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Effects of Phenanthrene on the Amazonian Fish Tambaqui *Colossoma macropomum*: LC_{50}, Growth and Haematology

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Abstract Oil spills have increased in frequency worldwide and biodiversity hotspots, such as the Amazon Basin, are under increasing threat of oil spills. The toxic effects of polycyclic aromatic hydrocarbons are poorly known for Amazonian fish species. The acute and sublethal effects of phenanthrene (Phe) on tambaqui (*Colossoma macropomum*), an important Amazonian food fish, were analysed in the present study. The Trimmed Spearman-Karber method was used to determine 96h-LC_{50} for Phe, which was 940 μg/L and represented roughly 70% of its regular water solubility at 30°C. Tambaqui specimens were exposed to 47, 235 and 470 μg/L, representing respectively, 5, 25 and 50% 96h-LC_{50} for Phe, for four weeks to analyse Phe effects on growth and haematological properties. Fish exposed to Phe showed high mucus production, expansion of lower lips, and the loss of both hydrodynamic balance and orientation. The condition factor has decreased by over 10% during the last two weeks of exposure to all Phe concentrations. After four weeks of exposure to 47 μg/L Phe (5% 96h-LC_{50}), there was observed a decrease of nearly 47% for final weight gain, 55% for specific growth rate, and 9% for blood haemoglobin. Additionally, plasma glucose increased up to 49% in animals exposed to 470 μg/L Phe (50% 96h-LC_{50}) and lactate increased up to 37% in animals exposed to 235 μg/L Phe (25% 96h-LC_{50}). The main finding was that tambaqui exposed to Phe even at the lowest level analysed (47 μg/L Phe, 5% of the 96h-LC_{50} Phe) exhibited disturbances of basic biological and physiological functions.

Keywords Amazon, Oil Spill, PAH, Zootechnical Performance, Haematology, MACeco

1. Introduction

The Amazon Basin has a variety of resources namely biodiversity, hydrocarbons, minerals and about 15% of the planet's fresh water. Hydrocarbons, such as those found in petroleum, represent an economic benefit to countries; however, these compounds also pose a risk to both ecosystems and human health when contaminating aquatic systems [1]. Some of the hazards of petroleum products are related, among others, to their water-soluble fractions (WSF), which contains polycyclic aromatic hydrocarbons (PAHs). Although PAHs have low water-solubility and are short-lived in the water column, persist in the sediment [2].

In the aquatic environment, PAHs dissolved in water or associated with particles in suspension or in the sediment are accessible to aquatic organisms, including freshwater fish. PAHs can cause severe behavioural, physiological and biochemical disturbances in fish [3]. Sixteen PAHs, among them phenanthrene (Phe), which is comprised of three fused benzene rings, have been identified as priority contaminants by the United States Environmental Protection Agency (EPA), the World Health Organization (WHO) and the European Economic Community (EEC) because aromatic hydrocarbons can bioaccumulate, result in contamination of the food chain and are toxic and potentially carcinogenic [4, 5]. The impacts of Phe on teleosts of the Amazon are unknown, despite Phe being an important toxic constituent of crude oil [6].

In the Amazon region, oil has been mined in the region of the Urucu River, a tributary of the Amazon River, approximately 700 kilometres from Manaus. The extracted oil is transported in pipelines to the city of Coari and shipped from there in oil barges to Manaus refinery. Even though strict safety protocols are observed, there is always some risk of an oil spill. In fact, several small accidents have occurred in the Brazilian Amazon, which have put the aquatic biota at risk [2, 7].

Fish are considered indicators of environmental quality and are used in ecotoxicological studies. Physiological and

haematological analyses are important tools for detecting the effects of chemical compounds on fish [3, 5]. In addition, the LC_{50} has been widely used in the assessment of water quality and the possible biological impacts of a given pollutant [8, 9]. Several pollutants have been described in waters of the Amazon, but their biological impacts have been insufficiently assessed [2, 7, 8, 10, 11].

In the Amazon, there are about 3,000 species of freshwater fish. However, only a few species (*Colossoma macropomum*, *Brycon amazonicus*, *Prochilodus nigricans*, and *Semaprochilodus* spp) account for more than 70% of the total fish landed in Amazonas State [12]. The tambaqui, *C. macropomum* (Characiformes, Serrasalmidae), is widely distributed in the Amazon basin, where its life cycle is closely associated with flood plains. Floodplain and floodplain lakes are habitats for larvae and young fish until reaching adulthood. Growing up to 90 cm and weighing up to 30 kg, the tambaqui is locally appreciated as a source of food.

