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Potential environmental toxicity of sewage effluent with pharmaceuticals

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Abstract

Sewage effluent effects on the biochemical parameters of *Astyanax bimaculatus* organs were investigateted. Treated sewage was collected in a treatment plant; 43 compounds, among them, pharmaceuticals and hormones, were investigated. Caffeine, ciprofloxacin, clindamycin, ofloxacin, oxytetracycline, paracetamol, sulfadiazine, sulfamethoxazole, sulfathiazole and tylosin waste was detected in the collected material. Fish were divided into four groups: control, TSE (treated sewage effluent), TSE + P (TSE with increased concentration of five pharmaceuticals) and PTSE (TSE + P post-treated with O_3/H_2O_2/UV). Biochemical parameters were evaluated in different organs after 14-day exposure. TBARS levels increased significantly in the brain of animals in the TSE and TSE + P groups in comparison to the control. There was significant reduction in TBARS levels recorded for the liver, muscle and gills of animals in the PTSE group in comparison to those of animals in the other groups. AChE activity reduced in the muscle of animals in the groups showing the highest pharmaceutical concentrations. CAT activity in the liver of animals in groups exposed to pharmaceutical effluent was inhibited. GST activity increased in brain of animals in the TSE + P and PTSE groups, whereas reduced levels of this activity were observed in liver of animals in the TSE group. Increased GST activity was observed in the brain of animals in TSE + P and PTSE groups. Based on integrated biomarker response values, the TSE + P group presented greater changes in the analyzed parameters. Results point out that pharmaceutical waste can cause oxidative stress, as well as affect biochemical and enzymatic parameters in *Astyanax* sp. Post-treatment can also reduce damages caused to fish, even in case of the likely formation of metabolites. Based on these results, these metabolites can be less toxic than the original compounds; however, they were not able to fully degrade the pharmaceutical waste found in the sewage, which can interfere in fish metabolism.

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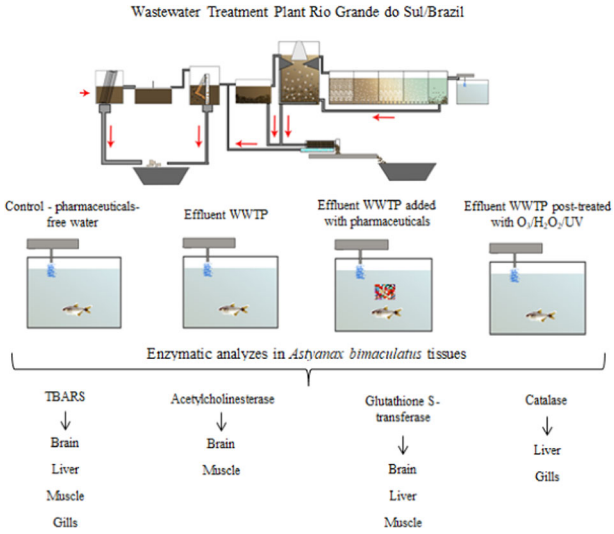
Graphical Abstract



Keywords Ecotoxicity · Enzyme activity · Antibiotics · Emerging pollutants · Sewage effluent

Introduction

Pharmaceuticals in aquatic environments has become a global concern due to adverse effects caused by them on both ecosystems and human health (Liu et al. 2018). The main disturbances are observed in the biochemical systems of living organisms and they are caused by highly stable and persistent substances that are discharged in the environment (Verlicchi et al. 2012; Cortes-Diaz et al. 2017). The introduction of complex wastewater mixtures from treatment plants presenting low pharmaceuticals' removal efficiency is the primary source of these compounds in the environment, especially in surface water (Li 2014; Knopp et al. 2016; Balakrishna et al. 2017; Cortes-Diaz et al. 2017; Sehonova et al. 2017; Felis et al. 2020). However, animal waste discharge in the soil and in water (Verlicchi et al. 2012), as well as clandestine domestic waste disposal, inappropriate disposal of medicines and packages, landfills and leaks in sewage network (Li 2014; Gavrilescu et al. 2015; Giebultowicz and Nałecz-Jawecki 2016) also contribute to contamination by pharmaceuticals.

Previous studies have described a series of (eco)toxicological effects caused by pharmaceuticals on the environment, which leads to reproductive and growth disorders in animals and humans (Carlsson et al. 2006; Galus et al. 2013; Liu et al. 2018) to damages to algae chloroplasts (Liu et al. 2018), water toxicity (Verlicchi et al. 2012; Galus et al. 2013; Ebele et al. 2016; Liu et al. 2017) and to the selection of multi-resistant microorganisms (Klatte et al. 2017) that pose global threat to human and animal health—many bacterial species have developed some resistance to antimicrobial agents (Felis et al. 2020; Booth et al. 2020). Antimicrobial resistance (AMR) is triggered by the bacteria's ability to become resistant to antibiotics through mutation, as well as by the acquirement of genes that provide resistance to antimicrobials. Accordingly, AMR accounts for thousands of deaths worldwide, on a yearly basis. These numbers tend to increase due to the increasingly expressive presence of pharmaceuticals in the environment (O'Neill 2016; Fies et al. 2020). Yet, (eco) toxicological effects are associated with the increased incidence of cancer in humans' reproductive system and of endometriosis (Aquino et al. 2013). According to Farré et al. (2008), Velicchi et al. (2012) and Li (2014), antidepressants, antibiotics, antipsychotics, cardiovascular drugs, antineoplastics, as well as natural and synthetic hormones, are the therapeutic classes of pharmaceuticals presenting the highest (eco)toxicological potential.

