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# TOXICITY OF WATER-SOLUBLE FRACTIONS OF BIODIESEL FUELS DERIVED FROM CASTOR OIL, PALM OIL, AND WASTE COOKING OIL

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**Abstract**—Concerns over the sustained availability of fossil fuels and their impact on global warming and pollution have led to the search for fuels from renewable sources to address worldwide rising energy demands. Biodiesel is emerging as one of the possible solutions for the transport sector. It shows comparable engine performance to that of conventional diesel fuel, while reducing greenhouse gas emissions. However, the toxicity of products and effluents from the biodiesel industry has not yet been sufficiently investigated. Brazil has a very high potential as a biodiesel producer, in view of its climatic conditions and vast areas for cropland, with consequent environmental risks because of possible accidental biodiesel spillages into water bodies and runoff to coastal areas. This research determined the toxicity to two marine organisms of the water-soluble fractions (WSF) of three different biodiesel fuels obtained by methanol transesterification of castor oil (CO), palm oil (PO), and waste cooking oil (WCO). Microalgae and sea urchins were used as the test organisms, respectively, for culture-growth-inhibition and early-life-stage-toxicity tests. The toxicity levels of the analyzed biodiesel WSF showed the highest toxicity for the CO, followed by WCO and the PO. Methanol was the most prominent contaminant; concentrations increased over time in WSF samples stored up to 120 d. Environ. Toxicol. Chem. 2011;30:893–897. © 2011 SETAC

**Keywords**—Toxicity Methanol Marine Risk evaluation Biodiesel

## INTRODUCTION

The continuous depletion of limited fossil resources and the pollution and greenhouse gases resulting from burning fossil fuel ([1]; http://www.ipcc.ch/), has led to an international race for the development of alternative energy sources, including biofuels [2]. Brazil is one of the most prominent producers of biofuels in the world. It has favorable climatic conditions, availability of vast areas for cropland (90 million ha), and technological capacity ([3]; http://publications.worldbank.org/).

Biodiesel consists of long-chain fatty acid esters, derived from renewable sources such as vegetable oils, waste greases, or animal fats, generally obtained via transesterification with methanol. It has received intensive attention as a potential diesel substitute, because of its comparable engine performance and environmental characteristics [4,5] such as reduced emissions of particulate matter, hydrocarbons, and carbon monoxide, and zero emissions of sulfur oxides (Sox) and aromatic compounds [6].

Many authors refer to biodiesel as being biodegradable and nontoxic [7,8]. In relation to greenhouse gases emissions, the total substitution of diesel fossil for biodiesel is environmentally beneficial. Sheehan et al. [9], for instance, showed that the overall life cycle emissions of $CO_2$ are lower for biodiesel and blends than for 100% petroleum diesel. Nevertheless, when used in diesel engines, biodiesel blends can generate toxic, mutagenic, and carcinogenic emissions, such as some carbonyl compounds, which are currently unregulated. These include compounds that can act as important precursors to free radicals, ozone, and peroxyacylnitrates [10–13]. Increased aromatic hydrocarbons (polycyclic aromatic hydrocarbons and monocyclic aromatic hydrocarbon) emissions, such as phenanthrene, ethyl-benzene, and trimethyl-benzene, have been demonstrated with the use of biodiesel blends [14]. In addition, the biodiesel soluble fractions resulting from accidental spillages into water or from the effluents of its production may contaminate the aquatic environment, generating methanol because of the reversibility of the transesterification reaction by hydrolysis ([15,16]; http://www.anp.gov.br/).

The introduction of biodiesel in the Brazilian Energy Matrix was made official (Law 11.097) in 2005 and mandatory in 2008. The substitution of diesel fossil reached 5% in 2010. The installed production of biodiesel is currently estimated at 3.8 billion L/year [17]. This brings environmental risks because of inefficient conversion technologies of second-generation biofuels production and impacts of increasing use of pesticides and fertilizers for crops expansion; accidental spillage and runoff to water bodies; and insufficient knowledge about the ecotoxicological impacts of products and effluents from the biodiesel industry.

