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Estimation of Biodiesel Cytotoxicity by Using Acid Phosphatase as a Biomarker of Lysosomal Integrity

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Abstract Biodiesel is promoted as environmentally less harmful than diesel fuel. Nevertheless its water-soluble-fraction (WSF) may contain methanol, which appears by a reversion of the transesterification reaction, when biodiesel contacts water. This paper evaluated the loss of the lysosomal membrane integrity in liver homogenate of juvenils Tilapia exposed to biodiesels-WSF, through the increase of the acid phosphatase activity, as an evidence of citotoxicity. Differences in the enzyme activity levels (3.4, 2.3 and 0.8 mU mg^{-1} total protein over the control value, which was 1.6 mU mg^{-1} total protein), found for castor oil, waste cooking-oil and palm oil-biodiesels, respectively, were indicative of their toxicity according to this decreasing trend. WSF-chromatograms suggest the cytotoxicity as related to methanol.

Keywords Biodiesel · Hepatotoxicity · Lysosomal biomarker · Acid phosphatase

Biodiesel is receiving special attention as potential substitute for diesel due to its physical–chemical characteristics and low global warming potential. Greenhouse gas emissions, originating from burning this fuel are significantly reduced in comparison with fossil diesel (Yang et al. 2000). Nevertheless this biofuel, chemically characterized as fatty acids esters, generally obtained via transesterification reaction of vegetable oils or animal fats with methanol may pollute soil and water bodies (Leite et al. 2011). The transesterification reaction is reversed by hydrolysis generating methanol, a potent hepatotoxic substance (Roel et al. 2000), prevalent in the water soluble fraction of this biofuel (Pereira et al. 2012).

The estimation of ecotoxicological effects based sole on chemical analysis of contaminants is not effective. Chemical analysis measure pollutants concentrations, but do not assess bioavailability or the possible interactions of effects resultant from exposure to toxic substances. These integrative actions can only be evidenced through biological responses such as toxicity tests or biomarkers. Biomarkers involve biological responses to stressors, evaluated at a biochemical, cellular or physiological levels (Nascimento et al. 2006). The first ecotoxicological effects resultant from interactions between the pollutant and the biota happen at a biochemical/cellular level (Nascimento et al. 1998; Fent 2001). Thus biomarkers can detect cyto-physiological alterations in organisms exposed to contaminants, previously to disease installation and death (Petrovic et al. 2004; Martins et al. 2005).

Alterations of the lysosomal membrane stability have been used as biomarker of generalized cellular stress pollution by Nicholson and Lam (2005). Lysosomes are multifunctional organelles rich in hydrolytic enzymes, responsible for breaking macromolecules that penetrate the cell via endocytosis. The lysosomal ability to sequester contaminants from sensitive intracellular sites is an essential protective mechanism, which makes the lysosomal membrane particularly susceptible to high concentrations of toxic compounds (Martins et al. 2005; Nazar et al. 2008). Under excess of exogenous substances, intralysosomal storage can increase the membrane permeability leading to the efflux of hydrolytic enzymes, and to the

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autolytic cell activity (Nicholson 2001). These effects are always associated with cellular dysfunction that may progress up to degenerative disease, apoptosis and cell death (Van Nierop et al. 2006). Different techniques have been used to the assessment of lysosomal stability as a biomarker. Techniques related to autophagy or endocytosis of stains, such as neutral red and cresil violet, were proved to reflect ecotoxicological responses associated with environmental changes (Martins et al. 2005). Other techniques use the efflux of the acid phosphatase (EC 3.1.3.2) as an effective biomarker. The activity of the acid phosphatase on different organisms has been used to determine pollutant effects on algae (Jonsson and Aoyama 2010; Pereira et al. 2012), *Daphnia magna* (Khangarot and Rathore 2003) and several species of fishes (Vijayavel and Balasubramanian 2007; Nazar et al. 2008). The acid phosphatase activity has also been taken as parameter for the evaluation of toxic effects of other chemicals and priority pollutants. The present research aimed to compare three different biodiesel-WSFs (B100) based on the assessment of lysosomal stability in liver homogenate of exposed *Oreochromis niloticus*, by using the acid phosphatase as a biomarker. The biodiesel samples have been obtained by methanol transesterification of *Ricinus communis* (castor oil), *Elaeis guinnensis* (palm oil) and waste cooking-oil (WCO).

