

Full Length Research Paper

Biodegradability of diesel and biodiesel blends

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The biodegradability of pure diesel and biodiesel and blends with different proportions of biodiesel (2% (commercial); 5% and 20%) was evaluated employing the respirometric method and the redox indicator 2,6-dichlorophenol indophenol (DCPIP) test. In the former, experiments simulating the contamination of natural environments (soil from a petrol station or water from a river) were carried out in Bartha biometer flasks (250 ml), and used to measure the microbial CO₂ production. With the DCPIP test, the capability of three inocula to biodegrade the blends was tested. Results show that although biodiesel is more easily and faster biodegraded than diesel oil, among the blends evaluated (2%, 5% and 20%), only the blend with higher concentration of biodiesel presented biodegradability significantly different from diesel and it was not verified an improvement on the biodegradation of the diesel by means of co-metabolism.

Key words: Biodiesel, diesel, blend, biodegradability, bioremediation.

INTRODUCTION

The introduction of the biodiesel into the Brazilian energetic matrix was determined by a federal law that establishes a compulsory blend of 2% of biodiesel in mineral diesel as of 2008 and 5% as of 2013. Currently Brazil produces approximately 750 million litres of biodiesel per year, a figure very close to the 840 million litres necessary to accomplish the 2% blend. Although either fuel presents the same function, they have very distinct origins and compositions. Biodiesel is composed of methyl or ethyl esters of fatty acids with low structural complexity as oleate, palmitate, estearate, linoleate, myristate, laureate and linolenate, derived from different vegetable oil sources such as soybean, sunflower, peanut, cotton, palm oil, coconut, babassu and castor oil and from animal fat (Pinto et al., 2005). Differently, diesel oil contains 2000 to 4000 hydrocarbons, a complex mixture of normal, branched and cyclic alkanes, and aromatic compounds obtained from the middle-distillate fraction during petroleum separation (Gallego et al., 2001).

Besides the recognised environmental benefits related to the biodiesel combustion (less emissions of CO₂, CO,

SO_x, volatile organic compounds and particulate material) (Pinto et al., 2005), the difference between the fuels compositions also influences their biodegradability. As occurs to the diesel oil, the commercialization of biodiesel or the biodiesel/diesel blend may cause environmental damages due to spills. The clean-up of these contaminated areas can be achieved with bioremediation, a technique based on the action of microorganisms, which turn hazardous contaminants into non toxic substances as CO₂, water and biomass. Here again, biodiesel presents advantages, since studies demonstrate that biodiesel is more easily biodegraded and less toxic than diesel oil (Koo-Oshima et al., 1998; Zhang et al., 1998; Makareviciene and Janulis, 2003; Pasqualino et al., 2006; Lapinskiené et al., 2006; Khan et al., 2007). Moreover, some of these works also show that biodiesel can promote and speed up the biodegradation of diesel by means of co-metabolism.

However, as there is a lack of studies that, besides the water contamination, also evaluate the soil contamination, here the aim was to verify in laboratory the effect of the addition of biodiesel on the biodegradability of the diesel oil in soil and in aquatic environment. In relation to the latter, differently from the previous works that used deionised water, experiments were carried out with water from a river next to potential sources of contamination.

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Table 1. Soil sample characteristics.

Parameter		Value	Parameter		Value								
pH (CaCl ₂)		6.7	K (mmolc/dm ³)		1.1								
Organic carbon (%)		0.29	Ca (mmolc/dm ³)		15								
Total nitrogen (%)		0.02	Mg (mmolc/dm ³)		2								
Available phosphorus (ppm)		2.0	H+Al (mmolc/dm ³)		10								
C:N:P		100 : 6.89 : 0.10	Al (mmolc/dm ³)		- ^b								
Moisture content (%)		12.7	CEC ^a (mmolc/dm ³)		28.7								
Grain size distribution (%)		Sand	Silt		Clay								
		81.4	7.3		11.3								
Micronutrients (ppm)					Heavy metals (ppm)								
S	Na	Fe	Mn	Cu	Zn	B	Co	Mo	Ba	Cd	Cr	Ni	Pb
12	13	19	3.0	0.6	7.3	0.15	0.56	- ^b	4.06	0.12	9.93	0.30	7.10

^aCation exchange capacity^bNot detected**Table 2.** Water sample characteristics.

