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Research paper

In situ evaluation of the toxicological impact of a wastewater effluent on the fish *Prochilodus lineatus*: biochemical and histological assessment

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ABSTRACT

Sewage effluents are the most important source of emergent pollutants in the aquatic environment. In the present study the toxicological impact of an untreated sewage effluent on the *Prochilodus lineatus* fish was assessed under field conditions using a caging experiment. The biomarkers which were measured here involved oxidative stress markers, hepatic function parameters, neurotoxicity indicators, energy reserves, histological alterations and brain cell proliferation. In addition, water quality parameters including the occurrence of some human pharmaceuticals were measured. Juveniles of *P. lineatus* were caged for 15 days at three sites: the effluent site, and 2 km upstream and downstream from the effluent discharge. Caffeine, atenolol, carbamazepine, enalapril and sildenafil were detected in water river samples. The increased activation of caspase-3 and the decreased cell proliferation in the diencephalon showed that the brain of fish caged at the effluent was affected. These fish also displayed a rise in hepatic transaminase activity, and oxidative stress in liver and gills which was evidenced by an increased lipid peroxidation and activation of antioxidant enzymes. At tissue level, increased glycogen and decreased lipid contents in liver as well as the highest indexes of hepatic lesions were also observed in the fish caged at the effluent. Both biochemical and histopathological findings demonstrated that effects were more severe on the liver of such fish than on their gills. The *in situ* exposure method carried out in our study makes it possible to observe the real effects of the sewage effluent on fish. Furthermore, our results also provide a better understanding of the harmful effects of wastewater effluents on the aquatic wildlife.

1. Introduction

During the last decades, global production of anthropogenic chemicals has increased from 1 to 400 million tons per year (Gavrilescu et al., 2015). Recently, concern about the impact of the so-called emerging contaminants (ECs) in aquatic environments has arisen and still little information is available to establish their potential environmental risks (Naidu et al., 2016). ECs encompass a wide range of manmade chemicals as pharmaceuticals, cosmetics, personal care products and pesticides, among others (Petrie et al., 2015). Within these substances, pharmaceuticals stand out as being one of the main groups of aquatic environmental contaminants (Hughes et al., 2013).

Municipal wastewater effluents are the most important source of ECs in the aquatic environment (Fent et al., 2006). Sewage treatments plants (STPs), even the most advanced ones, are not efficient enough to completely remove ECs from sewage. Thus, ECs are being continuously released into the environment (Hernando et al., 2006). This situation worsens in cities lacking STPs. Even though ECs are found at low concentrations (ng L^{-1} to μg L^{-1} range), the constant exposure to these compounds can lead to their accumulation in aquatic organisms (Khetan and Collins, 2007; Liu et al., 2015; Valdés et al., 2014, 2016; Vincze et al., 2015). Recent studies have shown that drugs such as the stimulant caffeine, β-blocker atenolol, the antiepileptic carbamazepine, the blood pressure regulator enalapril and the erectile dysfunction and pulmonary arterial hypertension sildenafil are the human pharmaceuticals most frequently found in wastewaters and receiving waters of Argentina (Elorriaga et al., 2013a,b).

The toxicological effects of many ECs on aquatic organisms have

Abbreviations: AChE, acetylcholinesterase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATE, atenolol; CAT, catalase; CBZ, carbamazepine; ECs, emerging contaminants; ENAL, enalapril; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione *S*-transferase; LPO, lipid peroxidation; ROS, reactive oxygen species; SIL, sildenafil; SOD, superoxide dismutase; STPs, sewage treatments plants; TBARS, thiobarbituric reactive substances

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been studied mainly under laboratory conditions (Brandão et al., 2013; Diniz et al., 2015; Guiloski et al., 2015; Islas-Flores et al., 2017; Li et al., 2010). Despite the fact that aquatic biota in the receiving environment is continually exposed to a complex mixture of ECs (Petrie et al., 2015), only a few field studies are available (Jasinska et al., 2015; Liu et al., 2015; Vincze et al., 2015). *In situ* studies using caging techniques are considered an appropriate approach to evaluate the connection between levels of contamination and adverse effects on exposed organisms (Oikari, 2006).

Previous caging experiments have demonstrated that sewage effluents cause several biochemical and physiological changes in fish. Endocrine disruption has been observed in *Oncorhynchus mykiss* and *Pimephales promelas* caged downstream from the discharge of STPs (Ings et al., 2011; Jasinska et al., 2015). Several authors have also observed the induction of biotransformation enzymes and oxidative stress markers in freshwater fish caged in rivers receiving wastewaters (Carney Almroth et al., 2008; Cazenave et al., 2014; Liu et al., 2015; Scarcia et al., 2014). Nevertheless, few studies on the effects of wastewater effluents across different levels of biological organization, such as metabolic, histological and behavioral responses are available (McCallum et al., 2017; Vincze et al., 2015).

Both laboratory and field studies have demonstrated that ECs adversely affect different organs of fish. Due to their role in xenobiotics detoxification and excretion, liver and gills are the most sensitive organs (Nunes et al., 2008; Ramos et al., 2014). However, brain, kidney, gonads and blood are also considered as potential target organs (Ings et al., 2011; Vieira et al., 2016). A high rate of reactive oxygen species (ROS) production and a relatively low antioxidant defense system make brain especially vulnerable to oxidative stress (Ballesteros et al., 2009; Matés, 2000; Xing et al., 2012). Cazenave et al. (2014) showed that *in situ* exposure to untreated sewage effluent caused lipid oxidative damage in the brain of *Prochilodus lineatus*. In mammals, oxidative stress and lipid peroxidation (LPO) in particular are associated with neurodegenerative diseases (Chiurchiù et al., 2016; Reed, 2011). Taking into account that neurogenic activity is higher in teleost fishes than tetrapods (Kaslin et al., 2008), fishes are valuable models for the evaluation of oxidative stress in brain. However, studies analyzing fish oxidative stress and neurodegeneration simultaneously remain scarce (Jiang et al., 2014; Wang et al., 2008).

