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Full Length Research Paper

Aerobic biodegradation of butanol and diesel oil blends

Adriano Pinto Mariano[1,2], Richard Clayton Tomasella[2], Clara Di Martino[3], Eduardo Beraldo Morais[4], Rubens Maciel Filho[1], Mirna Helena Regali Seleghim[3], Jonas Contiero[2], Sâmia Maria Tauk Tornisielo[4], Dejanira de Franceschi de Angelis[2]

[1]Laboratory of Optimization, Design and Advanced Control (LOPCA), School of Chemical Engineering - University of Campinas (UNICAMP), Av. Albert Einstein, 500, CEP 13083-852, Campinas, SP, Brazil.
[2]Department of Biochemistry and Microbiology - Institute of Biosciences São Paulo State University, UNESP, Brazil.
[3]Department of Ecology and Evolutionary Biology - Federal University of São Carlos, UFSCar. Brazil.
[4]Center of Environmental Studies, São Paulo State University, UNESP, Brazil.

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This work aimed to evaluate the aerobic biodegradation of butanol/diesel oil blends (5, 10, 15, 20%, v/v) in comparison to the biodiesel/diesel oil blend (20%, v/v). Respirometric experiments simulating the contamination of natural environments (soil and water from a river) were carried out in biometer flasks (250 mL) used to measure microbial carbon dioxide (CO_2) production. The automated turbidimeter Bioscreen C was used to follow the growth of *Pseudomonas aeruginosa* LBI on butanol/diesel oil blends. A redox indicator (2,6-dichlorophenol indophenol - DCPIP) test was used to evaluate the capability of four inocula to biodegrade the blends with 20% (v/v). The experiment which simulated the soil contamination demonstrated that butanol is less biodegradable than diesel oil, and for this reason the increase in the portion of butanol in the butanol/diesel blend from 5 to 20% had negative effects on biodegradation. While in soil the biodiesel/diesel blend was more easily biodegraded than the butanol/diesel blend, in water this order was the inverse. The insoluble fuels (diesel and biodiesel) were poorly biodegraded in water and the biodegradation of the butanol/diesel blend was favored by the water solubilization of the butanol, which enhances the bioavailability of this compound. On the other hand, initial concentrations of butanol in the water higher than 10 mL L^{-1} inhibited the cell growth of the tested microorganisms. Thus, butanol toxicity presumably had a significant effect on the degree of biodegradation of the fuel blends.

Key words: Butanol, biodiesel, diesel, biodegradation, blends, soil, water.

INTRODUCTION

Environmental concerns and the near-future shortage of oil have prompted several countries to adopt legislation concerning the addition of biofuels into the formulation of gasoline and diesel. Ethanol and biodiesel have been produced in large scale and in countries such as Brazil, flex fuel cars can be powered by either neat ethanol or any proportion of gasoline and ethanol. A comprehensive review on the use of biofuels, including their possible socio-economic, environmental and political implications can be found in Luque et al. (2008). Biobutanol is a 4-carbon alcohol derived from the fermentation of sugars and has many characteristics which make it a better fuel extender than ethanol. Butanol has higher energy content and is less explosive and corrosive than ethanol. Due to its non-polar characteristic, butanol can also be blended with diesel. Despite these advantages, inherent drawbacks related to the butanol fermentation make the production of biobutanol in industrial scale still not economically feasible (Ezeji et al., 2007). However, technology improvements for the production of biobutanol are in rapid pace and for this reason commercialization is expected for this decade (Qureshi, 2009). In 2007, DuPont (US) and BP (UK) announced their plans to produce biobutanol to be used as a fuel additive (www.butamax.com). Although the physicochemical characteristics of butanol make it a good

*Corresponding author. E-mail: adrianomariano@yahoo.com.br.

Abbreviations: But, Butanol; **BTEX,** benzene, toluene, ethyl-benzene, and xylenes; **DCPIP,** 2, 6-dichlorophenol indophenol.

Table 1. Soil sample characteristics.

