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Toxicity of biodiesel, diesel and biodiesel/diesel blends: comparative sub-lethal effects of water-soluble fractions to microalgae species

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Abstract The water-soluble-fractions (WSF) from biodiesel and biodiesel/diesel blends were compared to diesel in their sub-lethal toxicity to microalgae. Chemical analyses of aromatics, non-aromatics hydrocarbons and methanol were carried out in the WSF, the former showing positive correlation with increasing diesel concentrations (B100 < B5 < B3 < B2 < D). Biodiesel interacted with the aqueous matrix, generating methanol, which showed lower toxicity than the diesel contaminants in blends. The WSF caused 50% culture growth inhibition (IC50-96 h) at concentrations varying from 2.3 to 85.6%, depending on the tested fuels and species. However, the same species sensitivity trend (*S. costatum* > *N. oculata* > *T.chuii* > *P. subcapitata*) was observed for all the tested fuels.

Keywords Toxicity · Microalgae · Biodiesel · Blends

Biofuels are considered as one alternative to confront the depletion of fossil oil resources and to alleviate climate change problems (IPCC 2007). Brazil has a unique and leading position in the emerging global biofuels industry. Furthermore, it is also one of the few countries with the available arable land to expand production enough to become a major exporter (Rothkopf 2007). Large-scale production of biodiesel in Brazil started in 2003 and a 2% addition of biodiesel in diesel became mandatory in 2008. The National Council for Energy Policies further encouraged subsequently, a 3% and 5% biodiesel in diesel and a B5 blend became available at the petrol stations in 2010.

The studies comparing the toxicity of diesel, biodiesel and their distinct commercial blends are mostly related to gaseous emissions (Turrio-Baldassarri et al. 2004). Contamination from diesel, however, is a widespread event and a common source of public concern. When fossil fuels come into contact with a water-based matrix, several short-chained aromatic and non-aromatic hydrocarbons become bio-available. Benzene, toluene, ethyl-benzene and xylenes (BTEX) are often associated with fossil-fuels pollution, by causing high levels of toxic effects (Paixão et al. 2007). Biodiesel from soybean is virtually free from aromatics. Besides compatible to diesel, biodiesel is often considered non-toxic (Wang et al. 2000). Therefore, it is expected that blending diesel with biodiesel would reduce its toxicity. Biodiesel, however, can react with the water matrix and generate variable amounts of methanol, which is also toxic (Leite et al. 2011). There is no information related to the toxicological profile of the WSF from distinct biodiesel/diesel blends, even though, as previously reported (Tsai et al. 2010) their combustion emissions contain mutagenic and carcinogenic substances, not currently regulated within the biodiesel market. The aim of this work was to evaluate and compare the toxic profiles of the WSF obtained from neat biodiesel (B100) biodiesel/diesel blends (B2-2%, B3-3% and B5-5% biodiesel in diesel) and diesel (D), associated to contaminants present in these WSF. Marine and freshwater microalgae were used as test-organisms for this purpose.

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Materials and methods

The samples from B2, B3, B5 and D were obtained in gas stations from the fuel pump. Pure biodiesel (B100) was supplied by Petrobras-BR. At the Biomonitoring Laboratory, Institute of Biology, Federal University of Bahia, the biodiesel samples were treated 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 filtered and sterilized seawater or in distilled water (Milli-Q apparatus from Millipore®) respectively for marine and freshwater species. After stirring at constant speed (150 rpm) in closed Mariotte flasks for 20 h, part of the WSF was decanted, collected from the Mariotte flasks and chemically analyzed.

