was determined according to Beutler (1982). The enzymatic activity was calculated in terms of the sample protein content (Bradford, 1976), and is reported as nkat mg prot^{-1}. Each enzymatic assay was carried out by triplicate.

Lipid peroxidation (LPO) was determined by measuring the formation of thiobarbituric reactive substances (TBARS), according to Fatima et al. (2000). The rate LPO was measured spectrophotometrically at 535 nm and was expressed as nanomoles of TBARS formed per hour, per milligram of proteins (nmol TBARS mg prot^{-1}).

2.3.5. Energetic costs

In order to evaluate the consumption of energy associated to the effluent exposure, glycogen, lipid and protein content were measured in plasma, liver and muscle.

Glycogen was estimated according to Seifter et al. (1950). Briefly, 20 mg of hepatic and 60 mg of muscle tissues were treated with 1 ml KOH 30% and 0.5 ml KOH 60% at 100 °C. After alkaline tissue disruption, glycogen was precipitated by ethanol and glucose was determined using the anthrone reagent method. Lipid content was extracted using chloroform:methanol (2:1) by the method described by Folch et al. (1957) and total protein concentration in tissue was estimated according to Lowry et al. (1951) using bovine serum albumin as standard.

The quantitative determinations of glucose and protein plasma levels were performed with enzymatic colorimetric methods using the appropriate kits according to the protocols of the manufacturer (Winner Lab®). All biochemical analyses were measured in triplicate. Because blood volume was small, we could not get enough plasma for lipid assay.

2.4. Statistical analyses

Data were first tested for normality and homogeneity of variance using Shapiro Wilks and Levene's test, respectively. For statistical comparisons of data among 3 groups, 1-way ANOVA followed by Tukey's post-test was used for normally distributed data, and the Kruskal Wallis test for non-normally distributed data. Differences among means were considered significant when $P < 0.05$.

Additionally, in order to explore overall relationships between the different variables and to define the most important parameters involved in effluent exposure, we ran a PCA from a matrix data that included all investigated biomarkers. To minimize the number of empty spaces in the dataset, multivariate analysis was carried out taking into account five cases (individuals with 57 variables measured). Variables that had not a normal distribution were transformed using Log_{10} and tested again, prior to parametric analysis. All statistical analysis was performed by the InfoStat software (Di Rienzo et al., 2012).

Table 1
Survival and condition indexes of *Prochilodus lineatus* caged at upstream, effluent and downstream sites for 96 h. Values represent the median with (minimum–maximum).

	Upstream	Effluent	Downstream
Weight (g)	45.35 (33.17–55.73)	44.44 (35.20–60.40)	41.08 (37.12–55.22)
Total length (cm)	12.50 (11.00–13.00)	14.50 (11.00–16.00)	13.50 (11.50–16.50)
Condition factor	1.94 (1.17–2.66)	1.51 (1.20–2.79)	1.77 (1.15–2.73)
Liver somatic index	1.19 (0.94–1.61)	1.23 (0.98–1.43)	1.34 (1.04–1.95)
Survival (%)	100	70	100

Table 2
Physicochemical data measured at each caging site at the beginning (0 h), middle (48 h) and end (96 h) of the caging period. Values represent the median with (minimum–maximum).

