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Effects of the Water Soluble Fraction of Diesel Fuel Oil on Some Functional Parameters of the Neotropical Freshwater Fish *Prochilodus lineatus* Valenciennes

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Currently, leakage of oil transport pipelines and storage tanks are important contributors to the pollution of inland waters. Also, leakages of engine fuel, such as Diesel oil, from underground bulk storage tanks frequently constitutes a source of groundwater contamination, reaching the rivers and other aquatic ecosystems, producing serious pollution problems (Pacheco and Santos 2001a). However, little research has been done on the impact of petroleum hydrocarbons on freshwater ecosystems and their aquatic biota. The majority of studies examining the toxicity of petroleum hydrocarbons and its products have focused on marine species, and the toxic effects of petroleum hydrocarbons on freshwater species are relatively unknown (Pollino and Holdway 2003). Several laboratory experiments, with different organisms, have been carried out on the toxicity of diesel oil, but only a few concern fish (Pacheco and Santos 2001b). Among them, just some reports have been published concerning the effects of diesel oil exposure on morphological and physiological parameters in freshwater fish (Zhang et al. 2003) and there is a real lack of data concerning the effects of toxic agents on tropical freshwater fish species.

The neotropical freshwater fish *Prochilodus lineatus* (Valenciennes, 1847) represents a well suited species to toxicity tests because this fish has been shown to be sensitive to toxicants (Mazon and Fernandes 1999; Martinez and Souza 2002; Da Silva et al. 2004; Martinez et al. 2004; Almeida et al. 2005) and is considered a potential vertebrate for environmental monitoring (Cerqueira and Fernandes 2002; Camargo and Martinez 2006).

In this study, a suite of biological parameters was used to evaluate acute and subchronic induced effects/responses in *P. lineatus* exposed to the diesel water soluble fraction (DWSF). The following parameters were examined: a biotransformation enzyme (liver GST), an antioxidant enzyme (liver catalase), a hematological parameter (Hb content), metabolic parameters (blood glucose and proteins) and osmo-ionic parameters (blood Na^+, Cl^- and osmolality). These assays were designed to detect sublethal biochemical and physiological changes in freshwater fish exposed to DWSF. The utility of these methods lies in their ability to provide an early warning of diesel effects before community and ecosystem responses can be detected.

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MATERIALS AND METHODS

Static toxicity tests were carried out to evaluate DWSF effects to juveniles of *P. lineatus* (9.14 ± 1.32 g, mean ± SD, n=48). To obtain the DWSF one part of commercial diesel oil was added to 4 parts water in a glass container. The mixture was then exposed to sunlight for four days, simulating a diesel spill in tropical conditions. After that the upper insoluble phase was discarded and the remaining water phase was collected and diluted to 40% DWSF with well water. Experiments were performed in 100L glass aquaria with continuously aerated well water. Fish were divided in eight groups (6 fish in each), four groups were exposed to DWSF while the other four groups were exposed only to clean water, without diesel (control group). Water temperature, dissolved oxygen, pH and conductivity were continuously monitored. One experimental group plus one control group were terminally sampled at one of the following intervals: 24, 48, 96 h (acute exposure) and 15 days (subchronic exposure). During the subchronic experiment water (in control group) or water plus DWSF (in experimental group) was renewed after 7 days. Blood samples were taken from the caudal vein by means of heparinezed plastic syringes and subsequently fish were killed by cervical section and the livers were immediately removed. Livers were stored frozen at – 80°C.

A small amount of blood was used for hemoglobin determination by the cyanmethemoglobin method. Blood samples were then centrifuged (5 min, 12,000 *g*) and plasma samples were stored frozen (-20°C) until chemical analyses. Plasma sodium concentration was measured by flame photometry. Plasma osmolality was measured by freezing point depression. Plasma chloride concentration was determined by the thiocyanate method using a commercial kit (Analisa, Brazil). Plasma glucose was analysed using a colorimetric commercial kit (Glucox 500 - Doles Reagentes, Brazil) based on the glucose-oxidase reaction.