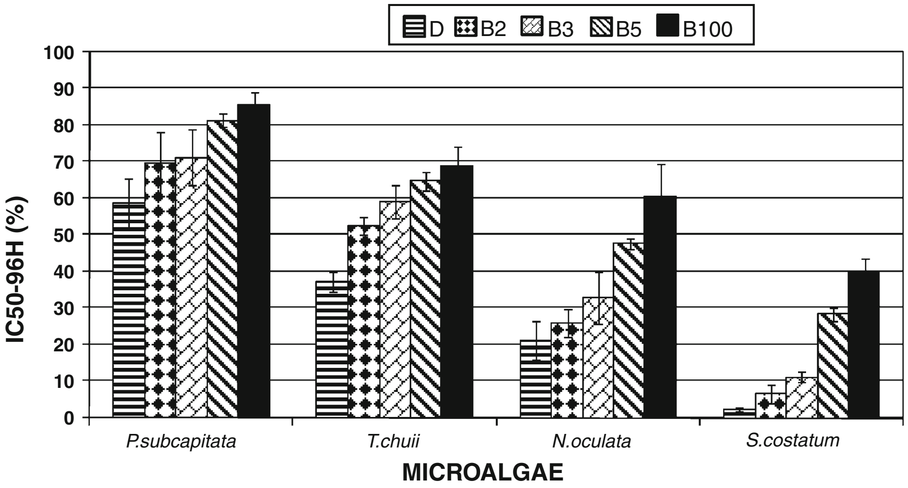
Chemical analyses were carried out at the LCQ (Quality Control Laboratory), at the Basic Petrochemical Unit, Braskem S. A., in Bahia, by following QA/QC procedures installed in the laboratory. All the samples were analyzed for C6–C8 mono-aromatics (BTEX), total heavy aromatic hydrocarbons (C9s+, representing all aromatic-HC having nine or more carbon atoms), and methanol.The chromatographic data (benzene, toluene, ethyl-benzene, xylenes and C9s+ aromatic HC) were obtained by using a gas chromatograph (Varian, CP3800 model) with a flame ionisation detector and a 50 m × 0.20 mm i.d. × 00.2 μm DB WAX capillary column, using hydrogen as the carrier gas. A purge and trap concentrator (Tekmar, 3000 model) with a Tenax column was used to quantify the low hydrocarbon levels. Electrical conductivity (Gehaka, CG 2200 model), pH (Metrohm, 654 model), and relative density 20/4°C (Anton Paar, 4500 model) analyses were also performed.

Toxicity tests were carried out using four species of microalgae collected from the Algae Bank located at the Biology and Biomonitoring Laboratory, Institute of Biology, Federal University of Bahia: a freshwater species, *Pseudokirchneriella subcapitata,* maintained in LC-Oligo medium and three marine species, *Tetraselmis chuii*, *Nannochloropsis oculata* and *Skeletonema costatum*, maintained at Conway medium, under standard conditions (temperature, 23 ± 2°C; illumination, 75 to 85μE/m^2/s). The tests were performed according to standardized ISO 8692 (2004) and ISO 10253 (1995) respectively for freshwater and saltwater species. Before testing, physical–chemical parameters (salinity, pH, temperature) were checked to the range accepted for the test species.The obtained 100%-WSF from the blends (B2, B3, B5), biodiesel (B100) and diesel (D) samples were dosed in a geometric dilution series of six loadings (0%, 4.6%, 10.0%, 22.0%, 46.0% and 100%), in triplicate vessels containing LC-Oligo and Conway medium, for, respectively, freshwater and salt-water species. Both controls and treatment flasks were inoculated with 10^4 cells mL^{-1} and incubated for 96 h in a rotary shaker under continuous illumination provided by fluorescent lamps. Coulter (Counter model ZI 991 3044-B) counting was used to evaluate the culture growth in comparison to controls (0% WSF). Each test was repeated three times and was fully randomized with regard to vials location during incubation and the order of cell counts. All the tests involved a positive (standard reference toxicant) and a negative (blank) control. A system of control-charts, based on dose-response results from the same species exposed to a reference toxicant (dodecyl sodium sulphate-DSS), was used for results accuracy. Toxic effects were estimated based on concentration-response curves and analyzed by the Trimmed Spearman Karber computer statistical method (Hamilton et al. 1977). Results were expressed as IC50 values (equivalent to the WSF-contaminants concentrations, causing 50% growth inhibition in the exposed cultures). Possible significant differences in toxicity among the various treatments were determined by ANOVA comparing the IC50 results followed by Tukey test (Graphpad Software 1997).

