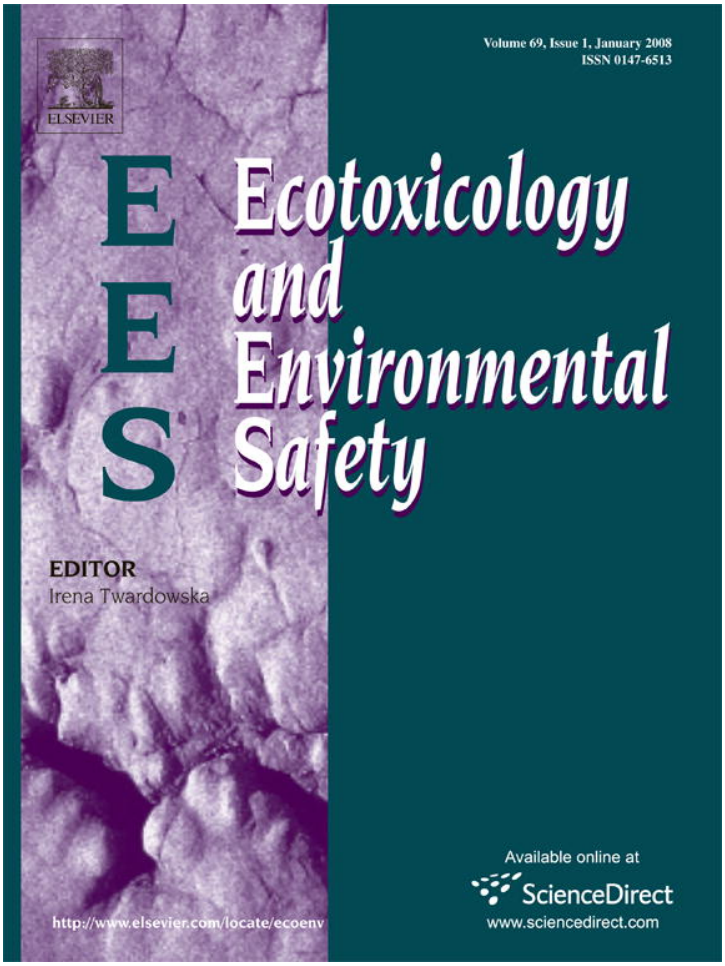

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Biochemical, physiological, and histological changes in the neotropical fish *Prochilodus lineatus* exposed to diesel oil

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Abstract

Toxicity tests were conducted simulating a diesel oil spill in a tropical environment and juveniles of *Prochilodus lineatus* were exposed to the water-soluble fraction of diesel oil (WSD) for 6, 24, 96 h, and 15 days. The results showed the activation of biotransformation pathways for xenobiotics, through a time-dependent increase of liver GST activity. WSD caused a decrease in hematocrit and hemoglobin content, very likely due to hemolysis. Furthermore, an increase in glucose levels was observed after acute exposure to WSD. A possible lack of cortisol response could also be associated with WSD, since a reduction in plasma cortisol was seen in fish exposed to the petroleum product for 15 days. Moreover, the occurrence of lesions in the gills and even more severe lesions in the liver, should lead to functional damage to both organs, interfering thus directly with fundamental processes for the maintenance of homeostasis in this fish.

Keywords: Petroleum products; Biochemical biomarkers; Hematology; Stress response; Histopathology; Freshwater pollution

1. Introduction

Among the different types of pollutants, petroleum products are one of the most relevant to aquatic ecotoxicology (Pacheco and Santos, 2001a). In freshwater ecosystems, one of the largest oil spills occurred in 2001 in Barigui River, in Paraná, southern Brazil, when 50.000 L of crude oil were accidentally discharged (Akaishi et al., 2004). Although these kind of large oil spills are widely covered in the media, it is believed that the principal source of inland waters contamination from petroleum and its derivatives is due to small and continuous leakages from underground bulk storage tanks, thereby reaching groundwater and later rivers (Tiburtius et al., 2005). However, little research has been done on the effects of petroleum products on tropical freshwater organisms (Pollino and Holdway, 2003; Akaishi et al., 2004).

Exposure to crude oil and derivatives can induce a variety of toxic symptoms in experimental animals. Petroleum hydrocarbons can act as a mediator in free radical generation in fish (Achuba and Osakwe, 2003). Studies with the goldfish *Carassius auratus* has shown an increase in antioxidant defenses in animals after exposure to different concentrations of the water-soluble fraction of diesel oil (WSD) for various experimental times (Zang et al., 2003, 2004). Other studies have also indicated that the exposure of fish to a water-soluble fraction of petroleum derivatives causes different effects in cortisol plasma concentrations (Alkindi et al., 1996; Pacheco and Santos, 2001a, b), suggesting that these contaminants might interfere in the fish stress response.

Some authors have shown a relationship between exposure to petroleum hydrocarbons and hemolysis and/or hemorrhage (Alkindi et al., 1996), while others have observed an increase in hematocrit of fish exposed to a

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WSD (Davison et al., 1992). Some works have also shown structural damage to organs and tissues related to the exposure of fish to petroleum derivatives (Engelhardt et al., 1981; Khan, 1998, 2003).

