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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