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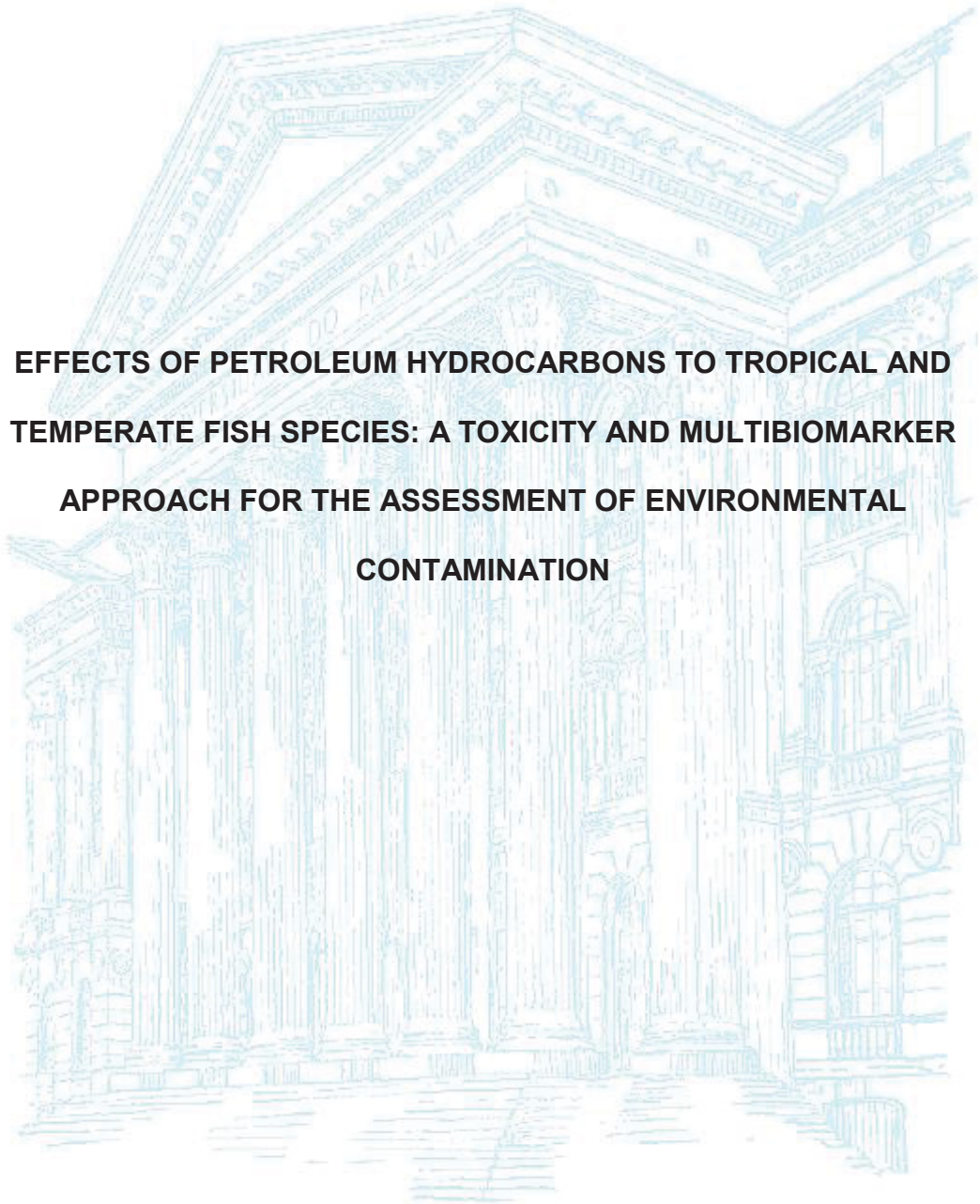
GIORGI DAL PONT



EFFECTS OF PETROLEUM HYDROCARBONS TO TROPICAL AND TEMPERATE FISH SPECIES: A TOXICITY AND MULTIBIOMARKER APPROACH FOR THE ASSESSMENT OF ENVIRONMENTAL CONTAMINATION

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Tese apresentada como requisito parcial à obtenção do grau de Doutor em Zootecnia, no Curso de Pós-Graduação em Zootecnia, Setor de Ciências Agrárias, da Universidade Federal do Paraná.

Supervisor: **Dr. Antonio Ostrensky**
Co-supervisor: **Dr. Luciana Rodrigues de Souza-Bastos**

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“…nothing can be great unless it cost you something…”

Viola Davis

RESUMO

Plantas industriais para o refino do petróleo são normalmente instaladas à uma distância considerável do local de extração do produto bruto. Assim, os derramamentos acidentais durante o transporte, como os ocorridos nos oleodutos OSPAR e OLAPA no estado do Paraná, sul do Brasil, em 2000 e 2001, respectivamente, continuam sendo uma importante ameaça ambiental. O vazamento contínuo de tanques de armazenamento e a descarga de resíduos industriais, municipais e domésticos também são considerados fontes de contaminação do ambiente aquático por petróleo e produtos derivados. A toxicidade desses produtos é geralmente atribuída à presença de hidrocarbonetos monoaromáticos (BTEX) e policíclico aromáticos (HPA). O impacto biológico da exposição ao BTEX e HPA já foi relatado. Quando essas mudanças biológicas podem ser mensuradas, elas são denominadas de biomarcadores. Entre os biomarcadores reconhecidos, utilizados para a avaliação da contaminação ambiental em peixes, podemos destacar as enzimas de biotransformação (fase I e II), estresse oxidativo, parâmetros genotóxicos, hematológicos, endócrinos, reprodutivos, fisiológicos, histológicos e morfológicos. Além disso, outras substâncias químicas podem alterar a respostas de biomarcadores, tais como alterações naturais nas características físicas e químicas em ambientes aquáticos tropicais. Apesar dessas evidências, nenhum estudo avaliou a associação entre variações abióticas, especialmente temperatura e pH, e a contaminação de hidrocarbonetos de petróleo. O objetivo desta tese foi estabelecer diferenças na toxicidade entre diferentes produtos derivados de petróleo e identificar possíveis alterações em biomarcadores induzidas por parâmetros fisicoquímicos da água durante a exposição à hidrocarbonetos de petróleo. Nossos resultados com *A. altiparanae*, uma espécie nativa da Bacia do rio Paraná (bacia que se estende do sul do Brasil ao norte da Argentina e Paraguai), demonstraram que a gasolina é o composto mais tóxico em comparação aos outros produtos testados. Também avaliamos a potencial influência dos parâmetros abióticos da água (temperatura e pH), em associação com a exposição à fração solúvel em água da gasolina (WSF_G), sobre biomarcadores de biotransformação (EROD e GST), estresse oxidativo (CAT e proteína carbonilada) e neurotóxicos (AChE) e respostas fisiológicas em *A. altiparanae*. Nossos resultados demostraram e influenciaram a temperatura e do pH em todos os biomarcadores testados. Em condições "normais" (25 ° C e pH 7), a WSF_G promoveu uma alteração das respostas de metabolização energética e hematológicas, devido a uma possível condição hipóxia metabólica. Quando a temperatura e o pH da água foram alterados, um conjunto secundário de mecanismos fisiológicos foi ativado para lidar com distúrbios osmorregulatórios e ácido-base induzidos. No caso da exposição à WSF_G, *A. altiparanae* foi capaz de manter a integridade das membranas celulares, indicando que os mecanismos reguladores mantiveram a homeostase. Usando a espécie *Carassius auratus*, identificamos as alterações da absorção de ureia e Mg^{+2} de absorção de O_2 (MO_2) resultantes da exposição aguda à WSF_G. Assim, os resultados obtidos neste trabalho fornecem uma importante base referente à toxicidade de diferentes produtos derivados de petróleo, bem como a possível influência das características abióticas da água na resposta de biomarcadores utilizados na avaliação da poluição por hidrocarbonetos de

petróleo e os possíveis mecanismos fisiológicos que são prejudicados agudamente.

Palavras-chave: Astyanax altiparanae, Carassius auratus, Fração solúvel em água, Poluição ambiental

ABSTRACT

Refinery plants can be located far away from the extraction sites. Hence, accidental spills during transportation, such as those occurred from the OSPAR and OLAPA pipelines at Paraná state, South Brazil, in 2000 and 2001, respectively, remain as an important environment threat. Non-stop leakage from commercial gas station storage tanks and the discharge of industrial, municipal and domestic waste are, also, considered sources of petroleum and refined products that could reach aquatic environments. The effects of contamination on aquatic ecosystems are normally linked with their chemical characteristics, as toxicity is usually attributed to the presence of monoaromatic (BTEX) and the polycyclic aromatic hydrocarbons (PAH). The biological impact of BTEX and PAH exposure has been reported over the years. When these biological changes can be analytically measured they are called biomarkers. Among all the well-known biomarkers used for the assessment of environmental contamination in fish, we can highlight the biotransformation enzymes (phase I and II), oxidative stress, genotoxic, haematological, endocrine, reproductive, physiological, histological and morphological parameters. Additionally, other parameters, only than BTEX and PAH, may alter biological responses/biomarkers, such as natural changes in physical and chemical characteristics in tropical aquatic environments. Despite those evidences, no study evaluated the association between abiotic variations, especially temperature and pH, and petroleum hydrocarbon contamination. The main goal of this thesis was to stablish the differences among petroleum and refined products toxicity and to identify possible biomarkers alterations induced by water physicochemical parameters during petroleum hydrocarbons exposure. Our results with *Astyanax altiparanae*, a tropical native fish species from the Paraná river Basis (massive watershed spread throughout South Brazil, North Argentina and Paraguay), demonstrated that gasoline presented the highest toxicity when compared to petroleum and other refined products. We also assessed the potential influence of water temperature and pH, in association with gasoline water-soluble fraction (WSF_G) exposure, to biotransformation (EROD and GST), oxidative stress (CAT and carbonyl protein) and neurotoxic biomarkers (AChE) and physiological responses (osmoregulation, acid-base, haematological and energetic metabolism) in *A. altiparanae*. Our results showed the influence of temperature and pH in all tested biomarkers. In “normal” (25 °C and pH 7.0) condition, WSF_G promoted an alteration in energetic metabolization, hematopoietic mechanism, due to a possible metabolic hypoxic condition. When water temperature and pH were changed, a secondary set of physiological mechanism were activated to cope with osmoregulatory and acid-base disorders, induced. In in normal or altered temperature or pH conditions, during WSF_G exposure, *A. altiparanae* sustain the integrity of cellular membranes, indicating that the trigged regulatory mechanisms were able to maintain general homeostasis. Using the temperate species *Carassius auratus* we identify alterations of urea and Mg^{+2} excretion rate and O_2 uptake (MO_2) resulting from WSF_G acute exposure. Thus, the results obtained in this work provides an important baseline for the toxicity of different refined petroleum products , as well the possible influence of water abiotic characteristics in the response of stablished biomarkers used for the assessment of petroleum hydrocarbon pollution and the possible physiological mechanisms that are acute impaired.

Keywords: Astyanax altiparanae, Carassius auratus, Environmental pollution, Water-soluble fraction

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LIST OF ABBREVIATIONS

∑ = Sum

°C = Degree Celsius

AA = Atomic absorption spectrophotometry

ACC = Carbonic anhydrase activity

AChE = Acetylcholinesterase

Ag = Silver

Al = Aluminum

Ba = Barium

BTEX = Monoaromatic hydrocarbons

Ca^{+2} = Calcium

CAT = Catalase

Cd = Cadmium

Cl^{-} = Chloride

Co = Cobalt

Cr = Chromium

Cu = Copper

CYP = Cytochrome

DO = Dissolved oxygen

EAT = Estimating Acute Toxicity

EDTA = Ethylenedeamine tetra acetic acid

EM = Emission

EROD = Ethoxyresorufin-*O*-deethylase

EX = Excitation

Fe = Iron

g = Gravity

GC-MS = Gas phase chromatography coupled to mass spectrometry

GST = Glutathione-*S*-transferase

H^+ = Proton

H_2O_2 = Hydrogen peroxide

HCO_3^- = Bicarbonate

HEPES = 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

Ht = Hematocrit

ICP-OES = Inductively coupled plasma optical emission spectrometry

IMO = Mineral insulating oil

ITPOF = International Tanker Owners Pollution Federation

J*amm* = Ammonia flux rate

J*urea* = Urea flux rate

K^+ = Potassium

KCl = Potassium chloride

L = Length

$LaCl_3$ = Lanthanum chloride

max = Maximum

MCV = Mean corpuscular volume

Mg^{+2} = Magnesium

$MgSO_4$ = Magnesium sulphate

min = Minimum

Mn = Manganese

MO_2 = Oxygen consumption rate

Na^+ = Sodium

NADPH = Dihydronicotinamide-adenine dinucleotide phosphate

NH_3 = Non-ionized or gaseous ammonia

NH_4^+ = Ionized ammonia

NH^4+NH_3 = Total ammonia

Ni = Nickel

O_2 = Oxygen

P = Phosphorus

PAH = Polycyclic aromatic hydrocarbons

Pb = Lead

PBS = Phosphate buffered saline

pH = potential of hydrogen

pHi = Internal pH

PO_2 = Oxygen pressure

PP = Total plasma protein

PTFE = Polytetrafluoroethylene

RBC = Red blood cell

ROS = Reactive oxygen species

rpm = Rotation per minute

RSD = Relative standard deviation

S.E.M = Standard error of the mean

SD = Standard deviation

Sr = Strontium

T = Duration of exposure

TMAO = trimethylamine oxide

U.S.EPA = United States Environmental Protection Agency

V = Experimental water volume

VA-DLLME = Vortex-assisted liquid-liquid dispersive microextraction

W = Weight

WBC = White blood cell

WSF = Water-soluble fraction

WSF_G = Gasoline water-soluble fraction

Zn = Zinc

ε = Molar extinction coefficient

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GENERAL THESIS PRESENTATION

From 1970 to 2009, approximately 10,000 accidents involving oil spill were recorded, which resulted in the release of 5.65 million tons of oil and refined products in the marine aquatic ecosystem (ITPOF, 2009). Among those large-scale disasters, the oil spill from Exxon Valdez, in 1989, and the massive crude oil launches in the Persian Gulf during the Gulf War, enticed international attention resulting in an increase in the environmental toxicology research around the world (KIRBY and LAW, 2010). Ever since, the scientific research with the focus on the evaluation of the fate of petroleum hydrocarbons on the aquatic environment, and theirs effects on fish biology, have drastically increased (KIRBY and LAW, 2010; MERHI, 2010).

Despite evidences indicating that the frequency of incidents has decreased in the past decades (HUIJER, 2005; BURGHERR, 2007; ITPOF, 2009), large-scale oil spills into the aquatic environment remain an important threat. The spill from the Prestige oil tanker on the Spanish coast, in 2002, (FRANCO et al., 2006; MARTÍNEZ-GÓMEZ et al., 2006), the explosion of the British Petroleum (BP) drilling platform in the Gulf of Mexico (USA), in 2010, resulting in the leakage of D*eepwater horizon* crude oil (MERHI, 2010; HARLOW et al., 2011) and, recently (November, 2017), crude oil spill from the Keystone XL pipeline, in the USA (LAWLOR and GRAVELLE, 2018), are examples of large-scale oil spill events.

Some authors suggest that the increase consumption of crude oil and derivative products accentuated the risks of environmental spills (KIRBY and LAW, 2010), mostly due to accidents that may occur during petroleum transportation (PURNELL, 2009). Non-stop leakage from commercial gas station storage tanks and the discharge of industrial, municipal and domestic waste are, also,

considered sources of petroleum hydrocarbons that could reach aquatic environments (ALBERS, 2003; BOWEN and DEPLEDGE, 2006; KIRBY and LAW, 2010).

In Brazil, the transport of crude oil through marine tankers and continental pipelines is a common procedure due to the presence of petroleum extraction plants in remote areas, as those from Urucu River province, in the Amazon region, and Campos Bay, in the southeast shoreline. Some of the refinery plants are located far away from the extraction plants. This is the case of Getúlio Vargas refinery plant (REPAR), located in the State of Paraná, South Brazil. In this area, happened one of the largest oil spill documented in Brazil´s history. In 2000, twenty-five thousand barrels of crude oil spilled from the OSPAR pipeline into the Barigui and Iguaçu rivers (OSTRENSKY *et al.*, 2001; BOEGER *et al.*, 2003), in an area severely impacted by anthropogenic actives. Only a year later, in 2001, a light refined oil spilled from the OLAPA pipeline, which is also connected to the REPAR refinery, into the Meio and Sagrado rivers. The accident occurred in a remote preserved forest area at Serra do Mar mountain range. The occurrence of minor-scale spills, is also a realistic scenario in the State of Paraná. In 2007, a diesel oil slick (100 m^2) was found at the Alegria River, in Medianeira (AEN-PR, 2007). The source of the oil spill was never determined. Another incident happened 2011, and 10,000 liters of lubricant oil was spilled during transportation into Cascavel River and caused interruption of water supply (CRUZ, 2011).

The effects of aquatic environmental contamination by petroleum and refined products are normally linked with their chemical characteristics. Petroleum is a complex mixture of several distinct organic compounds, mainly hydrocarbons, formed by the incomplete decomposition of organic matter (NEFF, 1979). The

composition of petroleum, and consequently, the composition of refined products, changes according to its origin. However, the organic petroleum hydrocarbon is considered to be the large group of compounds forming the oil (ALBERS, 2003). In this group, the toxicity is usually attributed to the presence of monoaromatic (BTEX) and the polycyclic aromatic hydrocarbons (PAH) substances (CONNELL and MILLER, 1981; BARRON, 2003). The toxicity of some refined products, such as gasoline and diesel, is mainly attributed to the BTEX (BARRON *et al.*, 1999), due to their ability to easily permeate the biological membranes (BARRON, 2003). Among all PAH, 16 are considered priority by the United States Environmental Protection Agency (U.S.EPA), because they are more persistent in the aquatic environment, although they are considered to be less toxic to fish than BTEX (BARRON *et al.*, 1999; RODRIGUES *et al.*, 2010).

The biological impact of BTEX and PAH exposure, after oil spill events, has been reported over the years (GLEGG *et al.*, 1999; OSTRENSKY *et al.*, 2001; BOEGER *et al.*, 2003; COLOMBO *et al.*, 2005; GONZÁLEZ-DONCEL *et al.*, 2008; MARTÍNEZ-GÓMEZ *et al.*, 2009). Changes in biological responses due to the exposure to an environmental pollutant are more frequent at cellular levels than high biological organization levels (VAN DER OOST *et al.*, 2003). When these biological changes can be analytically measured they are called biomarkers (PEAKALL, 1994). Among all the well-known biomarkers used for the assessment of environmental contamination in fish, we can highlight the biotransformation enzymes (phase I and II), heat shock proteins, oxidative stress, genotoxic, haematological, endocrine, reproductive, physiological, histological and morphological parameters. Usually, the acute and chronic exposure of fish to petroleum or refined products, or to their water-soluble fraction, may decrease food

efficiency, even with the increase in food consumption, demonstrating alterations on the energetic metabolism (VAN DER OOST *et al.*, 2003; OLSEN *et al.*, 2007). The metabolic alteration can cause a reduction in growth (VIGNIER *et al.*, 1992) and also lead to disturbances in respiratory ability, and damage to the immune system (GAGNON and HOLDWAY, 1999; COHEN *et al.*, 2001; COHEN *et al.*, 2005). As the alterations imposed by xenobiotic compounds move across the biological organization spectrum, a more generalized response will be observed and, thus, will reflect the general "health status" of the organism (HEATH, 1995) and the fish assemblage (MATTHEWS, 1998). However, not all the effects of petroleum hydrocarbons are understood (EPA, 2004; UNEP, 2004) and field work with fish communities indicate that oil spills not always cause significant damage to the fish assemblage structure (HORODESKY *et al.*, 2015; OSTRENSKY *et al.*, 2015; O. AGOSTINIS *et al.*, 2017).

In addition to contamination by petroleum hydrocarbons, other environmental variables may alter biological responses/biomarkers, such as natural physical and chemical changes in the aquatic environment (PEAKALL, 1994; VAN DER OOST *et al.*, 2003). Accentuated seasonal variations of the water temperature are common in the tropical aquatic environments (ESTEVES, 2011). In small tropical rivers or water streams, temperature can vary 16 °C from winter to summer (OYAKAWA *et al.*, 2006). Additionally, predictions of changes in the climatic scenario suggests the increase in the mean temperature of Paraná River Basin area (South Brazil) in 4.5 °C in 60 years (MARENGO *et al.*, 2012). The effects of temperature upon toxicity of xenobiotics to aquatic biota has been studied for years (CAIRNS *et al.*, 1975). Alterations inflicted by environmental temperature variations where attributed to impair osmoregulatory (MCCORMICK

et al., 1996; WOOD, 2001; WOOD *et al.*, 2017), energetic (DE ALMEIDA-VAL *et al.*, 2005; SCHULTE, 2015) and reproductive systems (ARANTES *et al.*, 2011) as well in swimming performance, metabolic rate and fish growth (BRETT and GLASS, 1973; CLARKE and JOHNSTON, 1999; AGUIAR *et al.*, 2002; GREEN and FISHER, 2004) and other biological systems. The natural or anthropogenic acidification of water bodies can also be considered as an important source of stress for fish (HEATH, 1995; WENDELAAR BONGA, 1997). Water pH in tropical rivers can vary from neutral (pH=7.0) to acid (pH=3.9) due to natural organic matter accumulation (OYAKAWA *et al.*, 2006; ESTEVES, 2011). The effects of water acidification in fish physiology in normal (MCDONALD, 1983; YE *et al.*, 1991; PEURANEN *et al.*, 1994; WOOD *et al.*, 2011) and heavy metal contaminated conditions (BOOTH *et al.*, 1988; CARVALHO and FERNANDES, 2006) has been studied in a small number of temperate and neotropical species. Despite this, no study evaluated the association between abiotic variations, especially temperature and pH, and petroleum hydrocarbon contamination in tropical freshwater environments.

Fishes of the genus *Astyanax* sp. successfully thrives the tropical rivers and water streams from South Brazil (GERRY, 1977; GARUTTI and BRITSKI, 2000; DE CARVALHO *et al.*, 2009). Species of the genus have been used as bioindicators of environmental contamination in field (OSTRENSKY et al., 2001; SCHULZ and MARTINS-JÚNIOR, 2001; BOEGER et al., 2003; AKAISHI et al., 2004; SILVA et al., 2009) and laboratory (DAL PONT, 2012; DE SIQUEIRA-SILVA *et al.*, 2015; BETTIM *et al.*, 2016; GALVAN *et al.*, 2016; TOLUSSI *et al.*, 2018) studies. A common species found in the State of Paraná is *Astyanax altiparanae*, popularly known as yellowtail tetra (GARUTTI and BRITSKI, 2000). Despite being

a small species, with average length of 10 cm when adult, have high ecological relevance due to their omnivorous food habit and because they (BENNEMANN and SHIBATTA, 2002) serve as food source for carnivorous species (PRIOLI et al., 2002). In addition to its ecological relevance, this species is commercially produced. The commercial accessibility of fingerlings and adult specimens has been fomenting their used laboratory tests. Nevertheless, a lot of scientific gaps regarding the species sensibility (toxicity) to different sources of petroleum hydrocarbons and their biomarkers responses in scenarios of water temperature increase or acidification remains to be studied.

The main goal of the thesis was to stablish the differences among petroleum and refined products toxicity and to identify possible biomarkers alterations induced by water physicochemical parameters during petroleum hydrocarbons exposure. To fill the scientific gaps described above, we incorporated toxicity, biochemical and physiological biomarkers assays and formulated two questions. How different is the acute toxicity (LC_{10} and LC_{50}) of petroleum and refined products to a native neotropical freshwater fish species? This first question was addressed in chapter 1. After asses the acute toxicity, in chapter 2 and 3, we questioned if well-established biomarkers, normally used for environmental contamination assessment, would behave differently if the exposure to petroleum hydrocarbon (gasoline water-soluble fraction) was associated with altered water temperature and pH. On chapter 1, 2 and 3 we used the native fish *A. altiparanae* as bioindicator (Figure 1A) in the laboratory trials. In addition to the two-formulated questions, based on alterations in the concentration of total ammonia in water, observed in chapter 2 and 3, in chapter 4 we assessed the effects of the acutely

exposure of the freshwater fish *Carassius auratus* (Figure 1B) to gasoline water-soluble fraction on nitrogen compounds excretion, ionic fluxes and metabolic rate.

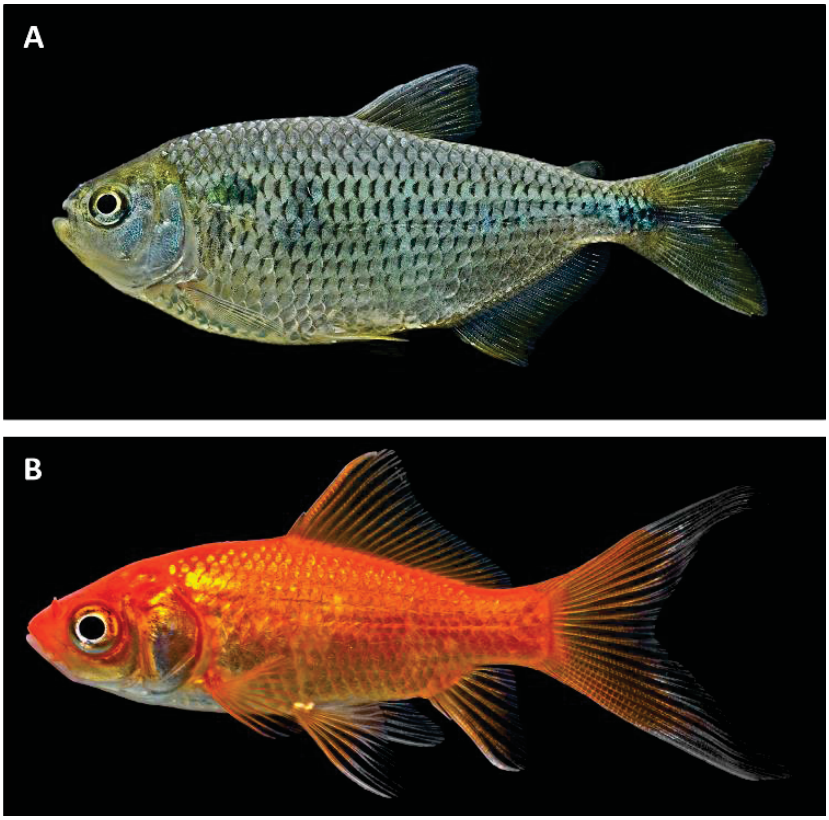

Figure 1. Specimen of (A) *Astyanax altiparanae* and (B) *Carassius auratus*. Pictures A by Diogo Barbalho Hungria and picture B from www.ncfishes.com.

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CHAPTER 1 – TOXICITY OF WATER-SOLUBLE FRACTION OF PETROLEUM, GASOLINE, DIESEL, MINERAL INSULATING AND LUBRICANT OIL TO THE NEOTOPICAL FRESHWATER YELLOW TETRA *Astyanax altiparanae*[1]

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ABSTRACT

Chemical properties of petroleum derivatives vary widely across its different classes and, consequently, at the final refined product. Here, we evaluated the toxicity (LC_{50} and LC_{10}) of WSF of petroleum and derivative products (gasoline, diesel, lubricant and insulating mineral oil (IMO) to *A. altiparanae*. The experiments were performed independently and for each procedure 110 fishes were exposed to different concentrations (n=10/concentration) of WSF (0 [control]; 0.25; 0.5; 1.0; 2.0; 4.0; 8.0; 16.0; 32.0; 64.0 and 100.0%) for 96h. Mortality was monitored every 24h. LC_{50} and LC_{10} (95% confidence intervals) were: gasoline= 2.65 (1.02-4.46) and 0.5 (0.35-0.91); diesel= 16.22 (11.73-21.09) and 2.75 (2.31-5.06); IMO^a = 17.01 (5.85-25.06) and 5.35 (3.33-8.65); petroleum= 23.59 (10.81-31.32) and 1.76 (1.39-3.86); lubricant= 27.65 (11.54-38.79) and 11.24 (6.45-16.78); IMO^b= 53.58 (22.98-127.92) and 7.84 (7.50-16.73). Due to higher polycyclic aromatic and monoaromatic hydrocarbons concentrations, gasoline presented the highest toxicity. Lubricant and IMO^b presented the lowest toxicity effects to *A. altiparanae* as it also presents elevated concentrations of high molecular weight petroleum hydrocarbons. On the other hand, IMO^a presented toxicity similar to diesel oil. This effect could be associated with the antioxidant inhibitor added to the oil for dielectric and insulating purposes, that could also present toxic effects. These results should be used as a basis for the sub chronic studies to evaluate the sub

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lethal effects of exposure of the species to the tested petroleum products and for environmental risk assessment programs.

Keywords: LC_{50}, LC_{10}, toxicity test, petroleum hydrocarbon, refined products

1.1. INTRODUCTION

After drilling, petroleum is transported to refinery plants to be transformed in more profitable sub product (JAHN *et al.*, 2008a). Chemical properties of petroleum vary widely across its different fuel classes and, consequently, at the final refined product (NEFF, 1979). Petroleum is constituted from a total of 18 different hydrocarbon series, of which the most common are the alkenes, naphthenic and aromatics (RIAZI, 2005). Generally, these chemical classes act as a guide to the commercial value of the refined products of the crude oil, with the lighter ends (shorter carbon chains) commanding more value (JAHN *et al.*, 2008b).

Gasoline and diesel are the most well-known petroleum light-refined products due to their world-wide use as automotive fuels (SPEIGHT, 2015a; SPEIGHT, 2015b). Light oils, normally used as fuels in combustion engines, are mainly constituted by short chains hydrocarbons – from 4 to 16 carbons (RIAZI, 2005). Those light refined products are rich in polar monoaromatic (volatiles) (BTEX - benzene, toluene, ethylbenzene and xylene) and polycyclic aromatic hydrocarbons (PAH). Although gasoline and diesel are fuels used in a daily basis and, thus, are more likely to contaminated aquatic environments (KIRBY and LAW, 2010), other types of petroleum derivatives can also be considered a potential environmental hazard, as the mineral insulating (IMO) and lubricant oils (ROGOWSKA and NAMIEŚNIK, 2010). The heavy portion resulting from the petroleum distillation process is also utilized for industrial purposes. Heavier

refined products, as lubricant oils, present mostly paraffinic, naphthenic, and other hydrocarbons with carbon-chains with more than 25 C (RIAZI, 2005). As those heavy hydrocarbons also presents higher boiling ranges, they are suitable for dielectric insulating and lubricating uses (ROUSE, 1998).

When petroleum, or any of its refined products, enter a freshwater or marine aquatic environment, through intentional or accidental disposal of domestic and industrial effluents and less often by oil spills (NEFF, 1979; HEATH, 1995), occurs the solubilization of polar compounds and lower molar mass compounds (BTEX and PAH, mainly), forming the water-soluble fraction (WSF) (ANDERSON, 1979). These low molecular weight petroleum hydrocarbons (128-300 g mol^{-1}) (EISLER, 1987), can be easily absorbed by gill and epidermal tissue and digestive tract of fishes (BARRON, 2003). Consequently, BTEX and PAH are considered the main petroleum hydrocarbons that cause toxic effects to inland and aquatic organisms (BARRON *et al.*, 1999; ALBERS, 2003). In the aquatic environment, BTEX and PAH are recognized by inducing a series of disturbances at several levels of the biological organization (FRENCH, 1991; HEATH, 1995; BARRON *et al.*, 1999; VAN DER OOST *et al.*, 2003). As mineral and lubricant oils present lower BTEX and PAH content, their toxicity is normally attributed to antioxidant substances added to the commercial product to increase dielectric characteristics (ROUSE, 1998). Some of this antioxidant inhibitors, however, did not caused toxicity to fish (MÓDENES *et al.*, 2018).

The acute and chronic alterations caused by BTEX and PAH have been widely studied in freshwater (SIMONATO *et al.*, 2004; SIMONATO *et al.*, 2008; SILVA *et al.*, 2009; SIMONATO *et al.*, 2011; DAL PONT, 2012; BETTIM *et al.*, 2016; GALVAN *et al.*, 2016; BARROS *et al.*, 2017) and marine fish species

(CLARCK-JR. and FINLEY, 1977; DOU *et al.*, 1998; STENTIFORD *et al.*, 2003; BOLOGNESI *et al.*, 2006; RODRIGUES *et al.*, 2010; AGAMY, 2013). However, the number of studies that have compared the toxic effects on survival of more than one hydrocarbon source are scarce. LOCKHART *et al.* (1987b), COHEN and NUGEGODA (2000), BHATTACHARYYA *et al.* (2003) and MÓDENES *et al.* (2018) tested the toxicity of different oils to temperate fish species. Unlike to the tropical fish species, the researches about toxicity of petroleum hydrocarbons on tropical biota are surprisingly limited. To our knowledge, the work performed by RODRIGUES *et al.* (2010) is the only one compared the toxicity of petroleum fuels to a tropical fish species - and information regarding IMO and lubricant toxicity to tropical fishes are non-existent.

