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Simulation of a surface spill of different diesel/biodiesel mixtures in an ultisol, using natural attenuation and bioaugmentation/biostimulation

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ABSTRACT

Accidents caused by leaks and/or spills on soils need to be addressed. Natural attenuation, biostimulation and bioaugmentation can be useful bioremediation strategies for decontamination processes in soils of diesel/biodiesel mixtures. The purpose of this study was to evaluate the degradation rate of the different fuels (B0, B20 and B100) in an ultisol under natural attenuation and biostimulation/bioaugmentation during 60 days of incubation in a controlled microcosm simulating a surface spill over soil. The degradation of different diesel/biodiesel mixtures was monitored for up to 60 days by dehydrogenase activity, respirometry by CO_2 release, the most probable number of heterotrophic and degrading microorganism and gas chromatography. The bacterial inoculum employed for biostimulation/bioaugmentation strategy consisted of *Bacillus megaterium, Bacillus pumilus, Pseudomonas aeruginosa,* and *Stenotrophomonas maltophilia*. The two bioremediation strategies have showed great degradation rates. The natural attenuation was effective for B0 and B20 treatments. The addition of the bacterial consortium and macronutrients contributed to the increased degradation of pure biodiesel in relation to natural attenuation, with higher rates for CO_2 release, enzymatic and degrading activity. It is suggested that the bacterial consortium has proven effective for presenting significant values for such parameters until the end of the 60-day incubation period.

Key words: bacterial consortium, bioremediation strategies, dehydrogenase, respirometry, soil spill.

INTRODUCTION

Biodiesel, for being a fuel from renewable sources and lipid nature, is getting more space in the Brazilian and world energy matrix, as an alternative for diesel replacement (Knothe 2010). In addition, this biofuel has many advantages compared to diesel, such as lower toxicity and higher biodegradation rates. Several countries are already adding biodiesel to diesel, helping to reduce the environmental problems caused by the presence of hydrocarbons (Silva et al. 2012, Brasil 2016). There

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are activities related mainly to the transport and storage of these fuels that may cause accidents such as leaks and/or spills, generating contamination in different environmental compartments (Tahhan et al. 2011).

Adding biodiesel to diesel may contribute to increased degradation of the hydrocarbon fraction, due to the phenomenon of co-metabolism (Fernandez-Alvarez et al. 2007, Gong et al. 2010, Yassine et al. 2013). Some bioremediation strategies can be used in case of accidents with these fuels, highlighting natural attenuation, biostimulation, bioaugmentation and their combinations. Although natural attenuation is one of the cheapest bioremediation strategies, it is not always the most effective. The bioaugmentation and biostimulation, especially their combination, can enhance the diesel and biodiesel degradation in soils (Fantroussi and Agathos 2005, Liu et al. 2009, Xu and Lu 2010, Tyagi et al. 2011, Meyer et al. 2014).

Bioaugmentation is a technique that helps to improve the ability of a contaminated area to remove contaminants by adding an isolated and identified strain or a consortium of potentially degrading microorganisms, evaluated technologically in laboratory conditions. Several researches report the use of consortia rather than a single isolate, since many microorganisms are only able to biotransform a toxic compound into intermediate compounds of the degradation pathway. Thus, the presence and interaction of other species are needed to complete the process, completely converting the organic contaminant into carbon dioxide by respiratory process (Fantroussi and Agathos 2005). The addition of nutrients can also accelerate the fuel-degrading process, giving nitrogen and phosphorus to microbial cell replication, optimizing the proliferation of indigenous or exogenous microbial community highly efficient for bioremediation (Tyagi et al. 2011), especially in stable sites with low nutrients ratio.

Many classic parameters such as respirometry techniques, most probable number of heterotrophic and biodegrading plate counts, enzyme activity quantification in soils and gas chromatography, may contribute to assess not only the microbial activity, but also to estimate the degradation rates of different diesel/biodiesel mixtures present in soil under different bioremediation strategies (Silva et al. 2012, Meyer et al. 2014). The purpose of this study was to evaluate the degradation rate for different fuels and mixtures (B0, B20 and B100) in an ultisol under two bioremediation strategies (natural attenuation and biostimulation/bioaugmentation) during 60 days of incubation in a controlled microcosm, simulating a surface spill.

