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Combined effects of exposure to microplastics and polycyclic aromatic hydrocarbons (PAH): Biochemical and histological changes in the neotropical species *Astyanax lacustris*

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Abstract

Contamination of aquatic environments by plastic particles and petroleum and its derivatives is a reality. Their presence in these environments occurs, both individually, and in combination. Microplastics (MP), of size ranging between 0.05 cm and 0.5 cm, are among the plastic particles most found in the aquatic environment. On the other hand, polycyclic aromatic hydrocarbons (PAH) are considered the most toxic and lipophilic part of oil and its derivatives. Studies evaluating the contamination of marine environments by these contaminants are ample, on the other hand, few studies have evaluated their effects in fresh water. The fish species *Astyanax lacustris*, popularly known as yellow-tailed lambari, are sensitive to environmental contamination and are commonly used as bioindicators. The work aimed to evaluate the toxicity of MP and PAH alone or in association, through the analysis of the damage caused by failures in biotransformation systems in target organs (protein carbonylation) and hepatic histological changes. Grade I and II liver histology were found, vacuolization, disarrangement of liver cords and deformation of the hepatic contour. In relation to carbonylated proteins, high concentrations of them were found in treatments with PAH. The liver damage found may be related to oxidative stress suffered by the organisms, caused by the pollutants to which they were exposed. It is concluded that PAH is a contaminant that generates liver damage due to oxidative stress caused in specimens of *A. lacustris.* MP does not alter the toxicity of PAH but has adsorption capability of the same.

Keywords: liver damage. cabonyl protein. biomarkers. oxidative stress.

Resumo

Efeitos combinados da exposição a microplásticos e hidrocarbonetos aromáticos policíclicos (PAH): Alterações bioquímicas e histológicas nas espécies neotropicais *Astyanax lacustris*

A contaminação de ambientes aquáticos por partículas plásticas e petróleo e seus derivados é uma realidade. Suas presenças nesses ambientes ocorrem de forma individual e em associação. Microplásticos (MP), com tamanhos variando entre 0,05 e 0,5 cm, são os MP mais encontrados no ambiente aquático. Por outro lado, os hidrocarbonetos policíclicos aromáticos (HPA) são considerados a porção mais tóxica e lipofílica do petróleo e seus derivados. Estudos avaliando a contaminação dos ambientes marinhos por esses contaminantes são abundantes, por outro lado, poucos são os trabalhos que avaliaram seus efeitos em ambientes dulcícolas. A espécie de peixes *Astyanax lacustris*, popularmente conhecida como lambari-do-rabo-amarelo, são sensíveis à contaminação ambiental, sendo comumente utilizados como biondicadores. Dessa forma, o presente projeto tem como objetivo avaliar a toxicidade dos MP e dos HPA, sozinhos ou em combinação, por meio da análise dos danos causados por falhas nos sistemas de biotransformação em órgãos alvo (carbonilação de proteínas) e alterações histológicas hepáticas. Foram encontradas histopatologias hepáticas de grau I e II, sendo elas, vacuolização, desarranjo dos cordões hepáticos e deformação do contorno hepático. Em relação a proteínas carboniladas foram encontradas altas concentrações delas nos tratamentos com HPA. Os danos hepáticos encontrados podem ser relacionados com estresse oxidativo sofrido pelos organismos, causado pelos poluentes a qual eles foram expostos. Conclui-se que HPA é um contaminante que gera dano hepático devido ao estresse oxidativo causado nos espécimes de *A. lacustris*. MP não altera sua a toxicicidade do HPA, mas possui capacidade de adsorção do mesmo.

Palavras-chave: dano hepático. proteína cabonilada. Biomarcadores. estresse oxidativo.

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ABBREVIATIONS AND ACRONYMS LIST

A.	-	*Astyanax*
cm	-	Centimeter
CT	-	Control without handling
CH	-	Control with handling
°C	-	Degrees Celsius
g	-	Gram
h	-	Hour
IAH	-	index of histopathological alterations
mg/L	-	Milligram per liter
Min.	-	Minute
ml	-	Milliliter
mm	-	Millimeter
mmol/L	-	Millimolar per liter
MP	-	Microplastic
MPC	-	Microplastic loaded with PAH
MVA	-	Mean value of alteration
N°	-	Number
nm	-	nanometre
PAH	-	Polycyclic aromatic hydrocarbon
PAH+MP	-	Polycyclic aromatic hydrocarbon in association with virgin microplastic
µL	-	Microliter
µg	-	microgram
µg/G	-	Microgram per liter
µg/L	-	Microgram per gram

SUMMARY

1. INTRODUCTION

Plastic particles that are between 0.05 cm and 0.5 cm are called microplastics (MP) (WU *et al.*, 2019). MP can be divided into primary MP and secondary MP, where primary MP are used by the cosmetics industries to make products such as toothpastes and exfoliants. Secondary MP are a product of the fragmentation of larger plastic (LAMBERT; WAGNER, 2018). Several studies evaluating the effects of MP in marine environments are widely found in the literature (COLE *et al.*, 2011). On the other hand, studies evaluating these effects in freshwater environments are short, even though MP can occur in freshwater in similar amounts with marine environments (DRIS *et al.*, 2015). The MP has different sources, entering freshwater environments through the disposal of industrial and domestic effluents. MP is used in industry as raw material for several products such as packaging film, pharmaceutical and hospital wrapping, toys (COUTINHO; MELLO; SANTA MARIA, 2003), while MP detected in domestic effluents comes from personal hygiene products and synthetic clothes. (CRAWFORD; QUINN, 2016; LI; LIU; PAUL CHEN, 2018). Regarding their composition, MP can be made of polyethylene, polystyrene, polyvinyl chloride, polypropylene, polycarbonate, polyamide or polychloroprene (HORTON *et al.*, 2017). The presence of MP in the aquatic environment can generate numerous environmental consequences, usually related to their high durability (LESLIE, H. A.; VAN DER MEULEN, M. D.; KLEISSEN, F. M.; VETHAAK, 2011), small particle size (MCINTYRE *et al.*, 1997), rough surface and the fact that MP stay on the surface of the water due to their buoyancy – characteristics that directly influence the adsorption processes of contaminants present in the environment (LI; ZHANG; ZHANG, 2018; TURNER; HOLMES, 2015). Therefore, in addition to the problems caused by the ingestion of MP (internal abrasion, ulcers and clogging of the gastrointestinal tract) (WRIGHT; THOMPSON; GALLOWAY, 2013), there are also problems related to the transporting of environmental contaminants by these particles (HARTMANN *et al.*, 2017).

