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An overview of biodiesel soil pollution: Data based on cytotoxicity and genotoxicity assessments

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ABSTRACT

Biodiesel production has received considerable attention in the recent past as a nonpolluting fuel. However, this assertion has been based on its biodegradability and reduction in exhaust emissions. Assessments of water and soil biodiesel pollution are still limited. Spill simulation with biodiesel and their diesel blends in soils were carried out, aiming at analyzing their cytotoxic and genotoxic potentials. While the cytotoxicity observed may be related to diesel contaminants, the genotoxic and mutagenic effects can be ascribed to biodiesel pollutants. Thus, taking into account that our data stressed harmful effects on organisms exposed to biodiesel-polluted soils, the designation of this biofuel as an environmental-friendly fuel should be carefully reviewed to assure environmental quality.

1. Introduction

The use of plant oils as a fuel in the compression ignition engine is as old as the engine itself [1,2]. Biodiesel, a mixture of mono-alkyl ester obtained from renewable sources of biologic origin, has gained importance in the recent past for its ability to replace fossil fuels [3]. The increasing production and consumption of biodiesel has encouraged environmental researchers to assess its hazard and fate in the environment.

The anthropogenic pollution of soil has become an issue of intensive scientific research since the end of the 20th century [4]. Genotoxic compounds in soil may affect living organisms' health, including human beings, by exposing them through different pathways, such as ingestion of plants that uptake soil pollutants and leaching of compounds from soil to ground and surface water used as drinking water [5]. Oil spillages have currently become as frequent as increasing industrialization rate and consumption of energy worldwide [6]. Thus, like other fuels, biodiesel can reach the environment through accidental releases and/or routine losses associated with the use of this biofuel.

Hazard identification of environmental pollutants is measured by employing different biological assays. Assessing the toxic contamination of a solid medium like soil is not a simple task, due to the sterile conditions required by most of the bioassays commonly applied to genotoxic screenings. Assays conducted with plants and bacteria, such as *Salmonella* mutagenicity test and genotoxicity *Allium cepa* test, are among the most widely used test systems to evaluate soil quality. However, other assays, like *in vitro* tests with cultured mammalian cells, have been successfully used to assess cytotoxic and genotoxic potentials of environmental samples, including soil samples [7].

The analysis of different endpoints is a requirement for an accurate environmental investigation. *Salmonella*/microsome assay is a widely accepted short-term test used to detect agents that can produce genetic damage that leads to gene mutation [8]. The *A. cepa* test is a plant test system capable of evaluating chromosomal aberration (CA) and micronucleus (MN) induction [9]. Since the MN results from acentric fragments or whole chromosomes

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that were not incorporated into the main nucleus during the cell division cycle [10], the MN test is a valid alternative to predict outcomes from the CA test, making the evaluation simpler and faster [11]. To improve the throughput capacity of the MN test more and more, a flow cytometry-based *in vitro* MN assay has recently been developed, allowing MN measurements in a large number of cells in a short period of time [11–13]. Cytotoxic evaluations are also important due to their association with environment-related disease [14] and the fact that cytotoxic effects can mask genotoxic potentials of test agents.

Aiming at predicting the harmful effects of soil contamination with biodiesel and their diesel blends on living organisms' health, the purpose of the present study was to assess the cytotoxic and genotoxic potentials of soil polluted with these fuels using different biological assays, such as detection of changes in mitochondrial membrane potential ($\Delta\Psi_m$); apoptosis recognition by Annexin V; *in vitro* MicroFlow® kit (Litron) assay; *Salmonella* mutagenicity assay and *A. cepa* test.

2. Materials and methods

2.1. Experiments and sample preparation procedures

The diesel (low sulfur diesel specified according to Brazilian standard ANP no. 42/09 [15]) and biodiesel (soy-based biodiesel produced by transesterification with methanol – quality specifications in agreement with domestic biodiesel standard ANP no. 07/08 [16]) used in this study were kindly provided by BioVerde (a biofuel company), Taubaté, SP, Brazil.

Spill simulations with biodiesel and their diesel blends in soil were performed according to Taylor and Jones [17], with modifications. Briefly, 250 mL of the mixtures B5 (5% biodiesel + 95% diesel), B20 (20% biodiesel + 80% diesel), B50 (50% biodiesel + 50% diesel) and the pure fuels B100 (biodiesel) and D100 (diesel), were added into distinct plastic containers (polyethylene, 5000 cm^3) containing 500 g of non-aseptic air-dried soil. Hereafter, the polluted soil (500 g) were moistened with ultrapure water (100 mL) and then placed in darkness 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 polluted soils were collected and stored at 4 °C for further sample preparation.

