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Genotoxicity assessment of water soluble fractions of biodiesel and its diesel blends using the *Salmonella* assay and the *in vitro* MicroFlow® kit (Litron) assay

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ABSTRACT

The designation of biodiesel as an environmental-friendly alternative to diesel oil has improved its commercialization and use. However, most biodiesel environmental safety studies refer to air pollution and so far there have been very few literature data about its impacts upon other biotic systems, e.g. water, and exposed organisms. Spill simulations in water were carried out with neat diesel and biodiesel and their blends aiming at assessing their genotoxic potentials should there be contaminations of water systems. The water soluble fractions (WSF) from the spill simulations were submitted to solid phase extraction with C-18 cartridge and the extracts obtained were evaluated carrying out genotoxic and mutagenic bioassays [the *Salmonella* assay and the *in vitro* MicroFlow® kit (Litron) assay]. Mutagenic and genotoxic effects were observed, respectively, in the *Salmonella*/microsome preincubation assay and the *in vitro* MN test carried out with the biodiesel WSF. This interesting result may be related to the presence of pollutants in biodiesel derived from the raw material source used in its production chain. The data showed that care while using biodiesel should be taken to avoid harmful effects on living organisms in cases of water pollution.

1. Introduction

Biodiesel is currently deemed a notable alternative to diesel oil, since it can be generated by domestic natural sources (e.g. soybean, rapeseed, etc.), contributing to a reduced dependence on petroleum-based fuels in countries where it is not produced (Balat and Balat, 2010). Apart from that, the biodiesel sector has also surged owing to the characterization of this biofuel as an environmentally friendly alternative to fossil fuels (Demirbas, 2009; Atadashi et al., 2010; Janaun and Ellis, 2010). However, the assessment of environmental impacts from the biodiesel industry refers mainly to air pollution (Yang et al., 2007; Hu et al., 2008; Karavalakis et al., 2009; EPA, 2010; He et al., 2010). There are few reports in the literature on its impacts on other biotic systems, such as aquatic environments (Khan et al., 2007; Leite et al., 2011; Nogueira et al., 2011).

Water polluted with harmful chemicals has constantly been both a current and worrying environmental issue. This is due to the need for better understanding how important water quality is for the health of living beings. In this context, and in view of the great incentive given to the biodiesel industry, some factors related to the production and the use of this biofuel must be taken into account in order to evaluate its actual hazard in case of undesirable environmental contamination.

One of the most relevant factors behind this issue refers to the use of biodiesel as a cleaning and bioremediation agent of crude oils (Miller and Mudge, 1997; Mudge and Pereira, 1999; Pereira and Mudge, 2004; Fernández-Álvarez et al., 2007), coal tar (Taylor and Jones, 2001) and PAH-contaminated soils (Gong et al., 2010). In this case, biodiesel acts as a "detergent," although less efficiently, acting as a dispersing and solubilizing agent, making chemical substances available and, consequently, enabling a more efficient action of degrading microorganisms (Mudge and Pereira, 1999; Taylor and Jones, 2001). Therefore, once the degrading action of microorganisms is not immediate, this bioavailability induction can cause deleterious effects on organisms as their exposure to hazardous pollutants increases.

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It is known that diesel oil contains about 2000 to 4000 hydrocarbons, including PAHs (Gallego et al., 2001), the ones which are known as dangerous environmental contaminants because of their noxious effects on the health of living organisms (Aina et al., 2006). Considering that a great deal of biodiesel is currently commercialized in diesel blends, the dispersing action of this biofuel may worsen the impacts of diesel water pollution.

Another fact that must be considered in this context refers to biodiesel quality certification. According to domestic and international standards, biodiesel quality assessment includes only parameters concerning the final quality that a biofuel must have before its use, such as viscosity, water content, ester content, total glycerol, and methanol or ethanol residues. (ANP, 2008). The presence of some contaminants from the raw material source used in the biodiesel production process – and which may negatively impact the environment –, is not currently considered when assessing final biodiesel quality.

Genotoxicity and mutagenicity assays are important to assess the safety of both chemical products and environmental samples. Different endpoints must be considered, such as point mutations and chromosome damage, which may be related to both numeric (polyploidy and aneuploidy) and structural alterations (breaks, losses, rearrangements, etc.). This way, genotoxic/mutagenic assessments require an elaborate analysis strategy in which *in vitro* assays have played an important role in this process over the last years (Flamand et al., 2006; Mun et al., 2009; Speit, 2009).

Among the several existing tests, the *Salmonella*/microsome assay and the *in vitro* micronucleus (MN) test are the ones most commonly used (Speit, 2009).