	Upstream	Effluent	Downstream
Ammonia (mg L^{-1} N-NH_3)	0.14^{a} (0.13–0.17)	0.28^{b} (0.20–0.31)	0.21^{a,b} (0.13–0.25)
Calcium (mg L^{-1})	8.00 (8.00–11.00)	13.00 (8.00–14.00)	9.60 (9.60–9.60)
Chemical oxygen demand (mg L^{-1})	5.00 (3.00–6.00)	5.00 (4.00–6.00)	7.00 (6.00–7.00)
Conductivity (μS cm^{-1})	81.50 (81.00–82.30)	81.60 (81.40–82.50)	80.80 (78.20–81.50)
Dissolved oxygen (mg L^{-1})	8.37 (7.39–9.35)	7.22 (7.12–7.31)	7.22 (7.00–7.44)
Hardness (mg L^{-1})	52.00 (40.00–60.00)	48.00 (44.00–48.00)	48.00 (40.00–52.00)
Magnesium (mg L^{-1})	8.00 (3.00–10.00)	4.00 (3.00–6.00)	6.00 (4.00–7.00)
Nitrates (mg L^{-1})	3.20 (3.00–3.40)	3.90 (3.10–4.00)	3.90 (3.80–5.00)
Nitrites (mg L^{-1})	0.02 (0.02–0.02)	0.02 (0.02–0.03)	0.04 (0.02–0.05)
pH	6.09 (6.05–6.35)	6.28 (6.16–6.30)	6.08 (5.96–6.45)
Phosphorus (mg L^{-1})	0.34 (0.32–1.34)	0.50 (0.32–0.84)	0.31 (0.28–1.00)
Temperature (°C)	19.80 (19.60–19.90)	19.90 (19.70–20.00)	19.90 (19.70–19.90)
Total coliforms (MPN·100 ml^{-1})	1300^{a} (500–1700)	24000^{c} (24000–24000)	3000^{b} (3000–4000)
Fecal coliforms (MPN·100 ml^{-1})	300^{a} (220–1100)	13000^{c} (8000–13000)	1700^{b} (1700–1700)
Transparency (cm)	0.20 (0.19–0.21)	0.18 (0.16–0.21)	0.19 (0.18–0.21)

Different letters (a,b,c) indicate significant differences at $p < 0.05$.

3. Results

3.1. Environmental variables

Physicochemical data on the water are given in Table 1. Most parameters were similar in the three caging sites. However, ammonia concentration at the effluent site was higher than at the reference site. Similarly, the highest densities of coliforms indicators were found at the effluent and downstream sites.

3.2. Biomarkers

3.2.1. Condition indexes

After 96 h of exposure, 6 individuals had died at effluent site. Survival and condition indexes for *P. lineatus* are shown in Table 2.

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Table 3
Hematological parameters in juveniles of *Prochilodus lineatus* caged at upstream, effluent and downstream sites. Values represent the median with (minimum–maximum).

	Upstream	Effluent	Downstream
RBC ($10^6/\mu l$)	2.08 (1.74–2.84)	2.12 (1.53–2.52)	2.14 (1.44–2.31)
Ht (%)	64.32[b] (58.62–71.97)	59.57[a] (47.77–72.92)	66.02[b] (60.98–76.39)
Hb ($g\,dl^{-1}$)	7.40 (5.10–8.84)	8.27 (6.88–9.16)	8.17 (5.63–8.81)
MCV (μm^3)	312.89 (232.08–354.33)	278.11 (189.56–378.82)	310.14 (263.98–530.49)
MCH (pg)	33.65 (25.11–43.37)	38.06 (36.08–44.97)	38.70 (24.38–42.45)
MCHC (%)	11.87 (7.89–14.41)	13.75 (11.44–14.91)	12.18 (7.89–14.44)
WBC (μl)	3468 (2436–5238)	3435 (1782–9020)	3502 (1792–7392)
Lymphocytes (%)	52.43 (30.00–71.43)	38.68 (12.07–67.59)	48.00 (28.16–69.14)
Neutrophils (%)	12.62[b] (4.67–26.50)	3.77[a] (0.00–14.78)	1.23[a] (0.00–19.00)
Eosinophils (%)	10.00 (2.52–21.05)	14.81 (3.45–19.81)	12.63 (5.50–29.00)
Monocytes (%)	18.42[a] (4.20–37.50)	35.65[b] (18.52–73.28)	26.00[a,b] (14.42–57.28)

Different letters (a,b) indicate significant differences at $p < 0.05$.

Overall, the morphometric endpoints for fish were similar among sites.

3.2.2. Hematology

Hematological parameters of *P. lineatus* caged at upstream, effluent and downstream sites are showed in Table 3. RBC, Hb, MCV, MCHC and WBC mean values did not differ among caging sites. In contrast, fish caged at the effluent showed lower Ht than upstream and downstream sites. On the other hand, leukocyte frequency was altered in fish caged at effluent and downstream sites, with a lower percentage of neutrophils and a marked increasing of monocytes (Table 3). Basophiles were not detected.