The soil samples were extracted according to a modified version of the protocol described by Pena et al. [18], which use the matrix solid phase dispersion (MSPD) method. Briefly, this method includes MSPD with aluminum oxide. The mixture is transferred to a column containing anhydrous Na_2SO_4, aluminum oxide and silica gel. 0.5 mL of KOH saturated in methanol is added to this column and, finally, the elution is done using 15 mL hexane:acetone (1:1, v/v). The extracts obtained were reduced in a rotary evaporator, dried in a gentle stream of pure nitrogen gas. The method presents an average recovery for the polycyclic aromatic hydrocarbons (PAHs) greater than 80%, in repeatability and intermediate precision conditions, respectively, with adequate precision (RSD from 1.0 to 14.0%), for all compounds. Two samples were treated in parallel: one for chemical analysis and the other for biological assay. For the former, the dry extract was resuspended in acetonitrile and kept at −20 °C until HPLC/Flu analysis. For the latter, the dry extracts were kept at 4 °C and resuspended in dimethyl sulfoxide (DMSO) just before accomplishing the bioassays. Besides the PAHs HPLC/Flu analysis, the extract was also submitted to GC/MS qualitative analysis aiming to identify other compounds, such as FAMEs and phytosterols.

The characterization of the soil used to carry out the simulations (soil control – SC) is showed on Table 1.

2.2. Cytotoxicity and genotoxicity testing

2.2.1. In vitro assays

Doses

For all *in vitro* bioassays carried out in the present study, the tested doses of soil extracts started from the highest concentration of 500 mg equivalents of soil sample [19].

2.2.1.1. Cultured mammalian cell assays. Cell culture

Chinese hamster ovary cells (CHO-K1) and Human T cell leukemia (Jurkat) (all from DSMZ GmbH, Germany) were grown in culture medium at 37 °C, 5% CO_2, in a humidified atmosphere. For routine culturing, cells were maintained at a density up to 0.5–1.5 × 10^6 cells/mL for Jurkat and ∼80% of confluence for CHO-K1. RPMI-1640 medium supplemented with 1% L-glutamine 200 mM and 10% of fetal bovine serum (FBS) and minimum essential medium EAGLE with 1% L-glutamine 200 mM, 1% L-proline and 10% of FBS (all from Sigma) were used, respectively, for Jurkat and CHO-K1.

Determination of $\Delta\Psi_m$

Changes in $\Delta\Psi_m$ were detected using tetramethylrhodamine ethyl ester perchlorate (TMRE, CAS No. 115532-52-0, Sigma). Jurkat cells previously exposed to soil extracts (treatment time 24 h) were washed with phosphate buffered saline (PBS) and incubated with 25 nM of TMRE for 10 min at 37 °C. TMRE fluorescence was measured using FL2 channel (585 nm) of FACSCalibur™ flow cytometry (BD Biosciences). The percentage of cells with intact $\Delta\Psi_m$ is given as well as the percentage of cells with decreased $\Delta\Psi_m$, and the fold increase of cells with decreased $\Delta\Psi_m$ over the solvent control (FI) was the criteria used to evaluate these data. FI was determined by dividing the treated percentage by the percentage obtained for the solvent control and a >2-fold increase over the solvent control value was classified as positive for that sample.

DMSO at 1% and Etoposide (CAS No. 33419-42-0, Sigma) at 0.5 μg/mL (final concentration) were used as negative and positive controls, respectively. The experiments were carried out in duplicate/dose and repeated three times.

Annexin V-FITC apoptosis detection kit I (BD Pharmingen™)

Phosphatidylserine (PS) externalization to the outer leaflet of plasma membrane of apoptotic cells was assessed using Annexin V-FITC Apoptosis Detection Kit I (BD Pharmigen™). Briefly, Jurkat cells previously exposed to test samples (treatment time 2 h) were washed twice with cold PBS and resuspended in 1× binding buffer [10 mM Hepes/NaOH (pH 7.4) 140 mM NaCl, 2.5 mM $CaCl_2$]. The new solutions were then incubated with 5 μL of each Annexin V-FITC and PI for 15 min at room temperature (25 °C) in the dark. 1× binding buffer was added again to each solution right before flow cytometry (FACSCalibur™, BD Biosciences) measurements were taken. Annexin V^-/PI^-, Annexin V^+/PI^- and Annexin V^+/PI^+ indicate, respectively, viable cells, early apoptosis and cell death. The criteria of FI described above for TMRE assay was also adopted here.

DMSO at 1% was used as negative control, 10 μM of Staurosporine (CAS No. 62996-74-1, Sigma) as positive control for apoptosis induction and 10 mM of $MnCl_2$ (CAS No. 7773-01-5, Sigma) as positive control for necrosis. The experiments were performed in a single well/dose and repeated twice.