Comparing the effects of diesel fuel with biodiesel (rapeseed oil transesterified with methanol) on a microbial community of aerated soils, Lapinskienè et al. [18] showed that the biodiesel was toxic at concentrations above 12% (w/w), whereas the diesel fuel exhibited toxic properties at lower concentrations (>3% w/w).

The scarcity of research on the effects of the effluents from the biodiesel production processes or the effects of the soluble fractions of spilled biodiesel on aquatic biota does not meet the precautionary principle, defined by Chapman [19] as a tool

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of policy; risk assessment, based on good ecological data, is needed to provide a sound basis for management decisions [20]. This study fills a data gap by determining the toxicity, to two Brazilian species, of the water-soluble-fractions (WSF) of three different biodiesels (B100), obtained by methanol transesterification of castor oil (CO), palm oil (PO) and waste cooking oil (WCO).

## MATERIALS AND METHODS

### *Sample preparation*

The biodiesel samples were supplied by the Experimental Energy Laboratory at the Federal University of Bahia (Brazil) as B100 (neat biodiesel), obtained via methanol transesterification of CO, PO, and WCO via, respectively, homogeneous NaOH catalysis, acid catalysis, and basic catalysis. At the Biomonitoring Laboratory, Institute of Biology, Federal University of Bahia, the biodiesel samples were treated according to the U.S. Environmental Protection Agency [21], to obtain the water-soluble-fractions (WSF). After homogenization (1,500 rpm), the samples were diluted (1:9 v/v) in filtered seawater and stirred at constant speed (150 rpm) in closed Mariotti flasks for 20 h. After decantation, part of the WSFs was collected from the Mariotti flasks and distributed in test containers, according to the specified concentrations required for the test protocols [22]. Before testing, physical-chemical parameters, which could be a source of false positives, were checked to the range accepted for the test species. Part of the WSF was incubated in closed dark bottles, maintained under refrigeration up to 120 d. Chromatographic analyses were conducted on the 1st, 60th, and 120th d samples.

### *Chemical analysis*

Part of the WSF obtained from the biodiesel samples diluted in seawater (36 ppt) was immediately used for the toxicity tests, whereas the remaining WSF was stored in closed amber-glass flasks and kept under refrigeration at 4 °C, for 60 and 120 d. All of the samples were analyzed by gas chromatography (GC) at days 1, 60, and 120 to determine the concentration of methanol and to provide a comparison of the degradation of biodiesel-WSFs with storage time.

All measurements were carried out using a Varian CP-3900 (automatic headspace sampler, Teledyne Tekmar HT3 capillary gas chromatograph). The analyte was adsorbed onto the solid-phase microextration fiber and then thermally desorbed in the inlet of a Varian CP-3900 GC, equipped with a split/splitless injection port and flame ionization detection system. The injector and detector temperatures (150 and 250 °C, respectively) were held constant during the analysis. The fused silica (100%-methyl silicone) capillary column used for separation was a 30-m-long, 0.32-mm inner diameter, 3-μm film thickness Varian CP-3900 (100%-methyl silicone). The GC oven was programmed for an initial temperature of 50 °C for 5.0 min and then increased to 250 °C for 10 °C/min. Nitrogen was used as the carrier gas at a constant flow rate of 2 ml/min, and the injector was operated in split mode (20:1 split ratio).

The 10-ml vials, containing 2 ml WSF from the three types of biodiesel B100 (originated from WCO, PO, and CO) were capped with Teflon-lined septum caps. The WSFs were then exposed to the headspace of the vial, and the volatile compounds were adsorbed. The retention time of methanol (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 solid-phase microextration and the GC conditions. Methanol peaks were clearly recognized by the use of the standard samples as controls. The analytical standards used were traceable to certified reference materials.

