Biochemical responses, morphometric changes, genotoxic effects and CYP1A expression in the armored catfish *Pterygoplichthys anisitsi* after 15 days of exposure to mineral diesel and biodiesel

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**ABSTRACT**

Despite being considered friendlier to the environment, biodiesel fuel can be harmful to aquatic organisms, especially when combined with petroleum diesel fuel. In this work we evaluated the effects of mineral diesel fuel containing increasing concentrations of biodiesel (5% and 20%, namely B5 and B20) and pure biodiesel (B100), at concentrations of 0.001 and 0.01 mL L⁻¹, after 15 days of exposure, in armored catfish (*Pterygoplichthys anisitsi*). Toxicity tests were also performed to estimate LC50 values (96 h) for each compound. Biotransformation enzymes [ethoxyresorufin-O-deethylase (EROD), and glutathione S-transferase (GST)] as well as oxidative stress markers (superoxide dismutase, SOD, catalase, CAT, glutathione peroxidase, GPx, and the level of lipid peroxidation) were measured in liver and gills after treatment. Genotoxic effects were also accessed in erythrocytes using the comet assay and by evaluating the frequency of micronuclei formation. Further, the mRNA of cytochrome P450 1A (CYP1A) was also measured in liver. Mortality was not observed even exposure to concentrations as high as 6.0 mL L⁻¹. EROD and GST activities were increased after B5 and B20 treatments; however, CYP1A mRNA induction was not observed. SOD and CAT activities were decreased, but GPx was significantly higher for all treatments in gills. There were no significant changes in lipid peroxidation, but genotoxicity markers revealed that all treatments increased comet scores. Fuels B5 and B20 increased micronuclei frequency. Our results indicate that despite being less toxic, biodiesel may cause sublethal alterations in fish that may alter long term health.

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1. Introduction

Petroleum-derived diesel oil is being gradually replaced by biodiesel as an automotive fuel, especially in Brazil in which 5% biodiesel (B5) is required as a constituent within petroleum diesel by the Brazilian government since 2010 ([ANP, 2008]). Despite being a renewable alternative for fossil fuels, biodiesel is considered friendlier to the environment since it is more biodegradable and emits fewer amounts of greenhouse gases ([IPCC, 2007; Leduc et al., 2009]). According to these data, it could be expected that diesel toxicity would decrease as the biodiesel concentration in the mixture increases. Nevertheless, it has been recently demonstrated that despite its lower direct effects to exposed organisms, biodiesel can have harmful effects ([Leme et al., 2012; Poon et al., 2009; Nogueira et al., 2011b, 2013; Cavalcante et al., 2015]).

Toxic effects of biodiesel have been attributed to the presence of contaminants from the raw matter used to produce biodiesel ([Nogueira and Almeida, 2012]), the presence of methanol or ethanol in biodiesel due to reversion of the trans-esterification reaction that originates biodiesel ([Leite et al., 2011; Cruz et al., 2012; Cavalcante et al., 2015]), and the presence of different additives in biodiesel to increase its quality for using in different kinds of motors ([Nogueira and Almeida, 2012]). Moreover, in previous studies we showed that biodiesel contains significant amounts of malondialdehyde (MDA), a typical product of unsaturated fatty acid oxidation by reactive oxygen species (ROS) ([Nogueira et al., 2012]).

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These data indicate that methanol-esterified fatty acids from biodiesel are catalyzing auto-oxidation reactions producing reactive compounds and potentially accounting for the toxicity of biodiesel. In fact, fish exposed to biodiesel derived from animal fats for two and seven days presented higher levels of lipid peroxidation products in gills when compared to unexposed animals (Nogueira et al., 2011b, 2013).

Several factors could contribute to biodiesel toxicity. For example, biodiesel can be obtained from very diverse sources and used with different proportions with petroleum diesel and, thus, contains different amounts and kinds of additives as well as constituents. Most studies evaluating biodiesel toxicity in the past used acute durations of exposure (Khan et al., 2007; Nogueira et al., 2011b; Leme et al., 2012; Nogueira et al., 2013), and few have examined longer term exposures. We have recently published results on the biochemical responses of the suckermouth armored catfish *Pterygoplichthys anisitsi* after short term exposure (two and seven days) to diesel, biodiesel and biodiesel blends (B0, B5, B20 and B100) (Nogueira et al., 2013). In order to know how *P. anisitsi* responds after a longer exposure to these contaminants, in the present work we evaluated the effects of pure (B100) or mixed biodiesel (B5, B20) after 15 days of semi-static exposure on a series of biochemical responses in liver and gills: the expression of CYP1A transcripts, the activities of biotransformation enzymes ethoxyresorufin-O-deethylase (EROD), and glutathione S-transferase (GST), as well as the activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and lipid peroxidation. Genotoxic effects were also evaluated by means of the comet assay and the quantification of micronuclei and other nuclear abnormalities in peripheral erythrocytes. In addition, all endpoints were compared to survival of the fish after exposures.