Along with the effort to understand the effects of petroleum hydrocarbons to native species, the neotropical *Astyanax altiparanae* (CHARACIFORMES, CHARACIDAE) have been frequently used as bioindicator model in ecotoxicological studies developed in southern Brazil (AKAISHI *et al.*, 2004; SILVA *et al.*, 2009; HORODESKY *et al.*, 2015; OSTRENSKY *et al.*, 2015; BETTIM *et al.*, 2016; GALVAN *et al.*, 2016; BARROS *et al.*, 2017). *A. altiparanae* is wide distributed and successfully thrives in the freshwaters from the upper Paraná River basin, in South America (GARUTTI and BRITSKI, 2000; BENNEMANN and SHIBATTA, 2002). The use of the species as environmental bioindicator is fomented due to its pollution tolerance (SCHULZ and MARTINS-JÚNIOR, 2001) and aquaculture availability for laboratorial studies (DE CARVALHO *et al.*, 2009). Thus, the main goal of this study was to compare the toxicity (LC_{10} and LC_{50}-96h) of petroleum and refined products (gasoline, diesel, mineral insulating and lubricant oil) water-soluble fraction for the neotropical fish *Astyanax altiparanae*.

1.2. METHODS AND MATERIALS

Fingerlings specimens of *A. altiparanae* (n= 1000; W = 1.87 ± 0.12 g; L = 4.85 ± 0.06 cm) were obtained from a commercial fish facility in Curitiba, Paraná, Brazil and taken to the Integrated Group for Aquaculture and Environmental Studies (GIA/UFPR) laboratory for acclimation. They were maintained for 2 weeks in a flow system tank (1000 L) supplied with dechlorinated water, continuous air pumping (dissolved oxygen= 6.33 ± 0.27 mg L^{-1}) and controlled temperature (25 ± 2 °C). Fishes were fed twice a day with commercial dry food (Kowalski®, Brazil, crude protein = 47%). Feeding was stopped 48 h before the start of the LC_{50}-96h experimental procedures. Additionally, the fish were not fed during the whole experimental period to avoid the increase of the concentrations of nitrogen residues in the water.

Gasoline and diesel oil were acquired direct from commercial gas station, storage and transported in sealed glass Erlenmeyer flasks (2 L) covered with aluminum foil, 24 h before the starting the preparation of the soluble fraction. The insulating mineral oil (IMO^a), was purchased from a private company and storage in steel barrel for 60 days prior use. The other insulating mineral oil (IMO^b), the petroleum (blend of LAMR and MS40 crude oil – 25.6° API) and the lubricant oil were donated by private oil-company's. All the donated products were storage in steel sealed barrels. The water-soluble fraction (WSF) of the six products cited above were prepared daily, according the methodology described by ANDERSON *et al.* (1974). Briefly, one part of the product was added to nine parts of water and stirred for 22 h at room temperature (25 ± 1 °C) in a sealed Mariotte bottle (20 L) covered with aluminum foil. At the end of the period, the stirring was switched-off and the mixture remained standing for 30 min for the complete separation of water

soluble fraction and the insoluble oil fraction. The water-soluble fraction obtained and immediately used in the experiments.

The procedure for each LC_{50} trial consisted in acclimating 140 fishes in a 100 L static system tank for 72 h. During this first acclimation period the mortality rate observed was ≤ 5%. Then, fishes were separated in 4 groups and conditioned in 14 L glass aquaria, where they were held for 48 h. After this period, fishes were grouped (n=10; ~ 1.0 g of fish L^{-1}) in 4 L glass aquaria for the acclimation to the experimental condition for 24 h. In both acclimation and experimental glass aquaria (14 and 4 L, respectively) no mortality was observed. Eleven WSF test concentrations were used: 0 (control), 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 and 100%. This standard experimental series is recommended by the United States Environmental Protection Agency (EPA, 2002) for measuring acute toxicity for aquatic organisms. The tests were performed in semi-static conditions, i.e., water and WSF of petroleum and derivative products was renewed daily (80% of the total volume) and fish mortality was recorded every 24 h over a period of 96 h.

Water temperature and oxygen concentration (YSI® Pro 20) was monitored every 6 h during the first 12 h of exposure. At same time, water samples were collected for immediate pH analysis (Horiba Scientific® B-713) and posterior total ammonia (VERDOUW *et al.*, 1978) determination. After 12h of experimentation, measurements of the same parameters were performed daily until the end of 96h. Samples of undiluted WFS (100%) and control treatments were collected daily in amber flasks for polycyclic aromatic (PAH) monoaromatic hydrocarbons (BTEX) analysis.

Daily mortality data and nominal WSF concentrations (%) were used for the calculation of LC_{50} and LC_{10}-96h for each oil to *A. altiparanae*. For this, the Risk

Assessment Tools Software were used in the Estimating Acute Toxicity (EAT) mode (MAYER *et al.*, 2009). The calculation method is based in the standard Probit analysis (FINNEY, 1971).

Both PAH and BTEX analysis were carried out utilizing gas phase chromatography coupled to mass spectrometry (GC-MS). For PAH, we used a Thermo Fisher Scientific® gas chromatograph (Focus GC Polaris Q model), equipped with a Thermo® AS3000 auto-sampler for liquid injection and for BTEX a Shimadzu® gas chromatograph (model 2010) coupled to mass spectrometer (TQ8040) in tandem system (GC-MS/MS), equipped with a Shimadzu® AOC-5000 autosampler for headspace analysis. For the PAH determination, water samples were submitted to a vortex-assisted liquid-liquid dispersive microextraction (VA-DLLME) procedure adapted from REZAEE *et al.* (2006) and ZHANG and LEE (2012) methodologies. One mL of the extraction solution ($CHCl_3$ + C_3H_6O) was quickly added in 5 mL of the sample and followed by agitation (1 min) and centrifugation (2800 rpm for 10 min). An aliquot of the highest density fraction (50 µL) was transferred to a chromatographic insert contenting 10 µL of the internal PAH standard solution (mix of deuterated PAH at 100 µg L^{-1}) and injected into GC-MS. The procedure used for BTEX extraction was based on the headspace methodology described by FERNANDES *et al.* (2014). WSF_G sample (5 mL) was heated (60 ºC) and stirred (300 rpm) for 5 min to promote the volatilization of the BTEX. Instrumental parameters used are detailed in Table 1.

Table 1. Instrumental parameters utilized for determination of polycyclic aromatic hydrocarbons (PAH) and monoaromatic hydrocarbons (BTEX) in aqueous samples via gas phase chromatography coupled to mass spectrometry (GC-MS).

PAH	Column	DB5-ms (Agilent®) 30 m x 0.25 mm x 0.25 µm
	Carrier gas	Analytical helium 5.0 (99.999%) with a flow rate of 1.2 mL min^{-1}
	Injector	Splitless, injection volume 1.0 µL
	Temperature	Column: 40 ºC (5 min) Ramp 1: 5 ºC min^{-1} to 230 ºC Ramp 2: 2 ºC min^{-1} to 250 °C Ramp 3: 5 ºC min^{-1} to 300 ºC (8 min) Detector: 300 ºC Injector: 270 °C Transfer line: 270 ºC Ions source: 270 ºC
	SIM* mode	Scan 1 (5 min): (m/z 128, 136, 152, 154, 162, 164, 166, 178) Scan 2 (30 min): (m/z 178, 202, 228, 244) Scan 3 (47 min): (m/z 228, 236 240, 252, 260, 264, 276, 277, 278, 279)
BTEX	Column	SH-Rtx-5ms (Shimadzu®) 30 m x 0.25 mm x 0.25 µm
	Carrier gas	High-pure helium (99.99999%) with a flow rate of 1.02 mL min^{-1}
	Injector headspace	Injection volume: 500 µL
	Temperature	Column: 35 ºC (4 min), Ramp 1: 10 ºC min^{-1} to 100 ºC Ramp 2: 30 ºC min^{-1} to 200 ºC Detector: 250 ºC Injector: 180 °C Syringe: 60 ºC Sample: 60 ºC Transfer line: 250 ºC Ions source: 250 ºC
	Agitation	Sample: 300 rpm
	SIM* mode	Scan (m/z): 78; 91 and 106

Analytical curves were build in triplicate for PAH (0.5 to 4.0 µg L^{-1}) and BTEX (5.0 to 1000.0 µg L^{-1}) determination. High purity analytical standards of BTEX (benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene and *p*-xylene) and the 16 priority PAH suggested by U.S.EPA (2001) were used: naphthalene (Naf); acenaphthylene (Aci); acenaphthene (Ace); fluorene (Flu); phenanthrene (Fen); anthracene (Ant); fluorantene (Fla); pyrene (Pyr); benzo(*a*)anthracene (BaA); criseno (Cris); benzo(*b*)fluoranthene (BbF); benzo(*k*)fluoranthene (BkF); benzo(*a*)pyrene (BaP); indene(1,2,3-*cd*)pyrene (Ind); dibenzo(*a*,*h*)anthracene (Dib); benzo(*g*,*h*,*i*)perylene (Ben). Five deuterated internal standards (AccuStandart®) were also used: Naphthalene (NafD8); Acenaftene (AceD10); Fenantrene (FenD10); Chrysene (CrisD12) and Perylene (PerD12). The stock mix standard solutions of PAH and BTEX were prepared in dichloromethane and methanol (J.T. Baker®), respectively, at concentrations of 5.0 mg L^{-1}, and kept under controlled temperature (-18 °C) until use. Work solutions where prepared daily. The accuracy was determined by a recovery test by spiking three known concentrations of PAH (0.35; 1.2 e 5.0 µg L^{-1}) and BTEX (8.0; 120.0 e 450.0 µg L^{-1}) compounds in a water sample from the same source used in the experimental procedure. The recovery test was performed in three replicates for each concentration level studied. The repeatability of both methods was studied by analyzing six replicates of a standard solution of PAH and BTEX and expressed as relative standard deviation (RSD %).

1.3. RESULTS AND DISCUSSION

The mean ± SD values of water quality parameters monitored during the toxicity trials are shown in Table 2. Temperature, dissolved O_2 and pH did not vary

between trials ($p>0.05$). Although the total ammonia concentrations in the experimental trials with IMO[b] and lubricant oil were higher than trials with gasoline, diesel and petroleum ($p<0.05$), the absolute concentration remained below the LC_{50}-96h established for *A. altiparanae* (DAL PONT, 2012).

Table 2. Mean ± SD of water temperature, dissolved oxygen (O_2), pH and total ammonia (NH_4+NH_3) among petroleum and refined products toxicity trials (LC_{10} and LC_{50}-96h) for the neotropical fish *Astyanax altiparanae*.

Water Parameter	Gasoline	Diesel	Petroleum	IMO[a]	IMO[b]	Lubricant
Temperature (°C)	24.23 ± 1.08	25.06 ± 0.92	25.5 ± 0.32	25.1 ± 0.45	25.0 ± 0.40	25.1 ± 0.50
O_2 (mg L^{-1})	6.89 ± 2.21	7.04 ± 1.24	7.00 ± 0.39	6.70 ± 0.86	6.70 ± 0.40	7.90 ± 1.10
pH	6.12 ± 1.10	6.64 ± 0.38	7.00 ± 0.32	6.90 ± 0.35	7.00 ± 0.40	6.90 ± 0.40
NH_4+NH_3 (mmol L^{-1})	0.059 ± 0.037[a]	0.109 ± 0.050[a]	0.060 ± 0.043[a]	0.114 ± 0.043[ab]	0.158 ± 0.80[b]	0.194 ± 0.178[b]

As BTEX and PAH are considered the most toxic petroleum hydrocarbons (BARRON *et al.*, 1999; ALBERS, 2003), their content in the 100% WSF, of each tested product, was determinate. Both BTEX and PAH concentrations were different among the tested WSF. This result was expected due to the recognized characteristics of each petroleum refined product (JAHN *et al.*, 2008b; SPEIGHT, 2015b; d; c). Gasoline presented higher values of both BTEX and PAH. The same trend was detected in the WSF produced by RODRIGUES *et al.* (2010), BETTIM *et al.* (2016) and GALVAN *et al.* (2016). In contrast, for the tested mineral insulation and lubricant oils, BTEX was not detected and the sum of PAH were about ten times lower than petroleum WSF (Table 3). Diesel WSF presented lower BTEX than petroleum. On the other hand, the concentrations of naphthalene, phenanthrene and anthracene were higher. These results are in accordance with the PAH concentrations obtained by RODRIGUES *et al.* (2010) and DAL PONT (2012). The toxicity threshold of WSF of refined products to freshwater organisms can be strongly associated with the of individual petroleum hydrocarbons content, specially the lower molecular weight hydrocarbons, as benzene and naphthalene (LOCKHART *et al.*, 1987a). Naphthalene exhibits high bioaccumulation potential (ANDERSON, 1979), and is rapidly adsorbed (24 h) by several fish tissues (DOMINGOS *et al.*, 2011). The same characteristics are attributed benzene (MEYERHOFF, 1975).

Table 3. Mean monoaromatic (BTEX) and polycyclic aromatic hydrocarbons (PAH) concentrations in water-soluble fraction (WSF) of petroleum and refined products during toxicity tests (LC_{10} and LC_{50}-96h) for the neotropical fish *Astyanax altiparanae*.

WSF Parameter	**Toxicity Trials**					
	Gasoline	**Diesel**	**Petroleum**	**IMO[a]**	**IMO[b]**	**Lubricant**
BTEX (µg L^{-1})						
Benzene	44830.00	977.14	2873.00	< 5.0	< 5.0	< 5.0
Toluene	42200.00	679.86	1279.50	< 5.0	< 5.0	< 5.0
Ethylbenzene	15960.00	126.77	118.00	< 5.0	< 5.0	< 5.0
m,*p*-Xylene	18020.00	151.71	321.00	< 5.0	< 5.0	< 5.0
o-Xylene	16480.00	158.17	341.00	< 5.0	< 5.0	< 5.0
∑ BTEX	*137490.00*	*2093.66*	*4932.50*	< 5.0	< 5.0	< 5.0
PAH (µg L^{-1})						
Naphthalene	3.89	9.19	3.58	ND	ND	ND
Acenaphthylene	< 0.35	ND	ND	ND	ND	ND
Acenaphthene	< 0.35	ND	< 0.35	ND	ND	ND
Fluorene	ND	ND	0.46	< 0.35	ND	ND
Phenanthrene	ND	7.17	ND	ND	ND	ND
Anthracene	ND	7.09	0.59	ND	ND	ND
Fluoranthene	2.90	ND	< 0.35	< 0.35	< 0.35	< 0.35
Pyrene	2.50	1.00	ND	ND	ND	ND
Benz[*a*]anthracene	3.80	0.65	0.44	0.46	0.41	< 0.35
Chrysene	4.60	0.60	0.38	0.40	0.36	< 0.35
Benzo[*b*]fluoranthene	5.50	1.26	< 0.35	< 0.35	< 0.35	< 0.35
Benzo[*k*]fluoranthene	5.60	1.08	3.91	< 0.35	< 0.35	< 0.35
Benzo[*a*]pyrene	4.20	1.04	< 0.35	< 0.35	< 0.35	< 0.35
Indeno[1,2,3-*cd*]pyrene	6.70	1.24	< 0.35	< 0.35	< 0.35	0.46
Dibenz[*ah*]anthracene	8.90	1.20	0.352	< 0.35	< 0.35	0.43
Benzo[*ghi*]perylene	6.70	1.10	< 0.35	< 0.35	< 0.35	0.43
∑ PAH (µg L^{-1})	*55.29*	*32.63*	*9.70*	*0.86*	*0.77*	*1.32*
∑ (µg L^{-1})	**137545.29**	**2126.28**	**4942.20**	**0.86**	**0.77**	**1.32**

The deleterious effects of petroleum and its refined products on aquatic organisms can occur by toxic action (direct absorption of the petroleum components by the organism), physical action (reduction of incident light) and/ or habitat modification (modification of the water quality parameters and/or decreased

food availability) (BARRON, 2003; VAN DER OOST *et al.*, 2003). In the tested LC_{50}-96h condition, only the toxic action was tested. In this scenario, gasoline presented the highest toxicity to *A. altiparanae* [LC_{50}= 2.65 (1.02-4.46)]. WSF_G was 6 times more toxic than diesel [LC_{50}= 16.22 (11.73-21.09)], 6.4 times than OMI[a] [LC_{50}= 17.01 (5.85-25.06)], 8.9 times than petroleum [LC_{50}= 23.59 (10.81-31.32)], 10 times than lubricant [LC_{50}= 27.65 (11.54-38.79)] and 20 times more toxic than IMO[b] [LC_{50}= 53.58 (22.91-127.92)] (Figure 2). Our results are in accordance with the toxicity threshold obtained by RODRIGUES *et al.* (2010). The authors compared the toxicity of gasoline, diesel and petroleum to larvae of *Odontesthes argentinensis* and obtained higher toxicity with WSF of gasoline. In fact, the toxicity of gasoline was reported to be so elevated, that LC_{50} of gasoline effluents is around 8.9% (v/v) (ALVES *et al.*, 2017). MOS *et al.* (2008) DAVISON *et al.* (1992) and RODRIGUES *et al.* (2010) also found diesel LC_{50} similar with the values obtained to *A. altiparanae*. As our results are comparable with other fish species, they are suitable to be use as guidance values for sub-chronic tests using tropical fish species.

The toxicity of WSF of diesel was lower than petroleum and similar to IMO[a]. In this scenario, it is possible to assume that the acute toxicity threshold of gasoline, diesel and petroleum WSF are intimate attached to the BTEX and PAH concentrations. Lubricant and IMO[b] presented the lowest toxicity. On the other hand, even with lower BTEX and PAH in the WSF, IMO[a] presented toxicity similar to diesel oil. IMO is a term used to refer to all oils that are obtained from crude oil dewaxed paraffin and mixed with additives substances to provide antioxidant inhibition and dielectric characteristics (ALUYOR and ORI-JESU, 2009). The substances used to promote antioxidant characteristics can also present toxicity

propriety (WILHELM *et al.*, 2018) and, thus, potentialize the toxicity of the tested IMO. SALAM *et al.* (2016) compared the toxicity of inhibited IMO (with antioxidant substance, in this case butylated hydroxytoluene) and non-inhibited IMO to zebrafish and did not detected any combined additional toxicity threshold. Thus, although we did not measure in the present work, we suggest that the differences on IMO toxicity for *A. altiparanae* could be associated to use of different types of antioxidant substances by the manufacturing company.

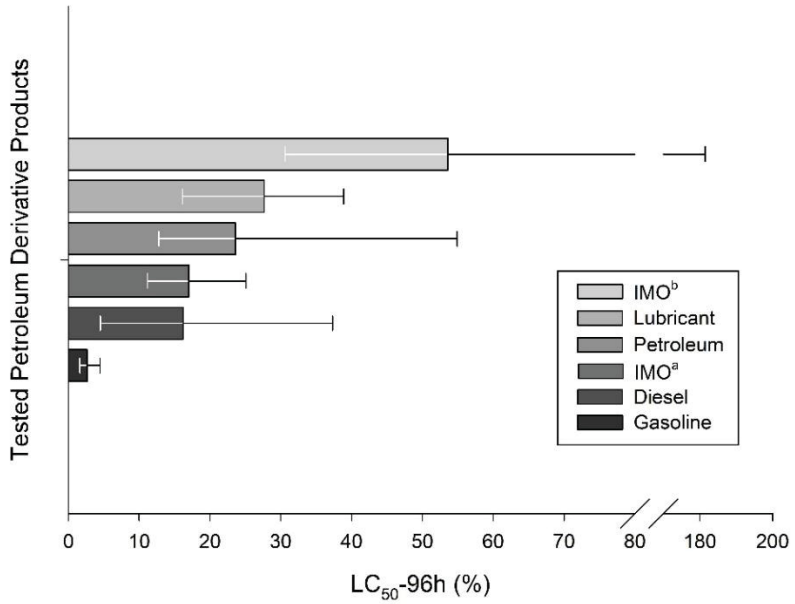

Figure 2. Petroleum and derivative products [gasoline, diesel, insulation mineral oil (IMO[a] and IMO[b]) and lubricant oil] toxicity (LC_{50}-96h) to the neotropical freshwater fish *Astyanax altiparanae*. Bars represent the mean observed LC_{50} values and whiskers represent LC_{50} upper and lower values.

Another important feature related to the contamination of aquatic environments by petroleum hydrocarbons is attributed to the chronic effects in the biological levels (VAN DER OOST *et al.*, 2003). To perform such experiments and assess biological alterations, researches relied on values of no-observed-effect concentration (NOEC) of toxicants for many years (CHAPMAN *et al.*, 1996). The

NOEC values correspond to the highest tested concentration that does not lead to significant deviation from the control response (JAGER, 2012). However, the lack of a statistically significance does not mean that the effect does not exist or is no effect (FOX and LANDIS, 2016). Another source of safe concentrations of environmental pollutants for chronic tests was suggested by SPRAGUE (1971). The author proposed that the secure range is between 1 and 40% of the tested LC_{50}-96h. However, this range is wide and lethal concentrations can be wrongly adopted. Thus, a more reliable safe concentration that have been adopted is the EC_{10} or LC_{10} value (BEASLEY *et al.*, 2015). Thus, we also determinate the LC_{10}-96h the tested products to *A. altiparanae*. The LC_{10}-96h pattern among the tested WSF products changed in comparation with the results from LC_{50} (Figure 3). The calculated LC_{10}-96h was 0.5% (0.35-0.91) to gasoline, 2.75 (2.31-5.06) to diesel, 1.76 (1.39-3.86) to petroleum, 5.35 (3.33-8.65) to IMO^a, 7.84 (7.50-16.73) to IMO^b and 11.24 (6.45-16.78) to lubricant oil. The most significant modified result, compared to the LC_{50} value, was from petroleum WSF. The calculated LC_{10}-96h of petroleum to *A. altiparanae* corresponded to only 7% of the observed LC_{50}. The lubricant oil, IMO^a, gasoline, diesel, and IMO^b WSF LC_{10}-96h corresponded to 40.8, 31.5, 18.9, 17.0 and 14.6% of the correspondent observed LC_{50}, respectively. Although all the calculated LC_{10} values were within the secure range suggested by SPRAGUE (1971), the results show that the variation among the different refined products needs to be considered in chronic exposure trials. BETTIM *et al.* (2016) and GALVAN *et al.* (2016) used the NOEC values for the chronic exposure of *A. altiparanae* to gasoline. The adopted value (NOEC= 1.5% *v/v*) was three times higher than the LC_{10}-96h calculated here to the same species.

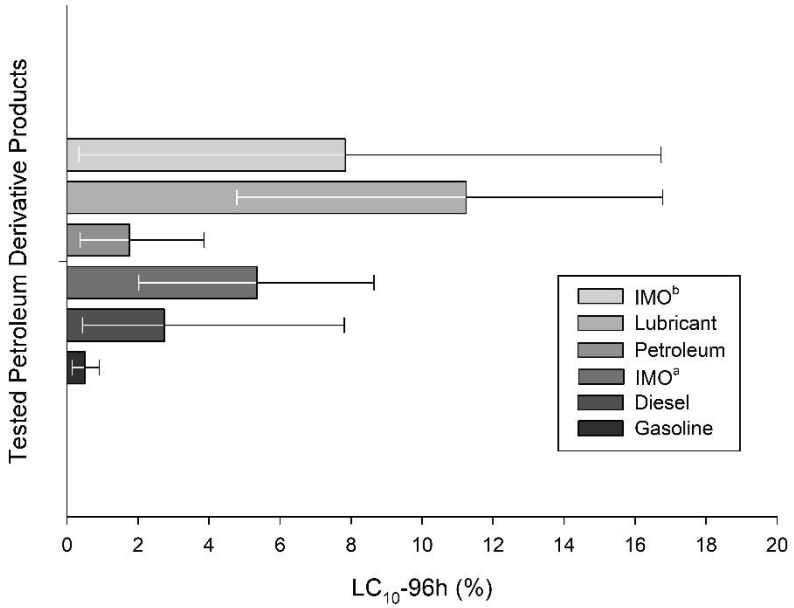

Figure 3. Petroleum and derivative products [gasoline, diesel, insulation mineral oil (IMO^a and IMO^b) and lubricant oil] toxicity (LC_{10}-96h) to the neotropical freshwater fish *Astyanax altiparanae*. Bars represent the mean observed LC_{50} values and whiskers represent LC_{50} upper and lower values.

Our results highlight the first detailed investigation of acute petroleum and refined products WSF toxicity to a neotropical fish specie. Further, the obtained results confirmed that BTEX and PAH environmental concentrations plays an important role on the toxicity to *Astyanax altiparanae*. Beyond that, other chemical substances added to the commercial products can also contribute to the distinct observed sensibility. The knowledge of the precise LC_{10} values for petroleum and for the different refined petroleum classes to a native fish specie, can be of great use for laboratorial use and for the establishment of conservational water quality criteria in tropical aquatic environments.

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CHAPTER 2 – DOES WATER TEMPERATURE AND PH MODIFY THE RESPONSES OF BIOMARKERS OF FISH EXPOSED TO GASOLINE WATER-SOLUBLE FRACTION?

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ABSTRACT

The oil and oil products spill are responsible for the input of monoaromatic (BTEX) and polycyclic aromatic (PAH) petroleum hydrocarbons into the aquatic environment. Biomarkers responses used to identify the primary effects of BTEX and PAH in biochemical levels and are extensively used in contamination scenarios. However, the effects of pollutants on fish biomarkers could also can be influenced by the characteristics of the aquatic environment. Thus, we aimed to assess EROD, GST, CAT, protein carbonyl and AChE biomarker responses during water-soluble fraction of gasoline (WSF_G) contamination (96 h) and recovery (96 h), in altered water (temperature: 25 and 30°C; pH: 7.0 and 4.0), using the neotropical fish species *Astyanax altiparanae* (N= 140; W= 16.21 ± 3.87 g; Lt = 10.02 ± 0.66 cm). All tested biomarkers responded either to the alterations in water temperature or pH alone or in association with WSF_G exposure. In the 25°C+pH7.0, liver EROD activity increased when compared to control group. This increase reached a two-fold rise after recovering for 96 h in clean water. The alteration of water temperature, from 25 to 30 °C, inhibited the enzyme activity

and, in pH 4.0, EROD did not change with the WSF_G exposure. GST activity presented different regulation (increased and decrease) according to the presence o WSF_G and water abiotic characteristic. The activity CAT was changed only by water parameters as the content of protein carbonyl in the muscle tissue. Brain and muscle AChE presented significant alterations as a result of WSF_G and temperature e pH exposure. Our results demonstrated that the biotransformation, oxidative stress and neurotoxicity biomarkers in *A. altiparanae* are influenced by water parameters, in this case, high temperature and low pH. Even EROD, considered the most effective biomarker to petroleum hydrocarbon exposure, had its activation inhibited. Thus, we recommend carefulness during the use of those biomarkers in environmental pollution assessment and in laboratorial studies as they can display changes in their responses patterns.

Key-words: AChE, CAT, EROD, GST, Petroleum hydrocarbons, protein carbonyl; WSF; gasoline

2.1. INTRODUCTION

Among the different industrial processes that petroleum and refined products are submitted from its extraction to final utilization, the transport is considered one important source of accidental introduction of hydrocarbons to the aquatic environment (NEFF, 1979; ALBERS, 2003; KIRBY and LAW, 2010). Several accidental spills occurred in marine (CLARCK-JR. and FINLEY, 1977; BARBER *et al.*, 1995; GLEGG *et al.*, 1999; HUIJER, 2005; ITPOF, 2009; HARLOW *et al.*, 2011; SCHMIDT-ETKIN, 2011) and continental environments (OSTRENSKY *et al.*, 2001; BOEGER *et al.*, 2003; ROSE, 2004; ALBARELLO, 2012) during transportation. These accidents are responsible for dumping tons of petroleum products into the aquatic environment (SCHMIDT-ETKIN, 2011). Among the different components of petroleum, the monoaromatic (BTEX) and polycyclic aromatic (PAH) hydrocarbons are recognized as the most toxic for biota (CONNELL and MILLER, 1981; BARRON *et al.*, 1999) due to the ability of these compounds to easily cross biological membranes (ALBERS, 2003).

Compared to the crude oil, light refined fuels, like gasoline and diesel, presents higher concentrations of BTEX and PAH and, consequently, are considered more toxic to aquatic organisms (BARRON *et al.*, 1999; ALBERS, 2003; RODRIGUES *et al.*, 2010).

The investigation of the biological effects of BTEX and PAH exposure is normally conducted through the assess of biomarkers responses. Several biomarkers were developed to identify the effects of the different sources of contamination (VAN DER OOST *et al.*, 2003). Some of these biomarkers are used to identify the primary effects in biochemical levels and are extensively used in petroleum hydrocarbon contamination scenarios (HEATH, 1995). The biochemical activity of some biotransformation enzymes is normally used as biomarkers due to their role in the transformation of lipophilic pollutants to more water-soluble compounds (VAN DER OOST *et al.*, 2003). One example is the CYT P450 enzymatic group, usually measured by the activity of ethoxyresorufin *O*-deethylase (EROD) - widely utilized as biotransformation biomarker for petroleum hydrocarbon (WHYTE *et al.*, 2000). The increase of hepatic EROD activity in fish undergoing polluted environments by organic compounds was demonstrated by several studies (GAGNON and HOLDWAY, 2000; PACHECO and SANTOS, 2001; ABRAHAMSON *et al.*, 2007; PAL *et al.*, 2011; SIMONATO *et al.*, 2011; BETTIM *et al.*, 2016; DUARTE *et al.*, 2017). Some fish species, however, showed the inverse response, decreasing EROD activities after exposure to organic (HAHN *et al.*, 1993; FENT and BUCHELI, 1994) and inorganic contaminants (VIARENGO *et al.*, 1997). The variation on EROD response can be attributed to biotic (species, age, sex, nutritional status) and abiotic (temperature and pH tolerance) characteristics of the organism or aquatic

environment, respectively (WHYTE *et al.*, 2000). Another biotransformation enzyme, responsible for the conjugation reaction with other polar chemical groups to facilitated excretion of the pollutants, is the glutathione-*s*-transferase (GST) (KEEN *et al.*, 1976). Although GST plays an important role in the conjugation of reactive species, the enzyme is also recognized by metabolic activation of halogenated substances (VAN DER OOST *et al.*, 2003).

The biotransformation reaction of many pollutants can lead to the formation of reactive oxygen species (ROS) (NETO, 2014). Catalase (CAT) is one of several enzymatic antioxidant defenses that is extensively reported as a petroleum hydrocarbon exposure biomarker in fish (GAGNON and HOLDWAY, 1999; SIMONATO *et al.*, 2008; SIMONATO *et al.*, 2011; SADAUSKAS-HENRIQUE *et al.*, 2017), due to their role in the protection of the organism against oxidative damage induced by ROS (VAN DER OOST *et al.*, 2003; RAY *et al.*, 2012; SIES, 2015). In addition, during oxidative stress conditions, the increase of metabolic rate can lead to modification of amino acids in proteins, rising the protein carbonyls content (PARVEZ and RAISUDDIN, 2005), which has also been studied as biomarker of oxidative stress in polluted aquatic environment (AHMAD *et al.*, 2000; PARVEZ and RAISUDDIN, 2005; LORO *et al.*, 2012; TKACHENKO *et al.*, 2014). In the same way, the activity of acetylcholinesterase (AChE) is also considered as an important biomarker for petroleum hydrocarbon exposure (PAYNE *et al.*, 1996) due to is essential role in the normal functioning of sensory and neuromuscular neuro systems, operating the deactivation mechanism of acetylcholine at nerve endings (ČOLOVIĆ *et al.*, 2013). Fish AChE activity in brain and muscle is normally inhibited by several

environmental contaminants (KIRBY *et al.*, 2000; ČOLOVIĆ *et al.*, 2013) including petroleum hydrocarbons (VIEIRA *et al.*, 2008; BETTIM *et al.*, 2016).

