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Microbial activity in two soils with different clay content contaminated by different diesel/biodiesel mixtures

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Biodiesel is an alternative energy source that has a high biodegradability potential and low toxicity, contributing to ecosystem impact reductions. The aim of this study was to determine, by the natural attenuation technique, the microbial activity of two soils: one clayey (CLA) and the other sandy (SAN), contaminated with different concentrations of biodiesel blended with diesel (B0, B5, B20 and B100) simulating a surface spill. The respirometry, fluorescein diacetate (FDA) hydrolysis and cultivable heterotrophic bacteria and actinobacteria count techniques were used to determine the microbial activity in the different microcosms at up to 48 days of incubation. For the respiration activity, the CLA soil was most active at all mixed fuel concentrations (B0, B5, B20 and B100), as compared to the SAN. Furthermore, the biodiesel addition to the two soil types contributed to the microbial activity increase, and higher CO_2 release values were found in the B20 and B100. For the FDA activity, it was found that the CLA soil showed higher activity at the B5 and B20 concentrations, and heterotrophic count showed a tendency towards a CFU g^{-1} decrease as the incubation time increased. This indicates that the CLA soil, due to a higher amount of nutrients, clay, organic matter and CEC, was associated with the addition of biodiesel and showed higher microbial activity. The results obtained in this study contribute to future studies of surface contamination by different mixtures of diesel/biodiesel in soils with similar physical and chemical characteristics.

Key words: Biodiesel, diesel, biodegradation, natural attenuation, clayey and sandy soils.

INTRODUCTION

The search for alternative energy sources has been encouraged in recent decades due to the disadvantages presented by the use of diesel and other petroleum products. These disadvantages can be exemplified by the high levels of contamination in different environmental compartments and the dependence on non-renewable energy sources, such as the use of petroleum-based fuels. On the other hand, the biodiesel from renewable sources, is produced from vegetable oils or animal fats which are transformed into long-chain esters by various esterification or transesterification processes (Demirbas, 2008; Dias et al., 2014).

The use of biodiesel has been widely stimulated in many countries. Brazil has a regulatory framework that

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authorizes the commercial use and which promotes the growing addition of this biofuel to diesel oil by the fuel distributors (MME, 2014). Depending on the biodiesel addition percentage in the diesel/biodiesel blend, a nomenclature is given to the mixture. For example, B5 has 5% biodiesel; B20, 20%; until reaching B100 corresponding to pure biodiesel (Silva and Freitas, 2008).

The use of biodiesel has many environmental advantages, since this fuel has high biodegradability potential and low toxicity (Lisiecki et al., 2014). This is due to the presence of fatty acids and two oxygen atoms which make up the ester function, which allows greater molecule reactivity (Chao et al., 2010, Bücker et al., 2011). In the absence of sulfur and polycyclic aromatic hydrocarbons in the composition, low CO_2, CO, SO_2 and particulate material atmospheric emission levels are observed during biodiesel combustion (Canakci et al., 2006), biodiesel being considered a non-toxic fuel for ecosystems and the biota that live in them. This proves of great interest because spills, leaks and accidents with fuel used in the country can generate serious contamination events, especially in soils.

One of the most widely used techniques for the decontamination of affected environments is bioremediation. This technology uses the physiological competence of micro-organisms (native or introduced), to completely degrade a contaminant in addition to it being a low cost-effective and environmentally acceptable alternative (Jacques et al., 2007). Among bioremediation strategies, natural attenuation is considered an advantageous process, because the reduction of the contaminant may effectively occur using only the potential of the native microbial population without the addition of nutrients to the soil (Horel and Schiewer, 2011). Thus, a positive point of biodiesel is its accelerated biodegradation when compared with other fuels such as diesel (Sorensen et al., 2011).

Due to the addition of an ever-increasing percentage of biodiesel to diesel, it is essential to assess the behavior of these different mixtures, simulating a surface spill of such fuels in soils. The aim of this study was to determine the whole microbial activity from the soil, by the passive bioremediation technique (natural attenuation), performed by microbial respiration, microbial activity of degradative enzymes and quantification of heterotrophic bacteria in two soil types: 1) Oxisol, basaltic substrate (CLA) and 2) Rhodic Paleudalf (RP), sandy substrate, (SAN) contaminated with different biodiesel/diesel blends (B0, B5, B20 and B100).