Despite these previous investigations carried out on petroleum derivatives effects on fish, some toxicological response levels in fish remain poorly understood, revealing the lack of data regarding the stress mechanism, as well as biotransformation and genotoxic responses (Pacheco and Santos, 2001a). In particular, there are only few reports concerning the effects of diesel oil exposure on morphological and physiological parameters in freshwater fish (Zang et al., 2003; Simonato et al., 2006) and there is a real need of information about the effects of this fuel oil on Neotropical freshwater fish species.

The fish species *Prochilodus lineatus* (Valenciennes, 1847) (= *P. scrofa* Steindachner, 1881) is native to the south and southeast regions of Brazil and represents a well suited species to environmental monitoring as it is a bottom feeder fish which is in contact with xenobiotics in water and in sediment and also has been shown to be sensitive to variations in water quality (Mazon and Fernandes, 1999; Martinez and Souza, 2002; Da Silva et al., 2004; Martinez et al., 2004; Almeida et al., 2005; Camargo and Martinez, 2006).

Thus, considering the growing cases of environmental accidents involving spills of petroleum distillate products into continental waters in the last years in Brazil, the aims of the present study were to investigate biochemical, physiological, and histopathological parameters of *Prochilodus lineatus* exposed to diesel oil as potential biomarkers to assess pollution by these petroleum products and accordingly to get information about the threat imposed by these spills to this neotropical fish species.

2. Materials and methods

2.1. Animals

Juvenile specimens of *Prochilodus lineatus* (Characiformes, Prochilodontidae), weighting 29.1 ± 14.7 g (mean $\pm$ SD, $n = 114$), were supplied by the Universidade Estadual de Londrina hatchery station. Prior to the toxicity tests, fish were acclimated to laboratory conditions for a minimum of 7 days in a 600-L tank with dechlorinated water ($T \cong 21.3$ °C; pH $\cong 7.35$; OD $\cong 7.79$ mgO_2 L^{-1}; conductivity $\cong 110\,\mu S\,cm^{-1}$; $Na^+ \cong 0.086$ mM; $K^+ \cong 0.030$ mM; $Cl^- \cong 0.103$ mM; hardness $\cong 80$ mg L^{-1} $CaCO_3$). During this period, fish were fed with commercial pellet food (32% of protein) each 48 h.

2.2. Preparation of WSD

To obtain the WSD, one part of commercial diesel oil was added to four parts water in a glass container. The mixture was then exposed to intense sunlight for 6 h, simulating a diesel spill in tropical conditions (Nicodem et al., 1998). After that the upper insoluble phase was discharged and the remaining water phase was collected and diluted to 50% WSD with dechlorinated water. WSD (before and after dilution) was examined spectrofluorimetrically for the presence of mono- and polyaromatic hydrocarbons.

2.3. Tests for acute and sub-chronic toxicity

Fish were submitted to acute (6, 24, and 96 h) and subchronic (15 days) static toxicity tests, performed in glass aquaria of 100 L, each containing eight fish. One control group, consisting of eight animals exposed only to water (the same as that used for acclimation), was sampled at each experimental interval along with the experimental groups exposed to water plus WSD. Replicates were carried out for each experimental time. During the tests water was continuously monitored for temperature, dissolved oxygen, pH, and conductivity.

2.4. Sampling

Immediately after removing the fish from the aquaria, they were anesthetized with benzocaine ($0.1\,g\,L^{-1}$), and blood samples were taken from the caudal vein by means of heparinized plastic syringes. Subsequently, fish were killed by cervical section and their livers and gills immediately removed. Blood was then centrifuged for 5 min at 3000*g* and plasma samples were stored frozen (−20 °C). One part of the liver and the gills were fixed for histological analysis and the other part of the liver was frozen at −80°C for biochemical analysis.

2.5. Physiological parameters

Hematocrit values were determined by blood centrifugation (5 min, 5000*g*) in glass capillaries, using a microhematocrit centrifuge. Total hemoglobin content of the blood was measured by the cyanomethemoglobin method using commercial available kit (Analisa, Brasil) in spectrophotometer at 540 nm. Plasma osmolarity was determined with a freezing point osmometer. Plasma Na^+ and K^+ were measured in diluted samples (1:100) against known standards by flame photometry. Plasma chloride concentration was determined by the thiocyanate method using a commercial kit (Labtest, Brazil) in spectrophotometer at 470 nm. Cortisol was analyzed with a commercial immunoenzymatic kit (Active® Cortisol EIA, Diagnostic Systems, USA) and the readings carried out in a microplate reader at 450 nm. Plasma glucose was analyzed using a colorimetric commercial kit (GLUCOX 500-Doles Reagentes, Brazil), based on the glucose-oxidase reaction, in spectrophotometer at 505 nm. Total protein concentration was determined according to Lowry et al. (1951), using bovine serum albumin (BSA) for the calibration curve. All samples were analyzed in duplicate.

2.6. Biochemical assays

Liver samples were weighed, homogenized in 10 volumes of 0.1 M phosphate buffer, pH 7.0, and then centrifuged for 20 min at 14,700*g* (4 °C) to obtain the supernatant for glutathione-*S*-transferase (GST) and catalase analyses. GST activity was determined as described by Keen et al. (1976) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The change in absorbance was recorded at 340 nm and the enzyme activity was calculated as nM CDNB conjugate formed min^{-1} mg^{-1} protein using a molar extinction coefficient of $9.6\,mM\,cm^{-1}$. Catalase activity was estimated from the rate of consumption of hydrogen peroxide levels (Beutler, 1975). Change in absorbance was recorded at 240 nm and enzyme activity was expressed as μM H_2O_2 consumed min^{-1} mg^{-1} protein. Total plasma and liver proteins were measured by the method of Lowry et al. (1951) with BSA as standard. All samples were analyzed in duplicate.