MATERIALS AND METHODS

SOIL SAMPLES

Representative surface soil samples were collected from an area without history of contamination at 0-20 cm depth (Horizon A), classified by the Brazilian Soil Classification System (EMBRAPA 2006) as Typical Red Dystrophic Argisol (ADL), sandstone substrate, being collected in an area next to the BR 386 Federal Highway, geographically located in city of Triunfo, Coxilha Velha area, RS (29° 44’ 09’’ S, 51° 37’ 44,7’’ W) respectively. After collection, the material was sieved through a 2 mm mesh screen. Soil moisture was standardized, maintaining field capacity around 60%. The physicochemical properties of the soils were determined by laboratory analysis, according to the methods described by Tedesco et al. (1995).

FUEL

Metropolitan diesel (B0) and soy methyl biodiesel (B100) samples were used provided by the Ipiranga Petroleum Distributor and BSBios companies, respectively. The fuels used were pure diesel, pure biodiesel and B20 (20% biodiesel and 80% diesel) (V/V). These fuels were added to the soil. The B20

was previously prepared in the laboratory using a graduated cylinder. The fuels were stored in dark bottles, in order to be protected from light, avoiding photo-oxidation.

SOIL BIOREMEDIATION EXPERIMENTS

300 g of soil were incubated in respirometric flasks (1 L), keeping the rest of the volume for gaseous changes between microcosms and inner atmosphere. The moisture was adjusted and kept to 60% of field capacity (capability of 60% soil micropores turn full of water). The deliberate contamination of soils was conducted under experimental design characterized by four different fuel mixtures (control, B0, B20 and B100) and two bioremediation strategies (natural attenuation and bioaugmentation/biostimulation) in triplicate, according factorial scheme 4x2, totaling twenty-four experimental plots. 9 mL of fuel were added to moist soil (30 $mL.Kg^{-1}$, simulating a surface fuel spill on a 0.5 hectare soil by a tank-truck able to charge 30.000 L of fuel), mixing with a spatula to make the content of each microcosm as homogeneous as possible. The microcosms were assembled according to the following layout: two bioremediation strategies, being natural attenuation and bioaugmentation/biostimulation, and control treatment (without fuel). In order to evaluate the microbial activity during degradation, were performed respirometry experiments, evaluation of dehydrogenase activity and heterotrophic/degrading bacteria plate count during period of incubation, as well as gas chromatography, to estimate the percentage of fuel that was degraded at the end of incubation. The experiments were performed in triplicate and incubated for a period of 60 days. In order to estimate by the natural attenuation biodegradation activity, it was considered only the indigenous microorganisms in the soil, with no nutrient addition. For the bioaugmentation/biostimulation strategy, an inoculum was added by pipetting and mixed to the soil, consisting of four bacterial isolates at a final concentration of 2.10^8 UFC g^{-1} (modified from Bento et al. 2003): *Bacillus megaterium*, *Bacillus pumilus*, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* (Meyer et al. 2012), in addition to a nutrient solution of NH_4NO_3 and KH_2PO_4 at variable concentrations, so that the C:N:P ratio was adjusted to 120:10:1 for each treatment (performing the amount of macronutrient composed and required by the bacterial cell for organic matter degradation), according to the composition of the soil, as suggested by Jiménez et al. (2007). The carbon source provided was exclusively from the fuel.

RESPIRATORY ACTIVITY

The microbial respiratory activity in closed microcosms containing soil contaminated with diesel and/or biodiesel was evaluated by cumulative liberation of CO_2 (Stotzky 1965). A capturing system for CO_2 was assembled in each microcosm. The capturing system consisted of a plastic cup containing 20 mL of NaOH 0.75 $mol.L^{-1}$ suspended in a metal frame. Flasks without soil were used as blanks in order to quantify the CO_2. The flasks were kept tightly closed and only periodically opened for basal respiration determination. The analyses were performed using 3 mL of $BaCl_2$. $2H_2O$ 30% and 3 drops of phenolphthalein 1% were added to the NaOH solution. The residual amount of NaOH was titrated with HCl 0.5 $mol.L^{-1}$. The carbonic gas produced was calculated by the equation:

$$C\text{-}CO_2 \text{ generated } (mg.Kg^{-1} \text{ soil}) = (V_B - V_A).(M_C/2).M_{HCl}.(FC/m) \quad \text{Equation 1}$$

where: V_B is volume in mL of HCl 0.5 $mol.L^{-1}$ used to titrate the blank proof; V_A is volume in mL of HCl 0.5 $mol.L^{-1}$ used to titrate the treatment; M_C is the carbon molar mass in $g.mol^{-1}$; M_{HCl} is the concentration in $mol.L^{-1}$ of the standardized HCl

solution; *FC* is the correction factor for the molarity (M_{HCl}/M_{NaOH}) and *m* is the mass (kg) of dry soil in the flask.

