
11 Culturable Microbiome and Biodegradation Activity of Diesel B5 and Biodiesel (B100) in a Contaminated Soil Bioremediation Study

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11.1 INTRODUCTION

11.1.1 BIODIESEL IN THE ENERGY LANDSCAPE AND ITS ENVIRONMENTAL FATE

The global impacts due to environmental pollution have driven the development of technologies towards substitution of petroleum in the energy landscape, with the focus on renewable sources (Vianna 2006). Some of the most promising alternatives are fuels obtained from plant biomass, such as biodiesel (Rathman et al. 2005).

This fuel, basically composed of fatty acid methyl esters (FAME) mainly produced by vegetable oils or animal fats transesterification, has given rise to environmental advantages, such as cleaner burning, higher biodegradability potential and lower environmental toxicity (Suarez et al. 2007). Therefore, it emerges as a potential substitute for mineral diesel, in principle partially through its inclusion in blends (diesel/biodiesel), called diesel B. The biodiesel percentage is expressed, for example, as diesel B5 (95% diesel and 5% biodiesel) up to B100 (100% biodiesel).

Many countries have presented public incentives for the production and use of biodiesel. The main world program is in the European Union (EU), the largest consumer and producer, with rapeseed as the main source (68%). Studies have shown that the final aim of these countries is to achieve blends with 20% biodiesel by 2020 (Sorda et al. 2010; Klessmann et al. 2011).

Thereby, biodiesel and its blends with diesel are subject to the same kind of accidents commonly associated with the total petroleum hydrocarbons (TPH) industry, which can compromise the environment, through soil and water pollution. In contrast to diesel, biodiesel, as a low structural complexity compound, has been identified as more susceptible to degradation by microbial action (Fernández-Alvarez et al. 2007). However, further studies are needed for a satisfactory understanding of the environmental fate of leaks or spills of pure biodiesel, as well as of diesel/biodiesel blends, at different concentrations.

11.1.2 (Bio)DIESEL BIOREMEDIATION

Several technologies are available for remediation of contaminated soils by spills or fuel leaks toward reduction of concentration and/or toxicity contaminants reduction (USEPA 2012). An efficient alternative suggested for the remediation of organic pollutants in soils is the use of bioremediation techniques. The treatment of contaminated areas using biological processes can present lower cost and

equivalent efficiency to physicochemical methods, besides being environmentally safe (Khan et al. 2004).

Bioremediation is the degradation of organic pollutants soil, water or sediment by the microorganisms catabolism of complex molecules, either by mineralizing them or by transforming them into less toxic compounds (Alexander 1999; Crawford and Crawford 1996). Depending on the microorganisms involved in the process and the environmental conditions in the contaminated site, bioremediation may occur under aerobic, anaerobic or anoxic conditions. In general, the aerobic treatment is more conducive to the contaminant mineralization and reduces the degradation time (Österreicher-Cunha et al. 2012). However, for the fuels studied in this chapter, additional information is still needed for the effective application of this process, especially in relation to the microbial populations involved, biodegradation time and favorable environmental conditions.

Autochthonous microbial communities can conduct intrinsic bioremediation (IB), which can promote the removal or reduction of contaminants naturally by the action of microbial metabolism associated with other spontaneous physico-chemical routes (Singh et al. 2009). Filamentous fungi and, mainly, bacteria (due to faster growth and enzymatic complex system) are more efficient for contaminants degradation. Not only the microbial diversity, but also the population density and the contaminated site conditions are key points to the success of biodegradation. In addition, there is no doubt about the importance of understanding the microbial community interactions into the contaminated site during the process (Dragun 1998; Watanabe 2001; Singh et al. 2006; Li et al. 2008; Muyzer and Stams 2008).

Diesel B bioremediation features in soil, including the optimum nutritional and environmental conditions for biodegradation, as well as an extensive microbial description and dynamics (bacteria and fungi) are not yet fully established. For example, some studies conducted in soil laboratory micro/mesocosms suggest an increase in the biodegradation of blends due to biodiesel addition. However, they diverge about the influence of biodiesel concentration, microbial diversity, its biomass density throughout the process and soil characterization (Junior et al. 2009; Horel and Schiewer 2011; Silva et al. 2012).

11.2 OBJECTIVES AND STUDY DESIGN

This chapter discusses the characterization of *ex situ* (bio)degradation of FAME components of biodiesel, as well as TPH associated with FAME in the B5 blend, at different soil depths. Parameters related to the reduction of the contaminant concentration and the culturable degrader microbiota dynamics (fungi and bacteria) present throughout the test will be examined. The NA bioremediation efficiency will be evaluated by the remaining ecotoxicity degree in the contaminated soil at the end of the incubation period.

The aim is to contribute to a better understanding of the participation of the native microbiota in terrestrial ecosystems contaminated by diesel/biodiesel blends and to advance bioremediation action strategies.