2.7. Histological analyses

For histological studies, the liver and gills were first fixed in a solution containing alcohol, formalin, and acetic acid (ALFAC) and then stored in 70% alcohol. The organs were embedded in paraffin, sectioned (5 μm), and the slides were stained with hematoxylin and eosin (HE). The sections were examined by light microscopy, using as reference Takashima and

Hibiya (1995), and photographed using a digital camera. The presence of histological alterations for each organ was evaluated semi-quantitatively by the degree of tissue change (DTC), which is based on the severity of the lesions. For DTC calculation (modified from Poleksić and Mitrović-Tutundžić, 1994), the alterations in each organ were classified in progressive stages of damage to the tissue: stage I alterations, which do not alter the normal functioning of the tissue; stage II, which are more severe and impair the normal functioning of the tissue; and stage III, which are very severe and cause irreparable damage. A value of DTC was calculated for each animal by the formula: $DTC = (1 \times \Sigma I) + (10 \times \Sigma II) + (100 \times \Sigma III)$ where I, II and III correspond to the number of alterations of stages I, II and III, respectively. DTC values between 0 and 10 indicate normal functioning of the organ; values between 11 and 20 indicate slight damage to the organ; values between 21 and 50 indicate moderate changes in the organ; values between 50 and 100 indicate severe lesions and values above 100 indicate irreversible damage to the organ (Poleksić and Mitrović-Tutundžić, 1994).

2.8. Statistical analysis

The results obtained for each experimental group were compared with that of the respective control group utilizing Student's parametric *t*-test or the non-parametric Mann-Whitney test, depending on the distribution of the data. Values of $P \leqslant 0.05$ were considered significant.

3. Results

3.1. Water analysis

Analysis of WSD by spectrofluorimetry showed the presence of monoaromatic and polyaromatic hydrocarbons at all the exposure times. Fluorescence emission peaks indicated a predominance of benzene, toluene, xylene, naphthalene, fluorine, and phenanthrene. The physical-chemical characteristics of the water for all the experimental periods remained stable. The mean values obtained for the experimental groups were (mean ± SE): temperature, 22.43 ± 0.14 °C; pH, 6.96 ± 0.04; DO, 6.52 ± 0.12 mg $O_2\,L^{-1}$; and conductivity, 107.89 ± 3.22 μS cm^{-1}. For the control groups, the values obtained were: temperature, 22.82 ± 0.12 °C; pH, 6.98 ± 0.04; DO, 6.65 ± 0.12 mg $O_2\,L^{-1}$; and conductivity, 116,24 ± 3.36 μS cm^{-1}.

3.2. Hematocrit and hemoglobin

The hematocrit and hemoglobin content of fish exposed to WSD for 96 h and 15 days were significantly lower in relation to the respective control groups. Potassium showed an increase for these same experimental times and also for 6 h in fish exposed to diesel oil (Fig. 1).

3.3. Cortisol and glucose

Animals exposed to WSD for 15 days showed a significant reduction in plasma cortisol concentration, in relation to the respective control, whereas plasma glucose in fish exposed to WSD for 24 and 96 h was significantly greater compared to the respective control groups (Fig. 2).

