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Biochemical biomarkers in Nile tilapia (*Oreochromis niloticus*) after short-term exposure to diesel oil, pure biodiesel and biodiesel blends

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ABSTRACT

Fossil fuels such as diesel are being gradually replaced by biodiesel, a renewable energy source, cheaper and less polluting. However, little is known about the toxic effects of this new energy source on aquatic organisms. Thus, we evaluated biochemical biomarkers related to oxidative stress in Nile tilapia (*Oreochromis niloticus*) after two and seven exposure days to diesel and pure biodiesel (B100) and blends B5 and B20 at concentrations of 0.01 and 0.1 mL L^{-1}. The hepatic ethoxyresorufin-*O*-deethylase activity was highly induced in all groups, except for those animals exposed to B100. There was an increase in lipid peroxidation in liver and gills in the group exposed to the higher concentration of B5. All treatments caused a significant increase in the levels of 1-hydroxypyrene excreted in the bile after 2 and 7 d, except for those fish exposed to B100. The hepatic glutathione-S-transferase increased after 7 d in animals exposed to the higher concentration of diesel and in the gill of fish exposed to the higher concentration of pure diesel and B5, but decreased for the two tested concentrations of B100. Superoxide dismutase, catalase and glutathione peroxidase also presented significant changes according to the treatments for all groups, including B100. Biodiesel B20 in the conditions tested had fewer adverse effects than diesel and B5 for the Nile tilapia, and can be suggested as a less harmful fuel in substitution to diesel. However, even B100 could activate biochemical responses in fish, at the experimental conditions tested, indicating that this fuel can also represent a risk to the aquatic biota.

1. Introduction

Petroleum derivatives are among the main compounds responsible for the contamination of aquatic ecosystems due to anthropogenic activities and environmental accidents like leaks and oil spills, being very harmful to aquatic organisms. Its insoluble fractions in water cover the body surface of fish, which can cause acute toxicity and mortality (Environmental Health Criteria, 1996). The soluble fraction of diesel oil contains many polycyclic aromatic hydrocarbons (PAHs) that can cause lesions in the liver and gill of fish (Simonato et al., 2008) and oxidative stress (Achuba and Osakwe, 2003; van der Oost et al., 2003; Zhang et al., 2003, 2004).

An alternative for the use of fossil fuels is biodiesel, which is a mixture of fatty acid methyl esters derived from the trans-esterification of animal fats and vegetable oils. Biodiesel is used to formulate a range of mixtures from B2 (2% biodiesel mixed with 98% fossil diesel) to B100 (100% biodiesel) (DeMello et al., 2007). Studies have shown that biodiesel is more biodegradable (Pasqualino et al., 2006; Prince et al., 2008) and produces fewer greenhouse gases than diesel (Lee et al., 2004; Balat and Balat, 2010). Furthermore, a study showed that biodiesel has lower acute toxicity to aquatic organisms (*Daphnia magna* and *Oncorhynchus mykiss*) than diesel oil (Khan et al., 2007). However, little is known about the toxicological effects caused by biodiesel and their mixtures in aquatic organisms.

Oxidative stress occurs in organisms when the rate of reactive oxygen species (ROS) production exceeds the rate of its decomposition by antioxidant systems, leading to an increase in oxidative damage to different cellular targets (Almeida et al., 2005). The biotransformation of xenobiotics is a factor that can lead to increased production of ROS (Zangar et al., 2004). The ethoxyresorufin-*O*-deethylase (EROD) activity is a catalytic measurement of cytochrome P450 isoform 1A and this enzyme is highly induced in the presence of PAHs (Whyte et al., 2000).

ROS can oxidize proteins, DNA and lipids of biological membranes. Thus, the state of oxidative stress may produce damage to tissues, inflammation, degenerative diseases and aging (Valavanidis et al., 2006).

The lipid peroxidation is a process in which cell membranes are oxidized by ROS and leads to the formation of secondary products

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such as malondialdehyde (MDA). The MDA has been largely measured as indicator of injury caused by ROS (Almeida et al., 2003, 2005, 2007).

To prevent oxidative stress, organisms have defense systems such as antioxidant enzymes (Almeida et al., 2007; Kopecka-Pilarczyk and Correia, 2009). The three major antioxidant enzymes are superoxide dismutase (SOD), which transforms superoxide anion into hydrogen peroxide, catalase (CAT) that decomposes hydrogen peroxide to molecular oxygen and water, and glutathione peroxidase (GPx) which reduce both hydrogen peroxide and lipid hydroperoxides (Nordberg and Arnér, 2001; Almeida et al., 2007). Glutathione S-transferases (GST) are phase II detoxification enzymes and catalyze the conjugation of reduced glutathione to electrophilic metabolites. GSTs also indirectly exert antioxidant function by removing reactive oxygen species and regenerates S-thiolated proteins (Sheehan et al., 2001).

Biochemical systems involved in the generation and detoxification of reactive oxygen species are widely used as biomarkers of aquatic contamination. The evaluation of these biochemical systems can be useful as biomarkers of aquatic contamination in resident indicator species, allowing for early detection of environmental problems (Frenzilli et al., 2004). The analysis of 1-hydroxypyrene (1-OH-Pyr) in bile can also be used as biomarker of PAH exposure, since PAH metabolites are excreted into the bile and concentrated (Aas et al., 1998).

Considering that diesel can significantly affect oxidative stress parameters and biotransformation enzymes in Nile tilapias (*Oreochromis niloticus*) (Nogueira et al., 2010), in this work we were interested to know if biodiesel is also able to affect these same parameters in these fish species. Our hypothesis was that biodiesel would have less harmful effects than diesel on the proposed parameters, and that blends of diesel with increasing concentrations of pure biodiesel could also decrease its toxic effects on fish. For this, we exposed some tilapias to pure diesel, pure biodiesel (B100), B5 and B20 for 2 and 7 d, and assessed the levels of lipid peroxidation by the measurement of MDA levels, and activity of enzymes EROD, GST, SOD, CAT and GPx in gill and liver. The levels of 1-hydroxypyrene (1-OH-Pyr) in the bile of the fish and the capacity of diesel elements to cause EROD inhibition were analyzed as well. This is the first work reporting the effects of biodiesel and its blends with petroleum diesel in biochemical biomarkers in an aquatic organism.