Tambaqui has been used in oil bioassays, and it can be considered a useful species for environmental monitoring [7, 13]. One of the characteristics of the tambaqui that makes it vulnerable to oil is its ability to expand its lower lips when exposed to low concentrations of dissolved oxygen. The tambaqui skims the thin superficial layer of the water column with the expanded lips to extract the water that is richer in oxygen, funneling it across the gills [14, 15]. However, this adaptation exposes tambaqui to more spilled oil, since the oil remains on the surface of the water column.

Considering the importance of the Amazon region and the potential environmental impacts involved in the oil production and transportation, in particular involving the aquatic biota, there is sufficient reason to study the effects of Phe on the growth and physiology of tambaqui. Thus, the hypothesis tested in the present study is that the exposure of tambaqui to Phe, even in low concentrations, causes disturbances that can be accessed by haematology and zootechnical parameters.

2. Materials and Methods

2.1. Experimental Animals and Conditions

Specimens of tambaqui weighing 31.88 ± 1.97 g were obtained from a fish farm (Sitio dos Rodrigues, Km 35, Rod. AM-010, Brazil). The animals were transported to the laboratory and were kept in outdoor tanks at 28 ± 1 ^{o}C, with a natural photoperiod for 2 weeks prior to the experiment. During this period, juveniles tambaqui were fed commercial feed containing 36% protein *ad libitum* twice a day (8AM and 4PM). The same ration was used during the growth experiments. For the LC_{50} experiment, fish feed was suspended 48 h before starting and during the trials. Water characteristics (pH, temperature and dissolved oxygen) were measured daily. All experimental procedures conformed to Brazilian national animal care regulations and were approved by INPA (Brazilian National Institute for Research of the Amazon).

Well water at INPA was vigorously aerated, as it had very high CO_2, prior to use in the experiments. This water was considered the "control (INPA)". This water has low ionic concentrations ($[Na^+]$ = 35 μM; $[Cl^-]$ = 28 μM; $[Ca^{2+}]$ = 11 μM; $[Mg^{2+}]$ = 1 μM; $[K^+]$ = 16 μM; pH 6.32; DOC = 1.1 mg.L^{-1}; O_2 6 mg.L^{-1}, temperature 27-30 ^{o}C) and was similar to the water from the Rio Negro, which is one of the natural habitats of tambaqui, although it did not have the same levels of dissolved organic carbon.

2.2. Determination of LC_{50}-phenathrene for Tambaqui

Before the experimental trials, the rate of decay of Phe in the experimental solutions was determined, since in addition to dissolving in the water, in small amounts, this PAH also volatilises and may bind to fish waste, diminishing its bioavailability. The rate of decay in the experimental aquarium at the various experimental conditions was determined (with/without fish, with/without aeration, with/without fish waste removal) and always considering the changes in control absorbance (see below). Then, 25% of the water in the experimental aquaria was replaced every 12 hours by solution containing Phe in order to replace the amount of Phe that was lost from the experimental solution. The replacement solution was prepared by diluting a stock solution less than 96h old. The ethanolic (water/ethanol 1:1) stock solution of Phe (1g/100 mL) was prepared using Phenanthrene 96% pure and was purchased from Sigma Chemical Co. (St Louis, USA).

During the experiments, spectrophotometric scanning between 200 and 300 nm was the only method used to check for Phe concentration, which had the highest absorbance at 254nm. A calibration curve (Phe concentration versus A254) was constructed. Then, water samples from the various experimental conditions were collected every 12 h, filtered in a 45 μm fiberglass filter and the A254 determined. The absorbance values were relativized to the control, averaged for every 12 h, and a curve of decay with time was made.

All trials were conducted in aerated 40-L aquariums, with a stocking density of one fish for every 2 L (16g/L) of experimental solution, in duplicate. Because initial trials with concentrations above 1.5 mg/L caused total mortality before 96 h, the trials were performed with concentrations lower than 1.5 mg/L, observing the Sprague geometric scale to define them [16]. All aquariums contained 20 fish and mortality was checked every 12 h over the 96-h experimental period. There was no mortality in the control aquarium. LC_{50} and its 95% confidence intervals were calculated as described below. Lip formation, mucus production and swimming behaviour were visually observed. The experimental water was collected and sent to an oil-waste cleaning company.