It is very difficult to identify (eco)toxicological effects due to lack of acute toxicity observation in organisms, even at significant concentrations (Morley 2009), since toxicity does not follow a uniform standard applicable to global assessments (Guo et al. 2020). In addition, in order to reduce or eliminate pharmaceuticals from effluent, it is essential applying advanced treatment systems to retain or oxidize pharmaceuticals. Recent studies have indicated that advanced oxidative processes (AOPs) used to associate ultraviolet radiation, hydrogen peroxide and ozone, among others, can be highly efficient (99%) in removing certain pharmaceuticals in aqueous matrices (Anjali and Shanthakumar 2019)

and also reduce toxicological risks (Alvim et al. 2020; Guo et al. 2020). However, unknown effects of metabolites can be generated by oxidation; their toxicity can be lower, higher or equivalent to that of the original compounds (Verlicchi et al. 2012; Gavrilescu et al. 2015; Azuma et al. 2016; Giannakis et al. 2017). Therefore, it is essential assessing the toxicity rates in order to estimate the potential risks of treating wastewater through AOPs (Guo et al. 2020). Nevertheless, biochemical changes in organisms use to be the first responses to environmental changes that can be detected and quantified, mainly those at enzymatic activity level (Clasen et al. 2014; Pérez et al. 2018).

Thus, assessing biomarkers in fish is an alternative to understand chronic (eco)toxicological effects related to fauna exposure to different chemical substances and to emerging pollutants likely found in aqueous matrices (Clasen et al. 2014; Braz-Mota et al. 2015; Cortes-Diaz et al. 2017; Guiloski et al. 2017; Pérez et al. 2018) that have been included in environmental improvement programs (Liu et al. 2018). Biomarkers belonging to different biochemical processes, such as the level of substances reactive to thiobarbituric acid, used to assess oxidative damage, the activity of acetylcholinesterase enzymes to determine neurotoxicity, and the catalase and glutathione S-transferase in antioxidant and detoxification activity are important tools to provide broad answers to contaminants' action mechanisms in organisms.

Oxidative stress processes result from unbalance between oxidatant and antioxidant compounds (Braz-Mota et al. 2015) that can trigger the generation of reactive species or the inability to defend and/or to change the antioxidant profile of living organisms (Clasen et al. 2014; Samanta et al. 2018), to compromise cell and tissue biological functions, as well as homeostatic balance (Halliwell and Whiteman 2004; Cortes-Diaz et al. 2017). Enzymatic and non-enzymatic activities are essential as antioxidant defense mechanisms to neutralize the effects of reactive oxygen species (ROS).

Reaching a general conclusion about the severity of stressors can be a challenging process, mainly when multiple stressors are taken into consideration; therefore, Integrated Biomarker Response (IBR) (Guerlet et al. 2010) was used in the present study. IBR use allows combining different biomarker responses observed in the different analyzed organs and, consequently, provides more comprehensive and integrative understanding about wastewater effects on the assessed treatments (Maulvault et al. 2018). In addition, it enables comparing deviations between biomarkers of specimens collected from polluted and treated sites to information recorded for the reference site. Parameters were plotted in star plot to represent the reference deviation of each assessed biomarker (Olivares-Rubio et al. 2013).

The aim of the current study was to investigate the sublethal effects of sewage effluent on the biochemical parameters and oxidative stress observed in *Astyanax bimaculatus* organs by taking into account the constant exposure of fish to pharmaceutical waste discharged into surface waterbodies, given their importance to the food chain and wide geographic distribution in the neotropical region.

Materials and methods

Origin and collection of effluent with pharmaceuticals

Assays were conducted with sewage effluent from an effluent treatment plant (ETP) located in the metropolitan area of Porto Alegre, Rio Grande do Sul State, Brazil. The assessed system operates with flow of $2250 \pm 250\ L\ s^{-1}$, on average, which serves ~600,000 individuals and consists in a mechanical preliminary treatment unit to screen and desanding effluent. These procedures were followed by biological treatment in UASB reactors and sludge activated to allow the cyclic treatment at aeration and sedimentation stage, and disinfection with hydrogen peroxide (H_2O_2). This hydrogen peroxide is easily decomposed into oxygen and water in the presence of catalytic impurities found in the effluent, and under increased temperature, pH above 5 and exposure to light, eliminating it before carrying out the post-treatment.

Treated samples were collected every hour from 07:00 a.m. to 12:00 a.m. until getting the total volume of 80 L required for the tests. Non-toxic and high-density polyethylene bottles (HDPE) (20-L capacity)—which were previously sanitized and rinsed—filled with samples were used at collection time. The aliquot of 1 L of sample was collected with amber bottle to determine the pharmaceuticals and hormones in it; the sample was refrigerated at ±4 °C until analysis time.

Part of the collected effluent was used to prepare two other treatments, which were described in the experimental project. These treatments were also subjected to analysis of biochemical parameters and oxidative stress.

Determining the presence and concentration of pharmaceuticals and hormones in effluent

The treated effluent sample was subjected to analysis focused on 43 compounds, including human prescription pharmaceuticals, veterinary drugs and hormones, at the Pesticide Residue Analyses Laboratory (LARP), UFSM. The aliquot of 100 mL of sample was pre-concentrated through solid phase extraction (SPE) with Strata®-X

cartridge—adapted from Jank et al. (2014). After sample preparation, the presence of pharmaceuticals and hormones was determined through ultra-high performance liquid chromatography coupled to Waters' Mass Spectrometry (UHPLC-MS/MS) (USA). In summary, liquid chromatograph; triple quadrupole type MS detector, model Xevo TQ; electrospray ionization source; Waters Acquity UPLC® BEH C18 analytical column (50 × 2.1 mm, 1.7 µm) (USA) and MassLynx 4.1 software data acquisition system (Waters, USA) were used in the study. Other effluent samples with pharmaceuticals were subjected to the same determination process.