The effects of pollutants on fish biomarkers could also be influenced by the characteristics of the aquatic environment. Those influences are normally linked with variations on water physicochemical parameters resulting from natural and anthropogenic changes (WHYTE *et al.*, 2000; ALMEIDA *et al.*, 2014). Accentuated seasonal variations of the water temperature are common in tropical aquatic environments (ESTEVES, 2011). In small tropical rivers or water streams, temperature can vary 16 °C from winter to summer (OYAKAWA et al., 2006). Additionally, predictions of changes in the climatic scenario suggests the increase in the mean temperature of Paraná River Basin area (South Brazil) in 4.5 °C in 60 years (MARENGO et al., 2012). The natural or anthropogenic acidification of water bodies can also be considered as an important source of stress for fish (HEATH, 1995; WENDELAAR BONGA, 1997). Water pH in tropical rivers can vary from neutral (pH=7.0) to acid (pH=3.9) due to natural organic matter accumulation (OYAKAWA et al., 2006; ESTEVES, 2011).

The responses of multiple biomarkers to environmental petroleum hydrocarbons pollution (COHEN *et al.*, 2001; MARTÍNEZ-GÓMEZ *et al.*, 2006; SIMONATO *et al.*, 2008; VIEIRA *et al.*, 2008; KATSUMITI *et al.*, 2009; MARTÍNEZ-GÓMEZ *et al.*, 2009; SILVA *et al.*, 2009; SIMONATO *et al.*, 2011; BETTIM *et al.*, 2016) and the effects of altered physicochemical characteristics of the aquatic environment to normal physiological responses in fish (LOMHOLT and JOHANSEN, 1979; TOEWS *et al.*, 1995; WOOD, 2001; SOLLID *et al.*, 2003; LUSHCHAK *et al.*, 2005; LUSHCHAK and BAGNYUKOVA, 2006; 2007; FATHIMA I. IFTIKAR *et al.*, 2010; DE OLIVEIRA and VAL, 2017) has been

studied mainly separately. Although scarce, some work evaluating the association of pollutant exposure in modified water environment has been published (PACHECO and SANTOS, 2001; CARVALHO and FERNANDES, 2006; SADAUSKAS-HENRIQUE *et al.*, 2016; BRAZ-MOTA *et al.*, 2017).

In the present work, we aimed to assess biochemical biomarkers responses during WSF_G contamination, in normal and altered temperature and pH conditions, using the neotropical fish species *Astyanax altiparanae*. Fishes of the genus *Astyanax* successfully thrives the tropical rivers and water streams from South Brazil (GERRY, 1977; GARUTTI and BRITSKI, 2000; DE CARVALHO et al., 2009). and *A. altiparanae* have been used as bioindicators of environmental contamination in field (OSTRENSKY et al., 2001; SCHULZ and MARTINS-JÚNIOR, 2001; BOEGER et al., 2003; AKAISHI et al., 2004; SILVA et al., 2009) and laboratory (DAL PONT, 2012; DE SIQUEIRA-SILVA et al., 2015; BETTIM et al., 2016; GALVAN et al., 2016; TOLUSSI et al., 2018) studies. We hypothesize that the modification of water parameters would modify the expected response of biomarkers typically used for the assessment of environmental petroleum hydrocarbons pollution.

2.2. MATERIAL AND METHODS

2.2.1. Animal Maintenance and Ethical Note

Adults of *Astyanax altiparanae* (GARUTTI and BRITSKI, 2000) (N= 140; W= 16.21 ± 3.87 g; Lt = 10.02 ± 0.66 cm) were bought from a commercial fish facility (Peixes e Peixes®) located in Curitiba, Paraná, Brazil. Fishes were transported for 1 h in plastic bags filled with freshwater ($^1/_2$) and air ($^2/_3$) to the

laboratory of the Integrated Group for Aquaculture and Environmental Studies (GIA/UFPR). To avoid parasitic infestation after transport, fishes were submitted to a prophylactic salt bath (6 g NaCl L^{-1}) for 2 h (ZENI *et al.*, 2015). Then, were placed in a main holding tank (1000 L) equipped with flow through system filled with dechlorinated tap water from Curitiba (pH= 7.24 ± 0.66; alkalinity= 40.0 ± 12.4 mg $CaCO_3$ L^{-1}). For the acclimation to the laboratory conditions, fishes were kept under controlled temperature (25.4 ± 0.7 °C) and continuously airflow supply (dissolved oxygen= 6.13 ± 0.72 mg L^{-1}) for 4 weeks prior experimentation. Pellets of dry commercial food (Kowalski®, crude protein = 47%) were offered *ad libitum* twice a day. During this period the observed mortality rate was 2%.

All experimental procedures were approved by the Ethics Committee for Animal Use of the Federal University of Paraná (protocol #874).

2.2.2. Gasoline Water-Soluble Fraction

Erlenmeyer flasks covered with aluminum foil were used to store 4 L of regular gasoline obtained from commercial gas station. During the experimentation period flasks were held sealed at room temperature (25 °C) and light-free conditions. The WSF_G was obtained daily by stirring 9 parts of water and 1 part of gasoline for 22 h (ANDERSON *et al.*, 1974) in a 22 L Mariotte Flask, also covered with aluminum foil. Stirring was kept constant to achieve a vortex corresponding to $^1/_3$ of the water + fuel column height. Thirty min before use, the stirring was stopped to allow proper separation of insoluble droplets of gasoline suspended in the WSF_G.

2.2.3. Experimental Conditions

The experimental conditions were designed to assess the effects of water temperature or pH during WSF_G exposure. The procedure was divided in three 96 h phases (total= 288 h): acclimation, exposure and recovery (Figure 4A and B). For acclimation, 40 fishes were transferred from the main flow through tank to two static systems (100 L - 20 fishes/tank) and kept in this condition for 72 h (daily renewal of 80% of the water volume). Then, fishes were individually transferred to 4 L glass aquaria (experimental condition) and held in clean freshwater for 24 h. Preceding the 96 h exposure period, 80% of the water volume was replaced. A group of twenty fishes were exposed to 0.5% (*v/v*) of WSF_G (LC_{10}-96h of WSF_G for *A. altiparanae* established at chapter 1) for 96 h. At same time, other 20 fishes were maintained as control groups in clean freshwater. At the end of exposure, 20 fishes (control N=10; WSF_G N=10) were anaesthetized (MS222 0.2 g ml^{-1}) for blood and tissue sampling. The remaining twenty fishes (control N=10; WSF_G N=10) were submitted to recovery for 96 h in clean freshwater (renewal of 100% of water) until sampling of the tissues.

In this condition, three independent experimental trials were performed (Figure 4A and B). First, water temperature and pH were kept at 25 °C and 7.0, respectively, during acclimation, exposure and recovery. On the second trial, temperature was slowly raised from 25 °C to 30 °C (0.62 °C each 12 h) during the acclimation period, and pH remained at 7.0. Finally, at the third experiment, water pH was lowered from 7.0 to 4.0 (in 4 days), using 5 and 25% HCl solutions, during the acclimation period and maintained during the experiment. Temperature was held at 25 °C.

To avoid fluctuation in water temperature, all trials were performed in temperature-controlled water baths. Individual experimental aquaria were supplied with continuous airflow through all experimental period.

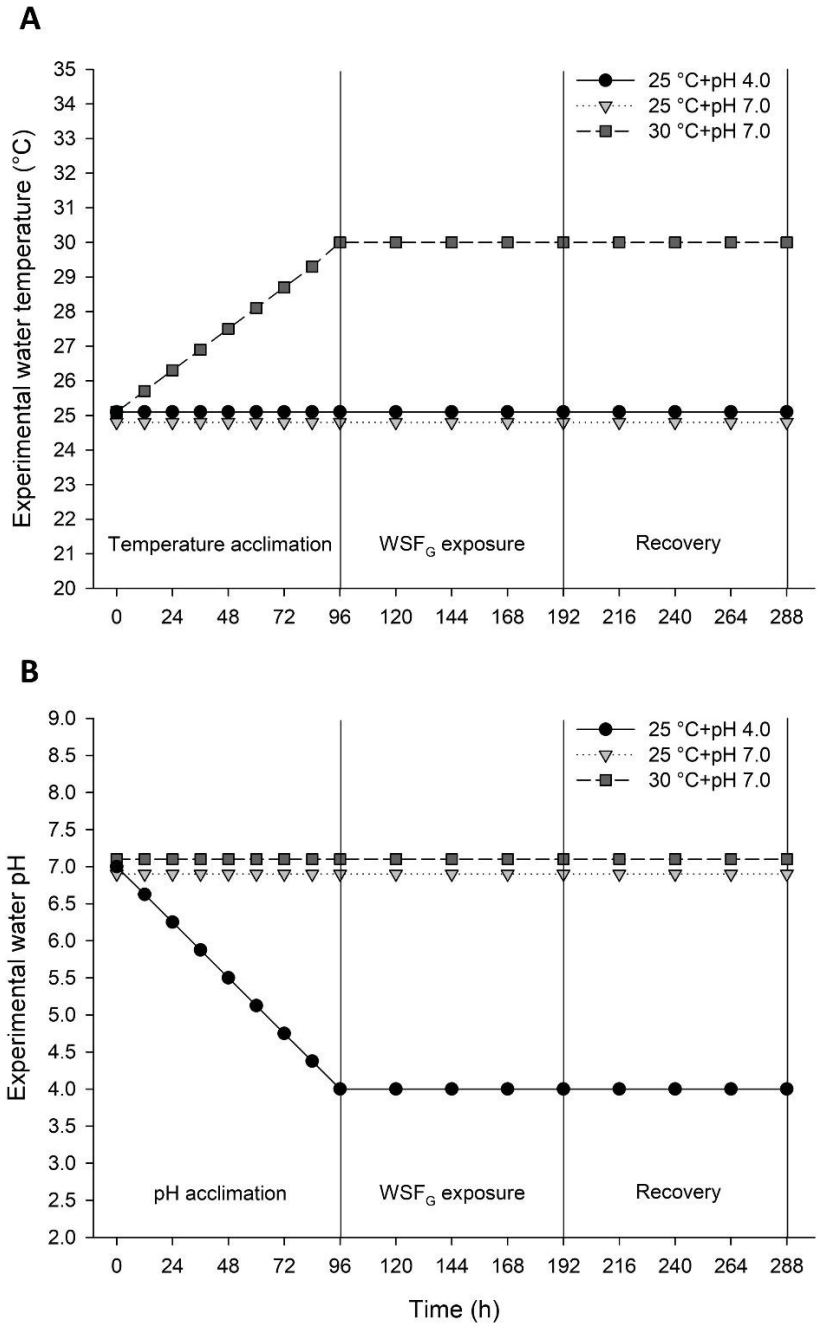

Figure 4. Acclimation and experimental procedures adopted for three independent experimental trials with *Astyanax altiparanae* during exposure to 0.5% of water-soluble fraction of gasoline (WSF_G) under temperature (25 and 30 °C) or pH (7.0 and 4.0) alteration. A) Temperature variation

during acclimation, exposure and recovery periods and B) pH variation during acclimation, exposure and recovery periods.

2.2.4. Water and Tissue Sampling

One liter of 100% WSF_G (daily prepared) and water from one aleatory controlled group were daily sampled in amber flasks for PAH and BTEX determination. Water were sampled, before (2 mL) and after (2 mL) water change, from all controlled and experimental aquaria for immediate pH analysis and than frozen (-20 °C) for posterior ammonia and Cl^- analysis.

Before euthanasia by medullar section, fishes were anesthetised with buffered MS222 0.2 g L^{-1} (OSTRENSKY *et al.*, 2015) for gill, liver, brain and muscle sampling. All tissues were immediately frozen in liquid nitrogen and held at -80 °C until the moment of the analyzes. EROD activity was determinate on the hepatic tissue. GST and CAT activity was measured in gill and liver tissue. The content of carbonyl protein was determined in the muscular tissue. Muscle and brain were used for AChE activity analysis.

2.2.5. Analytical Protocols

2.2.5.1. Water quality parameters

Temperature and dissolved oxygen concentration (YSI® - Pro 20) were analysed directly in the experimental aquaria and pH (Horiba Scientific® B-713) were analysed immediately after water sampling. Defrosted water samples were used for colorimetric determination of total ammonia (595 nm) (VERDOUW *et al.*, 1978) and chloride (480 nm) (ZALL *et al.*, 1956).

2.2.5.2. Petroleum Hydrocarbons

Both PAH and BTEX analysis were carried out utilizing gas phase chromatography coupled to mass spectrometry (GC-MS). For PAH, we used a Thermo Fisher Scientific® gas chromatograph (Focus GC Polaris Q model), equipped with a Thermo® AS3000 auto-sampler for liquid injection and for BTEX a Shimadzu® gas chromatograph (model 2010) coupled to mass spectrometer (TQ8040) in tandem system (GC-MS/MS), equipped with a Shimadzu® AOC-5000 autosampler for headspace analysis. Instrumental parameters used are detailed in Table 4.

Table 4. Instrumental parameters utilized for determination of polycyclic aromatic hydrocarbons (PAH) and monoaromatic hydrocarbons (BTEX) in aqueous samples via gas phase chromatography coupled to mass spectrometry (GC-MS).

PAH	Column	DB5-ms (Agilent®) 30 m x 0.25 mm x 0.25 µm
	Carrier gas	Analytical helium 5.0 (99.999%) with a flow rate of 1.2 mL min^{-1}
	Injector	Splitless, injection volume 1.0 µL
	Temperature	Column: 40 ºC (5 min) Ramp 1: 5 ºC min^{-1} to 230 ºC Ramp 2: 2 ºC min^{-1} to 250 °C Ramp 3: 5 ºC min^{-1} to 300 ºC (8 min) Detector: 300 ºC Injector: 270 °C Transfer line: 270 ºC Ions source: 270 ºC
	SIM* mode	Scan 1 (5 min): (m/z 128, 136, 152, 154, 162, 164, 166, 178) Scan 2 (30 min): (m/z 178, 202, 228, 244) Scan 3 (47 min): (m/z 228, 236 240, 252, 260, 264, 276, 277, 278, 279)
BTEX	Column	SH-Rtx-5ms (Shimadzu®) 30 m x 0.25 mm x 0.25 µm
	Carrier gas	High-pure helium (99.99999%) with a flow rate of 1.02 mL min^{-1}
	Injector headspace	Injection volume: 500 µL

Temperature	Column: 35 ºC (4 min), Ramp 1: 10 ºC min^{-1} to 100 ºC Ramp 2: 30 ºC min^{-1} to 200 ºC Detector: 250 ºC Injector: 180 °C Syringe: 60 ºC Sample: 60 ºC Transfer line: 250 ºC Ions source: 250 ºC
Agitation	Sample: 300 rpm
SIM* mode	Scan (m/z): 78; 91 and 106

Analytical curves were build in triplicate for PAH (0.3; 0.5; 0.8; 1.2; 1.5; 2 and 4 μg L^{-1}) and BTEX (5; 10; 25; 50; 100; 500 and 1000 μg L^{-1}) determination. High purity analytical standards of BTEX (benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene and *p*-xylene) and the 16 priority PAH suggested by U.S.EPA (2001) were used: naphthalene (Naf); acenaphthylene (Aci); acenaphthene (Ace); fluorene (Flu); phenanthrene (Fen); anthracene (Ant); fluorantene (Fla); pyrene (Pyr); benzo(*a*)anthracene (BaA); criseno (Cris); benzo(*b*)fluoranthene (BbF); benzo(*k*)fluoranthene (BkF); benzo(*a*)pyrene (BaP); indene(1,2,3-*cd*)pyrene (Ind); dibenzo(*a*,*h*)anthracene (Dib); benzo(*g*,*h*,*i*)perylene (Ben). Five deuterated internal standards (AccuStandart®) were also used: Naphthalene (NafD8); Acenaftene (AceD10); Fenantrene (FenD10); Chrysene (CrisD12) and Perylene (PerD12). The stock mix standard solutions of PAH and BTEX were prepared in dichloromethane and methanol (J.T. Baker®), respectively, at concentrations of 5 mg L^{-1}, and kept under controlled temperature (-18 °C) until use. Work solutions where prepared daily.

The accuracy was determined by a recovery test by spiking three known concentrations of PAH (0.35; 1.2 and 5 μg L^{-1}) and BTEX (8; 120 and 450 μg L^{-}

^{1}) compounds in a water sample from the same source used in the experimental procedure. The recovery test was performed in three replicates for each concentration level studied. The relative standard deviation (RSD %) were calculated to determinate analytical repeatability.

2.2.5.3. Biotransformation biomarkers

For the EROD activity analysis, liver samples (approximately 0.3 g) were homogenized (ultrasonic homogenizer Eco-Sonics® Ultronic QR300) in a 25% HEPES buffer (HEPES [4.77 mg mL^{-1}]; KCl [11.18 mg mL^{-1}]; pH 7.5). Liver S9 fraction were immediately obtained after 12,000 *g* centrifuging for 20 min (4 ºC). 20 µL of ethoxyresorufin (0.03 mg mL^{-1}) was added to a mix solution (1250 µL of HEPES buffer [23.83 mg mL^{-1}]; 10 µL of $MgSO_4$ [154 mg mL^{-1}]; 50 µL of bovine serum albumin [40 mg mL^{-1}] and 30 µL of *β*-NADPH [30 µg mL^{-1}]) containing 50 µL of S9 fraction. After 2 min, the reaction was stopped with 2.5 mL of methanol. The supernatant was separated through centrifugation (3000 rpm) for 5 min and read at fluorescence spectrophotometer (Molecular Devices® SpectraMax M2 - EX= 530 nm and EM= 585 nm). All samples were read in duplicate and the results expressed as ρmol of resorufin min^{-1} mg of $protein^{-1}$ according to the method established by GAGNON and HOLDWAY (2000).

The activity of GST was measured in supernatant obtained after homogenization in Tris-base/EDTA buffer pH 7.6 (Tris base [0.121 mg mL^{-1}]; EDTA [0.372 mg mL^{-1}]; 1,4-dithiothreitol [0.192 mg mL^{-1}]; sucrose [0.342 mg mL^{-1}] and KCl [0.074 mg mL^{-1}]) followed by 30 min centrifuging (2000 rpm and 4 ºC). The kinetic enzymatic formation of GST, catalyzed by L-glutathione reduced (7.66 mg ml^{-1}) in presence of substrate (1-chloro-2,4-dinitrobenzene [10.13 mg

ml^{-1}], was read in fluorescence spectrophotometer at 340 nm wavelength (KEEN *et al.*, 1976). The absorbance delta of triplicate samples was used to calculate the enzyme activity (U min^{-1} g $protein^{-1}$) using a molar extinction coefficient (ε) of 9.6 mM cm^{-1}.

2.2.5.4. Oxidative stress biomarkers

For CAT determination, the tissues were homogenized in a Tris-base/EDTA pH 7.6 buffer. Quartz cuvette (1 mL) was used to mix 10 µL of the liver homogenate or 15 µL of the gill homogenate and 990/985 µL of the reaction media (H_2O_2 solution [100 µL of H_2O_2 30% in 100 mL of distilled water] + 1 M Tris-Base, 5 mM EDTA, pH 8.0, 25 °C buffer). After gentle mix of the sample homogenate and the reaction media, the enzymatic degradation of H_2O_2 was measured at 240 nm for 1 min. CAT activity was expressed as µmol H_2O_2 mg^{-1} protein min^{-1} (BEUTLER, 1975).

Protein carbonyls was colorimetric measured (358 nm) in muscle homogenate (homogenization in PBS pH 7.2 buffer followed by centrifuging at 12000 *g* [4°C] for 20 min) according to the 2,4-dinitrophenylhydrazine reaction with carbonyl groups to form 2,4-dinitrophenylhydrazone method, described by LEVINE *et al.* (1994). Results were expressed as nmol of protein carbonyl mg $protein^{-1}$.

2.2.5.5. Brain and muscle AChE assay

The activity of AChE was determined on brain and muscle tissue (ELLMAN *et al.*, 1961). Phosphate buffer pH 7.4 (in 20% glycerol solution) was used for

homogenization (ultrasonic homogenizer Eco-Sonics® Ultronic QR300). Supernatant were obtained by 12,000 *g* centrifugation (4 °C) for 15 min. Acetylcholine iodide (Sigma®) was used as substrate for the hydrolysis reaction. The color resulting from the production of the yellow anion 5-thio-2-nitro-benzoic acid was measured at 415 mn for 6 min. Results were calculated using 13.6 nmol min^{-1} mL^{-1} as the molar extinction coefficient (ε). All results were expressed as nmol of AChE min^{-1} mg $protein^{-1}$.

2.2.5.6. Protein assay

Total content of protein of each analysed tissue was determined by colorimetric following the method described by BRADFORD (1976) using Sigma® reagent and BSA as standard.

2.2.6. Statistical Analysis

Normality and homogeneity of the data was analysed through Shapiro-Wilks and Bartlett's tests, respectively. One-way ANOVA analysis of variance, followed by *post-hoc* Tukey, and Kruskal-Wallis test, followed by *post-hoc* Dunns, were used to determinate significant differences among parametric and non-parametric data ($p<0.05$). Differences between exposure or recovery treatments and their respective control group was established through t-test and Mann-Whitney U test for parametric and non-parametric data, respectively (ZAR, 1984). Data were analyzed using SigmaStat® 3.5 and graphically plotted using SigmaPlot® 11.0 (both by Systat Software Inc.) and presented as mean ± SD or median (min-max).

2.3. RESULTS

Both experiments performed at 25 °C (pH 7.0 and 4.0) presented the same mortality rate (12.5%). Changes in water temperature (30°C+pH7.0) increased the mortality to 17.5%. Measured temperature, pH and dissolved oxygen (DO) did not presented significant variation among treatments ($p>0.05$) through the experimental periods (Table 5). Total ammonia concentrations were below of the toxic level for *A. altiparanae* in all experimental trials (DAL PONT, 2012). However, NH_4+NH_3 values tended to raise only in the control groups before the daily water change. After the water change, concentrations were similar among the treatments. Cl^- concentrations were lower in 25°C+pH7.0 and 30°C+pH7.0 trials, when compared to the 25°C+pH4.0 trial (Table 5).

The concentrations of the measured petroleum hydrocarbons (BTEX and PAH) presented variation among the 100% WSF_G used in the experimental trials, with higher concentrations of BTEX (Table 6). In the control groups of all experimental procedures, the presence of BTEX and PAH weren't detected.

Table 5. Mortality [absolute number (number of fish)] and water physicochemical parameters during exposure of the neotropical fish *Astyanax altiparanae* to 0.5 % of water-soluble fraction of gasoline (WSF_G) in different experimental water temperature (25 and 30°C) and pH (7.0 and 4.0) conditions.

Trials	Period	Treat.	Mort.	Measured Temp. (°C)	Measured pH	O_2 (mg L^{-1})	NH_4+NH_3 (mmol L^{-1})		Cl^- (mmol L^{-1})	
							Before	After	Before	After
25°C+ pH7.0	96h	Control	1 (10)	25.24 ± 0.63	6.69 ± 0.21	6.84 ± 0.21	0.098 (0.012-0.257)	0.029 (0.009-0.059)	0.35 (0.27-5.10)	0.55 (0.27-0.89)
		Exposure	3 (10)	25.49 ± 0.65	6.60 ± 0.46	6.33 ± 0.56	0.026 (0.010-0.205)*	0.022 (0.007-0.079)*	0.38 (0.29-0.68)	0.65 (0.22-1.03)
	192h	Control	1 (10)	25.30 ± 0.68	6.64 ± 0.56	6.74 ± 0.27	0.070 (0.013-0.207)	0.023 (0.006-0.098)	0.37 (0.24-1.85)	0.64 (0.26-2.35)
		Exp.+ recovery	1 (10)	25.15 ± 0.48	6.89 ± 0.26	7.05 ± 0.57	0.026 (0.008-0.099)*	0.014 (0.006-0.048)*	0.33 (0.24-1.64)	0.56 (0.07-1.54)
30°C+ pH7.0	96h	Control	0 (10)	30.30 ± 0.43	6.90 ± 0.23	6.26 ± 0.17	0.048 (0.017-0.134)	0.016 (0.006-0.064)	0.74 (0.26-1.85)	1.43 (0.60-1.75)
		Exposure	2 (10)	30.50 ± 0.38	6.80 ± 0.26	5.98 ± 0.36	0.031 (0.000-0.245)*	0.008 (0.001-0.030)*	0.85 (0.41-1.95)	1.35 (0.44-1.83)
	192h	Control	2 (10)	30.40 ± 0.37	6.80 ± 0.28	6.00 ± 0.38	0.063 (0.019-0.231)	0.017 (0.007-0.055)	0.63 (0.44-2.08)	0.68 (0.02-2.92)
		Exp.+ recovery	3 (10)	30.60 ± 0.44	6.80 ± 0.32	6.10 ± 0.32	0.045 (0.002-0.239)*	0.015 (0.002-0.093)	0.78 (0.46-1.98)	0.62 (0.37-3.15)
25°C+ pH4.0	96h	Control	1 (10)	25.50 ± 0.17	4.00 ± 0.33	7.00 ± 0.14	0.138 (0.065-0.412)	0.055 (0.004-0.185)	9.01 (4.29-11.36)	7.75 (5.36-9.30)
		Exposure	1 (10)	25.60 ± 0.25	4.00 ± 0.25	7.20 ± 0.44	0.055 (0.001-0.514)*	0.056 (0.004-0.241)	7.62 (4.46-13.30)	7.28 (3.90-9.63)
	192h	Control	1 (10)	25.30 ± 0.37	3.90 ± 0.19	7.10 ± 0.19	0.185 (0.037-0.656)	0.050 (0.014-0.202)	8.14 (4.63-12.10)	7.41 (3.62-9.30)
		Exp.+ recovery	3 (10)	25.60 ± 0.46	3.90 ± 0.21	7.30 ± 0.35	0.096 (0.002-0.479)*	0.055 (0.002-0.358)	8.07 (5.39-13.06)	8.05 (5.46-9.19)

* Indicates difference to the corresponding control group (*Mann-Witney* test, $p<0.05$). When significant differences were not detected ($p>0.05$ symbols were omitted.

Table 6. Concentrations of monoaromatic (BTEX) and polycyclic aromatic hydrocarbons (PAH) in 100% water-soluble fraction of gasoline (WSF_G) used during the different exposure trials with the neotropical freshwater fish *Astyanax altiparanae*.

Aromatic Petroleum Hydrocarbons	**Experimental Trials**		
	25°C+pH7.0	30°C+pH7.0	25°C+pH4.0
BTEX (µg L^{-1})			
Benzene	11996.2	9922.6	10344.7
Toluene	11161.6	9335.2	9472.3
Ethylbenzene	817.5	645.9	686.5
m,*p*-Xylene	1608.2	1204.7	1339.9
o-Xylene	1487.8	1120.0	1284.1
∑ *BTEX*	*27071.3*	*22228.4*	*23127.5*
PAH (µg L^{-1})			
Naphthalene	<0.5	<0,5	<0,5
Acenaphthylene	< 0,5	< 0,5	< 0,5
Acenaphthene	< 0,5	< 0,5	< 0,5
Fluorene	nd	nd	nd
Phenanthrene	nd	nd	nd
Anthracene	nd	nd	nd
Fluoranthene	1.5	2.1	2.3
Pyrene	1.8	1.9	2.0
Benz[*a*]anthracene	3.8	1.2	3.4
Chrysene	4.6	2.0	4.1
Benzo[*b*]fluoranthene	5.5	3.7	4.4
Benzo[*k*]fluoranthene	5.6	3.9	4.7
Benzo[*a*]pyrene	4.2	3.4	3.7
Indeno[1,2,3-*cd*]pyrene	5.4	2.8	3.7
Dibenz[*ah*]anthracene	5.3	2.5	3.2
Benzo[*ghi*]perylene	5.4	4.7	4.3
∑ *PAH*	43.0	24.2	35.7

Quantification limit = BTEX < 5.0 µg L^{-1} and PAH < 0.5 L^{-1}. BTEX= Monoaromatic hydrocarbons. PAH= Polycyclic aromatic hydrocarbons.

All tested biomarkers responded either to the alterations in water temperature or pH alone or in association with WSF_G exposure. In the 25°C+pH7.0, liver EROD activity exhibited, during petroleum hydrocarbon exposure, increasing of 0.7 times when compared to control group ($p<0.05$). This

increase reached a two-fold variation after recovering for 96 h in clean water ($p<0.05$). The alteration of water temperature, from 25 to 30 °C, inhibited the enzyme activity. In pH 4.0, EROD activity did not change with the WSF_G exposure (Figure 5).

The exposure of *A. altiparanae* to WSF_G promoted markedly changes in the branchial GST activity. In 25°C+pH7.0, the exposure to WSF_G induced significant increase in the enzyme activity ($p<0.05$). In acid water, however, GST activity was inhibited by the WSF_G ($p<0.05$). In 30 °C, an inhibition ($p<0.05$) was detected but only during recovery in freshwater. Branchial GST activity also responded acutely to the alterations in water parameters. Among control groups, the rise in temperature and reduction in pH induced a 3 and 2.25-fold increase at the enzyme gill activity ($p<0.05$), respectively. The same differences were also detected among the 192 h control groups ($p<0.05$) (Figure 6A). Although the activity in the hepatic tissue was lower than the values detected in the gill, some alterations were induced by both WSF_G and water parameters. Oppositely to the gill activity, in the liver, WSF_G promoted GST inhibition in 25°C+pH 7.0 and activation in pH 4.0 trials ($p<0.05$) (Figure 6B). This pattern was also observed during recovery in freshwater ($p<0.05$).

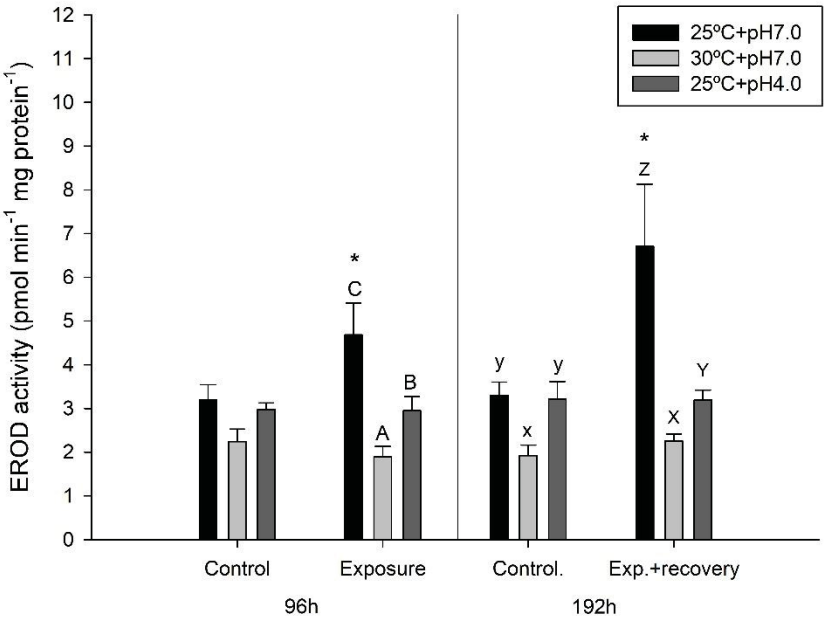

Figure 5. Mean ± S.E.M values of ethoxyresorufin-*O*-deethylase (EROD) activity in liver of *Astyanax altiparanae* after 96 h exposure to 0.5% of water-soluble fraction of gasoline (WSF_G) and after 96 h of recovery in clean freshwater (192 h). Lowercase letters indicate difference in the 96 h (a, b and c) and 192 h (x, y and z) control groups among the different experiment trials (One-Way ANOVA, $p<0.05$). Capitalized letters indicate difference in the 96 h-exposure (A, B and C) and 192 h-recovery (X, Y and Z) groups among the different experiment trials (One-Way ANOVA, $p<0.05$). ∗ Indicates difference to the corresponding control group (*t* test, $p<0.05$). When significant differences were not detected ($p>0.05$), letters and symbols were omitted.