2. Material and methods

2.1. Chemicals

All reagents were purchased from Sigma Chemical (St. Louis, MO, USA). Commercial diesel oil was obtained from a gas station and neat biodiesel was obtained from Biodiesel Division of JBS S.A., Lins, Sao Paulo State, Brazil. Some of the components of the biodiesel used in the present study are shown in Table 1.

2.2. Analyses of PAH in diesel and biodiesel

In order to evaluate the presence of PAH in diesel and biodiesel, samples were analyzed using a Shimadzu Prominence liquid chromatograph equipped with a fluorescence detector and a 20 μL loop injector. The analytical system included a ZORBAX Eclipse PAH column (5 μm particle size, 4.6 mm × 260 mm I.D.) and a mixture of acetonitrile and water as the mobile phase. The separations were performed under gradient conditions (flow rate: 1.3 mL min^{-1}, 0–9 min acetonitrile 40%; 9–13 min acetonitrile 40–80%; 13–18 min acetonitrile 80%; 18–20 min acetonitrile 80–90%; 20–37 min acetonitrile 90%). Fluorescent detection was performed by applying the following excitation (Ex) and emission (Em) wavelength program: 220/322 nm (determination of naphthalene to fluorene), 240/398 nm (determination of phenanthrene to benzo(g,h,i)perilene), and 300/498 nm (determination of indene(1,2,3-cd)pyrene). The identification of PAHs in diesel and biodiesel were done comparing the retention time of the peaks with those from authentic PAH standards, and by spiking samples with the same standards under the same conditions. PAH analyses and sample preparation were done according to the procedure described by Obuchi et al. (1984). A preparative solid-phase (SPE) extraction C18 cartridge was conditioned with 5 mL of acetronitrile/water (85:15, v/v), and then 50 μL of diesel or biodiesel was added into the column. The fuels were eluted with acetronitrile/water (85:15, v/v) and the first 10 mL were collected in a volumetric glass. A volume of 20 μL of the samples was sampling and injected into HPLC-FD for PAHs identification. In the case of diesel, samples had to be diluted 20 times in acetronitrile/water (85:15, v/v) before injections.

Table 1
Some components of biodiesel used in the present study, according to information from the manufacturer.

Compound	Amount
Water content	256 mg kg^{-1}
Total contaminants	22 mg kg^{-1}
Carbon residues	0.02%
Ester content	98.6%
Sulfated ash	0.01%
Total sulfur	5.65 mg kg^{-1}
Sodium + potassium	1.15 mg kg^{-1}
Calcium + magnesium	1.00 mg kg^{-1}
Phosphorus	1.5 mg kg^{-1}
Free glycerin	0.01%
Total glycerin	0.21%
Monoglycerides	0.62%
Diglycerides	0.20%
Triglycerides	0.0%
Methanol	0.0%

2.3. Test organisms

The specimens of *O. niloticus* were obtained from the Monte Aprazivel's agricultural school (state of Sao Paulo, Brazil). The average weight was 57.12 g, and the average length was 11.82 cm. Both males and females were used. The animals were placed in individual tanks (20 L) with dechlorinated water at a controlled temperature (25 °C). Before the exposure began, the animals went through a period of 3 d of acclimatization. The fish were fed with commercial fish food once a day during the experimental period.

This work has permission from the Ethics Committee for Animal Use in research of the "Universidade Estadual Paulista" (CEUA-IBILCE/UNESP).

2.4. Experimental procedure

Fish were exposed in tanks without contaminant (controls) and with addition of diesel, B5, B20 and B100 at concentrations of 0.01 mL L^{-1} and 0.1 mL L^{-1}. Each group was composed by five animals exposed individually to the treatment in aquariums of 17 L (five real replicates for each experimental group). After 2 and 7 d of exposure, five fish were removed from each experimental group and anesthetized with benzocaine (45 mg L^{-1} water) to remove liver and gills. The organs were stored immediately at −80 °C. In total 90 animals were used, with 45 fish per exposure. The concentrations used in this work were based on a previous study done by our research group in which tilapia were exposed to concentrations of 0.1 and 0.5 mL L^{-1} of diesel oil for 2 and 7 d respectively (Nogueira et al., 2010). However, fish exposed to higher

concentration died after 4 d of experiment. Thus, we adopted a concentration of 0.1 mL L^{-1} and a tenfold lower concentration (0.01 mL L^{-1}) for this work to guarantee the survival of animals until the end of the experiment.

2.5. Preparation of samples for analysis of EROD, GST, SOD, CAT and GPx

Liver and gill tissues were homogenized (1:4, w/v) in Tris buffer 20 mM (pH 7.4), sucrose 0.5 mM, KCl 0.15 mM and 1 mM protease inhibitor (PMSF). The samples were then centrifuged at 10 000*g* for 20 min at 4 °C. To obtain the cytosolic fraction, the supernatant portions were collected and re-centrifuged at 50 000*g* for one additional hour at 4 °C. The supernatant obtained after this second centrifugation was used for the analyses of SOD, CAT, GPx and GST, and the pellet of the liver samples was re-suspended in 100 μL of Tris buffer (100 mM, pH 7.5), containing EDTA 1 mM, dithiothreitol 1 mM, KCl 100 mM, and 20% glycerol, and used for the analysis of EROD activity.

2.6. Enzymatic assays and protein quantification

EROD activity was measured using the Burke and Mayer method (1974), but with some modifications. 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. The reaction was observed for 3 min at 30 °C. EROD activity (pmol min^{-1} mg^{-1} of protein) was calculated based on a previously prepared resorufin standard curve. GST activity was determined by measuring the increase in absorbance at 340 nm, incubating reduced glutathione (GSH) and 1-chloro-2, 4-dinithrobenzene (CDNB) as substrates, following Keen et al. (1976). SOD activity was evaluated by the inhibition of cytochrome c reduction in the presence of the hypoxanthine/xanthine oxidase $O_2^{\cdot}$ generator system at 550 nm (McCord and Fridovich, 1969). CAT activity was quantified at 240 nm by the H_2O_2 decomposition according to Beutler's method (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). Protein levels were measured by the method of Bradford (1976) using bovine serum albumin as standard.