Materials and methods

The biodiesel samples (B100) obtained by methanol transesterification of castor oil (CO), palm oil (PO) and waste cooking-oil (WCO) were treated at the Biomonitoring Laboratory, Institute of Biology, Federal University of Bahia, according to Anderson et al. (1974) to obtain the water-soluble-fractions (WSF). After homogenization (1,500 rpm), the samples were diluted (1:9 v/v) in distilled water (Milli-Q apparatus from Millipore®) and stirred at a constant speed (150 rpm) in closed Mariotti flasks for 20 h. After decantation, part of each WSF was collected and distributed in test containers by following the previously established standard operation procedures (SOPs) inserted in the QA/QC Laboratory Program (Nascimento et al. 2002; Nazar et al. 2008). These SOPs are based on the results of tests previously performed with different species. The tests comprise of exposing such species to a serial dilution of the tested material (0 %, 4.6 %, 10.0 %, 22.0 %, 46.0 %, 100 %) aiming to determine their effective concentrations (Martins et al. 2005; Paixão et al. 2007; Nazar et al. 2008; Nascimento et al. 2009; Leite et al. 2011; Pereira et al. 2012). All these tests involve a positive (standard reference toxicant) and a negative (blank) control. A system of DSS-control charts, based on dose–response results, is used to check for accuracy.

Based on the results of the LOEC (lowest observed effect concentration), juvenile fishes were exposed to 10 %-WSF/freshwater (v/v) for 24 h, at a constant temperature (22° C) and without any food. One control, in which fishes were held in similar aquaria containing only freshwater, was set up for each treatment. Physical and chemical parameters were evaluated at the beginning and at the end of each experiment. Following the ethical guidelines for euthanasia AVMA (AVMA—Association Veterinary Medical American - Guidelines on Euthanasia 2007), after exposure, the fishes were anesthetized by immersing them, for 5 min, in a solution of benzocaine (40 mg/L) previously diluted in ethanol (1:10; v/v), and were sacrificed by cervical section (Simonato et al. 2008). The liver was rapidly removed from each fish, and washed in a proportion of 1 g of tissue to 10 mL of a buffered solution (Manitol 25×10^{-2} M, TRIS 2×10^{-3} M, EDTA 4×10^{-4} M, disodium phosphate 10^{-2} M and monopotassium phosphate 10^{-2} M), adjusted to pH 7.4. After suspended in the same buffer free of phosphate, they were homogenized in a Potter–Elvejhem apparatus, at 250 rpm for a maximum of 2 min, in a bath of melting ice. The various components of the hepatic homogenate were separated by fractioned centrifugation (Sorvall Evolution®, at 4°C, from 900 to 22,000*g*).

To determine the acid phosphatase activity, the pellet originated from centrifugation at 22,000g, which contained most of the lysosomes, was re-suspended into the same quantity of the buffered solution as the initial homogenized material, and divided into 2 tubes. A 4×10^{-3} M digitonine solution was added to one of them and both were incubated in ice for 20 min. After this, they were centrifuged at 22,000g for 20 min to settle the intact lysosomes. The stability of lysosomal membranes was evaluated by determining the acid phosphatase activity, measured according to Roy et al. (1971), and expressed as mU mg^{-1} of total proteins, determined according to classical technique of Bradford (1976). The results obtained with the exposed fishes were compared to the controls using ANOVA and the Tukey–Kramer multiple comparison tests. Values were considered significant if $p < 0.05$.