### 2. Material and methods

#### 2.1. Chemicals

All chemicals used in this work were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Commercial diesel (B5) was purchased from a Petrobras gas station, and the pure biodiesel (B100) was obtained from the Industry Fertibom, Catanduva, São Paulo, Brazil, and was made from animal fat. The B20 mixture (20% biodiesel on 80% diesel oil) was prepared by mixing B5 with B100 in the laboratory. It should be mentioned that there was no information about the composition of biodiesel in the B5 diesel obtained from the gas station. Consequently, it is likely to have different constituents than the B100 used for the other treatments (B100 and B20). Thus, in this study we are only considering the effects of general biodiesel formulations on fish.

#### 2.2. Test organisms

Armored catfish *Pterygoplichthys anisitsi* (144.83 ± 57.98 g and 18.30 ± 2.71 cm) were obtained from a fish farm located at Gua-piaçu (Sítioca Bom Jesus, Rodovia Assis Chateaubriant, São Paulo, Brasil). Due to the absence of external sexual dimorphism of this species, male and females were used. This work was approved by the Ethics Committee for Research Use of Animals of the Sao Paulo State University.

#### 2.3. Toxicity tests

Acute toxicity tests (96 h) were carried out in 18 L glass aquarias, with one fish per aquarium, under constant aeration, pH (8.0 to 8.5) and temperature (26 °C), and a 12/12 h cycles of dark/light. The tests were done for each mixture of diesel and biodiesel (B100, B20 and B5), at the following concentrations: 6.0, 4.5, 3.0, 1.5, 1.125, 0.75, 0.375 and 0.188 mL L⁻¹, in tap water. Six fish were used for each concentration. After 96 h of exposure, the mortality rate of fish were registered for each concentration for the calculation of LC50 (96 h) values.

#### 2.4. Exposure experiments

Exposures were carried out in 42 glass aquaria of 18 L with one fish per aquarium, totaling seven groups of six replicates, at controlled temperature (26 °C) and pH (8.0–8.5), and under constant aeration. Fish were acclimated for one month in tanks of 500 L, and for five days in their respective aquaria before exposures. During the exposure period, fish were fed every two days, and the water (with the specific contaminants) were changed every five days, in order to avoid ammonia accumulation. The treatment groups included one control without diesel exposure, and other six groups which received B5, B20 and B100 at 0.01 and 0.001 mL L⁻¹. The water pH and levels of unionized ammonia during the experiments were measured daily during the experiment and did not surpass 8.5 and 1.13 mg L⁻¹, respectively (Table 1). The reported LC50 values for NH₃ levels at pH 8.0 for most catfish species such as the channel catfish *Ictalurus punctatus* (24 h) and the silver catfish *Rhamdia quelen* (96 h) were above 1.80 mg L⁻¹ (Tommasso et al., 1980; Sheehan and Lewis, 1986; Miron et al., 2011). Thus, NH₃ levels measured in this work should not substantially interfere with biochemical responses in *P. anisitsi*. There were no differences in NH₃ levels between treatments and control. After 15 days of exposure, all fish were collected and anesthetized with benzocain (45 mg L⁻¹), and had their blood, liver and gills collected for biochemical analyzes. The tissues were immediately frozen at −80 °C and the blood was immediately used for genotoxicity analyzes. Pieces of liver from control fish and fish exposed to the highest B5, B20 and B100 concentrations were

### Table 1

Morphometric data (weight, length, HI and K) of *Pterygoplichthys anisitsi* exposed to 0, 0.001 and 0.01 mL L⁻¹ of diesel containing 5% of biodiesel (B5), diesel containing 20% of biodiesel (B20) or pure biodiesel (B100), measured at the end of the exposure period, and mean values of pH, temperature and unionized ammonia measured every five days during 15 days of exposure.