2.3. Determination of Growth and Zootechnical Parameters

Forty fish were selected with a minimum variation in weight. After the selection, two fish were placed in each of the 20 plastic aquariums with 4 L of water from the "control (INPA)" well. After 24 h of recovery in the aquariums, Phe was added to make experimental solutions with 47, 235 and 470 µg/L, equivalent to 5%, 25% and 50% of LC_{50} concentrations (all nominal concentrations), respectively. Five replicates were used for the control and each of the three concentrations for four weeks.

The experimental Phe solutions were partially renewed every 12 h. One litre of the solution in each aquarium (equivalent to 25%) was replaced with freshly prepared solution to recompose the amount of Phe (similar to LC_{50} trials) and to recompose the water quality, thus reducing the effect of the excretion products. This was done with the help of a manual vacuum pump (Peters & Russell Inc., USA).

The amount of feed administered daily was readjusted after two weeks from the start of the experiment, according to increase in the fish mass. The mass gain of the fish was calculated by subtracting the fish mass at the beginning of the experiment from the fish mass after two weeks of exposure. The final mass was obtained at the end of the four-week experiment. The intermediate mass, i.e. the mass after two weeks, was measured indirectly by weighing the aquarium plus the fish. For the final sampling, fish were anaesthetised with neutralised MS222. Anaesthesia, measurement and sampling usually lasted about 3 min/fish.

The food consumed was calculated by subtracting the food not consumed after 30 min from the total offered. The unconsumed granules were counted and multiplied by their dry average mass (50 granules were weighed in triplicate to obtain their average mass). The following zootechnical parameters were calculated according to the equations below, where Wt and W0 were the final and initial mass values, respectively per aquarium and t was the time in days.

(1) Mass gain (WG) = [(final mass - initial mass)]
(2) Condition factor (CF) = [wet mass (g)/total length $(cm)^3$] x 100
(3) Hepatosomatic index (HSI) = [liver mass (g)/body mass (g)] x 100
(4) Percentage mass gain (%WG) = [(final mass - initial mass)/initial mass] x 100
(5) Specific growth (SGR) = [(lnWt - lnW0)/t] x 100
(6) Apparent feed conversion = dry mass feed consumed/mass fed by the animal
(7) Food efficiency (FE) = (mass gained by animal/dry mass food consumed) x 100

2.4. Haematology

At the end of the fourth experimental week, haematological parameters were measured for fish from each treatment and control. Blood was collected from the caudal vein using heparinised syringes from previously anesthetised fish. The haematological parameters measured were: plasma glucose using the Glucox 500 enzymatic colorimetric kit (Doles S.A., Goiânia, Brazil), following the manufacturer's instructions; plasma lactate using the enzyme lactate dehydrogenase (Sigma Chemical Co., St. Louis, USA); haematocrit using the microhaematocrit technique; concentration of haemoglobin by the cyanometahaemoglobin method [17]; red cell count using the Neubauer chamber. In addition, VCM (mean corpuscular volume), HCM (mean corpuscular haemoglobin) and MCHC (mean corpuscular haemoglobin concentration) were calculated according to Brown [18].

2.5. Statistical Analysis

For the calculation of the LC_{50}, the mortality data for the 24, 48, 72 and 96-hours periods were analysed by the LC_{50} Programs JS Pear Test based on the Trimmed Spearman-Karber method [19]. Zootechnical and haematological parameters were tested for normality and homoscedasticity, expressed as mean and standard error of the mean (SEM) and were statistically checked for mean differences by analysis of variance (ANOVA). When there was a significant difference between the treatments at the 5% level, the Tukey test was used to discriminate the differences, using Sigma Stat statistical software.

3. Results

3.1. Water Conditions in Experimental Trials

There were no significant differences in water quality among different aquariums, either for determination of LC_{50} or for chronic exposure for growth analysis. The mean oxygen, pH and temperature for all experimental situations, measured every 12 h, were 6.1 ± 0.2 $mg.L^{-1}$; 7.0 ± 0.1; 27 ± 1 °C, respectively. Ammonia levels, which were measured every 24 h behaved in the same way, presenting mean values of 23 ± 2 µM, without significant differences among the aquariums.