Results and discussion

All the analyzed WSF originated from fuels or blends promoted growth inhibition (IC50–96 h) to all the tested species (Fig. 1), showing a common trend: increasing toxicity from the WSF-B100 to B5, B3, B2 and D, as evidenced by the corresponding decrease in CI-50 values, which represent the joint actions of contaminants concentrations in the WSF, responsible for determining 50% growth inhibition to the microalgae cultures. Results showed that biodiesel imposed a significantly lower ($p < 0.05$) toxicological impact than diesel to all the microalgae species. The soluble organic fractions of diesel contain mostly PAHs and these components have been proven toxic to microalgae and other organisms in levels depending on their concentrations (Paixão et al. 2007; Zhang et al. 2004). On the other hand, even though many authors refer to biodiesel as being biodegradable and non-toxic (Leung et al. 2006), its toxicological potential to aquatic and terrestrial organisms has been previously reported (Lapinskiene et al. 2006; Leite et al. 2011).

The response (IC50–96 h) of the distinct microalgae exposed to different WSF is compared in Fig. 1. *P. subcapitata* (freshwater) and *N. oculata* (seawater) did not respond to chemical differences (ANOVA, $p > 0.05$) found in the treatment B2 and B3, when compared to diesel-WSF treatment. For the other two saltwater species (Fig. 1), the WSF of the blends B2 and B3 showed to be less toxic than the diesel-WSF. Similarly, the IC50–96 h values for *T. chuii* and *P. subcapitata* did not statistically vary ($p > 0.05$) when exposed to biodiesel-WSF (B100) and the highest biodiesel ratio in blends (B5-WSF). In all cases, the WSF from the blend B5 caused less noxious



MULTIPLE RANGE TEST: Values united by dashed line are not siginicantly different (p>0.05)

T.chuii

Products	B100	B5	B3	B2	D
Means	68.7	64.8	59.2	52.5	37.3

!--------------------------! !-------! !-----------!

N..oculata

Products	B100	B5	B3	B2	D
Means	60.3	47.7	32.9	25.9	21.3

!---------! !--------! !----------------------------!

P.subcapitata

Products	B100	B5	B3	B2	D
Means	85.6	81.4	71.2	69.5	58.7

!-----------------! !-----------------------------!

S.costatum

Products	B100	B5	B3	B2	D
Means	39.6	28.5	11.2	6.6	2.3

!---------! !--------! !---------------! !---------!

Fig. 1 Microalgae responses to toxic effects of contaminants present in WSF (water-soluble-fractions) of diesel (D), biodiesel/diesel blends (B2, B3, B5) and neat biodiesel (B100)

effects than diesel-WSF and B2-WSF (different at a level $p < 0.05$), proving a higher benefit of the blend B5 over the blend B2, in lowering the diesel toxicity. The same trend in species sensitivity to the effects of biodiesel and diesel blends (*S. costatum* > *N. oculata* > *T.chuii* > *P. subcapitata*) was observed for all the tested fuels. Most of the currently available literatures on the effects of different chemicals on microalgae have relied almost exclusively on *Pseudokirchneriella subcapitata* because its widespread use in routine toxicity testing. Moreover there is relatively little information on other microalgae species that could be equally, if not more, appropriate for many toxicity tests. In the present work, as reported elsewhere (Nascimento et al. 2009), *S. costatum* was the most sensitive to the tested fuels-WSF, showing a comparatively stronger growth inhibition. As growth rate is closely related to energy production, it is possible that the contaminants in WSF have channeled the algal metabolism to cope with the stress, by producing energy reserves instead of directing them to meet growth requirements, as was previously reported by Yang et al. (2002) for this species, when exposed to 2,4-dichlorophenol. Even though the metabolism redirection toward energy storing of lipids is a common response for Diatoms and Chlorophyta under stress (Hu et al. 2008), *S. costatum* sensitivity to fuel contaminants can be an indication of its use as surrogate for marine species in toxicity studies (Pavlic et al.2005).

The concentrations of the distinct BTEX in the tested WSF are shown in Fig. 2. Benzene contributed with the highest concentrations in all the blends-WSF, while toluene was comparatively higher in the WSF from diesel. As expected, any BTEX was detected in biodiesel-WSF. The concentrations of aromatics of higher molecular weight (C9s+), found below detection limits (<1 μg/L) in B100-WSF (Fig. 2), correlated negatively ($r^2 = 0{,}951$) with increasing biodiesel ratios in blends, while showing the highest values in diesel-WSF (383 ± 18.9 μg/L). The higher average value of non-aromatics hydrocarbons in B100-WSF differed significantly (ANOVA, $p < 0.05$) from all the other analyzed samples. Increases in the concentration of methanol correlated ($r^2 = 0.0885$) with the increase of biodiesel in blends (Fig. 2), the highest value being observed in the B100-WSF (87 mg/Lfor B100).