MATERIALS AND METHODS

Soils

Surface samples of two types of soil were collected from an area without contamination history at a depth of 0-10 cm (The Horizon), classified by the Brazilian System of Soil Classification (EMBRAPA, 2006 as: 1) Oxisol, basaltic substrate (clayey - CLA) and 2) Rhodic Paleudalf (RP), sandy substrate (sandy - SAN) collected in areas along the Federal Highway BR 386, geographically located in the municipalities of Fazenda Vilanova, RS (29° 34' 43.6'' S, 51° 50'33.8'' W) and Triunfo, in Coxilha Velha, RS (29° 44' 09'' S, 51° 37' 44.7'' W), respectively. The soil choice was based on differences in the organic matter, clay and cation exchange capacity (CEC) levels. After collection, the material was sieved through 2 mm mesh. Soil moisture was standardized, presenting field capacity around 60%. During the experiment, distilled water was added to respirometric bottles to maintain the same standard of humidity during the incubation process. The physical and chemical properties of the soil were determined in the Soil Science Department Analysis Laboratory of the Faculdade de Agronomia of the Federal University of Rio Grande do Sul and by the Soil Laboratory of the Fundação Estadual de Pesquisa Agropecuária de Porto Alegre, RS, according to the methodologies described by Tedesco et al. (1995) (Table 1).

Table 1. Physical and chemical characterization of two soils evaluated.

Evaluated parameter	Depth (0-10 cm)	
	Sandy (SAN)[a]	Clayey (CLA)[b]
Clay (g kg^{-1})	140	480
Organic Matter $^{-1}$)	10	24
CEC ($mmol_c$ dm^{-3})	7.4	10.8
pH (H_2O)	4.5	5.2
SMP Index	5.6	6.0
Available P (mg dm^{-3})	2.8	2.6
Available K (mg dm^{-3})	39	52
Zinc (mg DM^{-3})	0.7	5.8
Copper mg DM^{-3})	0.8	4.5
Manganese (mg dm^{-3})	25	94
Exchangeable aluminum (mmolc dm^{-3})	1.5	0.4

[a]Rhodic Paleudalf; [b]Oxisol.

Fuels

Samples of metropolitan diesel and soy biodiesel were used, provided by the Ipiranga Oil Distributor and BSBios companies, respectively. Sterilization of fuels (diesel and biodiesel) was performed by vacuum filtration through a membrane with 0.22 µm pores, using a sterile *kitassato* flask. After this procedure, the fuels were stored in sterile flasks and sealed. To avoid photo-oxidation, the flasks were protected from light with aluminum foil and stored at room temperature. Different diesel/biodiesel mixtures were prepared: 100% diesel fuel (B0); 95% diesel and 5% biodiesel (B5); 80% diesel and 20% biodiesel (B20) and 100% biodiesel (B100).

Respirometric analysis

The methodology used was described by Bartha and Pramer (1965), which quantifies the carbon dioxide released via microbial respiration in the soil. Soil samples (300 g) were added in 1000 ml glass jars and were then infected with 15 ml from fuel (B0, B5, B20 or B100) per kg^{-1} dry soil. The flasks were equipped with a CO_2 capture apparatus, containing plastic cups with 20 ml from a 0.75 M

NaOH solution and were hermetically sealed. The flasks were slightly open every 12 days, removing the container with the NaOH solution and mixing 3 ml of $BaCl_2.2H_2O$ 30% in order to precipitate the CO_2 in solution. Afterwards, 200 µL from phenolphthalein indicator were added, and finally the solution was titrated with 0.5 M HCl until the solution changes the color. A soil sample was removed with moisture equal to that present in the experiments to determine the mass of dry soil in kg, expressing the production of C-CO_2 in mg.Kg^{-1} dry soil. The experiments were conducted in triplicate and incubated at 28°C for 48 days. The negative control corresponded to the soil samples (CLA and SAN) in which there was no fuel addition (NC). The production of C-CO_2 was determined according to Stotzky (1965), the values in mg CO_2 determined by the formula below:

$$\text{mg C-CO}_2 = \frac{[(B - T) \times eq \times M\ HCl \times CF]}{Mc}$$

Where: B = volume (ml) of HCl solution used to titrate the negative control (without soil); T = volume (ml) of HCl solution used to titrate the treatment; eq = gram-equivalent of C (= 6); M = molarity of standardized HCl solution; CF = correction factor for acid/base normality = M HCl/M NaOH; Mc = soil dry mass (kg).