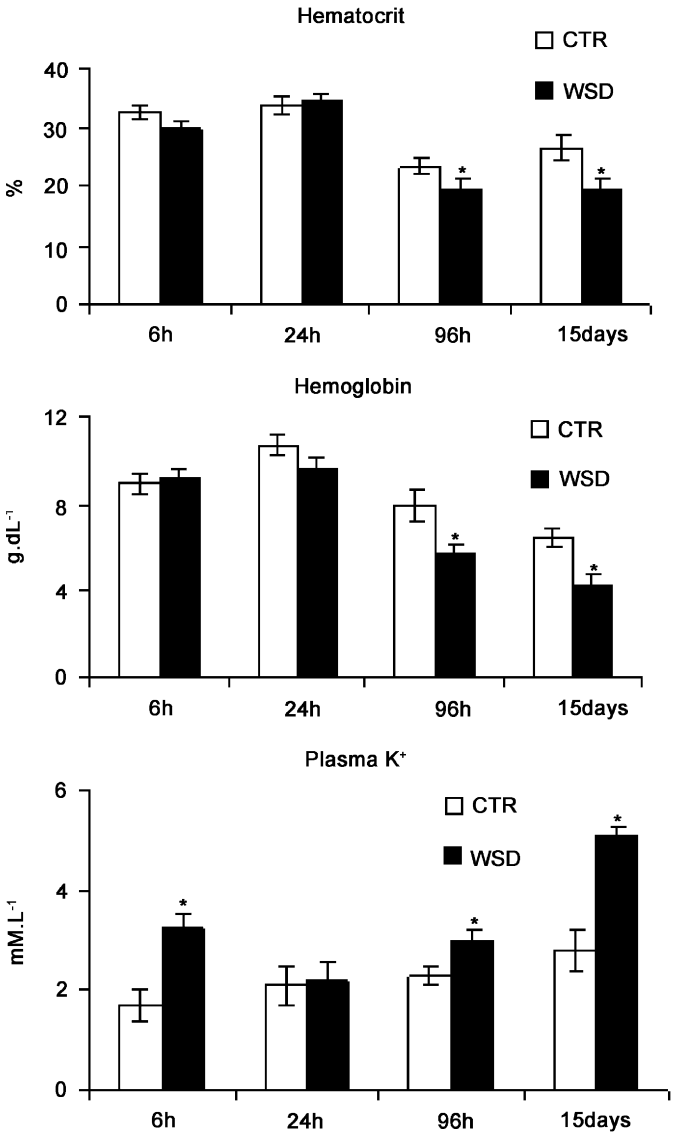

Fig. 1. Hematocrit (%), hemoglobin content ($g\,dL^{-1}$) and plasma potassium (mML^{-1}) in *Prochilodus lineatus* exposed to water-soluble fraction of diesel (WSD) or only water (CTR) for different experimental periods. Bars represent means and vertical lines the SE. *Significantly different from respective control ($P < 0.05$).

3.4. Hepatic catalase and GST activities

Hepatic catalase activity did not vary significantly in the groups exposed to WSD compared to the respective control groups. On the other hand, hepatic GST activity showed a time-dependent increase and it was significantly greater in fish exposed to WSD for 96 h and 15 days, in relation to the respective control groups (Fig. 3).

3.5. Metabolic and osmo-ionic parameters

Plasma total protein levels in fish exposed to WSD for 15 days were significantly reduced in relation to the control group. In animals exposed to WSD for 6 h, the plasma sodium concentration was significantly lower compared to

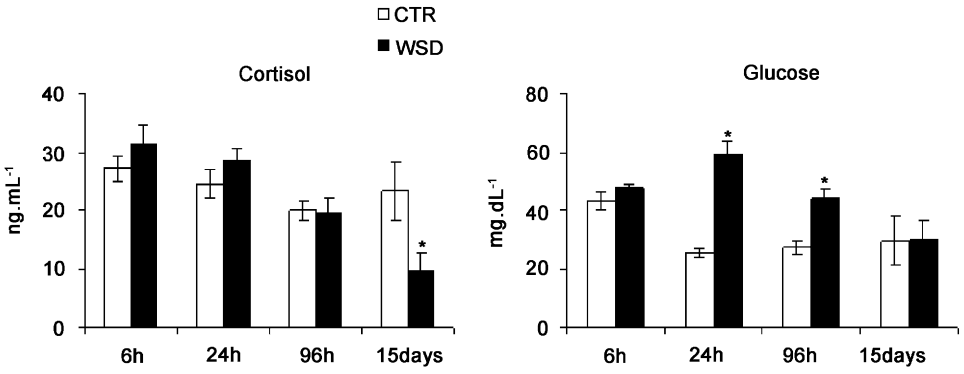

Fig. 2. Plasma cortisol (ng mL^{-1}) and glucose (mg dL^{-1}) concentrations in *Prochilodus lineatus* exposed to a water-soluble fraction of diesel (WSD) or only water (CTR) for different experimental periods. Bars represent means and vertical lines the SE. *Significantly different from respective control ($P<0.05$).

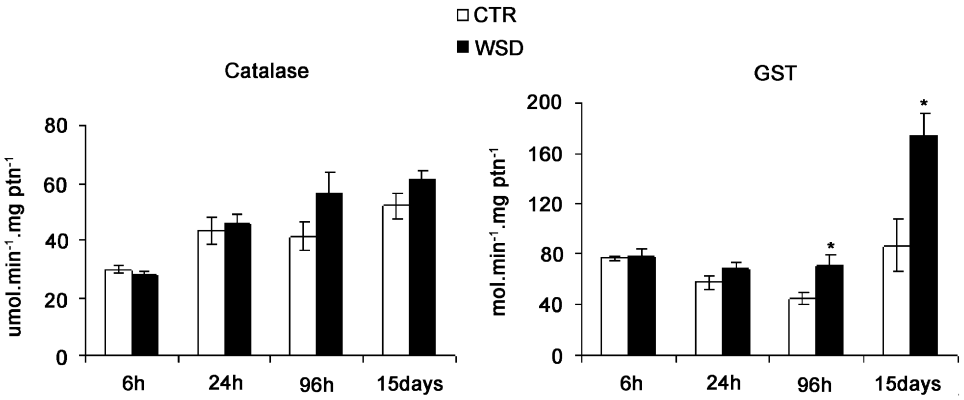

Fig. 3. Hepatic activity of catalase and glutationa-*S*-transferase (GST) of *Prochilodus lineatus* exposed to water-soluble diesel (WSD) or only water (CTR) for different experimental periods. Bars represent means and vertical lines the SE. *Significantly different from respective control ($P<0.05$).