Fish

A fish farm provided 72 *A. bimaculatus* specimens presenting mean weight of 8.0 ± 1.5 g and mean length of 8.2 ± 0.7 cm to be used as test organisms. Animals were allowed to acclimate for ten days in 250 L non-toxic polyethylene tank filled with clean water dechlorinated through intense aeration, in static system conditioned to laboratory conditions under natural photoperiod (12 h dark/12 h light). Fish fed on commercial Supra® twice a day (42% crude protein), in amount equivalent to 5% of their body weight, during the acclimation and experimental periods.

Experimental project

Fish were randomly divided into four groups after the acclimation period. Each group comprised 18 fish ($n = 6$, in triplicate) distributed in 40-L plastic tanks subjected to constant aeration. The control group was kept in tank with pharmaceutical-free dechlorinated water. The second group (TSE) was exposed to treated sewage. The third group (TSE + P) was exposed to the same effluent from group two (TSE) added with ciprofloxacin (11.44 µg L^{-1}), oxytetracycline (7.93 µg L^{-1}), paracetamol (151.17 µg L^{-1}), sulfamethoxazole (188.69 µg L^{-1}) and trimethoprim (30.65 µg L^{-1}) at concentrations higher than the ones predicted to avoid adverse effects (PNEC) on the most sensitive fish species, micro-crustaceans or algae (Li 2014). PNEC did not provide the upper concentration limit for a given medicine or for other chemicals that have some sort of toxic effect; however, it intends to point out concentrations that may pose risk to the species. The greater persistence and/or occurrence in the sample investigated and observed in other studies in the literature (Martín et al. 2012; Dinh et al. 2017; Hu et al. 2018; Bisognin et al. 2018) are other reasons to investigate the increased concentration of these pharmaceuticals. The fourth group (PTSE) was exposed to the effluent from group 3 (TSE + P) post-treated with O_3/H_2O_2/UV (0.5 mgO_3 mgCOD^{-1}/25 mgH_2O_2 L^{-1}/15 min UV). This treatment resulted from the combination of techniques described by Zimmermann et al. (2011), Afonso-Olivares et al. (2016) and Alvim et al. (2020). The association of these processes is also justified by the study by Xu et al. (2017), who highlighted that cations (Fe^{3+}, Cu^{2+}, NH_4^+) and anions (NO_3^-, HCO_3^-, HPO_4^{2-}), as well as high total organic carbon (TOC) levels inhibit target compound removal in more polluted matrices, such as effluents. Thus, higher oxidants doses and/or oxidant association are required and demand longer to fully remove pharmaceuticals from complex natural samples in order to avoid the formation of more toxic intermediate products.

The experiment was conducted for 14 days; during this period, the following mean daily parameters were monitored: dissolved oxygen 5.6 ± 0.8 mg L^{-1}, temperature 23.2 ± 2.6 °C, pH 6.8 ± 0.2, electrical conductivity 462.0 ± 13.6 (µS cm^{-1}) and alkalinity 92.4 ± 3.2, ammonia and nitrite.

Fish were euthanized through medullary section after the experimental period, and their body mass and length were measured. Subsequently, the brain, gills, liver and muscle were collected for biochemical tests.

Biochemical parameters

Reagents used in the assays were purchased at Sigma Chemical Co. (St. Louis, MO, USA)—they had high purity degree (95–99%).

Oxidative damage assay (TBARS)

Lipid peroxidation was estimated in TBARS assay carried out based on malondialdehyde reaction (MDA) with 2-thiobarbituric acid (TBA). Results were read in spectrophotometer, according to Buege and Aust (1978). The assay was conducted with the gills (50 mg), brain (50 mg), liver (50 mg) and muscles (250 mg). The organs were homogenized in Potassium Phosphate (20 mM) and centrifuged at 5000 × *g* for 10 min (min). Subsequently, 10% trichloroacetic acid (TCA) and 0.67% thiobarbituric acid were added to homogenates in order to adjust the sample to the final volume of 1 mL. Reaction mixtures were incubated at 95 °C for 30 min. Blends were centrifuged at 5000 × *g* for 15 min after cooling; the optical density was measured through spectrophotometry, at 532 nm. TBARS levels were expressed as nmol MDA mg $protein^{-1}$.

Acetylcholinesterase (AChE) activity assay

AChE activity was determined based on the method described by Ellman et al. (1961). Brain (30 mg) and muscle (50 mg) extracts were prepared and homogenized in 50 mM sodium phosphate buffer, at pH 7.2 and 1% Triton X-100. The homogenate of each tissue was centrifuged for 10 min at 3000 × *g* at 5 °C; the supernatant was used as

enzyme source. Aliquots of 50 µL of brain extract supernatant and 100 µL of muscle extract were incubated at 30 °C for 2 min in buffer solution added with 100 mM sodium phosphate, at pH 7.5, and using 10 mM DTNB as chromogen. After incubation, reaction was triggered by acetylcholine (ACh: 0.5 mM) addition as substrate to the reaction mixture—final volume was 2.0 mL. Absorbances were determined through spectrophotometry at 412 nm for 2 min. Enzymatic activity was expressed in µmol ACh hydrolyzed min^{-1} mg $protein^{-1}$.