To validate the toxicity results, chemical analyses were performed by Head-Space-Gas Chromatography carried out on fresh biodiesel-WSFs and on preserved samples. These WSFs were maintained in dark closed bottles at 4°C for 60 and 120 days, to identify the prevalent toxic agent and its degradation in water along the incubation period. A Varian CP-3900 (Automatic Headspace Sampler, TELEDYNE TEKMAR HT3™ Capillary Gas Chromatograph) was used for this purpose. The GC was programmed for an initial temperature of 50°C held for 5 min and increased to 250°C at 10°C min^{-1}. Nitrogen was used as carrier gas, at a constant flow rate of 2 mU mg^{-1} and the

injector was operated in split mode (20:1 split ratio). The 10 mL vials, containing 2 mL of WSF from the three types of biodiesel B100 were capped with Teflon lined septum caps. The WSF were then exposed to the headspace of the vial, and the volatile compounds were adsorbed. The retention time of methanol (Rt = 1.30 min) was determined by direct injection of neat methanol. The actual amount of methanol present in the WSF was read as 1×10^{-4} % mass/mass. This determination was performed using the described headspace SPME, and the GC conditions. Methanol peaks were clearly recognized by the use of a standard sample as control.

Results and Discussion

Pollutants can affect fish metabolism at various levels and cause physiological dysfunction, structural changes in organs and tissues, and alterations, which can impair growth and reproduction (Simonato et al. 2008). Several biochemical parameters have been used to estimate fish responses to toxic substances. Among the most investigated are enzymes present in liver tissue, most of them involved in the detoxification of xenobiotics and their metabolites (Van der Oost et al. 2003). As the main organ for metabolism, the liver plays important role in the storage, accumulation, biotransformation and excretion of xenobiotics in fish (Jimenez and Stegeman 1990), what explains the use of the hepatic homogenate as the target for the present study.

Based on the results of the present study, among the analyzed biodiesel-WSF, the one produced from castor oil (CO) was the most toxic, followed by those produced from waste-cooking oil (WCO) and palm-oil (PO) (Fig. 1). The observed values of acid phosphatase activity were 3.4, 2.3 and 0.8 mU mg^{-1} above the control (1.6 mU mg^{-1} of total proteins), respectively. The toxic effects of the WSF from

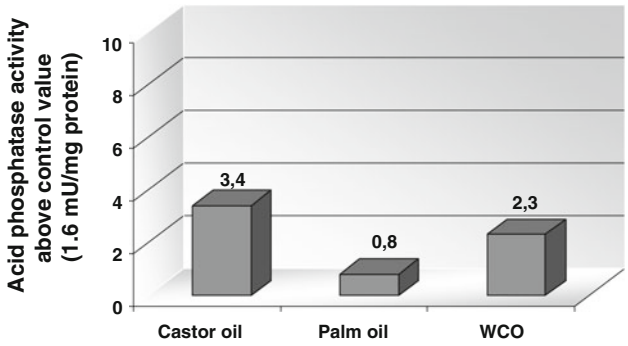

Fig. 1 Values of acid phosphatase activity over control value (1.6 mU/mg protein), measured in the liver lysosomal fraction of fishes (Tilapia), exposed to the biodiesel-WSFs from castor oil (CO), palm oil (PO) and waste-cooking-oil (WCO)

CO and WCO were significantly different ($p < 0.05$) between themselves, being also individually different from the control, while the results for PO-WSF did not differed significantly ($p > 0.05$) from the control. Except for the biodiesel-WSF from palm oil, the others tested WSFs showed to be hapatotoxic to fishes, since the results are indicative of a loss of stability of lysosomal membranes, thus causing cellular dysfunction by altering its biochemical capacity for response and defense.