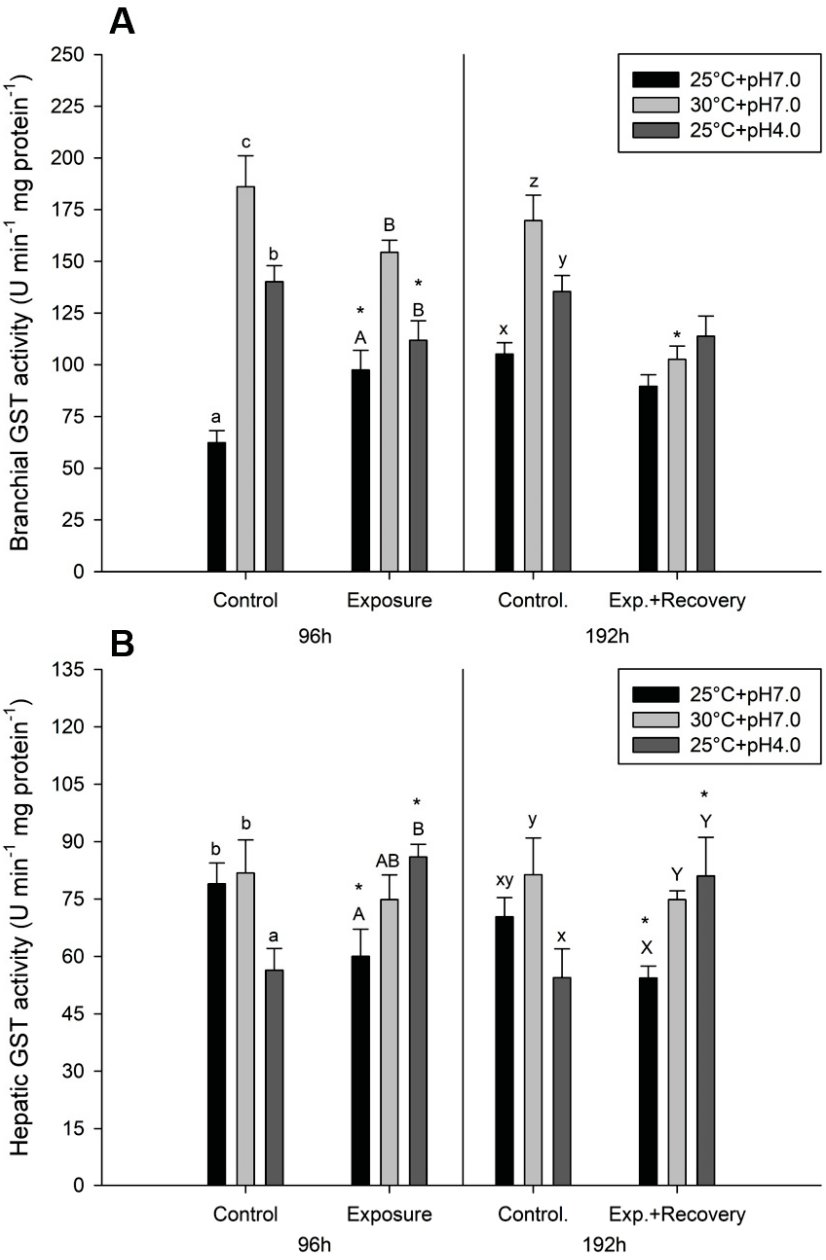

Figure 6. Mean ± S.E.M values of glutathione-S-transferase (GST) activity in branchial (A) and liver (B) tissue of *Astyanax altiparanae* after 96 h exposure to 0.5% of water-soluble fraction of gasoline (WSF_G) and after 96 h of recovery in clean freshwater (192 h). Lowercase letters indicate difference in the 96 h (a, b and c) and 192 h (x, y and z) control groups among the different experiment trials (One-Way ANOVA, $p<0.05$). Capitalized letters indicate difference in the 96 h-exposure (A, B and C) and 192 h-recovery (X, Y and Z) groups among the different experiment trials (One-Way ANOVA, $p<0.05$). ∗ Indicates difference to the corresponding control group (*t* test,

$p<0.05$). When significant differences were not detected ($p>0.05$), letters and symbols were omitted.

No differences were observed in branchial CAT activity during WSF_G exposure. The activity of this enzyme was changed only by water parameters, with an increased activity induced by the lower pH condition ($p<0.05$). The pH also induced the hepatic CAT activity (0.75-fold increase) in both control and exposure treatments ($p<0.05$) (Figure 4A).

An increase ($p<0.05$) in protein carbonyl content was detected after 96 h exposure to WSF_G in the low pH condition (Figure 8). While WSF_G did not severally affect the oxidative degradation of protein in the white muscle, temperature and pH plays an important role in this metabolic mechanism. The mean protein carbonylation detected in *A. altiparanae* in the 30 °C trial was 6.3 times higher than 25 °C ($p<0.05$). In the low pH trial, the difference was even higher reaching a 12.6-fold increase when compared to the neutral pH (25°C+pH7.0) trial ($p<0.05$) (Figure 8).

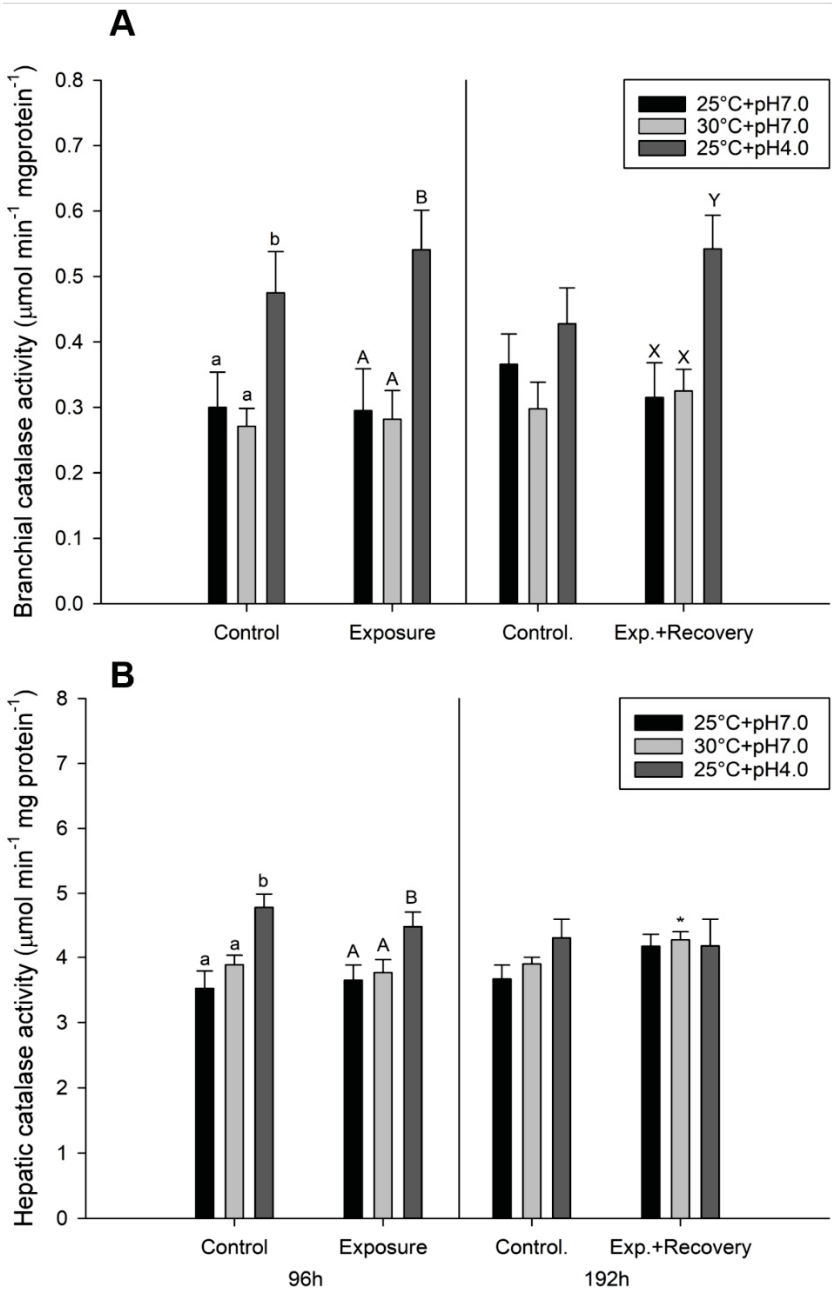

Figure 7. Mean ± S.E.M values of catalase (CAT) activity in branchial (A) and liver (B) tissue of *Astyanax altiparanae* after 96 h exposure to 0.5% of water-soluble fraction of gasoline (WSF_G) and after 96 h of recovery in clean freshwater (192 h). Lowercase letters indicate difference in the 96 h (a, b and c) and 192 h (x, y and z) control groups among the different experiment trials (One-Way ANOVA, $p<0.05$). Capitalized letters indicate difference in the 96 h-exposure (A, B and C) and 192 h-recovery (X, Y and Z) groups among the different experiment trials (One-Way ANOVA, $p<0.05$). ∗ Indicates difference to the corresponding control group (*t* test, $p<0.05$). When significant differences were not detected ($p>0.05$), letters and symbols were omitted.

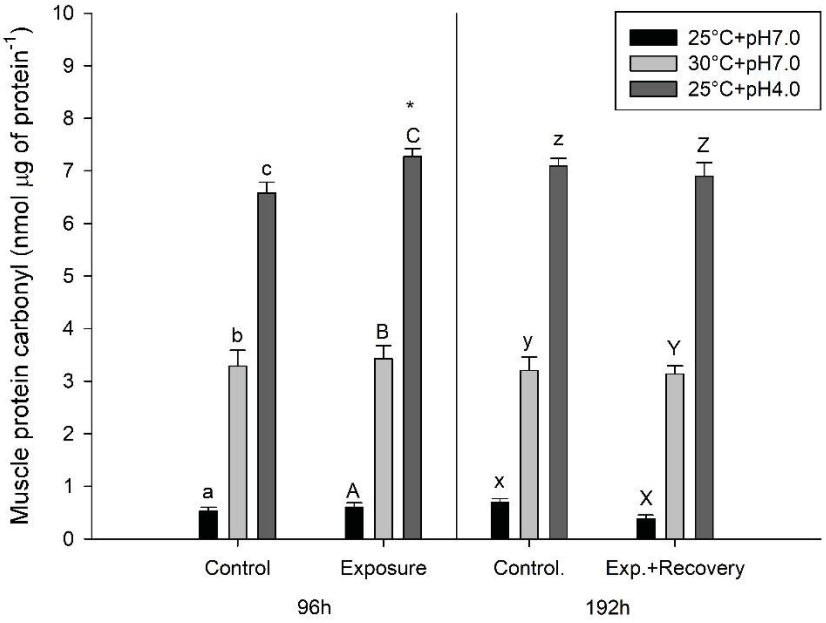

Figure 8. Mean ± S.E.M values of protein carbonyl content in muscle of *Astyanax altiparanae* after 96 h exposure to 0.5% of water-soluble fraction of gasoline (WSF_G) and after 96 h of recovery in clean freshwater (192 h). Lowercase letters indicate difference in the 96 h (a, b and c) and 192 h (x, y and z) control groups among the different experiment trials (One-Way ANOVA, $p<0.05$). Capitalized letters indicate difference in the 96 h-exposure (A, B and C) and 192 h-recovery (X, Y and Z) groups among the different experiment trials (One-Way ANOVA, $p<0.05$). * Indicates difference to the corresponding control group (*t* test, $p<0.05$). When significant differences were not detected ($p>0.05$), letters and symbols were omitted.

The activity of AChE in brain and muscle of *A. altiparanae* are showed in Figure 9. The exposure to WSF_G in acidified water inhibited the activity ($p<0.05$) of this enzyme in the brain, while in the muscle, the AChE activity increased after recovery in the 25°C+pH7.0. A significant inhibition was also detected during the recovering period at 30 °C and pH 4.0 (Figure 9A). Brain and muscle AChE activity presented significant increase under 30 °C and pH 4.0 in 96 and 192 h. In the white muscle, the activity reached a 3-fold increase at 30 °C.

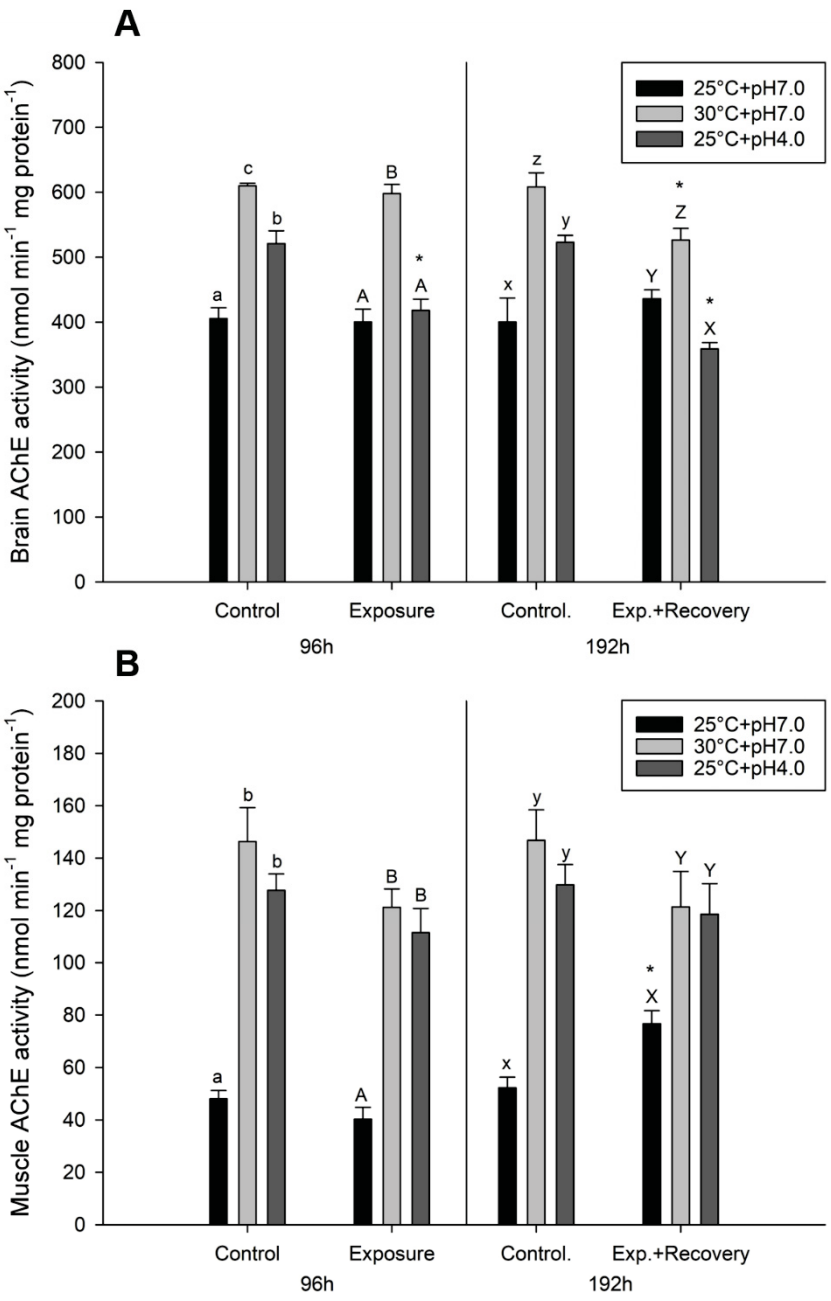

Figure 9. Mean ± S.E.M values of acetylcholinesterase (AChE) activity in brain (A) and muscle (B) of *Astyanax altiparanae* after 96 h exposure to 0.5% of water-soluble fraction of gasoline (WSF_G) and after 96 h of recovery in clean freshwater (192 h). Lowercase letters indicate difference in the 96 h (a, b and c) and 192 h (x, y and z) control groups among the different experiment trials (One-Way ANOVA, $p<0.05$). Capitalized letters indicate difference in the 96 h-exposure (A, B and C) and 192 h-recovery (X, Y and Z) groups among the different experiment trials (One-Way ANOVA, $p<0.05$). ∗ Indicates difference to the corresponding control group (*t* test,

p<0.05). When significant differences were not detected (p>0.05), letters and symbols were omitted.

2.4. DISCUSSION

The role of biotransformation, oxidative and neurotoxic indicator enzymes as biomarkers of petroleum hydrocarbons exposure was tested in a number of tropical and temperate fish species, as reviewed by VAN DER OOST *et al.* (2003). However, BENGTSON and HENSHEL (1996) have been inquiring how biomarkers might be useful if changes in the environmental water parameters, as a result of natural or anthropogenic alterations, alter their expected responses. Here, we measured the effects of water temperature and pH, during the exposure of *A. altiparanae* to 0.5% of WSF_G, in the response of EROD, GST, CAT and AChE activity and protein carbonyl content.

A significant EROD induction in liver of the *A. altiparanae* was detected after exposure to 25°C+pH7.0 in association with WSF_G and this up-regulation pattern was maintained after recovery (i.e. without WSF_G). Increases in the EROD activity is widely describe in the literature for fish exposed to petroleum hydrocarbons (GAGNON and HOLDWAY, 2000; PAL *et al.*, 2011; SIMONATO *et al.*, 2011), including *A. altiparanae* (BETTIM *et al.*, 2016). This biotransformation enzyme is considered the most sensitive to petroleum hydrocarbon exposure in fish (see the reviews by WHYTE *et al.* (2000) and VAN DER OOST *et al.* (2003). The increases in water temperature during the WSF_G treatment altered the response of *A. altiparanae*, resulting in EROD inhibition, while for low pH no alterations were observed. The inhibition of hepatic EROD during environmental or laboratory exposure to organic toxicants had already

been described for fish (HAHN *et al.*, 1993; FENT and BUCHELI, 1994; VAN DER OOST *et al.*, 2003). Although the alterations of water temperature and pH could increase ROS generation (LUSHCHAK and BAGNYUKOVA, 2006; RAY *et al.*, 2012), that may lead to inhibition of EROD (SADAUSKAS-HENRIQUE, 2014), the exact mechanism in *A. altiparanae* remain to be studied. Our data, however, is the first evidence demonstrating that *A. altiparanae* inhibits EROD activity as a result of the association between petroleum hydrocarbon and low pH, or high temperature. Anyway, it is important to note that, when fish were challenged by WSF_G under low pH and high temperature, the EROD did not respond, where increases in the activity of the GST seems to play a central role in detoxifying the WSF_G products in liver under those conditions.

The measurement of GST in liver tissue showed a down-regulation pattern in the normal temperature and pH condition. The result agrees with enzyme response in *A. altiparanae* in laboratory conditions (BETTIM *et al.*, 2016). The essential role of GST is the detoxification process, as this enzyme catalyze the conjugation of chemical pollutants with glutathione (GSH) (BENGTSON and HENSHEL, 1996), in a defence mechanism against oxidative cellular stress (VAN DER OOST *et al.*, 2003). Thus, it was expected that GST also presented an increased activity, the same way as EROD, once it acts in conjugation with exogenous compounds derived or not from the biotransformation phase (EROD) (SADAUSKAS-HENRIQUE *et al.*, 2017). The opposite behavior of hepatic GST (down-regulated) and EROD (up-regulated) was already described by other authors (BRAZ-MOTA *et al.*, 2015; SADAUSKAS-HENRIQUE *et al.*, 2016; SADAUSKAS-HENRIQUE *et al.*, 2017). For example, SHAILAJA and D'SILVA (2003) described this same pattern in EROD and GST activity of *Oreochromis*

mossambicus exposed to 25% refinery effluent (SHAILAJA and D'SILVA, 2003). These authors attributed the observed divergence to alterations in the kinetic induction of phase I (bioactivation) and phase II (conjugation/detoxification) systems. Consequently, that could lead to the accumulation of reactive metabolites that could cause tissue damage. Although the GST activity was down-regulated in the liver, at 25°C+pH4.0 it was up-regulated in the gills, suggesting that branchial GST could play an important role in the petroleum hydrocarbon detoxification in *A. altiparanae*.

In our study, the exposure of *A. altiparanae* to WSF_G changed the hepatic GST response, increasing the activity in acidified water. On the other hand, branchial GST was inhibited in the acid condition. In both brachial and hepatic tissues, the activity of GST seems to act in reverse ways under normal water temperature and pH and in acid condition. The increase of water temperature activated the brachial GST when comparted to 25°C trial, but in the liver, it persisted at control levels. VIEIRA *et al.* (2008) investigated the effects of a fuel and two individual PAH (Benzo[*a*]pyrene and anthracene) in *Pomatoschistus microps* and found hepatic GST induction after exposure to benzo[a]pyrene and fuel and GST inhibition after anthracene exposure. The authors suggested that the observed differences could be related to the affinity of Ah-R receptors, as Ah-R ligands promote GST activity (GOKSØYR and HUSØY, 1998), induced by the presence of lower molecular weight individual hydrocarbons. However, this hypothesis doesn't appear to fit the pattern showed by *A. altiparanae* in the present study, as the concentrations of individual PAH were similar. Other hypothesis is associated to changes promoted by the temperature and pH alterations on physiological mechanisms, which could alter membrane diffusional

gradients (WOOD, 2001) and, consequently, facilitated or inhibit the individual PAH uptake. Additionally, SADAUSKAS-HENRIQUE *et al.* (2016) reported that acclimation of *Colossoma macropomum* to Rio Negro water, which presents low pH and high concentrations dissolved organic compounds, prior to dispersed crude oil exposure, inhibited GST response. Thus, it seems plausible to assume that changes in water temperature and pH could affect the chemical speciation of water substances that could both interfere in the individual PAH availability (FORTUNY *et al.*, 2007) and membrane diffusional gradients and, therefore, affect kinetic of the biotransformation enzymes in *A. altiparanae*. Thus, in a scenario of water temperature increase or acidification – which have already been reported in the area where species of the *Astyanax* genus thrives (OYAKAWA *et al.*, 2006), EROD and GST could not be considered as reliable biomarkers.

The WSF_G caused increases in the hepatic CAT activity of *A. altiparanae* only during the recovery period of the 30 °C trial, indicating a delayed oxidative metabolization of ROS (LUSHCHAK, 2011). This lack of CAT activity induction by petroleum hydrocarbon, in the remain treatments, is in accordance with the study performed by Bettim et al., (2016), where no increases in CAT activity was observed for *A. altiparanae* exposed to WSF_G (BETTIM et al., 2016). CAT enzyme is responsible for the reduction of the hydrogen peroxide to oxygen and water (H_2O_2 to O_2 and H_2O). The hydrogen peroxide molecules derive, specially, from the dismutation of superoxide anions into hydrogen peroxide to oxygen and water by the superoxide dismutase (SOD) (VAN DER OOST et al., 2003). This cascade of reactions occurs in an attempt to neutralize the highly reactive oxidative group, like hydroxyl radical (OH), to prevent the oxidation of important biological molecules as lipids, DNA and proteins. The increased concentration of

carbonyl proteins in the white tissue of *A. altiparanae* indicates that oxidative damage was induced by the formation of OH radicals under high water temperature and low pH. In the present work, once the protein carbonylation was severely affected by the water temperature and pH, we assume that the antioxidant defense in *A. altiparanae* was inefficient to cope with these water physicochemical alterations, once these additional challenges severely impairs the *A. altiparanae* when exposed to WSFG.

The activity of AChE plays an important role in the cholinergic synapses due to is rapid hydrolysis of acetylcholine to choline and acetate (HEATH, 1995; BENGTSON and HENSHEL, 1996). Also, the activity of this enzyme is known by its inhibition under organic pollutants exposure been widely used as bioindicator of organic pollutants contamination (ASSIS and MELA, 2014). In the present work, the activity of AChE during *A. altiparanae* exposure to WSF_G in 25°C+pH7.0 did not change in both brain and muscle tissue. However, when the water temperature increased, or the water pH was reduced, occurred the up-regulation of both brain and muscle AChE activity. The same way, BARROS *et al.* (2017) found increases of the brain AChE of the *Astyanax* sp. sampled in five rivers with differences in the water temperature. They attributed the cerebral AChE increases to an over compensatory synthesis due to a stressful condition. On the other hand, the low pH caused inhibition of the brain AChE activity when fish were exposed to the WSF_G exposure. Likewise, AKAISHI *et al.* (2004) detected AChE inhibition in *Astyanax* sp. exposure for 96 h to petroleum WSF (15 and 33%). Anyway, the concentrations of petroleum WSF adopted by the authors were significantly higher than those used by BETTIM *et al.* (2016) an in the present study.

Although *A. altiparanae* presents neurotoxic resilience to the tested WSF_G, the results suggest that, as it was hypothesized, that AChE activity, and the other biomarkers tested, in *A. altiparanae* are not only modulated by the presence of petroleum hydrocarbons but by the association with water temperature and pH.

2.5. CONCLUSIONS

The present study demonstrated that the biotransformation and oxidative stress biomarkers in *A. altiparanae* are influenced by water parameters, as high temperature and low pH. Even EROD, considered the most effective biomarker to petroleum hydrocarbon exposure, had its activation inhibited. We also verified that the stress condition, caused by the alteration of water physicochemical parameters, severely increased the fish oxidative stress response. This altered metabolic condition, however, was not detectable through the measurement of CAT activity, suggesting that other oxidative mechanisms could be activated by *A. altiparanae*. Thus, we recommend carefulness during the use of those biomarkers in environmental pollution assessment and in laboratorial studies as they can display changes in their responses patterns.

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CHAPTER 3 – THE ROLE OF WATER TEMPERERATURE AND PH ON PHYSIOLOGICAL AND HAEMATOLOGICAL PARAMETERS IN FISH ACUTELY EXPOSED TO WATER-SOLUBLE FRACTION OF GASOLINE

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ABSTRACT

Continental aquatic environment has undergone chemical pollution due to the increase of anthropogenic activities. Among those chemical substances, petroleum hydrocarbons are considered a potential hazard for the biodiversity in aquatic environments, with some effects been evaluated on basic physiological mechanisms. Additionally, water abiotic characteristics, as dissolved oxygen, temperature and pH also impose a stress condition that can lead to altered physiological responses. The goal of the present work was to evaluate the effects of water-soluble fraction of gasoline (WSF_G) exposure, during temperature or pH water modification, to the physiological responses of the neotropical freshwater specie *Astyanax altiparanae*. After exposure to WSF_G for 96 h and recovery in freshwater for the 96 h, the obtained results highlight the first evidence indicating the physiological alterations related to WSF_G contamination in altered water temperature and pH in *A. altiparanae*. In “normal” (25 °C and pH 7.0) condition, WSF_G promoted an alteration of energetic metabolization, hematopoietic

mechanism, due to a possible metabolic hypoxic condition. When water temperature (30 °C) and pH (4.0) were changed, a secondary set of physiological mechanism were activated to cope with osmoregulatory and acid-base disorders, induced by a severely metabolic hypoxic condition. In in normal or altered temperature or pH conditions, during WSF_G exposure, *A. altiparanae* sustain the integrity of cellular membranes, indicating that the trigged regulatory mechanisms were able to maintain general homeostasis.

Keywords: environmental pollution; osmoregulation; acid-base regulation; plasmatic ions; ATPases; carbonic anhydrase; WSF; Gasoline

3.1. INTRODUCTION

Continental aquatic environment has undergone physical, chemical and biological changes due to the increase of anthropogenic pollution. This scenario is a result of rapid and high demographic and industrial growth (PACHECO and SANTOS, 2001; HUIJER, 2005), which is leading to the environmental contamination by several toxic chemicals (HEATH, 1995). Among those toxic substances, petroleum hydrocarbons contamination is considered a potential hazard for the biodiversity in aquatic environments (ANDERSON, 1979; CONNELL and MILLER, 1981; KPONEE *et al.*, 2015) due to its atmosphere accumulation and a large number of accidental spills into aquatic environments (KIRBY and LAW, 2010) Petroleum and its refined products are a complex mixture of aliphatic, cyclic, and aromatic hydrocarbons (HEATH, 1995), and its individual components can induce toxic effects to the aquatic biota (BARRON *et al.*, 1999; ALBERS, 2003). Among these components, the monoaromatic (BTEX) and polycyclic aromatic (PAH) hydrocarbons are considered the most toxic to fishes due to their ability to cross biological membranes (NEFF, 1985; BARRON, 2003; SROGI, 2007; LIN and TJEERDEMA, 2008). The percentage of the total

content of BTEX and PAH increase during the refining processes (HEATH, 1995). Thus, lighter refined products, as gasoline and diesel, are considered to be more toxic to fishes (CONNELL and MILLER, 1981; RODRIGUES *et al.*, 2010).

Several biomarkers were stablished for the evaluation of fish responses to toxic substances (PEAKALL, 1994; BENGTSON and HENSHEL, 1996). Among these markers, biotransformation enzymes, oxidative stress parameters, genotoxic parameters, biotransformation products, hematological, immunological, endocrine, physiological, histological, morphological and reproductive parameters are highlighted (VAN DER OOST *et al.*, 2003). Although the assessment of the effects caused by the exposure to petroleum hydrocarbons have been focused on biotransformation and oxidative stress processes (BENGTSON and HENSHEL, 1996; WHYTE *et al.*, 2000), some works have been evaluating its effects on basic physiological mechanisms, as osmoregulation, acid-base balance and energetic metabolism (MCKEOWN and MARCH, 1978; ENGELHARDT *et al.*, 1981; BOESE *et al.*, 1982; BRAUNER *et al.*, 1999; MATSUO *et al.*, 2005a). SIMONATO *et al.* (2008) used a multibiomarker approach to evaluate the effects of diesel WSF to *Prochilodus lineatus* and detected alterations on hematocrit, glucose, cortisol and ions plasmatic content. The acute exposure of *Colossoma macropomum* to crude oil and chemical dispersants caused osmoregulatory alterations (DUARTE et al., 2010).

Although these physiological parameters can be affected by petroleum hydrocarbons, others water abiotic characteristics, as dissolved oxygen, temperature and pH also impose a stress condition that can lead to altered adaptive responses (CAIRNS *et al.*, 1975; WOOD, 1989; SOLLID and NILSSON,

2006; WOOD *et al.*, 2017). In tropical aquatic environments, accentuated seasonal variations of the water temperature are fairly common events (ESTEVES, 2011). In small tropical rivers or water streams, temperature present seasonal variations. Influenced by annual seasons, for example, the temperature of water streams in South Brazil can vary between 14 and 30 °C (OYAKAWA *et al.*, 2006). Additionally, the natural or anthropogenic acidification of water bodies should also be considered as an important source of stress for fish (HEATH, 1995; WENDELAAR BONGA, 1997). Water pH in tropical rivers can vary from neutral (pH=7.0) to acid (pH=3.9) due to natural organic matter accumulation (OYAKAWA *et al.*, 2006; ESTEVES, 2011). Although water temperature and pH could influence the responses of physiological biomarkers in fish during petroleum hydrocarbon exposure, to our knowledge, the cumulative assessment of this environmental stressor have never been performed.

Among the tropical bioindicator fish species commonly used in ecotoxicological studies we can highlight *Prochilodus lineatus* (MARTINEZ and SOUZA, 2002; SIMONATO *et al.*, 2004; VANZELLA *et al.*, 2007; SIMONATO *et al.*, 2008; SIMONATO *et al.*, 2011), *Colossoma macropomum* (MATSUO *et al.*, 2005b; DUARTE *et al.*, 2010; BRAZ-MOTA *et al.*, 2015; ROBERTSON *et al.*, 2015; SADAUSKAS-HENRIQUE *et al.*, 2016; DE OLIVEIRA and VAL, 2017; SADAUSKAS-HENRIQUE *et al.*, 2017; WOOD *et al.*, 2017) and species from the *Astyanax* genus (SCHULZ and MARTINS-JÚNIOR, 2001; MARTINEZ and SOUZA, 2002; AKAISHI *et al.*, 2004; LEMOS *et al.*, 2008; SILVA *et al.*, 2009; DAL PONT, 2012; DE SIQUEIRA-SILVA *et al.*, 2015; GALVAN *et al.*, 2016; BARROS *et al.*, 2017). Wide distributed in the freshwaters from the upper Paraná River basin (GARUTTI and BRITSKI, 2000; BENNEMANN and SHIBATTA,

2002), and adapted to water streams were temperature and pH changes frequently (OYAKAWA *et al.*, 2006), the use of *Astyanax altiparanae* as environmental bioindicator is been fomented due to its pollution tolerance (SCHULZ and MARTINS-JÚNIOR, 2001) and aquaculture availability for laboratorial studies (DE CARVALHO *et al.*, 2009).

The goal of the present work was to evaluate the effects of WSF_G exposure, during temperature or pH water modification, to the physiological responses of the neotropical freshwater specie *Astyanax altiparanae*.