Catalase activity assay (CAT)

CAT activity was determined in gill (50 mg) and liver (50 mg) tissues through ultraviolet spectrophotometry, according to Nelson and Kiesow (1972). Tissues were homogenized in Potter tissue homogenizer—10 volumes (w/v) of 20 mM potassium phosphate buffer—at pH 7.5 and centrifuged at 10,000 × *g* for 10 min at 4 °C. Assay mixture comprised 2.0 mL of potassium phosphate buffer (50 mM, pH 7.0), 50 µL of H_2O_2 (0.3 M) and 10 µL of gill homogenate. The same procedure was applied to liver tissue. Change in H_2O_2 absorption within 60 s was measured at 240 nm in quartz cuvettes. Catalase activity was calculated and expressed in µmol min^{-1} mg $protein^{-1}$.

Glutathione-S-transferase activity (GST) assay

GST activity was assessed in brain (50 mg), muscle (250 mg) and liver (50 mg) tissue, based on the procedure described by Habig et al. (1974) by using 1-Chloro-2,4-dinitrobenzene (CDNB) as substrate. The aliquot of 2.5 mL of 20 mM potassium phosphate buffer at pH 6.5 and 50 µL of the homogenate (from each tissue) were added to a glass cuvette. Subsequently, 300 µL of 0.1 mol L^{-1} of GSH and 150 µL of 0.1 mol L^{-1} of CDNB were added to ethanol. Enzymatic activity was determined based on changes in absorbance at 340 nm, by adopting molar extinction coefficient of 9.6 mM cm^{-1}. GST defines the amount of enzyme catalyzing the formation of 1 µmol of GS-DNB per minute at 25 °C, at pH 6.5. It was expressed in µmol GS-DNB min^{-1} mg $protein^{-1}$.

Integrated biomarker response (IBR)

The "Integrated Biomarker Response Index" version 2 (IBRv2) was calculated based on results recorded in previous assays to feature the effects of different water conditions and/or pharmaceutical–exposure levels. IBR values were calculated through log transformation and represented in star plots (Beliaeff and Burgeot 2002; Sanchez et al. 2013).

Statistical analysis

Results were subjected to analysis of variance (ANOVA) between groups, which was followed by Tukey's test—all biomarkers met results recorded through the normality and variance homogeneity tests. The value $p \leq 0.05$ was statistically significant in analysis results based on procedures available in the statistical package R, version 3.5.0 (R Core Team 2016).

Results and discussion

Pharmaceutical waste determination in treated sewage effluent was performed before the experimental period. The concentrations of active principles detected in the three effluent groups are shown in Table 1.

Aside from caffeine, which is psychostimulant, and paracetamol, which is analgesic and antipyretic, the other compounds detected in the sewage effluent are antibiotics, which is one of the classes presenting greater (eco)toxicological potential (Farré et al. 2008; Velicchi et al. 2012; Li 2014; Liu et al. 2018).

Many (eco)toxicity studies carried out with antibiotic waste detected in different test organisms were compiled by Liu et al. (2018). These authors described a series of disturbances caused by some of the compounds detected in the effluent samples in the current study, mainly in fish. Sulfamethoxazole, for instance, can change ethoxyresorufin-O-deethylase (EROD) enzyme levels in hepatocytes, which suggests changes in the capacity to synthesize protein in the

Table 1 Residual pharmaceutical concentrations detected in sewage effluent samples based on *A. bimaculatus* specimens exposed to them

Pharmaceutical	MDL (µg L^{-1})	MQL (µg L^{-1})	TSE (µg L^{-1})	TSE + P (µg L^{-1})	PTSE (µg L^{-1})
Caffeine	0.006	0.020	0.966	0.966	0.031
Ciprofloxacin	0.006	0.020	0.092	11.443*	0.258
Clindamycin	0.006	0.020	0.071	0.071	n.d.
Ofloxacin	0.006	0.020	0.025	0.025	n.d.
Oxytetracycline	0.060	0.200	1.154	7.929*	0.847
Paracetamol	0.012	0.040	1.170	151.170*	0.056
Sulfadiazine	0.006	0.020	0.078	0.078	n.d.
Sulfamethoxazole	0.006	0.020	0.255	188.692*	0.391
Sulfathiazole	0.006	0.020	0.070	0.070	n.d.
Tylosin	0.006	0.020	0.051	0.051	n.d.
Trimethoprim	0.006	0.020	n.d.	30.647*	0.798

MDL method detection limit, *MQL* method quantification limit, *n.d.* not detected, *TSE* treated sewage effluent, *TSE + P* TSE added with pharmaceuticals, *PTSE* TSE + P post-treated with $O_3/H_2O_2/UV$