Fish livers were homogenized in ten volumes (w/v) of ice-cold 0.1M K-phosphate buffer (pH 7.0) and centrifuged (14000 X *g*) for 20 min at 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 the substrate. The change in absorbance was recorded at 340 nm and enzyme activity was calculated as nM CDNB conjugate formed.min^{-1}.mg $protein^{-1}$ 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 $protein^{-1}$. Total plasma and liver proteins were measured by the method of Lowry et al. (1951) with bovine serum albumin as standard. All samples were analyzed in duplicate.

For each parameter analysed differences between the control group and the DWSF group, for each exposure period, were tested for significance by Student-t test. Means were considered significantly different where $P < 0.05$.

RESULTS AND DISCUSSION

All fish exposed to DWSF survived the 15 days exposure and no significant differences were observed between control and DWSF-exposed fish among the following parameters: catalase liver activity, hemoglobin content and plasma concentrations of ions and proteins (Table 1). This water soluble fraction contains polar and low-molecular mass compounds, namely, aromatic hydrocarbons included in two toxicologically relevant groups: BTEX compounds (benzene, toluene, ethyl benzene and xylene isomers) and small polyaromatics hydrocarbons (PAH), in particular naphthalenes (Pacheco and Santos 2001a).

Table 1. Catalase liver activity, hemoglobin content (Hb), plasma concentrations of total proteins and ions (Na^+ and Cl^-) of *Prochilodus lineatus* exposed to clean water (CTR) or diesel water soluble fraction (DWSF) during different periods.

Parameter	Group	Experimental Periods			
		24h	48h	96h	15 days
Liver CAT	Control	38.4 ± 2.8	38.3 ± 3.2	54.1 ± 6.2	46.8 ± 4.1
($\mu mol.min^{-1}.mg ptn^{-1}$)	DWSF	40.6 ± 3.9	33.6 ± 2.5	42.4 ± 2.9	54.7 ± 8.1
Hb	Control	5.7 ± 0.2	7.7 ± 0.6	5.6 ± 0.3	5.5 ± 0.3
($g.dL^{-1}$)	DWSF	4.9 ± 0.3	6.5 ± 0.5	5.3 ± 0.4	5.1 ± 0.2
Proteins	Control	32.9 ± 7.1	27.5 ± 5.3	27.8 ± 3.6	29.8 ± 4.6
($mg.mL^{-1}$)	DWSF	29.9 ± 5.1	21.9 ± 6.9	23.9 ± 2.7	28.5 ± 3.6
Na^+	Control	159.6 ± 5.9	141.7 ± 6.4	144.7 ± 4.3	154.2 ± 8.3
(mM)	DWSF	156.8 ± 13.7	137.8 ± 7.5	144.2 ± 5.4	160.3 ± 5.2
Cl^-	Control	108.2 ± 5.4	125.8 ± 4.2	115.0 ± 3.1	104.3 ± 20.8
(mM)	DWSF	102.2 ± 6.1	115.9 ± 1.7	115.0 ± 5.1	104.3 ± 2.7

All data are expressed as mean ± SD. N = 5 - 6.

Glutathione-*S*-transferases (GST) are a group of enzymes that catalyze the conjugation of reduced glutathione (GSH) with a variety of electrophilic metabolites, and are involved in the detoxification of both reactive intermediates and oxygen radicals (Van der Oost et al. 2003). In the present study liver GST activity was significantly increased only after 15 days exposure to DWSF (Fig.1). This GST induction observed after diesel exposure probably reflects a response to the chemical, resulting in a conjugation process, where diesel compounds or its metabolites are combined with endogenous molecules to form conjugates that could be more easily excreted. It has been demonstrated that the activity of this enzyme may be enhanced in the presence of polycyclic and polychlorinated

hydrocarbons (Zhang et al. 1990) and even low level organic contamination can lead to increased hepatic GST activity (Machala et al. 1997).