Our study was aimed at assessing the toxicological impact of a sewage effluent on *Prochilodus lineatus* ("sábalo") juveniles at different levels of biological organization. As regards the biochemical level, oxidative stress markers (the antioxidant enzymes glutathione *S*-transferase, gluthatione reductase, catalase, superoxide dismutase, and the oxidative lipid damage), biomarkers related to hepatic function (transaminases), and indicators of neurotoxic effects (acetylcholinesterase, caspase-3) were selectively assessed in gills, liver and brain. At tissue level, liver and muscle energy reserves, gill and liver histopathology and brain cell proliferation were examined. In addition, water quality parameters including coliform load and the levels of human pharmaceuticals commonly found in Argentinean wastewaters were measured at the caging sites.

2. Material and methods

2.1. Study sites, fish and caging experiment

The Colastiné River (Argentina) is one of the most important tributaries of the Middle Paraná River. It flows 35 km over a sandy floodplain, it has a mean depth of 11 m and a mean discharge of $\approx 1700\ m^3\ s^{-1}$ (Iriondo, 1975; Amsler et al., 2007). This river receives untreated domestic wastewaters from Santa Fe city (525,093 inhabitants). The flow of the effluent discharge oscillates between $1.00–1.20\ m^3\ s^{-1}$. This effluent is characterized by a high fecal coliform load and elevated levels of certain metals (As, Cr, Ni, Cu, Pb, Ni, Zn) (Cazenave et al., 2014; Eberle et al., 2015).

The caging experiment was carried out at three exposure sites: 0.2 km from the discharge of a sewage effluent (Effluent Site, 31°67′303″S; 60°63′522″W), 2 km upstream from such effluent (Upstream Site, considered as reference site, 31°67′156″S; 60°61′114″W); and 2 km downstream from the same effluent (Downstream Site, 31°67′915″S; 60°64′595″W). The experiment was conducted in May 2014 (wet season; average monthly water level of 3.95 m; average monthly flow of $2{,}236.46\ m^3\ s^{-1}$) (SRHN, 2017).

Prochilodus lineatus is a neotropical fish that represents a large part of the total ichthyomass of this region (Bonetto et al., 1970) and it has already been used in biomonitoring studies (Camargo and Martinez, 2006; Cazenave et al., 2009, 2014; Troncoso et al., 2012; Vieira et al., 2016). Juveniles ($n = 70$; 7.01 ± 0.57 g; 8.84 ± 0.33 cm total length) were obtained from a local farm, and maintained under laboratory conditions (aerated dechlorinated water, 23 ± 1 °C temperature, 12:12 h light-dark cycles) for 7 days. After this period, a group of fish ($n = 10$; basal group) were sampled to be used as a baseline for biomarkers. The remaining fish were transported from the laboratory to the exposure sites (by boat for < 1 h) in plastic bags (100 L) with oxygenated water. Two cages ($n = 10$ fish/cage) were placed at each site. Perforated polyethylene cages ($0.60\ m \times 0.30\ m \times 0.36\ m$, 65-$dm^3$) were completely immersed (depth ≤ 1.5 m) to allow water circulation and sediment contact for fish feeding. After a 15-day exposure, fish were retrieved and rapidly transported (in aerated river water) back to the laboratory for sample processing. Experiment was conducted in accordance with the national and institutional guidelines for the protection of animal wildlife (CONICET, 2005).

2.2. Water quality and occurrence of pharmaceuticals

Both at the beginning and the end of our field experiment water samples were taken from each site for water quality parameters and human pharmaceuticals analysis. The measured water quality parameters involved: temperature (°C), pH, conductivity ($\mu S\ cm^{-1}$), dissolved oxygen ($mg\ L^{-1}$), transparency (cm), alkalinity ($mEq\ L^{-1}$ $CaCO_3$), hardness ($mg\ L^{-1}$ $CaCO_3$), dissolved organic matter (PtCo), ammonia ($mg\ L^{-1}$), nitrates ($mg\ L^{-1}$), nitrites ($mg\ L^{-1}$), bicarbonates ($mg\ L^{-1}$), total phosphorus ($\mu g\ L^{-1}$), calcium ($mg\ L^{-1}$) and magnesium ($mg\ L^{-1}$), and total and fecal coliforms (MPN) (APHA and AWWA, 1998).

The analyzed human pharmaceuticals such as atenolol, caffeine, carbamazepine, enalapril and sildenafil were measured in the dissolved fraction following the methods established by Elorriaga et al. (2013a) with some modifications. Pharmaceutical standards (99% purity) were obtained from Parafarm (Saporiti, Argentina) and the isotopically labeled standard of carbamazepine-d_{10} (98%) was purchased from Cambridge Isotope Lab (USA). At each sampling site, 150 mL of water were filtered *in situ* through 0.45 μm pore size 47 mm cellulose filters, placed into a dark bottle, and spiked with 150 μL of the labeled standard ($3.3\ \mu g\ L^{-1}$). Samples were stored in ice, transported to the laboratory, and once there transferred to a freezer (-20 °C). Solid phase extraction (SPE) was performed on OASIS HLB® cartridges (60 mg–3 mL from Waters Corp.). The SPE cartridge was preconditioned with 5 mL pure methanol followed by 5 mL nanopure water. Then, 60 mL of filtered sample were diluted with 60 mL of nanopure water and loaded at a rate of 5 mL/min, washed with 5 mL of 5% methanol, and finally eluted with 2×5 mL of pure methanol. Extracts were taken to dryness under a gentle flow of nitrogen and resuspended in 300 μL ($200\times$ concentration) of mobile phase (50% A and 50% B). Samples were