*Active principles at increased concentration for the TSE + P exposure test

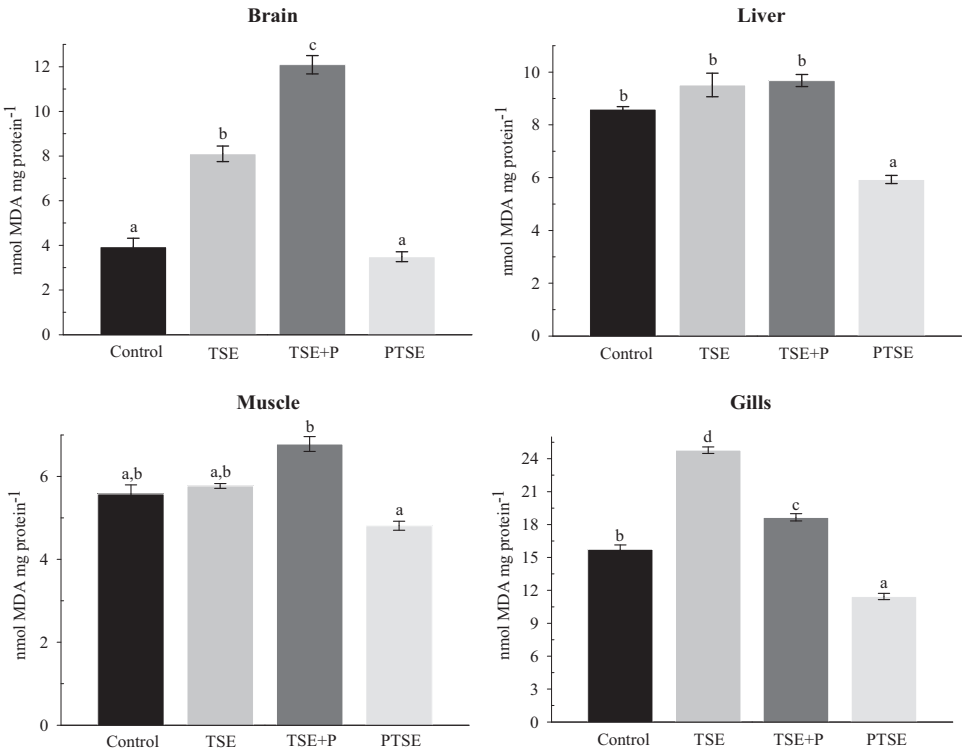


Fig. 1 TBARS levels in the brain, liver, muscle and gills of *A. bimaculatus* exposed to treated sewage effluent (TSE), TSE added with pharmaceuticals (TSE + P), and TSE + P post-treated with $O_3/H_2O_2/UV$ (PTSE), for 14 days. Data represent the mean and standard error (±). Different letters (a–c) between bars indicate statistical difference between treatments in the Tukey test, at 5% significance level. Values recorded for the muscle tissues of animals in the control and TSE (a, b) groups did not differ from the TSE + P treatment, nor from the PTSE

liver, whereas quinolone antibiotics, such as ciprofloxacin, clindamycin and ofloxacin, tend to persist in the body long after the exposure period and to increase the risk of bioaccumulation. Ciprofloxacin can inhibit the activity of cytochrome P450 enzymes that act in synthesizing hormones in most tissues in organisms. The other detected antibiotics belonged to the tetracycline and sulfonamide groups; they cause pro-oxidative effects, change the enzymatic activity, as well as toxicity, teratogenesis and genotoxicity.

Accordingly, based on the TBARS assay, lipid peroxidation is widely used as oxidative damage biomarker in studies on fish exposure to xenobiotics (Lenártová et al. 1997; Clasen et al. 2014; Sehonova et al. 2017; Guiloski et al. 2017; Gonçalves et al. 2018; Samanta et al. 2018). TBARS levels recorded in the current study for different organs of *A. bimaculatus* are depicted in Fig. 1.

Lipid peroxidation is an important consequence of oxidative stress; thus, the TSE and TSE + P treatments have evidenced significant increase in TBARS levels in the brain and gills as response to the control group. However, TBARS levels in brain and muscle tissue of animals in the group exposed to the post-treated sewage effluent (PTSE) did not statistically differ from the control. The liver and gills of animals in the PTSE group presented reduced lipid peroxidation levels in comparison to the other groups. In summary, TBARS levels in all analyzed organs of animals in the PTSE group were lower than that of the others or, at least, they were not significant to levels observed for the control group. This outcome points out the efficiency and importance of the treatment applied to this group, which showed reduced oxidative damages in different organs of fish exposed to the effluent post-treatment.

Lipoperoxidation process was performed through chain reaction; it highlights the ability of a single radical species to outspread several deleterious biochemical reactions (Aguirre-Martínez et al. 2016). Nunes et al. (2015) assessed the (eco)toxicological risks posed to fish exposed to water from Santa Maria River, in Brazil. This river is a sewage receiver. Although these authors did not feature the water from this river, they observed significant increase in TBARS levels in fish muscle in comparison to the control group.

Some authors have reported lack of TBARS level response in fish exposed to medical products (Brandão et al. 2013; Rodrigues et al. 2019; Sehonova et al. 2019); however, these studies have evaluated the effects of different pharmaceutical classes, in separate. Accordingly, Ebele et al. (2016) reported that the complex pharmaceutical mixture presents greater (eco)toxicity and potential to disturb or change organisms.

The activity of the AChE enzyme, which is related to the physiological functions of the fish (Dutta and Arends 2003) and is often reduced in the presence of xenobiotics (Fossi et al. 1995; Clasen et al. 2014), is another parameter used in toxicological evaluations. AChE activity results are depicted in Fig. 2.

AChE activity was analyzed in order to determine whether the tested compounds showed neurotoxic effect on