DEHYDROGENASE ACTIVITY

The dehydrogenase activity was determined according to the method proposed by Thalmann (1968) and Alef (1995) with modifications. 2 g of moist soil were collected from every respirometric flasks and added to 2 ml 2,3,5-triphenyl tetrazolium chloride (TTC) 0.9% in Tris-HCl pH 7.8 buffer. The mixture was placed in a tube and incubated at 30 °C for 24 h. Soon after, 16 mL of acetone P.A. were added to stop the enzymatic reaction, leaving the homogenate in the dark for 2 hours. The soil suspension was then filtered and the absorbance of the filtrate evaluated at 546 nm in a spectrophotometer. A standard curve was constructed to quantify the amount of 2,3,5-triphenyl formazan of each sample (unit for $TFP.ml^{-1}$) at 0, 7, 21, 35, 49 and 60th day of incubation.

FUEL EXTRACTION FROM SOIL SAMPLES

In order to verify the biodegradation percentages of the hydrocarbons and esters (B0, B20 and B100) present in respirometric flasks with 300 g of soil, two chromatographic analyses were conducted, the first at time zero and the second at the end of the experiment (60 days). Always before collection, the soil was turned over with a spatula to oxygenize and ensure mixture homogeneity. Subsequently, composite samples from five equidistant points within each flask were collected, and 5 g of soil was taken at each point, in order to complete 25 g. Soil samples remained at -18 °C until fuel extraction. Thereafter, the samples underwent a Soxhlet extraction process for 12 hours using methanol/dichloromethane grade pesticide (1:3) (V/V) as organic solvents (USEPA 1996). The extracted material was concentrated by rotary evaporator (RV 05 - ST IKA - WERKE) at 50 rpm. In turn, the amount of residual water in the sample was removed by adding anhydrous sodium sulphate. The extract was then washed with dichloromethane grade pesticide and transferred to a new vial. Subsequently, the extract remained evaporating in a fume hood until constant weight. The samples were stored at -18 °C until submitted to chromatographic analysis.

FUEL DEGRADATION

The degradation of fuels of different treatments was evaluated by TPH (total petroleum hydrocarbon) measurements for B0 and B20 concentrations and total esters for B20 and B100 at the beginning (time zero) and at the end of the experiment (after 60 days of incubation). For pure diesel and the diesel fraction in B20, the hydrocarbon band between C_8 – C_{28} was evaluated. The analyses were compared to the primary standard of the same fuel batches used in the biodegradation experiments. The calculation of the degradation percentage after 60 days of incubation was according to the formula:

$$(DTF - DTI) / DTI \cdot 100 \qquad \text{Equation 2}$$

where DTF: fuel concentration at the final time (60 days), DTI: fuel concentration at time zero. For the determination of total esters was used a Dani GC 1000 Digital Pressure Control (DPC) chromatograph, FID detector, Linear Programmed Temperature Vaporizer injection (L-PTV), with temperature and pressure programming during a 1:15 split injection (L-PTV injector: 118 °C/0.75 min., aq 999 °C/min. 175 °C/20min.) and a DB-Wax column (30 m x 0.32 mm x 0.25 um); an initial oven column temperature of 50 °C/6 min. and final temperature of 230 °C with rate of 5 °C/min.; 6 min retention time; detection temperature 260 °C; N_2 gas flow at 10 mL/min. and run time of 50 min., according to ASTM D 6584 (2012) and EN 14110 (2003) methods. The Total Petroleum Hydrocarbons were determined

by the USEPA 8015B (1996) method. Soil samples were extracted with hexane and dichloromethane in 1:1.5 proportion and an aliquot of the extract (2μL) was injected in a gas chromatograph (Dani GC1000) with flame ionization detector (FID) and capillary column VF-5MS (30 m x 0.53 mm x 1.5 μm). Nitrogen was employed as carrier gas and the hydrocarbon concentration was quantified using external standardization. The method detection limit was 0.05 $mg.kg^{-1}$.