3.2. Phenanthrene (LC_{50})

The analysis of the Phe concentration in the experimental solution revealed a continuous decay over time (Figure 1). The decay curve was expressed as Y = 0.22273 - 0.00381 * X (r^2 = 0.94, P <0.0001). Using this equation, it was determined that the rate of replacement to maintain a nearly constant concentration of Phe was 205 µg/L every 12 h.

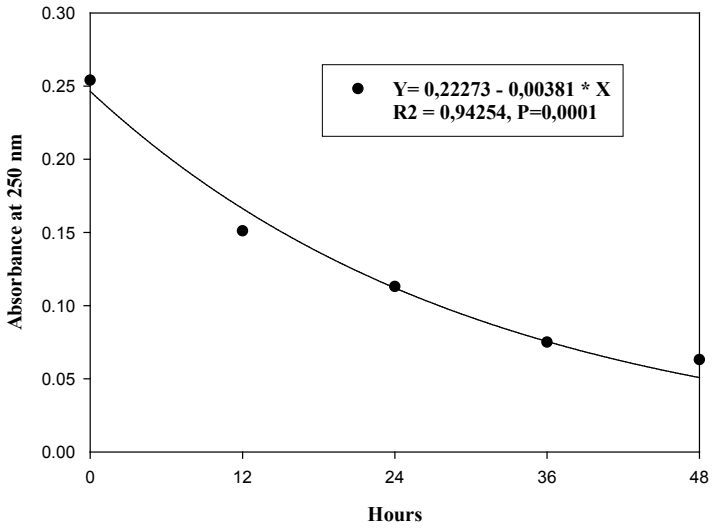

Figure 1. Reduction of phenanthrene (1 µg/mL) absorbance at 250 nm for 48 hours and the resulting decay equation

The concentration of Phe that resulted in 10% survival was 1.50 mg/L. From this concentration, the survival of the animals was determined over the following concentrations: 650, 720, 800, 890, 980, 1090, 1220 and 1350 µg/L. The LC_{50} of Phe for tambaqui (*Colossoma macropomum*) calculated by the 96-hour Trimmed Spearman-Karber method was 940 µg/L and ranged from 910 to 970 µg/L, with both values at a significance level of 95%. The computer program "LC_{50} Programs JS Pear Test" also was used to calculate the LC_{50} values for Phe every 24 h (Figure 2). The highest recorded value was at 24 h (being 1090 µg/L, ranging from 1050 to 1130 µg/L).

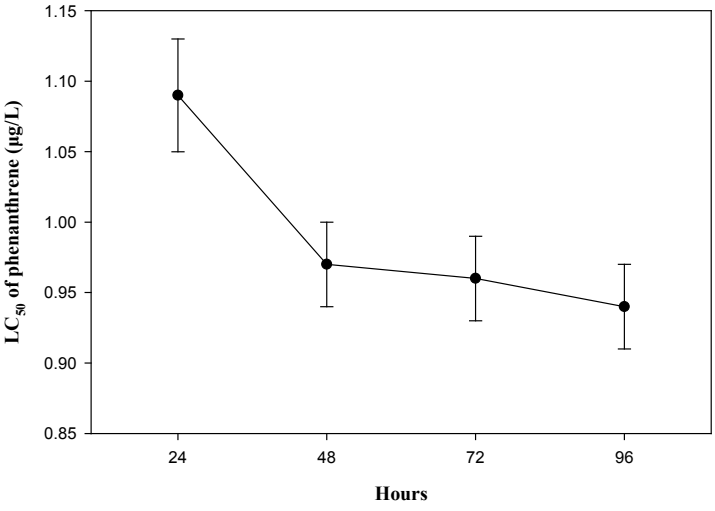

Figure 2. LC_{50} values (µg/L) over time for *Colossoma macropomum* exposed to phenanthrene in well water

Except for the lowest concentration of Phe, an increase of lip formation, mucus production, erratic swimming and disorientation was visually observed for all Phe concentrations tested. These effects were greatest at the highest Phe exposure.