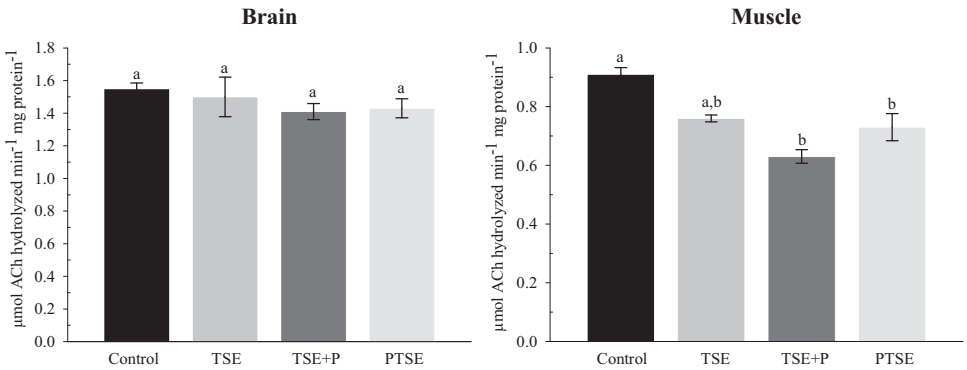


Fig. 2 AChE enzyme activity in the brain and muscle tissues of *A. bimaculatus* exposed to treated sewage effluent (TSE), TSE added with pharmaceuticals (TSE + P) and TSE + P post-treated with O_3/H_2O_2/UV (PTSE), for 14 days. Data represent the mean and standard error (±). Different letters between bars indicate statistical difference between treatments in the Tukey test, at 5% significance level. Enzyme activity assessed in the muscle tissue of animals in the TSE group did not differ from the control, or from the other treatments

A. bimaculatus. There was no significant change in the AChE activity in brain tissue between the control and the other groups. However, AChE activity inhibition was observed in the TSE + P group in comparison to the control; it remained in the PTSE group. According to Liu et al. (2018), Norfloxacin, alone or combination to sulfamethoxazole, can reduce AChE in aquatic organisms.

The present results are in compliance with those described by Nunes et al. (2015), who observed significant AChE activity reduction in muscle tissue of *Astyanax* sp. exposed to water-diluted sewage effluent in comparison to the control. Similarly to the present study, the authors did not find significant decrease in AChE enzyme activity in fish brain tissue in comparison to the control.

The reduced AChE activity in muscle tissue of organisms exposed to chemical substances can be explained by Dutta and Arends (2003), who reported that this enzyme is directly responsible for muscle contraction and relaxation stimuli. Based on the current results, medicines found in the analyzed treatments have neurotoxic potential. The inhibition is related to the action mode of these medicines. Caffeine is known to inhibit the AChE activity (Mohamed et al. 2013; Pohanka and Dobes 2013). Changes in TBARS levels associated with AChE activity inhibition in fish organs suggest oxidative damage trend in groups exposed to the highest concentrations of medical waste.

CAT and SOD (which were not evaluated in the current study) are the main antioxidant enzymes used to neutralize ROS; converting them into metabolites is less harmful to organisms (Clasen et al. 2014). CAT enzyme activity results are shown in Fig. 3.

CAT activity in the liver significantly decreased in groups exposed to medical waste in comparison to the control. CAT activity inhibition was reported by Bayni et al. (1996) after they analyzed fish exposed to domestic and industrial sewage. According to Zhang et al. (2015) and

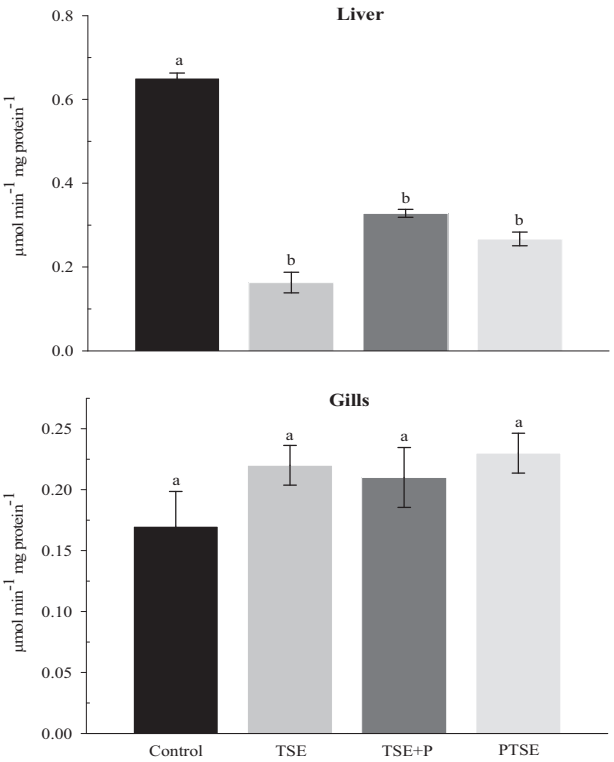


Fig. 3 CAT enzyme activity in the liver and gills of *A. bimaculatus* exposed to treated sewage effluent (TSE), TSE added with pharmaceuticals (TSE + P) and TSE + P post-treated with O_3/H_2O_2/UV (PTSE), for 14 days. Data represent the mean and standard error (±). Different letters between bars indicate statistical difference between treatments in the Tukey test, at 5% significance level. Gills did not show significant difference between treatments

Gobi et al. (2018), any decrease in the activity of this enzyme points toward direct damage inflicted to protein structure. Gills did not show CAT activity change in any of the analyzed treatments in comparison to the control group. This outcome suggests that, although hydrogen peroxide