After leakages and spills, fuels can cause significant environmental impact in natural systems (Lapinskiene et al. 2006; Leite et al. 2011). Some of such impact is often attributed to the bioavailability of water-soluble compounds such as BTEX and other aromatics of lower molecular-weight. The present investigation showed that contamination of water by diesel could generate 400 μg/L of aromatics (C9s+). Similarly, biodiesel can produce significant amounts of methanol when in contact with an aqueous-matrix (87 mg/L, Fig. 2), which appears as a result of hydrolysis, causing the reversion of the transesterification reaction. Methanol, present in Biodiesel-WSF in concentration as low as $1.10^{-4}\%$, was pointed out as toxic to biota (Leite et al. 2011).

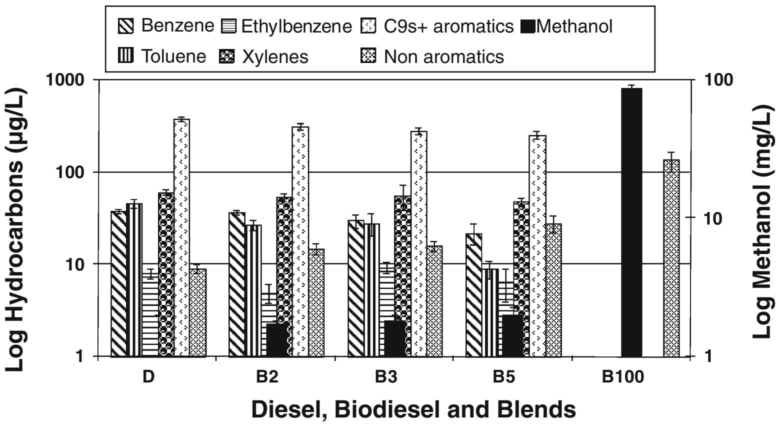


Fig. 2 Mean and standard deviations of BTEX concentrations and aromatics (C9s+), non-aromatic hydrocarbons and methanol in the WSF (water soluble fractions) of diesel (D), neat biodiesel (B100) and blends biodiesel/diesel (B2, B3, B5)

MULTIPLE RANGE TEST: Values united by dashed line are not different (p>0.05)

<u>Benzene</u>
Products D B2 B3 B5
Mean 38±2,0 37±1,5 30±5,0 22±5,5
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<u>Toluene</u>
Products D B3 B2 B5
Mean 46±4,6 28±7,6 27±1,0 9±1,0
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<u>Ethyl-benzene</u>
Products B3 D B5 B2
Mean 9,3±0,6 8±1,0 6,5±2,5 5±0,3
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<u>Xylenes</u>
Products D B3 B2 B5
Mean 61±4,0 56±17,0 54±3,0 48±1,5
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<u>C9s+ Aromatics Hydrocarbons</u>
Products D B2 B3 B5 B100
Mean 383±18,9 317±9,5 282±9,5 256±13,5 1,0±0,1
!----------!!----------!!----------------------!!--------!

<u>Non Aromatics Hydrocarbons</u>
Products B100 B5 B3 B2 D
Mean 136±33 28±1,4 16±1,9 15±2,0 9,2±0,3
!---------!!----------------------------!!----------!

<u>Methanol</u>
Products B100 B5 B3 B2
Mean 87±2,6 2,0±1,0 1,8±0,2 1,7±0,1
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The overall result from this work showed that biodiesel blended to diesel promotes a decreasing in toxicity of this fossil fuel, directly correlated with the resultant decrease in total aromatic hydrocarbons. Diesel alone is significantly more toxic for all species than the respective blends. Regarding the blends biodiesel/diesel-WSF there is also enough evidence to suggest that, in spite of the fact that B5 contained the highest concentrations of methanol, it showed the lowest levels of toxicity for all tested species. Therefore, methanol did not synergistically increase the toxic effect of the diesel in blends-WSF to the four different microalgae used as test organisms.

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