3.3. Growth and Zootechnical Parameters

The values for condition factor, hepatosomatic index (HSI) and absolute weight gain are shown in table 1. Although the liver is the site of detoxification reactions, there was no significant variation in HSI after four weeks of exposure for any of the tested Phe concentrations. This contrasted with the significantly lower absolute mass gain after four weeks for all Phe concentrations tested. It also contrasts with the decrease in the condition factor after four weeks for the higher

concentrations of Phe, but not after two weeks of exposure. The relative mass gain (Figure 3A) and the specific growth (Figure 3B) confirmed the above observations for the absolute mass gain, since those parameters showed a significant decrease in relation to the control. This is evidence that exposure of tambaqui to environments contaminated with Phe, even at only 5% of its 96-h LC_{50} and even for short periods (two weeks), caused reductions in the growth and mass of the animals. This reduction in growth, both in length and mass, can be explained by the increase in feed conversion after four weeks of exposure to Phe (Figure 4A) and the consequent decrease in feed efficiency (Figure 4B).

Table 1. Effect of Phe on the condition factor (0, 2 and 4 weeks), hepatosomatic index and mass gain of tambaqui (*Colossoma macropomum*). Different lowercase letters indicate differences between treatments and different capital letters indicate differences between exposure times for the same concentration of Phe for the condition factor ($P < 0.05$)

Parameters	Exposure period (weeks)	Phenanthrene concentration (%CL_{50})			
		Control	5	25	50
Condition Factor	0	1.65 ± 0.03^{A}	1.58 ± 0.05^{A}	1.61 ± 0.04^{A}	1.69 ± 0.02^{A}
	2	1.71 ± 0.03^{A}	1.67 ± 0.04^{B}	1.73 ± 0.05^{B}	1.79 ± 0.05^{B}
	4	1.71 ± 0.05^{A}	1.52 ± 0.02^{C}	1.58 ± 0.02^{C}	1.58 ± 0.04^{C}
Hepatosomatic Index	4	2.56 ± 0.13^{a}	2.40 ± 0.07^{a}	2.31 ± 0.14^{a}	2.31 ± 0.17^{a}
Mass Gain (g)	4	13.44 ± 1.83^{a}	7.17 ± 1.39^{b}	6.99 ± 1.80^{b}	7.08 ± 1.13^{b}

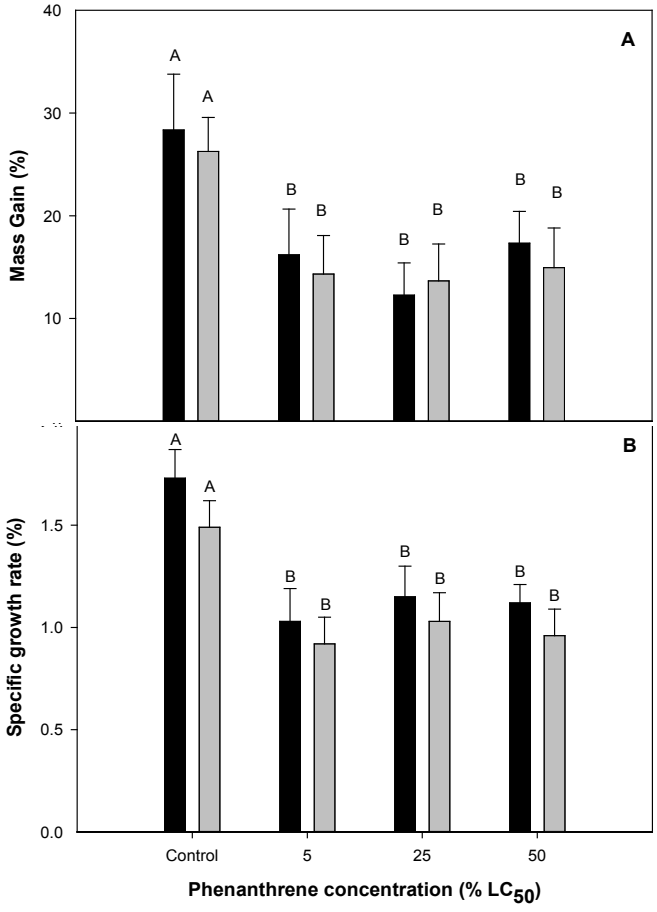

Figure 3. Effect of phenanthrene on mass gain (A) and specific growth (B) of *Colossoma macropomum*. The columns and vertical bars represent means ± SEM (n = 6). Different uppercase letters indicate difference from control ($P<0.05$). Solid bars = 14 days; grey bars = 28 days