Parameter	Value	Parameter	Value	Parameter	Value
pH	7.38	Ammonia (mg/L)	1.65	Bacteria (CFU/mL)	2.6 . 10 ³
BOD (mg/L)	3.33	Chlorate (mg/L)	5.9	Filamentous Fungi (CFU/mL)	17
COD (mg/L)	29.12	Cyanate (mg/L)	0.006	Yeast (CFU/mL)	3
DO (mg/L)	6.58	Phenols (mg/L)	- ^a	Toxicity (EC50) ^b	- ^a
Conductivity (μS/cm)	82.9	Volatile solids (mg/L)	0.045		
Acidity (mg/L)	7.76	Fixed solids (mg/L)	0.092		
Alkal. HCO ₃ (mg/L)	23.21	Soluble solids and in suspension (mg/L)	0.137		
Nitrite (mg/L)	0.043	Sedimentation	<0.1		
Nitrate (mg/L)	1.009				

^aNot detected^b*Daphnia similis*

MATERIAL AND METHODS

Soil and water sampling and their characteristics

The soil sample was collected at a petrol station (Rio Claro/SP/Brazil) during the replacement of underground pipes (0.50 m depth). This sample showed low level of contamination (104 mg/Kg) by unknown fuel, possibly due to leaks in the pipes and ground infiltrations (Mariano et al., 2008a,c). Until performing the biodegradation experiments, the sample was stored at 5°C. Table 1 summarizes some of the soil physicochemical characteristics. Values of heavy metals concentrations are not above the more restricted levels set by the Cetesb (São Paulo Environmental Agency – Brazil) and by the Dutch list (Cetesb, 2005).

The soil physicochemical analyses were performed by the laboratory "Instituto Campineiro de Análise de Solo e Adubo (ICASA)", according to the methodology proposed by Embrapa (1997), except the following parameters; total nitrogen (laboratory "PIRASOLO – Laboratório Agrotécnico Piracicaba", according to Embrapa (1997)) and the moisture content (obtained by the oven drying method).

The water sample was collected at the Jaguari river (22° 42' 00" S / 47° 08' 06" W) located in Paulínia (SP/Brazil). The composite sample was obtained at the river surface along a transect perpendicular to the flow direction. Nearby the sampling location, an oil

refinery (Replan/Petrobras) and roads represent potential sources of contamination. Table 2 summarizes some of the water characteristics (APHA, 1998).

Respirometric experiment

Biodegradation experiments simulated soil and water contaminations, respectively from the petrol station and the Jaguari river. The soil and water contamination was carried out by adding fuel (50 ml/Kg of soil; 10 ml/L of water) with the following volume percent compositions of the biodiesel/diesel blend: 0/100; 2/98; 5/95; 20/80 and 100/0, respectively denominated: B0, B2, B5, B20 and B100. The diesel oil and the B2 blend were obtained at petrol stations (respectively, BR and ALE distributors) in Rio Claro (SP/Brazil). The other blends were prepared in laboratory combining the diesel oil with a biodiesel produced from castor oil.

Biodegradation experiments were carried out in Bartha biometer flasks (250 ml) used to measure the microbial CO₂ production (Bartha and Pramer, 1965; Régis and Bidoia, 2005; Mariano et al., 2007a, 2008a,b,c). The CO₂ produced is proportional to the carbons consumed by microorganisms from the test substrate. Thus, the amount of CO₂ is proportional to the percentage of substrate bio-degraded. Mineralization studies involving measurements of

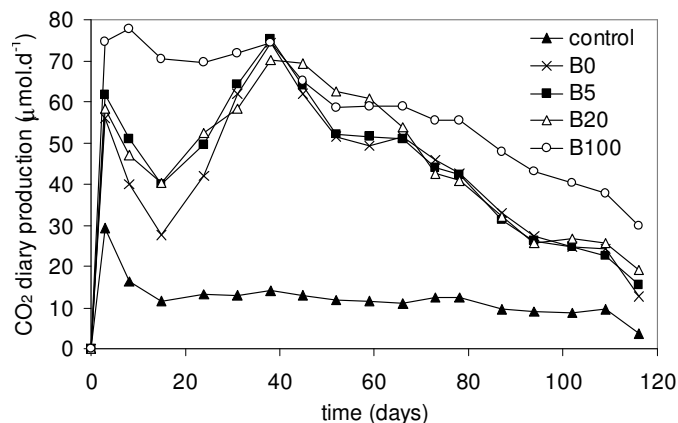


Figure 1. Daily CO₂ production during incubation of the first respirometric experiment with the soil contamination.