3.2. MATERIAL AND METHODS

3.2.1. Animal Care and Ethical Note

Sixty adult of *Astyanax altiparanae* (Garutti & Britski, 2000) (W= 16.21 ± 3.87 g; Lt = 10.02 ± 0.66 cm) were bought from a commercial fish company (Peixes e Peixes®) located in Curitiba, Paraná, Brazil. Fishes were transported for 1 h in plastic bags filled with freshwater ($^1/_2$) and air ($^2/_3$) to the laboratory of the Integrated Group for Aquaculture and Environmental Studies (GIA/UFPR). To avoid parasitic infestation after transport, fishes were submitted to a prophylactic salt bath (6 g NaCl L^{-1}) for 2 h (ZENI *et al.*, 2015). Than, were placed in a main holding tank (1000 L) equipped with flow through system filled with dechlorinated tap water (pH= 7.24 ± 0.66; alkalinity= 40.0 ± 1.4 mg $CaCO_3$ L^{-1}). Fishes were kept under controlled temperature (25.4 ± 0.7 °C) and continuously airflow supply (dissolved oxygen= 6.13 ± 0.72 mg L^{-1}) for 4 weeks prior experimentation. Pellets of dry commercial food (Kowalski®, crude protein = 47%) were offered *ad libitum* twice a day. During the acclimation period the observed mortality rate was 2%.

All experimental procedures were conducted according to the Brazilian regulations of animal care and approved by the Ethics Committee for Animal Use of the Federal University of Paraná (protocol #874).

3.2.2. Gasoline Water-Soluble Fraction

Erlenmeyer flasks covered with aluminum foil were used to store 4 L of regular gasoline obtained from commercial gas station. During the experimentation period flasks were held sealed at room temperature (25 °C) and light-free condition. The WSF_G was obtained daily by stirring 9 parts of water and 1 part of gasoline for 22 h (ANDERSON *et al.*, 1974) in a 22 L Mariotte Flask, also covered with aluminum foil. Stirring was kept constant to achieve a vortex corresponding to $^1/_3$ of the water + fuel column height. Thirty min before use, the stirring was stopped to allow proper separation of insoluble droplets of gasoline suspended in the WSF_G.

Samples of 100% WSF_G were obtained daily for polycyclic aromatic hydrocarbons (PAH) and monoaromatic hydrocarbons (BTEX) determination.

3.2.3. Experimental Conditions

The experimental condition was designed to assess the effects of water temperature and pH during WSF_G exposure. The procedure was divided in three 96 h (total= 288 h) phases: acclimation, exposure and recovery (Figure 4). For acclimation, 40 fishes were transferred from the main tank to two static systems (100 L - 20 fishes/tank) and kept in this condition for 72 h (daily renewal of 80% of the water volume). Than, fishes were individually transferred to 4 L glass

aquaria (experimental condition) and held in clean freshwater for 24 h. Preceding the 96 h exposure period, 80% of the water volume was replaced. A group of twenty fishes were exposed to 0.5% of WSF_G (LC_{10}-96h of WSF_G for *A. altiparanae* established at chapter 1) for 96 h. At same time, other 20 fishes were maintained as control groups in clean freshwater. At the end of exposure, 20 fishes (control N=10; WSF_G N=10) were anaesthetized (MS222 0.2 g ml^{-1}) for blood and tissue sampling. The remaining twenty fishes (control N=10; WSF_G N=10) were submitted to recovery for 96 h in clean freshwater (daily renewal of 100% of water) until sampling.

As described above, the fish were subjected to three independent trials. First, water temperature and pH were kept at 25 °C and 7.0, respectively, during acclimation, exposure and recovery. On the second trial, temperature was slowly raised from 25 °C to 30 °C during the acclimation period (Figure 4A), and pH remained at 7.0. Finally, at the third experiment, water pH was gradually lowered from 7.0 to 4.0 (using 5 and 25% HCl solutions) was maintained during the experiment. Temperature held at 25 °C (Figure 4B). To avoid fluctuation on water temperature, all trials were performed in temperature-controlled water baths (dimensions= 3400 cm x 80 cm x 25 m). The individual experimental aquaria were supplied with continuous airflow through all experimental period.

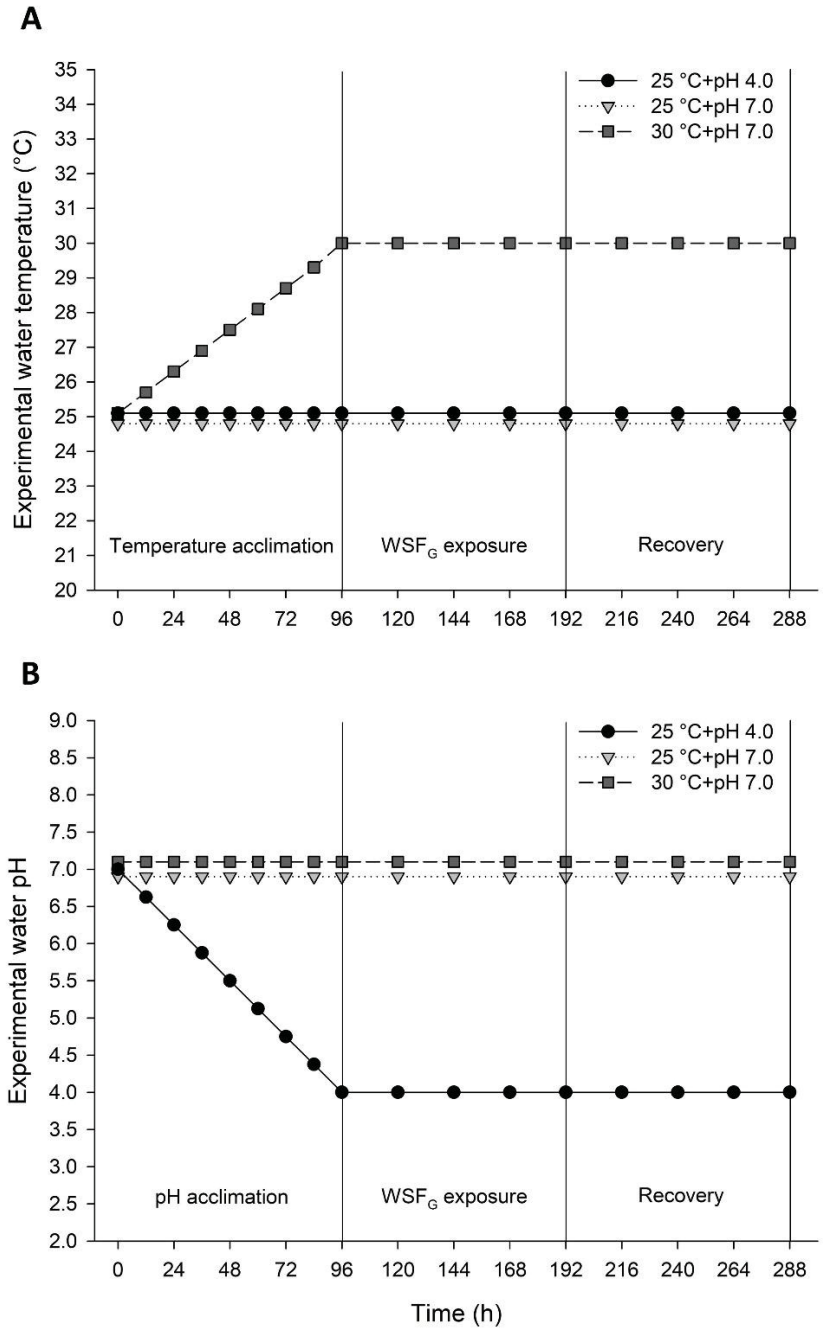

Figure 10. Experimental conditions used for *Astyanax altiparanae* exposure to water-soluble fraction of gasoline. A) Temperature acclimation prior exposure. B) pH acclimation prior experimental exposure.

3.2.3.1. Water sampling

One liter of water from controlled and exposed conditions were daily sampled in amber flasks for PAH and BTEX determination. Water samples were obtained before and after the daily water change for immediate pH analysis and, then, frozen (-20 °C) for posterior analysis of chloride and total ammonia.

3.2.3.2. Blood and tissue sampling

Before blood sampling, fishes were anesthetised with buffered MS222 (0.2 g L^{-1}) (OSTRENSKY *et al.*, 2015). Blood was obtained through puncture of caudal vein with heparinized syringes. Approximately 100 µL of blood was immediately used for haematological analysis. The remaining sample was centrifuged (3000 rpm, 5 min, 4ºC) for plasma separation. Plasma was kept under -20 °C condition until glucose and ions analysis. Fishes were euthanized (medullar section) for gill and muscle sampling. All tissues were immediately frozen in liquid nitrogen and held at -80 °C until the moment of the analyzes.

3.2.4. Analytical Protocols

3.2.4.1. Water Analysis

Temperature and dissolved oxygen concentration (YSI® - Pro 20) were analysed directly in the experimental aquaria and pH (Horiba Scientific® B-713) were analysed immediately after water sampling. Defrosted water samples were use for colorimetric determination of total ammonia (595 nm) (VERDOUW *et al.*, 1978) and chloride (480 nm) (ZALL *et al.*, 1956).

3.2.4.2. WSF_G Analysis

BTEX and PAH extraction were performed according to the headspace methodology described by FERNANDES *et al.* (2014) and the vortex-assisted liquid-liquid dispersive microextraction (VA-DLLME) methodology described by REZAEE *et al.* (2006) and ZHANG and LEE (2012), respectively. Both PAH and BTEX analysis were carried out utilizing gas phase chromatography coupled to mass spectrometry (GC-MS) as already described in chapter 1.

PAH extraction were performed in a vortex-assisted liquid-liquid dispersive microextraction (VA-DLLME) procedure according to the method was adapted from the work of REZAEE *et al.* (2006) and ZHANG and LEE (2012). One mL of the extraction solution ($CHCl_3$= 0.075 mL + C_3H_6O= 0.925 mL) was quickly added in 5 mL of the aqueous sample, or the PAH standard solution, followed by agitation in a vortex for 1 min and centrifugation at 2800 rpm for 10 min. Thus, an aliquot of the highest density fraction (50 µL) was transferred to a chromatographic insert contenting 10 µL of the internal PAH standard solution (mix of deuterated PAH at 100 µg L^{-1}) and injected into GC-MS.

The procedure used for BTEX extraction was based on the headspace methodology described by FERNANDES *et al.* (2014). WSF_G sample (5 mL) was added in a 20 ml vial sealed with PTFE/silicone septa and aluminum seal. The sealed vail was heated (60 ºC) and stirred (300 rpm) for 5 min to promote the volatilization of the BTEX by displacing the equilibrium to the gas phase, located in the headspace of the vial.

Both PAH and BTEX analysis were carried out utilizing gas phase chromatography coupled to mass spectrometry (GC-MS). For PAH, we used a Thermo Fisher Scientific® gas chromatograph (Focus GC Polaris Q model),

equipped with a Thermo® AS3000 auto-sampler for liquid injection and for BTEX a Shimadzu® gas chromatograph (model 2010) coupled to mass spectrometer (TQ8040) in tandem system (GC-MS/MS), equipped with a Shimadzu® AOC-5000 autosampler for headspace analysis. Instrumental parameters used are detailed in Table 7.

Table 7. Instrumental parameters utilized for determination of polycyclic aromatic hydrocarbons (PAH) and monoaromatic hydrocarbons (BTEX) in aqueous samples via gas phase chromatography coupled to mass spectrometry (GC-MS).

PAH	Column	DB5-ms (Agilent®) 30 m x 0.25 mm x 0.25 µm
	Carrier gas	Analytical helium 5.0 (99.999%) with a flow rate of 1.2 mL min^{-1}
	Injector	Splitless, injection volume 1.0 µL
	Temperature	Column: 40 ºC (5 min) Ramp 1: 5 ºC min^{-1} to 230 ºC Ramp 2: 2 ºC min^{-1} to 250 °C Ramp 3: 5 ºC min^{-1} to 300 ºC (8 min) Detector: 300 ºC Injector: 270 °C Transfer line: 270 ºC Ions source: 270 ºC
	SIM* mode	Scan 1 (5 min): (m/z 128, 136, 152, 154, 162, 164, 166, 178) Scan 2 (30 min): (m/z 178, 202, 228, 244) Scan 3 (47 min): (m/z 228, 236 240, 252, 260, 264, 276, 277, 278, 279)
BTEX	Column	SH-Rtx-5ms (Shimadzu®) 30 m x 0.25 mm x 0.25 µm
	Carrier gas	High-pure helium (99.99999%) with a flow rate of 1.02 mL min^{-1}
	Injector headspace	Injection volume: 500 µL
	Temperature	Column: 35 ºC (4 min), Ramp 1: 10 ºC min^{-1} to 100 ºC Ramp 2: 30 ºC min^{-1} to 200 ºC Detector: 250 ºC Injector: 180 °C Syringe: 60 ºC Sample: 60 ºC Transfer line: 250 ºC

	Ions source: 250 ºC
Agitation	Sample: 300 rpm
SIM* mode	Scan (m/z): 78; 91 and 106

Analytical curves were build in triplicate for PAH (0.3 to 4 µg L^{-1}) and BTEX (5 to 1000 µg L^{-1}) determination. High purity analytical standards of BTEX (benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene and *p*-xylene) and the 16 priority PAH suggested by U.S.EPA (2001) were used: naphthalene (Naf); acenaphthylene (Aci); acenaphthene (Ace); fluorene (Flu); phenanthrene (Fen); anthracene (Ant); fluorantene (Fla); pyrene (Pyr); benzo(*a*)anthracene (BaA); criseno (Cris); benzo(*b*)fluoranthene (BbF); benzo(*k*)fluoranthene (BkF); benzo(*a*)pyrene (BaP); indene(1,2,3-*cd*)pyrene (Ind); dibenzo(*a*,*h*)anthracene (Dib); benzo(*g*,*h*,*i*)perylene (Ben). Five deuterated internal standards (AccuStandart®) were also used: Naphthalene (NafD8); Acenaftene (AceD10); Fenantrene (FenD10); Chrysene (CrisD12) and Perylene (PerD12). The stock mix standard solutions of PAH and BTEX were prepared in dichloromethane and methanol (J.T. Baker®), respectively, at concentrations of 5.0 mg L^{-1}, and kept under controlled temperature (-18 °C) until use. Work solutions where prepared daily.

The accuracy was determined by a recovery test by spiking three known concentrations of PAH (0.35; 1.2 and 5 µg L^{-1}) and BTEX (8; 120 and 450 µg L^{-1}) compounds in a water sample from the same source used in the experimental procedure. The recovery test was performed in three replicates for each concentration level studied. The repeatability of both methods was studied by analyzing six replicates of a standard solution of PAH and BTEX and expressed as relative standard deviation (RSD %).

3.2.4.3. Blood and Plasma Analysis

The hematocrit was determinate in heparinized microhematocrit capillary tubes centrifuged at 3000 *g* for 15 min (Benfer® DMH2), and the volume percentage occupied by the red blood cells was read through a standardized hematocrit scale. The counting of erythrocytes and leukocytes in the Neubauer chamber was performed using the Rees-Ecker method (VIVAS, 2014). The mean corpuscular volume (MCV) was obtained using the formula proposed by (FERNANDES JUNIOR *et al.*, 2010):

$$\text{MCV (fL)} = \frac{(\text{Hematocrit x } 10)}{\text{Count of erythrocites}}$$

Plasmatic Cl^- concentrations were colorimetrically determined (480 nm) using the methodology described by (ZALL *et al.*, 1956). Analysis of plasmatic Na^+ and K^+ were performed in plasma samples diluted (1:100) with ultrapure water (Milli-Q® - Merck Millipore). The ions concentrations were determined by flame photometry (CELM® FC - 180) using a 150 mM NaCl and 5 mM KCl standard solution.

3.2.4.4. Branchial Enzymatic Analysis

Na/K ATPase, H^+ ATPase and total ATPase were analysed simultaneously in a 96-well microplate according to the methodology of KÜLTZ and SOMERO (1995). Gill tissue was homogenized (0.01 g of deoxycholic acid in 10 mL of SEI buffer [150 mM of sucrose; 50 mM imidazole; 10 mM EDTA; pH

7.5]) in an ultrasonic homogenizer (Eco-Sonics® Ultronic QR300) and the supernatant separated by centrifuging (5 min; 2000 *g*; 4 °C). The assay was performed immediately after homogenization. The coupling of ATPase activity to pyruvate kinase and lactate dehydrogenase was measured at 340 nm for 10 min in the absence and presence of ouabain and N-ethylmaleimide (NEM). The Na/K, H^+ and Total ATPase activity is expressed as mmol of ADP mg^{-1} of protein h^{-1}.

Branchial carbonic anhydrase specific activity (CAA) was determined according to the methodology described by VITALE *et al.* (1999). For that, tissue homogenization was performed with phosphate buffer 10 mM, pH 7.4 and the homogenate obtained after centrifugation at 12,000 *g* for 5 min. The decrease of the sample pH in presence of the reaction buffer (mannitol 225 mM; sucrose 75 mM; tris base 10 m; NaH_2PO_4 10 mM; pH 7.4) was measured for 20 s (pH meter Horiba Scientific® - F74BW). The slope values of the linear regression curves (determination coefficient [R^2] > 0.98) were calculated using the pH values over time. The CAA was expressed as mg $protein^{-1}$.

3.2.4.5. Energetic Substrates Analysis

Cortisol was measured in muscle. One mL of phosphate buffer (PBS, pH 7.2) was used for the homogenization of 100 mg of wet muscle in an ultrasonic homogenizer. Obtained homogenate was submitted to a cortisol extraction with 8 mL of diethyl ether (BERTOTTO *et al.*, 2010). The dried homogenate was resuspended with 1 mL o PBS. Hormone analysis was performed by radioimmunoassay (RIA). A 96-well microplate cortisol Elisa kit (Diagnostics Biochem Canada® - CAN-C270) coated with anti-rabbit *γ*-globulin serum was used. To increase assay quality control, cortisol standards (high cortisol= 51.0 µg

dL^{-1}; low cortisol= 9.0 µg dL^{-1}) were used among the tested samples. Absorbance was read at 450 nm.

Glucose levels were measured in plasma with a colorimetric commercial kit (Labtest® - 133-2/500). The method is based on the glucose oxidation by glucose oxidase. This reaction generates H_2O_2, which reacts with 4-aminoantipyrine and phenol in presence of peroxidase forming antipyrilquinonimine. The increased of color conferred by antipyrilquinonimine was measured at 520 nm and the glucose concentration was expressed as mg dL^{-1}.

Glycogen content was measured in muscle according to BIDINOTTO *et al.* (1997). Samples (100 ± 10 mg) were digested in 1 mL of KOH 6N in boiling water for 4 min. After cleansing the sample with ethanol 95%, K_2SO_4 10%, and centrifuging (3000 rpm; 3 min), the colorimetric reaction was performed with phenol 3% and H_2SO_4 and read at 480nm.

3.2.4.6. Tissue Hydration Analysis

Immediately after collection, gill and muscle tissue were weighed (wet weight) on a precision balance (Shimadzu® - AUY220). For dehydration measurement, samples were kept in a stove (J.Prolab®-JP 1000) for 24 h at 60 °C. Then, dry weight was measured and the loss of water (hydration) was demonstrated by the percentage of initial sample weight.

3.2.5. Gill Histology

The second left branchial arch was sampled, fixed in Davidson's AFA (Alcohol, formalin and acetic acid) solution (33% of 95% ethanol, 22% formaldehyde, 11.5% acetic acid and 33.5% distilled water) and preserved in 70% ethanol. The fixed gill arches were dehydrated (sequence of ethanol solutions and xylol) and embedded in histological paraffin (Leica® TP1020). Sections of the tissue were performed (5 µm thickness - Leica® RM 2125 RT) and the permanent slides were stained with H&E (Leica® Auto Stainer XL) (TELOSA *et al.*, 2003). To support the physiological results, identification of histopathological lesions was performed under optical microscopy (Olympus® BX41).

3.2.6. Statistical Analysis

Normality and homogeneity of the data was analysed through Shapiro-Wilks and Bartlett's tests, respectively. One-way ANOVA analysis of variance, followed by Tukey post-hoc, and Kruskal-Wallis test, followed by Dunns post-hoc, were used to determinate significant differences among parametric and non-parametric data ($p<0.05$), respectively. Differences between exposure or recovery treatments and their respective control group was established through t-test and Mann-Whitney *U* test for parametric and non-parametric data, respectively (ZAR, 1984). Data were analyzed using SigmaStat® 3.5 and graphically plotted using SigmaPlot® 11.0 (both by Systat Software Inc.) and presented as mean ± S.E.M (water temperature, pH, O_2 and Cl^-; plasmatic Na^+, Cl^-, K^+ and glucose; branchial Na/K, H^+ and total ATPases, ACC and hydration;

muscle glycogen, cortisol and hydration; Ht and PP) or median (min-max) (total ammonia in water; WBC, RBC and MCV).

3.3. RESULTS

3.3.1. Characterization of WSF_G

The measured petroleum hydrocarbons analytes were not detected or presented concentrations below the analytical quantification limit in the control groups. The concentrations of monoaromatic (BTEX) polycyclic aromatic (PAH) of the 100% WSF_G are presented in Table 8. Daily preparation of WSF_G resulted in slightly variations among the independent trials. Nevertheless, the analytes with higher concentrations exhibited the same proportions. The sum of benzene and toluene, for example, corresponded to 85% of the total BTEX in all experimental trials.

Table 8. Concentrations of monoaromatic (BTEX) and polycyclic aromatic hydrocarbons (PAH) in 100% water-soluble fraction of gasoline (WSF_G) used during different the exposure trials with the neotropical freshwater fish *Astyanax altiparanae*.

Aromatic Petroleum Hydrocarbons	**Experimental Trials**		
	25°C+pH7.0	30°C+pH7.0	25°C+pH4.0
BTEX (µg L^{-1})			
Benzene	11996.2	9922.6	10344.7
Toluene	11161.6	9335.2	9472.3
Ethylbenzene	817.5	645.9	686.5
m,*p*-Xylene	1608.2	1204.7	1339.9
o-Xylene	1487.8	1120.0	1284.1
∑ *BTEX*	*27071.3*	*22228.4*	*23127.5*
PAH (µg L^{-1})			
Naphthalene	<0.5	<0,5	<0,5
Acenaphthylene	< 0,5	< 0,5	< 0,5
Acenaphthene	< 0,5	< 0,5	< 0,5
Fluorene	nd	nd	nd
Phenanthrene	nd	nd	nd
Anthracene	nd	nd	nd
Fluoranthene	1.5	2.1	2.3
Pyrene	1.8	1.9	2.0
Benz[*a*]anthracene	3.8	1.2	3.4
Chrysene	4.6	2.0	4.1
Benzo[*b*]fluoranthene	5.5	3.7	4.4
Benzo[*k*]fluoranthene	5.6	3.9	4.7
Benzo[*a*]pyrene	4.2	3.4	3.7
Indeno[1,2,3-*cd*]pyrene	5.4	2.8	3.7
Dibenz[*ah*]anthracene	5.3	2.5	3.2
Benzo[*ghi*]perylene	5.4	4.7	4.3
∑ *PAH*	43.0	24.2	35.7

Quantification limit = BTEX < 5.0 µg L^{-1} and PAH < 0.5 L^{-1}
nd= not detected.
BTEX= monoaromatic hydrocarbons.
PAH= Polycyclic aromatic hydrocarbons.

3.3.2. Water Parameters and Survival Rate

Mortality of 12.5% of was observed in both experiments performed at 25 °C (pH 7.0 and 4.0). When the temperature was raised to 30 °C, mortality also increased (17.5%). Measured temperature, pH and dissolved oxygen (DO) do not presented significant variation among treatments ($p>0.05$) (Table 9). Although NH_4+NH_3 were below toxic concentrations for *A. altiparanae* in all experimental trials, in the treatments contaminated with WSF_G, its concentrations were significative lower ($p<0.05$) than the control groups among all trials realized. Cl^- concentrations were lower in the water of the 25°C+pH7.0 and 30°C+pH7.0 trials, when compared to the 25°C+pH4.0 trial (Table 9)

Table 9. Mortality [absolute number (number of fish)] and water physicochemical parameters during exposure of the neotropical fish *Astyanax altiparanae* to 0.5 % of water-soluble fraction of gasoline (WSF_G) in different experimental water temperature (25 and 30°C) and pH (7.0 and 4.0) conditions.

Trials	Period	Treat.	Mort.	Measured Temp. (°C)	Measured pH	O_2 (mg L^{-1})	NH_4+NH_3 (mmol L^{-1})		Cl^- (mmol L^{-1})	
							Before	After	Before	After
25°C+ pH 7.0	96h	Control	1 (10)	25.24 ± 0.63	6.69 ± 0.21	6.84 ± 0.21	0.098 (0.012-0.257)	0.029 (0.009-0.059)	0.35 (0.27-5.10)^{a}	0.55 (0.27-0.89)^{a}
		Exposure	3 (10)	25.49 ± 0.65	6.60 ± 0.46	6.33 ± 0.56	0.026 (0.010-0.205)*	0.022 (0.007-0.079)*	0.38 (0.29-0.68)^{A}	0.65 (0.22-1.03)^{A}
	192h	Control	1 (10)	25.30 ± 0.68	6.64 ± 0.56	6.74 ± 0.27	0.070 (0.013-0.207)	0.023 (0.006-0.098)	0.37 (0.24-1.85)^{x}	0.64 (0.26-2.35)^{x}
		Exp.+recovery	1 (10)	25.15 ± 0.48	6.89 ± 0.26	7.05 ± 0.57	0.026 (0.008-0.099)*	0.014 (0.006-0.048)*	0.33 (0.24-1.64)^{X}	0.56 (0.07-1.54)^{X}
30°C+ pH 7.0	96h	Control	0 (10)	30.30 ± 0.43	6.90 ± 0.23	6.26 ± 0.17	0.048 (0.017-0.134)	0.016 (0.006-0.064)	0.74 (0.26-1.85)^{a}	1.43 (0.60-1.75)^{b}
		Exposure	2 (10)	30.50 ± 0.38	6.80 ± 0.26	5.98 ± 0.36	0.031 (0.000-0.245)*	0.008 (0.001-0.030)*	0.85 (0.41-1.95)^{B}	1.35 (0.44-1.83)^{B}
	192h	Control	2 (10)	30.40 ± 0.37	6.80 ± 0.28	6.00 ± 0.38	0.063 (0.019-0.231)	0.017 (0.007-0.055)	0.63 (0.44-2.08)^{y}	0.68 (0.02-2.92)^{x}
		Exp.+recovery	3 (10)	30.60 ± 0.44	6.80 ± 0.32	6.10 ± 0.32	0.045 (0.002-0.239)*	0.015 (0.002-0.093)	0.78 (0.46-1.98)^{Y}	0.62 (0.37-3.15)^{X}
25°C+ pH 4.0	96h	Control	1 (10)	25.50 ± 0.17	4.00 ± 0.33	7.00 ± 0.14	0.138 (0.065-0.412)	0.055 (0.004-0.185)	9.01 (4.29-11.36)^{b}	7.75 (5.36-9.30)^{c}
		Exposure	1 (10)	25.60 ± 0.25	4.00 ± 0.25	7.20 ± 0.44	0.055 (0.001-0.514)*	0.056 (0.004-0.241)	7.62 (4.46-13.30)^{B}	7.28 (3.90-9.63)^{C}
	192h	Control	1 (10)	25.30 ± 0.37	3.90 ± 0.19	7.10 ± 0.19	0.185 (0.037-0.656)	0.050 (0.014-0.202)	8.14 (4.63-12.10)^{z}	7.41 (3.62-9.30)^{y}
		Exp.+recovery	3 (10)	25.60 ± 0.46	3.90 ± 0.21	7.30 ± 0.35	0.096 (0.002-0.479)*	0.055 (0.002-0.358)	8.07 (5.39-13.06)^{Z}	8.05 (5.46-9.19)^{Y}

Temperature, pH and O_2 are expressed as mean ± S.E.M and were analysed through One-Way ANOVA ($p<0.05$). Total ammonia (NH_4+NH_3) and Cl^- concentrations are expressed as mean (min-max) and were analysed with Kruskal-Wallys ($p<0.05$). Small letters indicate difference in the 96 h (a, b and c) and 192 h (x, y and z) control groups among the different experiment trials. Capitalized letters indicate difference in the 96 h-exposure (A, B and C) and 192 h-recovery (X, Y and Z) groups among the different experiment trials. * Indicates difference to the corresponding control group (Mann-Whitney, $p<0.05$)

3.3.3. Haematological Responses

Difference on erythrocyte count were only detected in fish exposed to WSFG in 25°C+pH7.0 condition ($p<0.05$). However, the period of recovery in freshwater was enough to return RBC to the control levels (Table 10). An increase of erythrocytes count was induced by temperature and pH ($p<0.05$). Comparing the values obtained with fish exposed to 25°C+pH7.0 for 96 h, the rise of 5 °C, in the 30 °C trial, increased the RBC in 107%. At low pH, the increase reach 156%. This pattern was observed in the 96-h control group, and 192 h control and recovery groups. A down drop on Ht values was detected only in the recovery groups of all experimental trials ($p<0.05$) (Table 10). Total plasmatic protein increased in both exposure and recovery treatments at the 25°C+pH7.0 trial ($p<0.05$) and reduce at pH 4.0, ($p<0.05$). The comparation of MCV between experimental trials detected lower values only in the control and WSF exposure treatments at pH 4.0 ($p<0.05$). *A. altiparanae* showed a significant reduction after recovery in the 25°C+pH7.0 and pH 4.0 trials but showed increase in the WBC count at WSF_G+30 °C+pH7 trial the increased (Table 10).

Table 10. Blood parameters of *Astyanax altiparanae* exposed to 0.5% of water-soluble fraction of gasoline (WSF_G) in association with water temperature or pH alteration. White blood cells (WBC), red blood cells (RBC) and mean corpuscular volume (MCV) are expressed as mean (min-max). microhematocrit (Ht) and total plasmatic protein (PP) are showed as mean ± S.E.M.

Trials	Time	Treat.	Blood Parameters				
			WBC ($_x10^4$ mm^{-3})	RBC ($_x10^5$ mm^{-3})	Ht (%)	PP (g dL^{-1})	MCV (fL)
25°C+pH7.0	96h	Control	0.70 (0.10-22.50)^{a}	7.05 (3.10-18.80)^{a}	31.62 ± 1.77^{a}	5.48 ± 0.50^{a}	390.62 (159.66-842.10)^{b}
		Exposure	1.35 (0.45-2.80)	16.50 (6.70-22.40)^{A}*	30.83 ± 1.83	7.33 ± 0.38^{B}*	181.81 (116.07-567.16)^{B}
	192h	Control	0.60 (0.25-1.10)^{x}	11.60 (3.30-17.00)^{x}	25.14 ± 2.24^{x}	5.62 ± 0.61^{x}	230.26 (115.64-575.75)^{y}
		Recovery	0.25 (0.10-1.00)^{X}*	6.10 (0.40-22.80)^{X}	20.25 ± 1.96^{X}*	7.00 ± 0.45*	284.09 (105.26-633.33)^{Y}
30°C+pH7.0	96h	Control	1.35 (0.85-34.50)^{ab}	14.60 (1.00-21.00)^{ab}	35.77 ± 1.10^{b}	6.76 ± 0.22^{a}	235.29 (166.68-522.38)^{ab}
		Exposure	2.70 (1.00-6.85)	17.20 (6.70-26.20)^{A}	33.25 ± 1.20	6.07 ± 0.04^{AB}	189.50 (110.68-522.38)^{B}
	192h	Control	2.10 (0.35-3.60)^{y}	11.40 (4.80-36.10)^{xy}	36.00 ± 1.12^{y}	6.25 ± 0.19^{xy}	250.87 (108.03-708.33)^{y}
		Recovery	4.25 (2.30-5.00)^{Y}*	17.10 (4.70-29.80)^{XY}	26.00 ± 1.57^{Y}*	6.52 ± 0.28	181.28 (93.96-617.02)^{Y}
25°C+pH4.0	96h	Control	2.45 (1.90-28.00)^{b}	18.10 (2.40-30.80)^{b}	35.55 ± 1.71^{b}	7.50 ± 0.67^{b}	183.57 (123.37-248.61)^{a}
		Exposure	1.90 (0.70-11.25)	21.75 (11.40-25.30)^{B}	31.37 ± 1.32	5.47 ± 0.28^{A}*	150.86 (134.38-217.94)^{A}
	192h	Control	1.35 (0.80-2.50)^{xy}	24.60 (20.20-36.70)^{y}	36.37 ± 1.75^{y}	7.82 ± 0.46^{y}	141.46 (95.36-207.92)^{x}
		Recovery	0.65 (0.15-1.15)^{X}*	24.00 (5.70-30.30)^{Y}	27.33 ± 0.76^{Y}*	5.95 ± 0.33*	118.41 (95.71-438.59)^{X}

Small letters indicate difference in the 96 h (a, b and c) and 192 h (x, y and z) control groups among the different experiment trials (One-Way ANOVA or Shapiro-Wilks, $p<0.05$). Capitalized letters indicate difference in the 96 h-exposure (A, B and C) and 192 h-recovery (X, Y and Z) groups among the different experiment trials (One-Way ANOVA or Shapiro-Wilks, $p<0.05$). * Indicates difference to the corresponding control group (*t* test or Mann-Whitney, $p<0.05$).