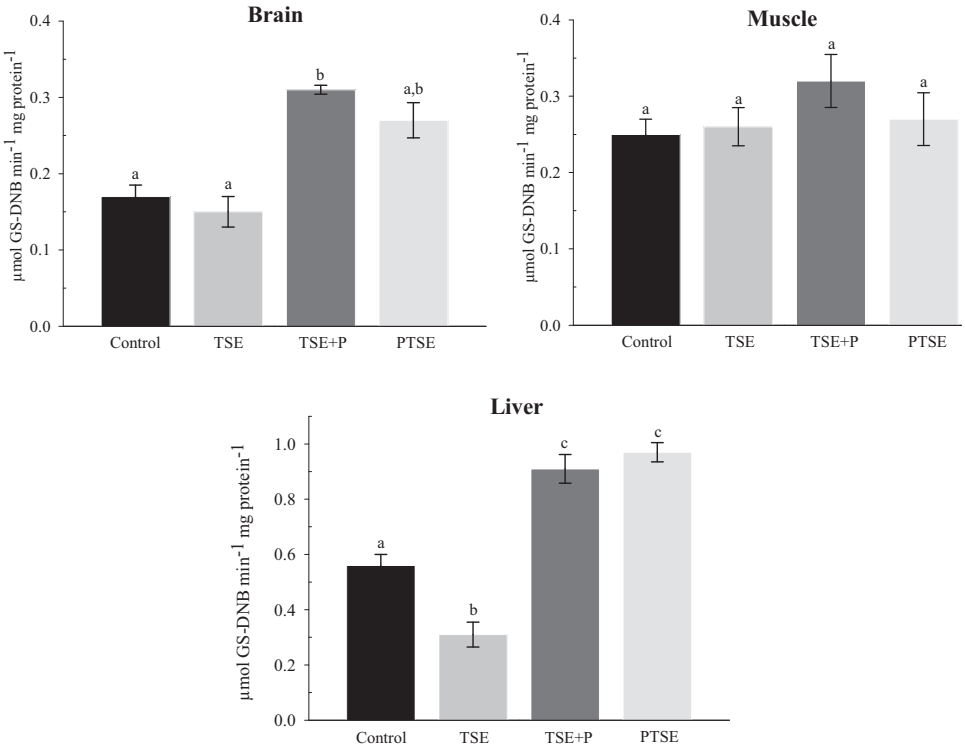


Fig. 4 GST enzyme activity in the brain, muscle and liver of *A. bimaculatus* exposed to treated sewage effluent (TSE), TSE added with pharmaceuticals (TSE + P) and TSE + P post-treated with O_3/H_2O_2/UV (PTSE), for 14 days. Data represent the mean and standard error (±). Different letters (a–c) between bars indicate statistical difference between treatments in the Tukey test, at 5% significance level. Bars followed by letters b and c indicate statically difference from the control group

was produced by the exposure to different treatments with medical waste, catalytic degradation was not followed by increased catalase activity. This hydrogen peroxide likely accounts for the increased TBARS levels observed in gill tissue.

However, despite the reduction in ROS removal ability evidenced by CAT reduction, lack of activity may be the consequence of the enhanced protection of other antioxidant defense mechanisms such as GST. The GST activity protects from oxidative stress, which was significantly higher in brain and liver tissue of fish exposed to EST + F (Fig. 4). Such an increased GST activity and reduced CAT activity, suggest antioxidant defense system flaws. The increased GST activity was observed in the liver of animals in the ESPT group in comparison to the control and EST groups. This outcome assumingly highlights metabolite formation resulting from the treatment process the effluent was exposed to. By-product formation during pharmaceuticals' degradation by advanced oxidative processes was reported by several authors (Gavrilescu et al. 2015; Azuma et al. 2016; Giannakis et al. 2017). Resulting metabolites may be less, equivalent or more toxic than the original compounds, depending on the treatment process and on the conditions of the environmental matrix where they are found (Xu et al. 2017).

GST behavior in the brain of *Astyanax* sp., reported by Nunes et al. (2015) was the same as the one recorded in the current study. These authors observed increased enzymatic activity in the group exposed to water contaminated with urban effluents and pesticides. The study conducted by Guiloski et al. (2017) reinforced the hypothesis of increased GST enzyme activity in liver subjected to detoxification conditions, since the exposure of *Rhamdia quelen* to the concentration of $2.5\,\mu g\,L^{-1}$ of paracetamol recorded increased enzymatic activity in this organ. Guiloski et al. (2015) also reported increased GST activity in the liver of *Hoplias malabaricus* exposed to 0.3 and $3\,\mu g\,kg^{-1}$ of dexamethasone; the study by Sehanova et al. (2017), who assessed the larval-embryo exposure of *Cyprinus carpio* individuals to $50\,\mu g\,L^{-1}$ of sodium naproxen and tramadol hydrochloride mixture.

Increased lipid peroxidation levels in brain tissue, associated with decreased AChE enzyme activity in muscle tissue and CAT in liver tissue, as well as increased GST activity in liver tissue of fish in the TSE + P and PTSE groups, can be explained by the combination of antibiotics belonging to the fluoroquinolones group (ciprofloxacin and ofloxacin present in this study) to tetracyclines (oxytetracycline, also found in the current study) that potentiate toxicity, cardiotoxicity, immunotoxicity and disordered locomotion behavior development in antagonistic actions (Liu et al. 2018). Fatty acids are essential for the proper functioning of the nervous system in fish. However, their composition and metabolism can be changed by the presence of xenobiotics, mainly in mitochondria where ROS is produced; it can change the physiology of the nervous system. This process turns the brain into one of the organs most susceptible to changes in the metabolic activity (Olivares-Rubio et al. 2020).

