UNIVERSIDADE SANTA CECÍLIA
PROGRAMA DE PÓS-GRADUAÇÃO EM SUSTENTABILIDADE DE ECOSSISTEMAS COSTEIROS E MARINHOS

JUAN MARTINS DE CAMPOS

Biochemical and physiological responses of the neotropical fish species, *Astyanax lacustris,* exposed to microplastic and polycyclic aromatic hydrocarbons (PAH)

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Dissertação apresentada a Universidade Santa Cecília como parte dos requisitos para obtenção de título de mestre no Programa de Pós-Graduação em Sustentabilidade de Ecossistemas Costeiros e Marinhos, sob a orientação da Profa. Dra. Helen Sadauskas-Henrique e coorientação do Profa. Dra. Luciana Rodrigues de Souza-Bastos.

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DEDICATÓRIA

Dedico este trabalho aos meus avós (José Paulo e Elza), meus pais e familiares que me incentivaram e apoiaram nesse grande processo pelo qual eu tive que passar na minha vida.

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ABSTRACT

The materials of plastic origin are widely used, it has multiple utilities and forms with low cost, with brings practicality and consequent abundance of this material around the world. Plastic can reach the aquatic ecosystem through industrial and domestic discharges, where, due the weathering, can became microplastics (MP), or be discharged as MP. The MP have the capacity to carry adsorbed toxic compounds, such as polycyclic aromatic hydrocarbons (PAH), which are the most toxic portion of the crude oil. To evaluate the possible toxic effects of the virgin MP, PAH and virgin MP and PAH in association, we performed experiments with the *Astyanax lacustris*, widely used as a fish model. Environmental relevant concentration of MP (10 mg L^{-1}) and 20% of the LC50-96 h of the crude oil for the A. lacustris (2.28 µg L^{-1}) were used along 96 h of exposure. Fish were exposed to virgin MP; PAH; MPC (MP loaded with PAH); PAH+MP (virgin MP in association with PAH) and the control without (CT) and with handling (CH). After the 96 h, blood was removed for erythrocytic nuclear abnormalities (ENA) analysis and osmoregulation parameter (plasma osmolality; Na^{+}, K^{+}, Cl^{-2}, Mg^{+2}; glycose and lactate); gills for analysis of histopathological and the osmoregulation enzymes Na^{+}, K^{+} ATPase and carbonic anhydrase; and muscle samples were used to evaluate the glycogen as energetic substrate. Virgin MP was able to cause ENA and depletion of muscle glycogen, which indicates that changes in the metabolism was caused by the virgin MP. However, the MP loaded with PAH and the virgin MP in association with PAH were more toxic than the PAH and virgin MP alone, with no differences between the treatments MPC and PAH+MP. However, there were no differences in the toxicity of the MP loaded with PAH and the virgin MP in association with PAH.

Keywords: Microplastic. PAH. Tetra cardinal. Biochemical. Physiological. Alteration.

RESUMO

Respostas bioquímicas e fisiológicas da espécie de peixes neotropicais, *Astyanax lacustris*, expostas a hidrocarbonetos aromáticos microplásticos e policíclicos (PAH)

Os materiais de origem plástica são amplamente utilizados, possui múltiplas utilidades e formas de baixo custo, com traz praticidade e consequente abundância desse material em todo o mundo. O plástico pode chegar ao ecossistema aquático através de descargas industriais e domésticas, onde, devido ao intemperismo, pode se tornar microplástico (MP), ou ser descarregado como MP. A MP tem a capacidade de transportar compostos tóxicos adsorvidos como hidrocarbonetos aromáticos policíclicos (HPA), que são a porção mais tóxica do petróleo bruto. Para avaliar os possíveis efeitos tóxicos da MP virgem, HPA e virgem MP e HPA em associação, realizamos experimentos com os *Astyanax lacustris*, amplamente utilizados como modelo de peixe. Foram utilizadas concentrações ambientais relevantes de MP (10 mg $^{L-1}$) e 20% do LC50-96 h do petróleo bruto para o *A. lacustris* (2,28 µg $^{L-1}$) ao longo de 96 horas de exposição. Os peixes foram expostos à MP virgem; HPA; MPC (MP carregado com HPA); HPA+MP (MP virgem em associação com HPA) e o controle sem (TC) e com manuseio (CH). Após as 96 h, o sangue foi removido para análise de anormalidades nucleares eritrocíticas (ENA) e parâmetro de osmorregulação (osmolaridade plasmática; Na^+, K^+, $^{Cl-2}$, Mg^{+2}; glicose e lactato); brânquias para análise das enzimas histopatológicas e osmorreguladoras Na^+, K^+ ATPase e anidrase carbônica; e amostras musculares foram utilizadas para avaliar o glicogênio como substrato energético. A Virgem MP foi capaz de causar ENA e esgotamento do glicogênio muscular, o que indica que as alterações no metabolismo foram causadas pela MP virgem. No entanto, o MP carregado com HPA e a MP virgem em associação com o HPA foram mais tóxicos do que o HPA e a MP virgem sozinho, sem diferenças entre os tratamentos MPC e HPA+MP. No entanto, não houve diferenças na toxicidade do MP carregado com HPA e da MP virgem em associação com o HPA.

Palavras-chave: Microplástico. HPA. Tetra Cardinal. Bioquímico. Fisiológico. Alteração.

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LIST OF ABBREVIATIONS AND ACRONYMS

CT	-	Control without handling
CH	-	Control with handling
MP	-	Virgin Microplastic
PAH	-	Polycyclic aromatic hydrocarbons
MPC	-	Microplastic loaded with PAH
PAH+MP	-	Polycyclic aromatic hydrocarbons in association with virgin Microplastic

Summary

1. Introduction

Plastic products are materials widely used all over the world. They have several formats and utilities, and are low cost, but it cannot be denied that this practicality generates an abundance of this material, making its management more difficult. According to The United Nations Environment Programme (UNEP) only 9% of all plastic waste ever produced has been recycled in the world Worldwide. (PARKER, 2018). While in Brazil only a small portion (less than 20%) are reused (GOMES *et al.*, 2008).This material is of industrial and domestic origin, most of these plastic wastes are used and disposed of on land and in environments close to rivers, lakes and lagoons, subjecting these freshwater environments to contamination by plastic materials (HORTON, ALICE A.; WALTON, ALEXANDER; SPURGEON, DAVID J.; LAHIVE, ELMA; SVENDSEN, 2017). Fishing activities are also responsible for the plastic wastes, such as the use of nylon. Through the freshwater environment, these residues can reach the estuarine and marine environments through the discharge of effluents and later through rainy seasons that allow the transportation of these plastic materials to other environments (DANTAS; BARLETTA; DA COSTA, 2012). Microplastics can have several compositions such as polyethylene, polystyrene, polypropylene, polycarbonate, polyamide and polychloroprene (HORTON, ALICE A.; WALTON, ALEXANDER; SPURGEON, DAVID J.; LAHIVE, ELMA; SVENDSEN, 2017). These materials are available for a wide chain of organisms such as fish, aquatic mammals, birds and are able to induce several effects including physical damage (COLE, MATTHEW; LINDEQUE, PK; HALSBAND, 2016), having high persistence in the environments, and capacity to carry adsorbed toxic compounds (GEYER; JAMBECK; LAW, 2017; ZHANG *et al.*, 2018) such as polycyclic aromatic hydrocarbons (PAHs).