3.3.3. Physiological responses

The exposure of *A. altiparanae* to WSF_G in 25°C+pH7.0 do not promoted alterations in the plasmatic concentrations of Na^+ and Cl^- (Figure 11 and B), but, after 96 h, there was a decrease in the plasmatic K^+ in the 0.5% WSF_G group ($p<0.05$). After the removal of the pollutant (i.e., in the recovery situation), this loss of K^+ was recovered, returned to control levels (Figure 11C). When exposure to the association of WSF_G and temperature rise or lower pH, A. altiparanae presented an increase of plasmatic Na^+ ($p<0.05$) (Figure 11A). Chloride concentration in the plasma was also affected by temperature and pH alterations, with a reduction of 29.5 and 36.3% of total Cl^- content, respectively. In the acid scenario, the exposure to WSF_G potentiated the Cl^- loss ($p<0.05$), reaching 50% decrease in Cl^- plasmatic concentration after WSFG exposure and recovery (Figure 11B).

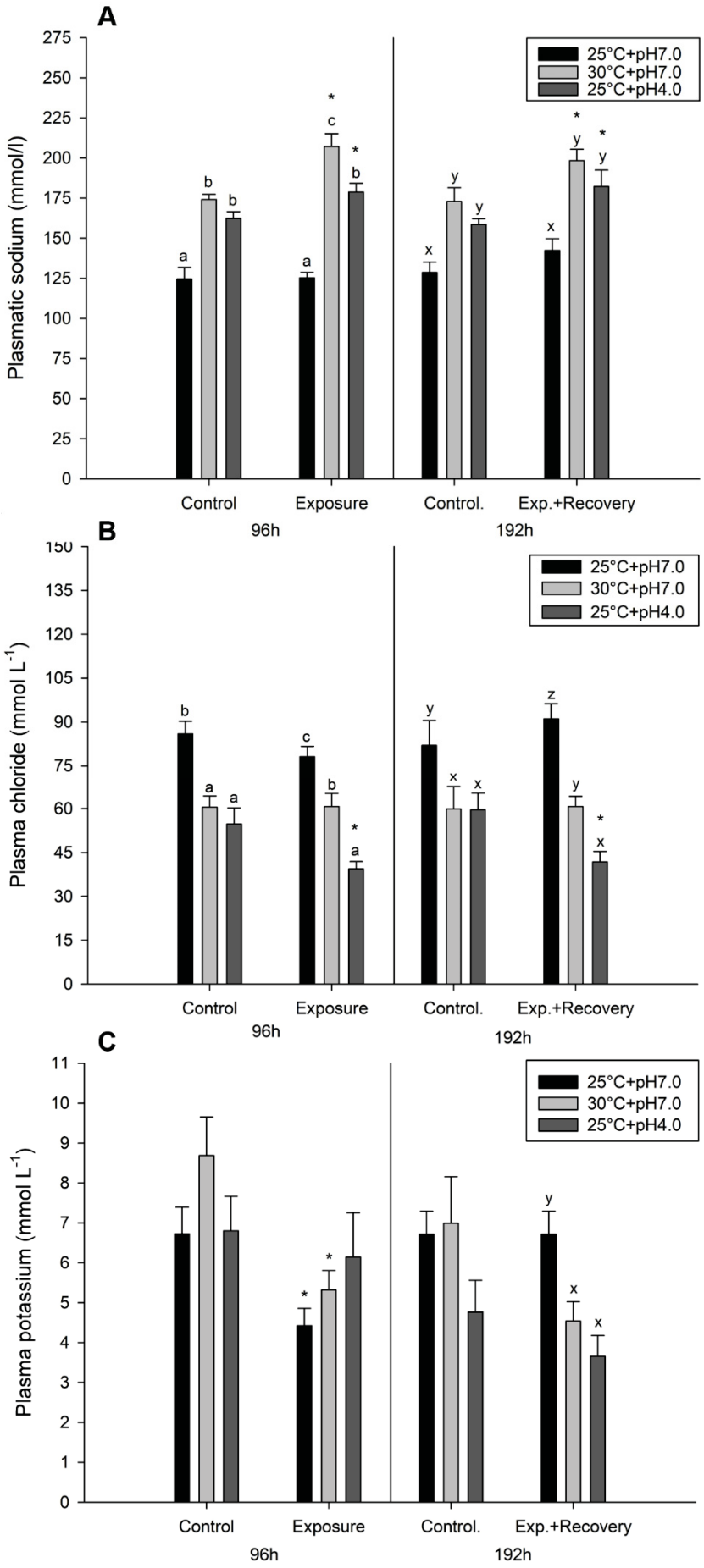
A
Plasmatic sodium (mmol/l)
25°C+pH7.0
30°C+pH7.0
25°C+pH4.0
Control
Exposure
Control.
Exp.+Recovery
96h
192h
B
Plasma chloride (mmol L-1)
C
Plasma potassium (mmol L-1)

Figure 11. Mean ± S.E.M values of plasmatic sodium (A), chloride (B) and potassium (C) in *Astyanax altiparanae* after 96 h exposure to 0.5% of water-soluble fraction of gasoline (WSF_G) and after 96 h of recovery in clean freshwater (192 h). Lowercase letters indicate difference in the 96 h (a, b and c) and 192 h (x, y and z) control groups among the different experiment trials (One-Way ANOVA, $p<0.05$). Capitalized letters indicate difference in the 96 h-exposure (A, B and C) and 192 h-recovery (X, Y and Z) groups among the different experiment trials (One-Way ANOVA, $p<0.05$). ∗ Indicates difference to the corresponding control group (t test, $p<0.05$). When significant differences were not detected ($p>0.05$), letters and symbols were omitted.

The activity of branchial Na/K ATPase (Figure 12A) and H^+ ATPase (Figure 12B) were not altered by WSF_G exposure in 25°C+pH7.0. However, both enzymes presented, increase when *A. altiparanae* was exposed to higher temperature (30°C+pH 7.0) or reduction in the pH (25°C+pH4.0). The branchial activity of H^+ ATPase in the control groups of 30 °C and pH 4.0 was 75% higher than 25°C+pH7.0. For Na/K ATPase, the same condition promoted a 65% increase. As both Na/K and H^+ ATPase presented a raise in their activity, total ATPase also increased (Figure 12C).

The branchial carbonic anhydrase activity (CAA) (Figure 13) increased (75% higher) in fish exposed to WSF_G at 30°C ($p<0.05$) when compared to the WSF_G + 25°C+pH7.0 experimental trial. Water acidification boosted the CAA activity with a 2-fold increase ($p<0.05$), when compared to the control group.

The exposure of *A. altiparanae* exposed to WSF_G, at 25°C+pH7.0, increased the muscular cortisol concentration ($p<0.05$). Temperature and pH also increased the stress response. In this scenario, however, the addition of 0.5% of WSF_G did not act as an additional stressor (Figure 14A).

The content of muscular glycogen of the *A. altiparanae* declined as a result of the WSF_G exposure (Figure 14B) in the 25° and 30° C trials, when compared to its respective control groups ($p<0.05$). This energetic substrate mobilization

represents almost 50% of the muscle glycogen. After recovery in freshwater for 96h, glycogen returned to control levels. The initial glycogen content was affected by temperature and pH. When compared to the 25°C+pH7.0 trial, the alteration on the water parameters induced the mobilization of 30% of the storage glycogen ($p<0.05$). After the recovery period, the concentrations returned to control levels.

Plasma glucose decreased in the fish submitted to the WSF_G treatment at 25°C+pH7.0 ($p<0.05$) (Figure 14C). Temperature and pH also reduced glucose in the control groups ($p<0.05$). After recovery period, glucose remained lower than 25 °C an pH 4.0 trials.

The branchial (Figure 15A) and muscular (Figure 15B) water content (tissue hydration) were not affected by WSF_G singly or in association with temperature and pH alterations ($p>0.05$).

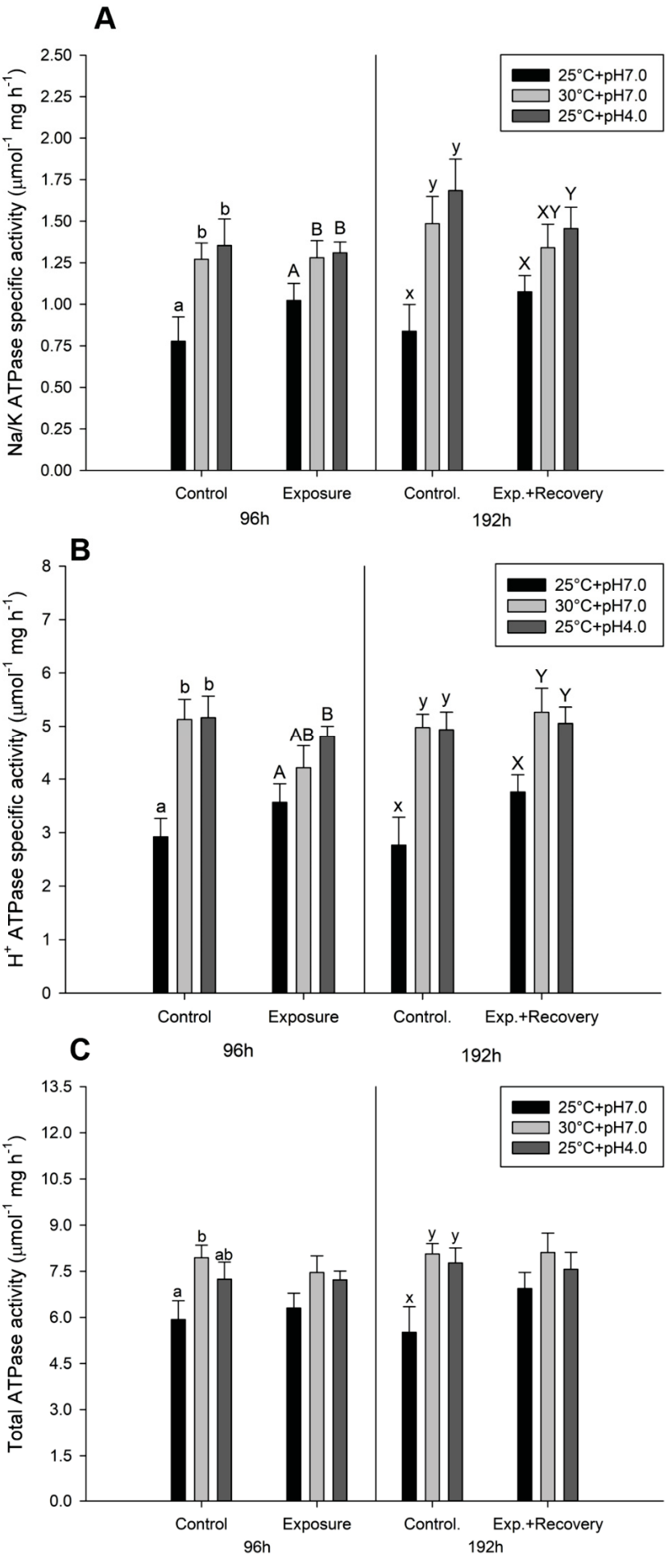
A
Na/K ATPase specific activity (µmol-1 mg h-1)
25°C+pH7.0
30°C+pH7.0
25°C+pH4.0
Control
Exposure
Control.
Exp.+Recovery
96h
192h
B
H+ ATPase specific activity (µmol-1 mg h-1)
C
Total ATPase activity (µmol-1 mg h-1)

Figure 12. Mean ± S.E.M values Na/K ATPase (A), H^+ ATPase (B) and total ATPase (C) activity in brachial tissue of *Astyanax altiparanae* after 96 h exposure to 0.5% of water-soluble fraction of gasoline (WSF_G) and after 96 h of recovery in clean freshwater (192 h). Lowercase letters indicate difference in the 96 h (a, b and c) and 192 h (x, y and z) control groups among the different experiment trials (One-Way ANOVA, $p<0.05$). Capitalized letters indicate difference in the 96 h-exposure (A, B and C) and 192 h-recovery (X, Y and Z) groups among the different experiment trials (One-Way ANOVA, $p<0.05$). ∗ Indicates difference to the corresponding control group (t test, $p<0.05$). When significant differences were not detected ($p>0.05$), letters and symbols were omitted.

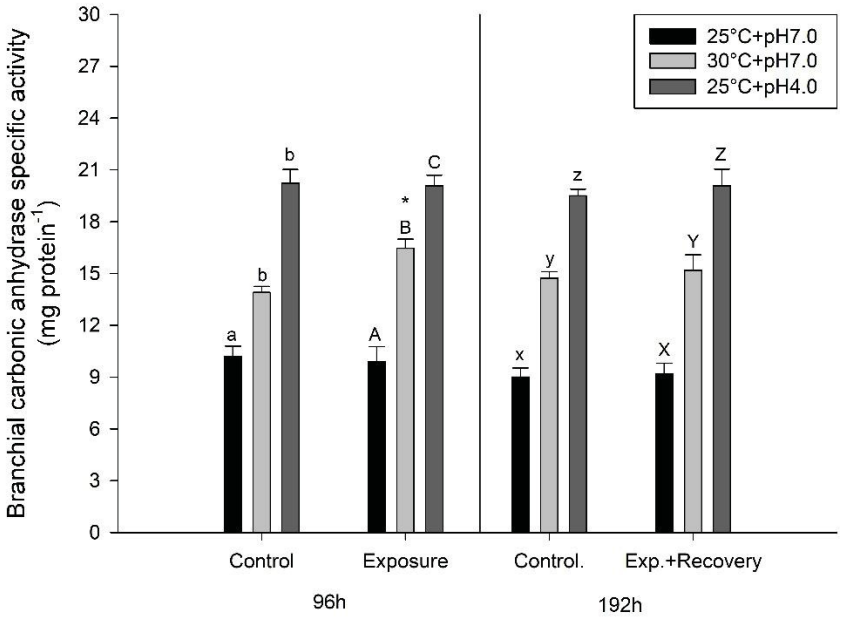

Figure 13. Mean ± S.E.M values branchial carbonic anhydrase specific activity (CAA) of *Astyanax altiparanae* after 96 h exposure to 0.5% of water-soluble fraction of gasoline (WSF_G) and after 96 h of recovery in clean freshwater (192 h). Lowercase letters indicate difference in the 96 h (a, b and c) and 192 h (x, y and z) control groups among the different experiment trials (One-Way ANOVA, $p<0.05$). Capitalized letters indicate difference in the 96 h-exposure (A, B and C) and 192 h-recovery (X, Y and Z) groups among the different experiment trials (One-Way ANOVA, $p<0.05$). ∗ Indicates difference to the corresponding control group (t test, $p<0.05$). When significant differences were not detected ($p>0.05$), letters and symbols were omitted.

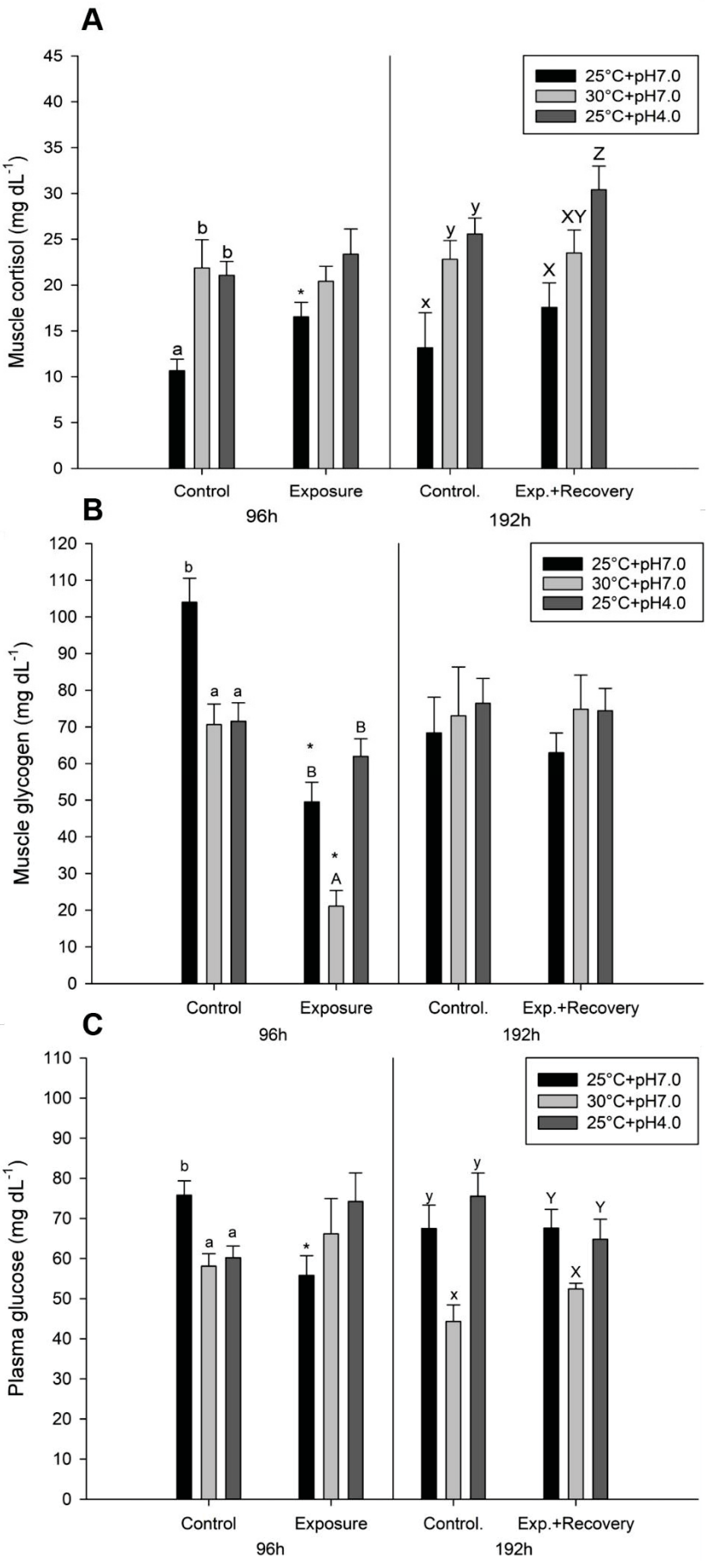
A
Muscle cortisol (mg dL-1)
25°C+pH7.0
30°C+pH7.0
25°C+pH4.0
Control
Exposure
Control.
Exp.+Recovery
96h
192h
B
Muscle glycogen (mg dL-1)
C
Plasma glucose (mg dL-1)

Figure 14. Mean ± S.E.M values of muscle cortisol (A), muscle glycogen (B) and plasmatic glucose (C) in *Astyanax altiparanae* after 96 h exposure to 0.5% of water-soluble fraction of gasoline (WSF_G) and after 96 h of recovery in clean freshwater (192 h). Lowercase letters indicate difference in the 96 h (a, b and c) and 192 h (x, y and z) control groups among the different experiment trials (One-Way ANOVA, $p<0.05$). Capitalized letters indicate difference in the 96 h-exposure (A, B and C) and 192 h-recovery (X, Y and Z) groups among the different experiment trials (One-Way ANOVA, $p<0.05$). ∗ Indicates difference to the corresponding control group (t test, $p<0.05$). When significant differences were not detected ($p>0.05$), letters and symbols were omitted.

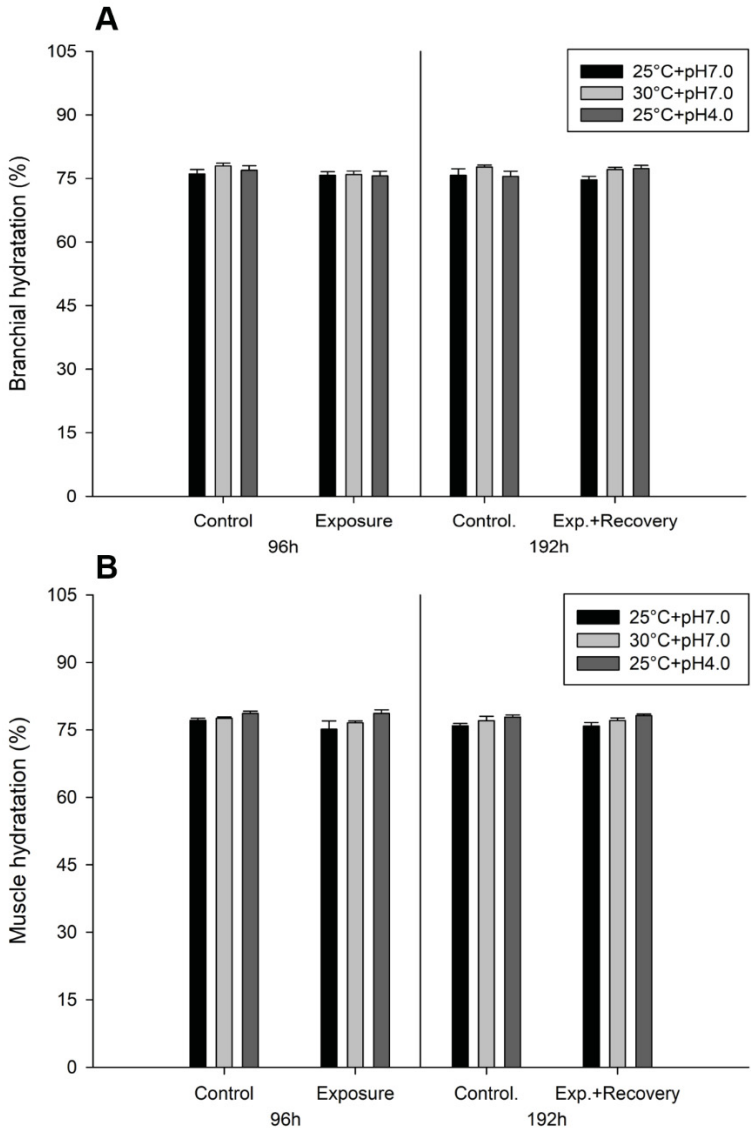

Figure 15. Mean ± S.E.M values of branchial (A) and muscle hydration (B) in *Astyanax altiparanae* after 96 h exposure to 0.5% of water-soluble fraction of gasoline (WSF_G) and after 96 h of recovery in clean freshwater (192 h).

3.4. DISCUSSION

The chemical composition of petroleum and refined products are considered the main factor defining its toxicity to fish (HEATH, 1995; BARRON, 2003). Normally, this toxicity is associated to the concentrations of BTEX and PAH (BARRON *et al.*, 1999; ALBERS, 2003) solubilized in the WSF (ANDERSON, 1979; EVANS, 1987; WOOD, 2001). Comparing the concentration of BTEX and PAH in the WSF_G obtained in the present work (Table 8) with other published data, we can detect a few differences, mainly in the total sum of hydrocarbons (BTEX+PAH). Even though BETTIM *et al.* (2016) and GALVAN *et al.* (2016) obtained higher concentrations of BTEX and PAH in the 100% WSF_G, the proportion individual BTEX (benzene, ethylbenzene, toluene and xylenes) was similar to those obtained here. The proportions of PAH, on the other hand, was quite different, with 98% of the total PAH been accounted to naphthalene alone. Such variations on WSF composition can be attributed to the fuel source (HEATH, 1995) and to the methodology of WSF extraction (ANDERSON *et al.*, 1974), leading to differences in the biomarkers responses (BENGTSON and HENSHEL, 1996; WHYTE *et al.*, 2000).

Temperature and pH were kept within the proposed range for each experimental group. The continuous and controlled oxygen supply held the dissolved concentrations around optimum values for fish (HEATH, 1995), despite the fact that in all treatments of the pH 4.0 trials, concentrations were slightly higher. Chloride concentrations in water increased at pH 4.0 trial due to the use of HCl to modify and maintain water pH to the tested levels. Thus, Cl^- concentration in the pH 4.0 trial was higher than average concentration in tropical aquatic environments (BECKER and BALDISSEROTTO, 2014). NH_4+NH_3

remained below the concentrations considered toxic to *A. altiparanae* (DAL PONT, 2012). In water sampled before the daily replacement, however, fish contaminated with WSF_G showed lower NH_4+NH_3 concentrations (Table 9). Here, two approaches could explain the total ammonia decrease. Nitrogen normally occurs in the aquatic environment in the fixed inorganic form (ammonia, nitrite and nitrate) which are degraded by anaerobic bacterial populations in water (KUYPERS *et al.*, 2003). Thus, the decrease of total ammonia observed here, could be a result of bacterial degradation. Other possibility that could explain the inhibition is related to alterations in the mechanisms of ammonia excretion as a result of WSF_G exposure. Environmental alterations can modify osmoregulatory and acid-basic mechanisms and lead to inhibition of ammonia excretion (WILKIE and WOOD, 1991; RANDALL, 2011). The mechanisms of ammonia excretion vary between fish species, and mainly involves NH_3 passive diffusion (modulated by pH) and NH_4^+/Na^+ exchange (RANDALL and WRIGHT, 1987) through the gills. Plasmatic pH alteration (alkalosis and/or acidosis) is known to decrease ammonia excretion in freshwater fishes (HEATH, 1995) due to reduction of the diffusion gradient across the gills and an alteration on nitrogen excretion towards the formation of urea (WILKIE and WOOD, 1991). The shift of nitrogen excretion from ammonia to urea is a result of the attempt to reduce the concentrations of NH_4+NH_3, which is more toxic to fish than urea (RANDALL and TSUI, 2002), or due to alterations resulting from a metabolic hypoxic condition (unpublished data – Chapter 4). These data, obtained with goldfish (*Carassius auratus*) exposure to different levels of WSF_G (0, 0.25, 5, 10 and 25%), reveled that acute exposure imposed a negative effect on the capacity for O_2 uptake, which led to a metabolic hypoxic condition and, consequently, increase in urea excretion. As fish species

presents different metabolic approaches to environmental alterations that can change ammonia excretion (RANDALL and WRIGHT, 1987; ROBERTSON *et al.*, 2015), the actual mechanism affected by WSF_G in *A. altiparanae* needs to be investigated.

As the branchial tissue is in direct contact with the aquatic environment and is considered an important organ for the regulation of water, ionic and acid-base balance in fish (EVANS, 1987; WOOD, 2001), waterborne toxic agents are well known for their ability to impair hydromineral regulation at gills (WENDELAAR-BONGA and LOCK, 2008). In the present work, WSF_G was not able to change plasmatic concentrations of the principal osmoregulation ions (Na^+ and Cl^-) and the physiological enzymatic mechanisms associated (Na/K ATPase) in *A. altiparanae* held in normal temperature (25 °C) and neutral pH (7.0). The only ionic alteration detected after WSF_G exposure was the decrease of plasmatic K^+. A similar result of plasmatic Na^+ and Cl^- concentrations was obtained with *Pleuronectes flesus* exposed to WSF of petroleum (ALKINDI *et al.*, 1996). Na^+ and Cl^- (also osmolarity) have not been altered were observed in *Prochilodus lineatus* exposure to the accommodated water-soluble fraction of diesel for 6, 24 and 96 h (SIMONATO *et al.*, 2008). The authors, however, detected an increase of plasmatic K^+, which is in contrast with the result obtained for *A. altiparanae*. The alterations in plasmatic K^+ of *Prochilodus lineatus* have been considered as a secondary effect resulting from hemolysis. Other authors, however, have attributed the alteration to electrolytic and osmoregulatory unbalance induced by stress mechanisms (WENDELAAR BONGA and LOCK, 1991; WENDELAAR BONGA, 1997; WENDELAAR-BONGA and LOCK, 2008) or to the cation migration to/from erythrocyte in hypoxic (SILKIN and SILKINA, 2005)

or NO_2^- experimental condition (MARTINEZ and SOUZA, 2002). Here, WSF_G induced cortisol increase in *A. altiparanae*, suggesting that the K^+ response in this species may be linked to the acute stress response.

The influence role of petroleum hydrocarbons exposure (ANDERSON, 1979) and water physicochemical characteristics, such as temperature and pH (CAIRNS *et al.*, 1975; WOOD, 1989), on the physiological mechanisms of fishes is considered for many years. When *A. altiparanae* was submitted to conditions of temperature increase and pH reduction, plasmatic Na^+ increased, Cl^- decreased and the measured enzymatic osmoregulatory and acid-base regulator systems were activated. The observed increase of Na/K and H^+ ATPase activity at the gill tissue support the rise of plasmatic Na^+ under temperature and pH stress conditions (HEISLER, 1993). PARKS *et al.* (2008) reviewed the uptake mechanisms of Na^+ by freshwater fishes and defended the existence of an coupling apical Na^+ channel and H^+ ATPase, along with a basolateral membrane Na^+/HCO_3^- co-transporter and Na/K ATPase. Thus, as more Na^+ would be uptake from water by the apical membrane, protons (H^+) would be excreted from the intracellular compartment. Than, Na^+ would cross the basolateral membrane into the plasma and carrying HCO_3^-. This mechanism, would be crucial for acid-base regulation in a scenario of metabolic acidosis, which could be induced by water acidification (HEATH, 1995) and blood of PO_2 reduction (WOOD, 2001), which are normal responses to water temperature rise (CLARKE and JOHNSTON, 1999). Additionally, the altered temperature and pH promoted a reduction of plasmatic Cl^-. In fact, changes in the unidirectional fluxes of Na^+ and Cl^- can be dynamically adjusted by the fish to achieve acid-base balance (WOOD, 1988; 1991). Although we did not measure blood pH, the associated activation of CAA,

enzyme responsible to produce H^+ and HCO_3^- by the hydration of CO_2, and the Cl^- decrease concentrations in plasma, suggests that HCO_3^- and H^+ are been produced to neutralize an metabolic acidosis condition (PARKS *et al.*, 2008) induced by CO_2. To compensate for the decrease in internal pH (pHi), the cell could reduce the rate of secretion of HCO_3^- and, because HCO_3^- secretion is linked to Cl^- uptake (WOOD, 2001), the rate of Cl^- uptake would be reduced (FENWICK *et al.*, 1999).

The exposure of *A. altiparanae* to WSF_G, increased Na^+ concentrations in the plasma above to its correspondent control groups (30 °C and pH 4.0), suggesting that under petroleum hydrocarbons influence, another uptake mechanism was triggered. In goldfish, exposed to WSF_G impaired branchial O_2 uptake (unpublished data – see chapter 4) which could lead to a metabolic hypoxic condition. In a severely hypoxic condition, *Trachurus mediterraneus ponticus* increase the content of Na^+ erythrocyte (SILKIN and SILKINA, 2005). The authors attributed this mechanism to the activation of Na^+/H^+ exchanger to reduce erythrocyte $[H^+]$ and promoted internal alkalinisation, which reduce O_2 and hemoglobin affinity (BURGGREN and ROBERTS, 1991; WOOD, 1991) and improve of oxygen supply to tissues. In acute hypoxic conditions, the successful increase of erythrocyte pHi can lead to a secondary HCO_3^- efflux in exchange to Cl^- (JENSEN *et al.*, 1993), resulting in the reduction of plasma Cl^-. In the acid condition, the exposure o *A. altiparanae* to WSF_G promoted an decrease of Cl^- on plasma, even after the recovery period. WOOD (1989) also suggested that in acid condition, the Cl^- uptake can be depleted due a damage caused by the action of the low pH at the Cl^-/HCO_3^- exchanger in the apical membrane. Thus, the mechanism of Cl^- clearance in the plasma of *A. altiparanae* could be interpret as

a result of erythrocyte influx, due to a possible metabolic hypoxic condition, and by an additive function loss of the Cl^-/HCO_3^- exchanger at the apical membrane. The osmoregulatory and acid-base imbalance promoted by temperature rise, pH dropdown and WSF_G exposure in *A. altiparanae* is, apparently, a secondary effect caused by the loss of O_2 uptake ability. The secondary effect is corroborated by the lack of cellular membrane disruption and in gill and muscle losses in hydration, indicating that the stress condition wasn't enough to cause severe physiological disturbances.