3.2.3. Transaminases and alkaline phosphatase

As shown in Fig. 2, no significant differences in ALT and ALP activities were found in the liver and kidney of fish. On the contrary, hepatic and renal AST measured in caged fish exhibited higher activities in the effluent and downstream sites, compared to the reference site.

3.2.4. Oxidative stress

Following 96 h of exposure, a clear effect of effluent on several antioxidant enzyme activities could be observed in liver (GST, GR), gills (CAT, GR) and brain (GR, GPx) (Table 4).

On the other hand, fish caged at the effluent site showed higher LPO levels in liver, brain and kidney (Fig. 3). High LPO levels were also observed in the same tissues of fish caged at downstream site.

3.2.5. Energetic costs

There was an obvious impact of the effluent on the glycogen reserves of the caged fish (Table 5). The percentage of decrease in glycogen levels after effluent exposure reached 73% and 38% in the liver and muscle, respectively. No significant differences were observed on lipid and protein tissue concentrations.

3.3. Integrated analysis

Fig. 4 shows that 32.3% of overall variance is explained by the first two principal components. According to Legendre and Legendre (1979), interpretation of principal components may be done for eigenvalues of the data matrix higher than 1. The PCA indicated that 14 eigenvalues were higher than 1; moreover correlation coefficients are significant when they are higher than $\sqrt{d/n}$, d being the number of principal components and n the number of variables. Therefore, correlation coefficients >0.5 were indicative of a good representation of the variables with principal component axes.

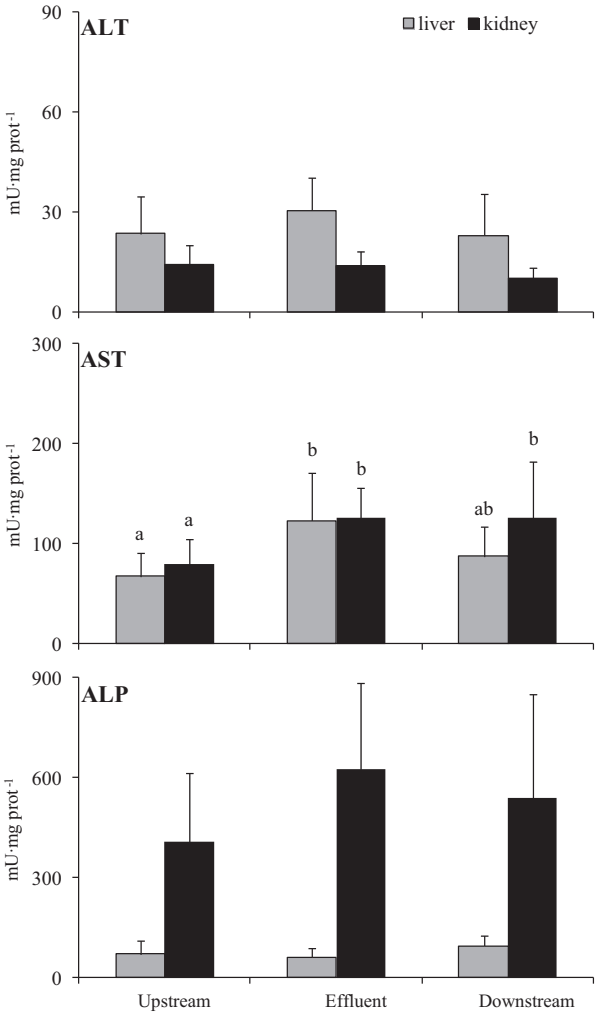

Fig. 2. Alanino-aminotransferase (ALT), aspartate-aminotransferase (AST), and alkalinephosphatase (ALP) activities measured in liver and kidney of *Prochilodus lineatus* caged at upstream, effluent and downstream sites. Data are shown as mean ± SD. Means not sharing the same letter (a,b) are significantly different at $p < 0.05$.