The PAHs are a subgroup of persistent organic pollutants (POPs) (RODRIGO ORNELLAS MEIRE; ANTÔNIO AZEREDO ; MARCIA DE SOUZA PEREIRA ; JOÃO PAULO MACHADO TORRES, 2007). They present two or more condensed aromatic rings, and can have nitrates and oxygenates in their composition, in accordance with their organic origin. The PAHs can have natural or anthropogenic origin, where the last one can be a consequence of its extraction, industrial activities and petroleum refining (YUNKER *et al.*, 2002). The PAHs have several origins such as the processes of combustion of organic material, the burning of coal, cigarette smoke and industrial

processes such as aluminum production (PEREIRA NETTO *et al.*, 2000). Also, the increases of PAHs in the aquatic environment can impair the health of the aquatic organisms due to the mutagenic and carcinogenic properties of this class of xenobiotics (YU. V. PASHIN AND L. M. BAKHITOVA, 1979). Alterations on behavior (BROWN *et al.*, 2016; GUVEN *et al.*, 2018; PULSTER *et al.*, 2020), immunotoxicity (COLLIER *et al.*, 2013; JIANG; YANG; FANG, 2020; MARYAM KHANIYAN, NEGIN SALAMAT, ALIREZA SAFAHIEH; DEPARTMENT, 2014), oxidative stress (JIANG; YANG; FANG, 2020; PRITSOS *et al.*, 2017; SOLTANI *et al.*, 2019) and histopathological alterations like neoplasia were already reported for aquatic organisms exposed to these (KAMMANN *et al.*, 2017; MATSCHE *et al.*, 2020; SNYDER *et al.*, 2015).

In this sense, biomarkers act as a tool to diagnose chemical exposure and their effects to the aquatic organisms (CORMIER; DANIEL, 1994). Changes can be measured at low levels of the biological organization, like biochemical, physiological, histological and behavioral levels (PARVEZ; RAISUDDIN, 2005), generating information that may predict the effects of the contaminants in the high levels of the biological organization, like population and communities (WENDELAAR BONGA, 1997).

Several are the effects caused by exposure to contaminants, these begin with the contact of the gills with the aquatic environment. The gills are an important respiratory organ that presents high permeability and vascularity, also the gills present a central function in the processes of ionic and osmotic homeostasis responding directly to any type of alteration in the aquatic environment where this animal is located (RODRIGUES; BASTOS, 2011). The changes that may occur in the gills due environmental stressor can be morphological (primary) and physiological (secondary). Some specific effects to be highlighted are variations in the morphology and number of mitochondria rich cells, damages such as hyperplasia, lamellar fusion, and hypertrophy. Metabolic changes such as glucose concentration, lactate and glycogen are also consequences of stress, acting as secondary response, hormones released after being exposed to stressors generate metabolic changes to meet the animal's energy demand. Changes of this nature was already described for fish species exposed to PAHs (GROSELL; PASPARAKIS, 2021; HONDA; SUZUKI, 2020; ROCHMAN *et al.*, 2013a).

Astyanax lacustris, popularly known as tetra cardinal, is a fish species present in southern Brazil (GARUTTI; BRITSKI, 2000). These animals have been widely used for studies evaluating the aquatic contamination, as well as, as a model fish species for laboratory studies (DESTRO *et al.*, 2021; MUÑOZ-PEÑUELA *et al.*, 2021; OSTRENSKY; PEDRAZZANI, 2016; TINCANI *et al.*, 2019). This fish species also have ecological relevance for their omnivorous habit (SHIBATTA *et al.*, 2002) and for being predated by different species of predators (GIORGI DAL PONT, 2018).

In this sense, the present study aimed to understand the toxicity of the PAHs and MPs, separated and in association, to the neotropical fish species *Astyanax lacustris* through the analysis of the biochemical, physiological, and histological responses.

Specific objectives:

- Evaluate the toxicity of virgin microplastics; PAH; MP loaded with PAHs and PAH and virgin MP in association, through the measurement of the osmoregulatory parameters in plasma (concentration of ions Na^+, K^+, Cl^-, Mg^+; and osmolality) and gills (Na^+, K^+ ATPase and carbonic anhydrase activities).
- Evaluate the toxicity of virgin microplastics; PAH; MP loaded with PAHs and PAH and virgin MP in association, through the measurement of the energetic substrates (lactate and glycogen concentrations in muscle and plasma glucose concentration).
- Evaluate the toxicity of virgin microplastics; PAH; MP loaded with PAHs and PAH and virgin MP in association, through the measurement of the presence of erythrocytic nuclear abnormalities.
- Evaluate the toxicity of virgin microplastics; PAH; MP loaded with PAHs and PAH and virgin MP in association, through the measurement of the gill histological damage.

2. Material and methods

The experiments were performed in the Grupo Integrado de Aquicultura e Estudos Ambientais (GIA) located in the Universidade Federal do Paraná (UFPR) (Curitiba, Paraná, Brasil). *Astyanax lacustris* specimens (9.08 ± 0.35 grams; mean 14.51 ± 0.73 centimeters) were acclimated in 20 L tanks for one week inside the experimentation room (controlled air temperature 25°C). The animals were fed daily

with commercial food pellets during the acclimatization process (Kowalski®, Brazil, crude protein = 47%).

2.1. Experimental design

After one week of acclimatization, the animals were divided into 6 semi-static experimental treatments (n=12) according to Figure 1. The experimental groups were, CT: control without handling; CH: control with handling; MP: virgin microplastic; PAH: polycyclic aromatic hydrocarbons; MPC: virgin MP loaded with PAH and PAH+MP: PAH and virgin MP in association. The animals were individually exposed in glass aquariums (5 L) wrapped in black plastic and with constant aeration supply. During the 96 h of the experiment, daily exchanges of experimental solutions were performed, according to item 3.1.3. Fish feeding was suspended one day before the beginning of the experiments.