In vitro MicroFlow® kit (Litron) assay

The flow cytometry-based *in vitro* MN assay was applied to CHO-K1 cells previously exposed to soil extracts (treatment time 24 h) according to the *in vitro* MicroFlow® kit (Litron) manual. Essentially, upon completion of the chemical treatment of the test, the cells were first stained with a photo-activated dye (Dye 1 – EMA) and then washed, lysed and stained with lysis solutions containing RNase, nucleic acid Dye 2 (SYTOX Green) and the counting beads. Samples were protected from light and kindly resuspended right before flow cytometry measurements (FACSCalibur™, BD

Table 1
Characterization of the soil used to carry out the spill simulations with biodiesel and its diesel blends.

Sample	Soil texture											Organic matter (g/kg)	Electrical conductivity (dS/m)
	Sand (g/kg)						Silt (g/kg)	Clays (g/kg)		Flocculation (%)	Textural class		
	VCS	CS	MS	FS	VFS	TS		With dispersant	Water				
SC (soil control)	6	16	119	257	72	469	79	451	100	78	Clay	26	146.7

VCS, very coarse sand; CS, coarse sand; MS, medium sand; FS, fine sand; VFS, very fine sand; TS, total sand.

Bioscience). The percentages of relative survival, EMA-positive, hypodiploid events and MN were determined based on the acquisition of at least 10,000 gated nuclei per sample. The calculations were performed according to Bryce et al. [13,20], using Excel (Microsoft, Seattle, WA). Positive results were expressed herein as mean fold increased related to concurrent solvent control. Significant induction of MN and EMA-positive events was taken to be ≥3-fold mean increase over the mean observed for solvent control [21].

DMSO at 1% was used as a negative control and Mitomycin (MMC, CAS No. 50-07-7, Sigma) at 0.1 μg/mL and Vinblastine (VB, CAS No. 143-67-9, Sigma) at 10 ng/mL as positive controls. The experiments were carried out in duplicate/dose and repeated three times.

2.2.1.2. Bacterial assay. Salmonella/microsome preincubation assay

Samples were tested in the preincubation *Salmonella*/microsome assay using *Salmonella 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 30-min preincubation at 37 °C [8,22]. The S9 mixture was freshly prepared according to Maron and Ames [22] before each test using lyophilized Araclor-1254-induced rat liver S9 fraction (Moltox, Boone, NC), resulting in 4% (v/v) of S9 fraction in the mixture. The background was carefully evaluated and the colonies were counted by hand. Results were statistically analyzed by the Salanal computer program, which uses the Bernstein model [23]. A sample was considered positive when there was a significant positive dose response and a significant statistical difference between the tested doses and the negative control (ANOVA). The mutagenic potency was expressed in revertants/mL equivalent of water sample. The tester strains which showed positive results had their assays repeated three times to confirm the data.

The positive controls were carried out with 4-nitroquinoline-*N*-oxide (4-NQO, CAS No. 56-57-5, Sigma) at 0.05 μg/μL (TA98 and TA100 −S9); 2-aminoanthracene (2-AA, CAS No. 613-13-8, Aldrich, Seelze, Germany) at 0.25 μg/μL (TA98 and TA100 +S9); Sodium azide (NaN_3, CAS No. 26628-22-8, Sigma) at 0.05 μg/μL (TA1535 −S9); 9-aminoacridine (9-AAc, CAS No. 90-45-9, Sigma) at 0.50 μg/μL (TA1537 −S9); 2-AA at 0.10 μg/μL (TA1535 and TA1537 +S9). DMSO (Sigma) was used both to prepare the positive controls and as negative control.

Ames microplate fluctuation protocol (MPF) assay

The Ames MPF assay with the tester strains TA98, TA100, TA1535 and TA1537 was performed in liquid media in 24-well plates during sample exposure and in 384-well plates for revertant growth, according to 'Ames MPF Instructions for Use' (Xenometrix AG, Allschwil, Switzerland). Shortly, bacteria were grown overnight, diluted in Exposure Medium and exposed to test samples in 24-microwell plates for 90 min at 37 °C with agitation in either the presence or absence of 4.5% Aroclor 1254-induced rat liver S9 (Moltox, Boone, NC). The exposed cultures were then diluted in Indicator Medium and the contents of each 24-well culture were distributed into 48 wells of a 384-well plate (50 μL per well). After 48-h incubation at 37 °C, the plates were scored by eye for yellow wells. The criteria used to evaluate the MPF results were the fold increase in number of positive wells over the solvent control baseline (FIB), and the dose dependency. The fold increase of revertants was determined by dividing the mean number of positive wells at each dose by the solvent control baseline. The solvent control baseline was defined as the mean number of positive wells in the solvent control plus 1 SD. All solvent controls from an experiment with identical conditions were combined. An increase of >2-fold in relation to the baseline was classified as positive for that dose. Positive responses of >2-fold in relation to the baseline at more than one dose with a dose–response led the test sample to be classified as positive. A test sample was classified as negative where no response >2-fold the baseline and no dose–response was observed.