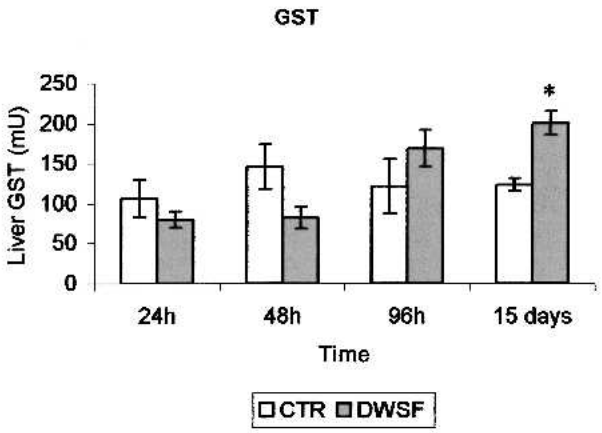

Figure 1. Liver activity of Glutathione-S-Transferase of *Prochilodus lineatus* exposed to clean water (CTR) or the diesel water soluble fraction (DWSF) during different experimental periods. The bars indicate mean and the vertical lines SE. * indicates difference in relation to control ($P < 0.05$).

Glucose concentrations for fish acutely exposed to DWSF were significantly higher than those measured for control fish (Fig.2), indicating a stress-induced mobilization of energy reserves. This elevation in blood glucose increases the amount of energy-sources available to the organism, to satisfy the extra demand caused by the stressor (Bracewell et al. 2004). This stress-related hyperglycemia, reported in many species of teleosts, is mediated mainly by the effects of catecholamines on glucose release from the liver and glycogenolysis is the main process accounting for this glucose release (Wendelaar Bonga 1997). Conversely, in fish sub-chronically exposed to DWSF no significant alteration in blood glucose was observed. The impaired ability to keep increased blood glucose after 15 days of exposure to DWSF may reflect the depletion of glycogen reserves or a reduced capacity to respond to stress after a longer exposure to DWSF, making the organism incapable of responding and consequently less able to survive (Wendelaar Bonga 1997; Martinez et al. 2004).

P. lineatus exposed to DWSF maintained good osmotic balance throughout the 96h study period once plasma osmolality, sodium and chloride concentrations were stable. However, after 15 days of exposure, plasma osmolality was increased significantly (Fig.3). Clearly more work is required to understand the significance of this rise in view of stable monovalent ions and total proteins.

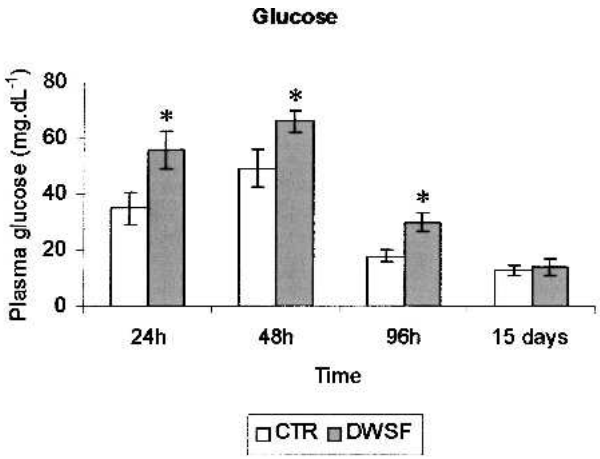

Figure 2. Plasma glucose concentrations of *Prochilodus lineatus* exposed to clean water (CTR) or the diesel water soluble fraction (DWSF) during different experimental periods. The bars indicate mean and the vertical lines SE. * indicates difference in relation to control ($P < 0.05$).

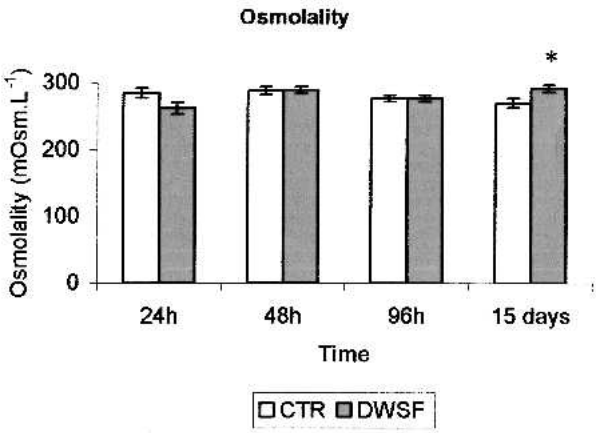

Figure 3. Plasma osmolality of *Prochilodus lineatus* exposed to clean water (CTR) or the diesel water soluble fraction (DWSF) during different experimental periods. The bars indicate mean and the vertical lines SE. * indicates difference in relation to control ($P < 0.05$).