Reduction of O_2 uptake by fish, in scenarios of environmental contamination, is normally attributed to decrease in functional branchial surface area, increases in mean blood-to-water diffusion distance, or decreases in mean water-to-blood O_2 pressure gradient (WOOD, 2001). The occurrence of some histopathological lesions, as epithelial lifting, hyperplasia of epithelial cells and hypertrophy of mucous cell, in fish gill exposed to petroleum hydrocarbons are considered an adaptive mechanism, as they inhibit the entrance of xenobiotics across the tissue (VAN DER OOST *et al.*, 2003) by the increase of blood-to-water diffusion distance. SIMONATO *et al.* (2008) identify epithelial lifting in *Prochilodus lineatus* exposed to diesel. The same histological alterations were detected in *Astyanax* sp. exposed to petroleum hydrocarbons (AKAISHI *et al.*, 2004; SILVA *et al.*, 2009; DAL PONT, 2012; BARROS *et al.*, 2017). Although not quantified, we also identified some histopathological alterations in the gill tissue, related to PAH and BTEX exposure, that could have contributed to the loss of O_2 uptake capacity in *A. altiparanae* (Figure 16). NERO *et al.* (2006) exposed *Perca flavescens* and *Carassius auratus* to oil-contaminated for three weeks and detected an extensive proliferation of mucous cells on the gill tissue of both

species. Although the proliferation of mucous cell may be beneficial in reducing pollutant entry across the gill epithelia (ULTSCH and GROS, 1979), the exposure to the contaminant for a chronic period could compromise the efficiency of gas exchange and lead to a metabolic hypoxic condition, as a result of increased distance for gas exchange along the secondary lamellae (HEATH, 1995). This protection mechanism seems to be activated by the fish in the initial hours of exposure. In acute conditions (4 h), the exposure of goldfish to WSF_G compromised O_2 uptake (MO_2) (unpublished data – Chapter 4). Thus, it appears reasonable to postulate that the physiological mechanisms activated in *A. altiparanae*, in the present work, is mainly trigged by the inability to maintain blood PO_2. The physiological data of energetic metabolism and the haematological data also support this hypothesis.

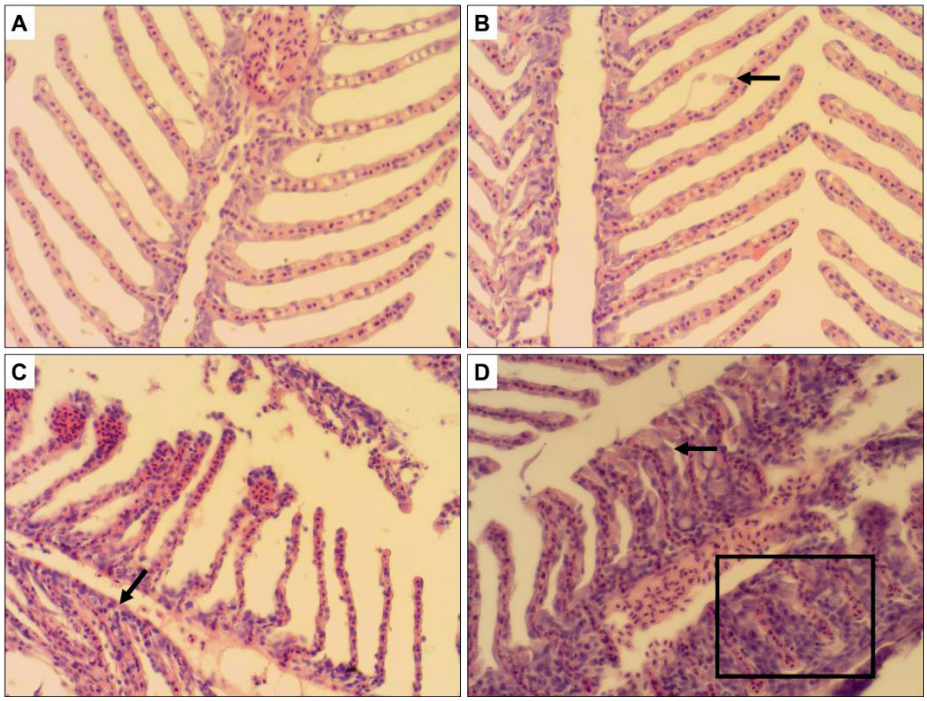

Figure 16. Histology of *Astyanax altiparanae* gill tissue. (A) Normal gill histology, (B) arrow indicates epithelial lifting, (C) arrow indicates lamellar adhesion and (D) arrow indicates moucus cell hypertrophy and square indicates cellular hyperplasia. H.E stain and 1000x magnification.

Petroleum hydrocarbons are considerate examples of stressor substances to fish (WENDELAAR BONGA, 1997). However, the classic physiological stress response, induced by petroleum and its individual constituents, with the increase of cortisol followed by a hyperglycemic is absent or minimal (HEATH, 1995). Our data shows the increase of muscular cortisol of *A. altiparanae* exposed to WSF_G in the 25°C+pH7.0 experimental condition. This typical stress response was followed by the degradation of muscle glycogen. Normally, this energetic mobilization mechanism results in the increase of plasmatic glucose (WENDELAAR BONGA, 1997). However, our data shows a decrease of plasmatic glucose in this treatment. In the 30 °C and pH 4.0 trials, alone or associated with WSF_G exposure, the increase of muscle cortisol induced glycogen degradation, but the plasmatic glucose values remained equal or below the controls groups. This response may be explained by the activation of anaerobic degradation of glycogen storages. The effects of petroleum hydrocarbons to fish are attached to the increase of lactic acid in blood and white muscle (HEATH, 1995). The same mechanism is activated by tropical Amazonian fish species to regulate physiological mechanisms to help then to cope with low O_2 and high temperature conditions (DE ALMEIDA-VAL *et al.*, 2005). Even the restorage of muscular glycogen content, detected in the recovery period, can be explained by this anaerobic energetic activation. WOOD (1991) reviewed the metabolism interactions in fish after exhaustive exercise and posted that metabolic acidosis has generally been attributed to H^+ generation and lactate production by ATP breakdown in white muscle. As soon the stress condition is ceased, fish can increase O_2 consumption to restore aerobic demands, promote lactate clearance and resynthesizes ATP and glycogen at the muscular tissue.

Thus, under a condition of metabolic hypoxia the most important mechanism regulated by the fish is the improvement of the O_2 uptake by the blood and delivery to the tissues.

The attempt to increase O_2 uptake capacity can be evaluated through the measurement of hematological parameters (VAN DER OOST et al., 2003; HRUBEC and SMITH, 2010). In our experimental conditions, *A. altiparanae* increased the RBC count when exposed to WSF_G (Table 10). Then, the changes in water temperature and pH also promoted an severe increase in RBC count (Table 10). GALVAN et al. (2016) exposed *A. altiparanae* to WSF_G (1.5% *v/v*) and do not detected alterations in RBC after 96 h of exposure and recovery period. The results obtained by the authors, however, were normalized and expressed as percentage of total blood cell content (erythrocytes, leukocytes, thrombocytes and mastocytes) and, thus, could neglect changes in the total count of RBC. Erythrocyte is the most abundant blood cell and are engaged in the transportation of O_2 and CO_2 (SILKIN and SILKINA, 2005; HRUBEC and SMITH, 2010). This overall increase in RBC lead to Ht increase, mainly in the 30 °C and pH 4.0 experimental conditions. In fishes inhabiting acid aquatic environments, the count of RBC and Ht are normally higher (WOOD, 1989) due to the lower affinity of between hemoglobin and oxygen in acid pHs. Hence, *A. altiparanae* is clearly activating hematopoietic mechanism (erythrocyte release from the spleen) to reduce metabolic hypoxia. The increase of PP (loss of plasma water) in the 25°C+pH7.0 trial, also indicates that an hemoconcentration condition was induced by the petroleum hydrocarbons exposure, even after the recovery period. Hemoconcentration resulted from increased RBC, RBC swelling or reduction of plasma volume by the increase of PP (HEATH, 1995) can cause the increase of

blood viscosity and, eventually, lead to circulatory collapse (HEATH, 1995; WILSON, 2011). The opposite response was detected in the fishes of pH 4.0 exposed to WSF_G. The reduced MCV, detected and pH 4.0 trial, associated with the RBC count, Ht and PP, suggests that the RBC are smaller due to cell shrink (WOOD, 1989). The change of *A. altiparanae* hematocrit, in the tested experimental conditions, is a combinate result of RBC count increase, RBC swelling, RBC shrinking and PP alterations. Thus, the evaluation of *A. altiparanae* hematological responses, in environmental pollution scenarios or laboratory condition, should be carefully interpreted.

The changes in the count of WBC was also influenced by the alteration in water physicochemical parameters. It was suppressed at 25°C+pH7.0 and pH 4.0. GALVAN *et al.* (2016) also do not detected a variation on WBC in *A. altiparanae* after WSF_G exposure, with only a tendency of reduction after recovery. WBC count is a sensitive biomarker for petroleum hydrocarbons exposure (PEAKALL, 1994; HEATH, 1995) and is normally associated to the non-specific immunity in fish (WENDELAAR BONGA, 1997). This specific hematological/immunity response in *A. altiparanae*, however, seems to be highly influenced by water temperature, as WBC significantly increased at 30 °C. The influence of temperature in fish immunological responses is normally linked to the optimal physiological temperature range for each fish species (MARTINS *et al.*, 2011), but increase of optimal non-specific immunity function was also identified in temperature range different of the physiological optimal (XU *et al.*, 2011).

3.5. CONCLUSION

The results obtained here with *A. altiparanae*, highlight the first evidence indicating the physiological mechanisms related to WSF_G contamination in altered water temperature and pH. In “normal” (25 °C and pH 7.0) condition, WSF_G promoted an alteration of energetic metabolization, hematopoietic mechanism, due to a possible metabolic hypoxic condition. When water temperature (30 °C) and pH (4.0) were changed, a secondary set of physiological mechanism were activated to cope with osmoregulatory and acid-base disorders, induced by a severely metabolic hypoxic condition. In in normal or altered temperature or pH conditions, during WSF_G exposure, *A. altiparanae* sustain the integrity of cellular membranes, indicating that the trigged regulatory mechanisms were able to maintain general homeostasis.

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CHAPTER 4 – ACUTE EXPOSURE TO WATER-SOLUBLE FRACTION OF GASOLINE (WSF_G) IMPAIR NITROGEN-WASTE EXCRETION DUE TO THE ACTIVATION OF ANAEROBIC METABOLISM IN GOLDFISH *Carassius auratus*

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ABSTRACT

Ammonia is the main nitrogenous compound produced and excreted by teleost fishes. The diffusional mechanism, related to membrane permeability and which allows NH_3 to be easily excreted into or absorbed from the environment, is also considered a weak point in environmental stress conditions, i.e., during the exposure to petroleum hydrocarbons. The goal of this work was to evaluate the effects of water-soluble fraction of gasoline (WSF_G) on nitrogen excretion, osmoregulatory and metabolic regulation of goldfish *Carassius auratus*. Specimens (N= 8/treatment) were individually acclimated (4 h) in a 260 mL and then exposure (6 h) to 0, 0.25, 5, 10 and 25% of WSF_G. Three, experimental trials were performed to assess total ammonia (*Jamm*), urea (*Jurea*), Na^+, K^+, Ca^{+2} and Mg^{+2} flux rates, plasma urea and Mg^{+2} concentrations, muscular Mg^{+2} and O_2 uptake (MO_2). *Jamm* was not affected by the exposure to WSF_G. *Jurea*, however, increased along with the WSF_G concentration and the same pattern of dose/response was observed for the Mg^{+2} net flux rate. This result could be linked to the activation of anaerobic energetic metabolism, through the breakdown of ATP and other adenylates substrates. Plasmatic urea and Mg^{+2} remained unaffected. MO_2 was influenced by the WSF_G exposure and presented a

compensatory increase after the pollutant removal. Our results suggest that, although WSF_G did not alter ammonia excretion, acute exposure was sufficient to trigger metabolic changes in *C. auratus*, such as increased Mg^{2+} and urea excretion due to metabolic alteration, probably regarding the inability, inferred by BTEX and PAH, to maintain the mechanism of O_2 uptake at the gills.

Keywords: BTEX, Environmental pollution, Ionic flux rates, PAH, N-waste, Osmoregulation, Oxygen uptake

4.1. INTRODUCTION

Teleost fishes break down proteins and amino acids from their diet to obtain carbon skeleton (RANDALL and WRIGHT, 1987). The nitrogenous compounds (N-wastes) resulted from this catabolic cycle are excreted as ammonia, urea, trimethylamine oxide (TMAO), creatinine and uric acid (FORSTERS and GOLDSTEIN, 1969; WOOD, 2001a). Due to differences in diffusion rate across biological membranes, N-wastes exhibit variances on their excretion pathway. Ammonia, urea and TMAO have high diffusion rate and are primarily excreted through branchial tissue (FORSTERS and GOLDSTEIN, 1969) compared to creatinine and uric acid (CAMPBELL, 1991; WOOD, 1993), which are excreted by the kidney.

Ammonia is the main nitrogenous compound produced and excreted by teleost fishes (CHEW *et al.*, 2005). Its high diffusive rate reduces the necessity of the use of energy consumption to detoxify and store ammonia as urea or uric acid, as it occurs with mammalian or birds and reptiles, respectively (ANDERSON, 1995). In aqueous solutions, such as the intracellular environment and the aquatic environment, ammonia occurs in two chemical forms: ionized (NH_4^+) and non-ionized or gaseous (NH_3); and the sum of both forms is

denominated total ammonia (WOOD, 1993). Intracellular, extracellular or environmental pH and temperature are the main factors contributing to the NH_4^+ dissociation in NH_3, showing a direct relationship of this factors with the NH_3 concentration (RANDALL and TSUI, 2002). Additionally, NH_4^+ is poorly permeable in biological membranes when compared to NH_3. For this reason, ammonia most often crosses biological membranes, in both directions, as NH_3 (CHEW *et al.*, 2005). The presence of Rhesus (Rh) glycoproteins enhance the diffusional rate of NH_3 across membranes of fish gills (WRIGHT and WOOD, 2009). NH_3 is also permeate through K^+ channels (RANDALL, 2011). The excretion of NH_4^+ is known to be actively transported through membranes (RANDALL, 2011), but the actual mechanism in the in the apical and basolateral membranes remains debatable (RANDALL and WRIGHT, 1987; WILKIE, 1997; RANDALL, 2011). The diffusional mechanism, related to membrane permeability and which allows NH_3 to be easily excreted into or absorbed from the environment, is also considered a weak point in environmental stress conditions (WENDELAAR BONGA and LOCK, 1991). These situations can be caused by several stress sources and can lead to alterations in the osmoregulation mechanisms (WENDELAAR BONGA and LOCK, 1991). In metabolic acidosis, that could be induced by an hypoxic condition, the N-waste excretion would decrease as NH_3 would combine with H^+ and be trapped inside internal extracellular or intracellular environment (RANDALL, 2011). To compensate an excretory or diffusional osmoregulatory misbalance, normally associated with ion losses, and to eliminate metabolic waste products, fish accumulate ions (Na^+ and Cl^-) in exchange for NH_4^+, H^+ and HCO_3^- (WENDELAAR BONGA and LOCK, 1991; WOOD, 1991).

Among the main sources of stress, different chemical substances like petroleum hydrocarbons, are known for their high toxicity and have been considered one of the main cause of changes in physiological responses in fish (HEATH, AG, 1995). Petroleum monoaromatic (BTEX – Benzene, toluene, ethylbenzene and xylene) and the polycyclic aromatic hydrocarbons (PAH) are known to cause biochemical, physiological, morphological and behavioral alterations in fish (PEAKALL, 1994; VAN DER OOST *et al.*, 2003). For instance, petroleum hydrocarbons are known for the ability to cause injuries to the O_2 uptake due to narcotic actions (CORREA and GARCÍA, 1990) or morphological gill damage (SOLANGI and OVERSTREET, 1982; WINKALER *et al.*, 2001; SOLLID *et al.*, 2003; STENTIFORD *et al.*, 2003; SIMONATO *et al.*, 2008; AGAMY, 2013), resulting in decline of plasma O_2 content (ALKINDI *et al.*, 1996). In hypoxic condition, fish tend to present low efficiency of ATP production as result of the alterations on the metabolism (HOCHACHKA, 1980). Although a great number of studies have investigated the effects of petroleum hydrocarbons in different responses of the fish (SPIES, 1987; GAGNON and HOLDWAY, 1999; INCARDONA *et al.*, 2004; SIMONATO *et al.*, 2008; RODRIGUES *et al.*, 2010; HORODESKY *et al.*, 2015), the observations regarding its effects on N-wastes excretion, and the interaction with osmoregulatory mechanisms and metabolic regulation in acute exposure is limited (ENGELHARDT *et al.*, 1981; CORREA and GARCÍA, 1990) and need to be better clarified.

The goal of this work was to evaluate the effects of water-soluble fraction of gasoline (WSF_G) acute exposure on nitrogen excretion, osmoregulatory and metabolic regulation of goldfish *Carassius auratus*. This temperate freshwater fish species is widely used in laboratory experimentation because of its high

capacity to withstand hypoxic and osmotic challenge conditions (VAN DEN THILLART and KESBEKE, 1978). Our hypothesis is that, as petroleum hydrocarbons could impair the morphology and, consequently, the gill function, this may lead to a loss in the ammonia excretion process in *C. auratus* and, consequently, increased plasma retention of this metabolite, followed by osmoregulatory and metabolic damages. Thus, to test the effects of these compounds on these physiological aspects of *C. auratus* we evaluate the flux rate and plasmatic concentrations of the main ions and N-wastes and measure O_2 uptake.

4.2. MATERIAL AND METHODS

4.2.1. Animals Maintenance

Goldfish specimens (N= 100; W= 3.49 ± 1.19 g) were purchased from Noah`s Pet Shop, Vancouver, British Columbia, Canada. At the laboratory, fish were held in 200 L tanks filled with dechlorinated Vancouver tap water (pH= 7.23; $[Na^+]$= 97.30 µmol L^{-1}, $[K^+]$= 4.85 µmol L^{-1}, $[Ca^{2+}]$= 161.19 µmol L^{-1} and $[Mg^{2+}]$= 7.30 µmol L^{-1}) under flow through conditions (dissolved O_2 ≥ 75.0% saturation; room temperature= 20.0 ± 0.5 °C; controlled photoperiod= 12h light: 12h dark) for two weeks prior experimentation. Commercial flakes of dry food (45% crude protein) were offered once a day and suspended 24 h before starting and during experiments.

All experimental procedures were conducted in accordance with Canada Council for Animal Care and approved by the University of British Columbia Animal Care Committee (AUP# A14-0251).

4.2.2. Procedure for the Extraction of WSF_G

Regular gasoline where obtained in a commercial gas station (Vancouver, British Columbia) and stored in a 2 L autoclaving glass bottle cover with aluminum foil. Till use (5 days), it was kept in a second plastic container inside a sealed flammable cabinet to increase the protection from light incidence.

The extraction of the WSF_G was conduced according to the methodology described by ANDERSON *et al.* (1974) at room temperature (20.0 ± 1.0 °C). One part of gasoline and nine parts of water (1:9 ratio) where added into a 1 L Marriotte glass bottle and stirred in a magnetic plate. To standardize the procedure, the velocity of stirring was maintained to form a vortex corresponding to $^1/_3$ of the gasoline + water column height. After 22 h the stirring was stopped and the solution rest for 30 min to allowed proper separation of the fuel and WSF_G fraction phases. The obtained WSF_G was immediately used.

Samples of the WSF_G (100%) were collected for the determination of BTEX and PAH, heavy metals (Ag, Al, Ba, Cd, Co, Cr, Cu, Fe, Mn, Ni, P, Pb, Sr and Zn), nitrogen compounds (total ammonia and urea), and ions (Mg^{2+}, Ca^{2+}, K^+ and Na^+) content. Samples for PAH and BTEX, were stored in decontaminated amber flask (60 mL) prepared by three processes in ultrasonic washer: tap, bi distilled and milliQ® water; drying: 24 h at 105 °C; calcination: 4 h at 400 °C and kept under refrigeration (4 °C) for posterior chromatographic analysis. Water samples for metallic compounds determination were stored in amber flasks (60 mL) and preserved with 1% HNO_3 until analysis.

4.2.3. Experimental Design

Forty fishes were transferred from the main acclimation tank to 260 ml individual glass containers. In this static system condition, kept at room temperature (20.0 ± 1.0 °C) and continuously supplied with airflow, they were individually acclimated for 4 h. After acclimation, 80% of the water volume was replaced with clean freshwater (control) or freshwater containing the four concentrations of WSF_G (0.25, 5, 10 and 25% *v/v* – N= 8 fishes per treatment). The acclimation period, room temperature, airflow supply and WSF_G concentrations were kept the same through all the experimental procedures described below.

4.2.4. Experimental Procedures

4.2.4.1. Procedure 1: Measurement of Nitrogen and Ions (Na^+, K^+, Ca^{+2} and Mg^{+2}) *Flux Rate*

The main goal of this procedure was to evaluate nitrogen (ammonia and urea) excretion and, also, the flux rate of ions (Na^+, K^+, Ca^{2+} and Mg^{2+}) during exposure to WSF_G. At the end of 4 h acclimation period, fish were exposed for 6 h to four concentrations of WSF_G: 0.25, 5, 10 and 25% *v/v*. Water samples where collected at 15 min and 6 h after the beginning of the exposure. Temperature (YSI® 55) and pH (Fisher Scientific® AB15) were measured immediately and water samples for nitrogen compounds and ions determination were stored at -20 °C until analysis. At the end of the experimental trial, the exposed fish were euthanized with a neutralized MS222 solution overdose.

4.2.4.2. Procedure 2: Metabolic Rate During WSF_G Exposure

The second experimental procedure was designed to evaluate the oxygen consumption, plasma levels of urea and Mg^{2+}, and muscle Mg^{2+}. Fish were exposed to the four WSF_G concentrations described above for 6 h. After exposure, 80% of the water volume in each container was replaced with WSF_G and oxygen-saturated water. The initial oxygen partial pressure (PO_2) was measured (YSI™ 55) and the container was sealed with a latex rubber lid to avoid diffusion of O_2 from the atmospheric air to the water. The initial average (mean ± SD) PO_2 was 154.38 ± 1.93 Torr. Fish were kept in this condition for 60 min, then the lid was removed and the final PO_2 measurement was performed. For blood and muscle sampling, fish were anaesthetized with tamponed MS222 solution (5 g L^{-1}) added directly in the experimental container. Blood samples, acquired with heparinized (Sigma Aldrich® lithium heparin) syringes, were centrifuged for 6 min (6000 rpm) and the obtained plasma were immediately frozen (-80 °C) for posterior urea and Mg^{2+} determination. Fish were euthanized by medullar section and muscle tissue was sampled and immediately frozen in liquid nitrogen, then transferred to -80 °C freezer until Mg^{2+} determination.

4.2.4.3. Procedure 3: Metabolic Rate After WSF_G Exposure

The WSF_G exposure procedure used followed the same protocol describe above. However, for the oxygen consumption measurement, 100 % of the water + WSF_G volume was replaced with clean oxygen-saturated freshwater. The initial PO_2 was measured (YSI™ 55), the container was sealed, and fish were kept in this condition for 60 min. Then, final PO_2 measurement was performed.

4.2.5. Analytic Methods

4.2.5.1. Petroleum Hydrocarbons Analysis

Samples of 100% WSF_G were submitted to a vortex-assisted liquid-liquid dispersive microextraction (VA-DLLME) procedure prior PAH determination. The extraction method was adapted from the work of REZAEE *et al.* (2006) and ZHANG and LEE (2012). Consists in quickly adding 1 mL of the extraction solution ($CHCl_3$= 0.075 mL + C_3H_6O= 0.925 mL) in 5 mL of the aqueous sample, or the PAH standard solution, followed by agitation in a vortex for 1 min and centrifugation at 2800 rpm for 10 min. Thus, an aliquot of the highest density fraction (50 µL) was transferred to a chromatographic insert contenting 10 µL of the internal PAH standard solution (mix of deuterated PAH at 100 µg L^{-1}) and injected into GC-MS.

The procedure used for BTEX extraction was based on the headspace methodology described by FERNANDES *et al.* (2014). The 100% WSF_G samples (5 mL) were added in a 20 ml vial sealed with PTFE/silicone septa and aluminum seal. The sealed vail was heated (60 ºC) and stirred (300 rpm) for 5 min to promote the volatilization of the BTEX by displacing the equilibrium to the gas phase, located in the headspace of the vial.

Both PAH and BTEX analysis were carried out utilizing gas phase chromatography coupled to mass spectrometry (GC-MS). For PAH, we used a Thermo Fisher Scientific® gas chromatograph (Focus GC Polaris Q model), equipped with a Thermo® AS3000 auto-sampler for liquid injection and for BTEX a Shimadzu® gas chromatograph (model 2010) coupled to mass spectrometer (TQ8040) in tandem system (GC-MS/MS), equipped with a Shimadzu® AOC-

5000 autosampler for headspace analysis. Instrumental parameters used are detailed in Table 11.

Table 11. Instrumental parameters utilized for determination of polycyclic aromatic hydrocarbons (PAH) and monoaromatic hydrocarbons (BTEX) in aqueous samples via gas phase chromatography coupled to mass spectrometry (GC-MS).

PAH	Column	DB5-ms (Agilent®) 30 m x 0.25 mm x 0.25 µm
	Carrier gas	Analytical helium 5.0 (99.9%) with a flow rate of 1.2 mL min^{-1}
	Injector	Splitless, injection volume 1.0 µL
	Temperature	Column: 40 ºC (5 min) Ramp 1: 5 ºC min^{-1} to 230 ºC Ramp 2: 2 ºC min^{-1} to 250 °C Ramp 3: 5 ºC min^{-1} to 300 ºC (8 min) Detector: 300 ºC Injector: 270 °C Transfer line: 270 ºC Ions source: 270 ºC
	SIM* mode	Scan 1 (5 min): (m/z 128, 136, 152, 154, 162, 164, 166, 178) Scan 2 (30 min): (m/z 178, 202, 228, 244) Scan 3 (47 min): (m/z 228, 236 240, 252, 260, 264, 276, 277, 278, 279)
BTEX	Column	SH-Rtx-5ms (Shimadzu®) 30 m x 0.25 mm x 0.25 µm
	Carrier gas	High-pure helium (99.9%) with a flow rate of 1.02 mL min^{-1}
	Injector headspace	Injection volume: 500 µL
	Temperature	Column: 35 ºC (4 min), Ramp 1: 10 ºC min^{-1} to 100 ºC Ramp 2: 30 ºC min^{-1} to 200 ºC Detector: 250 ºC Injector: 180 °C Syringe: 60 ºC Sample: 60 ºC Transfer line: 250 ºC Ions source: 250 ºC
	Agitation	Sample: 300 rpm
	SIM* mode	Scan (m/z): 78; 91 and 106

Analytical curves were build in triplicate for PAH (0.3; 0.5; 0.8; 1.2; 1.5; 2 and 4 μg L^{-1}) and BTEX (5; 10; 25; 50; 100; 500 and 1000 μg L^{-1}) determination. High purity analytical standards of BTEX (benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene and *p*-xylene) and the 16 priority PAH suggested by U.S.EPA (2001) were used: naphthalene (Naf); acenaphthylene (Aci); acenaphthene (Ace); fluorene (Flu); phenanthrene (Fen); anthracene (Ant); fluorantene (Fla); pyrene (Pyr); benzo(*a*)anthracene (BaA); criseno (Cris); benzo(*b*)fluoranthene (BbF); benzo(*k*)fluoranthene (BkF); benzo(*a*)pyrene (BaP); indene(1,2,3-*cd*)pyrene (Ind); dibenzo(*a*,*h*)anthracene (Dib); benzo(*g*,*h*,*i*)perylene (Ben). Five deuterated internal standards (AccuStandart®) were also used: Naphthalene (NafD8); Acenaftene (AceD10); Fenantrene (FenD10); Chrysene (CrisD12) and Perylene (PerD12). The stock mix standard solutions of PAH and BTEX were prepared in dichloromethane and methanol (J.T. Baker®), respectively, at concentrations of 5 mg L^{-1}, and kept under controlled temperature (-18 °C) until use. Work solutions where prepared daily.

The accuracy of GC-MS analysis was determined by a recovery test by spiking three known concentrations of PAH (0.35; 1.2 e 5 μg L^{-1}) and BTEX (8; 120 e 450 μg L^{-1}) compounds in a water sample from the same source used in the experimental procedure. The recovery test was performed in three replicates for each concentration level studied. The repeatability of both methods was studied by analyzing six replicates of a standard solution of PAH and BTEX and expressed as relative standard deviation (RSD %).

4.2.5.2. Metals Analysis

Metal concentration of 100% WSF_G as measured through inductively coupled plasma optical emission spectrometry (ICP-OES) using a Thermo Scientific® (ICAP 6500) spectrometer operating with axial vision. The instrumental parameters are shown in Table 12. Argon with high purity (99.996%) was used.

Table 12. Operating parameters used for determination of metals in water-soluble fraction of gasoline (WSF_G) using inductively coupled plasma optical emission spectrometry (ICP-OES - Thermo Scientific®, ICAP 6500).

ICP-OES Operating Parameters	
Radiofrequency	40 MHz
Radiofrequency power	1150 W
Plasma gas flow rate	12.0 L min-1
Auxiliary gas flow rate	1.0 L min-1
Carrier gas flow rate	0.7 L min-1
Replicate read time	3 s
Time for stability	25 s
Replicates/sample	3
Torch	Quartz for axial view
Spectral lines	Ag: 328.068 (I)
	Al: 396.152 (I)
	Ba: 455.403 (II)
	Cd: 226.502 (II)
	Co: 230.786 (II)
	Cr: 267.716 (II)
	Cu: 327.395 (I)
	Fe: 238.204 (II)
	Mn: 257.610 (II)
	Ni: 231.604 (II)
	P: 213.618 (I)
	Pb: 220.253 (II)
	Sr: 407.771 (II)
	Zn: 213.856 (I)

(I) Atomic line; (II) ionic line.

4.2.5.3. Nitrogen Compounds

Ammonia content in water were determined colorimetrically (595 nm) according to the indophenol methodology described by VERDOUW *et al.* (1978). Determination of urea in water and plasma was based in the color reaction (read at 525 nm) with diacetyl monoxime and thiosemicarbazide in presence of acid solution (sulphuric acid, phosphoric acid and ferric chloride) under high temperature (RAHMATULLAH and BOYDE, 1980). Both ammonia and urea assays were read in the SpectraMax 340$_{PC}$ plate reader (Molecular Devices®).

4.2.5.4. Ions Analysis

Mg^{2+}, Ca^{2+}, K^{+} and Na^{+} in water were determined by atomic absorption spectrophotometry (AA). The AA instrument (Varian® *AA240FS*) operated in flame mode using acetylene as purging gas. Plasma samples were diluted (200x) with 1% lanthanum chloride ($LaCl_3$) solution prior AA analysis. Before Mg^{2+} determination in muscle, wet tissue was weight and digested in 1 ml of nitric acid (at room temperature) for 24h. Then, digested tissue samples were diluted (200x) in 1% $LaCl_3$ for AA determination.

Linear absorbance-concentration curve was build for each ion using a set of six standard solutions (plus blank sample) and considered suitable if presented $R^2 > 0.995$. Reading one of the standard solutions between every 15 experimental samples insured quality certification. The operation specifications and detection limits of the AA instrument are described in Table 13.

Table 13. Instrumental specifications for atomic absorption flame analysis (Varian® *AA240FS*) for determination of magnesium, calcium, potassium and sodium on water-soluble fraction of gasoline (100%) and experimental water, muscle and plasma samples after 6 h exposure experiment with goldfish *Carassius auratus*.

Instrumental	**Water**				**Plasma**	**Muscle**
Specification	***Mg²⁺***	***Ca²⁺***	***K⁺***	***Na⁺***	***Mg²⁺***	***Mg²⁺***
Lamp current (mA)	4.0	10.0	5.0	14.0	4.0	4.0
Wavelength (nm)	285.2	422.7	766.5	589	285.2	285.2
Slit width (nm)	0.5	0.5	1.0	0.5	0.5	0.5
Fuel flow (L min^{-1})	2.0	2.0	2.0	2.0	2.0	2.0
Air flow (L min^{-1})	13.5	13.5	13.5	13.5	13.5	13.5
Precision standard (%)	1.0	1.0	1.0	1.0	1.0	1.0
Precision sample (%)	1.0	1.0	3.0	3.0	1.0	1.0
Measurement time (s)	8.0	8.0	8.0	8.0	8.0	8.0
Calibration (µmol L^{-1})*	2.5-50.0[1]	10.0-200.0[2]	5.0-50.0[3]	25.0-200.0[4]	2.5-50.0[1]	2.5-50.0[1]
Modifier substance	None	None	None	None	$LaCl_3$	$LaCl_3$

*Standard solutions: [1]SCP Science® Mg^{2+} standard for AAs (1005 g Mg^{2+} L^{-1}); [2]Fluka® analytical Ca^{2+} standard for AAs (1000 g Ca^{2+} L^{-1}); [3]Fluka® analytical K^{+} standard for AAs (1.2 g K^{+} L^{-1}); [4]Fluka® analytical Na^{+} standard for AAs (1000 g Na^{+} L^{-1} in 1.1%).