Microbial activity by degradative enzymes in the soil

The microbial activity of the contaminated soil sample was evaluated according to the fluorescein diacetate (FDA) hydrolysis technique, wherein said substrate is mostly cleaved by esterases, lipases and dehydrogenases (Schnürer and Rosswall, 1982). Samples of 1 g of each soil type (sandy and clay) were distributed into centrifuge tubes in which 20 ml from a 60 mM sodium phosphate buffer was added. These tubes were incubated under agitation at 180 rpm and 28°C for 15 min. Afterwards, 100 µL from stock FDA solution was added into all tubes. The same procedure was used for negative controls (no added FDA) to subsequently subtract the turbidity values of the soil samples. All samples were incubated again at 28°C for 15 min under agitation at 180 rpm, promoting the stoppage of the enzymatic reaction by the addition of 20 ml of acetone p.a. The samples were centrifuged for 5 min at 6000 rpm, and the supernatant was filtered in Whatman No. 4 filter paper. The solutions were evaluated in a spectrophotometer (λ = 490 nm). To determine the standard curve, the following fluorescein concentrations were used: 0.00; 0.25; 0.75; 2.00; 3.74 and 7.48 µg.ml^{-1}. Microbial activity was evaluated at 0, 13 and 48 days and the experiment was conducted in triplicate.

Quantification of heterotrophics in the soil

To estimate the count of cultivable heterotrophic bacteria present in the soil samples, the plate count technique was used (APHA, 1998). The plate count agar (standard agar for counting) (PCA HiMedia) and starch casein agar (SCA) were used in order to determine the total heterotrophic bacteria and actinobacteria cell counts, respectively (CFU.ml^{-1}). The experiment was conducted in triplicate, and 1 g from soil samples (SAN and CLA) underwent serial dilutions in saline solution (0.85%), using an aliquot of 0.1 ml for spreading on the plates. For counting of bacteria and actinobacteria, the Petri dishes were incubated, respectively, for 24 h and 5 days at 28°C. The experiments were performed at 0, 13 and 48 days.

RESULTS

Soil physical and chemical characterization

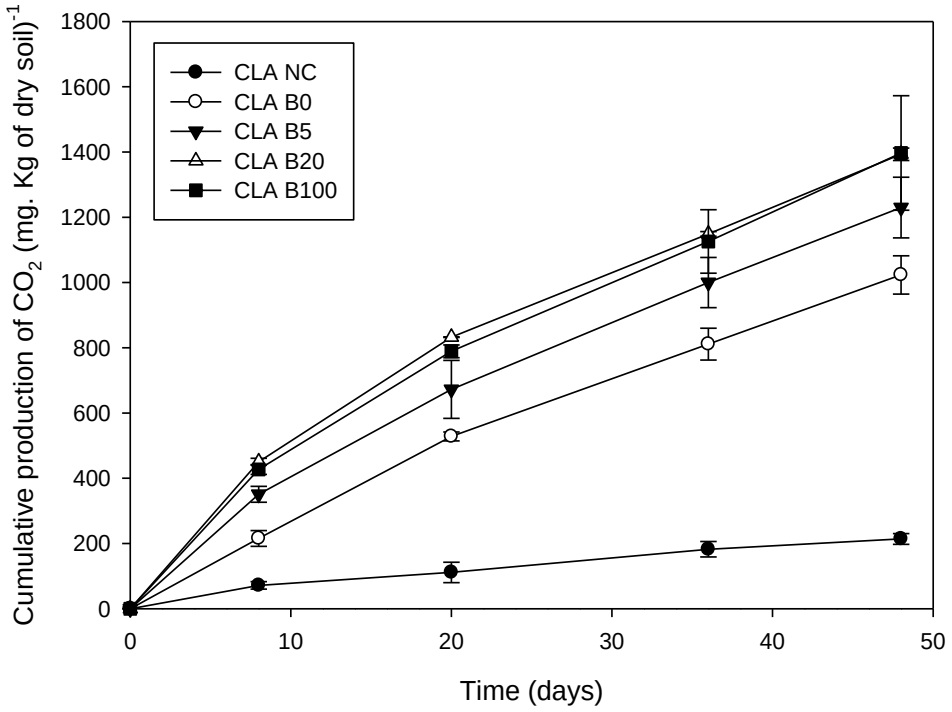
Table 1 shows the physical and chemical characterization of the soils. For all physico-chemical parameters evaluated, it can be seen that the clay soil (CLA) presents the highest values, such as those for clay, organic matter and cation exchange capacity, than the sandy soil (SAN). The clay content ranged from 480 g kg^{-1} in the clayey to 140 g kg^{-1} in the sandy, and the organic matter values ranged from 24 g kg^{-1} in the clayey to 10 g kg^{-1} in the sandy. The cation exchange capacity (CEC) was also higher in the clayey soil, with values ranging from 10.8 mmol dm^{-3} in clayey soil to 7.4 mmol dm^{-3} in the sandy soil. The clayey soil also showed higher amounts of available K (52 mg dm^{-3}), zinc (5.8 mg dm^{-3}), copper (4.5 mg dm^{-3}) and manganese (94 mg dm^{-3}), and the sandy soil presented a more acidic pH than the clayey soil.

