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

Juliana Delatim Simonato[a], Carmen L.B. Guedes[b], Cláudia B.R. Martinez[a,*,☆,☆☆]

[a]*Departamento de Ciências Fisiológicas, Universidade Estadual de Londrina, C.P. 6001, CEP:86051-990, Londrina, Paraná, Brasil*
[b]*Departamento de Química, Universidade Estadual de Londrina, C.P. 6001, CEP:86051-990, Londrina, Paraná, Brasil*

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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

*Corresponding author. Fax: +55 43 3371 4467.
E-mail address: cbueno@uel.br (C.B.R. Martinez).
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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 ($T \cong 21.3\,^{\circ}C$; 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 ($-20\,^{\circ}C$). One part of the liver and the gills were fixed for histological analysis and the other part of the liver was frozen at $-80^{\circ}C$ 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 $\mu M\ H_2O_2$ 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