In summary, this study shows that acute exposure of *P. lineatus* to DWSF caused significant physiological stress, resulting in elevated blood glucose levels. In the longer term (15 days) DWSF induced GST liver activity. Additional

investigations, including more biochemical, physiological and morphological parameters, are necessary to better define DWSF effects to *Prochilodus lineatus* and their suitability as biomarkers in ecotoxicological studies.

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REFERENCES

Almeida JS, Meletti PC, Martinez, CBR (2005) Acute effects of sediments taken from an urban stream on physiological and biochemical parameters of the neotropical fish *Prochilodus lineatus*. Comp Biochem Physiol 140C:356–363

Beutler E (1975) Red cell metabolism: a manual of biochemical methods. Grune & Straton, New York

Bracewell P, Cowx IG, Uglow RF (2004) Effects of handling electrofishing on plasma glucose and whole blood lactate of *Leuciscus cephalus*. J Fish Biol 64:65-71

Camargo MMP, Martinez CBR (2006) Biochemical and physiological biomarkers in *Prochilodus lineatus* submitted to in situ tests in an urban stream in southern Brazil. Environ Toxicol Pharmacol 21:61-69

Cerqueira CCC, Fernandes MN (2002) Gill tissue recovery after copper exposure and blood parameter responses in the tropical fish *Prochilodus scrofa*. Ecotoxicol Environ Saf 52:83-91

Da Silva MEF, Silva JA, Marangoni S, Novello JC, Meirelles, NC (2004) A new method to purify hepatic CYP1A of *Prochilodus scrofa*, a Brazilian freshwater fish. Comp Biochem Physiol 138C:67-74

Keen JH, Habig WH, Jakoby WB (1976) Mechanism for several activities of the glutathione-S-transferase. J Biol Chem 20:6183-6188

Lowry OH, Rosenbrough NJ, Farr AL, Randal RJ (1951) Protein measurements with the Folin phenol reagent. J Biol Chem 193:265-275

Machala M, Petřivalský M, Nezveda K, Ulrich R, Dušek L, Piačka V, Svobodová Z (1997) Responses of carp hepatopancreatic 7-ethoxyresorufin-*O*-deethylase and glutathione-dependent enzymes to organic pollutants – A field study. Environ Toxicol Chem 16:1410-1416

Martinez CBR, Souza MM (2002) Acute effects of nitrite on ion regulation in two neotropical fish species. Comp Biochem Phisiol 133A: 151-160

Martinez CBR, Nagae MY, Zaia CTBV, Zaia DAM (2004) Morphological and physiological acute effects of lead in the neotropical fish *Prochilodus lineatus*. Braz J Biol 64:797-807

Mazon AF, Fernandes MN (1999) Toxicity and differential tissue accumulation of copper in the tropical freshwater fish, *Prochilodus scrofa*. Bull Environ Contam Toxicol 63:797-804

Pacheco M, Santos MA (2001a) Biotransformation, endocrine, and genetic responses of *Anguilla anguilla* L. to petroleum distillate products and environmentally contaminated waters. Ecotoxicol Environ Saf 49:64-75

Pacheco M, Santos MA (2001b) Tissue distribution and temperature-dependence of *Anguilla anguilla* L. EROD activity following exposure to model inducers and relationship with plasma cortisol, lactate and glucose levels. Environ Int 26:149-155

Pollino CA, Holdway DA (2003) Hydrocarbon-induced changes to metabolic and detoxification enzymes of the Australian crimson-spotted rainbowfish (*Melanotaenia fluviatilis*). Environ Toxicol 18:21-28

Van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Pharmacol 13:57-149

Wendelaar Bonga SE (1997) The stress response in fish. Physiol Rev 77: 591-625

Zhang JF, Shen H, Xu TL, Wang R, Li WM, Gu YF (2003) Effects of long-term exposure of low-level diesel oil on the antioxidant defense system of fish. Bull Environ Contam Toxicol 71:234-239

Zhang YS, Andersson T, Förlin L (1990) Induction of hepatic xenobiotic biotransformation enzymes in rainbow trout by β-naphthoflanone. Time-course studies. Comp Biochem Physiol 95B:24-253