The positive controls were carried out with a 1:2 (v/v) mixture of 4-NQO at 2.5 μg/mL and 2-nitrofluorene (2-NF, CAS No. 607-57-8) at 50 μg/mL (TA98 −S9); 4-NOQ at 3333.3 ng/mL (TA100 −S9); N^4-aminocytidine (N4-ACT, CAS No. 57294-74-3) at 2.5 mg/mL (TA1535 −S9); 9-aminoacridine (9-AAc, CAS No. 90-45-9) at 375 μg/mL (TA1537 −S9); 2-AA at 125 μg/mL (for all tester strains used with S9) (all from Moltox, Boone, NC). DMSO was used to prepare the positive controls and as negative control.

2.2.2. In vivo assay

2.2.2.1. Allium cepa test. Unlike the assays above described, the *A. cepa* test was performed herein with the raw polluted soil (without extraction procedure), according to a modified version of the Grant's protocol [24]. Onion seeds (same stock and cultivar) were continuously exposed to test samples using distinct glass jars at 25 ± 2 °C. After reaching 2 cm in length the roots were collected and fixed in alcohol–acetic acid (3:1, v/v). The control tests were carried out with ultra-pure water (negative control) and methylmethane sulfonate (MMS, CAS No. 66-27-3, Sigma–Aldrich) at 10 mg/L (positive control). The slide preparation followed the procedure described by Leme and Marin-Morales [25]. 5000 cells were analyzed per treatment, being 500 cells per slide, totalizing 10 slides. To determine the potential induction of CA, all the possible chromosomal abnormalities observed in each slide were identified and scored. The genotoxic effects were also measured by counting the micronucleated cells in all slides of each treatment. The non-parametric test Kruskal–Wallis ($p < 0.05$) was used.

2.3. Chemical analysis

Identification and quantification of PAHs were done by liquid chromatography with fluorescence detection (HPLC-Flu) method, which allows for detecting the following PAHs: naphthalene, acenaphthylene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(e)pyrene, benzo(e)acefenantrilene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perilene, indene(123-cd)pyrene. The method presents an average recovery for the PAHs greater than 80%, in repeatability and intermediate precision

Table 2
PAHs detected in soil samples from the spill simulations.

PAHs (mg/kg)	SC	D100	B5	B20	B50	B100
Naphthalene	2.1	59.3	53.4	47.8	41.2	3.4
Acenaphthylene	0.1	1.4	1.3	1.2	0.9	0.2
Fluorene	0.2	9.8	8.8	7.6	7.1	0.8
Phenanthrene	1.3	37.7	29.9	27.1	24.3	2.1
Anthracene	0.2	1.3	1.2	1.1	1.1	0.7
Fluoranthene	0.1	0.6	0.6	0.5	0.5	0.6
Pyrene	0.1	4.5	4.0	3.8	3.2	0.4
Benzo(a)anthracene	nd	0.3	0.3	0.1	0.1	0.1
Chrysene	nd	2.7	2.4	2.3	2.2	0.6
Benzo(e)pyrene	nd	0.2	0.2	0.1	0.1	0.2
Benzo(e)acephenanthrylene	0.1	0.8	0.8	0.6	0.8	0.3
Benzo(k)fluoranthene	nd	1.1	1.0	0.8	0.9	0.9
Benzo(a)pyrene	nd	0.4	0.4	0.2	0.3	0.3
Dibenzo(a,h)anthracene	nd	0.2	0.2	0.1	0.1	nd
Benzo(g,h,i)perylene	nd	0.2	0.2	0.1	0.1	nd
Indene(123-cd)pyrene	nd	0.6	0.5	0.4	0.4	0.2
ΣPAHs	4.2	121.1	105.10	93.8	83.3	10.8

SC, soil control; D100 = pure diesel; B5 = blend 5% biodiesel + 95% diesel; B20 = blend 20% biodiesel + 80% diesel; B50 = blend 50% biodiesel + 50% diesel; B100 = pure biodiesel; nd, not detected (below detection limit, which ranges from 0.02 to 35.00 μg/L depending on the PAH analyzed).

conditions, respectively, with adequate precision (RSD from 1.0 to 14.0%), for all compounds.