analyzed using a 1100 Series LCMSD VL G1956A (Agilent Technologies Inc., USA) equipped with an electrospray ionization (ESI) interface. Chromatographic separation was achieved on an X-Select® CSH C18 column (Waters) 75 × 4.6 mm and 3.5 µm at 35 °C using 5 mM ammonium acetate (A) and methanol/acetonitrile 1:1 (B) as mobile phase. Flow was 0.5 mL min^{-1} and gradient was 25% to 100 of B in 9 min. Mass Spectrometer parameters as well as quantification and confirmation ions were the same described in Elorriaga et al. (2013a). Data were acquired and processed using the Agilent ChemStation (Agilent Technologies). Quantification was achieved using an external standard curve. Carbamazepine-d_{10} was used for estimating the recoveries and checking quantification of carbamazepine. Recovery was above 94% and the quantification limits of the method (LOQ) were 3.3, 9.4, 1.0, 1.5 and 3.0 ng L^{-1} for atenolol, caffeine, carbamazepine, enalapril and sildenafil, respectively.

2.3. Biochemical markers

All fish were anaesthetized with benzocaine (Parma de Croux, 1990) and their body weight (g) and total length (cm) were recorded. Fish were euthanized by cervical transection (AVMA, 2013) and gills, liver, brain and muscle were dissected and immediately stored at −80 °C for biochemical analyses.

Enzyme extracts from gills, liver and brain (N = 5 fish per site) were prepared according to Bacchetta et al. (2014). The following enzymatic activities were measured: glutathione *S*-transferase activity (GST, according to Habig et al., 1974); glutathione reductase (GR, Tanaka et al., 1994); catalase (CAT, Beutler, 1982); and superoxide dismutase (SOD, Misra and Fridovich, 1972). Hepatic aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined using a commercial kit (Wiener Lab®). Brain extract was also used to assess the activities of glutathione peroxidase (GPx, according to Drotar et al., 1985), and acetylcholinesterase (AChE, using a commercial test/kit of Wiener Lab®). Each enzymatic activity assay was carried out in triplicate and calculated in terms of total protein content in the enzymatic extract (Bradford, 1976).

Lipid peroxidation (LPO) in gills, liver and brain (N = 5 fish per site) was performed by measuring the formation of thiobarbituric reactive substances (TBARS), according to Fatima et al. (2000). Results were reported as nmol TBARS mg $prot^{-1}$.

The caspase-3 activity assay was used to determine apoptosis induction in the brain of fish (N = 5 fish per site). Whole brain extracts were obtained according to the manufacturer's protocol (Caspase-3 Colorimetric kit, Invitrogen). The enzymatic activity was measured spectrophotometrically using the colorimetric substrate DEVD-pNA, which consisted of chromophore *p*-nitroanilide (pNA), and a synthetic tetra peptide, DEVD (Asp-Glue-Val-Asp).

2.4. Energy reserves

Glycogen, lipid and protein contents were measured in liver and muscle (N = 5 fish per site). Glycogen was estimated according to Seifter et al. (1950). Lipid content was extracted following the method described by Folch et al. (1957) while the total protein concentration in tissue homogenates was estimated according to Lowry et al. (1951) using a bovine serum albumin as standard.

2.5. Histological markers

2.5.1. Gills and liver

A histopathological analysis was performed in gill and liver samples (N = 5 fish per site). Organs were removed and fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 16 h, dehydrated in a graded series of ethanol baths, and embedded in paraffin (Biopack, Argentina). Sections of 6 µm were stained with hematoxylin and eosin and examined under a light microscope (Leica DM2500). Histological alterations were evaluated in 10 non-consecutive sections from each organ of each fish. All the analyses we performed were blind. Observations were carried out at magnification 20×, 40× or 100×, depending on the tissue and alteration type.

In order to compare sites, histopathological changes were evaluated using a semi-quantitative method proposed by Bernet et al. (1999). Gill and liver alterations were classified into five reaction patterns (*rp*): circulatory disturbance (I_{rp1}), which results from a pathological condition of blood and tissue fluid flow (*e.g.* hemorrhage, aneurysm, edema); regressive changes (I_{rp2}), which result in a functional reduction or loss of an organ (*e.g.* architectural and structural alterations, plasma alterations, deposits, nuclear changes, atrophy, necrosis); progressive changes (I_{rp3}) or processes that lead to an increased activity of cells or tissues (*e.g.* hypertrophy, hyperplasia); inflammation (I_{rp4}),which includes changes associated with other reaction patterns (*e.g.* exudates; activation of the reticuloendothelial system, infiltration of leucocytes); and benign or malignant tumors (I_{rp5}), which are uncontrolled cell proliferation.

Then, reaction and organ indexes were calculated on the basis of two factors: the extension of a pathological change (score value, *a*) and its pathological importance (importance factor, *w*). The score value (1–6) was assigned according to the percentage of tissue exhibiting a certain alteration: 0: less than 5%; 1: between 5%–20%; 2: 21%–40%; 3: 41%–50%; 4: 51%–60%; 5: 61%–80%; and 6: 81%–100%. The importance factor (1–3) reflect the ability of the alteration to become reversible after the removal of the stressor (1: easily reversible; 2: reversible in most cases; 3: generally irreversible). This value was determined by the biological significance of the lesion for fish health, according to Bernet et al. (1999).

The reaction index expresses the quality of the lesions of an organ. It is calculated by the sum of the multiplied importance factors and the alteration score values of the corresponding reaction pattern. The reaction index (Irp) of an organ was calculated as:

$$I_{rp} = \sum_{alt} (a_{org\ rp\ alt} \times w_{org\ rp\ alt})$$

The organ index (I_{org}) was calculated as the sum of the five reaction patterns of an organ. It was determined as:

$$I_{org} = \sum_{rp} \sum_{alt} (a_{org\ rp\ alt} \times w_{org\ rp\ alt})$$

where: org is the organ (constant), rp: reaction pattern, alt: alteration, *a*: score value, and *w*: importance factor.