Table 1
Osmolarity, total protein, Na^+ and Cl^- concentrations in plasma of *Prochilodus lineatus* exposed to WSD or only to water (CTR) for different periods

Parameters		6 h	24 h	96 h	15 days
Osmolarity (mOsm L H_2O^{-1})	CTR	306.3±3.2(16)	263.6±4.2(11)	299.0±5.4(12)	278.7±25.2(3)
	WSD	320.4±10.9(16)	264.7±6.1(11)	307.1±5.3(14)	298.7±20.1(7)
Protein (mg mL^{-1})	CTR	23.3±0.4(7)	19.4±0.6(11)	21.0±0.7(12)	30.1±1.6(4)
	WSD	23.8±0.4(6)	20.0±0.6(12)	20.4±0.9(15)	22.6±1.2(6)*
Na^+ (mM L^{-1})	CTR	137.0±2.7(16)	140.0±2.7(11)	130.3±3.1(12)	132.0±2.2(4)
	WSD	128.1±2.1(16)*	137.2±2.0(11)	129.0±3.0(14)	133.7±4.2(8)
Cl^- (mM L^{-1})	CTR	99.0±1.7(8)	104.9±1.5(10)	102.9±2.4(12)	101.6±5.8(3)
	WSD	94.92±1.56(8)	106.4±1.7(11)	107.9±2.4(14)	104.2±5.5(5)

Values are mean±SE (*n*). *Significantly different from respective control ($P<0.05$).

the respective control. No significant alterations were found in osmolarity and plasma chloride concentration (Table 1).

3.6. Histopathological analyses

Fish exposed to WSD showed a series of histological alterations in the gills and liver (Table 2). The most relevant branchial changes were: epithelial lifting (Fig. 4C), hyperplasia with epithelial lifting (Fig. 4B), and aneurysm (Fig. 4D). The most relevant histological alterations observed in the liver of fish exposed to WSD were: biliary stagnation and nuclear and cellular degeneration (Fig. 5). Biliary stagnation was found in all animals examined, including the animals in the control groups (Table 2).

The DTC values determined for the gills of *Prochilodus lineatus* exposed to WSD for 24 and 96 h and 15 days were significantly greater than those of the respective control groups, with a mean value of 42, indicating moderate damage to the gills. Liver DTC values determined for fish

Table 2
Alterations found in the gills and liver of *Prochilodus lineatus* exposed to diesel water-soluble fraction and their respective stages of damage to the tissue

Gills	Liver
Stage I	
Hyperplasia of the gill epithelium	Nuclear hypertrophy
Sinus constriction	Irregular shaped nucleus
Hypertrophy of the gill epithelium	Nucleus in a lateral position
Blood congestion	Cellular hypertrophy
Dilation of the marginal channel	Cytoplasmic vacuolation
Epithelial lifting of lamellae	Cellular atrophy
Lamellar fusion	Irregularly shaped cells
Lamellar disorganization	Eosinophilic granules in cytoplasm
	Melanomacrophages aggregates
	Nuclear atrophy
	Peripheral nuclei
	Cytoplasmic vacuolation
Stage II	
Lamellar aneurysm	Nuclear vacuolation
Rupture of epithelial cells with hemorrhage	Cytoplasmic degeneration
Complete fusion of all the lamellae	Cell rupture
Rupture of pillar cells	Blood congestion
Rupture of the lamellar epithelium	Nuclear degeneration
	Pyknotic nucleus
	Bile stagnation

exposed to WSD were greater than those of the respective controls, in all exposure periods and the mean DTC value for liver of groups exposed to WSD was 73, indicating severe damage and possible impairment of hepatic function (Fig. 6).

4. Discussion

Diesel oil, as with other distilled products of petroleum, shows a low solubility in water, and therefore, laboratory investigations of its effects involve the preparation of water-soluble fractions. In the present work, fish were exposed to an experimental solution containing 50% of a WSD. The analyses conducted showed that this WSD contained polar compounds and aromatic hydrocarbons of two toxicologically relevant groups: BTX (benzene, toluene, and xylene) and polyaromatic hydrocarbons (PAHs) of low molecular weight such as naphthalene, fluorene and phenanthrene. A similar composition of a WSD has been previously reported by others (Anderson et al., 1974; Pacheco and Santos, 2001a).

The exposure of fish to WSD for 96 h and 15 days caused a decrease in hematocrit and hemoglobin content, which coincided with an increase in plasma K^+. Together, these results indicate that the increase in K^+ must have occurred due to hemolysis, which also accounted for the decrease in hematocrit and hemoglobin content. Therefore, prolonged exposure of more than 96 h to hydrocarbons present in WSD appears to have induced hemolysis in *Prochilodus lineatus*. Similar effects were found in flounder (*Pleuronectes flesus*) after 24 and 48 h exposure to a water-soluble