The chromatographic conditions used were optimized and included a Varian HPLC apparatus equipped with a 230 Controller pump, ProStar autosampler, and a 360 Fluorescence detector (FL) (Varían Inc.). The wavelength program was: excitation wavelength (λ_{ex}) 220 nm and emission wavelength (λ_{em}) 322 nm (0–9.6 min); 240 nm (λ_{ex}) and 398 nm (λ_{em}) (10.0–32.0 min); and 300 nm (λ_{ex}) and 498 nm (λ_{em}) (32–40.0 min). A Supelcosil LC-PAH column (250 mm × 4.6 mm × 5 μm) obtained from Supelco (St. Louis, MI, USA) was used at 22 °C. Gradient ACN: water elution began with 60% acetonitrile (5 min) and increased to 100% ACN in 20 min, remaining for 15 min in the latter condition. The flow rate used was 1.5 mL min^{-1}. The injection volume was 50 μL. The peaks were identified by comparison with the retention time for authentic PAH standards.

For GC–MS qualitative analysis, the extracts were analyzed using a gas chromatograph Varian model CP-3800 coupled to mass spectrometry detector ion trap Varian Saturn-2000 (GC–MS/MS), CP-8200 autosampler, capillary column fused silica: FactorFour VF-5ms, 30 m × 0.25 mm × 0.25 μm (5% phenyl, 95% methylpolisiloxane) from Varian Inc.

The instrument conditions used for analysis of extracts by GC–MS were as follows: acquisition mass range 30–450 Da, ionization mode by electronic impact (70 eV). The injection volume was 1.0 μL split (split ratio 10:1), injector temperature 300 °C, helium as carrier gas at 1 mL min^{-1}, linear velocity: 60 cm s^{-1}. Oven temp: 150 °C (hold 1.25 min) to 300 °C @ 8 °C/min (hold 10 min). Compound identification was done by fragmentogram study and comparison with NIST Spectra Library.

3. Results

Results of PAH chemical analysis are shown in Table 2. GC–MS analyses enabled the identification of some FAMEs as the methyl estheres from palmitic and stearic acids (C18:0) and from oleic acid (C18:1), besides the presence of β-sitosterol.

Our data showed cytotoxic effects to B5 and B50 soil extracts after *in vitro* exposure using mammalians cells. The results of Annexin V/PI assay pointed to an induction of early apoptotic events to Jurkat cells exposed to B5 soil extract at 5 mg/mL (Table 3). Depolarization of $\Delta\Psi_m$, also an indicative of apoptosis, was observed in Jurkat cells after their exposure to B50 soil extract at 5 mg/mL

Table 3
Measurements of apoptotic and necrotic events by Annexin V/PI assay on Jurkat cells exposed to different treatments.

Treatments	Annexin V^+/PI^-		Annexin V^+/PI^+	
	Early apoptotic cells		Late apoptosis or necrosis	
	% (Mean ± SD)	FI	% (Mean ± SD)	FI
DMSO	6.94 ± 0.90	1.00	9.48 ± 2.27	1.00
Staurosporine 10 μM	50.26 ± 13.70	**7.25**	18.81 ± 3.63	1.99
$MnCl_2$ 10 mM	8.12 ± 2.45	1.17	24.17 ± 4.74	**2.55**
SC 5 mg/mL	7.88 ± 1.52	1.14	10.82 ± 1.16	1.14
D100 5 mg/mL	8.84 ± 0.33	1.27	12.42 ± 0.11	1.31
B5 5 mg/mL	19.94 ± 4.49	**2.87**	15.96 ± 0.11	1.68
B20 5 mg/mL	10.60 ± 2.86	1.53	11.32 ± 1.19	1.19
B50 5 mg/mL	7.05 ± 1.32	1.02	8.61 ± 5.08	0.91
B100 5 mg/mL	7.85 ± 0.44	1.13	11.96 ± 0.54	1.26

SC, soil control; D100 = pure diesel; B5 = blend 5% biodiesel + 95% diesel; B20 = blend 20% biodiesel + 80% diesel; B50 = blend 50% biodiesel + 50% diesel; B100 = pure biodiesel. FI, fold induction over baseline (baseline = events % of solvent control – DMSO). Values in bold indicate FI greater than 2. Data obtained by measurements of 20,000 ungated events per treatment (10,000 ungated events per treatment in each experiments accomplished – two experiments).

using the lipophilic cationic fluorescent redistribution dye TMRE (Table 4). As the flow cytometry-based *in vitro* MN assay could also be used to evaluate cytotoxicity. The data of this test also revealed cytotoxic effects to B50 soil extract at the highest concentration tested (5 mg/mL) (Fig. 1). A lower dose of B50 extract (2.5 mg/mL) was included in this assay and the cytotoxic effect was then no longer observed for this sample (Fig. 1).

As for the genotoxic/mutagenic assessments, the flow cytometry-based *in vitro* MN assay did not show genotoxic potential for any tested extract (Fig. 1). On the contrary, the Ames test (preincubation procedure), which allows us to detect point mutations, pointed to mutagenic effects for B100, B20 and B50 extracts using the TA100 *Salmonella* strain in the absence of metabolic activation (S9) (Fig. 2). In accordance with the *Salmonella* mutagenicity assay, the results of *A. cepa* test showed a positive CA induction after exposure of this test organism to B100, B5, B20 and B50 soil samples, indicating a genotoxic potential to these soil extracts. Concerning the evaluation of MN induction in the *A. cepa* test, no significant frequencies were observed for any treatment accomplished, when compared to the negative control (Fig. 3).