In addition to MP, another class of contaminant of great importance, due to their environmental impacts, are the oil and derivatives, such as petroleum. Petroleum consists of the complex mixture of several distinct organic compounds, mainly hydrocarbons, formed by the incomplete decomposition of organic matter (NEFF, 1979). However, the

composition of petroleum, as well as that of its derivatives, varies according to its region of origin. Polycyclic aromatic hydrocarbons (PAH), despite being the smallest portion of petroleum and derivatives, are considered the most toxic compounds due to their lipophilicity, being considered carcinogenic and mutagenic (DIPPLE; CHENG; BIGGER, 1990; OSTRENSKY *et al.*, 2001). These compounds can go in the aquatic environment through the accidental spillage of oil and derivatives, they can come from the burning of fuels in vessels and can be leached by rain (SILVA, 2007). Fish encounter these substances in direct and indirect ways. Directly, it can occur through the absorption of these compounds by the gills and skin, and indirectly through food (bioaccumulation and trophic biomagnification). Once inside organisms, PAH can be biotransformed so that they are excreted, or they can accumulate in body (SADAUSKAS-HENRIQUE, 2014). Among all existing PAH, 16 were included in the legislation of the United States Environmental Protection Agency (U.S.EPA) (PERSISTENT, 2002) because they are considered more toxic in aquatic environments, due to greater solubility compared to other PAH (BARRON *et al.*, 1999; RODRIGUES *et al.*, 2010). The 16 are: naphthalene; acenaphthylene; acenaphthene; fluorene; phenanthrene; anthracene; fluoranthene; pyrene; benzo(a)anthracene; chrysone; benzo(b)fluoranthene; benzo(k)fluoranthene; benzo(a)pyrene; indeno(1,2,3-cd)pyrene; dibenzo(a, h)anthracene; benzo(g, h, i)perylene.

An effective method to assess the toxic effects of xenobiotics is through biomarker analysis. Biomarkers can be defined as measurable changes that show the exposure of organisms to contaminants, and the effects caused by this exposure, at different biological levels (Fish bioaccumulation and biomarkers in environmental risk assessment: A reviewVAN DER OOST; BEYER; VERMEULEN, 2003). Biomarkers are used to assess the effects of environmental contaminants, being widely used to assess the effects of PAHs on ichthyofauna (TUVIKENE, 1995). Biomarkers can be divided into exposure biomarkers, which consists of evaluating the activation of defense mechanisms, and effect biomarkers, which indicate damage resulting from the exposure of organisms to xenobiotics. One way of evaluating the absorption and metabolism of PAH in fish is through the analysis of the metabolites of these xenobiotics present in the bile, since PAH are biotransformed by the organism are stored in the gallbladder until their excretion (LIN;

CORMIER; TORSELLA, 1996; VUORINEN *et al.*, 2006). Among the effect biomarkers, the quantification of carbonyl proteins, thiobarbituric acid reactive substances (TBARS) and DNA damage are the most efficient biomarkers to assess protein and lipid damage (ALMROTH *et al.*, 2005). Histopathological damage to tissues such as gills, liver and intestine are also efficient biomarkers to assess the effects of contaminants on organisms.

Fish species of the genus *Astyanax*, popularly known as lambari, are easily found in freshwater bodies in southern Brazil (GARUTTI; BRITSKI, 2000). Species belonging to this genus have been widely used as indicators of aquatic contamination in both field and laboratory studies (TINCANI *et al.*, 2019). Among the various species belonging to this genus, *Astyanax lacustris* (DE LUCENA; SOARES, 2016), popularly known as yellow-tailed lambari, has been widely used in ecotoxicological studies (PONT, 2012). This fish species is a generalist species (omnivore), in addition serving as food for several other fish species in the trophic chain (PERETTI; ANDRIAN, 2008). In addition, this is a species produced in captivity, which makes it easier to obtain and maintain it for studies that assess the toxicity of environmental contaminants in endemic species.

Taking what has been presented into consideration, the present study seeks to answer questions such as: (i) Does virgin MP present toxicity to fish? (ii) do PAH without the presence of MP cause more or less toxicity? (iii) is there transfer of PAH adsorbed in MP to the water column? (iv) is virgin MP able to reduce the toxicity of PAH dissolved in water? To answer these questions, we will describe the main objectives and their respective methodologies in the topics below.

2. GENERAL OBJECTIVE

To characterize the virgin MP toxicity as well as the MP role as vectors of PAH contamination for the Neotropical fish species, *Astyanax lacustris*.

2.1. SPECIFIC OBJECTIVES:

(i) Evaluate the toxicity of virgin microplastics by evaluating the damage caused to proteins (carbonyl proteins) and histopathological damage to the liver.

(ii) **Hypothesis:** Virgin MP is not toxic to fish**.** Evaluate the toxicity of PAH in the absence of MP by evaluating the damage caused to proteins (carbonyl proteins) and histopathological damage to the liver.

(iii) **Hypothesis:** PAH are toxic to fish in the absence of MPs. To evaluate the toxicity of MP loaded with PAH (MP previously contaminated with PAH), through the evaluation of damage caused to proteins (carbonyl proteins) and histopathological liver damage.

(iv) **Hypothesis:** MP loaded with PAH causes greater toxicity to fish when compared with inert PAH and PAH diluted in water. Evaluate the toxicity of PAH when in the presence of virgin MP, through the analysis of damage caused to proteins (carbonyl proteins) and histopathological liver damage.

(v) **Hypothesis:** PAHs will be less bioavailable, causing less toxicity to fish.

3. MATERIAL AND METHODS

The experiments were carried out in the Laboratório de Pesquisa com Organismos Aquáticos (LAPOA) of the Grupo Integrado de Aquicultura e Estudos Ambientais (GIA/UFPR) – Curitiba. Specimens of yellow-tailed lambari (*Astyanax lacustris*) of approximately 16 g (15.9 ± 0.55) and 10 cm (10.00 ± 0.11) were acclimatized in 80 L aquariums for one week, in the interior of the experimentation room (air temperature controlled at 25 °C). These specimens were obtained from the GIA/UFPR maintenance greenhouse, were cultivated on site for approximately 2 years, prior to this, they were obtained from a commercial fish sales company (Peixes e Peixes®) located in Curitiba-PR. The environment related to the cultivation has a circulation system of dechlorinated water (pH= 7.24 ± 0.66; alkalinity = 40.0 ± 1.4 mg CaCO3/L), constant aeration (dissolved oxygen = 6.13 ± 0.72 mg L-1), low temperature variation rate (25.4 ± 0.7 °C) and daily feeding with commercial ration whose protein content can vary from 24 to 32%.