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (mL L⁻¹)</th>
<th>Fish weight (g)</th>
<th>Fish length (cm)</th>
<th>HI (%)</th>
<th>K</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>NH₃ (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>167.4 ± 62.8</td>
<td>19.8 ± 2.7</td>
<td>0.73 ± 0.02</td>
<td>0.40 ± 0.010</td>
<td>8.1 ± 0.3</td>
<td>23.1 ± 0.8</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>B5</td>
<td>0.001</td>
<td>133.0 ± 52.7</td>
<td>17.4 ± 2.8</td>
<td>0.69 ± 0.01</td>
<td>0.43 ± 0.013*</td>
<td>8.3 ± 0.4</td>
<td>23.2 ± 1.4</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>166.5 ± 71.0</td>
<td>18.0 ± 2.5</td>
<td>0.74 ± 0.04</td>
<td>0.43 ± 0.022*</td>
<td>8.0 ± 0.4</td>
<td>23.1 ± 1.4</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>B20</td>
<td>0.001</td>
<td>122.3 ± 49.9</td>
<td>16.6 ± 1.9</td>
<td>0.74 ± 0.03</td>
<td>0.42 ± 0.002</td>
<td>8.1 ± 0.4</td>
<td>23.2 ± 1.6</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>114.0 ± 9.4</td>
<td>17.1 ± 0.4</td>
<td>0.81 ± 0.19</td>
<td>0.43 ± 0.007*</td>
<td>8.3 ± 0.4</td>
<td>23.1 ± 1.5</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>B100</td>
<td>0.001</td>
<td>158.0 ± 86.1</td>
<td>19.1 ± 3.9</td>
<td>0.75 ± 0.14</td>
<td>0.39 ± 0.003</td>
<td>8.1 ± 0.4</td>
<td>23.3 ± 1.4</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>152.5 ± 49.6</td>
<td>20.2 ± 2.6</td>
<td>0.74 ± 0.09</td>
<td>0.41 ± 0.003</td>
<td>8.0 ± 0.4</td>
<td>23.3 ± 1.1</td>
<td>0.8 ± 0.3</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation.

* Statistical difference compared to the control group.
stored in RNAlater (Life Technologies, Carlsbad, CA, USA) at −20 °C for subsequent analyses of CYP1A transcripts.

2.5. Calculation of condition factor (K) and hepatosomatic index (HSI)

The condition factor (K, Le Cren, 1951) and length–weight relationship were determined by measuring total weight and total length, and the curve parameters a and b were determined by log transformation of raw data. The growth curves obtained from straight line for regression analysis between total weight and total length are log $W = \log a + b \log L$. Condition factor was assessed by the following expression: $K = W/L^b$, where $K$ is the condition factor; $W$ is the total weight; $L$ is the total length; $b$ the slope of the regression line in logarithmic form of weight and total length relationship (Froese, 2006). The HSI was calculated by liver weight/total body weight × 100 (Oguri, 1978).

2.6. CYP1A transcript quantification

Total RNA extraction was performed by the phenol:chloroform extraction using TRI Reagent (Life Technologies) and following manufacture’s protocol. The quality of the extracted RNA was evaluated by spectrophotometry (Nanodrop) and by agarose gel electrophoresis. The total RNA from each individual was used together with random primers to synthesize the complementary DNA (cDNA) following supplier instructions (High Capacity Reverse Transcriptase Kit, Life Technologies). Real time quantitative PCR was performed using a StepOnePlus thermal cycler, Power Reverse Transcriptase Kit, Life Technologies. Expression assays were performed in triplicate using the following primer pairs: TATTCTGGGGTCATG and beta-actin (normalizing control, F: GGCCA-YATTCTGGGGTCATG and beta-actin (normalizing control, F: TTGCCTCGAGAGGAGATGC and R: AGAGAAGCCTCGGGACACT). The expression assays were performed in triplicate using the following cycling parameters: 95 °C for 3 min and 40 cycles of 95 °C for 15 s and 62 °C for 25 s. The primer specificity was evaluated by a melting curve at the end of each run and by cloning and sequencing the amplicons. The amplicons were cloned in the pGEM-t Easy vector (Promega), which were used to transform competent E. coli DH5-alfa. Positive transformants were selected on a plate containing the appropriate antibiotic. Three positive colonies were grown for plasmid expansion, followed by miniprep (Qiagen). Plasmids containing inserted gene were sequenced at Macrogen (Rockville, USA). CYP1A mRNA expression was normalized against beta-actin transcripts and differences were calculated in relation to each control group by the delta–delta–Ct method (Livak and Schmittgen, 2001; Nolan et al., 2006).