The first axis explained 19.5% (PC1, 19.5%) of the global inertia in the data and it ordinates mainly the treatments (caging sites). Thus, on this first principal component, fish caged at the effluent site were clearly distinguished from fish exposed in other sites, showing significant positive loadings for the antioxidant enzymes in liver (GR, GST, GPx) brain (GR) and gill (CAT). Then, the increment in such biomarkers significantly differentiates the effluent site from the upstream and downstream groups in PC1. On the contrary, a negative correlation was found mainly for glycogen in muscle and liver, lymphocytes and Ht.

PC2 (12.8% of the total variance) showed mainly a high individual variation of effluent data. This axis appears to be positively correlated with antioxidant enzymes measured mainly in brain (CAT, GST) and kidney (GR, CAT, GST) and negatively for LPO and GR in gills, and LPO-muscle.

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Table 4
Antioxidant enzyme activity measured in brain, gills, intestine, kidney, liver and muscle of *Prochilodus lineatus* caged at upstream, effluent and downstream sites. Values represent the median with (minimum–maximum).

	Upstream	Effluent	Downstream
Brain			
GST	9.77 (4.01–11.87)	7.39 (4.68–13.37)	6.45 (3.70–8.30)
GR	0.62[a] (0.52–1.36)	1.51[b] (0.85–1.77)	0.54[a] (0.45–0.76)
GPx	7.60[a] (4.52–8.66)	4.28[b] (3.85–6.52)	5.06[a] (2.63–6.50)
CAT	175.2 (48.7–186.2)	123.2 (84.0–241.7)	105.6 (78.8–182.3)
Gills			
GST	3.27 (1.87–4.43)	5.26 (2.64–7.13)	2.35 (1.82–3.55)
GR	0.38[a] (0.26–0.70)	0.70[b] (0.37–1.15)	0.35[a] (0.24–0.51)
GPx	0.08 (0.06–0.14)	0.06 (0.03–0.09)	0. 04 (0.01–0.10)
CAT	28.84[a] (25.71–47.68)	104.7[b] (65.32–130.7)	39.16[a] (25.04–57.19)
Intestine			
GST	6.13 (2.69–7.31)	4.86 (4.03–5.29)	5.65 (4.50–7.15)
GR	0.45 (0.32–0.72)	0.55 (0.39–0.73)	0.46 (0.15–0.51)
GPx	2.09 (1.55–2.76)	2.31 (2.54–3.80)	2.77 (2.41–3.09)
CAT	60.15 (49.81–94.17)	85.10 (61.12–96.57)	47.97 (40.91–85.36)
Kidney			
GST	1.82 (1.68–2.29)	2.48 (2.09–3.10)	1.89 (1.70–3.29)
GR	0.48 (0.36–0.58)	0.43 (0.36–0.73)	0.48 (0.39–0.64)
GPx	2.53 (2.20–2.66)	2.10 (1.48–2.50)	2.01 (1.97–3.97)
CAT	134.2 (105.2–156.0)	82.75 (69.75–172.35)	84.09 (55.56–104.43)
Liver			
GST	3.92[a] (2.44–6.02)	6.22[b] (3.83–6.71)	3.34[a] (2.26–3.94)
GR	0.34[a] (0.33–0.41)	0.75[b] (0.37–0.87)	0.27[a] (0.23–0.39)
GPx	0.64 (0.39–0.93)	1.27 (1.14–1.84)	0.67 (0.37–0.98)
CAT	596.4 (284.0–813.7)	527.0 (445.4–704.9)	342.1 (233.9–758.2)
Muscle			
GST	0.83 (0.68–0.99)	0.82 (0.62–0.95)	0.90 (0.76–0.93)
GR	7.76 (5.68–10.34)	8.50 (6.79–13.59)	7.78 (5.92–9.86)
GPx	0.46 (0.35–0.54)	0.42 (0.40–0.53)	0.47 (0.43–0.52)
CAT	20.12 (16.92–24.55)	16.24 (14.35–19.90)	17.46 (14.57–19.13)

Different letters (a,b) indicate significant differences at $p < 0.05$. GST, glutathione S-transferease; GR, glutathione reductase; GPx, glutathione peroxidase; CAT, catalase.