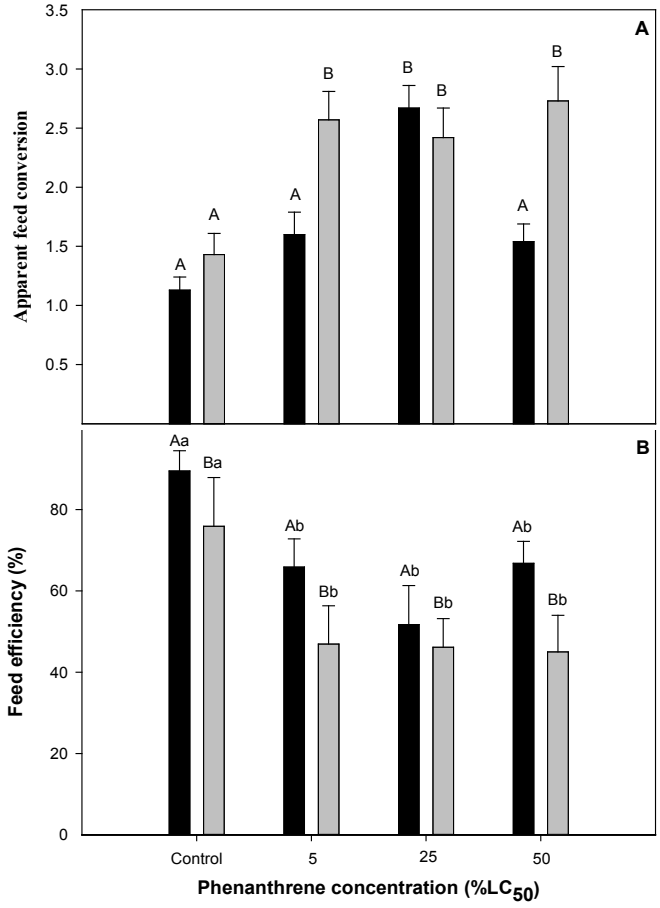

Figure 4. Effect of phenanthrene on apparent feed conversion (A) and feed efficiency (B) of *Colossoma macropomum*. The columns and vertical bars represent means ± SEM (n = 6). Different uppercase letters indicate difference from control (P<0.05). Different lowercase letters indicate difference between exposure time for a given Phe concentration (P < 0.05). Solid bars = 14 days; grey bars = 28 days

3.4. Haematology

The effect of different concentrations of waterborne Phe on haematological parameters after four weeks is shown in Table 2. Exposure to Phe at all concentrations caused mild anaemia, with a decrease in haemoglobin concentration but not haematocrit and number of circulating erythrocytes after exposure for four weeks. It is important to note that the corpuscular constants did not vary during exposure to Phe. In summary, the exposure to Phe led tambaqui to decrease haemoglobin concentration, without affecting the other blood parameters. Plasma glucose levels increased in a dose-dependent fashion and achieved a 40% increase over the control at the highest Phe water concentration, indicating that exposure to Phe was either a stressor for tambaqui or a metabolic disruptor. Lactate plasma levels are indicative of this effect, since exposure to the highest concentrations of Phe (235 and 470 µg/L) caused increases in lactate levels of 37% and 13%, respectively.

Table 2. Haematological parameters and plasma glucose and lactate of *Colossoma macropomum* after four weeks of exposure to Phe. Values represent means ± SEM (n = 6). Values sharing the same letter are not significantly different (P < 0.05)

	Phenanthrene Concentration (%LC_{50})			
Parameters	Control	5	25	50
RBC (10^6 cel/mm^3)	2.32 ± 0.09^a	2.26 ± 0.05^a	2.08 ± 0.10^a	2.25 ± 0.02^a
Hb (g/dL)	6.91 ± 0.11^a	6.08 ± 0.21^b	6.09 ± 0.10^b	6.33 ± 0.19^{ab}
Ht (%)	27.60 ± 0.68^a	27.00 ± 0.89^a	26.20 ± 1.15^a	26.40 ± 0.75^a
Glucose (mg/dL)	45.50 ± 0.77^a	55.48 ± 3.69^{ab}	57.90 ± 6.20^{ab}	63.15 ± 3.36^b
Lactate (mg/L)	39.06 ± 1.41^a	34.74 ± 4.25^a	53.36 ± 2.76^b	43.92 ± 2.91^{ab}
HCM (µm^3)	29.92 ± 0.99^a	26.86 ± 1.00^a	29.51 ± 1.35^a	28.15 ± 0.89^a
VCM (µg)	119.43 ± 3.96^a	119.31 ± 4.25^a	126.52 ± 4.61^a	117.31 ± 2.96^a
MCHC (%)	25.10 ± 0.85^a	22.57 ± 0.80^a	23.34 ± 0.75^a	24.05 ± 0.93^a