Soil characteristics	Values	Parameters	(mmolc dm^{-3})
pH ($CaCl_2$)	4.0	K	0.6
Organic matter (g dm^{-3})	8.0	Ca	1.0
Residual phosphorus (mg dm^{-3})	8.0	Mg	1.0
Moisture content (%)	7.4	TB[a]	2.7
		Al	1.0
		CTC[b]	27.7

Grain size distribution (%)								
					Sand		Silt	Clay
					86.0		4.1	9.9
Micronutrients (ppm)								
S	Na	Fe	Mn	Cu	Zn	B		
10	3.0	25	0.6	0.9	0.9	0.2		
Heavy metals (ppm)								
Ba	Cd	Cr	Ni	Pb				
14.4	<0.01	11.9	<0.01	<0.01				

[a] Total bases; [b] cation exchange capacity.

candidate to be used as a biofuel, studies concerning its effects on the environment are also necessary. As it is with any other fuel, failures during the many operations involved in the production and commercialization of butanol can result in contamination of soil and water due to spills.

Contaminated areas can be recuperated by the action of microorganisms which biodegrade the pollutant. Studies on the biodegradability of butanol aiming the removal of butanol from industrial waste streams were conducted by Heinze and Friedrich (1997) and Veeranagouda et al. (2006). USEPA (1989) reported that butanol was readily biodegraded in agricultural soils and biodegradation was the most important fate process for butanol in water. In our previous work (Mariano et al., 2009), the aerobic biodegradation of butanol/gasoline blends was assessed in comparison to the ethanol/ gasoline blend. Ethanol showed a much faster biodegradation rate than butanol, particularly in water, and the following order of degree of biodegradation was found: ethanol > butanol > gasoline. The addition of the alcohols to the gasoline resulted in positive synergic effects on the biodegradation of the fuels in soil and water matrices. Furthermore, butanol better enhanced the biodegradation of gasoline in soil than ethanol. These results prompted us to study the effects of butanol on the aerobic biodegradation of diesel oil having as reference the biodiesel/diesel blend. Since biodiesel has been in the fuel market for years, several works evaluating its effect on the biodegradation of diesel oil were published (Zhang et al., 1998; Makareviciene and Janulis, 2003; Pasqualino et al., 2006; Lapinskiené et al., 2006; Mariano et al., 2008a). These studies demonstrate that biodiesel is more easily biodegraded and less toxic than diesel oil and that biodiesel can promote and speed up the biodegradation of diesel by means of co- metabolism.

Thus, having in mind the possible use of biobutanol in the near future as a diesel oil extender and due to the lack of knowledge of the effects of butanol on the biodegradation of diesel oil, this work aimed to evaluate the biodegradation of a butanol/diesel oil blend in comparison to the biodiesel/diesel oil blend. Respirometric experiments were carried out simulating soil and water contaminations. Furthermore, the capability of different microorganisms to biodegrade the blends was evaluated by two methods: measurement of microbial growth by absorbance (automated Bioscreen C system) and by a redox indicator technique (Hanson et al., 1993). It should be noted that this is the first report on the effects of butanol on the biodegradation of diesel oil.

MATERIALS AND METHODS

Soil and water sampling and characterization

The soil sample was collected from the superficial layer of a non-contaminated site. Before performing the biodegradation experiments, the sample was stored at 5°C. 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). Table 1 summarizes some of the soil physico-chemical characteristics. Values of heavy metals concentrations were not above the restricted levels set by the Cetesb (São Paulo Environmental Agency–Brazil) and by the Dutch list (Cetesb, 2005).

The water sample was collected at Atibaia River located in Paulínia (SP/Brazil) (22°44'25.3"S/47°07'35.2"W) on 26 November 2008. The composite sample was obtained at the river surface along a transect perpendicular to the flow direction. Nearby the sampling location, an oil refinery (Replant/Petrobras) and highways represent potential sources of contamination. Table 2 summarizes

Table 2. Water sample characteristics

pH	7.78	Nitrate (mg L^{-1})	0.0	Bacteria (CFU mL^{-1})	54.50
BOD (mg L^{-1})	9.40	Ammonia (mg L^{-1})	0.71	Filamentous fungi (CFU mL^{-1})	11.8
COD (mg L^{-1})	11.90	Chlorate (mg L^{-1})	10.81	Yeast (CFU mL^{-1})	55.0
DO (mg L^{-1})	1.41	Sedimentation	<0.1		
Conductivity (µS cm^{-1})	140.90	Volatile solids (mg L^{-1})	0.056		
Acidity (mg L^{-1})	3.88	Fixed solids (mg L^{-1})	0.313		
Alkal. HCO_3 (mg L^{-1})	21.42	Soluble solids and in suspension (mg L^{-1})	0.369		
Toxicity (EC50)[a]	[b]				
Nitrite (mg L^{-1})	0.0				

[a] *Daphnia similis;* [b] not detected

Table 3. Respirometric experiments.