4. Discussion

Despite the fact that the flow cytometry-based *in vitro* MN test showed cytotoxicity for both B5 and B50 extracts, the data obtained by the other cytotoxic bioassays accomplished herein did not show a clear agreement between them. While the TMRE assay indicated

Table 4
Changes of $\Delta\Psi_m$ in Jurkat cells exposed to the soil extracts.

Treatments	Percentage of cell with disrupted $\Delta\Psi_m$ (Mean ± SD)	FI
DMSO	9.97 ± 1.65	1
Etoposide 0.5 μg/mL	81.14 ± 4.18	**8.14**
SC 5 mg/mL	8.85 ± 1.17	0.89
D100 5 mg/mL	8.41 ± 0.47	0.84
B5 5 mg/mL	16.48 ± 4.06	1.65
B20 5 mg/mL	9.65 ± 0.77	0.92
B50 5 mg/mL	24.63 ± 11.14	**2.47**
B100 5 mg/mL	9.19 ± 2.37	0.92

SC, soil control; D100 = pure diesel; B5 = blend 5% biodiesel + 95% diesel; B20 = blend 20% biodiesel + 80% diesel; B50 = blend 50% biodiesel + 50% diesel; B100 = pure biodiesel. FI, fold induction over baseline (baseline = % event solvent control – DMSO). Values in bold indicate FI greater than 2. Data obtained by measurements of 60,000 ungated events per treatment (10,000 ungated events per treatment in each experiments accomplished – three experiment in duplicate/treatment).

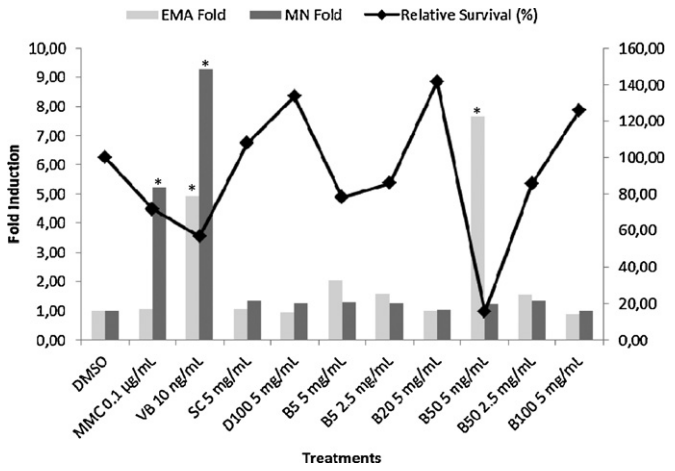

Fig. 1. Results of flow cytometry-based *in vitro* MN assay expressing the genotoxicity and cytotoxicity data observed. *Y*-axis shows fold increase values of EMA+ and MN. *YY*-axis showed the percentage of Relative Survival. DMSO = solvent control; MMC and VB = positive controls; SC = soil control; D100 = pure diesel; B5 = blend 5% biodiesel + 95% diesel; B20 = blend 20% biodiesel + 80% diesel; B50 = blend 50% biodiesel + 50% diesel; B100 = pure biodiesel. *≥3-Fold over the concurrent solvent control value.

that B50 extract is capable of causing changes in $\Delta\Psi_m$, the Annexin V assay showed that B5 induces apoptosis by detection of PS externalization. However, the positive effects obtained for B5 and B50 extracts may be an indicative that diesel contaminants are responsible for those cytotoxic effects.

Diesel oil is a complex chemical mixture of hundreds of chemical substances, including the PAHs [26]. PAHs are ubiquitous environmental contaminants recognized by their peril to human health [27]. PAHs can cause cytotoxicity directly or after their metabolic activation via cytochrome P450, depending on their molecular size. Two- and three-ring PAHs, such as naphthalene, acenaphthylene, acenaphthene, fluorene and phenanthrene, are recognized as direct cytotoxic inducers [28]. The PAHs chemically quantified in B5 and B50 extracts refer to these smaller PAHs, a fact that can explain the direct cytotoxicity observed. However, the differences between both the response intensity observed in the flow cytometry MN test and the mode of action detected by the assessments with TMRE and Annexin V can be related to the amount, interaction and bioavailability of the PAHs present in these samples.

Unlike the cytotoxic assessment, the genotoxicological evaluation promoted an accurate analysis with agreement among the data obtained.