3.1. EXPERIMENTAL PROTOCOL

The fish acclimatization process to the experimental protocol was carried out in three stages. The objective is for the change in density and shoal condition to be gradually altered since species of the genus *Astyanax* have shoal behavior. Initially, the individuals were placed in 100 L boxes containing 15 individuals for 96 h. Subsequently, four individuals were transferred to 15 L glass aquariums and kept in this condition for 48 h. Twenty-four hours before the beginning of the exposure to the experimental conditions, the specimens of *A. lacustris* were individually placed in 4 L glass fish tank. In this condition, the animals were divided into six experimental treatments (n=12) consisting of: Control without handling (CT), Control with handling (CH), virgin microplastic (MP), Polycyclic aromatic hydrocarbon (PAH), Microplastic loaded with PAH (MPC) and Polycyclic aromatic hydrocarbon and virgin MP in association (PAH+MP). For each treatment, 12 fish were used (n=12). During the 96 h of exposure, daily exchanges of the experimental solutions were performed, as described below. Fish feeding was suspended throughout the experimental period. During the entire acclimatization and experimental period, the animals were kept under conditions of constant aeration (OD ≥ 6.5 mg/L) and controlled temperature (25.0 ± 1.0 °C). To help control the temperature, the experiments were carried out in a water bath equipped with heaters and in a room with controlled room temperature (25.0 ± 1.0 °C).

The physical-chemical parameters of the water (pH, temperature, oxygen saturation) were measured daily and adjusted if necessary to pH 7.5; temperature of 25°C; and oxygen saturation 80-100%.

Daily, immediately after the water change in the fish tank, water and MP samples were collected for PAH measurements after 24 h of exposure. Water samples from loaded MP with PAH and MP solutions that were prepared daily were also collected for PAH analyses. Water and MP from the experiment were collected from each treatment (CT, CH, MP, PAH, MPC and PAH+MP) before the water exchange. Due to financial and logistical issues, only one aquarium was analyzed of each treatment.

After 96 h of exposure, the animals were anesthetized (MS-222) for blood collection and later euthanized (spinal cord section) and killed (hypovolaemia) for tissue samples (hepatic) collections, immediately frozen in liquid nitrogen and stored at -80 °C for

biochemical analysis. Liver samples were fixed in Davidson's solution (ALFAC) for 24 h and then preserved in 70° alcohol for further processing and histological analysis.

3.2. DEFINITION OF MP AND PAH CONCENTRATIONS AND PREPARATION OF EXPERIMENTAL SOLUTIONS

The MP concentration utilized in the present study was 10 mg/L. This concentration was defined based on the concentration already described in freshwater environments (LAMBERT; WAGNER, 2018-). Despite being low, it is a concentration that reflects what is found in the natural environment (environmentally relevant). The PAH concentration chosen was based on 20% of the LC_{50}-96h of oil found for *A. lacustris* (2.28 µg/L) (DAL PONT, 2018).

To reach this concentration of 2.28 µg/L, 114 µL of a supply solution with a concentration of 80 µg/mL were added to 4 liters of water. In the present study, we chose to use the Sulpeco® standard, which contains the 16 priority PAHs of the American Environmental Protection Agency (U.S.EPA). This supply solution was packaged in an amber bottle and stored at -20°C to avoid volatilization/degradation of light PAHs. For the treatment of MP loaded with PAH, a technique called “spiking” was performed, previously described in other works (BATEL *et al.*, 2016)). Spiking basically consists of contamination/marking of MP by the PAHs of interest. In the present work, the spiking process was carried out by stirring the MP (10 mg/L) in an ultrapure water solution containing 2.28 µg/L of the standard of the 16 priority PAH. The MP were kept under constant agitation for 24 h with the aid of a magnetic stirrer. At the end of the stirring period, the contaminated MP was filtered and dried 24 h at room temperature (25.0 ± 1.0 °C) in a glass desiccator containing silica gel. After drying, the loaded MP were used in the experiments. Samples of this contaminated MP were collected to determine the concentration of adsorbed PAH. These procedures were performed daily throughout the 96 h of the experiment.