Respiratory activity

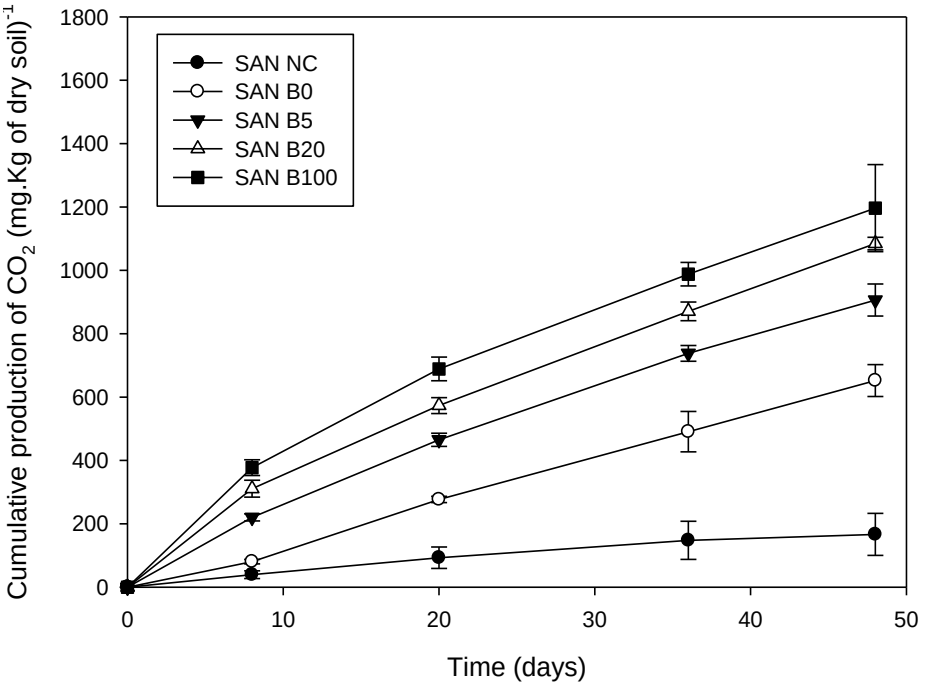
Figure 1A, B and Table 2 show the cumulative CO_2 release in Oxisol (CLA) and Rhodic Paleudalf (SAN), contaminated by different mixtures of diesel/biodiesel. By comparing the respirometric analysis of both soil samples, it can be seen that at all concentrations (B0, B5, B20 and B100), the clayey soil showed higher cumulative CO_2 release values than the sandy soil ($p < 0.05$). The highest cumulative CO_2 release values were observed in the clayey soil in the CLA concentration of B20 and CLA B100 at 48 days, the values ranging, on average, from 1393.14 to 1396.9 mg kg^{-1} dry soil, respectively. The sandy soil had the lowest average cumulative CO_2 release values under the same concentrations and the same incubation time (1084.46 and 1196.43 mg kg^{-1} dry soil, respectively). The lowest average values of cumulative CO_2 release were observed in sandy soil in the control treatment (NC SAN) and SAN B5 after 48 days of incubation (1023.36 and 166.63 mg kg^{-1} dry soil, respectively).