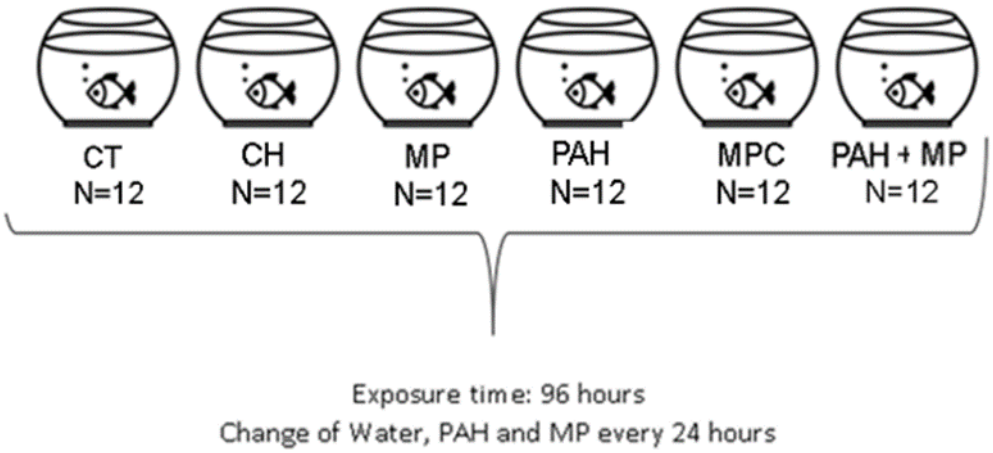

Figure 01. Scheme of experimental treatments and number of subjects (n) used in each treatment. CT: control without handling; CH: control with handling; MP: virgin microplastic; PAH: polycyclic aromatic hydrocarbons; MPC: virgin MP loaded with PAHs and PAH+MP: PAH and virgin MP in association.
Source: Elaborated by the author.

The experiments were conducted in a room with controlled temperature (25°C). The aquariums were all disposed into a water bath to maintain the temperature at 25°C using thermostats. The water physicochemical parameters of the aquariums (pH, temperature, dissolved oxygen) were measured daily and adjusted, if necessary, for pH 7.0; temperature of 25 °C; and dissolved oxygen 80-100% saturation. Daily, immediately after the water change in the fish tank, water and MP samples were collected for PAHs measurements after 24 h of exposure. Water samples from labeled

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 for collecting blood and tissue samples (plasma, gills, kidney, and muscle), the animals were euthanized (via medullary section). The tissue samples were collected and stored in a freezer -80°C where they were kept until the analysis was conducted for biological analysis that were described item 2.2.

2.1.2. Choice of MP and PAHs concentration used in the present study and preparation of experimental solutions.

The MP utilized in the present study were donated by the Brasquen®. The MP are composed by low density polyethylen (LDPE). Before its utilization in the experiments, the MP were passed through a sieve of 74 µm. The MP have rough surface and measures between 100-200 µm (Figure 2). It is known that the MP has the capacity of carrying compounds in its own surface (GEYER; JAMBECK; LAW, 2017; ZHANG *et al.*, 2018) in addition to this information, an electronic microscopy of the MP was performed so that it was possible to look at the structure of this material provided by the Brasquen®.

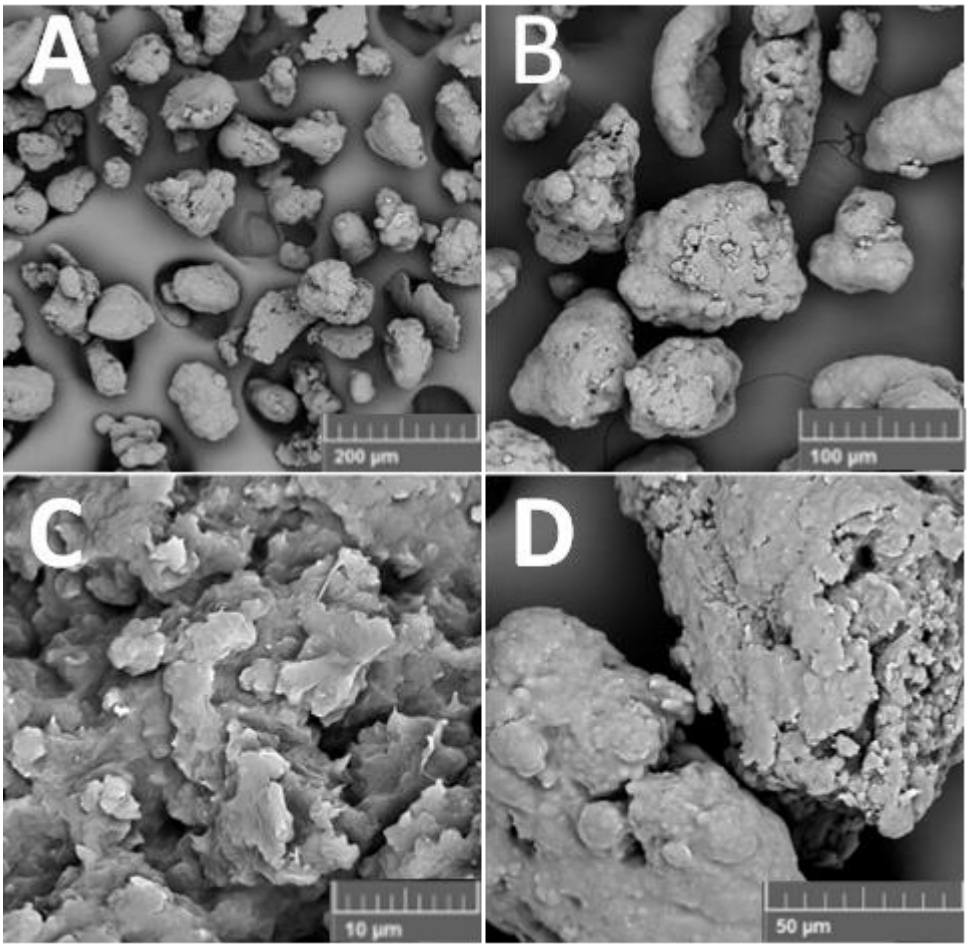

Figure 02. Photomicrographs captured by scanning electron microscopy (SEM) of the microplastics used in the present study. A) SEM HV: 15.0 Kv, SEM MAG: 200 x, View Field: 1.04mm, WD 9.98 mm, Det: BSE + SE, Date (m/d/y): 12/12/19; B) SEM HV: 15.0 Kv, SEM MAG: 400 x, View Field: 519 mm, WD 10.04 mm, Det: 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.