COUNTING OF TOTAL HETEROTROPHIC BACTERIA AND FUEL DEGRADER BACTERIAL COMMUNITY

Total heterotrophic bacteria's counting was measured using 1 g of soil sample from each respirometric flask, submitted to serial dilutions (10^{-1} to 10^{-9}). 100 μL of each serial dilution + 100 μL of TSB broth (Himedia) were added to 96 well plates, wrapped in aluminium foil and incubated at 28 °C for 48 h. Colony forming units (CFU) were measured by medium turbidity, according to FDA (2001), and represented by $CFU.g^{-1}$ dry soil. Fuel degraders counting was measured by 2,3,5-triphenyl tetrazolium chloride (TTC) redox indicator. The soil samples were also performed as described above. 100 uL of each serial dilution + 100 μL of basal mineral medium MM1 with TTC (Braddock and Catterall 1999) + 10 μL of B50 (diesel:biodiesel) were added to 96 well plates, wrapped in aluminium foil and incubated at 28 °C for 10-14 days. This technique looks for microorganisms capable to use B50 as a carbon source, detected by redox indicator (2,3,5-triphenyl tetrazolium chloride reduced to 2,3,5- triphenyl tetrazolium formazan, with colorless solution turned to rose color). CFU was also measured as described above.

STATISTIC ANALYSIS

The obtained results were analyzed by the Tukey test (comparison between mean values) and ANOVA, for a confidence level of 95% using the Sisvar program, version 4.6, (Build 60) (Ferreira 2011). The graphics were performed by SigmaPlot software, version 12.5.

RESULTS

The principal physicochemical attributes of a type of ultisol used to make up the microcosm are detailed in Table I.

When the cumulative CO_2 production after 60 days of incubation was evaluated, it was observed that all the analyzed results presented respiratory activity levels above their respective negative controls (no fuel). If considering the cumulative CO_2 production levels for different diesel/biodiesel (B0, B20 and B100) mixtures, it can be seen that, under natural attenuation, there was no increase in the CO_2 release rate as the biodiesel concentration was

TABLE I
Physicochemical attributes of ultisol evaluated.

Atribute	Depth (0- 20 cm)
Organic Matter ($g\ Kg^{-1}$)	11
Clay (%)	9
Fine Sand (%)	41
Coarse Sand (%)	42
Silt (%)	8
Organic Carbon ($g\ Kg^{-1}$)	5
Total Nitrogen ($g\ Kg^{-1}$)	0.5
Available P ($mg\ dm^{-3}$)	0
Available K ($mg\ dm^{-3}$)	68
Effective CEC ($mmol_c\ dm^{-3}$)[1]	2.3
CEC pH 7 ($mmol_c\ dm^{-3}$) [2]	5.4
Al ($mmol_c\ dm^{-3}$)	0.8
Ca ($mmol_c\ dm^{-3}$)	0.9
Mg ($mmol_c\ dm^{-3}$)	0.4
H + Al ($mmol_c\ dm^{-3}$)	3.9
pH (H_2O)	4.8
SMP Index	6.1

increased in the diesel/biodiesel mixture (Figure 1). However, for the bioaugmentation/biostimulation strategy, there are microbial activity differences among fuel mixtures in the soil (B100> B20> B0> NC) (Figure 1). It was also observed that there was higher respiratory activity for fuel types (B100, B20 and B0) when subjected to bioaugmentation/biostimulation, compared to natural attenuation.

The results of the dehydrogenase group enzymatic activities are summarized in Figure 2. For the natural attenuation strategy, in general, a decreasing dehydrogenase activity trend was observed over 60 days of incubation, with higher enzyme activity verified at the first two monitoring points (at 0 and 7 days of incubation). On the other hand, for bioaugmentation/biostimulation strategy, generally the significant enzyme values occur for the last two monitoring points (49 and 60 days of incubation), besides the B20 treatment at 21 days. It may be explained by an initial adaptation phase for microbial community added to the microcosms, when it enjoys the nutrients (N, P, K) provided to degrade simple carbon source from microcosm. After this period, the fuel carbon source degradation starts. In addition, for both bioremediation strategies, monitoring points that showed highest enzyme activity were in the B100 treatments.