Fluorescein diacetate (FDA)

Figure 1A, B and Table 2 show the sum of the average fluorescein activity in samples of the two soil types in the presence of different diesel/biodiesel mixtures. It can be seen that the highest total fluorescein activity averages were found in treatments CLA B5 and CLA B20 (104.1 and 101.2 mg of F g^{-1} dry soil h^{-1}, respectively). The same treatments in sandy soil had lower values than the clay soil (55.7 and 64.5 mg of F g^{-1} dry soil h^{-1}, respectively). The CLA, NC and CLA B0 control treatments of the clayey soil had the lowest average total fluorescein



(A)



(B)

Figure 1. (A) Cumulative CO_2 release in a Oxisol soil (CLA). ANOVA, tukey < 0.05 for the respective incubation times: 8 days: NCd (Negative Control), B0c, B5b, B20a, B100a; 20 days: NCd, B0c, B5b, B20a, B100a; 36 days: NCc, B0b, B5a, B20a, B100a; 48 days: NCc, B0b, B5ab, B20a, B100a. (B) Cumulative CO_2 release, in a rhodic paleudalf soil (SAN), contaminated by different mixtures of diesel/biodiesel. ANOVA, Tukey < 0.05 for the respective incubation times: 8 days: NC^{d} (Negative Control), $B0^{d}$, $B5^{c}$, $B20^{b}$, $B100^{a}$; 20 days:NC^{e}, $B0^{d}$, $B5^{c}$, $B20^{b}$, $B100^{a}$; 36 days: NC^{d}, $B0^{c}$, $B5^{b}$, $B20^{a}$, $B100^{a}$; 48 days: NC^{d}, $B0^{c}$, $B5^{b}$, $B20^{ab}$, $B100^{a}$

Table 2. Microbial activity in soils contaminated with different mixtures of diesel/biodiesel.

Fuel concentration	SAN		CLA	
	CO_2[1] Release	FDA[2] Activity	CO_2 Release	FDA Activity
NC	166.63^{Ad}	25.38^{A}	213.98^{Ac}	29.26^{A}
B0	652.11^{Bc}	24.56^{A}	1023.36^{Ab}	29.55^{A}
B5	906.31^{Bb}	18.57^{B}	1229.56^{Aa}	34.71^{A}
B20	1084.46^{Ba}	21.52^{B}	1393.14^{Aa}	33.73^{A}
B100	1196.43^{Ba}	16.41^{A}	1396.9^{Aa}	17.18^{A}

[1]Cumulative CO_2 production during the 48 days of incubation (mg of CO_2. Kg^{-1} dry soil.); [2]average FDA values for the three monitoring points (at times 0, 13 and 48 days) (mg of fluorescein. g^{-1} dry soil. h^{-1}). Uppercase letters: comparison of fuel concentrations between soils (Tukey <0.05); lowercase letters: comparison of fuel concentrations within each soil type (Tukey<0.05). NC: negative control.

activity, however, still higher than that in the sandy soil (87.8 and 88.6 mg of F g^{-1} dry soil h^{-1}, respectively). Thus, it can be seen that most of the treatments incubated in clayey soil showed higher microbial activity relative to the sandy soil, as compared to the same diesel/biodiesel mixtures. These results confirm the responses of the CO_2 release between the two soils, reaffirming that, in clayey soil, due to its physicochemical properties, the indigenous microbiota showed higher metabolic activity. Thus, it was expected that B100 treatment, in the clayey soil, as well as in the sandy, would present the highest average values and hence higher average total for fluorescein activity. However, this behavior did not occur (Figure 2).

Cultivable heterotrophic bacteria count

The cultivable heterotrophic bacteria and actinobacteria count in the diesel/biodiesel contaminated treatments in both CLA (clay) and SAN (sandy) soils are presented in Table 3. The results demonstrate a score reduction trend, with passing experiment incubation time (Table 3). Bacteria had higher CFU counts at baseline (0 days). Treatments B5 SAN and B100 CLA correspond to the samples with the highest counts (4870.7 g^{-1} and 512.14 CFU, respectively). At the 13th day of the experiment, a microbial population significant reduction can be observed, particularly in the sandy soil (SAN), with an average of 7.36 $CFU.g^{-1}$ among treatments SAN B0, B5, B20, B100 and NC. This trend was not observed in clayey soil (CLA) for treatments CLA B0, B5 and B20, it was only observed in the control treatment (NC CLA). However, at 48 days, there was a drastic reduction of the microbial population in all treatments (B0, B5, B20, B100 and control), with average values of 13.79 a 3.50 $CFU.g^{-1}$.

The count of actinobacteria colonies showed lower count values in relation to the total bacteria, and presented a count number in a progressive reduction. However, the CLA B5 treatment was an exception, as there was an increase of populations during the experiments, observing count values of 12.6 $CFU.g^{-1}$ at 13 days and 24.72 $CFU.g^{-1}$ at 48 days. At the end of the experiment, CLA B5 was the treatment with the highest actinobacteria count.