The concentration of MP chosen in the present study was 10 mg L^{-1}, this concentration was chosen because it is a concentration environmentally relevant, found for freshwater environments (WAGNER M, LAMBERT S, 2018). The concentration of PAHs chosen was based on the values of the 20% of the LC_{50}-96 h of crude oil found for *A. lacustris* (2.28 µg L^{-1}) (GIORGI DAL PONT, 2018). In the present study, we chose to use the Sulpeco standard® which contain the 16 priority PAHs of the EPA, since all PAHs are in the same concentrations, it was possible to verify which PAHs become most bioavailable for fish when in the presence of MP. The PAHs stock solution was stored in amber bottle sealed in -20°C to prevent the loss of light PAHs. For the treatment of MP previously contaminated with PAH, a technique called "spiking" were performed according to (ANNIKA BATEL,* FREDERIC LINTI,

MARTINA SCHERER, LOTHAR ERDINGER, 2016). Spiking consists of a technique of contamination/marking of the MP by PAH, for this reason, MP (at a concentration of 10 mg L^{-1}) were added in Erlenmeyer containing a solution of PAHs at a concentration of 2.28 ug L^{-1} (prepared in Milliq® water). For controls, virgin MP passed through the same process with Milliq® water only. After 24 hours in magnetic rotation, the MPs were dried in desicquer for 24 h (ANNIKA BATEL,* FREDERIC LINTI, MARTINA SCHERER, LOTHAR ERDINGER, 2016). Samples of the virgin MP and the marked MP were collected for analysis of PAHs. After drying, the virgin and the marked MP were used in the experiments. These procedures were performed daily throughout the 96-h experiment.

2.1.3. Exchange of experimental solutions:

Daily, during the 96 h experiment, the solutions of PAHs, virgin MP and MP marked with PAHs were renewed. For methodological reasons, it was decided to change all the contents of the aquariums (water and MP), so the renewal of the solutions was 100%. To this end, the fish were carefully transferred from one aquarium to another (containing new experimental solutions). This procedure was repeated for all treatments (including control). In addition, a control without handling were performed to isolate the effects of handling during the experimental solutions exchange.

2.1.4. Analysis of PAHs in water and microplastic

2.1.5. Protocol for the extraction of PAHs in water

The scheme of the procedure developed by vortex-assisted dispersive liquid-liquid extraction (VA-DLLME) and used in this work for PAHs extraction is presented in Figure 3.

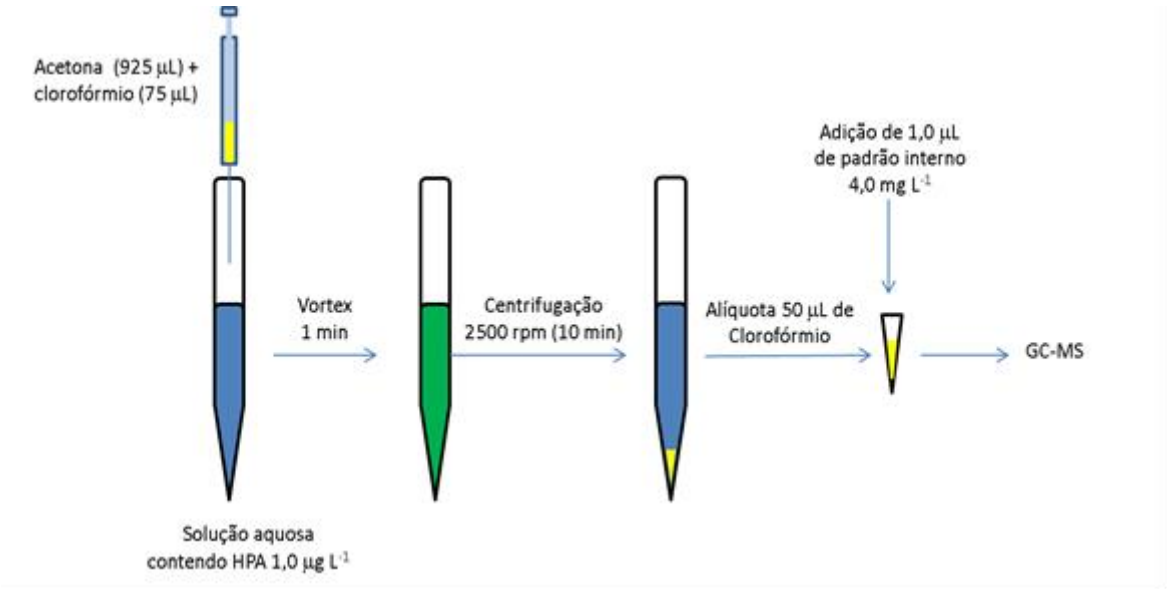

Figure 03. Schematic diagram of the procedure using vortex-assisted dispersive liquid-liquid microextraction (VA-DLLME).
Source: Elaborated by the author.

As illustrated in Figure 3 in VA-DLLME, 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).

2.1.6. Protocol for the extraction of PAHs in microplastic

Thirteen milligrams (13 mg) of MP were transferred to 8 ml glass tubes, along with 3.00 ml acetonitrile. These tubes were sonicated for 10 min, centrifuged for 5 min at 2800 rpm and a 2.50 ml supernatant aliquot were transferred to another glass tube and dried in a vacuum rotary evaporator. Complete evaporation of acetonitrile was performed in 20 min at 60 ºC. After evaporation, the samples were resolubilized in 250 µL hexane, transferred to 300 µL glass inserts and quantified in the gas

chromatography coupled to mass spectrometry (GC-MS). The determination of the concentration of the PAHs adsorbed in MP were performed in of MP samples contaminated with PAHs before entering the experiments.

2.1.7. Identification and quantification of PAHs extracted from water and MP.

After the extraction procedures, the PAHs were identified and quantified by GC-MS (EPA, 1996b). The identification of each compound was performed by comparison with standard solution injection, containing a mixture of the 16 PAHs, and consults the NIST mass spectra library of the equipment. The quantification was performed by analytical curve constructed in the concentration range of 5 to 1000 ng/ml, using as internal standard a solution containing five deuterate PAH (naphthalene-d8, acenaphthene-d10, fenantrene-d10, criseno-d12, perileno-d12) at a concentration of 100 ng/ml. As quality control, solutions were injected daily for curve verification, accepting maximum deviations of 10%.