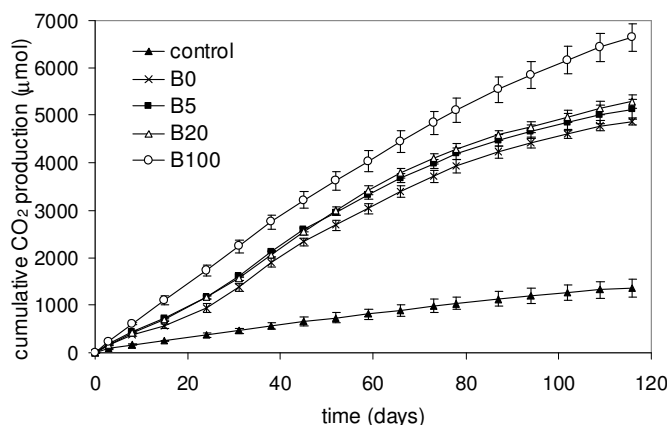


Figure 2. Cumulative total amounts of CO₂ produced by the first respirometric experiment with the soil contamination during incubation. Each error bar represents 1 SD of three replicate.

total CO₂ production can provide excellent information on the biodegradability potential of hydrocarbons (Balba et al., 1998). CO₂ evolution measures ultimate degradation (mineralization) in which a substance is broken down to the final products, while, for instance, the gas chromatography (GC) analysis measures primary degradation only in which the substance is not necessarily transformed to the end products.

For each experimental condition, the biometer flasks were prepared in triplicates (3 x 50 g of soil or 50 ml of water) and incubated at 27°C in the dark. The CO₂ produced was trapped in a 10.0 ml solution of KOH (0.2 N), located in the side-arm of the biometer. This solution was periodically withdrawn by syringe, and the amount of CO₂ absorbed was then measured by titrating the residual KOH (after the addition of barium chloride solution (1 ml; 1.0 N) used to precipitate the carbonate ions) with a standard solution of HCl (0.1 N). During this procedure, the biometers were aerated during 1.5 min through the ascarite filters.

Biodegradability test – DCPIP indicator

The biodegradability of the biodiesel/diesel blends was also verified using the technique based on the redox indicator 2,6-dichlorophe-

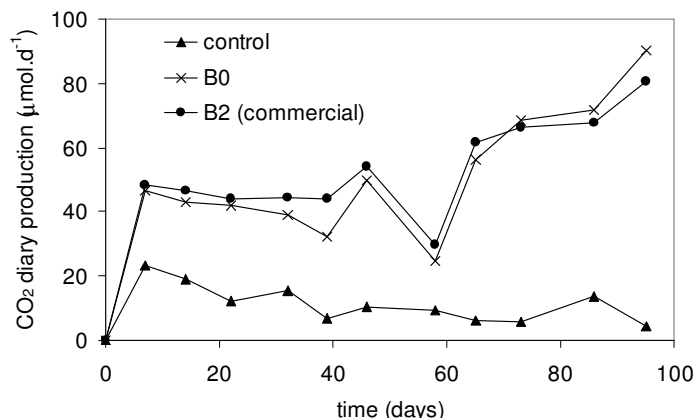


Figure 3. Daily CO₂ production during incubation of the second respirometric experiment with the soil contamination.

nol indophenol (DCPIP) (Hanson et al., 1993). The principle of this technique is that during the microbial oxidation of hydrocarbons, electrons are transferred to electron acceptors such as oxygen, nitrates and sulphate. By incorporating an electron acceptor such as DCPIP to the culture medium, it is possible to ascertain the ability of the microorganism to utilize hydrocarbon substrate by observing the colour change of DCPIP from blue (oxidized) to colourless (reduced). This Hanson et al. (1993) technique has been employed in several works (Cormack and Fraile, 1997; Roy et al., 2002; Mariano et al., 2007b, 2008a,c,d), but for the first time, this technique was used to evaluate the biodegradability of biodiesel/diesel blends.

The capability of three inocula to biodegrade the blends B0, B2, B5, B20, B50 and B100 was tested: consortium 1 (obtained from the soil at the petrol station); consortium 2 (from an uncontaminated soil collected at UNESP campus) and the culture *Pseudomonas aeruginosa* LBI (Benincasa et al., 2002).