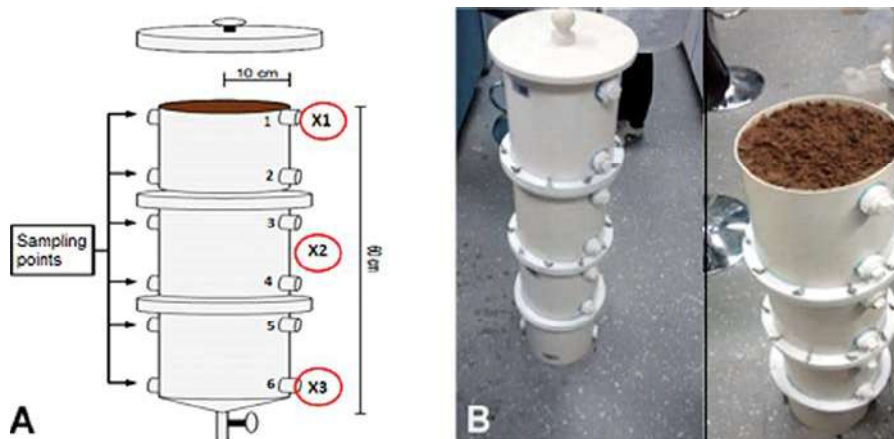


FIGURE 11.1 Microcosm experiments in PVC reactor columns. This system is composed of three cylindrical parts (cells) with 10 cm radius and 20 cm height each, placed on top of each other and allowing contact and fluid flow between adjacent compartments. Each cell is provided with four lateral openings, allowing samplings at six different depths. Samples were taken at three points, as indicated in the diagram: X1 (5 cm), X2 (30 cm, sample composed of points 3 and 4) and X3 (60 cm) (A). Incubation conditions: 21 kg clay loam soil, 20% humidity, 10% (v/m) contaminant concentration, outer temperature: room (25–28°C), incubation time: 60 days. Material previously mixed with microcosm assembly.

The bioremediation assays discussed herein were conducted in polyvinyl chloride (PVC) reactors as detailed in Figure 11.1. The soil used was from an urban area with no history of previous contamination. Granulometric, chemical and physico-chemical analyses classified the soil as clay loam and with conditions compatible with microbial activity. Fuels used as contaminants, biodiesel (B100) and diesel (B0), were donated by a distribution center for gas stations in the metropolitan region of Rio de Janeiro, Brazil. The diesel/biodiesel blend (B5) was produced in the laboratory.

Experimental tests and controls (abiotic degradation determination) are described in Table 11.1, which presents the substrate components of each microcosm. The

TABLE 11.1
Laboratory Microcosms

Assay	Contaminant	Description
EXP B5	B5	Soil contaminated by B5 blend (10% (v/w) contamination) <i>in natura</i>
EXP B100	B100	Soil contaminated by B100 blend (10% (v/w) contamination) <i>in natura</i>
CS B5	B5	Sterile soil contaminated by B5 blend* (10% (v/w) contamination)
CS B100	B100	Sterile soil contaminated by B5 blend* (10% (v/w) contamination)

*Soil sterilization: Three times autoclaving and 2% (v/w) formaldehyde. Fuel sterilization: 0.45 μ m filtration.

operational conditions were established so that they were compatible with the microbial activity and the contaminant percolation.

Each microcosm was assembled with the total amount of soil (sterile or contaminated), previously humidified. The test was set up to provide the least possible disturbance to the system during the bioremediation period. At the beginning and monthly during the treatment, the soils were analyzed for pH and moisture, always verifying the need to adjust the water content to 20% (v/m). The outer temperature was measured every two days, and the soil temperature at the beginning and after 30 and 60 days. No other intervention was performed throughout the experiments.

11.3 ANALYTICAL METHODOLOGY

During the experimental period (60 days), the soil samples were analyzed as summarized in Table 11.2. It is possible to observe the variation on the periodicity of the tests, whereas the ecotoxicity evaluation was carried out only at the end of the bioremediation assay.

11.3.1 QUANTIFICATION OF CULTURABLE MICROBIOME

In order to estimate the culturable microbiome biomass (bacteria and fungi), sample suspensions (pristine soil, fuels and contaminated soil) were obtained using saline solution (0.85% m/v) plus 0.1% of Tween™, homogenized at 300 rpm for 30 minutes. After obtaining this suspension, serial dilutions (1:10) were performed for cultivation assays.