Experiment 1	Soil contamination
1	Soil control (without addition of contaminants)
2	Soil + butanol (But100)
3	Soil + neat diesel (But0)
4	Soil + diesel (95%) + butanol (5%) (But5)
5	Soil + diesel (90%) + butanol (10%) (But10)
6	Soil + diesel (85%) + butanol (15%) (But15)
7	Soil + diesel (80%) + butanol (20%) (But20)
Experiments 2/3	**Soil/Water contamination**
1	Soil/water control (without addition of contaminants)
2	Soil/water + biodiesel (B100)
3	Soil/water + butanol (But100)
4	Soil/water + diesel (80%) + biodiesel (20%) (B20)
5	Soil/water + diesel (80%) + butanol (20%) (But20)
6	Soil/water + neat diesel (But0)

some of the water characteristics determined following the procedures described in APHA (1998).

Respirometric experiment

Table 3 summarizes the respirometric biodegradation experiments that simulated soil and water contaminations. Biodegradation experiments were carried out in Bartha biometer flasks (250 mL) used to measure the microbial carbon dioxide (CO_2) production (Bartha and Pramer, 1965; Mariano et al., 2007). The CO_2 produced is proportional to the percentage of substrate biodegraded. Mineralization studies involving measurements of total CO_2 production can provide excellent information on the biodegradability potential of hydrocarbons (Balba et al., 1998). CO_2 evolution measures ultimate degradation (mineralization) in which a substance is broken down to the final products. For each experimental condition, the biometer flasks were prepared in triplicates (50 g of soil or 50 mL of water) and incubated in the dark at 27°C. The quantity of fuel added to the soil and water was, respectively, 25 (experiment 1) and 50 (experiment 2) mL kg^{-1} of soil and 4 mL L^{-1} of water (0.4% (v/v)) (experiment 3). After the addition of the blends, the soil kept its moist consistency. Thus the absorption of the fuels to the soil particles did not cause it to become a thick paste. In relation to the water, a floating thin layer of insoluble fuels (diesel and biodiesel) was visible (no emulsion was formed), and butanol was completely solubilized in water.

The CO_2 produced was trapped in a 10 mL solution of potassium hydroxide (KOH) (0.2 M), located in the side-arm of the biometer. This solution was periodically withdrawn by syringe, and the amount of carbon dioxide absorbed was then measured by titrating the residual KOH (after the addition of barium chloride solution (1 mL; 0.5 M) used to precipitate the carbonate ions) with a standard solution of hydrochloric acid (HCl) (0.1 M). During this procedure, the biometers were aerated for 30 s through ascarite filters.

Monitoring of microbial growth using Bioscreen C

An automated turbidimeter (Bioscreen C, Lab systems Helsinki, Finland) was used to follow the growth of *Pseudomonas aeruginosa* LBI (Benincasa et al., 2002) in micro-titer plates. The working volume in the wells of the Bioscreen plate was 440 µL comprising 380 µL of Bushnell-Hass (BH) medium (Difco, 1984), 20 µL of inoculum, and 40 µL of carbon source (same fuel blends used in the respirometric experiment 1). The temperature was controlled at 27°C, and the optical density of the cell suspensions was measured automatically at 600 nm in regular intervals of 1 h, for 84 h. Before each measurement, the culture wells were automatically shaken for 60 s. The experiments were carried out in quadruplicates. The procedure to prepare the inoculum can be found in Mariano et al. (2009). *P. aeruginosa* LBI was chosen due to its known capability to