4. Discussion

The acute toxicity of Phe, in addition to its chronic effects, was determined under controlled laboratory conditions, where oxygen, pH and temperature were maintained at optimal levels. These conditions contrast with those observed in the natural habitats of the Amazonian tambaqui [14, 20, 21]. It is worthwhile to consider this aspect, since fish exposed to challenging conditions in their natural environment are more prone to intoxication with substances taken in mainly by the gill route due to increased ventilation with contaminated water [22]. Extreme hypoxia, with values below 1 mg O_2/L in warm waters, with temperatures above 32 ℃, is very common in water-bodies where the tambaqui lives. In several Amazonian locations these extreme conditions are further exacerbated by acidic pH, with values as low as pH 3.5 [14]. To cope with low oxygen availability in the water column, tambaqui expands the lower lips to capture the surface film of the water column, which is richer in oxygen [14, 15]. This adaptation acts against tambaqui in case of an oil spill, causing the animal to expose itself disproportionatelly to intoxicants dissolved in the water, as is the case of Phe. Therefore, the data obtained in this work under controlled experimental conditions could be exacerbated in the natural habitat of the species studied.

In addition, it is worth noting that water-dissolved Phe undergoes various chemical processes that need to be recognised in order to better understand the sensitivity of aquatic organisms to that compound. Among these processes is the decay of its concentration in water. Under conditions established herein, a decay of the Phe concentration in the order of 205 μg/L every 12 h was observed, which according to the literature, is related to volatilization, although generally occurring at low rates [23], photolysis, ozonisation, oxidation [24], and removal of Phe bound to fish waste, among other processes. Another process is related to the biodegradation of Phe in water and soils [23, 25].

The variation of the LC_{50} determined for tambaqui between 24 and 96 h showed a behaviour similar to that observed for other pollutants and fish species [8], that is, a decrease of the 96-h LC_{50} values for Phe as a function of the period of exposure (Figure 2). The 96-h LC_{50}-Phe for tambaqui under the described experimental conditions was 940 ± 30 μg/L Phe, which is equivalent to 5.3 ± 0.17μM. This value is similar to that determined for *Danio rerio*, which was 922.81 μg/L [26]. These values are 140 times higher than the MACeco (Maximum Acceptable Concentration for Ecosystems) [27]. Tambaqui is considered a resilient tropical species, able to survive hypoxic and acid environments [14, 28], and the data for acute exposure and chronic exposure to Phe suggest that this resilience does not extend to exposure to this PAH.

The concentrations established for chronic exposures (47, 235, 470 μg/L Phe) are equivalent to 7, 35 and 70 times the MACeco established by Verbruggen and van Herwijnen [27]. However, it is worthwhile to consider that the MACeco may vary between environments with different characteristics, such as between northern temperate and Amazonian waterbodies.

Under the chronic exposure conditions in the present study, except for the exposure to 47 μg/L Phe, there were increases in mucus production, lip formation and disorientation. In addition, a decrease in the condition factor value was observed for the experimental animals exposed to all Phe concentrations relative to the control. The condition factor decrease was accompanied by a 50% mass loss. That is, after four weeks of exposure to Phe the fish had approximately half the mass of the control fish.

Although there was no mortality during the chronic exposure period, the animals exposed to waterborne Phe, mainly in the higher concentrations, had lip swelling, erratic swimming and disorientation (personal observations) and, consequently, difficulty in catching food. Low growth due to exposure to Phe has also been observed for olive flounder exposed to a concentration equivalent to 0.3 times MACeco [29]. This adverse effect on tambaqui mass gain contrasts with the observation that no changes in hepatosomatic indices were observed (Table 1). That is, there was no hyperplasia or hepatic hypertrophy due to exposure to Phe in tambaqui specimens chronically exposed to Phe for four weeks. Increased hepatosomatic indexes have been observed in fish chronically exposed to organic contaminants, especially petroleum hydrocarbons such as Phe, both in contaminated environments and under experimental conditions [30].