4. Discussion

Fish can be severely impacted by the cumulative effects of multiple contaminant stressors. This study is the first assessment of biological effects of an untreated sewage effluent in the Colastine River in caged *P. lineatus*, a commercially and ecologically valuable species.

4.1. Environmental variables

Overall environmental variables did not present significant variations among sites during the experiment. Our results indicated only an increase in ammonia concentration and high densities of coliforms indicators at the effluent site, which are expected constituents of municipal waste. Ammonia is known to have harmful effects on growth, energy metabolism, ionic balance, gill structure and immunity of fish (Sinha et al., 2012). However, the ammonia levels observed in the effluent site were bellow guidelines for the protection of aquatic biota (SRHN, 2004). Thus, a direct toxicity of ammonia, in our experiment, is not likely. On the other hand, high density of microorganisms may break the immunological barriers of fish, penetrating different tissues and organs and they may survive and accumulate in fish, with the consequent risk involved in the consumption of contaminated fish (Guzmán et al., 2004).

Effluents from sewage have been shown to contain complex mixtures of chemicals (Gasperi et al., 2008; Bolong et al., 2009). Chemical identification of possible contaminants in aqueous effluents is a laborious, time consuming, and expensive task and of only limited use if no information exists about effects on aquatic life and environment (Al-Arabi et al., 2005). When aquatic organisms

Table 5
Energetic parameters of tissues (liver and muscle) and plasma of *Prochilodus lineatus* caged at upstream, effluent and downstream sites. Values represent the median with (minimum–maximum).

	Upstream	Effluent	Downstream
Liver			
Glycogen (μmol g^{-1} wt)	18.79[b] (11.37–41.22)	6.13[a] (2.99–9.82)	34.18[b] (6.31–71.26)
Lipid (μmol g^{-1} wt)	11.87 (9.36–27.60)	11.79 (7.66–24.83)	14.73 (12.66–34.54)
Protein (mg g^{-1} wt)	111.86 (69.54–139.18)	114.27 (60.96–137.04)	116.68 (94.18–160.07)
Muscle			
Glycogen (μmol g^{-1} wt)	0.36[b] (0.23–0.46)	0.18[a] (0.14–0.33)	0.44[b] (0.36–0.58)
Lipid (μmol g^{-1} wt)	8.88 (6.31–25.51)	12.10 (4.83–22.18)	9.27 (5.98–13.51)
Protein (mg g^{-1} wt)	57.95 (46.34–70.00)	52.59 (37.86–81.16)	68.22 (54.82–73.57)
Plasma			
Glucose (mg dl^{-1})	0.91 (0.55–1.66)	0.75 (0.51–1.86)	0.75 (0.39–1.23)
Protein (g dl^{-1})	3.23 (2.70–3.46)	3.17 (2.17–4.09)	3.28 (2.83–3.46)

Different letters (a,b) indicate significant differences at $p < 0.05$.

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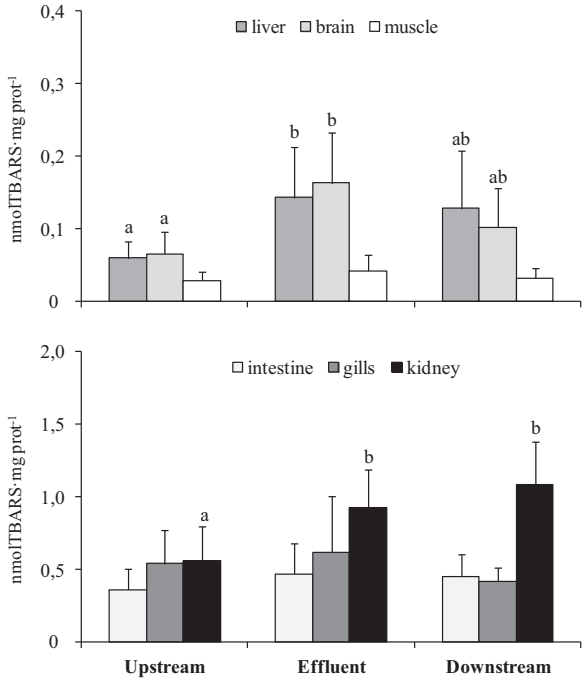