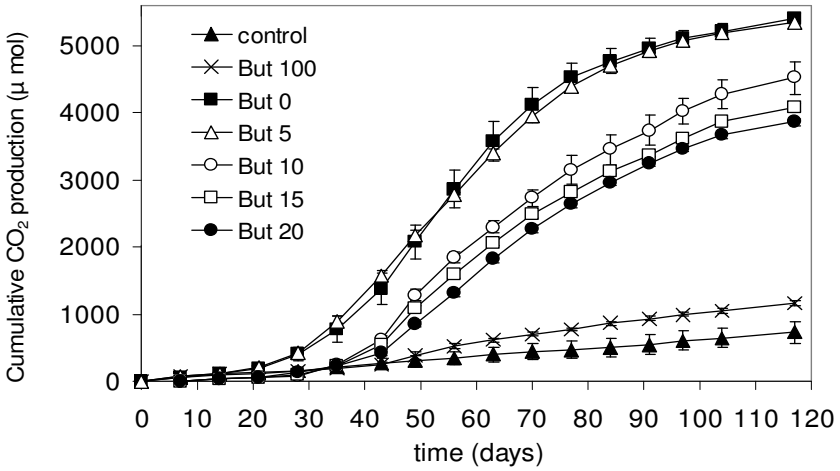

Figure 1. Cumulative total amounts of CO_2 produced in respirometric experiment 1 (soil contamination). Each error bar represents 1 SD of three replicates.

degrade petroleum hydrocarbons (Pirôllo et al., 2008).

Biodegradation test using the 2, 6-dichlorophenol indophenol (DCPIP) indicator

The biodegradation of the fuel blends used in the respirometric experiments 2 and 3 was also verified using the technique based on the redox indicator DCPIP (Hanson et al., 1993). The principle of this technique is that during the microbial oxidation of the carbon source, electrons are transferred to electron acceptors such as O_2, nitrates, and sulphates. By incorporating an electron acceptor such as DCPIP to the culture medium, it is possible to ascertain the ability of the microorganism to utilize the substrate by observing the color change of DCPIP from blue (oxidized) to colorless (reduced). This technique has been employed in other works including Cormack and Fraile (1997), Roy et al. (2002), Pirôllo et al. (2008), Mariano et al. (2008b, 2009), and Junior et al. (2009).

The capability of four inocula to biodegrade the fuel blends was evaluated: *P. aeruginosa* LBI (Benincasa et al., 2002); *Candida vismanathii* (isolated from the wastewater of the Replan/Petrobras oil refinery) (Junior et al., 2009); consortium 1 (obtained from the soil sample); consortium 2 (obtained from the water of the Atibaia River). The procedure to prepare the inocula can be found elsewhere (Mariano et al., 2009). Inocula (0.2 mL, concentration not determined) were added to essay tubes (duplicates) that contained sterile Bushnell and Haas (BH) medium (10 mL) and 1% (v/v) of the blends. The concentration of DCPIP was 0.14 mg mL^{-1}. The tubes were kept under agitation (60 rpm) at 27.0±1.0°C.

RESULTS AND DISCUSSION

The curves that represent the cumulative CO_2 production of the treatments of respirometric experiment 1 are shown in Figure 1. This experiment was designed to evaluate the effects of butanol concentration on the biodegradation of butanol/diesel oil blends in soil. After 117 days of contamination, the blend with 5% of butanol (But5) produced the same amount of CO_2 as the treatment with pure diesel oil (But0). However, as pure butanol (But100) had a poor biodegradation, the increase in butanol concentration resulted in a reduction of CO_2 production by 16.2, 24.3, and 28.5% (respectively, for blends But10, But15, and But20) in relation to But0. Butanol also affected the lag phase. While for But0 and But5 biodegradation started in the 21st day of experiment, the lag phase for the other blends was approximately 43 days.