The results of the degrading and heterotrophic microorganism counts obtained by the two bioremediation strategies in an ultisol are summarized in Figure 3. In most treatments, there was no significant increase in the population growth rate during the 60 days of incubation, although major differences were observed between heterotrophic and degrading microorganism counts for the bioaugmentation/biostimulation strategy, compared to the natural attenuation. For both bioremediation strategies, there was a reduction of degrading microorganism counts from the 35th day of incubation. In the natural attenuation, this

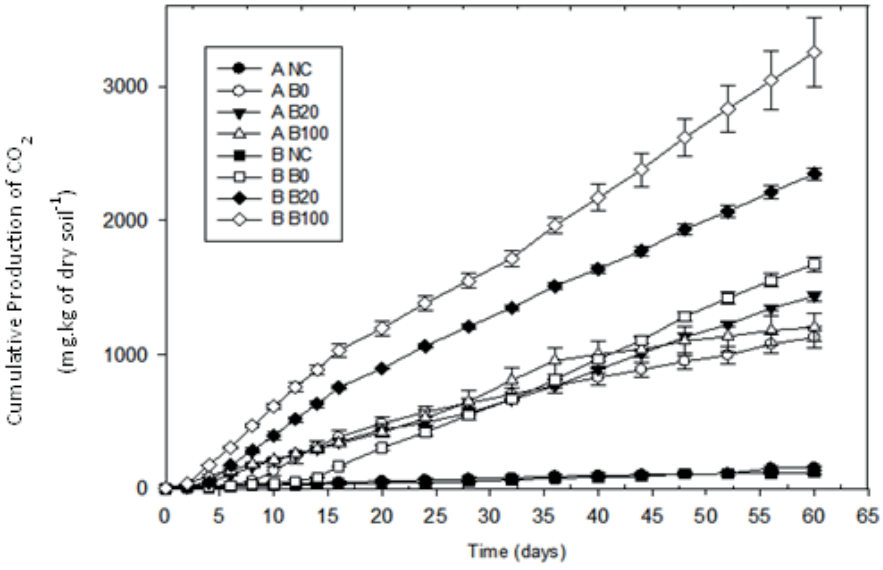

Figure 1 - Cumulative CO_2 production from an ultisol contaminated by different diesel/biodiesel mixtures (B0, B20 and B100) in two bioremediation strategies: natural attenuation (A) and bioaugmentation/biostimulation (B) during 60 days of incubation. A NC (A: natural attenuation, NC: control treatment without contamination). B NC (B: bioaugmentation/biostimulation, NC: control treatment without contamination). B0: pure diesel; B20: 20% biodiesel added to diesel; B100: pure biodiesel.

occurred for the B100 treatment, while the B0 and B20 treatments maintained a population rate of 10^7 CFU.g^{-1} soil by the end of incubation. As for the bioaugmentation/biostimulation, the B0 and B20 were the treatments that had their population reduced from 35th day of incubation, and the B100 was the treatment in which the population remained at 10^6-10^7 CFU.g^{-1} soil until the end of incubation.

The results of hydrocarbon and ester degradation after 60 days of incubation can be seen in Table II. The degradation percentage was calculated by the difference in concentration of hydrocarbons and/or esters detectable by gas chromatography from time zero, in relation to time 60 (days of incubation). For evaluating the diesel degradation, the concentration of TPH (Total Petroleum Hydrocarbon) in the soil was estimated by considering only the light fraction (C_8 - C_{28}) made up of a large proportion of aliphatic hydrocarbons and, to a lesser extent, aromatics. For the biodiesel fraction, the parameter used to evaluate the degradation was the detection of total esters. There was degradation for both bioremediation strategies. B0 and B20 treatments showed degradation rates about 90%, although there were significant differences in the degradation of the two diesel/biodiesel mixtures through adding the bacterial consortium and nutrients. Indeed, B100 was the only fuel in the soil that showed statistically significant differences between bioremediation strategies. The addition of bacterial inoculum plus nutrients contributed to its biodegradability (degradation rate of 59.77 and 74.53% for B100, respectively, under natural attenuation and bioaugmentation/biostimulation).