Fig. 3. Lipid peroxidation levels in liver, brain, muscle, intestine, gills and kidney of *Prochilodus lineatus* caged at upstream, effluent and downstream sites. Data are shown as mean ± SD. Means not sharing the same letter (a,b) are significantly different at $p < 0.05$.

are exposed to pollutants, a cascade of biological events takes place, if the concentration is high enough and/or the duration of exposure is sufficiently long to induce adverse effects (Wepener, 2008). During our experiment mortality was only observed in effluent site, indicating that the effluent have a lethal effect on juvenile *P. lineatus*. Besides, to obtain a holistic and integrative overview of how untreated sewage effluent exposure affects fish health, we assessed a battery of biomarkers.

4.2. Condition indexes

The morphometric indexes, such as CF and LSI, did not present significant variations among the different evaluated sites. This result coincides with previous reports for caged fish, in which no changes in condition indexes were observed between reference and polluted areas (Lindström-Seppä and Oikari, 1989; Soimasuo et al., 1995; Orrego et al., 2006; Maceda-Veiga et al., 2010). Thus, CF and LSI were not sensitive parameters for effluent exposure.

4.3. Hematology

Most red blood parameters did not show significant changes. Only Ht decreased in *P. lineatus* caged at the effluent site. The observed decrease might be explained by disorders in hemopoietic processes (inhibition of erythropoiesis) or an increased frequency of erythrocyte death (accelerated disintegration of RBC cell membranes) (Maceda-Veiga et al., 2010). However, the count of red blood cells was similar in fish from different sites, but MCV showed a trend to diminish in fish exposed to effluent site. So, it is likely that such decrease in Ht can be due to the higher rate of immature RBC (not determined in this study) in order to compensate disintegration of mature RBC.

On the other hand, we observed alteration in leukocyte frequency in fish caged at effluent and downstream sites. Similarly, previous field studies have reported monocytosis in fish exposed to sewage discharges or water contamination (Moiseenko et al., 2005; Maceda-Veiga et al., 2010). Monocytes (or macrophages) are active phagocytes and these cells are involved in the immune system (Takashima and Hibiya, 1995; Carlson and Zelidoff, 2008). Furthermore, infections commonly cause general increases in monocytes (Davis et al., 2008).

4.4. Transaminases

Enhancement activity of transaminases provides metabolic intermediates to meet the increased energy demand during imposed stress conditions in fish, and it is used to determine tissue