2.7. Lipid peroxidation

Lipid peroxidation levels were determined by measuring the product formed from the combination of malondialdehyde and thiobarbituric acid (TBA) through High Performance Liquid Chromatography (HPLC) and UV/Vis detection, and using a modified version of the method used by Almeida et al. (2004). Tissue (100 mg) was homogenized in 0.3 mL of Tris buffer 0.1 M, pH 8.0. Next, 40 mg of TBA was dissolved in 10 mL of HCl 0.2 M and 0.3 mL of this solution was added to the sample. The reaction mixture was then heated at 90 °C for 40 min. The colored derivative was then extracted with 1 mL of *n*-butanol 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. The HPLC system (ESA) consisted of ESA584 pump and an ESA526 UV/Vis detector. The column used was an ACE 5 C18 (250 × 4.6 mm, 5 μm). Chromatogram monitoring and peak identification and quantification were performed using the EZ Chrom Elite software (Agilent Technologies). The mobile phase was 0.05 M KH_2PO_4, pH 7.0, with 40% methanol, and was pumped at an isocratic flow of 1 mL min^{-1}.

2.8. Bile collection and quantification of 1-OH-Pyr

Bile was extracted from the fish with a hypodermic syringe, transferred to an Eppendorf microtube, which was then sealed and stored in ice until the analyses. The procedure for 1-hydroxypyrene determination followed the recommendations described by Ariese et al. (1993). A standard stock solution of 1-OH-Pyr (0.15 g L^{-1}) in dichloromethane was prepared. From this solution, another standard solution was prepared at 2.00 mg L^{-1} in ethanol/water 48% (v/v), for the construction of a standard calibration curve. The bile extracted from the tilapias was diluted in the proportion of 1:1000 with ethanol/water 48% (v/v), and measured by fluorescence in terms of a standard calibration curve of 1-hydroxypyrene from 1.0 to 20.0 μg L^{-1} similar to Ariese et al. (1993). Fluorescence was measured with a Cary Eclipse (Varian) spectrofluorimeter, which consisted of a xenon discharge lamp, two Monk–Gillieson monochromators, a Hamamatsu photomultiplier and quartz cells (1 × 1 cm). Slits for the excitation and emission monochromators were set at 5 nm, the photomultiplier voltage was adjusted to 600 mV, and the monochromator scan rates were 600 nm min^{-1}. The synchronous fluorescence peak of the 1-hydroxypyrene was located at 342 nm when $\Delta\lambda = 40$ nm.

2.9. In vitro EROD inhibition tests by diesel components

Ten liver samples of fish exposed to diesel were prepared as described above. The samples were then mixed during 30 s in a vortex and separated in 21 aliquots of 100 μL that were divided in seven groups of three aliquots (three replicates). The first group of three replicates was used to test the stability of EROD activity along time, by incubating the samples at 37 °C and measuring EROD activity at intervals of 10 min, during 60 min. The subsequent six groups were incubated during 30 min with 0.00% (control), 0.25%, 0.50%, 1.00%, 2.50% and 5.00% of pure diesel. After the incubations, EROD activity was measured in all samples.

2.10. Statistical analyses

Tests for normality (Shapiro–Wilk) and homogeneity of variances (Levene) were applied. Data were logarithmically normalized for CAT (gill and liver, 7 d), GST (liver, 2 and 7 d), 1-OH-pyrene (2 and 7 d) and MDA (gills, 2 and 7 d). Then, for comparisons between treatments of the same exposure period were made using one-way ANOVA followed by Fisher LSD test. To find differences between both exposure period for the same treatment (same concentration and contaminant) was applied the Student-*t* test. For non-parametric data Kruskal–Wallis test was used for the first case and Mann–Whitney in the second case. Significant differences were accepted only when $p < 0.05$. Analyses were performed with software Statistica 7.1.

3. Results

3.1. Analysis of PAH in diesel and biodiesel

The pure biodiesel chromatogram (Fig. 1A) revealed no peaks, indicating the absence of PAH on its composition. The pure diesel sample had to be diluted 20 times for the identification of PAH peaks (Fig. 1B), since the injection of pure diesel without dilution produced a chromatogram with very intense and undefined peaks (data not shown). The comparisons of diesel sample peaks with PAH standards (Fig. 1C) revealed the presence of specific PAH in diesel, like naphthalene, acenaphthene, fluorene, phenanthrene, fluoranthene, benz(a)anthracene, and chrysene.