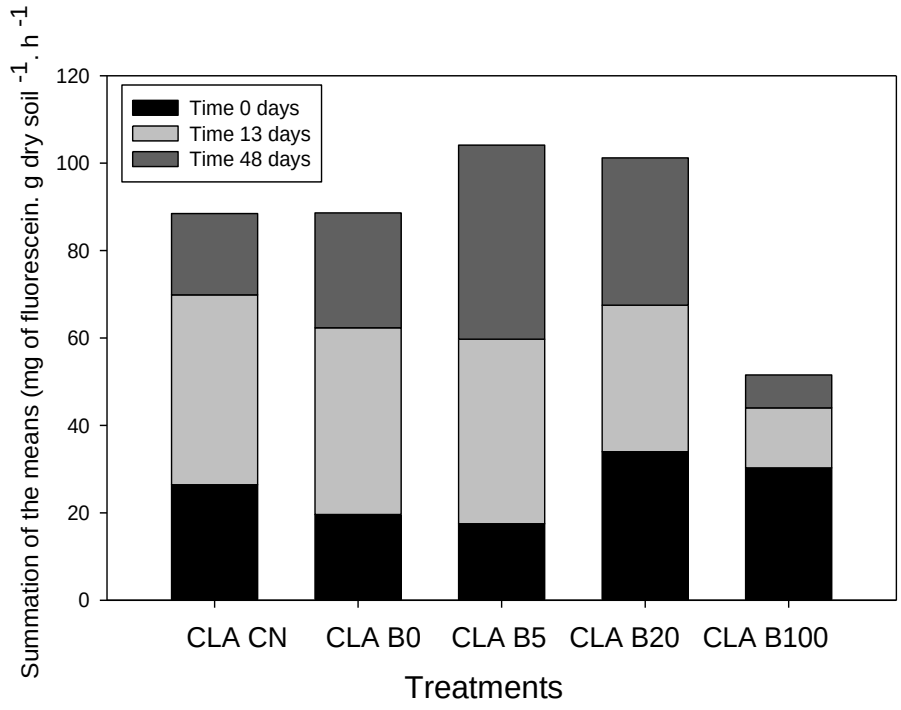
DISCUSSION

Physicochemical characterization of studied soils

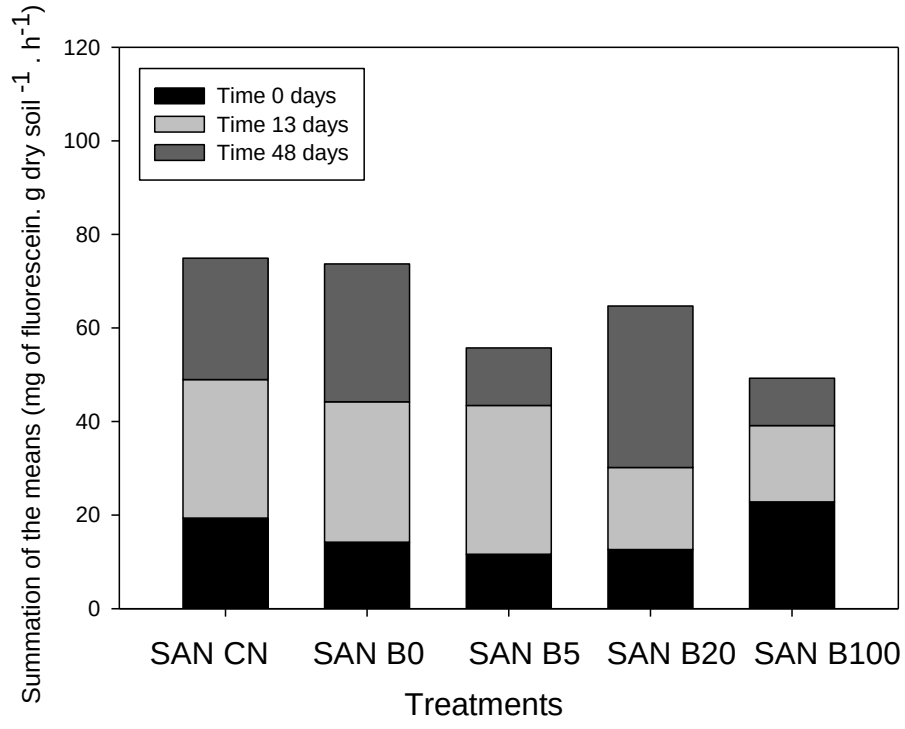
When evaluating the results of the cumulative CO_2 release and physicochemical characterization of the two soils, we can infer that some characteristics of the clayey soil (CLA) such as pH, CEC and organic matter content, may have contributed to the higher cumulative CO_2 release values in this soil. The pH of the clayey soil (CLA) was monitored with a value above 5.0, closer to neutrality in relation to the sandy soil (SAN). Soil pH is an important factor because it is responsible for controlling and maintaining the biological processes, and the pH range required for such activity is between 5.5 and 8.8. In addition, the soil CEC values and the organic matter content were higher in the clayey soil (CLA). These characteristics may have contributed to the growth and maintenance of the native microbiota present in this soil, aiding survival in the presence of the contaminant. Furthermore, this soil also had the highest K concentrations, and this macronutrient is considered essential for microbial growth. Melo et al. (2010), studying the covering soil attenuation process in a municipal solid waste landfill, observed that the samples containing higher values of MO, P and K were those that also showed the highest cumulative CO_2 values throughout the experiment. Thus, the physicochemical characteristics of impacted soils can positively or negatively influence the passive attenuation process.