2.7. Enzymatic assays and protein quantification

Liver and gills were homogenized (1:4, weight/volume) in Tris buffer 0.05 M (pH 7.4) containing sucrose 0.005 M, KCl 0.015 M and protease inhibitor (phenylmethylsulfonylfluoride) 0.001 M. The homogenized samples were centrifuged at 10,000g for 20 min at 4 °C, and then the supernatant fraction was collected and re-centrifuged at 50,000g for 1 h at 4 °C. The second supernatant fraction was used for GST, SOD, CAT and GPx assays, while the pellet, suspended in 100 μL of Tris buffer 0.1 M (pH 7.5), containing EDTA 0.001 M, dithiothreitol 0.001 M, KCl 0.1 M, and 20% glycerol, was used for EROD measurement.

SOD activity was evaluated by the inhibition of cytochrome c reduction in the presence of the hypoxanthine/xanthine oxidase $O_2^-$ generator system at 550 nm (McCord and Fridovich, 1969). CAT activity was quantified at 240 nm by the $H_2O_2$ decomposition according to the method described by Beutler (1975). GPx activity was assayed using the oxidation of NADPH (linked to GSSG reduction by excess glutathione reductase) at 340 nm, and using t-butyl hydroperoxide as substrate, as described by Sies et al. (1979). EROD activity was measured using the fluorimetric method described by Burke and Mayer (1974). The assay mixture contained 1950 μL of potassium phosphate buffer 80 mM (pH 7.4), 20 μL of 7-ethoxyresorufin 335 μM, 20 μL of NADPH 20 mM and 10 μL of microsomal liver extract. GST activity was determined by measuring the increase in absorbance at 340 nm, incubating reduced glutathione (GSH) and 1-chloro-2, 4-dinitrobenzene (CDNB) as substrates, following Keen et al. (1976). Protein levels were measured by the method of Bradford (1976), using bovine serum albumin as standard.

2.8. Lipid peroxidation

In order to assess the levels of lipid peroxidation, the product formed from the combination of malondialdehyde (MDA) and thiobarbituric acid (TBA) was detected by high performance liquid chromatography coupled to UV/vis detector (HPLC–UV/vis) (Almeida et al., 2003, 2004). For this analysis 100 mg of liver and gills samples was homogenized in 0.3 mL of Tris buffer 0.1 M (pH 8.0). Then 40 μg of TBA was dissolved in 10 mL of HCl 0.2 M and 0.3 mL of this solution was added to each sample. This mixture was heated at 90 °C for 40 min. Next 1 mL of n-butanol was added and samples were centrifuged at 3500 rpm for 3 min. The supernatant was collected and quantified by HPLC at 532 nm, in terms of a malondialdehyde (MDA) standard calibration curve that had been previously prepared using the same procedure used for the samples.

2.9. Comet assay, micronucleus and nuclear abnormalities quantification

Genotoxic effects of diesel and biodiesel in fish blood red cells were evaluated by quantifying micronuclei and nuclear abnormalities, and through the comet assay. The comet assay used the method described by Singh et al. (1988). Two slides were prepared per sample, and a total of 100 nucleoids were analyzed per animal on a Leica fluorescence microscope (40 × objective; filter B-34; $\lambda_{ex} = 420–490$ nm, $\lambda_{em} = 520$ nm). The nucleoids were visually classified according to the intensity of tail migration into classes from 0 to 3, according to the classification proposed by Kobayashi et al. (1995). Data are presented as Comet score, estimated by the following equation: Comet score = (Class 0 × 0) + (Class 1 × 1) + (Class 2 × 2) + (Class 3 × 3). For the evaluation of micronuclei, fish blood was smeared in two histological slides and kept in dark until dryness, and then fixed for 10 min in methanol. After fixation, the slides were dried at room temperature overnight. At the subsequent day, the slides were submitted to the Feulgen reaction, in two steps: an acid hydrolysis in HCI 1 M at 60 °C for 11 min, and a staining reaction with the Schiff reagent for two hours in dark at room temperature. A total of 3000 erythrocytes were analyzed per fish (1500 per slide) for micronuclei quantification in a Leica microscope (40 × objective). The same slides were used for the identification and quantification of the nuclear abnormalities (NA) which were categorized as blebbled, lobed, notched or broken eggs as proposed by Carrasco et al. (1990).