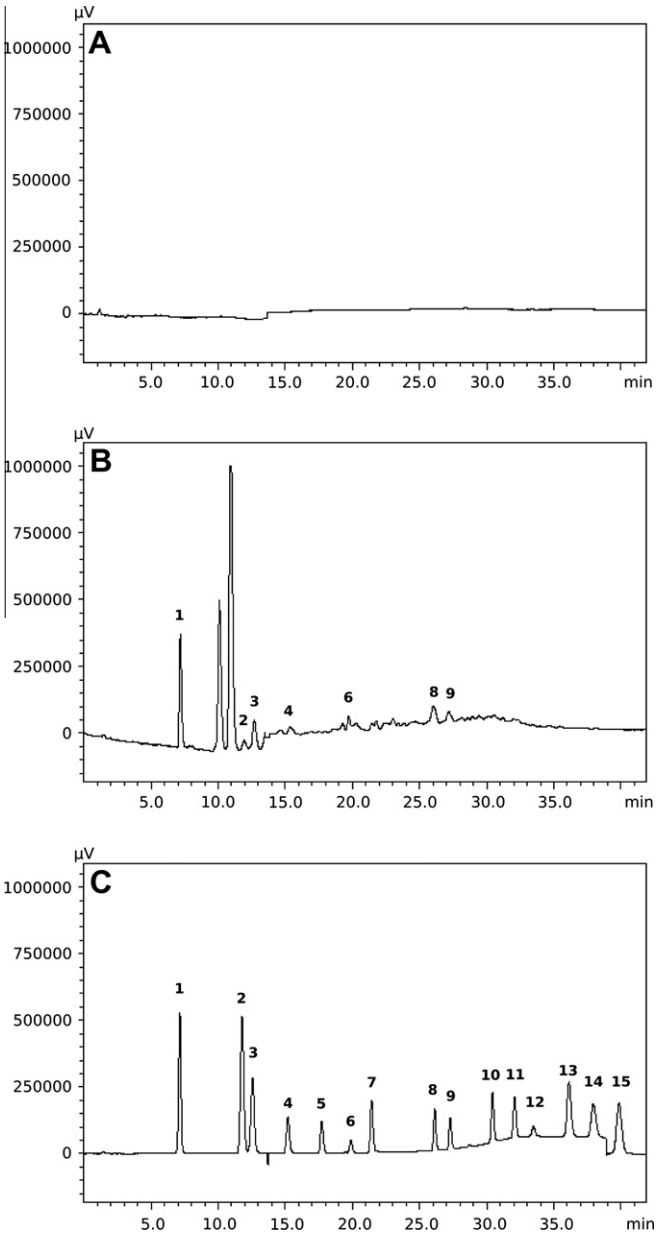

Fig. 1. HPLC-FD chromatogram of pure biodiesel (A), diesel diluted 20 times (B), and HPA standards (C). 1 – Naphthalene; 2 – acenaphthene; 3 – fluorene; 4 – phenanthrene; 5 – anthracene; 6 – fluoranthene; 7 – pyrene; 8 – benz(a)anthracene; 9 – chrysene; 10 – benzo(b)fluoranthene; 11 – benzo(k)fluoranthene; 12 – benzo(a)pyrene; 13 – dibenz(a,h)anthracene; 14 – benzo(g,h,i)perylene; 15 – indene(1,2,3-c,d)pyrene.

3.2. EROD activity

The EROD activity increased after 2 d of exposure to both concentrations of diesel and B20 and to the lower concentration of B5 when compared to control (Table 2). For the period of 7 d, there was increased activity in all treatments compared to control groups, except those exposed to pure biodiesel. Fish exposed to both concentrations of diesel oil and the concentration of 0.1 mL L^{-1} of B5 for 7 d showed higher EROD activity compared to animals exposed to the same treatment for 2 d. In both periods of exposure, EROD activity was higher in fish exposed to a concentration of 0.01 mL L^{-1} of B5 than in animals exposed to a concentration of 0.1 mL L^{-1} of that contaminant.

3.3. GST activity

In the liver, GST activity was lower in fish exposed to B100 0.01 mL L^{-1} for 7 d when compared to the control group and the same treatment for 2 d (Table 2). In the gill, the GST activity was lower in the group exposed for 2 d to B100 0.01 mL L^{-1} compared to control (Table 3). After 7 d of treatment, animals exposed to both concentrations of B100 showed lower GST activity than the control, unlike the groups exposed to diesel and B5 at a concentration of 0.1 mL L^{-1}, in which the enzyme activity was higher than in control group. Moreover, the GST activity was lower in gills of tilapias exposed to B5 0.01 mL L^{-1} than in fish exposed to B5 0.1 mL L^{-1}.

3.4. SOD activity

The hepatic SOD activity showed differences only in treatments for 2 d (Table 2). The activity was lower in groups exposed to both concentrations of B5, compared to respective control. The opposite happened with the group treated with pure biodiesel (0.1 mL L^{-1}) in which the enzyme activity was higher compared to the control and other treatments (Table 1). SOD in gills showed no differences between treatments (Table 3).

3.5. CAT activity

With the exception of fish exposed to 0.01 mL L^{-1} of diesel oil, liver catalase activity increased in all groups exposed for 7 d compared to the control and treated groups for 2 d. Furthermore, the enzyme activity was higher in tilapia exposed to 0.1 mL L^{-1} of diesel oil than 0.01 mL L^{-1} of that contaminant after 7 d of exposure (Table 2). Gill catalase activity remained unchanged in all groups (Table 3).

3.6. GPx activity

The hepatic GPx activity in fish exposed to diesel 0.01 mL L^{-1} was higher than in the control and animals exposed to diesel with a higher concentration after 7 d of treatment (Table 2). After 7 d of exposure to contaminants, the gill GPx activity in the groups treated with 0.01 mL L^{-1} of diesel and B5 was lower compared to control and those exposed to the same treatment for 2 d (Table 3).

3.7. MDA levels

The level of lipid peroxidation in liver was higher in tilapia exposed to 0.1 mL L^{-1} of B5 when compared to the control group, B5 0.01 mL L^{-1} and the group exposed to the same contaminant and the same concentration for 2 d (Table 2). The MDA concentration after 7 d was lower in the liver of animals exposed to higher concentrations of biodiesel, and this fact probably has no relation to the treatment.

In the gills, there was an increase in the concentration of MDA in the groups exposed to B5 0.1 mL L^{-1} and the B100 at both concentrations after 2 d when compared with the control group (Table 3). In this same period, the lipid peroxidation in animals exposed to B100 0.01 mL L^{-1} was higher in those who received treatment with diesel and B5 at a concentration of 0.01 mL L^{-1}. The same happened with the animals exposed to B100 0.1 mL L^{-1} than in group B20 of the same concentration.

After 7 d of exposure peroxidation in the gills of fish exposed to 0.1 mL L^{-1} of B100 was greater than in controls and fish exposed to other treatments except the lowest concentration of B100.

Table 2
Enzyme activity and MDA concentration in liver of *O. niloticus*.