2.5.2. Brain

In order to evaluate cell proliferation changes in the brain, immunocytochemical techniques were used in several specific forebrain areas. The identification of these sections in *P. lineatus* brain was made by comparing the neuroanatomy of other Characiformes (Gomes et al., 2013) and Cypriniformes (Wullimann et al., 1996).

Brains were partially dissected through skull opening and immersed overnight at 4 °C in PBS (pH 7.4) containing 4% paraformaldehyde (PFA). The following day, brains were removed, fixed in 4% PFA, and processed for paraffin inclusion. Transversal sections of 6 µm were mounted on 2% 3-Aminopropyl triethoxysilane (Sigma-Aldrich, USA) pretreated slides. Before being incubated with a primary antibody, sections were subjected to antigen retrieval in a sodium citrate buffer (pH 6) at 80 °C for 30 min, blocked in 5% skimmed milk powder diluted in PBS (0.1 M, pH 7.4) containing 0.3% Triton X-100, and then incubated overnight with a mouse anti-proliferating cell nuclear antigen

Table 1
Physicochemical parameters measured at each caging site at the beginning and the end (15 days) of the caging experiment. Values are expressed as mean ± SEM. Different letters indicate significant differences ($p < 0.05$).

	Upstream	Effluent	Downstream
Temperature (°C)	22.16 ± 0.05	22.00 ± 0.19	22.00 ± 0.19
pH	6.06 ± 0.12	6.29 ± 0.12	6.13 ± 0.02
Conductivity (μS cm^{-1})	73.83 ± 26.12	73.70 ± 18.95	72.56 ± 16.63
Dissolved oxygen (mg L^{-1})	7.89 ± 0.55	7.80 ± 0.01	7.85 ± 0.02
Transparency (cm)	18.50 ± 3.89	17.66 ± 7.12	19.50 ± 6.20
Alkalinity (mEq L^{-1} $CaCO_3$)	1.99 ± 0.13	1.44 ± 0.01	1.42 ± 0.02
Hardness (mg L^{-1} $CaCO_3$)	46.80 ± 8.66	46.80 ± 7.23	59.60 ± 14.68
Dissolved organic matter (PtCo)	65.00 ± 4.00	73.00 ± 5.80	69.00 ± 3.42
Ammonia (mg L^{-1})	0.19 ± 0.11	0.24 ± 0.08	0.26 ± 0.15
Nitrites (mg L^{-1})	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
Nitrates (mg L^{-1})	2.37 ± 1.39	2.25 ± 0.65	2.60 ± 1.32
Bicarbonates HCO_3^- (mg L^{-1})	106.43 ± 30.38	78.56 ± 18.80	76.93 ± 19.07
Total Phosphorus (mg L^{-1})	0.46 ± 0.75	0.24 ± 0.12	0.47 ± 0.61
Calcium (mg L)	10.43 ± 1.02	11.33 ± 4.10	9.26 ± 1.11
Magnesium (mg L^{-1})	5.20 ± 0.35	4.46 ± 4.02	8.73 ± 4.05
Total coliforms (MPN 100 mL^{-1})	766 ± 461[a]	23333 ± 3785[b]	16333 ± 5196[ab]
Fecal coliforms (MPN 100 mL^{-1})	110 ± 108[a]	15000 ± 5686[b]	6000 ± 1457[b]

(PCNA; 1:100; clone PC10; DAKO, Denmark) at 4 °C. Finally, sections were incubated with both a biotinylated secondary antibody and streptavidin conjugated to peroxidase, using a commercial kit (LSAB™2 Kits, DAKO, Denmark) and revealed with 3-3′-diaminobenzidine (Sigma-Aldrich, USA).

PCNA-immunoreactive cells were quantified using photographs of sections corresponding to specific brain regions or nuclei (telencephalic area, diencephalic nucleus, thalamic area). The quantification was performed using the ImageJ program (National Institutes of Health; http://rsbweb.nih.gov/ij/).

2.6. Statistical analysis

Data were tested for normality and homogeneity using Shapiro Wilks and Levene's test, respectively. Differences in biomarker responses among sites were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. When assumptions were violated, data were analyzed using Kruskal-Wallis nonparametric test. $P < 0.05$ was considered to be statistically significant. Statistical analysis was performed by the InfoStat software (Di Rienzo et al., 2015). All quantitative data are expressed as means with standard error of the mean (± SEM).

3. Results

3.1. Water quality and occurrence of pharmaceuticals

Table 1 shows the physicochemical parameters measured at each exposure site. Except for fecal indicators, which were higher at the effluent site, no significant changes were found in the parameters among sites.

Concentrations of human pharmaceuticals in water samples are presented in Table 2. In general, the concentrations of the total pharmaceuticals measured at the beginning and the end of the field

Table 2
Concentration of human pharmaceuticals in the Colastiné River (ng L^{-1}) measured at the beginning (first line) and the end (second line) of the caging experiment. LOD/LOQ: atenolol 0.7/3.3; caffeine 1.9/9.4; carbamazepine 0.2/1.0; elanapril 0.3/1.5; sildenafil 0.6/3.0.

Pharmaceutical	Upstream	Effluent	Downstream
Atenolol	4.8	3.5	7.4
	< LOQ	< LOQ	< LOQ
Caffeine	38.4	9.6	11.7
	21.1	16.5	< LOQ
Carbamazepine	< LOD	4,005.7	< LOD
	< LOD	13,768.8	10,578.8
Enalapril	96.3	2,114.0	48.6
	44.5	26,720.2	17,892.8
Sildenafil	19.7	237.8	20.9
	12.8	560.9	452.4

experiment were the highest at the effluent site and the lowest at the upstream site. The major contributors were enalapril and carbamazepine with concentrations usually above the μg L^{-1}, followed by sildenafil. Enalapril and sildenafil were detected in all analyzed samples. The concentrations of atenolol and caffeine were low and no clear differences were observed among sites.