TABLE 11.2
Methodological Analysis Applied to Monitored Natural Attenuation (MNA)
Bioremediation Assays

Parameter	Methodology	Objective	Period (days)
Culturable Bacteria	Aerobic and Anaerobic MPN	(An)aerobic biomass quantification of culturable heterotrophic bacteria	0/30/60
	<i>Spread plate cultivation</i>	Prevalent bacterial groups isolation–5 cm depth	
	16S rRNA gene sequencing of isolated cultures	Genera identification of putatively bacteria-biodegrader	0/30/60
	Mixed cultures <i>Denaturing Gradient Gel Eletrophoresis</i> (DGGE)	Community molecular profiles evaluation	0/60
Culturable Fungi	<i>Spread plate cultivation</i>	Biomass quantification and fungi/yeast isolation	0/30/60
	<i>ITS</i> gene sequencing of isolated cultures	Genera/species identification of putative biodegrader fungi	
Fuel Concentration	Gas Chromatography – Flame Ionization Detector	Fuel (bio)degradation assesment	0/60
Soil Toxicity	<i>Eisenia fetida</i> mortality tests	Soil (bio)remediation outcome	0/60

Biomass quantifications of culturable heterotrophic bacteria populations were estimated by Most Probable Number (MPN) technique in tubes with nutrient broth.¹ A similar method was employed to measure the bacterial density of culturable heterotrophic anaerobic biomass. For this, serial dilutions (1:10) were obtained in reducing solution² and the cultivation was performed in Fluid Thioglycollate Medium (SIGMATM), using sealed flasks. The anaerobic environment was maintained by N₂ purge. Incubation temperature was 30°C and the microbial growth verified after 48 hours. The results were expressed in MPN per gram of soil.

Fungal biomass estimation was carried out by direct counting of spread plate cultivation on Sabouraud medium (OXOIDTM), with chloramphenicol addition (SIGMATM), using 0.1 mL of the initial suspension and serial dilutions up to 10⁻⁵. Incubation was performed at 30°C for 7 to 14 days. After that period, the total and morphologically distinct and prevalent colony counts were performed, and the results expressed in terms of colony forming units (CFU) per gram of dry soil. Phenotypically distinct colonies were isolated in specific media, including Malt Extract Agar (MEA – OXOIDTM), Dextrose Potato Agar (BDA – OXOIDTM) and Czapek Yeast Extract Agar (CYA – Santos et al. 1998). Afterwards, microcultures were performed on slides for identification of characteristic structures of fungal genera. In relation to yeasts, only presence/absence and their identification were verified.

11.3.2 IDENTIFICATION OF BACTERIA AND FUNGI POPULATIONS

Prevalent aerobic bacterial group isolation on microcosm surfaces (5 cm) after 30 and 60 days and in pristine soil was carried out cultivating 1 mL of each serial dilution by spread plate on PCA (Plate Count Agar, HimediaTM), under the same incubation conditions as mentioned above.

After that, DNA extraction from isolated colonies was performed by thermal lysis: 10 sec inside liquid nitrogen, followed by 10 min 60°C dry bath at 3 cycles.

Molecular identification of the isolated microorganisms was followed by gene ribosomal 16S gene subunit (16S rRNA) amplification, approximately 1500 bp. Standard PCR (Thermocycler, Applied Biosystems) was employed PCR (Thermocycler PCR System 9700, Applied Biosystems) and Taq PCR Master Mix Kit (Qiagen) plus 0.2 µM of specific primers, Sadir (forward: 5'-AGAGTTTGATCATGGCTCAGA-3') and S17 (reverse: 5'-GTTACCTTGTTACGACTT-3'), following the cycling conditions: 94°C – 4 min; 94°C – 30 sec; 50°C – 30 sec; 72°C – 30 sec (30 cycles); 72°C – 5 min (Altschul et al. 1990).

DNA extraction from isolated fungi was performed using DNeasy Power Soil KitTM (Qiagen). The identification was followed by amplification of the gene encoding the 5.8S subunit ribosomal RNA and the ITS I and II regions (Internal Transcribed Spacers), fragment of approximately 600 bp. This procedure followed similar conditions as previously described, differing in the cycling conditions (94°C – 5 min; 94°C – 30 sec; 50°C – 30 sec; 72°C – 30 sec (30 cycles); 72°C – 30 sec), as well as in the primers employed: ITS5 forward 5'-GGAAGTAAAAGTCGTAACAAGG-3' and ITS4 (reverse) 5'-TCCTCCGCTTATTGATATGC-3' (White et al. 1990).

The amplicons were submitted for sequencing using BigDye Terminator v3.1 Cycle Sequencing StandardTM and using an automatic sequencer (AB 3500 Genetic Analyzer, Applied Biosystems). The final sequences, obtained after editing using Chromas LiteTM

and Bioedit softwares, were submitted to similarity comparison using BLAST and GenBank online (www.ncbi.nlm.nih.gov). The analysis provided the gene fragment identification (genus or species), considering a minimum of 98% of similarity.