### *Toxicity tests*

Toxicity tests were carried out using two organisms: the sea urchin *Echinometra lucunter* and the microalgae *Tetraselmis chuii*. Two different test protocols, the culture growth inhibition test, using microalgae, and the embryonic development test, using sea urchins [23], were applied. These tests, inserted in the quality assurance/quality control laboratory program, could guarantee the generation of precise and accurate results based on different end-points: the inhibition concentration (IC50) and the effective concentration (EC50), respectively, representing the WSF concentrations causing toxic effects (algae culture growth inhibition and sea urchin embryos abnormal development) to 50% of the exposed population. Both tests followed standard operating procedures developed previously for each test species, based on American Society for Testing and Materials [24] protocols and were performed by trained staff members from the Biomonitoring Laboratory. A system of control charts, based on dose–response results obtained from the same species exposed to a reference toxicant (dodecyl sodium sulfate), were used to determine the relative variability of repetitive data and the accuracy of results. All of the tests involved a positive (standard reference toxicant) and a negative (blank) control.

### *Sea urchin embryonic development test*

Adult sea urchin specimens were obtained from an area considered free of industrial and domestic wastes, at the coast of Salvador, Bahia, Brazil, and kept overnight in an aquarium containing filtered (20μm) natural seawater (36 ppt) taken from the same area. Before each test, gametes were collected from males and females (3–6 individuals) immediately after they started spawning under stimulation of a 0.5-M KCl solution injected into the body cavity [24,25]. The eggs were suspended in filtered (Whatman® Glassfiber filter, 47 mm ø, 1.2 μm pore) sterilized (129 °C; psi 1.5 $cm^{-2}$) seawater. Fertilization was accomplished by transferring 1 ml sperm to 100 ml of a dense egg suspension [22]. The necessary volume of the embryo suspension was calculated to provide a density of 20 embryos/ml in the test vessels.

The 100% WSF from each biodiesel sample obtained at the first day (corresponding to methanol concentrations of 2.1, 1.2, and 1.8 ppm for CO, PO, and WCO, respectively) was dosed in a dilution series of six loadings (0, 4.6, 10.0, 22.0, 46.0, and 100%), in triplicate vessels containing the same seawater used for fertilization, to which the embryos were pooled. Simultaneously, a reference toxicant-test (dodecyl sodium sulfate) was carried out to assure test precision [24]. After an exposure of 36 h, two 10 ml-samples were removed from each test vial, preserved in 5% buffered formalin, and later examined under a compound microscope. The first one hundred larvae or late embryos were counted as normal or abnormal [23,24], and the responses to the different treatments were recorded as the percentage of embryos failing to develop or developing in an abnormal manner [22,24]. Dissolved oxygen, salinity, temperature, and pH were maintained within the ranges of 6.5 to 6.9 ppm, 36 ppt, 25 ± 2 °C, and 7.0 to 8.5, respectively, according to the test protocol developed by Araújo and Nascimento [23].

Sea-urchin embryo responses to biodiesel WSFs were expressed as a percentage net risk of abnormality, using Abbot's formula [26]. Effects were calculated based on concentration–response curves and analyzed by the Trimmed Spearman Karber [27] computer statistical method. This was done to provide EC50 values, equivalent to the biodiesel WSF concentrations that may cause abnormalities in 50% of the exposed embryos. Means of the EC50 results and their standard deviations were calculated using the Graphpad InStat version 3.0 ([28]; http//www.graphpad.com).

*Microalgae growth-inhibition tests*

The microalgae were obtained from the culture collection of the Institute of Biology, Federal University of Bahia, where they had been maintained under appropriate controlled conditions. The tests involved the exposure of mono-specific *T. chuii*, until exponential growth phase, to dilutions of the different biodiesel WSFs and were carried out according to the methodology specified in the International Organization for Standardization 10253:1995, by using Conway medium and conditions previously applied to the species, to estimate [28] the toxicity of WSF of different gasoline formulations [29].

The same serial dilutions (0, 4.6, 10.0, 22.0, 46.0, and 100%) of biodiesel WSFs were applied to the algae culture growth test. The flasks were incubated for 96 h at a temperature of 24 °C in a rotary shaker under continuous illumination (4,000–4,800 lux) provided by fluorescent lamps (40W each). Coulter counting (Counter® model ZI 991 3044-B) was used to evaluate the reduction in number of cells (culture growth inhibition) when compared with the control (0% WSF). Each test was repeated three times and was fully randomized with regard to vial locations during incubation and the order of cell counts. Simultaneously, a reference toxicant (dodecyl sodium sulfate) test was conducted as described by the American Society for Testing and Materials [24] and the results checked against the laboratory dodecyl sodium sulfate control chart.