2.10. Statistical analyses

Statistical analyzes were performed to observe significant differences on averages between control and treated groups by means of the software Statistica 7.1. The normality and variance homogeneity of data were tested by the Shapiro–Wilks and Levene test, respectively. If data were normal and homogeneous, one-way
ANOVA was applied for comparisons between groups, followed by the Fisher LSD post-hoc test, to identify what groups presented significant differences. For non-parametric data, significant differences were detected by the Kruskal–Wallis test. Significant differences were accepted only when $p < 0.05$. Data are presented as mean ± standard deviation.

3. Results

3.1. Toxicity tests and morphological indexes

No mortality was observed for *P. anisitsi* even at concentrations as high as 6 mL L$^{-1}$ of B5. Also, no changes in HSI were observed after the exposure period ($p=0.6$). The parameter $b$ assessed for condition factor analysis and obtained from the regression between total weight and total length for all groups (control and treated) was lower than 3, indicating negative allometric growth for this species ($r^2=0.76$, $p<0.001$, $b=2.3$). Thus, large specimens became more elongated (Froese, 2006), in agreement with the body pattern of the *P. anisitsi*. The condition factor analysis indicated a difference in values between treatments ($p=0.0045$), with higher $K$ values in the animals treated with both concentrations of B5 and the higher concentration of B20.

3.2. Biotransformation enzyme activities and Cyp1a mRNA expression

EROD activity was statistically higher after 15 days of exposure to B20 0.01 mL L$^{-1}$ in both the gill and the liver, compared to the control group (Table 2). Moreover, hepatic EROD activity was also significantly higher in fish exposed to B5 at 0.01 and 0.001 mL L$^{-1}$. Despite EROD induction in hepatic microsomes, there was no significant increase in the levels of CYP1A mRNA in the liver following treatment with B5, B20 or B100 (Fig. 1). The sequences of the amplicons amplified with the primers for CYP1A and actin are shown in the supplementary material. The activity of GST was significantly higher in gills and livers of fish exposed to both 0.001 and 0.01 mL L$^{-1}$ of B20. GST in gills was also higher than controls in the fish exposed to 0.001 mL L$^{-1}$ of B100.