Respiratory activity

The release of CO_2 is an indirect measure used to evaluate microbial activity in the soil through which the respiration of existing microbiota is evaluated. The contribution of the presence of biofuel in the diesel/-biodiesel blend was confirmed in this study by the highest cumulative CO_2 release values in treatments B100 (100% biodiesel) and B20 in both soils. These results corroborate the results found by Mariano et al. (2008), although they found high cumulative CO_2 release values in the B5 and B20 blends as compared to diesel separately (B0). The highest values were also found in the B100 mixture. The biofuel presence in the diesel/biodiesel blend in soil may facilitate microbial



(A)



(B)

Figure 2. Total of the average fluorescein activity in Oxisol soil (CLA) (A) The sum of average fluorescein activity in a rhodic paleudalf soil (SAN); (B) contaminated by different mixtures of diesel/biodiesel.

Table 3. Count of cultivable heterotrophic bacteria and actinobacteria from various treatments contaminated with diesel/biodiesel mixtures in two types of soil.

Treatment	Actinobacteria Count (10^3 UFC. g^{-1} dry soil)			Bacteria Count (10^3 UFC. g^{-1} dry soil)		
	0 days	13 days	48 days	0 days	13 days	48 days
CLA NC	0.00	4.85	2.56	105.96	24.72	2.20
CLA B0	8.83	16.77	0.79	105.96	176.60	4.24
CLA B5	0.00	12.36	24.72	97.13	194.26	3.97
CLA B20	17.66	5.33	0.26	79.47	211.92	53.00
CLA B100	0	2.65	2.65	512.14	256.00	5.56
SAN NC	9.19	0.92	1.38	91.90	8.64	1.47
SAN B0	9.19	1.28	0.73	91.90	8.27	1.93
SAN B5	18.38	0.78	0.46	4870.70	6.98	0.32
SAN B20	1838	0.37	0.46	275.70	5.51	6.89
SAN B100	0.00	0.55	0.92	321.65	7.44	6.89

degradation of the fuel (Soares Junior et al., 2009). Biodiesel is more readily metabolized than diesel, first, because it is a labile compound consisting of fatty acids with oxygen atoms attached at one end. Therefore, when recognized, they are immediately attacked by enzymes such as acyl-CoA dehydrogenase (Balat, 2011; Yusuf et al., 2011). The highest CO_2 rate release estimated in the clayey soil treatments can be explained by probably having had variations in environmental factors that influence the bioavailability of the contaminant and the structure and density of the biodegrading community in the soil. Furthermore, it is important to note that clayey soils have a relatively higher CEC, among other physical and chemical factors, which helps in the breakdown of covalent bonds of a xenobiotic (abiotic degradation), thus allowing an increase in biotic degradation due to the hydrolysis process (Fay et al., 2008).