3.2. Biochemical markers

All fish survived till the 15th day of exposure and no significant differences in enzyme activities were detected between the basal group (baseline level) and the fish caged at the upstream site. Our results proved that the caging experiment is not stressful for fish and the upstream site can be considered as an appropriate reference site.

Biochemical markers are summarized in Table 3. Effluent exposure caused significant changes in brain caspase and CAT. Besides, an increase in gill GST and SOD was also observed. In liver, there were no significant differences in enzymatic activities, except for the AST activity which increased in the fish caged at the effluent. Lipid oxidative damage was recorded in gills and liver of fish exposed to the effluent.

3.3. Energy reserves

After a 15-day exposure at the effluent site, fish showed a significant increase in glycogen levels and a reduction in lipid levels in liver. Regarding muscle determinations, no differences were found (Table 4).

3.4. Histopathological examination

The main histological alterations found in gills consisted of aneurysms and hemorrhages, which were classified as the reaction pattern 1 (I_{rp1}). Progressive changes (I_{rp3}) such as hypertrophy or hyperplasia of epithelial cells, and partial or total lamellar fusion were also observed. The highest mean values of reactions (I_{rp1}; I_{rp3}) and organ indexes (I_{gills}) were obtained at the effluent and downstream sites. However, no significant differences among the calculated indexes were identified with respect to the upstream site (Table 5).

The most common liver histopathologies included circulatory disturbances (hemorrhages, I_{rp1}) and regressive changes (*e.g.*, irregular shape of hepatocytes nucleus, pyknosis and karyolysis, stagnation of the bile outflow from the hepatocytes and necrosis, I_{rp2}). In addition, major inflammatory processes (areas with lymphocytes and melanomacrophages infiltration, I_{rp4}) were only evident in some individuals exposed to the effluent site. Regressive changes index (I_{rp2}) and the general organ index (I_{liver}) increased significantly in fish exposed to the effluent (Table 5).

Table 3
Biochemical markers measured in different tissues of *P. lineatus* caged at upstream, effluent and downstream sites in the Colastiné River. Values are expressed as mean ± SEM. Different letters indicate significant differences ($p < 0.05$). The enzymatic activity is reported in nkat mg prot^{-1}, except for caspase-3 (arbitrary unit).

	Basal group	Upstream	Effluent	Downstream
Brain				
GST	2.12 ± 0.25	2.69 ± 0.08	2.28 ± 0.38	1.75 ± 0.19
GPx	7.47 ± 1.42	8.08 ± 0.77	8.81 ± 1.10	5.85 ± 0.48
GR	0.38 ± 0.07	0.35 ± 0.02	0.41 ± 0.06	0.29 ± 0.01
CAT	104.69 ± 22.34^{b}	114.85 ± 7.67^{b}	60.34 ± 5.50^{a}	81.68 ± 2.17^{ab}
SOD	1114.56 ± 135.53	1268.42 ± 120.02	1012.37 ± 119.02	977.86 ± 77.35
AChE	9.62 ± 1.20	7.80 ± 1.11	7.35 ± 1.44	5.56 ± 0.47
LPO	0.24 ± 0.09	0.22 ± 0.05	0.15 ± 0.02	0.21 ± 0.04
Caspase-3	0.044 ± 0.005^{a}	0.054 ± 0.003^{a}	0.086 ± 0.007^{b}	0.054 ± 0.005^{a}
Gills				
GST	1.04 ± 0.08^{a}	1.69 ± 0.11^{ab}	2.41 ± 0.26^{bc}	2.57 ± 0.29^{c}
GR	0.25 ± 0.03	0.21 ± 0.02	0.30 ± 0.03	0.30 ± 0.03
CAT	122.02 ± 16.67	134.69 ± 11.67	178.70 ± 34.51	190.21 ± 11.00
SOD	40.01 ± 5.50^{a}	62.01 ± 6.17^{a}	119.69 ± 15.50^{b}	82.35 ± 16.33^{ab}
LPO	0.04 ± 0.01^{a}	0.05 ± 0.01^{ab}	0.12 ± 0.02^{b}	0.04 ± 0.01^{a}
Liver				
GST	3.10 ± 0.84	1.77 ± 0.38	1.32 ± 0.23	1.32 ± 0.15
GR	0.52 ± 0.03	0.47 ± 0.13	0.71 ± 0.20	0.41 ± 0.08
CAT	196.21 ± 62.18	243.55 ± 25.17	358.57 ± 32.34	305.39 ± 37.34
SOD	1000.37 ± 383.91	864.17 ± 169.53	1027.04 ± 128.36	712.31 ± 164.53
ALT	362.91 ± 59.51	358.07 ± 94.19	461.59 ± 105.35	520.10 ± 62.35
AST	2.83 ± 0.33^{a}	3.67 ± 0.83^{a}	7.00 ± 0.50^{b}	4.17 ± 0.50^{a}
LPO	0.20 ± 0.03^{ab}	0.11 ± 0.02^{a}	0.33 ± 0.07^{b}	0.09 ± 0.02^{a}

Table 4
Energy reserves of *P. lineatus* caged at upstream, effluent and downstream sites in the Colastiné River. Values are expressed as mean ± SEM. Different letters indicate significant differences ($p < 0.05$). Glycogen and lipid content were expressed as μmol g^{-1} wt and protein levels as mg g^{-1} wt.