2.2. Biological analyses in *Astyanax lacustris*

2.2.1. Physiological parameters

2.2.2. Erythrocytic nuclear abnormalities (ENA)

Blood collection was performed with heparinized syringes (to avoid tissue coagulation), by caudal puncture or cardiac puncture, using heparinized hematocrits. A drop of the blood sample was used on a new blade, cleaned with alcohol, and previously identified; where a blood smear was performed, with the aid of another blade. After performing the smear and leaving them drying *over*night and at room *temperature,* the blades were fixed in 96% alcohol or P.A. for 15 minutes and allowed to dry again at room temperature. After that, it was used a Giemsa Conventional Coloring Method.

With the slides fixed and cored, the analyses are initiated, using optical microscope with an increase of 1000 times in the immersion lens, accounting for 3000 erythrocytes/lamina/per animal, as described in the procedures of Schmid (1975) and Carrasco *et al.* (1990).

In the optical microscope, the ideal imaging field containing the cells well apart from each other, with membrane and nucleus well visible for counting. In each image field, all the cells present in the field of view with the counter were counted. After counting the cells of this field of view, the abnormalities present in these cells (if they

have them) were counted and the field of view was later changed, and the counting process was restarted until reaching a total of 3000 cells/lamina. The abnormalities found were noted in a spreadsheet for statistical analysis.

2.2.3. Plasma osmolality, ions (Na^+, K^+, Cl^- e Mg^+), glucose and lactate

Plasma osmolality was determined in undiluted samples read in the vapor pressure microosmometer (WescorR 5520 VAPRO).

The determination of chloride (Cl^-) and magnesium (Mg^{2+}) ions was performed in plasma samples without dilution. These ions were determined through commercial colorimetric kits (Labtest®) with absorbance reading at 470 and 505 nm, respectively, in spectrophotometer (ULTROSPEC 2100 pro – Amersham Pharmacia Biotech). The ions Cl^-, when reacting with mercury thiocyanate in the presence of ferric nitrate, produce ferric thiocyanate of orange color proportional to the amount of chloride ions of the sample. Mg^{2+} ions react in alkaline medium with blue magon sulfonated, resulting in a pinkish complex proportional to the total concentration of magnesium ions in the sample. Sodium (Na^+) and potassium (K^+) ions were determined in diluted plasma samples (1:100) in ultra-pure water (Mili-Q) using a flame photometer (CELM FC - 180).

Glucose concentration was obtained through a commercial colorimetric kit (Labtest®) in undiluted plasma samples with absorbance reading at 505 nm (Molecular Devices® SpectraMax M2e). With the oxidation of glucose, through the action of glucose oxidase, hydrogen peroxide is formed which when reacting with 4- amino antipyrine and phenol forms the antipylquinonimine of red color proportional to the glucose concentration of the sample. The results were expressed as mg dL^{-1}.

Lactate concentration was determined through commercial colorimetric kit (Labtest®) in undiluted plasma samples with absorbance reading at 550 nm (Molecular Devices® SpectraMax M2e). The results were expressed as mg dL^{-1}.

2.2.4. Muscle glycogen

Glycogen content was measured in the muscle according to (PM BIDINOTTO, G MORAES, 1997). Samples (100 ± 10 mg) were degraded in 1 mL of KOH 6N in boiling water for a period of 4 minutes. After cleaning the samples with 95% ethanol, K2SO4 10%, and centrifugation (3000 rpm; 3 minutes), the colorimetric reaction was performed with phenol 3% and H_2SO_4 and read in 480nm.

2.2.5. Total proteins

The total protein of the homogenized was measured according to Bradford, (1976) using a spectrophotometer and albumin patterns. The readings were performed at a wavelength of 595 nm.

2.3. Statistical analyses

The data were represented as mean ± standard error of the mean. Variance analysis (ANOVA) was used to determine differences between the means. The Holm-Sidack Pos-hoc test was applied to identify the differences between the means of the analyzed biological variables, when existing. The programs used were SigmaStat 3.5 for statistical analysis and the SigmaPlot 11.0 program for the preparation of graphs.

3. Results

3.1. PAH in water and in MP particles

It can be observed in Table 1 the PAH concentrations of the working solution (µg L^{-1}) and in the MP (µg L^{-1}) 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^{-1}) and in the MP (µg g^{-1}) before and after the MP spiking procedure. Values with "<" symbol means that were below of the quantification limits.

PAH	Working PAH water solution (µg L^{-1})		MP (µg g^{-1})	
	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
Dibenzo[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 (CH); Polycyclic aromatic hydrocarbons (PAH); PAH and virgin MP (PAH+MP) and MP previously contaminated with PAH (MPC) after 24 h of exposure. Values with "<" symbol means that were below of the quantification limits.

PAH (µg L^{-1})	Experimental treatments					
	CT	CH	MP	PAH	MPC	PAH+MP
Naphthalene	<0.05	<0.05	<0.05	<0,05	<0.05	<0.05
Acenaphthylene	<0.05	<0.05	<0.05	<0,05	<0.05	<0.05
Acenaphthene	<0.05	<0.05	<0.05	<0,05	<0.05	<0.05
Fluorene	<0.05	<0.05	<0.05	<0,05	<0.05	<0.05
Phenanthrene	<0.05	<0.05	<0.05	<0,05	<0.05	<0.05
Anthracene	<0.05	<0.05	<0.05	<0,05	<0.05	<0.05
Fluoranthene	<0.05	<0.05	<0.05	<0,05	<0.05	<0.05
Pyrene	<0.05	<0.05	<0.05	<0,05	<0.05	<0.05
Chrysene	<0.20	<0.20	<0.20	<0,20	<0.20	<0.20
Benz[a]anthracene	<0.20	<0.20	<0.20	<0,20	1.74	0.28
Benzo[b]fluoranthene	<0.20	<0.20	<0.20	<0,20	10.96	9.07
Benzo[k]fluoranthene	<0.20	<0.20	<0.20	<0,20	10.80	9.14
Benzo[a]pyrene	<0.05	<0.05	<0.05	<0,05	<0.05	<0.05
Indeno[1,2,3-cd]pyrene	<1.00	<1.00	<1.00	<1,00	3.41	3.74
Dibenz[a,h]anthracene	<1.00	<1.00	<1.00	<1,00	4.36	4.17
Benzo[ghi]perylene	<1.00	<1.00	<1.00	<1,00	7.18	7.94
∑HPA (ng/L)	-	-	-	-	38.45	34.34

Source: Elaborated by the author.

3.2. Physiological parameters

3.2.1. Erythrocytic abnormalities (ENA)

The ENA values were higher for fish exposed to PAH, MPC and PAH+MP (0.013% for PAH and 0.011% for MPC and PAH+MPC), and the lowest values for fish under the controls CT and CH (0.003% and 0.005%, respectively) and MP (0.008%), without any significant differences between the treatments (Table 03).