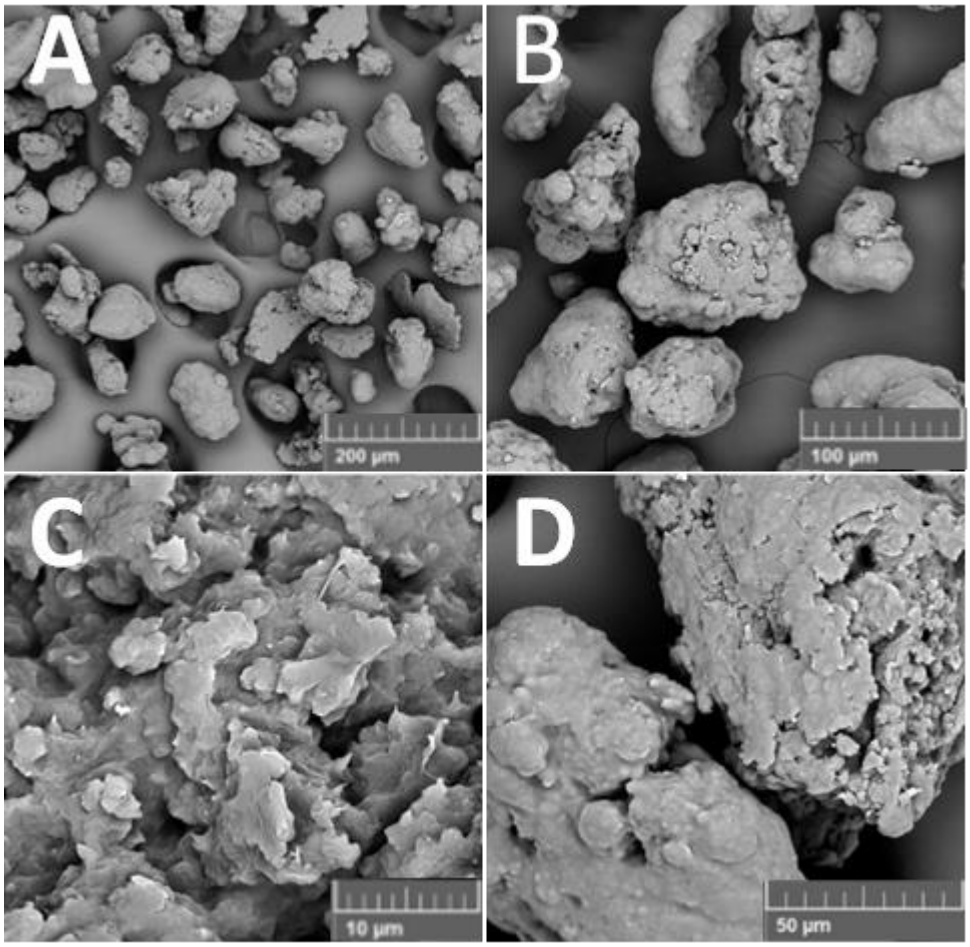

Figure 01. Photomicrographs captured by scanning electron microscopy (SEM) of the microplastics used in the present study. A) SEM HV: 15.0 Kv, SEM MAG: 200 x, View Field: 1.04mm, WD 9.98 mm, Det: BSE + SE, Date (m/d/y): 12/12/19; B) SEM HV: 15.0 Kv, SEM MAG: 400 x, View Field: 519 mm, WD 10.04 mm, Det: BSE + SE, Date (m/d/y): 12/12/19; C) SEM HV: 15.0 Kv, SEM MAG: 3.50 kx, View Field: 59.3 µm, WD 10.11 mm, Det: BSE + SE, Date (m/d/y): 12/12/19; D) SEM HV: 15.0 Kv, SEM MAG: 1.00 kx, View Field: 208 µm, WD 10.08 mm, Det: BSE + SE, Date (m/d/y): 12/12/19. All images made by MIRA3 TESCAN, Lactec- LAME.
Source: Elaborated by the author.

3.3. DAILY WATER CHANGE

Every day, during the 96 h of the experiment, the solutions of PAH, virgin MP and MP loaded with PAH were renewed, as well as the water of the controls. For methodological reasons, it was decided to change the entire contents of the fish tank (water and MP). Thus, the renewal of solutions was 100% in all treatments. For this, the fish were carefully transferred from one aquarium to another (containing new experimental solutions). This procedure was repeated for all treatments (including the control). In addition, it was carried out in a control treatment where the fish were not manipulated, so

that the possible effects of the manipulation of biological responses can be isolated from the responses related to treatments with MP and PAH.

3.4. PAH ANALYSIS IN WATER AND MICROPLASTIC

3.4.1 PAH extraction protocol in water:

The determination of the PAH concentration was performed in the samples at the end of the first exposure period (24 h). The concentration of the 16 PAH considered priorities by the EPA was determined by CG-MS (EPA, 1996c).

Tapered tubes are used in centrifugation glass, where an aliquot of 5.00 ml of standard solution or sample containing PAHs is transferred. This solution is made rapid injection, by means of micropipette, of 1.00 ml of chloroform solution or extractor (75 L) solubilized in 925 L acetone (disperser solvent). Rapid injection allows the formation of chloroform microgotes in the medium, immiscible with the aqueous phase. Subsequently, to increase the extraction efficiency, everything is stirring in vortex for 1.00 min, subsequent centrifugation for 10 min, favoring the formation of a sedimented phase (drop), which is removed quantitatively (50 L) and finally transferred to a chromatographic insert containing 1 L solution mix of internal patterns of deuterate PAH. The concentration of the 16 PAHs considered priority by the EPA were determined by liquid-liquid extraction (EPA, 1996c).

3.4.2. PAH extraction protocol in MP:

The determination of the concentration of PAH adsorbed on MP was performed on samples collected at the end of the first exposure period (24 h) and on MP samples contaminated with PAH before entering the experiments. An extraction was performed in sonicated tubes for 10 min, centrifuged for 5 min at 2800 rpm and an aliquot of the supernatant of 2.50 mL was transferred to another glass tube and dried in a rotary evaporator under vacuum. Complete evaporation of ACN was carried out in 20 min at 60 °C. After evaporation, the samples were resolubilized in 250 µL hexane, transferred to 300 µL glass inserts and quantified in the GC-MS system.

3.4.3. Identification and quantification of PAH extracted from water and MP:

The identification of each compound was performed by comparing it with the injection of a standard solution, containing a mixture of the 16 PAH, and consulting the NIST mass spectra library of the equipment. Quantification was performed using an analytical curve built in the concentration range from 5 to 1000 ng/mL, using as an internal standard a solution containing 5 deuterated PAH (naphthalene-d8, acenaphthene-d10, phenanthrene-d10, chrysene-d12, perylene-d12) at a concentration of 100 ng/mL. As a quality control, solutions were injected daily to verify the curve, accepting maximum deviations of 10%.

3.5. BIOLOGICAL ANALYSIS IN *ASTYANAX LACUSTRIS*

3.5.1. Protein Carbonylation:

The measurement of protein oxidation/carbonylation during the oxidative stress process was measured according to Levine et al. (LEVINE *et al.*, 1994). For this, the tissues were homogenized (1:10 mass:volume) in PBS pH 7.0 buffer and centrifuged (12,000g, 20 min, 4 °C). 200 µl of the supernatant, 500 µl HCl (2 M), 500 µl DPNH (10 mM) were added to it, then samples were incubated (30-37 °C) for 90 min. After cooling, 700 µL of TBA (28%) were added and centrifuged (9000 g) for 10 min. The supernatant was discarded, and the pellet was resuspended in 1 ml of ethanol-ethyl acetate (1:1), this procedure was repeated 2 more times. After that, the pellet was resuspended in guanidine chloride (6 M) and centrifuged again for 3 min. The supernatant was read at 358-370 nm wavelength. Values were expressed in nmol of carbonyls/mg of protein.

3.5.2. Total proteins

The total protein of the homogenates was measured (BRADFORD, 1976) using a spectrophotometer and albumin standards. Readings were taken at a wavelength of 595 nm.

3.5.3. Histological Analyses

For histological analysis, liver tissue was removed. This tissue, after its removal, was fixed in ALFAC for 24 hours. After fixation, the tissue was transferred to a 70% alcohol solution, where it remained until it was blocked. The blocks were trimmed, sectioned (5-10 µm), stained with periodic acid Schiff and counterstained with hematoxylin (PAS-H).

The slides were observed under a light microscope. The incidence and distribution of lesions were evaluated based on the following criteria: 0, absence of lesions (absence of lesions or lesions in up to 10% of the analyzed tissue); 0+, rarely present (occurrence of lesions in 11% to 25% of the total tissue analyzed); +, present (occurrence of lesions in 26% to 50% of the analyzed tissue); ++, frequent (occurrence of lesions in 51% to 75% of the analyzed tissue) and +++, highly frequent lesions (occurrence of lesions in 76% to 100% of the analyzed tissue). The mean value of alteration (MVA) for each animal was calculated according to Schwaiger et al. (1997) (SCHWAIGER *et al.*, 1997), but has been slightly modified to follow a respective numerical value: 0-1.0, no pathological changes; 1.1-2.0, mild focal changes; 2.1-3.0, moderately widespread lesions; 3.1-4.0, frequent injuries and 4.0-5.0, widely distributed injuries. The index of histopathological alterations (IAH) was calculated according to Camargo and Martinez (2007) (CAMARGO; MARTINEZ, 2007). The IAH was calculated based on the type, location and severity of the lesion. Liver lesions were classified into four groups: lesions in the structure of the hepatic parenchyma, including interstitial tissue; changes in hepatocytes, including cytoplasmic and nuclear changes; changes in blood vessels and necrosis. Injuries were classified into three degrees of damage based on whether the organ's normal structure would be restored. The IAH was calculated from the sum of the lesion types within each of the three stages multiplied by the stage index using the mathematical equation proposed by Poleksic and Mitrovic-Tutundzic (1994) (POLEKSIC; MITROVIC-TUTUNDZIC, 1994).