Period	Treatment	Concentration (mL L^{-1})	Biomarkers					
			EROD[A]	GST[B]	SOD[B]	CAT[B]	GPx[B]	MDA[C]
2 d	Control	–	12.62 ± 6.48	3.56 ± 0.36	85.55 ± 15.21	64.25 ± 3.02	0.04 ± 0.02	1.36 ± 0.93
	Diesel	0.01	35.83 ± 10.29[a,c]	4.49 ± 0,69	70.24 ± 9,91	67.08 ± 13,58	0.03 ± 0,01	1.25 ± 0,19
	Diesel	0.1	29.49 ± 7.57[a,c]	4.03 ± 0.51[c]	70.02 ± 15.16	73.05 ± 21.93[c]	0.04 ± 0.02	0.98 ± 0.27
	B5	0.01	39.91 ± 9.78[a,b]	4.05 ± 0.76	63.98 ± 11.31[a]	71.55 ± 14.65[c]	0.04 ± 0.02	2.15 ± 1.01
	B5	0.1	25.40 ± 10.59[c]	3.66 ± 0.71	62.04 ± 9.89[a,c]	66.93 ± 15.00[c]	0.04 ± 0.01	1.26 ± 0.57[c]
	B20	0.01	42.62 ± 14.33[a]	4.11 ± 0.82	73.15 ± 20.17	71.87 ± 13.68[c]	0.04 ± 0.02	2.29 ± 0.60
	B20	0.1	35.02 ± 14.66[a]	4.18 ± 0.53	86.90 ± 11.57	73.12 ± 11.55[c]	0.04 ± 0.01	2.03 ± 0.97
	B100	0.01	12.42 ± 8.02	3.71 ± 0.27[c]	90.19 ± 14.14[b]	78.30 ± 11.91[c]	0.02 ± 0.01[b]	1.53 ± 0.76
	B100	0.1	10.73 ± 3.65	4.06 ± 0.10	114.10 ± 15.09[a,c]	72.39 ± 17.30[c]	0.04 ± 0.02	1.83 ± 1.51
7 d	Control	–	7.33 ± 3.04	3.48 ± 0.42	72.21 ± 7.93	71.62 ± 7.25	0.04 ± 0.004	1.52 ± 0.41
	Diesel	0.01	47.84 ± 4.37[a]	4.39 ± 1.42	79.11 ± 15.12	95.09 ± 31.37[b]	0.06 ± 0.02[a,b]	0.99 ± 0.33[b]
	Diesel	0.1	44.33 ± 10.27[a]	4.84 ± 0.42[a]	60.64 ± 16.78	127.67 ± 20.40[a]	0.03 ± 0.01	2.01 ± 0.70
	B5	0.01	48.07 ± 8.90[a,b]	2.97 ± 0.87	64.58 ± 10.49	129.69 ± 24.38[a]	0.05 ± 0.01	1.78 ± 0.39[b]
	B5	0.1	39.00 ± 6.49[a]	3.61 ± 0.77	60.48 ± 13.03	103.75 ± 7.49[a]	0.03 ± 0.01	3.04 ± 0.52[a]
	B20	0.01	29.36 ± 3.33[a]	3.38 ± 0.45	54.90 ± 7.58	108.10 ± 10.21[a]	0.05 ± 0.02	1.69 ± 0.36
	B20	0.1	27.59 ± 3.89[a]	4.32 ± 1.02	55.61 ± 10.64	109.28 ± 20.65[a]	0.04 ± 0.003	1.38 ± 0.58
	B100	0.01	6.03 ± 5.18	2.07 ± 0.60[a,b]	64.34 ± 14.00	106.39 ± 17.73[a]	0.05 ± 0.01	1.06 ± 0.26
	B100	0.1	8.28 ± 6.98	3.25 ± 1.00	64.65 ± 17.39	128.13 ± 48.23[a]	0.04 ± 0.01	0.90 ± 0.31[a]

Note: Data expressed as mean ± standard deviation.
[A] Activity expressed as pmol min^{-1} mg^{-1} protein.
[B] Activities expressed as U mg^{-1} protein.
[C] Concentration expressed as nmol g^{-1} tissue.
[a] Significant difference compared to the control of the same period.
[b] Significant difference compared to the same contaminant exposed groups with the highest concentration.
[c] Significant difference between the exposure periods to the same group.

Table 3
Enzyme activity and MDA concentration in gills of *O. niloticus*.

Period	Treatment	Concentration (mL L^{-1})	Biomarkers				
			GST[A]	SOD[A]	CAT[A]	GPx[A]	MDA[B]
2 d	Control	–	0.24 ± 0.08	7.32 ± 1.07	6.43 ± 0.97	0.032 ± 0.010	2.13 ± 0.31
	Diesel	0.01	0.25 ± 0.08	9.00 ± 2.50	7.43 ± 1.06	0.025 ± 0.007[c]	1.87 ± 0.44
	Diesel	0.1	0.31 ± 0.07	8.39 ± 0.52	6.71 ± 1.07	0.028 ± 0.010	2.24 ± 0.42
	B5	0.01	0.32 ± 0.09	8.20 ± 0.31	6.45 ± 2.07	0.020 ± 0.003[c]	1.88 ± 0.44[b]
	B5	0.1	0.31 ± 0.09	8.07 ± 0.64	6.29 ± 0.86	0.022 ± 0.004	3.20 ± 1.20[a]
	B20	0.01	0.25 ± 0.07	9.32 ± 1.46	6.41 ± 1.92	0.029 ± 0.007	2.80 ± 0.28
	B20	0.1	0.24 ± 0.03	6.75 ± 1.42	7.12 ± 0.27	0.024 ± 0.005	2.11 ± 0.56
	B100	0.01	0.15 ± 0.02[a]	9.16 ± 1.43	6.24 ± 0.78	0.027 ± 0.006	3.27 ± 1.19[a]
	B100	0.1	0.18 ± 0.03[c]	8.98 ± 1.27	5.83 ± 1.56	0.022 ± 0.004	3.10 ± 0.90[a]
7 d	Control	–	0.19 ± 0.06	5.63 ± 0.33	6.46 ± 1.27	0.023 ± 0.010	2.06 ± 0.38
	Diesel	0.01	0.22 ± 0.03	6.44 ± 0.75	5.54 ± 1.51	0.011 ± 0.004[a,b]	1.94 ± 0.46
	Diesel	0.1	0.29 ± 0.08[a]	6.66 ± 1.77	7.96 ± 1.96	0.019 ± 0.005	1.91 ± 0.64
	B5	0.01	0.21 ± 0.07[b]	7.70 ± 1.59	6.48 ± 1.07	0.013 ± 0.003[a]	1.68 ± 0.28
	B5	0.1	0.33 ± 0.08[a]	7.78 ± 1.75	6.81 ± 1.89	0.017 ± 0.007	1.95 ± 0.20
	B20	0.01	0.14 ± 0.03	7.07 ± 0.57	5.87 ± 0.73	0.019 ± 0.004	2.62 ± 0.63
	B20	0.1	0.19 ± 0.04	7.03 ± 3.39	5.56 ± 0.80	0.020 ± 0.005	2.01 ± 0.33
	B100	0.01	0.11 ± 0.04[a]	8.60 ± 1.81	6.51 ± 0.73	0.017 ± 0.003	2.80 ± 0.87
	B100	0.1	0.09 ± 0.04[a]	7.12 ± 1.34	6.27 ± 1.01	0.016 ± 0.002	3.82 ± 1.32[a]