Effects were expressed as inhibition concentration values (equivalent to the biodiesel WSFs, which cause 50% algae culture growth inhibition), estimated by trimmed Spearman Karber [27], based on concentration–response curves from the culture growth exposure data. The coefficient of variation among tests was calculated to determine precision and repeatability. Possible significant differences in toxicity among the various biodiesel WSFs was determined by analysis of variance comparing the inhibition concentration results [28], followed by a Tukey test (parametric test of multiple comparison).

## RESULTS AND DISCUSSION

The WSF from CO showed the highest toxicity (EC50–36h = 4.22%) to *E. lucunter* embryos, followed by the WCO (EC50–36h = 8.95%), both differing significantly ($p < 0.05$) from the PO-WSF. The PO-WSF (EC50–36h = 22.25%) was the least toxic even though it was also significantly ($p < 0.05$) different from the control (Fig. 1). These results showed a slightly lower toxicity (higher EC50 values) but the same toxicity data trend as that determined by Nascimento et al. [15] for the same kind of biodiesel WSFs and test organisms (EC50 values: 2.45, 16.25, and 5.97% for CO, PO, and WCO-WSFs, respectively).

Embryos and larvae respond to many chemical variables by failing to develop or by developing in an abnormal manner [28,30]. The detection of any abnormality during embryonic development is a promising approach for estimating adverse effects of contaminants on ecosystems, based on the relationship between unsuccessful embryonic development and low larval recruitment [31]. This approach has only recently been applied to water-soluble fractions from biofuel products [15].

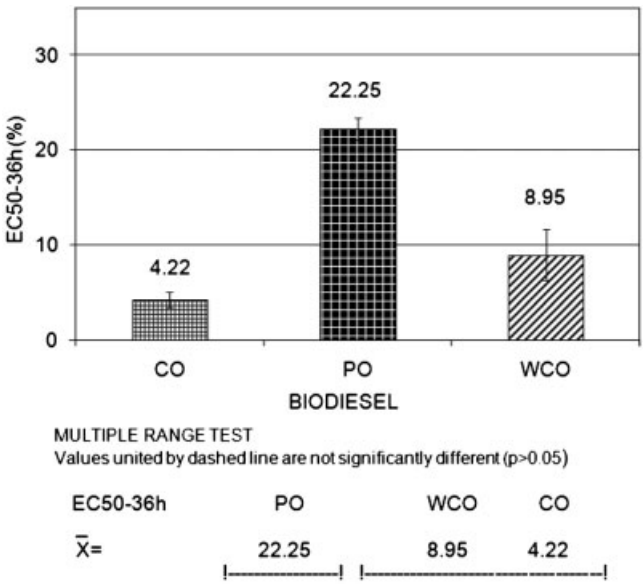


Fig. 1. Comparative responses of EC50 (effective concentrations that cause 50% abnormal embryo development) of *Echinometra lucunter*, exposed to water-soluble fractions of biodiesel fuels derived from castor oil (CO), palm oil (PO), and waste cooking oil (WCO).

Nascimento et al. [15] were the first authors to use this test protocol to evaluate the effects of biodiesel WSF exposure. However, larvae of various sea urchin species have been previously used to test the quality of waters and to provide data to estimate and prevent environmental harmful effects of effluents and products to be released in coastal areas [32–34].

The microalgae tests used in this research provided useful data to also infer the biological responses to chronic contamination; the tested WSFs (CO, PO, and WCO) inhibited the growth of *T. chuii* cultures, respectively, in concentrations (IC50–96h) of 48.3, 56.7, and 93.5% (Fig. 2). The data obtained for *T. chuii* in this study showed higher IC50–96 h values than previous data [15] from cultures of *Skeletonema costatum,* exposed to the same kind of WSFs, which indicates that *S. costatum* is more sensitive than *T. chuii* to the contaminant present in the biodiesel WSFs.