4. RESULTS

4.1. PAH ANALYSIS

It can be observed in Table 1 the PAH concentrations of the working solution (µg/L) and in the MP (µg/L) before and after 24 h of the MP spiking procedure. The PAH with low molecular weight (naphthalene until the chrysene, with exception of benzo[a]anthracene) had their concentrations decreased after the virgin MP spike procedure (Table 1). The virgin MP, after 24 h of the spinking procedure, had PAH concentrations bellow the quantification limits. After 24 h of PAH spiking procedure, the MP had values of the low molecular weight PAH, naphthalene; acenaphthylene;

acenaphthene and fluorene below the quantification limit, while the other PAH presented higher values than the virgin MP (Table 1).

Table 1. PAH concentrations in the working PAH water solution (µg/L) and in the MP (µg/g) before and after the MP spiking procedure. Values with "<" symbol means that were below of the quantification limits.

	Working PAH water solution (µg/L)		**MP (µg/g)**	
PAH	**Before virgin MP spiking**	**After virgin MP spiking**	**Virgin**	**Spiked**
Naphthalene	1.55	0.45	<0.04	<0.04
Acenaphthylene	3.08	<0.12	<0.04	<0.04
Acenaphthene	2.69	<0.10	<0.04	<0.04
Fluorene	1.82	0.28	<0.04	<0.04
Phenanthrene	1.97	0.36	<0.10	1.09
Anthracene	8.34	<0.20	<0.04	3.11
Fluoranthene	2.04	0.25	<0.04	27.4
Pyrene	2.52	0.28	<0.04	32.84
Benz[a] anthracene	3.25	2.45	<0.04	98.97
Chrysene	1.77	1.98	<0.04	88.5
Benzo[b] fluoranthene	1.83	7.38	<0.04	90.63
Benzo[k] fluoranthene	1.86	8.41	<0.04	85.42
Benzo[a]pyrene	<0.20	<0.20	<0.04	104.22
Indeno[1,2,3-cd] pyrene	3.5	3.09	<0.04	78.26
Dibenz[a,h] anthracene	2.95	4.07	<0.04	83.24
Benzo[ghi] perylene	2.79	2.46	<0.04	77.77
∑PAH	41.96	31.46		771.45

Source: Elaborated by the author

In Table 2 is showed the PAH concentration in the water of the treatments after 24 h, prior water exchange. It can be observed that in CT; CWH; MP and PAH, all the PAH were below the quantification limits. Also, for the PAH+MP and MPC, only the high molecular weight PAH were detected in the water after 24 h of exposure, they were, benzo[a]anthracene;benzo[k]fluoranthene;indene[1,2,3,cd]pyrene;

dibenzo[a,h]anthracene and benzo[ghi]perylene. The benzo[a]anthracene was below the detection limit for all treatments (Table 2).

Table 2. PAH concentration in the water of the treatments, Control (CT); Control with handling (CWH); Polycyclic aromatic hydrocarbons (PAH); PAH and virgin MP (PAH+MP) and MP loaded with PAH (MPC) after 24 h of exposure. Values with "<" symbol means that were below of the quantification limits.

PAH (µg/L)	Experimental treatments					
	CT	CH	MP	PAH	MPC	PAH+MP
Naphthalene	<0.05	<0.05	<0.04	<0,05	<0.05	<0.05
Acenaphthylene	<0.05	<0.05	<0.04	<0,05	<0.05	<0.05
Acenaphthene	<0.05	<0.05	<0.04	<0,05	<0.05	<0.05
Fluorene	<0.05	<0.05	<0.04	<0,05	<0.05	<0.05
Phenanthrene	<0.05	<0.05	<0.10	<0,05	<0.05	<0.05
Anthracene	<0.05	<0.05	<0.04	<0,05	<0.05	<0.05
Fluoranthene	<0.05	<0.05	<0.04	<0,05	<0.05	<0.05
Pyrene	<0.05	<0.05	<0.04	<0,05	<0.05	<0.05
Chrysene	<0.20	<0.20	<0.04	<0,20	<0.20	<0.20
Benz[a]anthracene	<0.20	<0.20	<0.04	<0,20	1.74	0.28
Benzo[b]fluoranthene	<0.20	<0.20	<0.04	<0,20	10.96	9.07
Benzo[k]fluoranthene	<0.20	<0.20	<0.04	<0,20	10.80	9.14
Benzo[a]pyrene	<0.05	<0.05	<0.04	<0,05	<0.05	<0.05
Indeno[1,2,3-cd]pyrene	<1.00	<1.00	<0.04	<1,00	3.41	3.74
Dibenz[a,h]anthracene	<1.00	<1.00	<0.04	<1,00	4.36	4.17
Benzo[ghi]perylene	<1.00	<1.00	<0.04	<1,00	7.18	7.94
∑HPA (ng/L)	-	-	-	-		34.34

Source: Elaborated by the author.

4.2. HISTOLOGICAL ANALYSES

The analysis of the hepatocytes revealed morphological alterations such as deformation of the cellular contour, disarrangement of the hepatic cords, nuclear vacuolization and cellular hypertrophy. As shown in Table 3, cellular hypertrophy was the least frequent histopathology, being seen only in the treatment of PAH and in small amounts. The other pathologies, deformation of the cellular contour, disarrangement of the hepatic cords, nuclear vacuolization and cellular hypertrophy were found several times and in greater quantity, but only in treatments with contaminants. The alterations found were grade I or II, not reaching grade III.

Table 2. Frequency of occurrence of the histopathologies present in liver tissue of *A. lacustris* exposed for 96 h to five different treatments (CT: Control; CWH: Control handling; MP: virgin microplastic; PAH: polycyclic aromatic hydrocarbons; MPC: MP loaded with PAH and PAH+MP: PAH and virgin MP in association.