Note: Data expressed as mean ± standard deviation.
[A] Activities expressed as U mg^{-1} protein.
[B] Concentration expressed as nmol g^{-1} tissue.
[a] Significant difference compared to the control of the same period.
[b] Significant difference compared to the same contaminant exposed groups with the highest concentration.
[c] Significant difference between the exposure periods to the same group.

3.8. Levels of 1-OH-Pyr in the bile

Data for 1-OH-Pyr concentrations found in the bile are shown in Fig. 2. For some groups the withdrawal of the bile was unable due to disruption of the gallbladder during dissection of the fish. All treatments caused a significant increase in the levels of 1-OH-Pyr excreted in the bile after 2 and 7 d of exposure, except for those fish exposed to B100. Considering the same concentration, animals exposed to 0.01 mL L^{-1} of B20 presented significantly higher 1-OH-Pyr in the bile compared to B5. Comparing the same treatment along the exposure days, there was a significant decrease in 1-OH-Pyr from 2 to 7 d of exposure, except for animals exposed to 0.01 mL L^{-1} of B5 and B20, and animals exposed to0.1 mL L^{-1} of B20.

3.9. EROD inhibition by diesel

The EROD activity was stable after the incubation of samples at 37 °C for 1 h. The enzyme activity also remained stable in all measurements taken every 10 min (data not shown). When proteic extracts were incubated *in vitro* during 30 min with increasing concentration of diesel, a significant concentration-dependent

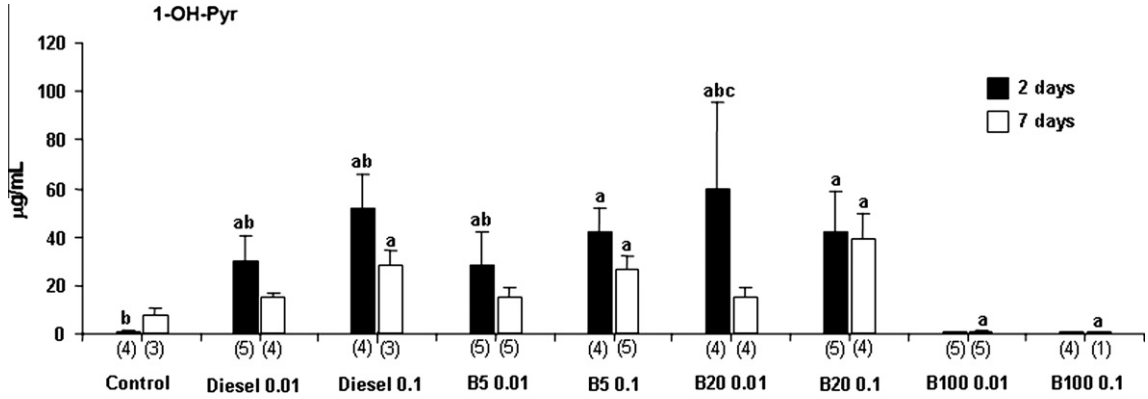

Fig. 2. 1-OH-Pyr levels in the bile of *Oreochromis niloticus*. The number in parenthesis represents the number of fish analyzed. [a]Significant difference compared to the control of the same period of exposure. [b]Significant difference comparing the same concentration of the same contaminant, between 2 and 7 d of exposure. [c]Significant difference compared to the group exposed to the same concentration of B5 after 2 d of exposure.

decrease in EROD activity was observed (Fig. 3). There was 70% inhibition of EROD activity in samples incubated with the lowest concentration of diesel (0.25% of the total extract volume), and about 100% inhibition was obtained by incubating the extracts with 1% of diesel.

4. Discussion

Although the use and production of biodiesel have been largely encouraged as a renewable and non-toxic alternative to petroleum diesel, the potential toxicity of this new fuel is almost unknown yet. To our knowledge, there is only one paper describing the toxic effects of biodiesel and its blends with petroleum diesel in aquatic animals, but just concerning the establishment of lethal concentrations for *D. magna* and *O. mykiss*, as already mentioned.

Polycyclic aromatic hydrocarbons are present in petroleum products and are described in the literature as aryl hydrocarbon receptor agonists in various organisms, including fish. The activation of these receptors promotes a chain of reactions that will synthesize the isoform of cytochrome P450 1A (CYP1A), leading to increased activity of this enzyme (Whyte et al., 2000), and often EROD activity is increased in fish exposed to PAHs (Kopecka-Pilarczyk and Correia, 2009; Trídico et al., 2010) and oil derivatives (Gold-Bouchot et al., 2006; Pathiratne et al., 2009). In our study, EROD activity was highly induced in all groups containing diesel and its blends with biodiesel, but not in fish exposed to B100. This enzyme was probably not induced in animals exposed to B100 because this compound does not contain PAHs, at least at significant concentrations, as shown in this work.