The monitoring of the growth of *P. aeruginosa* LBI in medium containing butanol/diesel oil blends (Figure 2) also demonstrated the negative effect of butanol on the biodegradation of butanol/diesel oil blends. Cell concentration decreased and lag phase increased with higher butanol concentration. For the blend, But20, and pure butanol, no cell growth was observed. At this point it is interesting to compare the results obtained with the butanol/diesel oil blends with those reported in our previous work (Mariano et al., 2009), in which butanol (But5, But10, But15 and But20 added in a concentration of 50 mL kg^{-1} of soil) had a positive effect on the biodegradation of gasoline. The explanation to this difference can be obtained by comparing the biodegradation of the pure compounds. While in Mariano et al. (2009) gasoline was less biodegraded than butanol (CO_2 production was 39.2% lower), in the present work diesel oil was more biodegraded than butanol (CO_2 production was 7.3 times higher). Thus as butanol is in the middle of the order of degree of biodegradation observed for the pure compounds (diesel > butanol > gasoline), its effect was positive on gasoline and negative on diesel oil. Respirometric experiments 2 and 3 were designed in order to compare the biodegradation of the blends butanol/diesel oil and biodiesel/diesel oil (blends of 20%) in soil and water (respirometric experiments 2 and 3, respectively). The effect of different concentrations of biodiesel other than 20% (B20) on the biodegradation of diesel oil can be found in Pasqualino et al. (2006), Mariano et al. (2008a), and Junior et al. (2009). Concerning the soil contamination (Figure 3), butanol and biodiesel had opposite effects. In

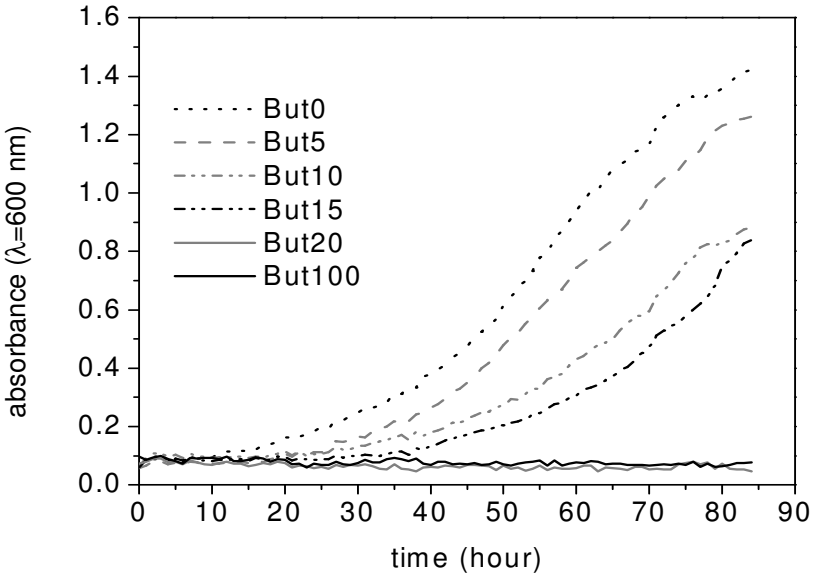

Figure 2. Growth curves of *Pseudomonas aeruginosa* LBI in medium containing butanol/diesel oil blends.

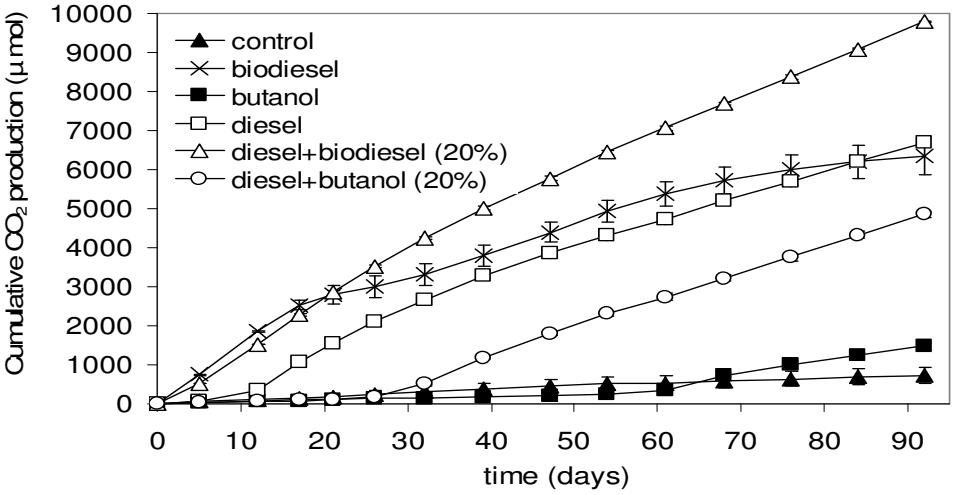

Figure 3. Cumulative total amounts of CO_2 produced in the respirometric experiment 2 (soil contamination). Each error bar represents 1 SD of three replicates.