Techniques that make use of the various components of the lysosomes are increasingly being used to monitor contaminants effects on aquatic biota. Lysosomes and cell membranes are the first pollutants targets (Jonsson and Aoyama 2010). The integrity of biological membranes is a prerequisite for the regulation of many cellular processes and its loss is recognized as cell damage (Strmac and Braunbeck 2002). When exogenous toxic substances act by weakening the lysosomal membrane, its rupture may occur and its enzymatic content, which has an acidic pH, is released into the cell protoplasm. In the present work, the higher acid phosphatase activity, in relation to the control, was associated to the degree of lysosomal membrane fragility, which was a consequence of lysosomal dysfunction in the liver of fishes exposed to biodiesels-WSF. The assumption that this technique represents an effective tool in the assessment toxicity has been previously claimed by several studies (Hwang et al. 2004; Petrovic et al. 2004; Martins et al. 2005; Okay et al. 2006), where the lysosomal membrane disfunction or disruption were evaluated by using the acid-phosphatase activity. This enzyme has been described as the most reliable among the marker enzymes (Petrovic et al. 2004; Van Nierop et al. 2006) for being involved in a number of cellular functions such as synthesis, transport, and metabolic regulation (Vijayavel and Balasubramanian 2007). Significant alterations in the stability of lysosomes isolated from tilapia hepatocytes when exposed to hydrocarbons present in the WSF of different gasoline formulations was also observed by using the acid phosphatase activity as a biomarker of lysosomal damages. Compared with the present results, the toxicity of the gasoline-WSF was higher than the biodiesel-WSF. This was resultant from the presence of aromatic hydrocarbons in the WSF of this fossil fuel (Nazar et al. 2008).

The samples used for ecotoxicological assessments were also analyzed by gas chromatography which revealed diverse methanol concentrations, according to the different biodiesels-WSF. This compound has been previously characterized as hepatotoxic (Roel et al. 2000; Pereira et al. 2012), and identified by other authors (Nascimento et al. 2009; Leite et al.2011), as the toxic agent in the biodiesel-WSF. The former results corroborate the data obtained in the present work (Fig. 2). Other oxidation by-products appeared in the WSF-chromatograms for all the samples