The inoculum *P. aeruginosa* LBI was prepared using bacterial cells transferred from the storage culture tubes and streaked onto the surface of Petri dishes containing PCA medium (Acumedia, EUA). To prepare the other two consortia, 10 g of respective soils were added to Erlenmeyer flasks (125 ml) containing 50 ml of sterile saline solution and kept under agitation for 1 min. After this period, the saline was streaked onto the surface of Petri dishes containing PCA medium. The Petri dishes were incubated during 24h at 35°C and then cells were harvested using sterile saline solution.

The inocula (1.0 ml, concentration not determined) were added to Erlenmeyer flasks (250 ml, duplicates) that contained sterile Bushnell-Hass (BH) medium (50 ml) and 1% (v/v) of the blends. The concentration of DCPIP was 20 mg/ml. Erlenmeyer flasks were kept under agitation (84 rpm) at 35.0 ± 0.5°C. The BH medium consists of, gL⁻¹: MgSO₄, 0.2; CaCl₂, 0.02; KH₂PO₄, 1.0; K₂HPO₄, 1.0; NH₄NO₃, 1.0; FeCl₃, 0.05 (Difco, 1984).

RESULTS

Firstly are presented the results related to the respirometric experiment with the soil contamination. Two experiments were carried out because the commercial blend (B2) was only obtained when the experiment with the other blends already had started. The CO₂ production is represented in daily (Figures 1 and 3) and cumulative (Figures 2 and 4) bases. In the first experiment (Figures 1

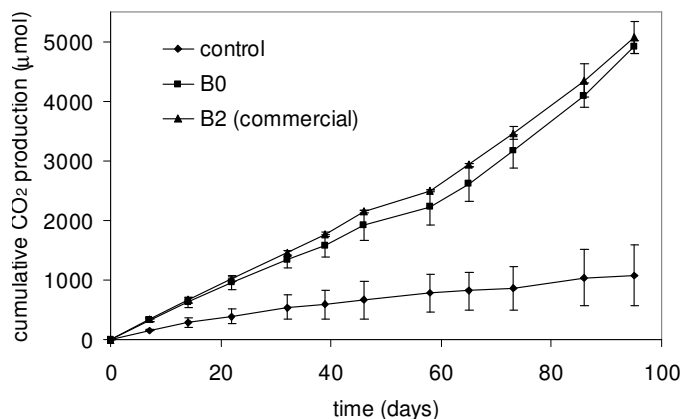


Figure 4. Cumulative total amounts of CO₂ produced by the second respirometric experiment with the soil contamination during incubation. Each error bar represents 1 SD of three replicate.

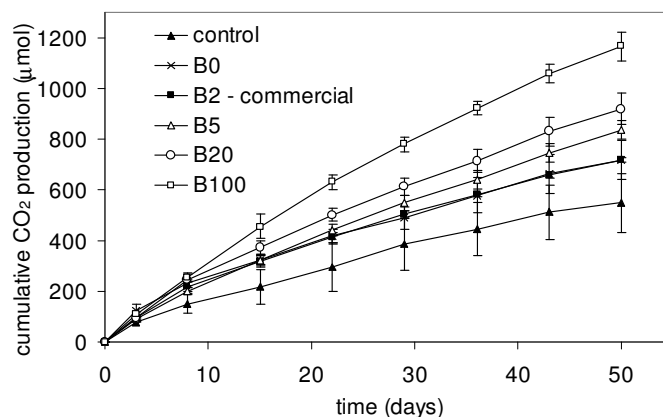


Figure 6. Cumulative total amounts of CO₂ produced by the respirometric experiment with the water contamination during incubation. Each error bar represents 1 SD of three replicate.