Treatments for both bioremediation strategies were: B100> B20> B0 for dehydrogenase enzyme activity as well as for CO_2 production. Furthermore, the microbial activity was higher for the bioaugmentation/biostimulation in relation to natural attenuation for diesel/biodiesel mixtures (B0, B20 and B100). For the biodegradation results, the B0 and B20 treatments had highest percentages, not presenting, however, statistic differences between the two bioremediation strategies. On the other hand, although B100 had presented a lower degradation percentage compared with B0 and B20 treatments, the addition of the bacterial consortium and nutrients contributed to an increase in the B100 treatment degradation percentage, compared to the same treatment under natural attenuation.

DISCUSSION

Many studies have demonstrated the success of natural attenuation in which the biodegradation of hydrocarbons can be efficient, mainly regarding the presence of bacteria and indigenous fungi (Bento et al. 2005, Sarkar et al. 2005, Margesin et al. 2007, Di Toro et al. 2008). This fact also occurred in B0 as well as in B20 mixture, since these two treatments showed no degradability differences when bacterial consortium and nutrients were added to the system. However, it is important to consider that the B0 treatment, even with decrease in the size of the degrading population from incubation in day 35, showed high degradation of the light diesel fraction (96.5%) (Figure 2 and Figure 3; Table II). On the other hand, the counting of degrading populations remained high and constant until the end of incubation for treatments A B0 and A B20, related to natural attenuation strategy (Figure 3). This shows that these populations probably exhibited favorable metabolic and genetic support, even under natural conditions to degrade hydrocarbons. It means that these bacteria would present specific enzymatic display for biodegradation of fuel contaminants. In general, for the natural attenuation strategy, high B20 and B0 treatment degradation rates were achieved, corresponding to a percent over 90 (Table II).

The B100 treatment showed less degradation when compared to the B0 and B20 mixtures for both bioremediation strategies. On the other hand, the B100 treatment showed highest degradation

TABLE II
Comparison of three different methods to assess microbial activity and biodegradation: dehydrogenase activity, cumulative CO_2 production and degradation percentage of the diesel/biodiesel (B0, B20 and B100) mixtures after 60 days, for the two bioremediation strategies [natural attenuation (A) and bioaugmentation/biostimulation (B)] in an ultisol.

Treatment	Dehydrogenase[1]		Cumulative CO_2 Production ($mg.Kg^{-1}$ dry soil) (60 days)		Biodegradation Percentage (%)	
	A	B	A	B	A	B
NC	7.04 Ab*	6.89 Ab	154.44 Ac	119.89 Ad	ND[2]	ND
B0	10.31Aab	5.31 Ab	1126.42 Bb	1674.02 Ac	96.47 Aa	98.15 Aa
B20	15.00 Bab	21.27 Aa	1202.91 Bab	2347.41 Ab	90.56 Aa	84.65 Aa
B100	18.07 Aa	23.90 Aa	1433.77 Ba	3255.73 Aa	59.77 Ab	74.53 Aa

*Averages followed by the same upper case in the lines, and lower case in the columns, do not present statistic difference from each other, according to Tukey test with 5% probability; [1]average of the 6 monitoring points during the 60 days of incubation; [2]TPH values not detected in the soil (ND).

percentages for bioaugmentation/biostimulation, compared to the natural attenuation. It was also observed that for B B100 treatment, the degrading microorganism counts showed constant (10^7 $CFU.g^{-1}$dry soil) to the end of the experiment during 60 days, besides presenting an increase of dehydrogenase activity at the last two monitoring points for that same treatment (Figure 2). Comparing the same mixture for natural attenuation, it is observed that the degrading microorganisms rates fell from the 35th day of incubation, reaching values close to 10^4 $CFU.g^{-1}$ dry soil. Additionally, this same treatment showed a similar reduction profile for the dehydrogenase activity from 21 days of incubation. These data confirm that the B100 treatment for the bioaugmentation/biostimulation strategy was more promising, compared to the natural attenuation, reinforcing the concept that the bacterial consortium may have retained their potential for resilience throughout the incubation period, favoring the increasing in degradability of the mixture, as shown in Table II. Furthermore, the addition of nutrients to the system may have favored the maintaining of microbial populations at high levels for B100. The population decrease for the same treatment under natural attenuation was probably due to the lack of nutrients available in the microcosm from that particular incubation period, associated with the absence of the bacterial consortium addition to the system. According to Fantroussi and Agathos (2005), the combination of bioaugmentation with biostimulation should prove to be a promising strategy, since the exogenous microorganisms, as well indigenous, can benefit from the addition of nutrients to the system. However, in the present study, it is interesting to note that the treatments containing B100, while showing a low breakdown percentage, were those which also demonstrated high enzyme activity and cumulative CO_2 production values. The release of CO_2 in soils is an indirect way to estimate the decarboxylation of organic compounds that are degraded in the system (Margesin et al. 2000). Moreover, a large dehydrogenases group participates in processes related to the assimilation and degradation of organic compounds by the citric acid cycle (Schinner et al. 1996, Margesin et al. 2000). These data also help confirm the higher degradation rate of treatment B B100 in relation to the A B100 (Table II).