The *Salmonella*/microsome assay, also known as the Ames test, uses strains of *S. typhimurium* derived from the parental LT2, histidine auxotroph (his^-), showing different mutations in the operon of this amino acid. This test is constructed to detect mutations such as frameshifts or base-pair substitution in the DNA (Maron and Ames, 1983). Since the creation of the Ames test, several changes in the initial protocol have been made to facilitate the execution of the method and increase the sensitivity of the test. Among the variations developed, the preincubation protocol has proven to be more sensitive in detecting mutagens than the plate incorporation assay. One reason might be the fact that, in this protocol, short-lived mutagenic metabolites have a better chance of reacting with the tester strains in the small volume of the preincubation mixture (Mortelmans and Zeiger, 2000). Recently, the Ames microplate fluctuation protocol (MPF) assay, also known as Ames II, has been indicated as a valid alternative to the standard Ames, once it requires a smaller amount of test samples and reagents to be carried out, besides being more automated (Flückiger-Isler et al., 2004; Kamber et al., 2009; Umbuzeiro et al., 2010).

The MN test has been regarded as a simple and effective tool to estimate genotoxic effects resulting from chromosome damage inflicted by chemicals (Leme and Marin-Morales, 2009). This is due to the fact that MN either result from damage, or are unrepaired or wrongly repaired in parental cells, being easily measured in daughter cells as a structure similar to the main nucleus, but in a reduced size (Fenech, 2000).

Although microscopy-based scoring of MN is relatively quick compared to other tests, e.g. the chromosome aberration (CA) test, automation of this process has been the focus of several studies (Shi et al., 2010). A flow cytometry-based *in vitro* MN assay has recently been developed to increase the throughput capacity of the test, allowing MN measurements to be made in a large number of cells within a short period of time (Nüsse et al., 1994; Shi et al., 2010). However, problems related to this method have initially been detected, such as false-positive response resulting from cytotoxicity (Nüsse and Mark, 1997). In an attempt to solve this problem, Litron lab (Rochester, NY, USA) has recently developed the *in vitro* MicroFlow® kit (Litron) assay, which differently labels the apoptotic events and those events arising from a genotoxic effect (Avlasevich et al., 2006; Bryce et al., 2007, 2008, 2010; Collins et al., 2008; Shi et al., 2010).

Although marketed products are toxicologically evaluated to avoid risks to living beings, further assessments are still necessary to really estimate how dangerous these products are, mainly when it comes to environmental issues. Considering the increase in output and commercialization of biodiesel within the next years, and shortage of data about its impacts on living organisms, genotoxicologic evaluation of both biodiesel and its diesel blends, by running spill simulations in water, is made relevant and necessary. Therefore, the purpose of this study was to assess the mutagenic and genotoxic effects of water contaminated with biodiesel and its different diesel blends, using the *Salmonella* and the flow cytometry-based *in vitro* MN test assays. Additionally, as regards the *Salmonella* assay, two different procedures (preincubation and MPF) were used herein with the aim of evaluating their sensitivity in detecting mutagens.

2. Material and methods

2.1. Experiments and sample preparation procedures

The diesel (low sulfur diesel) and biodiesel (soybean biodiesel produced by transesterification with methanol) used in this study were kindly provided by BioVerde (a biofuel company), Taubaté-SP, Brazil.

To evaluate the mutagenic effects of water polluted with biodiesel and its blends of diesel oil, spill simulations in water were performed according to Nicodem et al. (1998) and Vanzella et al. (2007), in laboratory conditions, with some modifications. Briefly, a concentration of 1.5% (v/v) of the blends B5 (5% biodiesel + 95% diesel), B20 (20% biodiesel + 80% diesel), B50 (50% biodiesel + 50% diesel) and pure fuels B100 (biodiesel) and D100 (diesel) was floating on the surface of 40 L of non-impacted water in distinct glass containers. The simulations were submitted to continuous circulation with submersible water pumps and placed in the dark for 13 h prior to exposure to low–medium solar light for 9 h, simulating spills in tropical conditions (mean temperature around 33.6–20.8 °C). After that, the upper insoluble phase was discharged and the remaining water phase was collected and stored at 4 °C to further sample preparation.

The water samples were extracted according to the US EPA method 550.1 (EPA, 1990), as follows: the samples underwent solid phase extraction with C-18 cartridge (500 mg/6 mL, Phenomenex) and were eluted with dichloromethane. The extracts obtained were reduced in a rotary evaporator and dried in a gentle stream of pure nitrogen gas. Two samples were parallelly treated: one for chemical analysis and the other for biological assay. For the former, the dry extract was resuspended in acetonitrile and held at −20 °C until HPLC/Flu analysis. For the latter, the dry extracts were held at 4 °C and resuspended in dimethyl sulfoxide (DMSO) just before performing the bioassays.

The water used in the simulations carried out was also extracted and designated as water control (WC) to assure its quality.

2.2. Salmonella/microsome preincubation assay

The samples were tested in the preincubation *Salmonella*/microsome assay using *S. typhimurium* TA98 (*his*D3052, *rfa*, Δ*bio*, *uvr*B, pKM101), TA100 (*his*G46, rfa, Δ*bio*, *uvr*B, pKM101), TA1535 (*his*G46, rfa, Δ*bio*, *uvr*B) and TA1537 (*his*C3076, *rfa*, Δ*bio*, *uvr*B). The assay was performed using five doses and triplicate plates/dose, both in the presence and absence of S9 using a 30-min