	Upstream	Effluent	Downstream
Liver			
Glycogen	50.82 ± 14.85^{a}	126.06 ± 20.47^{b}	79.98 ± 19.73^{ab}
Lipid	10.29 ± 0.64^{ab}	8.38 ± 0.52^{a}	11.45 ± 0.74^{b}
Protein	134.54 ± 13.20	100.76 ± 13.30	130.11 ± 8.93
Muscle			
Glycogen	0.89 ± 0.11	0.67 ± 0.10	0.96 ± 0.23
Lipid	3.09 ± 0.33	3.52 ± 0.58	3.21 ± 0.33
Protein	117.51 ± 13.09	132.95 ± 9.27	147.04 ± 6.69

Table 5
Categorical and total pathological indexes of *P. lineatus* caged at upstream, effluent and downstream sites in the Colastiné River. Values are expressed as mean ± SEM. Different letters indicate significant differences ($p < 0.05$). Reaction pattern: I_{rp1} circulatory disturbances; I_{rp2} regressive changes; I_{rp3} progressive changes; I_{rp4} inflammation. N.O.: Not observed effect.

	Upstream	Effluent	Downstream
Gills			
I_{rp1}	3.40 ± 1.50	5.40 ± 1.08	7.00 ± 0.32
I_{rp2}	N.O.	N.O.	N.O.
I_{rp3}	4.80 ± 1.39	5.60 ± 0.60	4.40 ± 1.66
I_{rp4}	N.O.	N.O.	N.O.
I_{gills}	8.20 ± 0.58	11.00 ± 1.10	11.40 ± 1.81
Liver			
I_{rp1}	0.50 ± 0.29	1.14 ± 0.26	1.00 ± 0.41
I_{rp2}	1.00 ± 0.71^{a}	9.14 ± 1.97^{b}	5.75 ± 1.25^{ab}
I_{rp3}	N.O.	N.O.	N.O.
I_{rp4}	N.O.	0.86 ± 0.46	N.O.
I_{liver}	1.50 ± 0.96^{a}	11.14 ± 2.16^{b}	6.75 ± 1.11^{ab}

In both organs, tumors were not recorded; therefore I_{rp5} has not been included in Table 5.

3.5. Brain proliferation

Fig. 1 shows the cell proliferation analysis of different forebrain regions in *P. lineatus*. A tendency to reduce the number of proliferative cells was observed in the telencephalic area of the fish exposed to the effluent and downstream sites (Fig. 1A). An increase of the PCNA immune reactive cells was observed in the anterior part of the preoptic area; though it was not statistically significant (Fig. 1B). Exposure to the effluent site caused a significant decrease in PCNA immunoreactive cells along the thalamus and periventricular zone of the posterior tuberculum in the diencephalon (Fig. 1C). No changes in cell proliferation were observed in the diencephalic nucleus of the lateral recess (Fig. 1D).

4. Discussion

Sewage effluents are complex mixtures of various contaminants. Even though chemical analyses allow us to register many pollutants present in wastewater they are still not capable of accounting for all of them. Moreover, chemical analyses alone are unable to demonstrate the impact of chemical pollution on the aquatic organisms. In order to evaluate the toxicological effects of a sewage effluent on the *Prochilodus lineatus* fish, we assessed biological parameters at different levels of organization.

In general, no significant differences in physico-chemical parameters among the caging sites were found, except for fecal indicators. As it was expected, the highest concentration of coliforms was found at the effluent site decreasing towards the downstream site, in agreement with a previous monitoring (Cazenave et al., 2014).

In addition, pharmaceuticals were reported for the first time in the Colastiné River. Caffeine, atenolol (ATE), carbamazepine (CBZ), enalapril (ENAL) and sildenafil (SIL) were detected in the water river samples, some of them (ENAL and SIL) were ubiquitous in all analyzed samples. High concentrations of pharmaceuticals were a good indicator of wastewater discharges. Concentrations of ENAL and CBZ in particular were very high in the Colastiné River, usually presenting values