Curiously, when the EROD results for only the groups exposed to B5 are compared, we noted that animals exposed to the lower concentration presented a significant higher EROD induction in comparison to those animals exposed to 0.1 mL L^{-1}. This result was the same for 2 or 7 d of exposure. It could be hypothesized that animals exposed to the higher concentration of diesel had a general impairment of metabolism, affecting their capacity to adequately induce EROD activity, while animals exposed to the lower concentration were able for a better EROD response. In fact, in a previous work (Nogueira et al., 2010) we observed that tilapias exposed to diesel at 0.1 mL L^{-1} presented a significant EROD induction compared to control animals, but this induction was not observed when the fish were exposed for 2 d to 0.5 mL L^{-1}, and all fish died when exposed to 0.5 mL L^{-1} for 7 d, indicating that higher diesel concentrations may contribute to an impairment of metabolism, and possibly leading to death.

Indeed, as already mentioned, the diesel fuel is a complex mixture of several compounds derived from petroleum (Vieira et al., 2007). Thus, there may be substances in this kind of fuel that at higher concentrations leads to an inhibition of the enzyme or in some stage of the pathway for the synthesis of cytochrome P450. Kopecka-Pilarczyk and Correia (2009) also observed that EROD induction was higher in animals exposed to the lower concentration of a PAH mixture (phenanthrene, pyrene and fluorene),

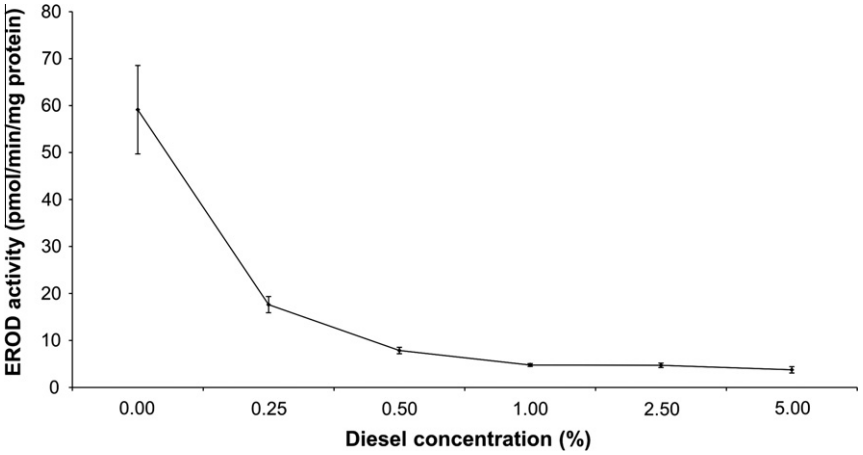

Fig. 3. EROD inhibition profile after incubation fish samples with increasing concentration of pure diesel.

compared to those animals that were exposed to a higher concentration, presenting lower EROD induction, in liver of *Sparus aurata*. Their results agreed with a previous work by Bucheli and Fent (1995) who noted that the activity of cytochrome CYP1A could be inhibited at higher concentrations of some inducers, such as β-naphtoflavone and certain PCBs.

To better clarify this response, we incubated some proteic extracts of those fish presenting high EROD activity with increasing concentrations of pure diesel. The results showed that diesel was able to strongly inhibit EROD activity, even at very low concentration as 0.25% (70% inhibition), thus confirming that diesel contain substances capable of inhibit EROD activity.

The levels of 1-OH-Pyr measured in the present study demonstrated that toxic PAHs are absorbed, metabolized and excreted in tilapias exposed to diesel and its blends with biodiesel, corroborating EROD results. Also, as our study was performed by using a single concentration of the contaminants, it was noted that levels of pyrene metabolite decreased significantly along the exposure period, being significantly higher after 2 d of exposure compared to 7 d of exposure, except for animals exposed to 0.1 mL L^{-1} of B20, which remained higher. This indicates that animals were able to deal with PAHs from diesel at the concentrations tested, and that PAH concentration decreased along the exposure period, as the fish eliminates it from water through their biotransformation processes.

The levels of the pyrene metabolite found in bile of different groups of fish did not differ between the groups diesel, B5 and B20, indicating that despite the proportion of biodiesel increase in these different treatments, the rate of 1-OH-Pyr excretion was the same. The only exception was the group B20 at 0.01 mL L^{-1}, which presented significantly higher metabolite compared to B5 at the same concentration in water after 2 d of exposure, and considering that the metabolite level did not decrease after 7 d of exposure. Considering that the amount of PAH is probably lower in B20 than pure diesel and B5, the lack of differences in 1-OH-Pyr in the bile or even higher concentrations in animals exposed to B20 confirm the possibility that biodiesel increase the rate of PAHs absorption from diesel by fish, or that it increases the capacity of fish to excrete the metabolites. However, this remains to be further confirmed.

Compared to fish exposed 7 d to B100, the control group after 7 d presented significantly higher 1-OH-Pyr in the bile, which was unexpected. We hypothesize that, as the control aquariums were placed near the aquariums contaminated with pure diesel, it is possible that some PAHs from diesel-treated aquariums migrated to the control aquariums through volatilization and atmospheric deposition along the exposure period. Nevertheless, the slight 1-OH-Pyr increase observed in controls after 7 d, compared to the control group after 2 d of exposure probably did not affect fish metabolism, since changes in EROD activity were not observed. This result is also important for planning future experiments, indicating that control aquariums should be placed away from those containing treatments.

Another interesting result was that the EROD response was concentration-dependent according to the proportion of biodiesel in diesel oil. Compared to controls, animals exposed to B100 had no differences in EROD activity. On the other hand, those animals exposed to B20 presented a significant EROD induction, compared both to controls and B100. However, the EROD induction was even significantly higher in animals exposed to B5 and pure diesel, compared to B20. This indicates that the use of B20 in substitution to pure diesel can be less deleterious to aquatic animals.

In our studies, increased hepatic GST activity only occurred on the seventh day for the groups exposed to higher concentration of diesel, which also showed a high EROD activity. Increases of GST jut after 15 d of exposure to diesel oil have been previously demonstrated (Simonato et al., 2008). This delay probably occurs because the induction of this enzyme is also related to the presence of metabolites generated by cytochrome P450 during the phase I of xenobiotic metabolism. Probably, if the exposure period of our experiments were larger, we would observe a greater induction of this enzyme.