relation to the pure diesel oil, the CO_2 production increased by 46.1% when biodiesel was added and decreased by 27.6% when butanol was present in the blend. It should be noted that although in respirometric experiment 1 the initial concentration of fuels (25 mL kg^{-1}) was half the concentration employed in respirometric experiment 2 (50 mL kg^{-1}), the decrease in the CO_2 production observed in the But20 blend in both experiments was practically the same (28.5 and 27.6%, respectively, experiments 1 and 2). According to the values of cumulative CO_2 production, the following order of degree of biodegradation was observed for the pure compounds in soil: diesel = biodiesel > butanol. In relation to the blends the order was: biodiesel/diesel > butanol/diesel.

In water, the insoluble fuels (diesel and biodiesel) were poorly biodegraded as indicated by the total amount of CO_2 produced (Figure 4), which was not statistically different from the control (Anova, $p = 0.05$). On the other hand, butanol had a production of CO_2 72% higher than the treatment with pure diesel oil. Consequently, the blend with butanol was more biodegraded than the blend with biodiesel (difference of 61.3% in CO_2 production). According to the values of cumulative CO_2 production, the following order of degree of biodegradation was observed for the pure compounds in water: butanol > biodiesel = diesel. In relation to the blends the order was:

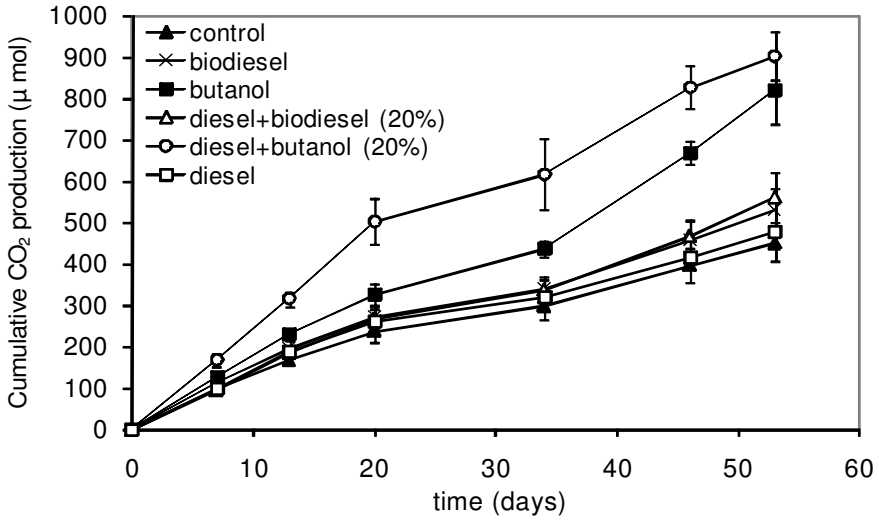

Figure 4. Cumulative total amounts of CO_2 produced in the respirometric experiment 3 (water contamination). Each error bar represents 1 SD of three replicates.

Table 4. Time (in hours) to decolourize the DCPIP indicator.

Microorganism	Diesel	Butanol	Biodiesel	Diesel+but (20%)	Diesel+biodiesel (20%)
Consortium 1[a]	220	-	37	91	211
Consortium 2[b]	-[c]	-	30	-	-
P. aeruginosa LBI	192	-	87	73	142
C. viswanathii	26	-	22	20	26

[a] From the soil; [b] from Atibaia river; [c] no decolourization during the experiment (220 h). During the experiment, no decolourization of the substrate controls (without inoculum) or of the inoculum controls (without fuel) was observed.