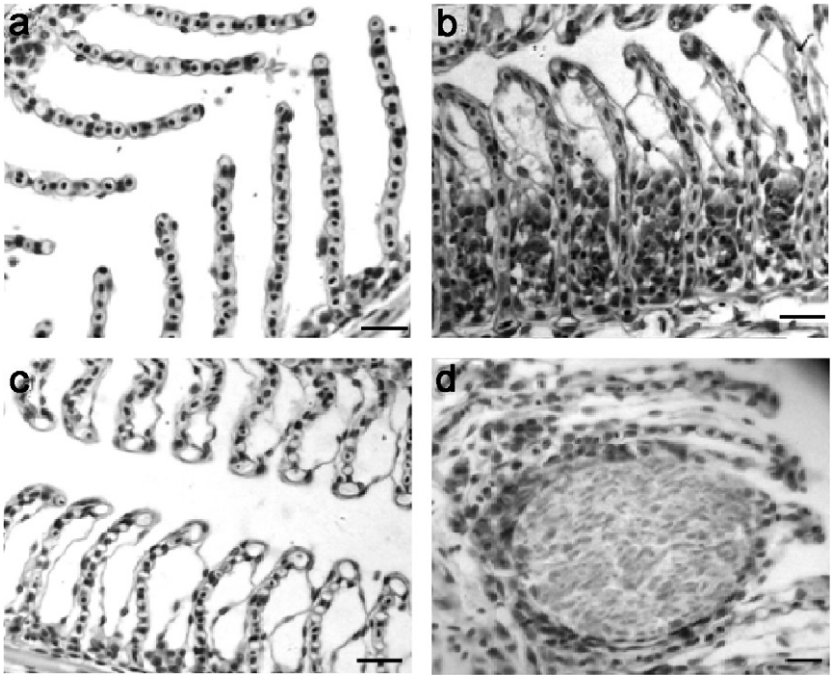

Fig. 4. Photomicrograph of gills of *Prochilodus lineatus*. (A) Animal exposed only to water (control); (B) animal exposed to WSD for 6 h, showing hyperplasia of the epithelium (arrow) and epithelial lifting; (C) animal exposed to WSD for 15 days, showing extensive epithelial lifting (arrow); and (D) animal exposed to WSD for 6 h, showing an aneurysm. HE. Scale bar corresponds to 50 μm.

fraction of crude oil, that is, a reduction in hematocrit and hemoglobin accompanied by an increase in plasma potassium concentration, without an effect on plasma concentrations of sodium or on plasma osmolarity (Alkindi et al., 1996).

On the other hand, different results were reported for the Antarctic fish *Pagothenia borchgrevinki* which showed an increase in hematocrit and hemoglobin content after acute exposure to a WSD (Davison et al., 1992, 1993). Thus, it is clear that exposure to the same chemical agent can induce different alterations (increase or decrease) in hematological parameters (Ranzani-Paiva and Silva-Souza, 2004), which can indicate adaptive responses to a stress agent or the direct effects of these contaminants on erythrocytes or their production.

Normally, after exposure to a stressor agent, a significant increase in glycemia occurs. In the present study, the animals showed a hyperglycemic response after 24 and 96 h exposure to WSD, indicating the provision of energy reserves for immediate utilization (Val et al., 2004). Similarly, Alkindi et al. (1996) also observed in flounder (*Pleuronectes flesus*) significantly elevated plasma glucose concentrations after 3 h exposure to the water-soluble fraction crude oil and an increase of more than 50% after 48 h. However, Pacheco and Santos (2001b) did not find significant differences in glucose values in eel (*Anguilla anguilla*) exposed to WSD for 3 and 4 h, in relation to the control groups. According to these authors, glucose may not be the most important fuel in energy metabolism in this species of eel.

The stress response involves a series of physiological changes, and their primary effects result in the release of catecholamines and corticosteroids into the circulation (Wendelaar Bonga, 1997). In teleost fish, an elevation in plasma cortisol, produced in the interrenal cells of the anterior kidney (Hontela, 1997), is known as the main hormonal response to stress (Wendelaar Bonga, 1997), and the elevation of plasma levels of this hormone is often used as a biomarker. In flounder (*Pleuronectes flesus*) exposed to a water-soluble fraction of crude oil for 3, 24, and 48 h, Alkindi et al. (1996) demonstrated an increase in plasma cortisol concentration. However, in the present work, plasma cortisol values varied only after 15 days exposure to WSD, when a significant decrease in this parameter was observed. Similarly, Pacheco and Santos (2001a) noted a decrease in plasma cortisol concentrations in eel (*A. anguilla*) exposed to WSD from 3 h to 3 days, and correlated this reduction to a possible direct cytotoxic action of WSD in interrenal cells. However, in another study in the same species exposed to WSD, Pacheco and Santos (2001b) observed an increase in plasma cortisol after 3 h. This shows that the response of cortisol not only occurs differently among distinct species but can also vary within the same species. Endocrine control of cortisol secretion in teleost fish is very complex (Wendelaar Bonga, 1997), and therefore, different types of acute stressors can cause distinct responses (Martins et al., 2000). In this study, since the release of cortisol appears to have been blocked, the increase in plasma glucose at 24 and 96 h must have

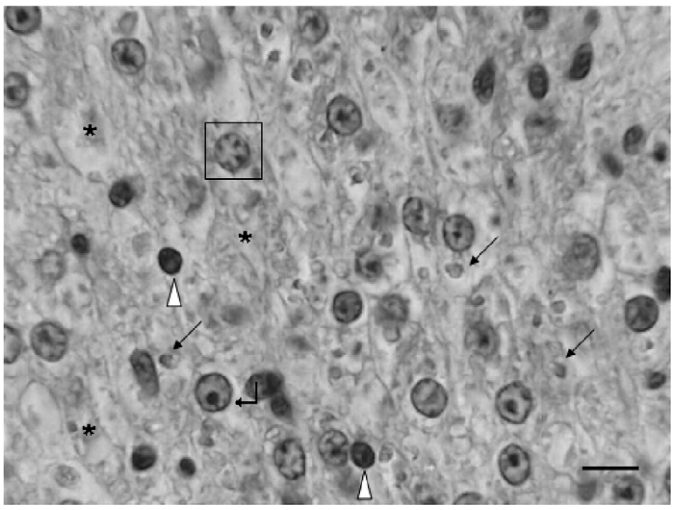

Fig. 5. Photomicrograph of liver of *Prochilodus lineatus* exposed to WSD for 15 days, showing biliary stagnation (arrow), pyknotic nuclei (arrowhead), cellular degeneration (*), nuclear degeneration (□), increased nuclear volume (↵). HE. Scale bar corresponds to 50 μm.