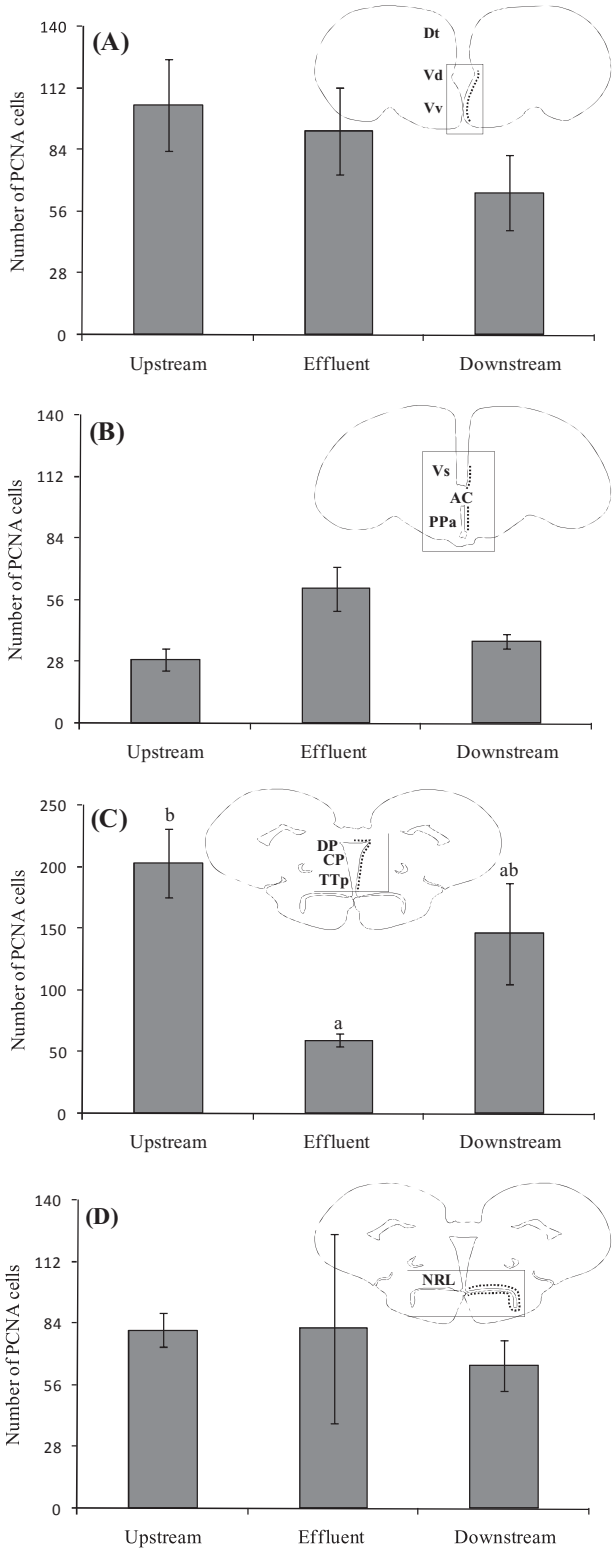

Fig. 1. Analysis of proliferating cell nuclear antigen (PCNA)-positive cells in different forebrain regions. Inset boxes show regions in which proliferation has been quantified: (A) telencephalic area (B) anterior part of the parvocellular preoptic nucleus (diencephalic nucleus) and supracommissural nucleus of telencephalic area (periventricular nucleus) (C) thalamic area, and (D) nucleus of the lateral recess (diencephalic nucleus). The number of proliferative cells is expressed as mean ± SEM. Different letters indicate significant differences ($p < 0.05$). Abbreviations: AC: anterior commissure; CP: central posterior thalamic nucleus; DP: dorsal posterior thalamic nucleus; Dt: Dorsal telencephalic area; NRL: nucleus of the lateral recess; PPa: anterior part of the parvocellular preoptic nucleus; TPp: periventricular nucleus of posterior tuberculum; Vd: dorsal nucleus of ventral telencephalic area; Vs: supracommissural nucleus of telencephalic area; Vv: ventral nucleus of ventral telencephalic area.

above the 2–27 μg L^{-1} at the downstream site. These concentrations were higher than those previously recorded in other water courses of Argentina (Elorriaga et al., 2013a,b; Elorriaga et al., 2013a,b; Valdés et al., 2014). A great variation in those pharmaceutical concentrations (equivalent to more than an order of magnitude) was observed at different sampling moments (at the beginning *vs.* the end) (Table 2). Several factors could have contributed to this variability, such as temporal variations in their usage, hydrological and climate changes (river flow, currents, rainfall), leading to a different spatial distribution of the plume. Similar findings have been observed for some ECs in previous studies, *e.g.* by Petrie et al. (2015).

On the other hand, caffeine concentration, a conventional indicator of anthropogenic pollution (Ferreira, 2005), and ATE were not clearly related with the discharge from the city sewage system (Effluent site). Drugs found at the upstream site may come from the substandard housing wastewater discharges along the banks of the Colastiné River.

The detection of pharmaceuticals in river waters has led to a growing concern about their potential effects on aquatic biota. The acute effects reported for pharmaceuticals were connected with concentrations usually 100–1000 higher than those found on the aquatic environment, but chronic adverse effects were seen in the range of wastewater concentrations (Fent et al., 2006; Li et al., 2010).

In our caging study, throughout the exposure period no mortality was recorded, suggesting that the survival of the exposed fish was not directly compromised by the wastewater effluent. However, several toxicological effects were observed and they should not be overlooked.

Oxidative stress may lead to enzymatic inactivation, lipid peroxidation (LPO), DNA damage and eventually cell death (van der Oost et al., 2003). In the present work, oxidative stress was observed as an increased LPO in liver and gills. In addition, a significant increase in GST and SOD activities was found in the gills of the effluent-exposed fish in comparison to the upstream- ones. The GST is involved in the phase II detoxification system and it also has an important antioxidant role in the reduction of a wide range of organic hydroperoxides by using glutathione (van der Oost et al., 2003). Besides, antioxidant enzymes such as SOD play a crucial role in maintaining a low rate of reactive and detrimental hydroxyl radicals. Despite the significant increase in GST and SOD activities, the lipid oxidative damage in gills could not be lessened by that antioxidant defense system. In a previous work, a short-term exposure (96 h) to the effluent caused an enhancement of antioxidant enzymes activities in gills, which could prevent lipid peroxidation (Cazenave et al., 2014). Nevertheless, our present results suggest that a longer exposure produces oxidative damage in gills of *P. lineatus*.

The effluent exposure also caused higher LPO levels in the liver of such fish. Similar results have also been observed in the liver of other fish species exposed to sewage effluents (Carney Almroth et al., 2008; Cazenave et al., 2014; Oakes et al., 2004; Vieira et al., 2016). Lipid peroxidation alters the bilayer structure and the physiological functions of cell membranes, playing a key role in the cellular membrane damage. In line with these results, a rise in the AST activity was also found in the liver of fish caged at effluent site. Stress induces a rise of the transamination pathway (Hori et al., 2006); and levels of AST and/or ALT become elevated in the liver of fish exposed to different environmental pollutants, reflecting hepatocellular damage both in the present study and the previous ones (Cazenave et al., 2014; de la Torre et al., 2005; Samanta et al., 2014; Van Campenhout et al., 2010).

A high fecal coliform load has been considered as an oxidative stress generator in the gills of mussels (Bianchi et al., 2014) and fish (Dautremepuits et al., 2009). Some pharmaceuticals such as CBZ, diclofenac, clofibrate and clofibric acid have also proved to induce oxidative stress in fish tissues (Brandão et al., 2013; Diniz et al., 2015; Islas-Flores et al., 2017; Li et al., 2010; Nunes et al., 2008). Therefore, it is possible that the exposure to coliforms, pharmaceuticals and many other xenobiotics in the effluent plume could be the responsible for inducting oxidative stress in the present experiment.