In the gill, the GST activity was elevated in fish exposed to diesel, and B5 (0.1 mL L^{-1}) after 7 d. However, as observed for hepatic GST in fish exposed for 7 d to B100 (0.01 mL L^{-1}), the GST in the gills was reduced compared to control groups exposed to pure biodiesel (0.01 mL L^{-1} of B100 in 2 d of treatment and for two concentrations of B100 after 7 d). It has been shown that GST activity is influenced by the levels of organic substrates, and induction or inhibition has been reported in several studies (van der Oost et al., 2003; Kopecka-Pilarczyk and Correia, 2009). Indeed, it has been demonstrated that some GST isoforms have peroxidase activity (Almeida et al., 2005), and increases in this enzyme, as seen in our study, can be also related to an antioxidant defense of the organism.

Exposure to diesel and its blends (not considering B100) for 7 d caused oxidative stress in the liver, evidenced by the increase in lipid peroxidation levels (B5 0.1 mL L^{-1}), GPx activity (diesel 0.01 mL L^{-1}), and CAT activity (diesel 0.1 mL L^{-1}; B5 and B20 both concentrations). The chain reaction of cytochrome P450 as well as redox-cycling reactions promoted by diesel components can be responsible for the formation of reactive oxygen species in tissues (ROS) leading to oxidative stress (Munro et al., 2007). Choi and Oris (2000) also observed increased lipid peroxidation in the liver of fish (*Lepomis macrochirus*) exposed to anthracene. Clams (*Ruditapes decussates*) from aquatic sites contaminated with PAHs also exhibit increased levels of MDA in the gills and digestive glands (Geret et al., 2003) indicating that these compounds can generate oxidative stress. In gills of fish from our study, oxidative stress was less evident; since no increases in lipid peroxidation neither antioxidant enzymes were observed, except for the group exposed to 0.1 mL L^{-1} of B5 for 2 d.

When the group B100 was compared to the control group, it was noticed that pure biodiesel can also be responsible for oxidative stress generation in liver and gill. Two days of exposure to B100 caused a significant increase in SOD activity in the liver and also a significant increase in MDA levels in the gill. After 7 d of exposure, CAT activity was significantly increased in the liver, being possibly responsible for the significant decrease in MDA levels at the higher B100 concentration. In the gill, there was also a significant increase in MDA levels after 2 and 7 d of exposure to B100, indicating that although biodiesel does not contain toxic PAHs in its composition, it can be also responsible in some way for oxidative stress generation.

As the biodiesel used in this study was made using animal fat as source, it can be also supposed that biodiesel can be absorbed through the gill, increasing the amount of fat acids in the gill tissue, contributing to an increase in the levels of lipid peroxidation. Also, it is possible that biodiesel contains MDA in its composition due to degradation processes along production, storage or even produced during the exposure experiment. To check this, some aliquots of the biodiesel were analyzed for the presence of MDA (data not shown) through the same procedure used for MDA measurements in fish samples (HPLC–UV), and it was noted that the biodiesel used in this work presented MDA at about 3.58 nmol mL^{-1}.

This indicates that biodiesel could contribute for the MDA levels in gills, since it is presented as a by-product in the biodiesel formulation and could be absorbed by the exposed animals. However, this is just a speculative explanation, mainly considering that just 0.01 and 0.1 mL L^{-1} of the biodiesel were used, corresponding to 0.358 and 3.58 nmol of MDA added to the aquarium for each liter of water, respectively. As the animals were exposed in aquariums

of 17 L, the effective amount of biodiesel possibly available in water may be 0.17 and 1.7 mL, respectively, corresponding to a maximum incorporation of 0.61 nmol and 6.1 nmol of MDA in the whole aquarium, which can be absorbed by the exposed animals. On the other hand, the lack of differences in animals exposed to B20 may be related to a protective effect of diesel on biodiesel oxidation. According to the National Renewable Energy Laboratory (2009), diesel decreases the oxidation rate of biodiesel, with a less generation of oxidative products. In their experiments, levels of acid compounds and peroxides generated due to auto-oxidation of B100 increased significantly along 12 weeks, while the test showed no indication that biodiesel oxidation is occurring in B5 blends, under the same test conditions.

Indeed, the gills represent a large contact surface with the environment, and it is very important in respiration, osmoregulation and excretion (Simonato et al., 2008). Because of this close contact with the environment and therefore with the contaminants, oxidative stress due to B100 exposure was more evident in this tissue.

The B5 was able to produce effects similar to diesel oil in the activity of EROD and catalase in liver and GST and GPx in the gills, and has generated more lipid peroxidation than diesel oil in both tissues, and also inhibited the liver SOD. Biodiesel is likely to increase the lipophilicity of the mixtures, causing an increase in absorption of toxic compounds present in diesel fuel. Because B20 has a lower concentration of diesel oil in its composition, enzymatic changes and hence the oxidative stress generated by this blend were smaller than the changes promoted by B5. Khan et al. (2007) observed in their acute toxicity tests with *D. magna* and *O. mykiss*, in general, the larger the fraction of diesel oil, the higher is the percentage of mortality. Thus, it was shown that B5 has a higher percentage of mortality than either B20 or B50 over time for these organisms.

5. Conclusions

Biodiesel B20 had fewer adverse effects than the diesel and B5 for the Nile tilapia, considering the experimental conditions used in this work. Thus, the use of B20 and probably mixtures with higher concentrations of biodiesel is more feasible by reducing the impact of this compound on the aquatic environment. However, our results, taking into consideration the fish species, the concentrations and exposure period used in the experiments show that biodiesel from animal fat and its blends with diesel oil also cause oxidative stress and enzymatic changes in *O. niloticus*. Therefore, although a more biodegradable fuel that emits less greenhouse gases, the results of this study show that biodiesel and its blends, at the conditions tested, also present hazards to aquatic biota. Thus, such compounds must be carefully handled to avoid spill and discharges into the environment. This highlights the importance of further studies relating to exposure of other aquatic species to biodiesel and its blends, in different concentrations and exposure periods, so we can better understand the mechanisms of toxicity that these compounds can generate.

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