The micro-nucleus was absent in controls (CT and CH) and had low percentage in MP, however the micro-nucleus percentage was higher in PAH in relation to controls (CT and CH) and MP, while the MPC and PAH+MP were higher than PAH (Figure 04).

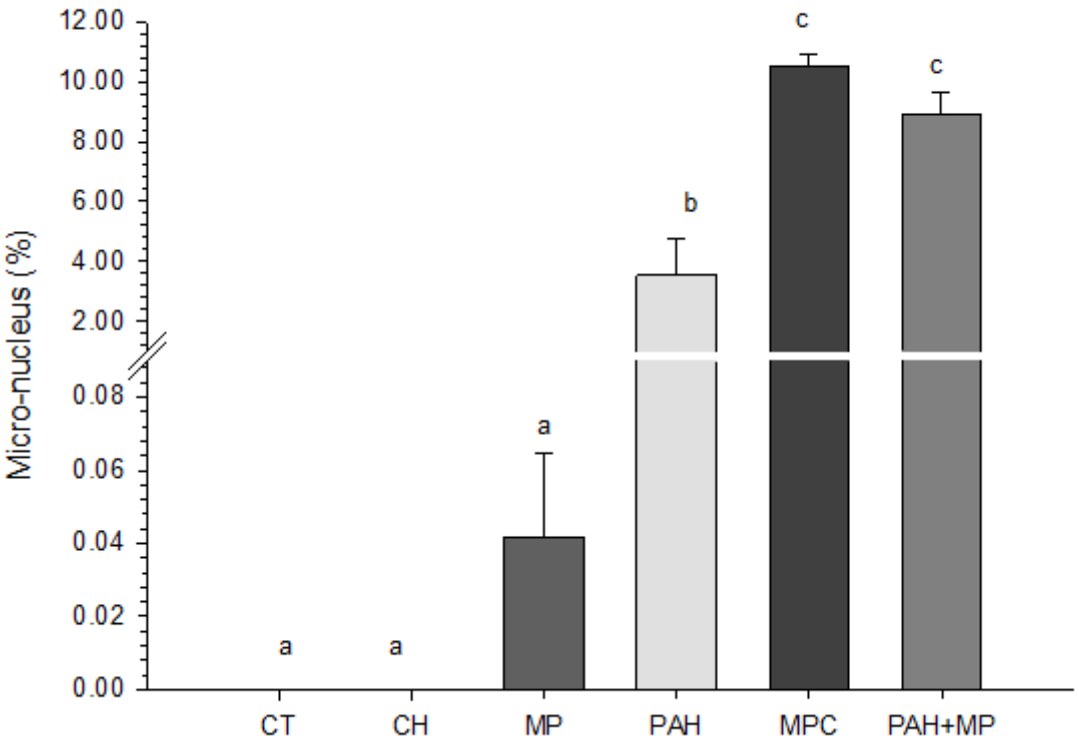

Figure 04. Mean ± standard deviation of the erythrocytic micro-nucleus of *Astyanax altiparanae* exposed to the distinct groups: control (CT); control with handling (CH); inert microplastic (MP); polycyclic aromatic hydrocarbons (PAH); microplastic contaminated with PAH (MPC); and PAH and inert MP (PAH+MP). Different letters indicate significant differences among the groups (P < 0,05). Source: Elaborated by the author.

3.2.2. Plasma osmolality, ions (Na^+, K^+, Cl^- e Mg^+), glucose and lactate

The osmolality concentration varies from 305 to 315 mOsm kg H_2O^{-1} without significant differences between the treatments. The lowest and the highest values was obtained for fish exposed to PAH+MP, and PAH and MPC treatments, respectively (Table 03).

The plasma Na^+ concentrations vary from 126 to 137 mMol L^{-1}. Fish exposed to MPC had lower plasma Na+ concentration in relation to CT, CH, and MP (Table 03).

The K^+ concentrations vary from 2.1 to 2.9 mMol L^{-1} without significant differences between the treatments. The lowest and the highest values was obtained for fish exposed to PAH+MP and PAH, respectively (Table 03).

The Cl^- concentrations vary from 97 to 106 mMol L^{-1} without significant differences between the treatments. The lowest and the highest values was obtained for fish exposed to CT and PAH+MP, respectively (Table 03).

The Mg^{+} concentrations vary from 1.98 to 2.01 mMol L^{-1} without significant differences between the treatments. The lowest and the highest values was obtained for fish exposed to CT and PAH+MP, respectively (Table 03).

The glucose and lactate concentrations vary from 98.6 to 103.7 mg dL^{-1}, and 39.4 to 48.2 mg dL^{-1}, respectively, without significant differences between the treatments. The lowest values for glucose and lactate were found for fish exposed to MPC and the highest values for glucose and lactate was found for fish under the control conditions CT and CH, respectively (Table 03).

Table 3. Mean ± standard deviation of the total blood ENA; and plasma glucose; lactate; Na^{+}; K^{+}; Mg^{2+}; Cl^{2-} concentrations and osmolality of *Astyanax altiparanae* exposed to the distinct groups: control (CT); control with handling (CH); inert microplastic (MP); polycyclic aromatic hydrocarbons (PAH); microplastic contaminated with PAH (MPC); and PAH and inert MP (PAH+MP). Different superscript letters indicate significant differences among the groups (P<0,05).

	ENA (%)	Gluc (mg dL^{-1})	Lact (mg dL^{-1})	Na^{+} (mMol L^{-1})	K^{+} (mMol L^{-1})	Mg^{+} (mMol L^{-1})	Cl^{-} (mMol L^{-1})	Osm (mOsm kg H_2O^{-1})
CT	0.005 ± 0.005	103.71 ± 4.34	39.63 ± 3.98	136.61 ± 2.09[a]	2.25 ± 0.166	1.98 ± 0.06	97.98 ± 4.13	312 ± 4.12
CH	0.003 ± 0.003	99.78 ± 4.47	48.23 ± 9.98	136.88 ± 1.88[a]	2.55 ± 0.304	2.02 ± 0.08	104.63 ± 11.80	308 ± 4.21
MP	0.008 ± 0.006	101.52 ± 3 .00	40.09 ± 2.92	135.15 ± 1.06[a]	2.42 ± 0.204	2.00 ± 0.07	99.79 ± 8.87	311 ± 2.22
PAH	0.013 ± 0.010	101.03 ± 5.23	44.16 ± 6.42	132.18 ± 1.55[ab]	2.88 ± 0.25	1.99 ± 0.06	105.53 ± 9.36	315 ± 4.52
MPC	0.011 ± 0.009	98.59 ± 5.13	39.44 ± 4.41	129.54 ± 1.94[b]	2.44 ± 0.44	2.00 ± 0.04	103.28 ± 11.43	315 ± 5.43
PAH+ MP	0.011 ± 0.011	101.31 ± 4.74	43.67 ± 6.30	125.60 ± 2.08[ab]	2.15 ± 0.132	2.01 ± 0.03	106.56 ± 6.52	305 ± 3.25

Note. ENA= erythrocytic abnormalities; Gluc= glucose; Lact= lactate; Osm= osmolality.