The *Salmonella* mutagenicity assay employed in the present study was conducted by two different protocols, aiming at comparing their sensitivity in detecting mutagens. The preincubation procedure showed clear significant dose-responses for B100, B20 and B50 soil extracts in TA100 assay without metabolic activation, indicating that these samples induced mutations by base-pair substitutions. On the other hand, the MPF protocol indicated an increase of <2-fold over the solvent control value only for a single dose of B20 extract (500 mg/mL) using TA1537 tester strain in the absence of S9. Nevertheless, as it did not meet all the criteria required by this method to predict the mutagenicity, it was not considered a positive result. The non-correlation data obtained in both *Salmonella* assay protocols used may be related to the mutagenic potentials obtained for these samples (0.4 rev/mg-equivalent to B20, 37.4 rev/mg-equivalent to B50, 378 rev/mg-equivalent to B100), which can be classified as weak mutagens. Thus, despite some studies stating that the Ames MPF assay is an efficient sorting alternative to the standard Ames test [29–31], even for weak mutagens [32], our data did not confirm this statement and showed

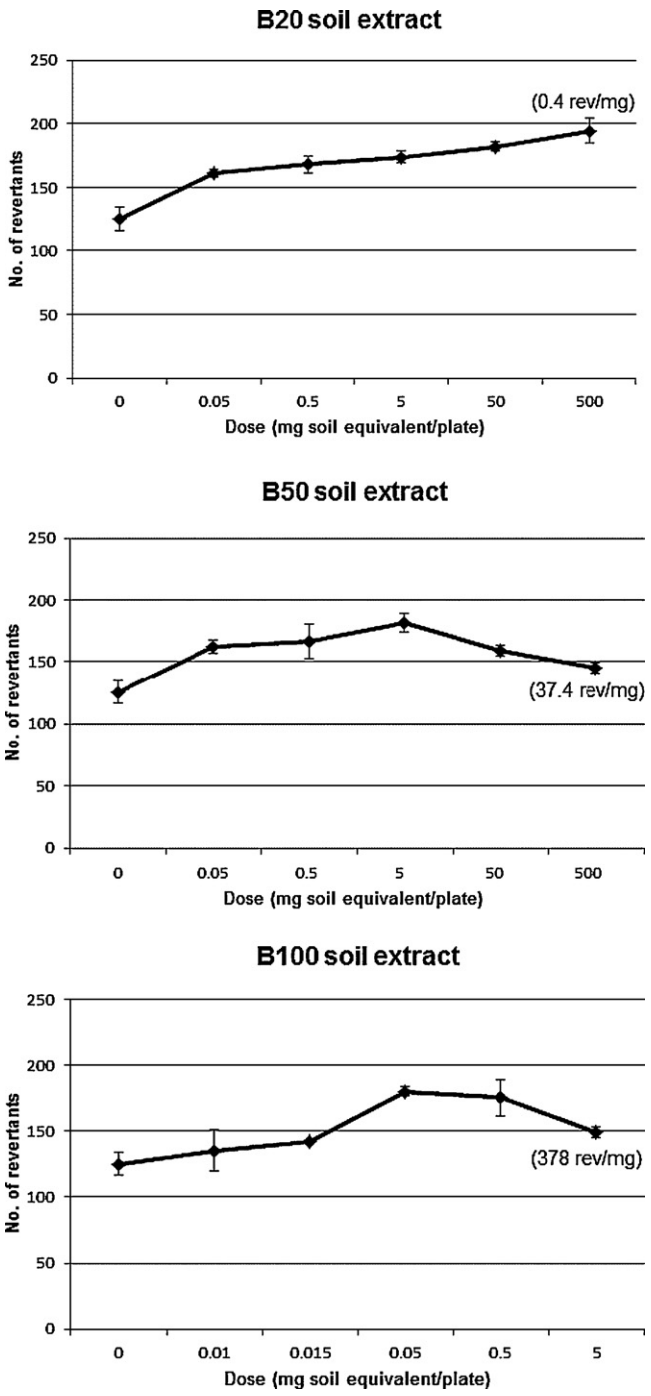

Fig. 2. Mutagenic activity detected in B20, B50 and B100 soil extracts by the *Salmonella*/microsome assay with TA100 strain in the absence of S9. Numbers in parenthesis represents the potencies, expressed in revertants/mg of soil equivalent.

a higher sensitivity in detecting mutagens for the preincubation procedure.

The evaluation of CA and MN frequencies in meristematic cells of *A. cepa* exposed to the polluted soils revealed a significant CA induction to B100, B5, B20 and B50 soil samples, indicating their genotoxic potentials. However, no significant MN values were observed in this assay, showing that the CA detected did not result, in this case, in MN. Likewise, the flow cytometry-based *in vitro* MN test did not show significant MN induction for any treatment accomplished.