Liver abnormalities	Level	CT	CH	MP	PAH	MPC	PAH+MP
Nuclear hypertrophy	I	0	0	0	0	0	0
Cellular hypertrophy	I	0	0	0	0+	0	0
Cell atrophy	I	0	0	0	0	0	0
Nuclear atrophy	I	0	0	0	0	0	0
Increase in the frequency of vessels	I	0	0	0	0	0	0
Deformation of the nuclear contour	I	0	0	0	0	0	0
Cell contour deformation	I	0	0	+++	+++	+++	+++
Disarray of the hepatic cords	I	0	0	+	+++	++	++
Nuclei at the periphery of the cell	I	0	0	0	0	0	0
Presence of melanomacrophages	I	0	0	0	0	0	0
Cytoplasmic vacuolization	I	0	0	0	0	0	0
Eosinophilic granules	I	0	0	0	0	0	0
Nuclear vacuolization	II	0	0	+	++	++	+
Nuclear degeneration	II	0	0	0	0	0	0
Cytoplasmic degeneration	II	0	0	0	0	0	0
Pyknotic nuclei	II	0	0	0	0	0	0
Cell disruption	II	0	0	0	0	0	0
Bile stagnation	II	0	0	0	0	0	0
Vessel rupture	II	0	0	0	0	0	0
Congestion	II	0	0	0	0	0	0
Focal necrosis	III	0	0	0	0	0	0
Total necrosis	III	0	0	0	0	0	0

0 Absence; 0+ Rare occurrence; + Present; ++ Frequent; +++ Very frequent.
Source: Elaborated by the author.

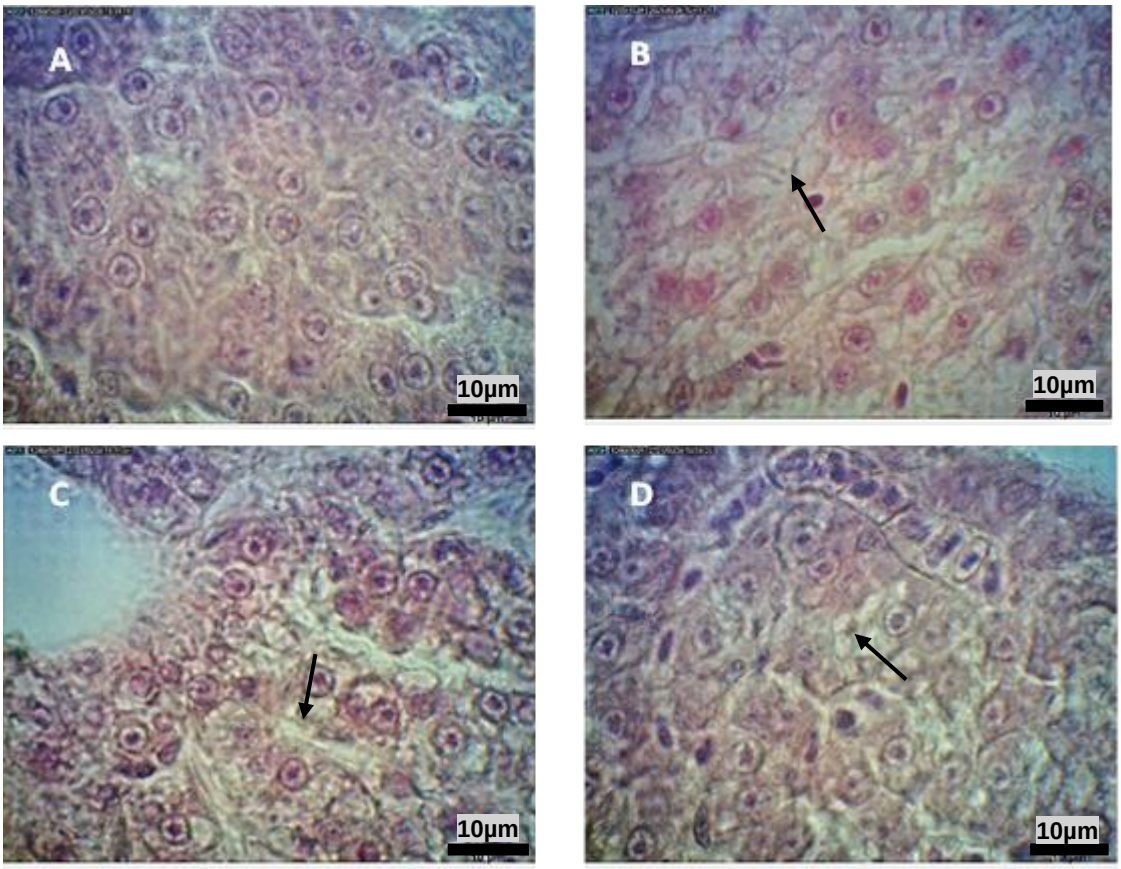

Figure 2. A. Normal liver and main hepatic histopathology found in A. lacustris. B. Vacuolation. C. Derangement of the hepatic cords. D. Cell contour deformation. Scale bar = 10 µm.
Source: Elaborated by the author

The mean value of alteration (MVA) (Figure 3) and histopathological index (HI) (Figure 4) for the control groups was 0, and for the treatments ranged from 2 to 3, and 2 to 6, respectively, indicating the presence of moderately widespread lesions. As shown in Figure 3 and 4, there is a significant difference between all treatments (MP, PAH, PAH+MP and MPC) with the two control groups (CT and CWH) for both indexes.