The toxicity results obtained in this research provide a basis for evaluating possible environmental risks associated with biodiesel WSFs to Brazilian marine organisms. Gas chromatography chromatograms carried out on the same biodiesel WSF samples used for the ecotoxicological estimates indicated that methanol, which is toxic to biota [35], was the most conspicuous contaminant in the samples. The reversion of the transesterification process, in the presence of water, can result in methanol production, which may increase during biodiesel degradation [15].

The methanol concentration in the incubated samples of the different biodiesel WSFs increased from day 1 up to day 120 (Fig. 3). Comparatively, the degradation (and consequently, the average methanol concentration) was significantly higher ($p < 0.05$) for the CO WSF, which leads to the conclusion that this ester is hydrolyzed more easily than in the biodiesel WSFs from palm oil or waste cooking oil, consequently indicating its higher relative hazard to the environment.

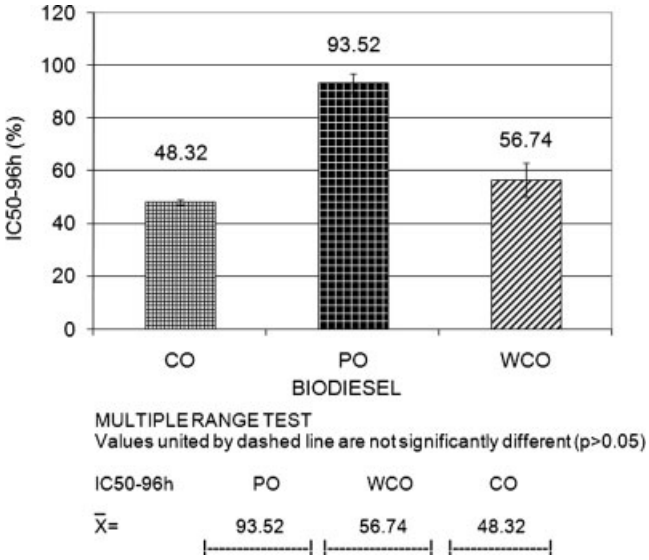


Fig. 2. Comparative responses of inhibition concentration (concentrations that cause 50% growth inhibition) of *Tetraselmis chuii* cultures exposed to water-soluble fractions of biodiesel fuels derived from castor oil (CO), palm oil (PO), and waste cooking oil (WCO).

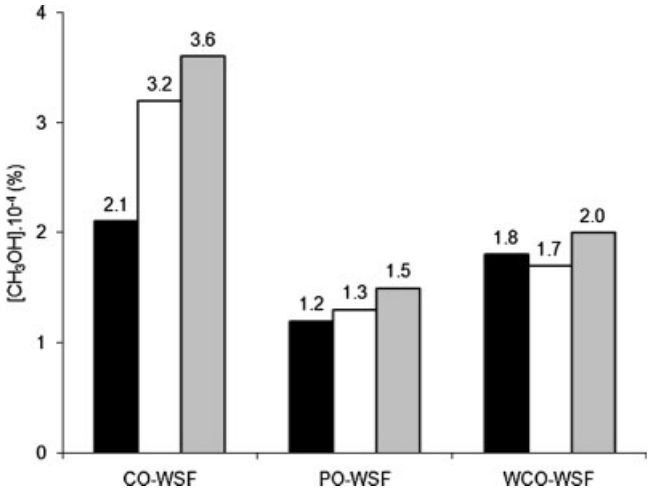


Fig. 3. Concentrations of methanol (%) obtained by gas chromatography. Chromatograms from the biodiesel-water-soluble fractions from castor oil (CO), palm oil (PO), and waste cooking oil (WCO), analyzed at different time periods (on the 1st, 60th, and 120th d of storage: Black, day 1; white, day 60; gray, day 120).

## CONCLUSIONS

By using local species, the sea urchin embryonic development test and the microalgae growth-inhibition test provided useful region-specific chronic toxicity data for the determination and prevention of biodiesel pollution. Methanol was shown to be the most conspicuous contaminant in the biodiesel WSF samples through GC chromatograms; both tests discriminated the different biodiesel samples according to concentration in the WSFs, pointing it out as responsible for the toxic responses. The findings of this study provide an important foundation for further detailed investigation into the effects of these products on coastal aquatic ecosystems.

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