The main criterion for choosing the bacterial consortium for the bioaugmentation strategy was determined from the optimum results for biodegradability for each isolate. Thus, evaluation of various enzyme activities in addition to the estimated production of high and low molecular

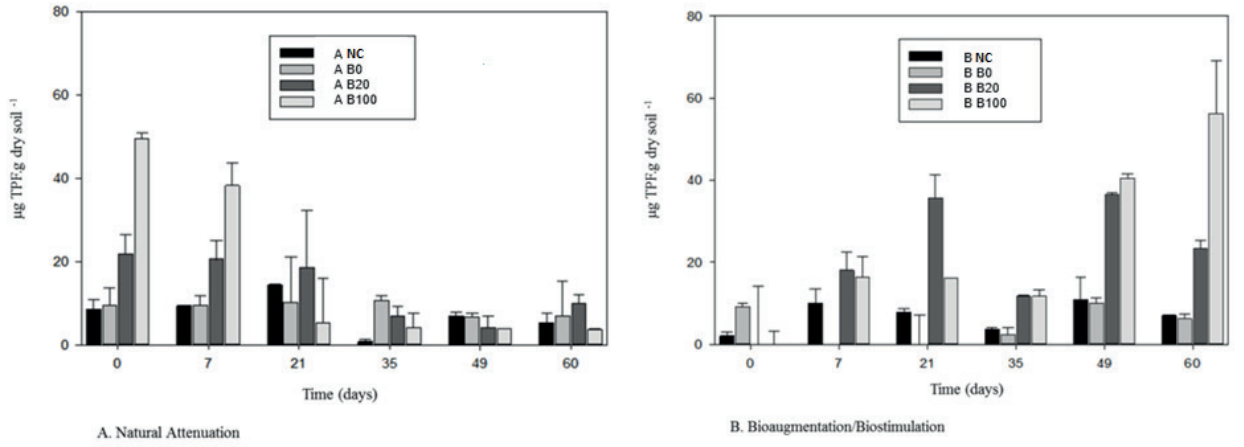

Figure 2 - Dehydrogenase activity in an ultisol contaminated with different fuel types (B0, B20 and B100) in two bioremediation strategies: natural attenuation (A) and bioaugmentation/biostimulation (B) during 60 days of incubation. A NC (A: natural attenuation, NC: control treatment without contamination). B NC (B: bioaugmentation/biostimulation, NC: control treatment without contamination). B0: pure diesel; B20: 20% biodiesel added to diesel; B100: pure biodiesel.