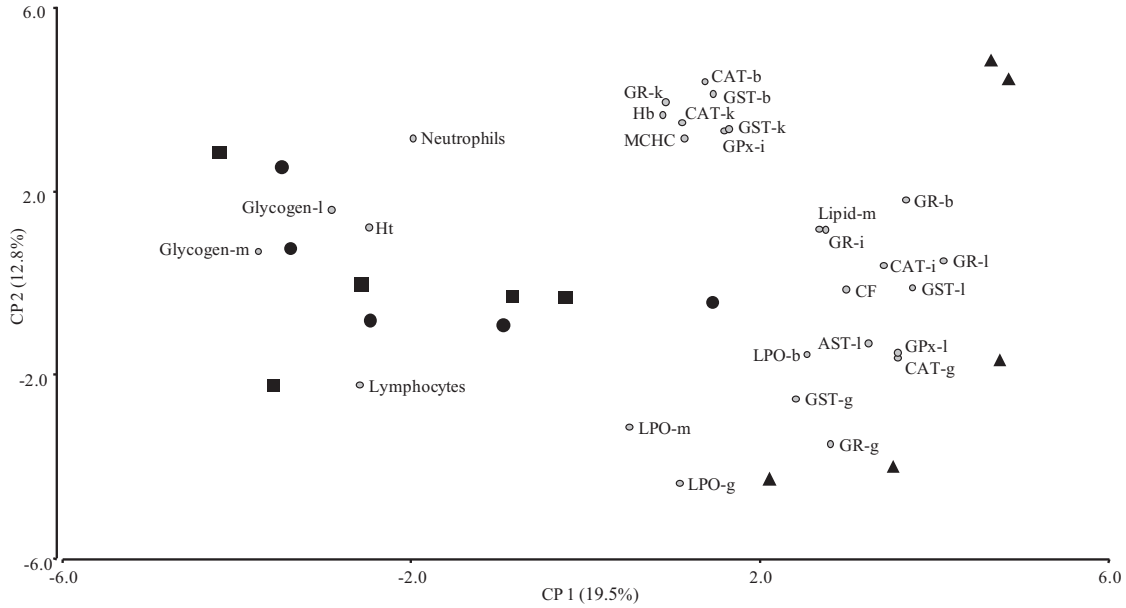

Fig. 4. Principal components analyses (PCA) of the biomarkers responses in fish caged at Colastine River. Individual scores are shown: circles (●) correspond to reference site (upstream), triangles (▲) to effluent site, and squares (■) to downstream site. Biomarkers with correlation coefficients >0.5 were represented in the PCA. Biomarkers abbreviations are explained in the text. Abbreviations for different tissues were followed by the corresponding letter. -b: brain; -g: gills; -i: intestine; -k: kidney; -l: liver; -m: muscle.

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damage in organs such as the liver and kidney (Rao, 2006; Ramaiah, 2007). The present study showed higher hepatic and renal AST levels in both the effluent and downstream sites, compared to reference site. According to our results, such increase was accompanied by the decrease in liver glycogen and rise in LPO levels, indicating the utilization of energy to overcome the stress condition and corroborating the occurrence of tissue damage. Similar increases in transaminases enzymes have been reported in fish exposed to pesticides, different forms of metal stress or polluted waters (de la Torre et al., 2007; Öner et al., 2009; Velisek et al., 2012).

4.5. Oxidative stress

Numerous environmental pollutants have been shown to impact organismal health via, at least in part, oxidative stress (Di Giulio and Hinton, 2008). Our results indicate that fish caged at the effluent site were indeed suffering from oxidative stress. This is evidenced by the significantly elevated enzyme activities and LPO levels observed in various tissues.

Brain, gills and liver showed enhanced enzymes activities in fish caged at the effluent site. Interestingly, increased GR activity was observed in these three tissues. The GR enzyme is responsible for maintaining the GSSG:GSH ratio and when its activity increases, more GSSG is converted in GSH, hence rising the free radical scavenging ability of a cell. Previous studies showed that GR can be considered a reliable marker for oxidative stress in field studies (Regoli et al., 2002; Stephensen et al., 2002).

On the other hand, hepatic GST increased after effluent exposure, which is an expected response because such enzyme is involved in the biotransformation of several pollutants, and the liver is known to be the primary organ where this process occurs (van der Oost et al., 2003). Induced GST activity indicates the role of this enzyme in protection against the toxicity of xenobiotic-induced lipid peroxidation (Rao, 2006).

Despite the increased activity of some antioxidant enzymes, effluent site clearly induced a situation of oxidative stress in brain and liver, with a significant increase in LPO. In other words, the observed antioxidant defenses were not able to prevent oxidative damage in these tissues. These results are in accordance with previous studies (Almroth et al., 2008) which found hepatic oxidative damage in fish caged near a sewage treatment plant. On the other hand, the brain is considered particularly prone to oxidative stress due to its high rate of oxidative metabolism, presence of autooxidizable neurotransmitters (dopamine and norepinephrine), high levels of highly unsaturated fatty acids, relatively modest antioxidant defenses, and presence of cytochrome P450s (Halliwell and Gutteridge, 2007).

On the contrary, gills exposed to effluent resist to oxidative stress through the elevation of GR and CAT activities and/or other antioxidant mechanisms, preventing an increase in LPO. This is in line with previous studies suggesting that the increased CAT activity can minimize MDA production and reduce the degree of oxidative stress that resulted from ROS (Kong et al., 2012).

On the other hand, we also observed lipid oxidative damage in the kidney of fish exposed to effluent. This could be possibly due to the absence of cell protection by unchanged antioxidant enzyme activities.