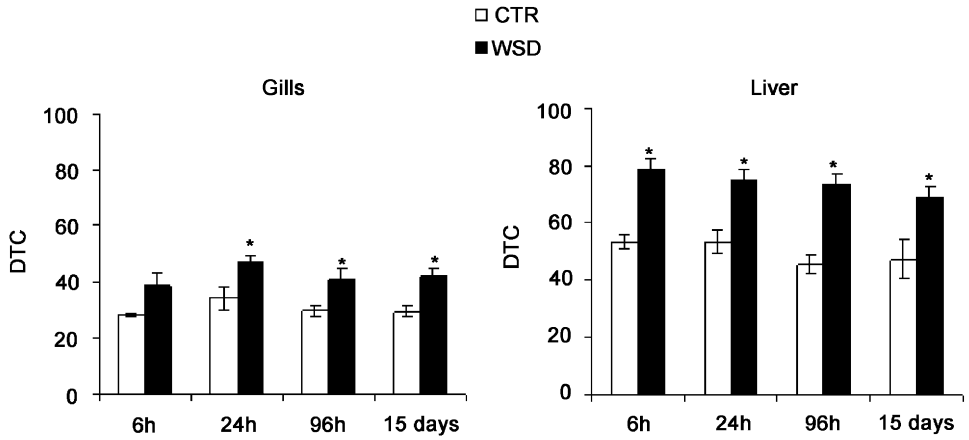

Fig. 6. Degree of tissue change (DTC) for gills and liver of animals exposed to WSD and their respective control groups (CTR). Bars represent means and vertical lines the SE (N gills = 5, N liver = 6). *Significantly different from respective control ($P<0.05$).

occurred through the catecholamine pathway, and the hyperglycemic state was not maintained with sub-chronic exposure due to the lack of a cortisol response.

Protein metabolism can provide information on the general energy mobilization of an animal and show relationships with effects of contaminants in these organisms (Adams et al., 1990). In the present study, fish exposed to WSD for 15 days showed a significant reduction in plasma protein concentration compared to the control group. It is known that the release of catecholamines and cortisol causes a variety of physiological and biochemical alterations, including hyperglycemia, glycogen depletion, and catabolism of plasma proteins, among others. These responses can be considered adaptive processes that help the organism with increased energy demand during exposure to stress factors (Martinez and Cólus, 2002). As indicated above, the animal exposed to WSD for 15 days did not show variation in glycemia in relation to control group. This could have been due the depletion of carbohydrate reserves because of the greater energy demand in this group of animals, which would resort to metabolizing proteins in response to a stressor agent.

The body fluids of freshwater fish are hyperosmotic compared to the aquatic medium. Therefore, these animals need to avoid water gain and loss of salts and contaminants presenting the water can interfere with this osmotic-ionic balance (Heath, 1995). Disturbances in the concentrations of plasma ions and/or in osmolarity have already been shown in various species of fish exposed to elevated concentrations of petroleum hydrocarbons (Engelhardt et al., 1981). However, in the present study, animals exposed to WSD did not show significant alterations in osmolarity and only little change in plasma chloride concentration. Davison et al. (1993) also found no significant alterations in plasma chloride and osmolarity after exposure of *Pagothenia borchgrevinki* to a WSD for 7 days, in relation to the control group. On the other hand, *Prochilodus lineatus* exposed to WSD for 6 h showed a reduction in plasma Na^+, which was re-established after 24 h exposure, indicating a transitory ionic alteration without major effects on osmotic–ionic homeostasis in these fish.

The liver in fish is an organ that performs various functions associated with the metabolism of xenobiotics (Jimenez and Stegeman, 1990). Hepatocytes like other cells are dependent on antioxidant enzymes for protection against reactive oxygen species (ROS), produced during the biotransformation of xenobiotics (Landis and Yu, 1995). Among the enzymes that comprise this defense system is catalase, responsible for the removal of hydrogen peroxide (H_2O_2) which is metabolized to O_2 and water (Van der Oost et al., 2003). Dose-dependent increases in catalase activity in the liver and other organs were found after 14, 21, and 28 days in African catfish (*Clarias gariepinus*) exposed to crude oil (Achuba and Osakwe, 2003). This showed that petroleum hydrocarbons are potent mediators of free radical formation in fish and that the increase in catalase activity in all the tissues examined can represent an adaptive response to protect the fish from the toxicity of free radicals induced by these hydrocarbons. Zang et al. (2003) exposed goldfish (*Carassius auratus*) to different concentrations of diesel oil for 40 days and also found that catalase activity increased significantly at one of the concentrations tested. However, in the present study, animals exposed to diesel did not show significant alterations in hepatic catalase activity in relation to the control group, although there was a tendency for an increase in enzyme activity with longer the longer exposure time. The fact that hepatic catalase activity was not induced does not exclude the possibility that there was ROS formation after exposure to WSD, because other enzymes such as glutathione peroxidase can metabolize hydroperoxides. This indicates the need to include more anti-oxidant parameters in the study of the possible effects of WSD on the generation of ROS and oxidative stress.