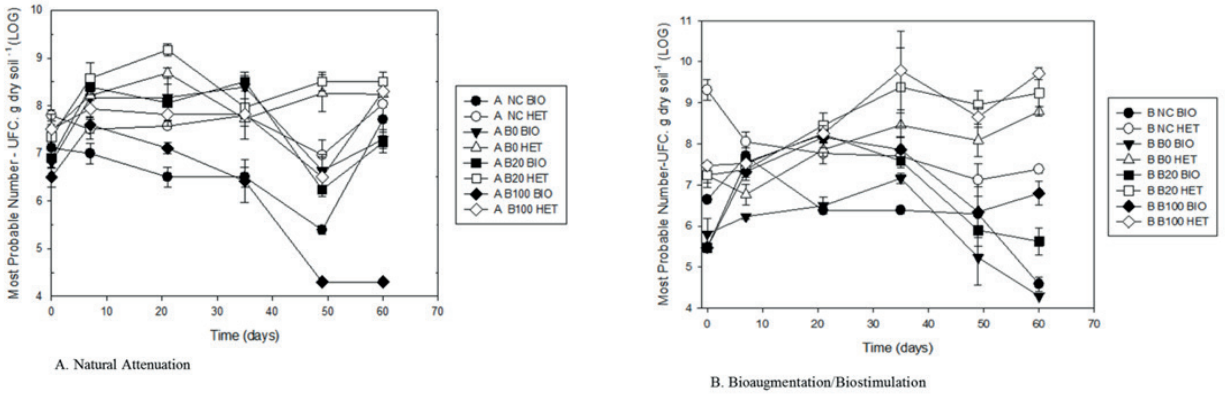

Figure 3 - Comparison of degrading (BIO) and heterotrophic (HET) microorganism counts in an ultisol contaminated by different mixtures of diesel/biodiesel (B0, B20 and B100) in two bioremediation strategies: natural attenuation (A) and bioaugmentation/biostimulation (B) during 60 days of incubation. A NC (A: natural attenuation, NC: control treatment without contamination). B NC (B: bioaugmentation/biostimulation, NC: control treatment without contamination). B0: pure diesel; B20: 20% biodiesel added to diesel; B100: pure biodiesel.

weight biosurfactants, associated with cell growth, were positive tools in order to elect four potentially degrading bacteria for the B0, B20 and B100 mixtures, all isolated from a landfarming cell located in a petrochemical industry in southern Brazil. Thus, according to the partial sequencing of the 16S region of ribosomal RNA, it was possible to identify the following isolates: *Bacillus megaterium* 1, *Bacillus pumilus* 2, *Pseudomonas aeruginosa* 5 and *Stenotrophomonas maltophilia* 6 (Meyer et al. 2012). It was found that these genera selected for the degrading consortium showed an intense relationship with the biodegradation processes of the fractions present in the diesel/biodiesel mixtures, according to the literature data published in the last decades (Atlas 1981, Leahy and Colwell 1990, Jacques et al. 2005, 2007, Chao et al. 2010, Thavasi et al. 2011, Maddela et al. 2016).

The bioaugmentation has no obvious limitations as to microbial degradation. However, this strategy may present flaws (Thompson et al. 2005). Considering the microbial ecology,

the selection of strains comes based on one key criterion in order to enable microorganisms to degrade, without regard to the potential of these strains in actively proliferating in different places where they were applied. Thus, as shown in some studies, the bioaugmentation with exogenous microorganisms usually ends up being effective only in the first days of incubation, due to the difficult task of adapting to the physical, chemical and biological characteristics of soil (Schwartz and Scow 2001, Bento et al. 2005, McKew et al. 2007).

Despite the inefficiency that exogenous microbial consortia may generate in relation to the degradation of diesel/biodiesel mixtures, since strains isolated from a soil can not necessarily be applied on another contaminated site because of the great complexity of these systems (Hosokawa et al. 2009), one should consider the possibility of investing in microbial consortia banks with degrading potential. This alternative may increase the chances of degradation success in areas contaminated by fuel, since the time required for the isolation and enrichment of microorganisms that are reintroduced in the impacted same location (autochthonous) is not always feasible in practice.

Thus, when organizing a database with detailed records of the physical, chemical and biological properties of impacted soil in which they were obtained, the chances of choosing a consortium or a bacterial strains mix compatible with the soil of interest to be decontaminated may be higher. Adding the inoculum several times throughout the incubation instead of a single application at the beginning can also contribute to the success of the exogenous population category and, therefore, increasing degradation of diesel/biodiesel mixtures in the soil, avoiding abrupt drops in population structure, caused mainly by competition from indigenous microorganisms and predation by protozoa (Schwartz and Scow 2001).

CONCLUSIONS

The goal of bioremediation was achieved in an ultisol submitted to surface contamination by B0, B20 and B100. Natural attenuation showed high biodegradation rates for the B0 and B20 mixtures, whereas the previously selected bacterial consortium, combined with the addition of nutrients, was effective to accelerate the biodegradation of pure biodiesel (B100). From the degrading counting and dehydrogenase activity, it was possible to suggest that the inoculated bacterial consortium may have remained in the system along the incubation period, contributing to greater B100 biodegradability.

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