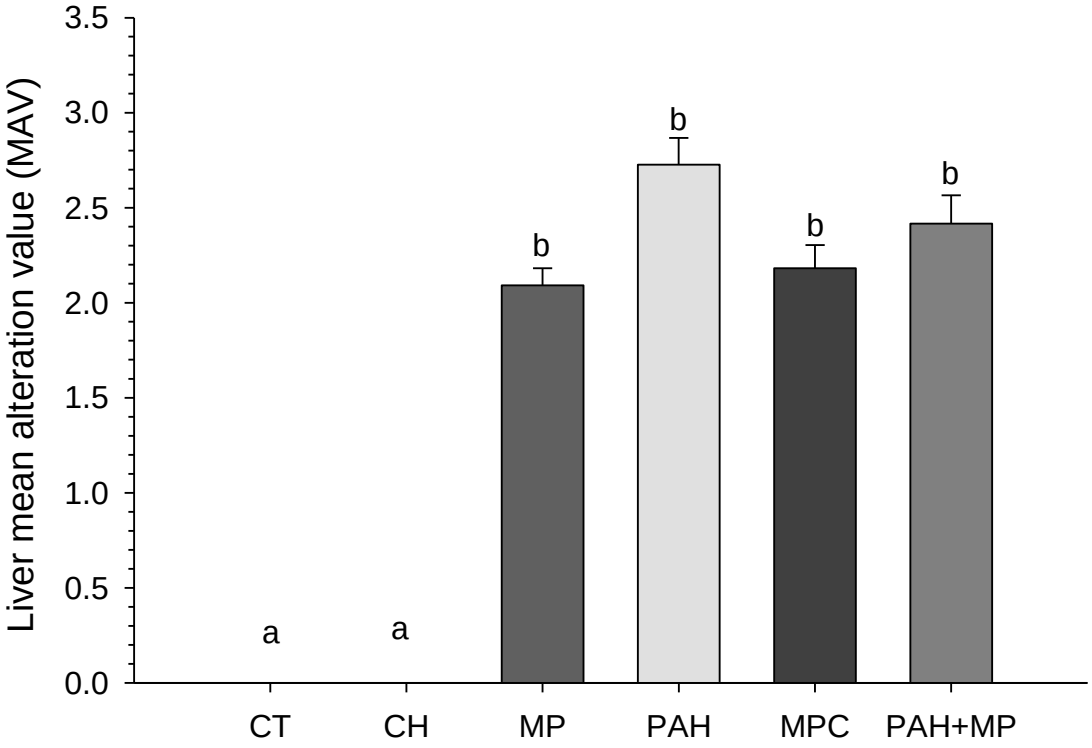

Figure 1 Liver mean alteration value (MAV) (mean ± std) of *A. lacustris*. exposed for 96 h to the five different treatments (CT: Control; CH: control with handling; MP: virgin microplastic; PAH: polycyclic aromatic hydrocarbons; PAH+MP: PAH and virgin MP and MPC: microplastic previously contaminated with PAH). Statistical differences between groups are represented by letters.
Source: Prepared by the author.

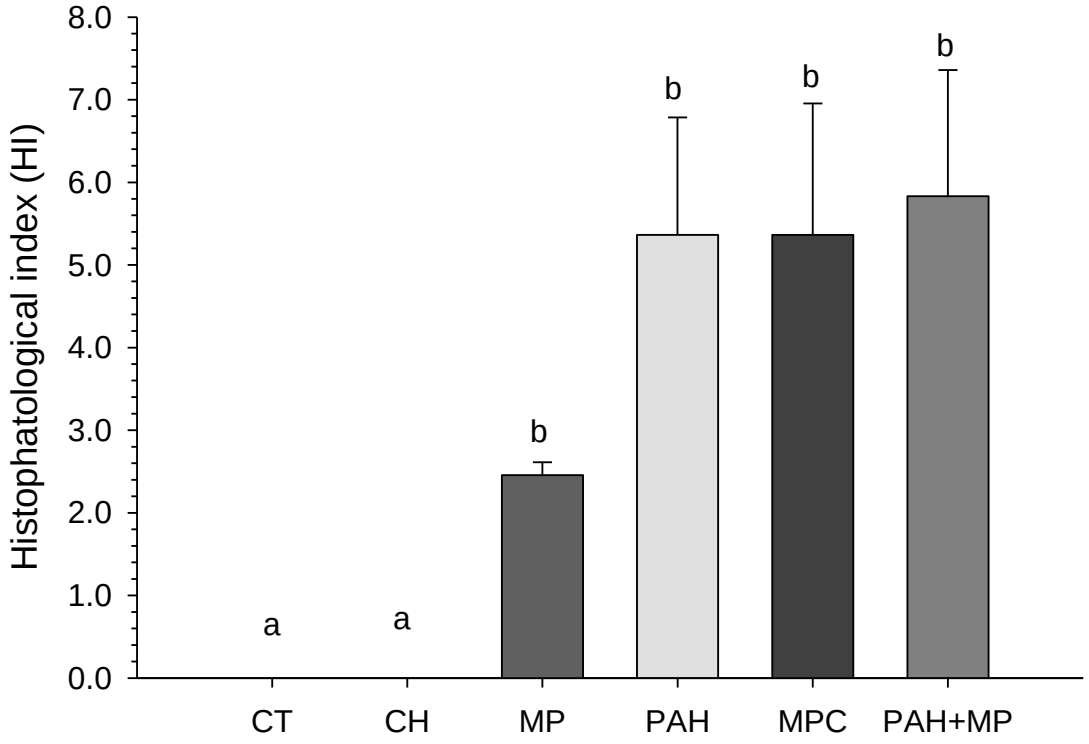

Figure 2. Histological index (HI) of A. lacustris exposed for 96 h to five different treatments (CT: control; CH: control with handling; MP: virgin microplastic; PAH: polycyclic aromatic hydrocarbons; PAH+MP: PAH andvirgin MP and MPC: microplastic previously contaminated with PAH). Statistical differences are shown by different letters.
Source: elaborated by the author.

4.3. PROTEIN CARBONYLATION

The exposure of *A. lacustris* to the contaminants used in this work promoted a quantitative change in the production of carbonyl proteins present in the liver tissue of the organism. As shown in Figure 5, the treatments show significant increases in relation to the control group. A greater increase can be observed in the treatments with PAH in relation to the treatment with MP. On the other hand, the MP treatment has changes in relation to the control, but not in relation to the control with handling. The treatments PAH, MPC, PAH+MP do not have significant differences between them.