A rich supply of non-specific enzymes, such as glutathione transferases, enables the liver to metabolize a large spectrum of organic substances (Landis and Yu, 1995). In the present study, GST showed a time-dependent increase in *Prochilodus lineatus* exposed to WSD with a significant increase after 96 h and doubled after 15 days exposure. This increase (15 days) was also demonstrated after exposure of *Carassius auratus* to a WSD (Zang et al., 2004). Other authors also found that the activities of detoxification enzymes, such as GST, are increased in the presence of polycyclic hydrocarbons (Stien et al., 1998; Van der Oost et al., 2003). Therefore, the increase in GST showed in the present study reinforces the important role of this enzyme in the biotransformation of compounds present in WSD and shows that GST activity can be a good biomarker for contamination by petroleum derivatives.

Gills are extremely important in respiration, osmoregulation, acid–base balance, and excretion of nitrogenous wastes in fish (Heath, 1995), and they represent the greatest surface area of the animal in contact with external environment. Therefore, their morphology can be very useful as a parameter in environmental monitoring (Schwaiger et al., 1997). In the present study, the gills of *Prochilodus lineatus* exposed to WSD showed the occurrence of histological alterations. Some of these alterations can be considered adaptive, since they protect the organism from the entrance of xenobiotics, such as in the case of epithelial lifting. Aneurysm, on the other hand, represents a lesion that can result from the rupture of pillar cells, and thus corresponds to the deleterious effect of xenobiotics on branchial tissue (Martinez et al., 2004). Semi-quantitative analysis of the lesions showed that the gills of animals exposed to WSD were more affected than respective controls, with DTC values indicating the occurrence of moderate damage. These results showed that diesel oil causes branchial lesions in *Prochilodus lineatus*, which, despite not having affected osmotic–ionic homeostasis, can compromise the other various functions of the gills and thereby cause functional disturbances in these fish. Khan (1998) also found in the gills of flounder (*Pleuronectes*

americanus) collected near an oil refinery, hyperplasia in the lamellae and in the inter-lamellar space, as well as epithelial lifting, and this author suggested that the lesions found were related to the oil spills. In another study with flounder collected at a location contaminated with PAHs, gills showed alterations such as hyperplasia and hypertrophy of the lamellar epithelium resulting in fusion and increase in mucus production (Khan, 2003). Rainbow trout (*Onchorrynchis mykiss*) exposed to two types of petroleum also showed gills with anomalies in the lamellar epithelium and drops of oil located between the lamellae (Engelhardt et al., 1981).

The liver can be considered a target organ and of great importance to fish, since it participates in processes such as the biotransformation and excretion of xenobiotics. Therefore, the liver can be studied in environmental monitoring due to its high sensitivity to contaminants (Thophon et al., 2003). Thus, alterations in its structure can be significant in the evaluation of the health of fish (Myers et al., 1998). Biliary stagnation, observed indistinguishably in the liver of control fish as well as of fish exposed to WSD, corresponds to an accumulation of bile inside hepatocytes, evidenced by the appearance of yellow cytoplasmic granules (Fanta et al., 2003). This alteration consists of the manifestation of a physiopathological condition caused by a lack of bile metabolism and excretion (Pacheco and Santos, 2002), in which bile secreted by hepatocytes remains inside cells and is not released into the digestive tract (Fanta et al., 2003). In the present study, the occurrence of this alteration was not associated with the presence of a xenobiotic, since it was also observed in the control group, and may reflect some nutritional problem resulting from the feeding of the fish in captivity.

In the present study, fish exposed to WSD showed diverse hepatic histological alterations, where some of these alterations such as increase in cellular and nuclear volume can be considered responses to a stressor agent, since they indicate functional activation of this organ. Others such as cellular and nuclear degeneration represent lesions that can culminate in the compromise of the organ, thus corresponding to damage to the tissue caused by exposure to xenobiotics. Quantitative analysis of the hepatic lesions showed that in animals exposed to WSD for all experimental times, the liver was extremely affected, with mean DTC values greater than 50, indicating severe damage to the organ. A study in flounder (*Pleuronectes vetulus*) exposed to sediment contaminated with PAHs, also revealed the presence of severe hepatic lesions, besides metabolites of these compounds in bile (Myers et al., 1998). Flounder (*Pleuronectes americanus*) collected near an oil refinery also showed various histological lesions in the liver which were considered indicative of the impact of the oil on the health of these fish (Khan, 1998, 2003).

In summary, the results obtained in this work showed clearly that the WSD can cause important alterations in *Prochilodus lineatus*, at the biochemical level up to the tissue level. At the biochemical level, there was activation of phase II biotransformation pathways for xenobiotics, through a time-dependent increase of GST. It could be shown that WSD causes a decrease in hematocrit and hemoglobin content, very likely due to hemolysis. Furthermore, an increase was observed in glucose levels after acute exposure to WSD, probably mediated by catecholamines. A possible lack of cortisol response could also be associated with WSD, since a reduction in plasma cortisol was seen in fish exposed to the petroleum derivative for 15 days; this effect can lead to the compromise of adaptive stress responses. Moreover, the occurrence of lesions in the gills and even more severe lesions in the liver, should lead to functional damage to both organs, interfering thus directly with fundamental processes for the maintenance of homeostasis in these fish.

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