butanol/diesel > biodiesel/diesel. Thus, the orders of degree of biodegradation in water were the opposite of that observed in soil. The biodegradation of butanol in water was favored by its solubilization, which enhances the bioavailability of this compound. Besides, the solubilization of the alcohol present in the blend can affect the biodegradation of the hydrocarbons. As reported for ethanol and methanol (Corseuil et al., 2004; Poulsen et al., 1992), it is very likely that butanol enhanced the water miscibility of the mono-aromatics benzene, toluene, ethyl-benzene, and xylenes (BTEX) present in the diesel oil. Solubilization of hydrocarbons increases their bioavailability, however, at the same time, the extension of the contamination increases. In cases of groundwater contaminations, studies demonstrated that ethanol can retard the biodegradation of petroleum contaminants, especially BTEX, by preferential degradation of the ethanol, causing consumption of electron acceptors and nutrients, and changes to microbial populations in favor of ethanol degraders (Powers et al., 2001; Corseuil et al., 2004; Niven, 2005). Another factor that favored the biodegradation of butanol in the respirometric experiment with water was the concentration of butanol in the water. While in this experiment butanol concentration was 0.4% (v/v) (4 mL L^{-1} of water) for But100, in the Bioscreen experiment concentration was 10% (v/v). For the respiremetric experiment with soil, based on the amount of fuel added (25 mL kg^{-1} of soil), a rough estimate gives a concentration higher than 2.5% (v/v). Depending on the concentration, alcohols such as ethanol and butanol can be toxic to microorganisms due to their devastating effects on cell membranes. For example, Veeranagouda et al. (2006) reported that the maximum specific growth rate of *Enterobacter* sp. VKGH12 on butanol varied from 0.27 h^{-1} when butanol concentration was 0.4% (v/v) to 0.05 h^{-1} at 1.2% (v/v). Another example of butanol inhibition can be found in the fermentation to produce butanol, in which cell growth inhibition and premature termination of the fermentation occur at a concentration of butanol of appro-ximately 1.6% (v/v) (Ezeji et al., 2007).

The results of the experiment with the redox indicator DCPIP (Table 4) show that none of the tested inocula was able to biodegrade pure butanol (But100) at an initial concentration of 1% (v/v) (10 mL L^{-1}). On the other hand, with the exception of consortium 2, biodiesel and diesel were biodegraded. When the initial concentration of butanol was 0.2% (v/v) in the case of But20, this blend

was more rapidly biodegraded than the biodiesel/diesel blend, as observed in the respirometric experiment with water. It should be noted that the inoculum which better biodegraded both blends was *Candida viswanathii*. In Junior et al. (2009) this microorganism was able to increase significantly the biodegradation in soil of biodiesel/diesel blends and biodiesel.

Finally, it is important to stress that the results presented here indicate the degree of biodegradation of fuel blends when exposed to different compartments (soil/water). Thus, besides the intrinsic biodegradability of each compound, factors such as solubilization and toxicity had effects on the degree of biodegradation of the fuels. For instance, in the experiments in which fuels were added to water (respirometric, microbial growth, and DCPIP), the effect of butanol in the biodegradation of diesel oil varied from beneficial to harmful according to the amount of fuel added. Thus butanol toxicity presumably had a significant effect on these studies.

Conclusion

This work compared the biodegradation of butanol/diesel and biodiesel/diesel oil blends. The experiment which simulated the soil contamination demonstrated that butanol is less biodegradable than diesel oil, and for this reason the increase in the portion of butanol in the butanol/diesel blend from 5 to 20% had negative effects on the biodegradation. It should be noted that the addition of 5% of butanol into diesel oil did not alter the biodegradation of the latter. While in soil the biodiesel/diesel blend (B20) was more easily biodegraded than the butanol/diesel blend (But20), in water this order was the inverse. The insoluble fuels (diesel and biodiesel) were poorly biodegraded in water and the biodegradation of the butanol/diesel blend was favored by the water solubilization of the butanol, which enhances the bioavailability of this compound. On the other hand, initial concentrations of butanol in water higher than 10 mL L^{-1} inhibited the cell growth of the tested microorganisms. Thus butanol toxicity presumably had a significant effect on the degree of biodegradation of the fuel blends. In this way, it is expected that areas contaminated by a high-volume spillage, the presence of butanol in the diesel can be negative for the biodegradation of the fuel blend due to the high toxicity of butanol.

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