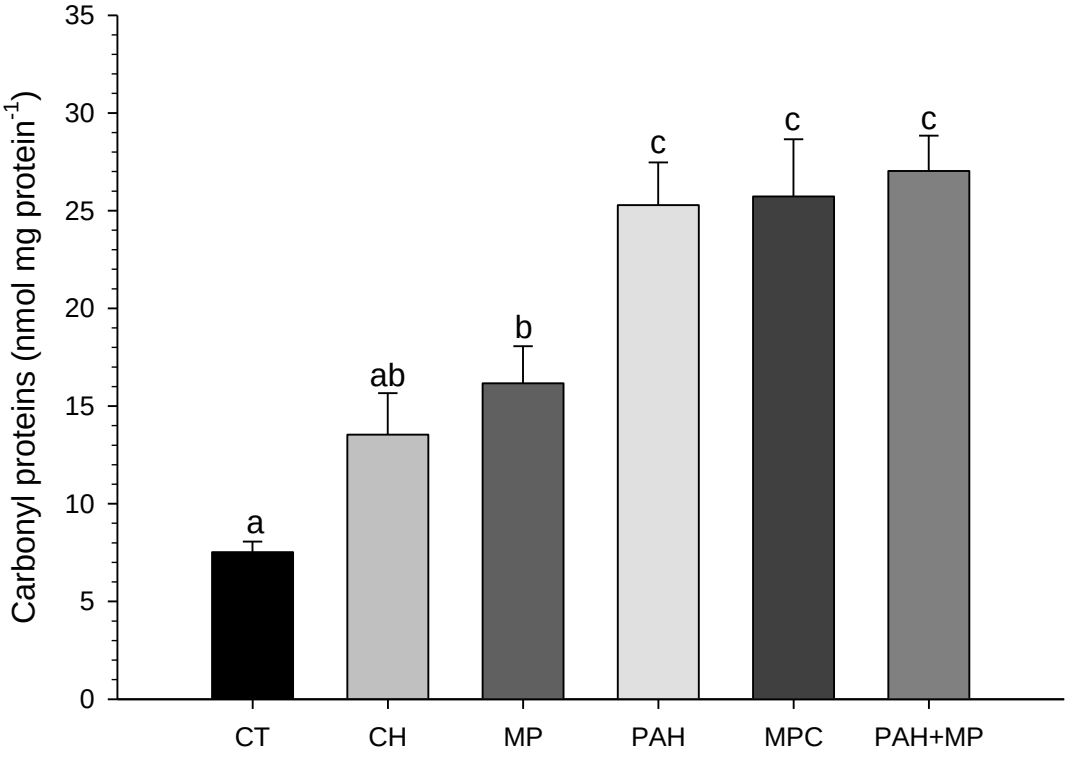

Figure 5 Carbonyl proteins in liver of A. lacustris exposed for 96 h to five different treatments (CT: control; CH: control with handling; MP: virgin microplastic; PAH: polycyclic aromatic hydrocarbons; PAH+MP: PAH andvirgin MP and MPC: microplastic previously contaminated with PAH). Statistical differences are shown by different letters.
Source: elaborated by the author.

5. DISCUSSION

The histopathologies found in the liver indicate that the fish suffered with physiological and biochemical changes due to the presence of the MP, MPC and PAH+MP. The liver is a biotransforming organ, and it suffers an increase in its biotransformation activity when organisms are exposed to toxic substances (SADAUSKAS-HENRIQUE *et al.*, 2017; VAN DER OOST; BEYER; VERMEULEN, 2003). In the present study, the most frequent histopathologies were vacuolization, disarrangement of the hepatic cords and deformation of the cell contour. Cytoplasmic vacuolization may be a sign of lipid damage, caused by oxidative stress due to the presence of contaminants (PAULINO *et al.*, 2014). The deformation of the nuclear contour was the histopathology with the highest frequency of occurrence in the organisms studied, other studies also described a frequent occurrence of this alteration as an indicator of

damages directly linked with the pollutants. According to Strmac and Braunbeck (2000), nuclear changes, such as deformation, indicate that the nucleus is one of the main sites of action of the contaminants. In the present study, fish exposed to the 20% of the LC_{50}-96 h and 10 mg/L of virgin MP, for 96 h, did not show changes of degree greater than II, indicating a reversibility in the pathological condition of the organ, once the contact with the pollutants is interrupted. Both virgin MP and PAH caused significant liver damage, since MVA (mean value of alteration) and HI (histopathological index) higher in PAH, MPC and PAH+MP than controls (CT and CH) (Figures 3 and 4). In this sense, the hypotheses that treatments with PAH and MP together would cause greater damage than MP and PAH alone were refuted. However, in the present study, very low concentrations of PAH (20% of LC_{50}-96 h) and MP (10 mg/L) were used, which may be influenced by the lack of observation of synergistic effects between the studied contaminants. Another study with PAH found hepatic alterations in livers of several fish species (i.e. *Labeo bata, Labeo rohita* and *Cirrhunus mrigal*) with several hepatic histopathologies found for *A. lacustris* in the present study. In a study performed with *Danio rerio*, they was able to found plastic particles inserted in the histological sections (COLE *et al.*, 2011). Anyway, in the present study, did not obtain the same results maybe because of the MP particle size (4 -6 µm for COLE *et al.*, 2011 against 0.05 - 0. 5 cm in the present study).

Corroborating the data on histopathological liver damage, in the present study increases in the concentration of carbonyl proteins were found in fish exposed to MP and PAH both alone and in association. Carbonyl proteins are a by-product of the physiological oxidative process. Oxidative stress, due to the increase in the body's metabolic activity, modifies the protein amino acids and increases the amount of this by-product generated, the accumulation of oxidative modification in biomolecules are linked to pathological changes in metabolism, induction of apoptosis and cell death. In the present study, fish exposed to virgin MP showed production of carbonylated proteins, however, fish exposed to treatments with PAH (PAH, MPC and PAH+MP) suffered greater oxidative damage to their liver proteins in relation to virgin MP and the controls (Figure 5). The increase in the amount of carbonyl proteins shows a damage or inefficiency of the body's antioxidant defense (DAL PONT, 2018), due to the stress caused by the pollutants (PAH and MP). Other studies also observed an increase in the concentration of carbonyl proteins in liver

tissues of fish exposed to other contaminants, such as pesticides (PARVEZ; RAISUDDIN, 2005), and observed a significant increase in these, indicating physiological damage directly related to the pollutant.

According to the review performed by BHAGAT *et al*., (2021), a synergistic effect between MP and contaminants, such as heavy metals; pesticides; drugs and plastic additives, were observed in 11 studies, while for 4 studies no interaction between MP and pollutants such as, florfenicol; mercury; phenanthrene and titanium dioxide, were observed, in a total of 15 studies. From this study we can see that there is a variation of compounds that exhibit synergy with MP, some of which are listed as having no interaction with a PAH, corroborating the results of the present work. A study carried out with fluoranthene and *Mytilus edulis* observed that the microplastics were able to carry the hydrocarbon, and it generated oxidative damage, which was measured with the analysis of enzymatic biomarkers. This study did not see a significant difference between treatment with fluoranthene alone and treatment with microplastic contaminated with fluoranthene (MAGARA *et al.*, 2019) although this study refers to another bioindicator organism, we can see a trend in results with respect to the present work. In another work, this contaminant transfer to water was seen, only through nanoplastics (TREVISAN; UZOCHUKWU; DI GIULIO, 2020) being an indication that even a particle smaller than those of the present study would cause damage.

6. CONCLUSION

It can be concluded that the presence of PAH generates liver damage that was measured through histological damage and amount of carbonyl proteins. The presence of virgin MP does not influence the toxicity of PAH, as it was toxic in all treatments that contained it. In treatments that contained both MP and PAH, there was no statistical difference between them. Thus, it can be said that the PAH adsorbed on the loaded MP were indeed toxic to the organisms, considering that only the inert MP did not cause the damage that the contaminated MP did. On the other hand, the toxicity of inert MP, at the concentrations used in this study, did not cause liver damage in relation to histology and concentration of carbonyl proteins.

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