Fluorescein diacetate (FDA)

The diacetate fluorescein (FDA) hydrolysis method is considered a simple and quick procedure to evaluate microbial activity since the FDA is hydrolyzed by various enzymes (lipases, proteases and esterases) produced by the microorganisms. Biodegradation of biodiesel begins with methyl or ethyl ester hydrolysis from lipase or esterase action (Boczar et al., 2001). However, the results obtained by analysis of the fluorescein diacetate hydrolysis in the B100 treatment for both soils were not consistent, pointing to probable improper use of this indicator in this study. Orantas (2013) also detected a similar problem when studying the effect of oily sludge in isolation in the soil. The authors reported that the results indicated that the hydrolysis of FDA would not be a good microbial activity indicator related to hydrocarbon degradation because probably the lipases, proteases and esterases hydrolyzed were less related to hydrocarbon degradation process than dehydrogenases. Furthermore, the presence of sludge interfered with the detection of this enzyme, thus there was no good standard of evaluation. A similar problem was detected in 100% biodiesel in the present study: possibly the presence of this compound may have caused an interference in the detection of fluorescein activity in both soils. It can be inferred that in the first 13 days of treatment, there were significant changes in enzyme production (lipases and esterases). However, as the biodiesel concentration in the diesel/biodiesel mixture increased, the enzyme activity decreased. These results were not expected since biodiesel contains long chain fatty acids in its composition and thus can be considered a good stimulator for the synthesis of lipase and esterase. However, it can be seen that at lower concentrations, the addition of diesel fuel in the soil will probably have stressed the native microbiota, stimulating the enzyme production in an attempt to degrade the xenobiotics. Some studies have argued that biodiesel, when added to diesel/biodiesel blends, acts as a co-metabolizing agent, enhancing the degradation rate of the most recalcitrant compound by leading to an increase in the microbial population and stimulating the production of degradative enzymes (Pasqualino et al., 2006; Schleicher et al., 2009; Horel and Schiewer, 2011).

Cultivable heterotrophic bacteria count

Regarding the cultivable heterotrophic bacteria, it should be pointed that the two soil samples used in this study did not have a contamination history. Thus, the tested bacteria were subjected to an intense adaptation process and probably top an intensive reorganization of the structure and composition of the microbial population. It is

still important to note that, due to the addition of the diesel/biodiesel blend, the bacterial community may have undergone physiological stress and led through an intense rehabilitation process. In turn, due to the fact that the native heterotrophic bacteria belong to a soil without a contamination history, a large part may have been selected, with potentially degrading microbiota, from the added xenobiotic, surviving for the most part.

In the CLA B100 treatment, microbial reduction was observed whose control had more emphatic difference, and that progressively maintained itself until the end of the 3rd processing, with the possibility of depletion of readily available carbon sources. Similar results were found by Silva et al. 2012. The same was not observed in CLA treatments B0, B5 and B20, which showed an increase of the bacterial population until of the 2nd processing, probably by selection/physiological adaptation for the biodegradation of the contaminant, either by expression of specific enzymes, adjustments at a genic level, or by selection of organisms suitable for specific degradation activity (Leahy and Colwell, 1990). However, there was a sudden reduction in the count of bacteria and actinobacteria on the SAN treatments, as compared to count of CLA treatment. Consistent with other studies (Margesin and Schinner, 2001), we observed a decrease in heterotrophic count during the incubation time, probably due to increased degradation of the different treatments, including the negative control, exhausting the sources of carbon and other nutrients of an inorganic nature. In other hand, another study of biodegradation that presented the same concentrations of diesel and biodiesel from the brazilian tropical forest soil showed that the whole heterotrophic bacteria populations had a tendency for increasing until the half incubation time and afterwards the populations kept in highest count after the end of the experiment, totaling 60 days (Silva et al., 2012). This fact shows that several factors are responsible for those different results. It is clear that the structural and nutritional composition of the soil has some fundamental role, as observed in the CLA treatments, whose soil presents the highest clay and organic matter content. In general, the soils that show this profile tend to adsorb both nutrients as contaminants for more time, turning them available bit by bit to the microbiota from the site (Labud et al., 2007). Presumably, these slow and gradual processes may contribute to the high adaptability of the micro-organisms that act on the natural attenuation, as compared to those of the SAN treatment. Although natural attenuation is a very slow bioremediation strategy as compared to other treatments, it becomes efficient, considering it for a long time period, besides its low-cost and its *in situ* facilities.

Conclusion

In a general way, the biodiesel and the clay soil contributed to stimulation of the native microbiota activity. Therefore, the biodiesel can present as a potential replacement regarding diesel fuel. Microbial activity was observed for the natural attenuation along the incubation time. However, the reduction of the native population in both soils along the experiment was not an unexpected factor, since this strategy was not favorable for the establishment of the population for a long period of time (nutrients lack), especially to the sandy soil. The results obtained in this paper contribute to future studies of surface contamination by different mixtures of diesel/biodiesel in soils with similar physical and chemical characteristics.

Conflict of interests

The authors did not declare any conflict of interest.

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