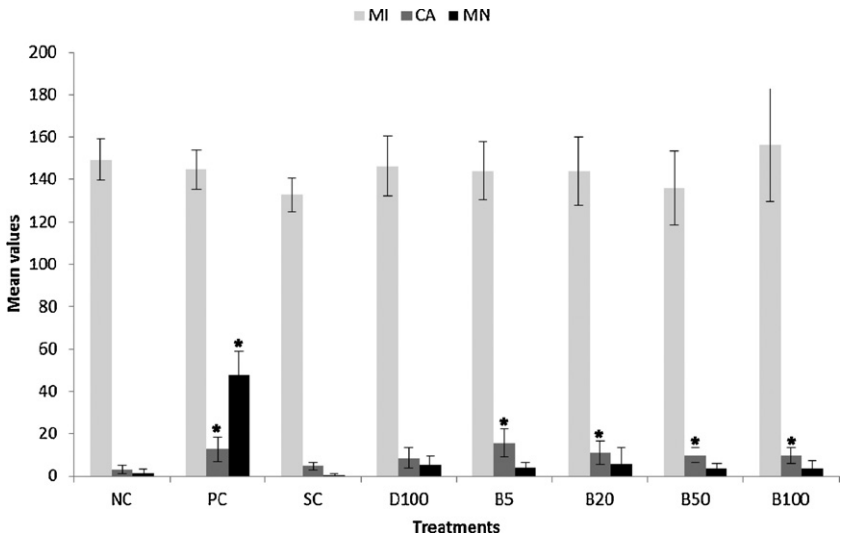

Fig. 3. Results of mitotic index (number of dividing cells), CA and MN in *A. cepa* meristematic cells after treatment with soil extracts from the spill simulations accomplished. 5000 cells analyzed per treatment. NC = negative control; PC = positive control; SC = soil control; D100 = pure diesel; B5 = blend 5% biodiesel + 95% diesel; B20 = blend 20% biodiesel + 80% diesel; B50 = blend 50% biodiesel + 50% diesel; B100 = pure biodiesel. Mean ± S.D. *Significantly different from negative control ($p < 0.05$), according to Kruskal–Wallis test.

The positive genotoxic/mutagenic results obtained for biodiesel and their diesel-blend polluted soils may have an interesting explanation. Unlike cytotoxic effects, which here were related to diesel pollutants, the genotoxicity/mutagenicity observed can be related to biodiesel contaminants, since only the soil extracts with pure biodiesel and their diesel blends showed these positive responses. This hypothesis becomes more reliable when mutagenic potencies obtained in the *Salmonella* mutagenicity assay are analyzed. According to our data, the mutagenic potencies increase as the percentage of biodiesel in the samples goes up (0.4 rev/mg-equivalent to B20 < 37.4 rev/mg-equivalent to B50 < 378 rev/mg-equivalent to B100). Our group also detected similar genotoxic/mutagenic effects to biodiesel polluted water [33].

Considering that the genotoxicity/mutagenicity observed herein are associated with the biodiesel contamination, these effects can be related to both fatty acid methyl esters (FAMEs), i.e. the biodiesel itself, or impurities (e.g. free sterols) from the feedstock used in the biofuel production, all of which are present in the marketed biodiesel. Soybean, one of the main sources of biodiesel production [34], is known for its high concentration of phytoestrogens and phytosterols [35,36]. Yang et al. [37] showed that several phytosterols are present in biodiesels and their concentrations in biofuel are highly dependent on carboxylic acid sources. GC–MS data from our group also corroborate this assertion, showing the presence of β-sitosterol in soy-based biodiesel. The harmful effects of phytosterols, plant sterols structurally quite similar to cholesterol, have been intensively investigated by food administration studies and, in general, these agents have shown to raise no obvious concerns when it comes to human safety so far. However, considerable attention has been given to phytosterol oxides. Owing to their chemical structures, phytosterols can oxidize and produce a wide variety of oxidation products that have controversial biological effects [38,39]. Oxidation of phytosterols is accelerated by high temperatures, exposure to ionizing radiation, light, among other conditions [39,40]. Fuel spillages in both water and soil are influenced by several environmental factors, which can promote changes in the chemical structures of pollutants. The spill simulations performed herein were carried out in the summer, during which the exposure to high temperatures and solar radiation may induce some reactions, such as the photoxidation of phytosterols present in the soy-based biodiesel used.

In conclusion, although biodiesel is considered an eco-friendly alternative to petroleum-based diesel, our data serves as a warning to this assertion. The soils contaminated with biodiesel and their diesel blends promote here genotoxic/mutagenic effects by inducing CA and base-pair substitution mutations. Nevertheless, it is still unclear which to hold responsible for these positive responses, i.e. FAMEs or impurities from the feedstock present in the marketed biodiesel. Therefore, further studies combining both biological and accurate chemical analyses are necessary to elucidate it.

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