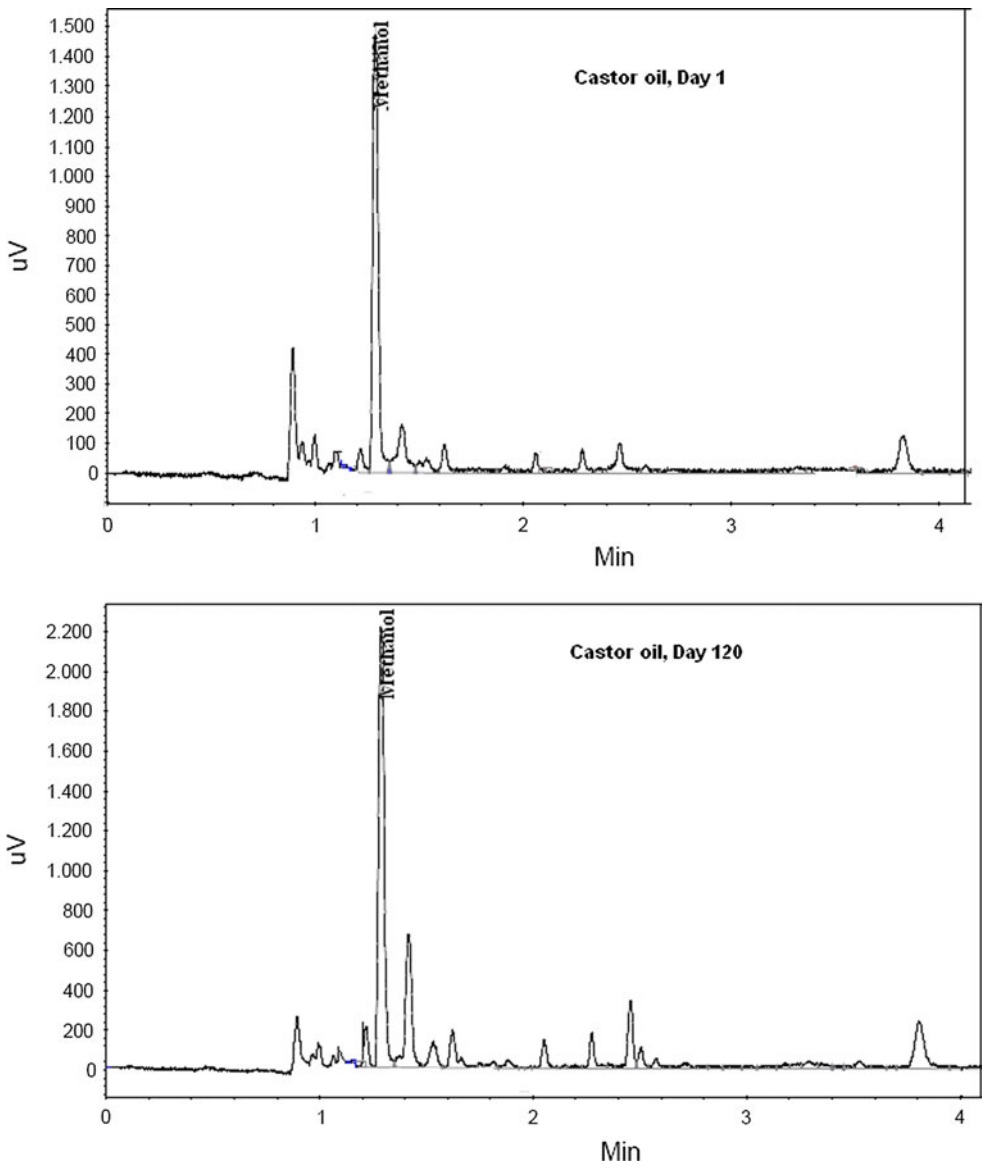

Fig. 2 CG-chromatograms of castor oil biodiesel-WSF, in freshwater at day 1 (*above*) and at day 120 (*below*)

analyzed, but were in very low quantities as compared to methanol. The methanol concentration values were significantly ($p < 0.05$) higher for castor-WSF than for PO and WCO-WSF, while not significantly different ($p > 0.05$) for the last ones (Fig. 3). The highest amount of methanol found in the CO-chromatograms can explain the highest toxicity of this product in relation to the other tested biodiesel-WSF.

Biodiesel water-soluble-fractions may reach the pluvial collecting system and, by runoff, can reach nearby freshwater or coastal ecosystems. Considering the reversibility of the transesterification process by hydrolytic reaction and the toxicological results of the present study, the biodiesels-WSF may became more toxic with time due to methanol formation. This would exacerbate environmental

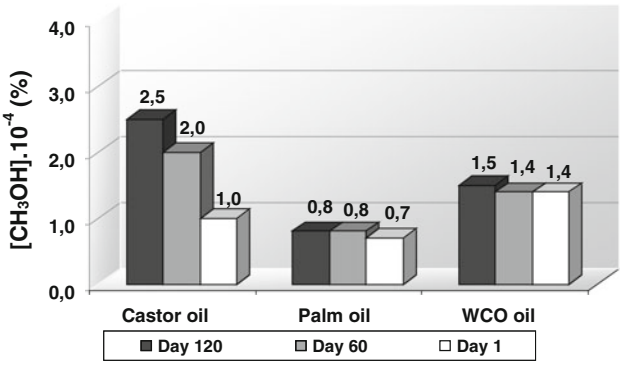

Fig. 3 Concentrations of methanol (%) obtained in CG-Chromatograms from castor oil, palm oil and WCO biodiesel-WSFs on freshwater, analyzed at different periods (1, 60 and 120 days) of incubation

risks and such fact has not been properly addressed. If biodiesel is to be used as a primary source of energy, these findings may represent a potential challenge for aquatic ecosystems.

The analyzed biodiesels-WSF from castor and waste-cooking oil were found to be toxic to fishes, indicating that biodiesel may be not strictly a biocompatible fuel. The environmental risk associated with the runoff of biodiesel water-soluble-fractions, should be addressed, considering the perspectives of replacing fossil fuels with biofuels in a higher extent. Methanol was identified as the most conspicuous contaminant in the biodiesel-WSF GC chromatograms, varying in quantity among the different biodiesel WSF analyzed. The partial reversion of the transesterification reaction, in the biodiesel- WSF may lead to a crescent methanol concentration that, in the present work, was higher for the castor oil, and lower, for palm oil. Recognizing that few investigations have been made on toxicity and degradation of WSF-biodiesel, the findings of this study provide an important foundation for further detailed investigation.

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