So far, our current results suggest the probable existence of xenobiotics in the effluent discharges, which could exert oxidative stress in essential functionally tissues (brain, liver and kidney) of *P. lineatus*. It is important remark that fish caged at downstream site also exhibited oxidative damage in those tissues. So, according to the lipid peroxidation data, downstream site may be also impacted by effluent discharges in concentrations enough to affect health conditions of fish.

4.6. Energetic costs

Glycogen is the main reserve source of energy for animals during normal metabolism and their content in liver and muscle may indicate the health condition of the fish (Hori et al., 2006; Saravanan et al., 2011). Glycogen depletion is a stress-induced response and our data show that exposure to effluent provoked a reduction in liver and muscle glycogen content of *P. lineatus*. Similar to other studies, the decrease was greater in liver than in muscle, as liver is the principal site of glycogen synthesis and storage (Begum, 2004; Cattaneo et al., 2008).

Our results indicate the utilization of stored glycogen to meet extra energy demand in the metabolism under the effluent stress. Finally, this depletion of energy reserves may have its consequences for higher levels of biological organization, like diminished growth, survival probability and reproduction (Sancho et al., 2010).

Glycogen levels in fish living under environmental stress may reflect an increased glucose levels in the serum (Öner et al., 2008). However, we did not detect a significant increase of plasma glucose in fish exposed to effluent for 96 h. This may be due to utilization of glucose by the fish as an energetic substrate to carry out the repair processes to counter damages caused by chemicals, and homeostatic mechanisms involving glycogenolysis and gluconeogenesis tend to maintain normal blood glucose (Ricard et al., 1998).

4.7. Integrating analysis

A number of approaches have been applied to analyze large biomarker data sets. The most promising application involves multivariate statistical techniques, which also identify those biomarkers that are responsible for clearly distinguishing contaminated sites from clean sites (Wepener, 2008). In our study, PCA analysis indicated that fish caged at the effluent site clustered separately from those transplanted upstream and downstream, suggesting a differential physiologic profile among organisms. Data obtained in fish placed in cages demonstrate the high sensitivity of certain biochemical parameters. Especially strong responses were seen in oxidative stress markers and glycogen content. Thus, fish exposed to effluent showed antioxidant enzyme activation and had an extra energy demand as indicated by liver and muscle glycogen.

Nevertheless, in field studies it is difficult to determine whether a physiological response reflects an adverse environmental condition or an adaptational adjustment in response to environmental stress (Maceda-Veiga et al., 2010). Taking into account these considerations, the observed mortality, lipid oxidative damage and glycogen depletion in fish caged at the effluent support the hypothesis that the physiological response of *P. lineatus* reflects adverse environmental conditions. These responses may not be sufficiently low to preserve the physiological integrity of sensitive species, especially the young stages.

5. Conclusions

This study demonstrates the utility of an integrative approach based on fish biomarkers combined with a caging experimental strategy to assess potential biological effects of the exposure to waters receiving untreated sewage effluent. Thus, according to our results sewage discharges in the Colastine River may constitute an ecotoxicological hazard to fish populations, since they induced stress responses in caged fish. Specifically, mortality, monocytosis, transaminase increase, antioxidant enzyme activation, lipid oxidative damage and glycogen depletion were observed in juvenile *P. lineatus* exposed to effluent site. According to multivariate analysis, oxidative stress markers and metabolic parameters were key biomarkers to contribute in separating fish caged at effluent site

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from those caged at upstream and downstream sites. So, we suggest the use of those biomarkers in future environmental monitoring assessments. On the other hand, of all organs investigated, the liver showed the highest degree of alteration, which corroborates its importance as a target organ in fish toxicology studies.

In spite of the fact that the effluent had an impact on several biomarkers, a subsequent recovery was observed at downstream site, suggesting a decontamination or dilution effect. Although this study did not provide evidence as to which particular contaminants are responsible for the biomarker responses in the fish, sewage effluents discharges in the outfall discharge area are implicated. However, it is important to consider that the scenario reported in our study may be worse in the future because sewage discharge will become more concentrated during a dry season.

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