4.2.6. Calculations

The net flux rates of ammonia-N (J_{amm} [µmol N kg h^{-1}]) and urea-N ($J_{urea\text{-}N}$ [µmol N- kg h^{-1}]) were calculated according to the equations (1) and (2).

$$Jamm = \frac{(ammfinal - amminitial)\,.V}{W\,.\,T} \qquad (1)$$

$$Jurea - N = \frac{ureafinal - ureainitial\,.V}{W\,.\,T}\,.2 \qquad (2)$$

Where $amm_{initial}$ (1) and $urea_{initial}$ (2) are the initial water ammonia (mmol L^{-1}) and urea (mmol N-urea L^{-1}) concentrations; amm_{final} (1) and $urea_{final}$ (2) are

the final water ammonia (μmol L^{-1}) and urea (mmol N-urea L^{-1}) concentrations; *V* is the experimental water volume (L); *W* is the weight of the animal (kg); and *T* is the duration of the flux period (h).

The saturation of O_2 (%) in the water were obtained by converting PO_2 values using the solubility constants described by BOUTILIER *et al.* (1984). Oxygen consumption rate (MO_2 [μmol O_2 kg h^{-1}]) were calculated using the equation (3) presented below:

$$MO2 = \frac{(O2, initial - O2, final) \, . V}{W \, . \, T} \qquad (3)$$

The $O_{2,initial}$ and $O_{2,final}$ (3) represents the oxygen concentrations in the water (μmol L^{-1}) at the start and end of the experiment, respectively, and the other variables (*V*, *W* and *T*) represents, exactly, what was described for the above equations.

4.2.7. Statistical Analysis

Normality and homogeneity of the data was analysed through Shapiro-Wilks and Bartlett's tests, respectively. One-way ANOVA analysis of variance, followed by Tukey test, was used to determinate significant differences among treatments of all obtained data ($p<0.05$). Data were analyzed using SigmaStat® 3.5 and graphically plotted using SigmaPlot® 11.0 (both by Systat Software Inc.) and presented as mean ± SD.

4.3. RESULTS

4.3.1. Characterization of WSF_G

The 100% WSF_G used in the experimental trials presented higher concentration monoaromatic hydrocarbons (BTEX) when compared to polycyclic aromatic hydrocarbons (PAH) compounds. Benzene and toluene were the most abundant BTEX. Naphthalene, acenaphthylene, phenanthrene concentrations were below the method quantification limit - and anthracene was not detected (Table 14). Aluminum, iron, strontium and zinc concentrations were detected in the 100% WSF_G (Table 15).

Table 14. Concentrations of monoaromatic (BTEX) and polycyclic aromatic hydrocarbons (PAH) of 100% water-soluble fraction of gasoline (WSF_G) used during for the exposure trials with goldfish *Carassius auratus*.

BTEX (µg L^{-1})	**100% WSF_G**
Benzene	1336.1
Toluene	5562.6
Ethylbenzene	227.6
m,*p*-Xylene	429.9
o-Xylene	407.7
∑ *BTEX*	*7963.8*
PAH (µg L^{-1})	
Naphthalene	<0.5
Acenaphthylene	<0.5
Acenaphthene	1.1
Fluorene	0.9
Phenanthrene	<0.5
Anthracene	nd
Fluoranthene	3.1
Pyrene	3.0
Benz[*a*]anthracene	5.4
Chrysene	7.1
Benzo[*b*]fluoranthene	8.0
Benzo[*k*]fluoranthene	8.7
Benzo[*a*]pyrene	6.0
Indeno[1,2,3-*cd*]pyrene	7.9
Dibenz[*ah*]anthracene	7.9
Benzo[*ghi*]perylene	8.7
∑ *PAH*	*67.8*
∑	***8031.58***

Quantification limit = BTEX < 5.0 µg L^{-1} and PAH < 0.5 L^{-1}. BTEX= monoaromatic hydrocarbons. PAH= Polycyclic aromatic hydrocarbons.

Table 15. Metallic compounds concentrations of 100% water-soluble fraction of gasoline (WSF_G) used during the exposure trials with goldfish *Carassius auratus*.

Metallic Compound	**100% WSF_G (mmol L^{-1} x10^{-4})**
Ag	<0.92
Al	7.16
Ba	<0.72
Cd	<0.89
Co	<1.70
Cr	<1.92
Cu	<1.57
Fe	4.12
Mn	<1.82
Ni	<1.70
P	<3.23
Pb	<0.48
Sr	2.13
Zn	3.14

4.3.2. Nitrogen and Ions Flux Rate

The total ammonia net flux rate was not affected by the exposure to WSF_G (Figure 17A). On the other hand, the excretion o urea significantly increased ($p<0.05$) along with the WSF_G concentration (Figure 17B). When compared to the control group, the excretion of urea increased 0.6 times at 0.25% ($p>0.05$) and 2.15 times at 25% of WSF_G ($p<0.05$). The same pattern of dose/response significant increase ($p<0.05$) was observed for the Mg^{+2} net flux rate (Figure 18), reaching 3.5 times at 25% of WSF_G when compared to the control group. The Na^+, Cl^-, K^+ and Ca^{+2} flux did not present significant alterations ($p>0.05$) (Figure 19A, B, C, and D, respectively).

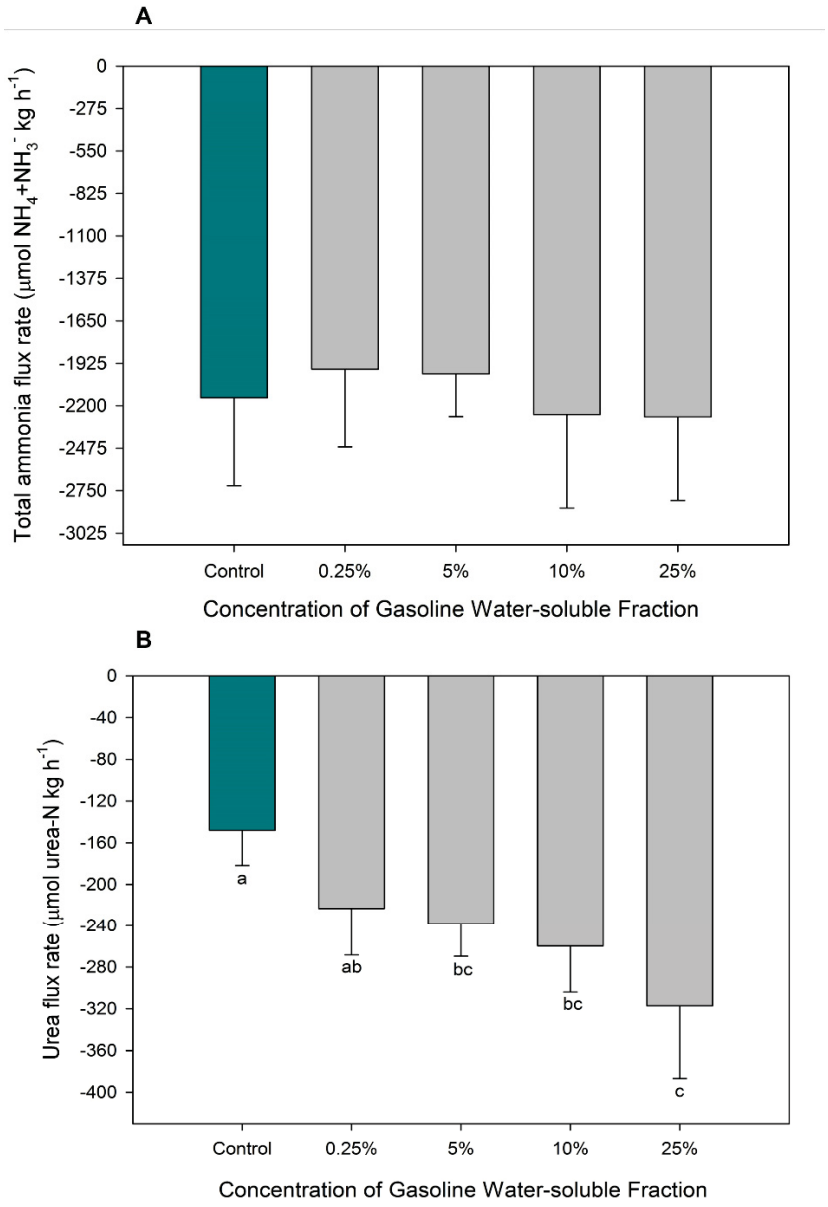

Figure 17. Mean ± SD values of (A) total ammonia and (B) urea flux rate of goldfish *Carassius auratus* after 6h exposure to gasoline water-soluble fraction (WSF_G). Letters indicate significant difference among treatments (One-way ANOVA, Tukey, $p<0.05$).

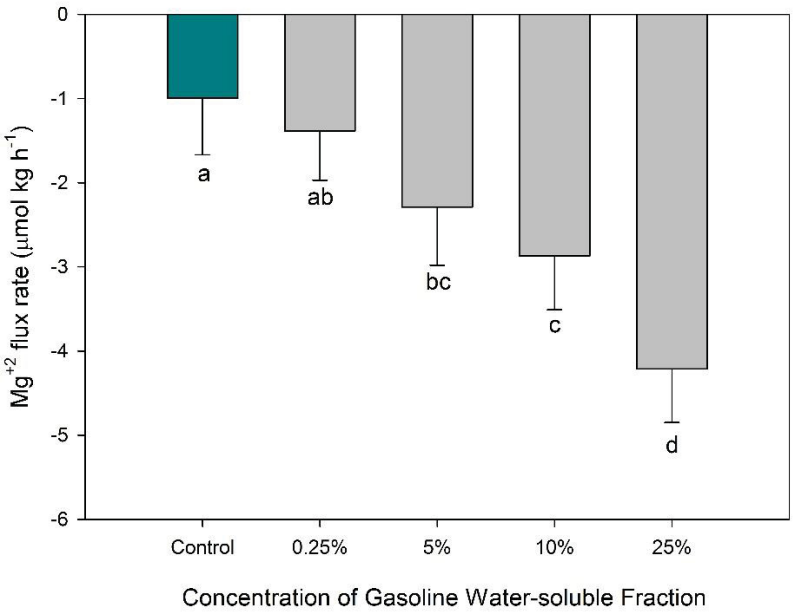

Figure 18. Mean ± SD values of Mg^{+2} flux rate of goldfish *Carassius auratus* after 6h exposure to gasoline water-soluble fraction (WSF_G). Letters indicate significant difference among treatments (One-way ANOVA, Tukey, $p<0.05$).

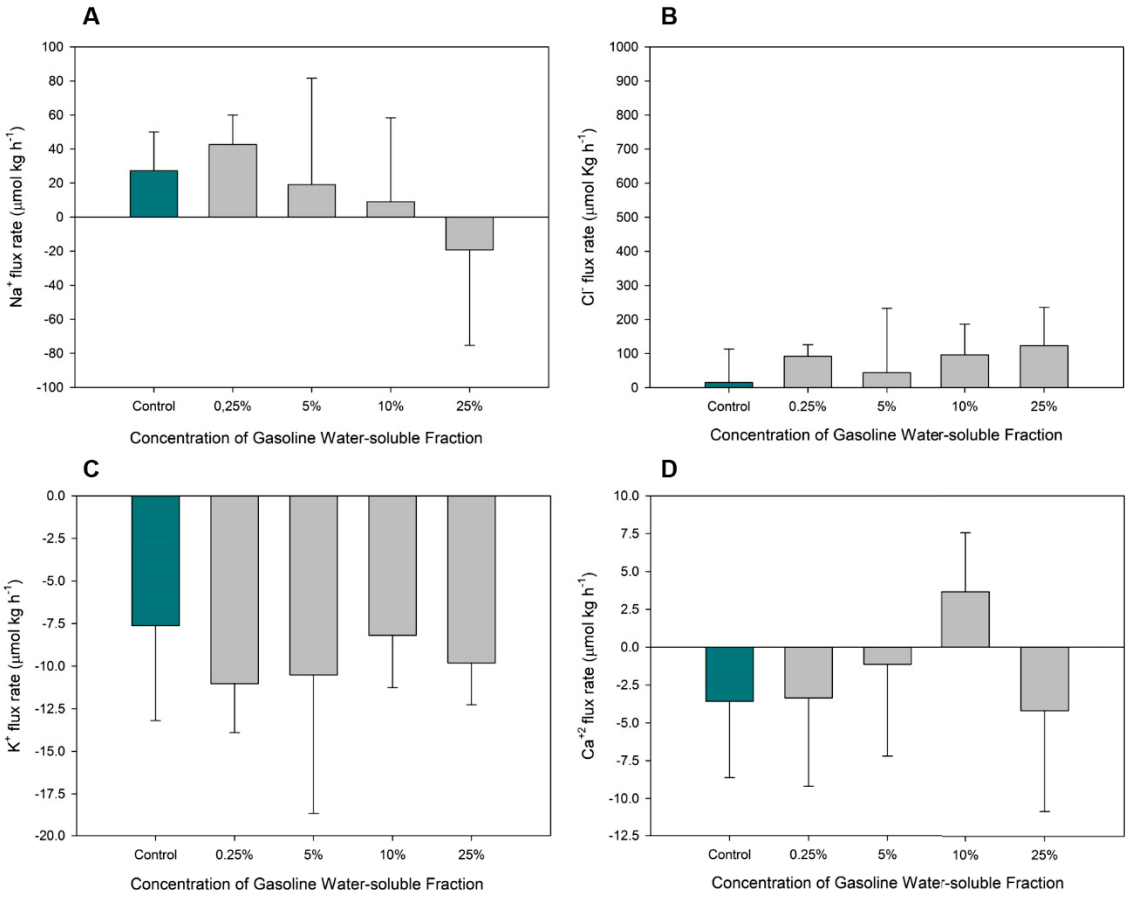

Figure 19. Mean ± SD values of (A) Na^{+}, (B) Cl^{-}, (C) K^{+} and Ca^{+2} flux rate of goldfish *Carassius auratus* after 6h exposure to different concentrations of gasoline water-soluble fraction (WSF_G). Letters indicate significant difference among treatments (One-way ANOVA, Tukey, $p<0.05$).

4.3.3. Plasma and Muscle Mg^{+2}, Plasma Urea and MO_2

Differently from the increase observed on the flux rate of urea and Mg^{+2}, their plasmatic concentrations (urea - Figure 20 and Mg^{+2} - Figure 21A) were not significantly affected by the WSF_G exposure ($p>0.05$). The muscular concentrations of Mg^{+2} slightly increased ($p<0.05$) at 25% of WSF_G.

The oxygen uptake was significantly ($p<0.05$) influenced by the WSF_G (Figure 22). Compared to the control group, the MO_2 mean was 25.3% lower at 0.25% of WSF_G and reached its lowest values at 5%, with a significative ($p<0.05$)

reduction of 30.5%. At 10% and 25% of WSF_G, the increase towards the control value were 19% and 7%, respectively.

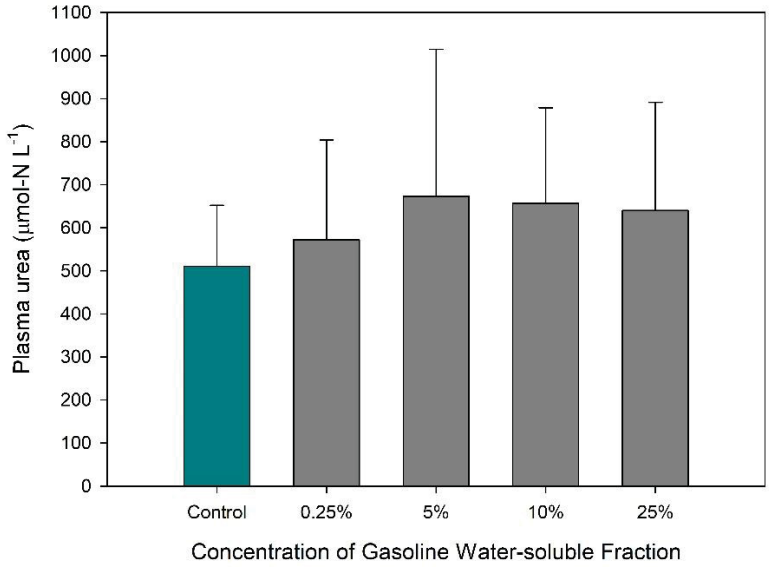

Figure 20. Mean ± SD values of plasmatic concentrations of urea in goldfish *Carassius auratus* after the second experimental procedure (6h exposure to different concentrations of gasoline water-soluble fraction (WSF_G). Letters indicate significant difference among treatments (One-way ANOVA, Tukey, $p<0.05$).

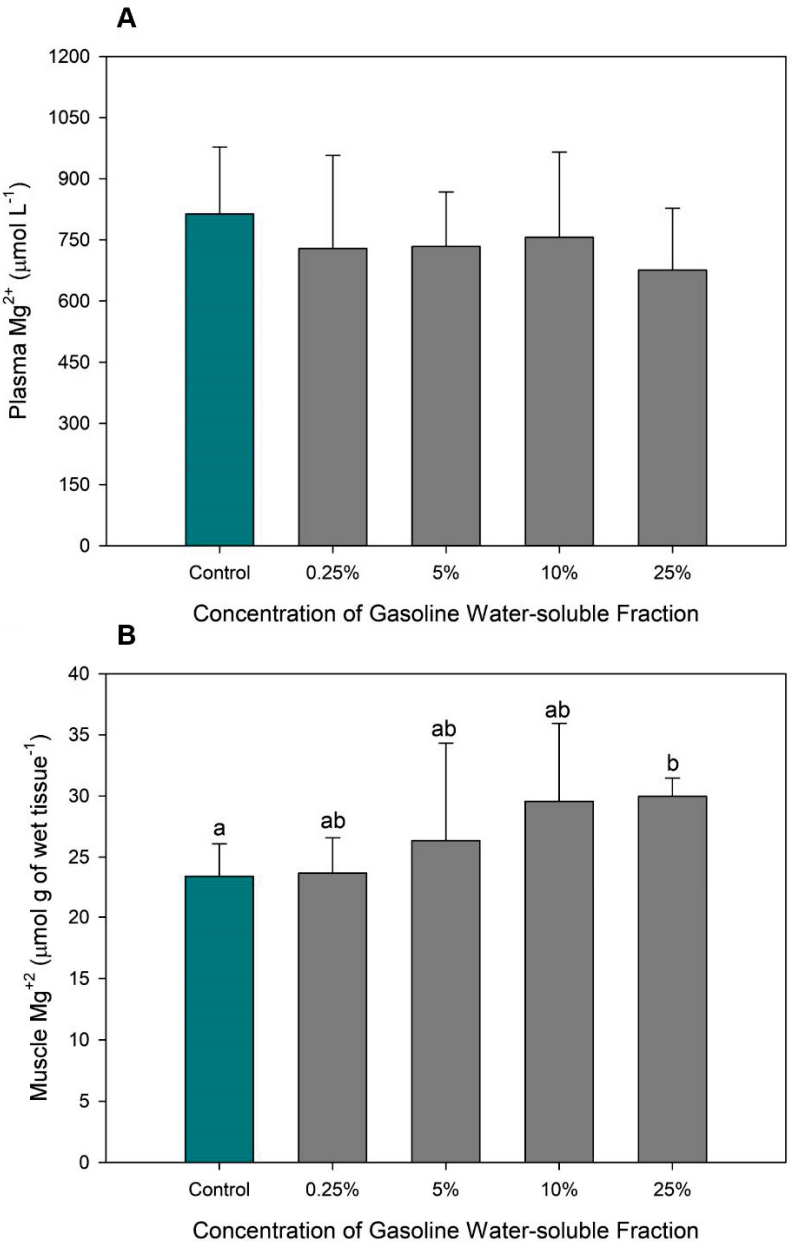

Figure 21. Mean ± SD values of (A) plasmatic and (B) muscular concentration of Mg^{+2} in goldfish *Carassius auratus* after 6h exposure to different concentrations of gasoline water-soluble fraction (WSF_G). Letters indicate significant difference among treatments (One-way ANOVA, Tukey, $p<0.05$).

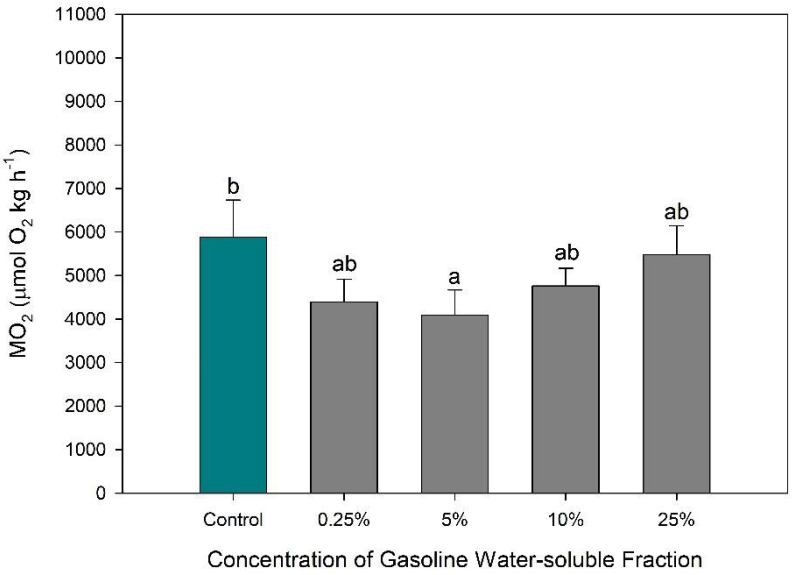

Figure 22. Mean ± SD values of oxygen uptake by the gills (MO_2) of goldfish *Carassius auratus* after 6h exposure to different concentrations of gasoline water-soluble fraction (WSF_G). The 1 h respirometry measurement was performed using the same WSF_G concentrations used during the exposure trial. Letters indicate significant difference among treatments (One-way ANOVA, Tukey, $p<0.05$).

4.3.4. Metabolic Rate Before WSF_G Exposure

Goldfish presented a different MO_2 pattern of response when the measurement was performed in clean freshwater (Figure 23). In this scenario, the oxygen gill uptake progressively increased through WSF_G experimental concentrations ($p<0.05$). At 0.25%, the MO_2 was 32% ($p<0.05$) higher than the control group and the gradual increased reached 92% at the 25% group ($p<0.05$).

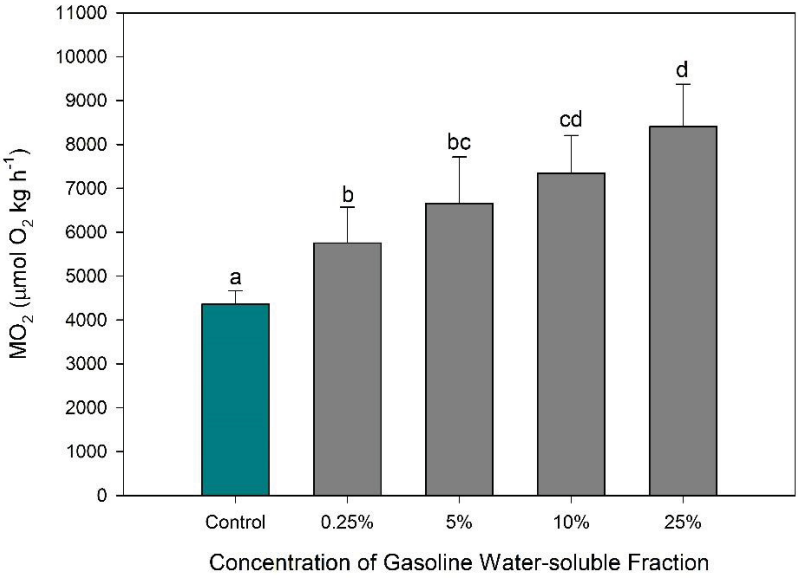

Figure 23. Mean ± SD values of oxygen uptake by the gills (MO_2) of goldfish *Carassius auratus* after 6h exposure to different concentrations of gasoline water-soluble fraction (WSF_G). The 1 h respirometry measurement was performed in clean-fresh water. Letters indicate significant difference among treatments (One-way ANOVA, Tukey, $p<0.05$).

4.4. DISCUSSION

The toxicity of petroleum hydrocarbons to fish increases as the individual molecular weight decrease (ALBERS, 2003). The WSF_G is known to present higher concentrations of monoaromatic hydrocarbons (BTEX) in detriment to polycyclic hydrocarbons (PAH) (SAEED and AL-MUTAIRI, 1999). Due to this chemical characteristic, gasoline is considered the most toxic petroleum refined product to fish (see results in chapter 1 for *Astyanax altiparanae*) (BARRON *et al.*, 1999; RODRIGUES *et al.*, 2010). Here, the individual petroleum hydrocarbon concentration range, detected in the 100% WSF_G, are in accordance with the international literature and can be considerate as an effective toxic source of BTEX and PAH (RODRIGUES *et al.*, 2010; BETTIM *et al.*, 2016; GALVAN *et al.*,

2016; PILATTI *et al.*, 2016). Although Al, Fe and Zn were detected in the 100% WSF_G, they are only considered harmful to fish in higher concentrations and lower water pH (WOOD, 2001b). The water pH in the tested conditions, however, remained among neutral values. The toxicity of Sr is also kwon to be very low for the aquatic biota (MCPHERSON *et al.*, 2014). Thus, the metallic compounds that presented concentrations above the analytical quantification limit, in the 100% WSF_G, are unlikely to be impose a significant toxicological hazard. Thus, BTEX and PAH are the main substances in the tested WSFG that could induced the observed toxic effects.

Despite the high toxicity of the WSF_G to marine (RODRIGUES *et al.*, 2010) and freshwater (unpublished data – see chapter 1) fish species, ammonia flux rate of *C. auratus* of was not altered by the 6h exposure. This result refutes our hypothesis and diverge from the result found by CORREA and GARCÍA (1990) with juveniles of the marine *Mugil curern* exposed to benzene (0 to 10 ml L^{-1}). The authors detected a 40% reduction on ammonia excretion during 24h exposure to the higher tested benzene concentration. The ability to uphold normal ammonia flux rate, in environmental stress conditions (air, fresh water or ammonia exposure), was observed by SOUZA-BASTOS et al. (2014) in *Lipophrys pholis*, a marine fish specie that lives at intertidal pools and is considered very tolerant species to intense physiological challenges. This contrasting results could be attributed to the differences in time exposure and to the resilient characteristic of *C. auratus* in conditions of environmental challenge (VAN WAARDE and DE WILDE-VAN BERGE HENEGOUWEN, 1982), considering that this is the first work to verify the action of petroleum hydrocarbons on the excretion of ammonia in this species.

In this case, the lack of ammonia alterations is more likely to be a result of changes in N-wastes metabolism (CAMPBELL, 1991; CHEW et al., 2005; RANDALL, 2011). Some fish species presents the ability to reduce the ammonia toxicity through the synthesis of less toxic N-wastes when exposed to situations of environmental challenge (RANDALL, 2011; SOUZA-BASTOS *et al.*, 2014). The similar dose-response presented by urea and Mg^{2+} fluxes, on the other hand, strongly suggests that the response to acute WSF_G could be linked to the activation of anaerobic energetic metabolism, through the breakdown of ATP and other adenylates in the muscle (WOOD, 1993). In low dissolved oxygen conditions, the anaerobic ATP breakdown leads to the production of IMP [releasing two moles of Mg^{2+} (BENDER, 2012)], from which its purine ring component is broken down to uric acid, and then converted to urea in the process of uricolysis (CAMPBELL, 1991; WOOD, 1993; RANDALL, 2011). This anaerobic pathway is energy-expensive, as the synthesis of one mole of urea requires 5 moles of ATP (HOCHACHKA, 1980). The significant rise of muscular Mg^{2+} concentrations, observed here for *C. auratus*, can also explain the anaerobic breakdown of ATP in the white tissue. In a chronic high environmental ammonia exposure, for example, the ammonia fluxes of goldfish remained unchanged while urea excretion significantly increase (FROMM, 1970). The absence of significant differences in plasmatic urea and Mg^{2+}, as well the maintenance of Na^+, Cl^- and K^+ fluxes, also supports the idea that *C. auratus* presents the ability to cope with the physiological challenge imposed by the exposure to WSF_G.

This hypothetic state of metabolic hypoxia, suggested by the urea and Mg^{2+} fluxes, as well by the increased muscle Mg^{2+}, as a result of the ATP anaerobic breakdown, was confirmed by the O_2 gill uptake (MO_2) measured

during WSF_G exposure. Thus, the metabolic mechanism adopted by *C. auratus* can not be considered an effective physiological regulation unless goldfish was, indeed, experience and metabolic hypoxic condition. This fast-triggered anaerobic mechanism, can be one of the reasons why goldfish successfully survived the high toxic environmental challenge imposed by the WSF_G. The results obtained by CORREA and GARCÍA (1990), regarding the oxygen consumption during benzene exposure, suggest that fish increase O_2 uptake as it increases respiration rate as result of the increase of opercula movement. Even though we did not measured ventilation in our experiments with goldfish, it is well-known that fish can increase opercula movement in hypoxic conditions to compensate low water O_2 partial pressure (PO_2) (LOMHOLT and JOHANSEN, 1979) or to cope with low gas diffusion rates induced by environmental contaminants (WOOD, 2001b). In a stress-induced scenario, the changes triggered by the action of catecholamines and cortisol (WENDELAAR BONGA, 1997) can modify the gill membrane permeability and affect the capacity of gas exchange through the gill (CARNEIRO et al., 2005). The inability to uptake water O_2, presented by *C. auratus* during WSF_G exposure, was compensated 1h after the removal of the stress source (see Figure 23). After stress or intensive exercise circumstances, fish focus on restore its original metabolic state through the increase of O_2 consumption (WOOD, 1991). Thus, we believe that the compensating O_2 uptake presented by *C. auratus* is been used to restore metabolic substrates sources and recovery general normal homeostasis.

4.5. CONCLUSIONS

The results presented in this study suggest that, although WSF_G did not alter ammonia excretion, exposure for 6 h was sufficient to trigger metabolic changes in *C. auratus*, such as increased Mg^{2+} and urea excretion as a result of metabolic alteration, probably regarding the inability, inferred by BTEX and PAH, to maintain the mechanism of O_2 uptake at the gills.

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OVERALL CONCLUSIONS

This thesis has addressed several challenges inflicted to tropical and temperate fish species in the field aquatic environmental contamination by petroleum hydrocarbons. Our data, obtained with the native neotropical species *Astyanax altiparanae*, showed that the toxicity of refined petroleum products differs along with the content of BTEX and PAH in the WSF.

Further, using a laboratory approach to assess the interaction of petroleum hydrocarbon exposure during water alteration of abiotic parameters (temperature and pH), we demonstrate that *A. altiparanae* responses of biotransformation (EROD and GST), oxidative stress (CAT and protein carbonyl) and neurotoxic biomarkers were changed under the tested conditions, with or without the contamination of WSF_G. Thus, we recommend carefulness for the use of those biomarkers in field-environmental pollution assessment and in laboratorial studies as they can display changes in their responses patterns.

In the same laboratory procedures, we also demonstrated that basic osmoregulatory and acid-base regulation mechanisms were activated to cope whit the WSF_G exposure under temperature and pH altered conditions. Our data also suggests that *A. altiparanae* can sustain the integrity of cellular membranes, indicating that the physiological mechanisms, trigged by the WSF_G exposure and temperature and pH alteration, were able to maintain general homeostasis.

Finally, using the temperate fish *Carassius auratus* as bioindicator species, we were able to demonstrate that the initial acute response to WSF_G exposure is related to the impairment of O_2 uptake mechanisms, which activate the anaerobic metabolism and leads to a ATP degradation and increase of urea and Mg^{+2}.

Thus, the results obtained in this work provides an important baseline for the toxicity of different refined petroleum products, as well the possible influence of water abiotic characteristics in the response of stablished biomarkers used for the assessment of petroleum hydrocarbon pollution and the possible physiological mechanisms that are acutely impaired.

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