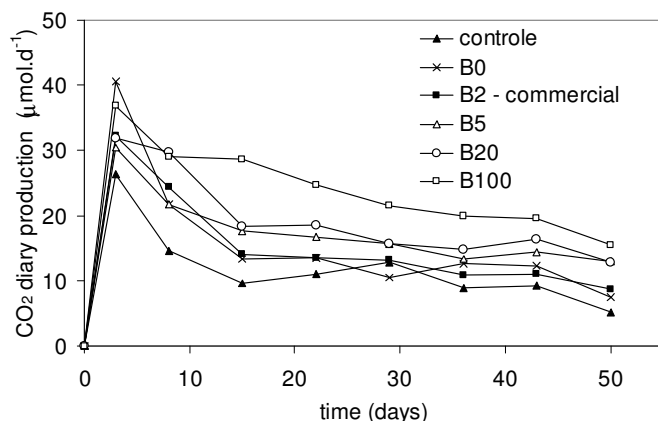


Figure 5. Daily CO₂ production during incubation of the respirometric experiment with the water contamination.

and 2) the total CO₂ produced (µmol/ (Kg/day)) from B0, B5, B20 and B100 were 804.4, 882.6, 911.7 and 1114.9, respectively. In the second experiment (Figures 3 and 4), these values for B0 and the blend B2 (commercial) were 1034.5 and 1069.3, respectively. These values and the curves in the graphics show that the CO₂ production increased as more biodiesel were present in the blend. Statistically (Anova, $p=0.05$) only the blend B20 and the pure biodiesel (B100) differed from B0.

The daily and cumulative CO₂ productions during the respirometric experiment with the water contamination are shown in Figures 5 and 6, respectively. The total CO₂ produced (µmol/(Kg/day)) from B0, B2, B5, B20 and B100 were 287.1,; 287.5, 334.0, 367.8 and 466.4, respectively. Again, as in the soil contamination, these values and the curves in the graphics show that the CO₂ production increased as more biodiesel was present in the blend. Statistically (Anova, $p=0.05$) only the blend B20 and the pure biodiesel (B100) differed from B0.

The results obtained with the biodegradability test using the redox indicator DCPIP (Table 3) show that the time necessary to decolorization of the DCPIP indicator decreased with the increase of the concentration of biodiesel in the blend.

DISCUSSION

Previous works related to the biodegradation of biodiesel and diesel blends mainly focused on the water contamination (Zhang et al., 1998; Makareviciene and Janulis, 2003; Pasqualino et al., 2006) with the exception of the work by Lapinskiene et al. (2006), which evaluated the microbial transformation of these compounds in soil. These works demonstrated that biodiesel and the biodiesel/diesel blends are more easily and faster biodegraded than diesel oil. In the present work, similar results were obtained now with the contamination of soil from a petrol station and water from a river. Experimental evidences are the CO₂ production in the respirometric experiments that increased as more biodiesel was present in the blend and the time necessary for decolorization of the indicator in the biodegradability test using the redox indicator DCPIP, which decreased with the increase of the concentration of biodiesel in the blend.

Zhang et al. (1998) explain that biodiesel is more easily metabolized than diesel because the former is a natural product consisting of pure fatty acids that are hydrocarbon chains with two oxygen atoms attached at one end, which are very biologically active, being recognised and attacked immediately by enzymes such as acetyl-CoA dehydrogenase. The biodegradation of diesel, which consists of a large amount of alkanes (hydrocarbon chains from C10-C20) without oxygen attached, demands adapted microorganisms able to produce enzymes that recognise these molecules.

Table 3. Time (in hours) to decolourization of the DCPIP indicator.

Microorganism	first experiment					second experiment	
	B0	B5	B20	B50	B100	B0	B2 (commercial)
consortium 1 ^a	36	29	27	24	22	120	90
consortium 2 ^b	68	44	24	20	20	64	41
<i>P. aeruginosa</i> LBI	28	13	9	8	3	72	20

^aFrom the soil at the petrol station

^bFrom a non-contaminated area

Obs: During the test, no decolourization of the substrate control (without inoculum) or of the inoculum control (without oil) was observed.

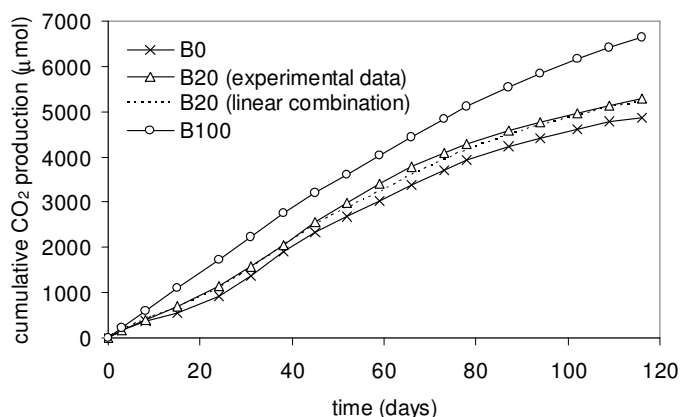


Figure 7. Evaluation of the synergic effect (co-metabolism) for the blend B20 (first respirometric experiment with the soil contamination).