### Table 2

Enzymatic activities of ethoxyresorufin-O-deethylase (EROD), glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and malondialdehyde (MDA) levels in gills and liver of *Pterygoplichthys anisitsi* exposed to 0, 0.001 and 0.01 mL L$^{-1}$ of diesel containing 5% of biodiesel (B5), diesel containing 20% of biodiesel (B20) or pure biodiesel (B100) for 15 days.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Treatment</th>
<th>Concentration (mL L$^{-1}$)</th>
<th>Biochemical Biomarkers</th>
<th>EROD$^{A}$</th>
<th>GST$^{D}$</th>
<th>SOD$^{B}$</th>
<th>CAT$^{D}$</th>
<th>GPx$^{D}$</th>
<th>MDA$^{A}$</th>
</tr>
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</tr>
<tr>
<td></td>
<td>Control</td>
<td>0</td>
<td></td>
<td>22.6 ± 16.4</td>
<td>23.4 ± 7.7</td>
<td>1.89 ± 0.11</td>
<td>0.9 ± 0.3</td>
<td>7.3 ± 1.4</td>
<td>0.61 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>B5</td>
<td>0.001</td>
<td></td>
<td>24.2 ± 19.6</td>
<td>23.8 ± 5.3</td>
<td>1.05 ± 0.05</td>
<td>0.6 ± 0.1*</td>
<td>11.5 ± 2.1*</td>
<td>0.59 ± 0.02</td>
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<td>0.01</td>
<td></td>
<td>48.9 ± 34.0</td>
<td>24.4 ± 3.3</td>
<td>1.00 ± 0.02</td>
<td>0.7 ± 0.1*</td>
<td>12.0 ± 0.9*</td>
<td>0.38 ± 0.01</td>
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<td></td>
<td>Gills</td>
<td>B20</td>
<td>0.001</td>
<td>30.4 ± 21.6</td>
<td>30.8 ± 6.8*</td>
<td>1.09 ± 0.03</td>
<td>0.9 ± 0.2</td>
<td>9.3 ± 2.2*</td>
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<tr>
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<td></td>
<td>71.6 ± 21.4*</td>
<td>29.9 ± 4.6*</td>
<td>0.89 ± 0.02*</td>
<td>0.9 ± 0.2</td>
<td>9.7 ± 1.0*</td>
<td>0.28 ± 0.01</td>
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<tr>
<td></td>
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<td>B100</td>
<td>0.001</td>
<td>44.9 ± 37.8</td>
<td>32.3 ± 4.0*</td>
<td>0.74 ± 0.01*</td>
<td>0.9 ± 0.2</td>
<td>10.3 ± 1.2*</td>
<td>0.29 ± 0.01</td>
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<tr>
<td></td>
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<td></td>
<td>42.5 ± 22.2</td>
<td>25.7 ± 4.1*</td>
<td>0.84 ± 0.02*</td>
<td>0.9 ± 0.2</td>
<td>11.6 ± 1.7*</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0</td>
<td></td>
<td>14.9 ± 0.8</td>
<td>298.9 ± 84.6</td>
<td>34.25 ± 5.66</td>
<td>184.9 ± 13.8</td>
<td>48.6 ± 4.3</td>
<td>0.79 ± 0.21</td>
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<tr>
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<td>B5</td>
<td>0.001</td>
<td></td>
<td>64.0 ± 6.0*</td>
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<td>26.01 ± 3.61*</td>
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<td>25.9 ± 17</td>
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<td>0.01</td>
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<td>94.0 ± 9.1*</td>
<td>298.8 ± 54.3</td>
<td>20.18 ± 4.18*</td>
<td>146.8 ± 7.0</td>
<td>35.4 ± 4.0</td>
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<td>47.8 ± 1.2</td>
<td>367.1 ± 38.9*</td>
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<td>97.6 ± 5.3*</td>
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<td>B100</td>
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<td>311.2 ± 67.3</td>
<td>29.99 ± 1.36*</td>
<td>174.2 ± 6.6</td>
<td>43.6 ± 4.0</td>
<td>0.88 ± 0.16*</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation (parametric data); **Median ± standard error (non-parametric data); pmol/min/mg protein; U/mg protein; nmol/g tissue; mL/mg protein. 
* Statistical difference compared to control ($p < 0.05$).
* Statistical difference compared to the same contaminant at lower concentration.

3.3. Antioxidant enzymes

SOD activity was lower in gills of fish exposed to B20 0.01 mL L$^{-1}$ and to B100 0.001 and 0.01 mL L$^{-1}$, compared to the control group (Table 2). In the liver, the SOD activity was also lower in fish exposed to B5 and B20 at both concentrations used. The activity of CAT was lower in the gills of fish exposed to the two concentrations of B5 compared to the control group. In the liver, alterations in CAT activity were not observed. With respect to GPx activity, it was higher in gills of fish exposed to all treatments in comparison to control values, but no differences were observed in the liver.

3.4. Lipid peroxidation

Lipid peroxidation levels were lower in gills of fish exposed to B20 0.01 mL L$^{-1}$ and to B100 0.001 mL L$^{-1}$, when compared to control animals (Table 2). In the liver, MDA levels were significantly lower compared to control for fish exposed to both concentrations of B5, and to 0.001 mL L$^{-1}$ of B20 and B100.

3.5. Comet assay, micronuclei formation and nuclear abnormalities

When compared to control values, the comet scores in blood
cells were significantly higher in fish exposed to both concentrations of B5 and B100, and to the higher concentration of B20 (Fig. 2). With respect to micronuclei quantification, a significant increase was observed only for fish exposed to the lower concentration of B20 (Table 3). Nevertheless, the amount of Notched, Lobed and Broken-eggs nuclei were significantly higher in blood cells of fish exposed to all treatments (Notched), to the lower concentrations of B5 and B20 (Lobed), and to the higher concentration of B5 and the two concentrations of B20 (Broken-eggs), when compared to the control group. No differences were observed for the Blebbed nuclear abnormality among treatments (Table 3).