The relative mass gain (Figure 3A) and the specific growth (Figure 3B) confirmed the above observations for the absolute mass gain, since these parameters showed a significant decrease relative to the control, verifying that tambaqui exposure to Phe-contaminated environments, even at concentrations as low as 47 μg/L (5% of 96-h LC_{50} or 7% of MACeco) and even for short periods (two weeks) caused reductions of growth and mass. This reduction in growth, length and mass, is explained by the increase in feed conversion after four weeks of exposure to waterborne Phe (Figure 4A) and consequent decrease in feed efficiency (Figure 4B). It has been hypothesised that detoxification mechanisms in fish exposed to Phe require energy mobilization for the functioning of these detoxification mechanisms [31-33]. If this occurs, energy mobilization simultaneously requires the use of energy reserves, which can result in weight loss, particularly when food is not sufficient to maintain organic homeostasis. These factors occurring together result in an increase in apparent feed conversion and consequent decrease in feed efficiency, exactly what was observed for tambaqui chronically exposed to waterborne Phe for four weeks.

After four weeks of exposure to waterborne Phe, blood indices were stable, except for a decrease in haemoglobin concentration compared to control animals. Even exposure

to higher concentrations did not cause changes in blood indices. In olive flounder, Jee and Kang [34] did not observe variations for red cell count, haematocrit and haemoglobin concentration for animals exposed to 89 µg/L, even after four weeks, which was similar to that observed for tambaqui. However, those authors observed significant variations for those parameters after two weeks at the highest concentration (356 µg/L) and after four weeks for the animals exposed to the intermediate concentration (178 µg/L). In the study of Jee and Kang [34] the animals were exposed for the same period and at concentrations of Phe only slightly lower than those used here in the present study (235 and 470 µg/L). In the comparison of these two species, tambaqui and flounder, it was observed that haemoglobin concentration was the most affected blood index in both species. Decreased haemoglobin concentration may occur by reduction in its synthesis or by increases in its destruction. In both cases, the process can be reflected in the other blood indexes (RBC counts, Ht, VCM, CHCM and HCM), which did not occur in tambaqui.

Glucose and lactate levels are classic indicators of stress. A dose-dependent increase in plasma glucose levels was observed; that is, the higher the Phe levels were in water, the higher the plasma glucose levels were. Similar results were observed for tambaqui exposed to crude oil and oil dispersants [2, 35]. An increase in plasma glucose levels in animals exposed to pollutants, such as Phe, is common. As it is an energetic metabolite, the organism mobilises the glucose from its reserves in order to produce the energy needed to face physiological challenges imposed by contaminated environments. This increase in glucose can be metabolised aerobically or anaerobically. In aerobic conditions, lactate plasma levels remain without significant differences in relation to the control, as was observed in *Liza aurata* [31]. However, if the animal needs to activate anaerobic metabolism, there are significant increases in lactate levels, as has been observed for tambaqui exposed to the highest concentrations (235 and 470 µg/L) of Phe. This suggests that this PAH causes disturbance in the glycolytic metabolism, either by limiting the availability of oxygen at the tissue level or by a direct effect on lactate dehydrogenase, and these conditions were not evaluated in the present study.

In summary, although tambaqui is a species resistant to several environmental challenges, it is vulnerable to Phe, an oil-derived PAH, exposure. After four weeks of exposure to Phe, bio-indexes such as condition factor, final mass gain, specific growth and feed efficiency were negatively affected. Exposure to Phe also caused reduced haemoglobin concentration, which could reduce oxygen transfer to tissues, required for xenobiotic metabolism and increases plasma levels of glucose and lactate, which are indicators of stress, confirming our hypothesis. Although there is no comprehensive survey for Phe concentration in Amazonian waters, several accidents with oil spills have been registered and have extended over small bodies of water, including marginal lakes, a habitat of the species studied here. Thus, the present study warns of the vulnerability of *C. macropomum* juveniles to chronic exposure to this crude oil derivative.

Author Contributions

CCV and ALV conceptualized the study, CACV performed the experiments, ALV and CCV performed the formal data analysis, ALV obtained the funding, CACV wrote the first draft of the manuscript, ALV reviewed and edited.

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Conflicts of Interest

The authors declare no conflict of interest.

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