Moreover, the presence of aliphatic cyclic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs) and alkylbenzenes, as well as their derivatives such as toluene, xylenes and PCBs (phenyl and biphenyls) gives the diesel a composition much more chemically complex.

Another point to be discussed is that Zhang et al. (1998) and Pasqualino et al. (2006) verified that biodiesel can promote and speed up the biodegradation of diesel by means of co-metabolism, that is a term used to describe the process in which microorganisms use a second substrate (readily degradable) as the carbon (energy) source to degrade the first substrate which otherwise is scarcely attacked by the microorganisms when it is the sole carbon source. Based on this concept, researchers verified that in some cases biodiesel can be applied in contaminated areas as an enhancement agent to bioremediation processes (Mudge and Pereira, 1999; Taylor and Jones, 2001; Obbard et al., 2004 and Fernández-Álvarez et al., 2006, 2007).

To determine how biodiesel can improve the biodegradability of the pure diesel, the synergic effect was evaluated for the mixtures according to the methodology proposed by Pasqualino et al. (2006), that is based on the measurement of CO₂ in a respirometric experiment. The total amount of CO₂ (cumulative value) produced

with a certain blend (B2, B5 and B20) was compared to a linear combination (LC) (Equation 1) of the total amount of CO₂ produced with the pure compounds (B0 and B100), as follows:

$$LC = D.(CO_2)_{B0} + B.(CO_2)_{B100} \quad (1)$$

Where D is the percentage of diesel in the blend, B the percentage of biodiesel in the blend, (CO₂)_{B0} the total amount of CO₂ (cumulative value) produced with B0 and (CO₂)_{B100} the total amount of CO₂ (cumulative value) produced with B100.

This linear combination was compared with the experimental values of the blends during the days of the experiments. Figure 7 shows the results for the soil contamination with the blend B20. This case exemplifies what was observed for all the other blends considering both soil and water contaminations. According to the methodology of analysis adopted, the curves of the experimental data and that of the linear combination are coincident, it indicates that, the biodiesel did not improve the biodegradation of the diesel by means of co-metabolism. It is important to comment that this methodology of analysis has limitations since it is not based on chromatographic analysis, which could indicate that only the biodegradation of certain compounds present in the diesel could be favoured by the co-metabolism as observed by Fernández-Álvarez et al. (2007).

The results obtained with the biodegradability test using the redox indicator DCPIP also indicate that the soil of the petrol station (from where was obtained consortium 1) had a microbiota adapted to degrade the fuels and the tests with consortium 2 show that the presence of hydrocarbonoclastic microorganisms in soils is ubiquitous, even in unpolluted soils (Venkateswaran and Harayama, 1995; Ron and Rosenberg, 2002 apud Lee et al., 2006; Mariano et al., 2008c).

The time demanded by inoculum 1 for the decolourization of the blend B0 in comparison to B5 decreased 19.4% and for *P. aeruginosa* LBI, 53.6% (Table 3). This experiment demonstrates that low concentrations of biodiesel in the blend (2% or 5%) enhance more significantly the biodegradation by single cultures than consortia. In bioremediation processes carried out by

mixed cultures, commensalisms play an important role since each species may have a specific function in the enzymatic reaction sequences, responsible for the breakdown of more complex molecules. Thus, consortia have less difficulty in biodegrading diesel. This fact is in agreement with the respirometric data, where the CO₂ produced with the blends B2 and B5 did not differ significantly from the pure diesel (B0).

Conclusion

Although biodiesel is more easily and faster biodegraded than diesel oil, among the blends evaluated (2%, 5% and 20%), only the blend with higher concentration of biodiesel presented biodegradability significantly different from diesel and it was not verified an improvement on the biodegradation of the diesel by means of co-metabolism.

In natural environments, as considered in this work, the commensalisms between different communities of microorganisms facilitates the biodegradation of diesel oil, for this reason, the addition of low quantities of biodiesel (2% or 5%) may not represent a gain when the biodegradability aspect is concerned.

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