4. Discussion

One of the goals of this work was to establish acute LC50 values (96 h) for all the proposed fuels (B5, B20 and B100). Surprisingly, no mortality was observed for any concentration tested, even after 120 h of exposure. No mortality was also recorded after 15 days of exposure of P. anisitsi to 0.001 and 0.01 mL L−1 of B5, B20 and B100. In addition, the K index of P. anisitsi was also not reduced by treatment. This index provides estimates of condition/fitness of the organism and was actually enhanced in animals exposed to both concentrations of B5 and the higher B20 concentration, suggesting that the more petroleum diesel that is present in the mixture, the more effective is the maintenance of energy storage in fish. The higher K index in fish exposed to higher petroleum diesel concentrations compared to fish exposed to mixtures containing higher biodiesel amounts could indicate that petroleum diesel may increase energy intake and storage. Petroleum diesel may have caused an increase in food intake, but unfortunately the feeding behavior of the fish was not monitored during the experiments to compare food intake between experimental groups. Thus, more studies are necessary to better address the mechanisms that led catfish to increase K index after petroleum diesel exposure.

Despite no mortality was observed for P. anisitsi in the toxicity tests, several biomarkers were responsive, indicating that this species could be used as sentinel organism in monitoring impacted areas. Although CYP1A mRNA expression was unaltered by treatment, we observed that EROD activity was higher in fish exposed to B5 and B20 than controls, particularly in the liver. In previous studies, Nogueira et al. (2013) also observed that EROD activity increased significantly in this species exposed to B5 and B20 after 2 and 7 days of exposure, and the results presented here showed that CYP1A remains active after 15 days of exposure to the fuels containing higher proportions of pure diesel, such as B5 and B20. EROD activity is commonly used to indicate exposure to planar aromatic hydrocarbons such as co-planar PCBs or petroleum-derived PAHs in fish (van der Oost et al., 2003). However, for the loricarid fish the significance of EROD activity induction by PAH is controversial, since very low activity was found for P. anisitsi (Nogueira et al., 2011a). In fact, previous studies did not detect EROD in two species of Brazilian catfish from the genus Hypostomus and another species of Pterygoplichthys even after exposure to pure diesel oil or β-naphthoflavone (Parente et al., 2009, 2011). P. anisitsi possesses at least one CYP1A gene, like other fish species. However some amino acid substitutions in comparison to many other fish CYP1A sequences may alter the protein structure whereas CYP1A protein and catalytic activities, due to the relatively large period of exposure of the diesel solution, CYP1A mRNA is rapidly produced following exposure to Ah receptor ligands whereas CYP1A protein and catalytic activities (EROD) remain induced after transcripts return to baseline (Haasch et al., 1993). Due to differences in translational regulation, only ~40% of the variation in protein concentration can be explained by knowing mRNA abundance in mammalian cells submitted to perturbations (Vogel and Marcotte, 2012). Consequently, measurements of CYP1A catalytic activity should include redundancy at either the protein or transcript level to gain a better understanding of the duration of exposure to likely inducers.

It should be noted that in gills the only group that presented

Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (mL L−1)</th>
<th>Genotoxic Biomarker</th>
<th>MN*</th>
<th>Notched†</th>
<th>Lobed*</th>
<th>Broken-eggs*</th>
<th>Blebbed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td></td>
<td>25.0 ± 19.1</td>
<td>63.7 ± 29.3</td>
<td>65.0 ± 2.3</td>
<td>18.5 ± 13.3</td>
<td>42.0 ± 2.3</td>
</tr>
<tr>
<td>B5</td>
<td>0.001</td>
<td></td>
<td>80.0 ± 29.1*</td>
<td>178.5 ± 8.8*</td>
<td>43.5 ± 7.0*</td>
<td>34.5 ± 12*</td>
<td>18.5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td></td>
<td>718 ± 16.9*</td>
<td>128.2 ± 77.4*</td>
<td>40.5 ± 5.6</td>
<td>38.5 ± 0.9*</td>
<td>35.0 ± 5.9</td>
</tr>
<tr>
<td>B20</td>
<td>0.001</td>
<td></td>
<td>99.2 ± 9.4*</td>
<td>128.7 ± 23.3*</td>
<td>28.5 ± 1.4*</td>
<td>48.5 ± 10.1*</td>
<td>15.5 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td></td>
<td>105.3 ± 63.45*</td>
<td>134.5 ± 39.2*</td>
<td>134.5 ± 39.2*</td>
<td>24.5 ± 19</td>
<td></td>
</tr>
<tr>
<td>B100</td>
<td>0.001</td>
<td></td>
<td>16.5 ± 12.0</td>
<td>101.2 ± 35.2*</td>
<td>56.0 ± 2.7</td>
<td>27.2 ± 13.3</td>
<td>36.0 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td></td>
<td>9.7 ± 8.9</td>
<td>124.5 ± 57.8*</td>
<td>60.5 ± 2.9</td>
<td>22.7 ± 9.7</td>
<td>94.5 ± 3.6</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation (parametric data); **Median ± standard error (non-parametric data).
†Significant difference compared to control.
‡Significant difference when comparing two concentration of the same contaminant.