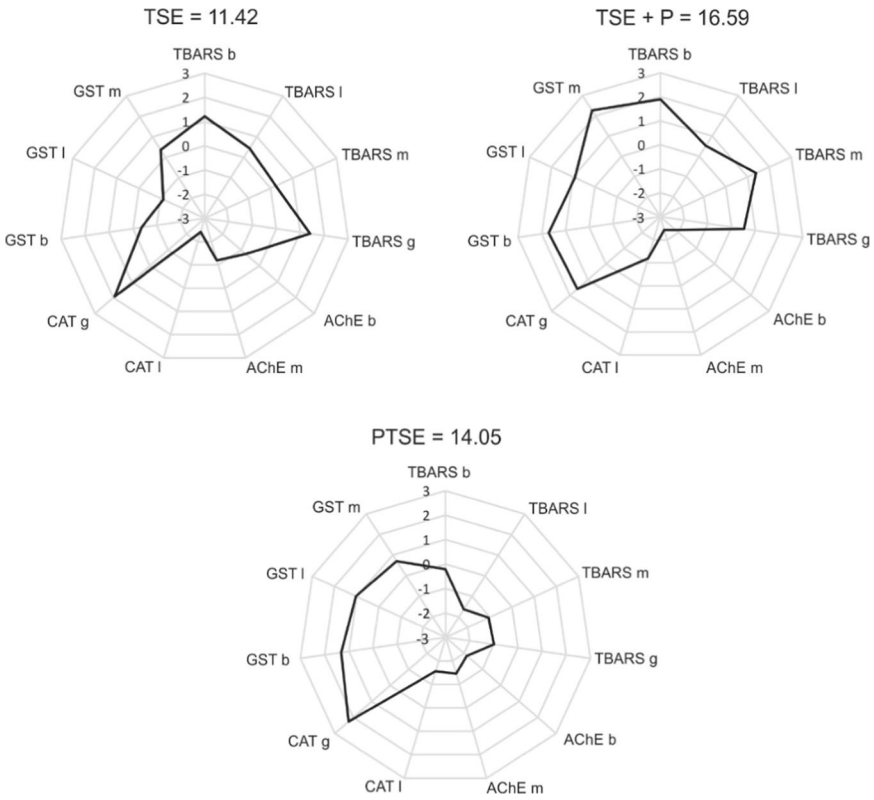


Fig. 5 Integrated Biomarker Response (IBR) index recorded for *A. bimaculatus* exposed to treated sewage effluent (TSE), TSE added with pharmaceuticals (TSE + P) and TSE + P post-treated with $O_3/H_2O_2/UV$ (PTSE), for 14 days. The spokes indicate the mean IBR index values of each treatment

Biomarker responses are not always clear and easy to interpret due to their different response patterns in different organs. Accordingly, the IBR index was calculated based on the selected biomarkers in order to integrate responses and to facilitate result interpretations (Fig. 5).

IBR values recorded for the TSE treatment show that TBARS levels in all analyzed organs, as well as the CAT activity in gill tissue, were the parameters undergoing major changes. The trend observed in the TSE group remained in the TSE + P group, and this finding confirmed that the observed effects were mainly caused by the presence of pharmaceuticals in the sewage.

Based on the IBR values, the TSE + P group presented the highest changes in the analyzed parameters, likely due to the higher concentration of pharmaceuticals in this group. In addition to TBARS and CAT parameters, GST levels in all analyzed organs were changed in the TSE + P group, and it showed that the higher concentration of antibiotics has activated the detoxification process in fish belonging to this group. AChE enzymatic activity inhibition showed neurotoxic effects on fish, after the addition of pharmaceuticals to sewage.

The IBR value recorded for PTSE was lower than that recorded for TSE + P; this outcome points out that the post-treatment was efficient in degrading pharmaceuticals found in sewage; however, it was not possible to fully degrade them, but it was possible observing AChE activity inhibition trend, even after the post-treatment. The GST activity shows the detoxification efficiency of the treatment over xenobiotic compounds evidenced by the decreased TBARS levels recorded for the PTSE group. However, the detoxifying system was activated and identified the possible formation of secondary metabolites due to the applied treatment. Thus, the IBR value recorded for PTSE was higher than that of TSE, in addition to the possible association with the presence and effects of trimethoprim, which is an active ingredient added to TSE + P that was not completely degraded in the post-treatment.

IBR is a tool used to understand how the set of analyzed biomarkers is influenced by the exposure to xenobiotics. Thus, it was possible identifying the most important biomarkers to weight IBR values under the tested conditions. TBARS levels in brain and muscle tissue, AChE activity in muscle tissue and GST in brain and muscle tissue of *A. bimaculatus* were more susceptible to the exposure to sewage added with pharmaceuticals; as well as to evaluate the efficiency of the post-treatment the sewage was subjected to. The TBARS levels in all tissues became more efficient after the addition of the GST activity in the muscle and brain tissue of the exposed fish. The present results corroborate the findings by Wang et al. (2010), who observed that the GST activity can be considered a biomarker sensitive to fish exposure to complex sewage-related contaminant mixtures.

Conclusions

Results have shown that the mixture of pharmaceuticals detected in the treated sewage effluent was capable of changing the biochemical parameters of *A. bimaculatus* in the assessed organs. Therefore, it can be concluded that, after conventional treatment, the sewage effluents presented compounds at concentrations capable of having toxic effects on *Astyanax* sp.

Changes in oxidative stress biomarkers were worsened when the concentration of pharmaceuticals increased in the effluent. TBARS levels in brain and gill tissues, AChE in muscle tissue, CAT in liver tissue and GST in brain and liver tissues showed significant changes in comparison to the control group. This outcome clearly highlights the risk and toxicity posed by the mixture of pharmaceuticals found in fish.

Effluent post-treated with O_3/H_2O_2/UV (PTSE) presented decreased lipid peroxidation levels in comparison to the control, and it indicates that metabolites likely formed during this process have lower toxicity than the original active principles; however, it was not possible to fully degrade the pharmaceuticals found in the sewage, which may be still interfering in the metabolism of fish.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The proposal of the present study was submitted to the Ethics Committee on the Use of Animals (CEUA) of the Federal University of Santa Maria (UFSM), having received the CEUA approval certificate no. 3824230418.

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