3.2.3. Muscle glycogen

The muscle glycogen concentrations vary from 1 to 40 ng dL^{-1}. Decreases in muscle glycogen were observed in fish exposed to PAH, MPC and PAH+MPC in relation to controls (CT and CH) (Figure 05).

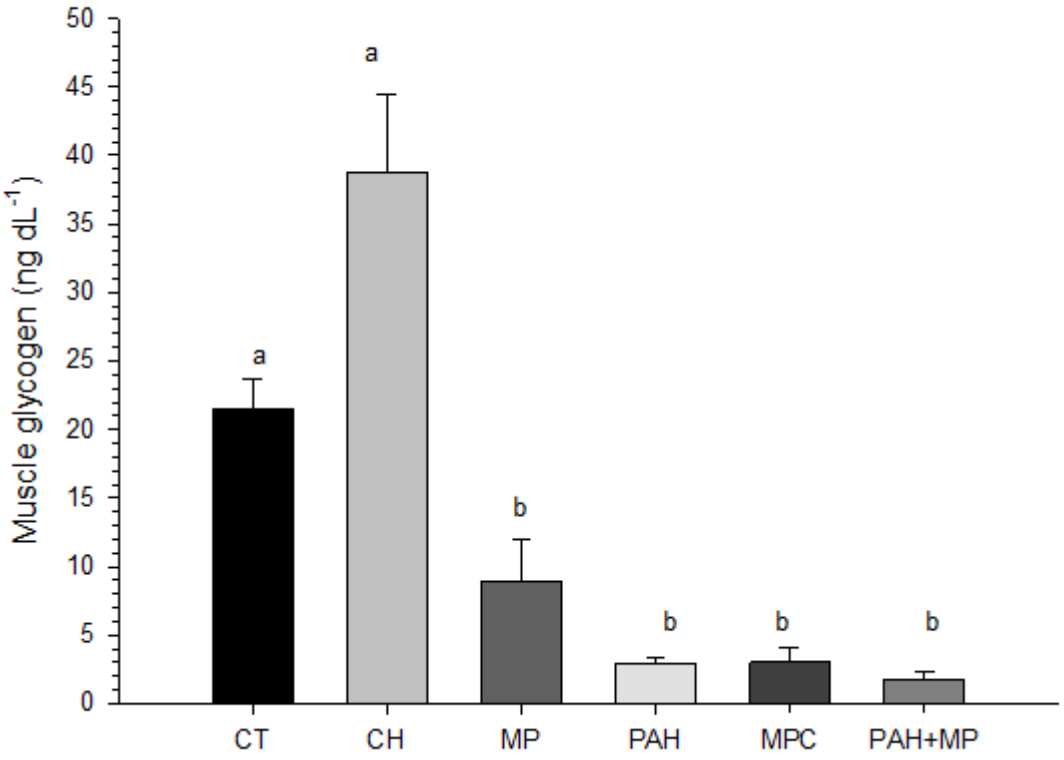

Figure 05. Mean ± standard deviation of the muscle glycogen of *Astyanax altiparanae* exposed to the distinct groups: control (CT); control with handling (CH); inert microplastic (MP); polycyclic aromatic hydrocarbons (PAH); microplastic contaminated with PAH (MPC); and PAH and inert MP (PAH+MP). Different letters indicate significant differences among the groups ($P < 0,05$).
Source: Elaborated by the author.

4. Discussion

In the present work, the PAH concentration, even corresponding to only 20% of the LC_{50}-96 h for *A. lacustris*, caused biochemical (ANE) and physiological effects (plasma Na^+ concentration and muscle glycogen), especially in the treatments where the PAH was in association with MP. The MP marked with PAH (MPC) presented similar biochemical and physiological effects of when the virgin MP was used in association with PAH (PAH+MP), once no differences was observed in the biochemical and physiological parameters between these groups. The virgin MP, used in freshwater relevant environment concentrations (WAGNER M, LAMBERT S, 2018), was also able to cause some disturbance, like increasing ENA formation and decreasing the energetic subtract in the muscle (glycogen). These and other results are discussed in the topics bellow.

4.1. PAH in water and in MP particles

The PAHs with a high molecular weight have higher octanol–water partition coefficients (Kow) and, as a consequence, they are highly lipophilic, tending to adsorb to particulate matter (BARRON *et al.*, 1999; HYLLAND, 2006). This may explain the fact that the low molecular weight PAH naphthalene, acenaphthylene, acenaphthene and fluorene were not detected in MP water after the spiking procedure, neither in the spiked MP. In fact, these PAH had their concentrations decreased after 24 h of spiking procedure. Due to their low molecular weight, i.e. low Kow, these PAH rapidly are lost from the aquatic environment due its high volatility (SADAUSKAS-HENRIQUE; SOUZA-BASTOS; SILVA, 2021) even in the presence of MP, this PAH was not detected in MPC and PAH+MP. Interesting to note that, in the PAH treatment, after 24 h of exposure, no one of the 16 PAH were detected in the water. This phenomenon could also be explained by the volatility of the PAH, when in the absence of MP, the PAH probably volatilized and/or adsorb to organic material (i.e., fish), making these PAH undetectable in the water. Anyway, in the present study, water exchange was performed each 24 h of exposure. Also, even the PAH been below to the detection limits, the fish exposed to PAH treatment experience disturbances in the measure parameters.