Effluent exposure also had an impact on the hepatic reserves by increasing glycogen levels and decreasing the lipid reserves, *i.e.* unfavorable environmental conditions affect metabolism leading to alterations in the energetic reserves of fish (Cattaneo et al., 2008; Menezes et al., 2015; Rossi et al., 2017). Thus, the reduction observed in the lipid content of the effluent-exposed fish indicates that the subchronic exposure to an untreated sewage triggers a mobilization of macromolecules to meet energy demands. The mobilization of energy reserves enables the fish to cope with the increased energy demand associated with stress (Cazenave et al., 2006; Wolf and Wolfe, 2005). Therefore, a decrease in hepatic glycogen levels would be expected. However, the response we found in the effluent-exposed fish was the opposite. Saravanan et al. (2011) reported that an increase in the liver and muscle glycogen content during sublethal lindane exposure may indicate a fish adaptation or impairment in the carbohydrate metabolism. In addition, due to their bottom feeding behavior (Bayo and Cordiviola de Yuan, 1996), *P. lineatus* were more likely to have higher food availability in the effluent site, leading to an increased glycogenesis in their liver.

Unlike most biochemical parameters, histological parameters are generally indicative of irreversible damage as they involve changes at higher levels of biological organization (Hinton et al., 1992). An impact of the effluent exposure at histological level was observed in the present study. Several alterations such as circulatory disturbances, lamellar fusion and hyperplasia or hypertrophy of epithelial cells, were observed in gills. These pathologies gradually affect the functions of gills and they are typical responses to acute and chronic exposure to a wide spectrum of contaminants (Ahmed et al., 2013; Cengiz and Unlu, 2006; Chovanec et al., 2003). Despite the highest mean values of gill lesion indexes obtained at both effluent and downstream sites, differences among them were not statistically significant.

On the contrary, marked differences in liver lesion indexes were observed. Regressive changes in particular were significantly higher in the liver of fish exposed to the effluent. This reaction pattern has been considered by Bernet et al. (1999) as a process which results in a functional reduction or loss of an organ. An increase in focal lymphocytic infiltration and macrophage aggregates was also observed. These changes could be associated with stress condition or infectious diseases (Agius and Roberts, 2003). Overall, our histological results are similar to those described in gills and liver of the brown trout (*Salmo trutta f. fario*) caged for 10 and 30 days downstream of STP discharges (Vincze et al., 2015).

Both biochemical and histopathological results have demonstrated that the physiological functions of the liver make this organ more adversely affected than the gills. The liver is not only a key organ for the basic metabolism of fish, but also a major place for biotransformation, accumulation and excretion of toxicants (Chovanec et al., 2003).

As regards fish brain some novel biomarkers of neurotoxicity were assessed in order to examine the effects caused by sewage effluents. To our knowledge, this is the first report relating sewage effluent exposure to the occurrence of apoptosis and cell proliferation. The increased activation of caspase-3 in the effluent site might account for the apoptosis in the fish brain. Similarly, an activation of caspase-3 in the brain of *Oreochromis niloticus* exposed to industrial effluents and pesticides was observed (Franco et al., 2010).

In mammals, the induction of apoptosis and neurodegenerative diseases are often associated with the production of ROS and the occurrence of LPO (Gandhi and Abramov, 2012). With the exception of a decreased CAT activity, no significant differences in brain oxidative stress markers were observed in the present study. CAT scavenges superoxide anion preventing pathological processes in cells (Matés, 2000). Thus, the intracellular conditions that trigger the apoptosis could be generated by CAT activity inhibition, the accumulation of superoxide anion and/or hydrogen peroxide, and probably other ROS.

In addition, a decrease in the cell proliferation in both the thalamus and the posterior tuberculum in the diencephalon of fish caged at the effluent was observed. It has been demonstrated that some chemicals usually present in wastewaters, such as estradiol, decreased cell proliferation in the telencephalon and in the mediobasal hypothalamus of adult *Danio rerio* (Diotel et al., 2013). The PCNA gene expression also tended to decrease in the *D. rerio* larvae treated with ethinylestradiol, suggesting that this pharmaceutical disrupts the proliferative activity in the brain (Pellegrini et al., 2016). Taking into account that proliferative cells can differentiate into neurons (Pellegrini et al., 2007), changes in the proliferative pattern could affect the subsequent neuronal differentiation. Burdon et al. (1996) showed that in a hamster fibroblasts culture, inhibitor agents of GPx and CAT not only depress proliferation rates but also lead to an increase in the appearance of apoptotic-like cells, similarly to the results obtained in the brain of *P. lineatus*.

5. Conclusions

In this study we assessed the biochemical and histological responses of *P. lineatus* under subchronic exposure to untreated sewage effluents. Our results have demonstrated that the effluent represents an important input of stressors in the Colastine River. A high coliform load and pharmaceuticals such as ATE, CBZ, ENAL and SIL were detected. However, a wide variety of other pollutants could be present in the effluent.

Our results also showed that the effluent has deleterious effects on several biomarkers, depending on the organ. The sewage effluent induces oxidative stress in gills and liver as it was evidenced by lipid peroxidation. In liver, both metabolic changes and histological damage were also observed. It is worth noting that the occurrence of apoptosis in the fish brain, caused by an increased activation of caspase-3 in the effluent site, as well as the changes in cell proliferation, becomes important aspects to be considered in further studies.

It is difficult to establish a cause-and-effect relationship in the environment as a mixture of toxicants might be acting simultaneously. However, the present study highlights the importance of understanding the realistic effects of *in situ* exposure to a sewage effluent on fish. The influence of some detected pharmaceuticals on the native fish species should be addressed in future laboratory studies using single and mixture exposures. Our findings also draw attention to the need to counteract this load of stressors that disturb several physiological processes and produce histological damage in the feral fish of the studied area and rivers where effluents are discharged.

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