Fig. 2. Comet scores in peripheral erythrocytes of Pterygoplichthys anisitsi exposed to 0 (control), 0.001 and 0.01 mL L−1 of diesel containing 5% of biodiesel (B5), diesel containing 20% of biodiesel (B20) or pure biodiesel (B100) for 15 days. * indicates significant difference compared to control group (p < 0.05).
significant EROD induction was the group exposed to the higher concentration of B20. Although B5 contains higher amounts of PAHs, significant EROD induction was not observed. Similar results were observed with GST activity. Nogueira et al., 2011 suggested that biodiesel B20 may contain surfactants that increase the absorption of petroleum diesel elements, such as PAH, resulting in a more pronounced EROD induction in the gills. Similar conclusions were also proposed by Leme et al. (2012). This hypothesis can be confirmed by monitoring the concentrations of PAHs absorbed by fish (in plasma or metabolites excreted in the bile) from petroleum diesel, in the presence or the absence of biodiesel formulation. The identity of compounds present in biodiesel that could be responsible for this effect, remain to be clarified, but warrant further study.

Although EROD is induced by planar aromatic hydrocarbons, some GST isoforms, especially GST-α class have peroxidase activity (Fiander and Schneider, 1999; Veal et al., 2002; Prabhu et al., 2001; Collinson and Grant, 2003), and thus the increase in GST activity could be also attributed to a protection against oxidative stress mediated by biodiesel elements. Exposure to diesel and biodiesel alone or mixed with diesel could be a response to ROS generation, and GST in our study would represent an additional antioxidant defense activated to deal oxidative stress. In a previous study diesel and biodiesel alone or blended increased SOD, CAT and GPx activities in P. anisitsi after 2 and 7 days of exposure (Nogueira et al., 2013). In the present study, GPx was also increased in gills of exposed fish. However, SOD and CAT activities were decreased, which contrast our previous findings. Explanations include that the longer exposure time (15 days) may have influenced oxidative enzymes and reduced overt oxidative damage. Consistent with this hypothesis the low SOD and CAT activities were mirrored by low MDA, suggesting that, if generated, ROS was intercepted by the antioxidant defense systems (GPx or GST) efficiently.

Genotoxic effects have been observed following exposure to biodiesel and its blends with petroleum diesel in Salmonella (Leme et al., 2012), a human cell line (Leme et al., 2011), and a fish cell line (Cavalcante et al., 2015). In this study, a significant increase in comet scores and nuclear abnormalities, especially notched nuclei, was found for most treatments, compared to control values, indicating that even pure biodiesel was genotoxic to fish erythrocytes. For B5 and B20 treatments, genotoxic effects can be attributed to PAHs and other toxic components from petroleum diesel. In the case of B100, the genotoxic effect could be also a response to unknown elements present in biodiesel composition, such as impurities, methanol from reversed transesterification reactions, or even reactive compounds originating from auto-oxidation of fatty acids present in biodiesel. In similar results, only biodiesel containing petroleum diesel caused increased levels of micronuclei, an effect not observed for B100, indicating a higher genotoxic potential for diesel-containing fuels. Taking together all data from the genotoxic markers, it can be concluded that B5, B20 and B100 were genotoxic to fish, and only B5 and B20 caused clastogenic effects. These results agree with previous findings, which reported an increased toxicity for mixtures containing greater proportions of petroleum diesel in biodiesel, compared to mixtures containing lower amounts of petroleum diesel (Khan et al., 2007; Leite et al., 2011).

5. Conclusion

Despite the resistance of P. anisitsi to the acute toxicity of the various fuels, our data clearly showed that B5, B20 and B100 may cause negative effects to the DNA of this fish. After 15 days of exposure, B5 and B20 produced more alterations in biochemical effects compared to B100. Increases in micronuclei incidence and comet responses were the most prevalent. The physiological impacts of these responses are unclear, but long term exposure may have negative effects to these particular species of fish.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2015.01.034.

References


ANP (The Brazilian National Petroleum, Natural Gas and Biofuel Agency), Resolution 2 from 29.1.2008.