Generally, low density polyethylene plastics are expected to be rubbery plastics (ROCHMAN *et al.*, 2013b). According to Zuo *et al.* (2019), the higher the rubber component of MPs, the stronger the sorption capacity for organic compounds. This is mainly due to the disordered arrangement of molecular chain segments in the rubbery MPs, and the existence of a large amount of free volume between the molecular chains, thus facilitating the sorption of hydrophobic organic compounds, like PAH (GUO *et al.*, 2012). In the present study, the SEM imagens of the MP (Figure 02) demonstrate particles with irregular surface, which also increases the PAH absorption (GUO *et al.*, 2012). In fact, the spiked MP presented a high concentration of PAH (771.45 µg g-1) adsorbed. This is a high value when compared with other studies like in Sharma et. al. (2020), which had an absorption of 110 µg g^{-1} for small polymers particles (> 0.1 µm) and 43 µg g^{-1} for bigger polymers particles (1-5 mm) for the PAH congeners- chrysene, benzo[a]pyrene and indeno[1,2,3-cd] pyrene. However, when we compared the leaching of the PAH from the MP, our study had lower values (38.45 µg L^{-1} for MPC and 34.34 µg L^{-1} for PAH+MP) when compared with the same study that have a leaching of 3170,00 µg L^{-1} (3.17 mg L^{-1}). Anyway, the leaching of PAH from

MP depends on several factor like, pH, water temperature, salinity, and Kow values (MEI *et al.*, 2020).

4.2. Erythrocytic abnormalities (ENA)

The presence of abnormal cells in blood smears of fish are useful to detect chromosomic damage produced by a wide range of toxic compounds, specifically by detecting binucleated or polynucleated cells containing micro-nucleus (AYANDA *et al.*, 2018). The micro-nucleus is extranuclear bodies that contain damaged chromosome fragments and/or whole chromosomes that were not incorporated into the nucleus after cell division. For instance, micro-nucleus is useful tool for evaluating the effects of genotoxic and mutagenic contaminants in fish species. In the present study, the virgin MP, at 10 mg L^{-1}, was not able to cause erythrocytic DNA damage in *A. lacustris*. On the other hand, Hamed *et al.* (2021) found dose-dependent (1; 10 and 100 mg L^{-1}) increases in nuclear abnormalities of the red cells of *Oreochromis niloticus*. The same way, Araújo *et al.* (2022) found that the erythrocyte DNA damage (comet assay) and the ENA in Danio rerio exposed to polyethylene MP alone were as pronounced as those observed in animals exposed to the mix of pollutant (alone or in combination with MP). According to Hamed *et al.* (2019) the MP additives may interact with erythrocytes and induce the interruption of plasma membranes, producing abnormally shaped blood cells. Despite this, in the present study, the virgin MP alone did not induce ENA in *A. lacustris*, the treatments with MP spiked with PAH and virgin MP in association with PAH caused increases in ENA, higher than those of fish exposed to the PAH alone. A reasonable explanation for this phenomenon, is that the higher production of reactive oxygen species (ROS) in erythrocytes could be caused by the direct interaction between MPs and erythrocyte plasma membranes (COSTA ARAÚLO *et al.*, 2020a) and by the PAH biotransformation process and consequent ROS production (SADAUSKAS-HENRIQUE *et al.*, 2017).

4.3. Plasma osmolality, ions (Na^+, K^+, Cl^- e Mg^{2+}), glucose and lactate

The gills are the main sites for gas exchange as well as acid–base and ionic regulation and are the first organs to contact the contaminants. Due to their very thin epithelia and large surface area that is in contact with water, the gills are the major route of uptake of waterborne pollutants by aquatic organisms (EVANS; PIERMARINI; CHOE, 2005). Pollutants, as PAH, have been shown to promote structural damage in

the branchial respiratory epithelium (EVANS; PIERMARINI; CHOE, 2005), affecting important gills functions, as osmoregulation (DAL PONT *et al.*, 2019; DUARTE; HONDA; VAL, 2010; RODRIGUES; BASTOS, 2011). Osmoregulation maintains the osmo-ionic concentration of the extracellular fluid, or internal environment of the animal as part of homeostasis and normal cellular functioning (AL-KINDI; BROWN; WARING, 2000). Hydromineral imbalance is an effect commonly caused by oils and byproducts on different species of marine or freshwater fish (AL-KINDI *et al.*, 1996; AL-KINDI; BROWN; WARING, 2000; BRAUNER *et al.*, 1999; DUARTE; HONDA; VAL, 2010; SOUZA-BASTOS; FREIRE, 2011; THERON *et al.*, 2014). In the present study, the hydromineral balance was not affected by the PAH and MP alone and in association, except for the Na^+ plasma concentration that was depleted in MPC in relation to CT, CH, and MP. *Colossoma macropomum* also experience decreases in Na^+ plasma concentration with was related with increases in the passive losses of Na^+ by the gills, indicating strong effects of the crude oil on gill ion permeability (DUARTE; HONDA; VAL, 2010).

4.4. Muscle glycogen

During the metabolization of contaminants, there is an increases of the basal metabolism (metabolic rate) due to the increases of the metabolic vias for homeostasis maintenance (WENDELAAR BONGA, 1997). In the present work, fish exposed to MP, PAH, MPC and HPA+MP had depletion of the muscle glycogen concentration in relation to CT e CH. The same way, Karami *et al.* (2016) found a significantly dose-dependent reduction in the muscle and liver glycogen contents of the catfish *Clarias garieoinus* exposed to the water soluble fraction petrol indicated that metabolism of carbohydrates was impaired. Fish usually increase their metabolic rates to metabolize and excrete aromatic hydrocarbons to allocate greater amount of energy to homeostatic maintenance than its storage, leading to a reduction in stored energy food reserves. Also, behavior changes during the exposure to environmental stressors, like PAH and MP, can increases the glycolysis and the cortisol release to the blood stream. In fish, high intensity exercise results in a near total depletion of white muscle glycogen stores. In fact, *A. lacustris* during the experiment presented changes of the behavior, in relation to CT e CH, where fish were restless. The increases of the natatory activity together with the stress of been exposed to the contaminants, with could increases the circulating cortisol, could be a feasible explanation for the depletion of the muscle

glycogen in MP, PAH, MPC and HPA+MP, in relation to CT e CH. In fact, according to Milligan (2003), the elevated plasma cortisol level appears to be inhibitory to glycogenesis, as there is no evidence of net muscle glycogen synthesis until cortisol levels begin to decline. Moreover, the inhibition of regulatory enzymes of glycogen synthesis might be responsible for the depleted glycogen content when fish are exposed to contaminants (RADHAIAH; JAYANTHA RAO, 1990).

5. Conclusion

In the present work, the virgin MP was able to cause ENA and depletion of muscle glycogen, which indicates that changes in the metabolism was caused by the virgin MP. However, the MP loaded with PAH and the virgin MP in association with PAH were more toxic than the PAH and MP alone, with no differences between the treatments with the MPC and PAH+MP. In this sense, with our results we can infer that there are no differences in the toxicity of the MP previously contaminated with the PAH and the virgin MP in association with PAH.

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