11.3.3 MOLECULAR CHARACTERIZATION OF CULTURABLE HETEROTROPHIC BACTERIAL COMMUNITY

Molecular characterization by DGGE used DNA extracted by thermal lysis (aforementioned) from aerobic and anaerobic liquid cultivation of pristine soil and after 60 days of bioremediation, at the three depths and in both microcosms (EXPB100 and EXPB5). The methodology was adapted from Muyzer and colleagues (1993), involving 16S ribosomal RNA subunit specific amplification. However, with insertion of a high GC “clamp” (5 min CGCCGCGCGCGCGGCGGGCGGGGCGGGG-CACGGGGG-3’) added to the 5 min portion of the U968 primer (forward: 5’-GCACGGGGGACGCGAAGAACCTTAC-3’), in addition to the other primer L1401 (reverse: 5’-GCGTGTGTACAAGACCC-3’). The cycling conditions used were 94°C – 3 min; 94°C – 30 sec; 50°C – 30 sec; 72°C – 30 sec (30 cycles); 72°C – 30 sec.

The amplified products were subjected to 6% polyacrylamide gel (acrylamide and bis-acrylamide) electrophoresis in a tris-acetate buffer (pH 8.3), with a denaturing gradient (urea and formamide, 40% to 60%), according to Aboim et al. (2004). Dendrograms were constructed based on the Jaccard similarity coefficient using Bionumerics v 6.0 (Applied Maths) software. Statistical analysis of non-metric multidimensional scaling (NMS) was also performed using online software “PAleontological STatistics - version 2.16”.

11.3.4 FUEL CONCENTRATION ANALYSIS

The soil contaminants were extracted using an adapted protocol from Schwartz, Ben-Dor and Eshel (2012) and USEPA 3550C (2007). In summary, 1 gram of soil was resuspended in n-hexane:acetone solution (50:50 v/v) followed by an ultrasonic bath for 60 min. After this, the final supernatant was filtered through Nylon™ membrane for injection in the gas chromatograph.

Chromatographic runs for TPH detection followed adapted protocols described in Weisman (1998), Petit (2004) and Institut Scientifique de Service Public (2010). Briefly, an extract aliquot was injected into a gas chromatograph (HP 5890 Series II, Hewlett-Packard™) equipped with a flame ionization detector (FID). The column used was a DB-5 (30 m by 0.25 mm) with hydrogen carrier gas at a flow rate of 20 mL min⁻¹ and air at a flow of 310 mL/min⁻¹. The temperature program used was 40°C 2 min⁻¹, 150°C 10 min⁻¹, 270°C 20 min⁻¹.

The FAME analysis was carried out in a gas chromatograph (ThermoQuest, ThermoFinnigan™) also equipped with a FID, based on methodology adapted from McCurry (2012), Huppel and Huybrighs (2012), Grama et al. (2014) and Standard EN14105:2011. It used a column Rt – 2560 (100 m by 0.25 mm) with helium carrier gas at a flow rate of 35 mL min⁻¹ and air at a flow of 350 mL min⁻¹. The temperature program applied was 80°C 25 min⁻¹, 175°C 10 min⁻¹, 205°C 10 min⁻¹; 225°C 20 min⁻¹.

The final chromatograms were analyzed according to molecular standards (SIGMA) of aliphatic hydrocarbons contained in the c8-c24 range, for TPH analyses;

and for EMAG, esters were used: c17: 0 (Marginic Acid), c16: 0 (Palmitic Acid), c18: 0 (Stearic Acid), c18: 1 (Oleic Acid), c1 (Linoleic acid) and c1 3 (α -Linolenic acid).

11.3.5 ECOTOXICITY EVALUATION

Ecotoxicity analyses were conducted in accordance with standard NBR 15.537 (ABNT, 2007), in triplicate and over a period of 14 days. The NA bioremediation efficiency was verified by comparing the following trials: pristine soil, B5 or B100 (10% m/v contamination) contaminated soil and B5 or B100 bioremediated soils, from the superficial and deepest cells of both microcosms.

11.4 RESULTS AND DISCUSSION

11.4.1 (BIO)DEGRADATION PROFILE OF TPH

The diesel B5 and B100 biodegradation in artificially contaminated clay loam soil was determined by estimating the total reduction percentages by discounting the abiotic ones obtained in the control microcosms. Figure 11.2 shows the percentage values of TPH contaminant reduction discriminated for abiotic control and total degradation (experimental reactor), with the difference attributed to biodegradation.

It was observed that the bioremediation process was only effective at the intermediate depth (30 cm), with the microorganisms contributing to the hydrocarbon content reduction by only around 10%. This rate could be due to the retention of compounds of higher molecular mass and great recalcitrance on the reactor surface. In this way, intermediate (partially degraded) compounds and other fractions with lower molecular mass would be percolated, increasing the concentration of more easily biodegradable compounds in such depth. It could also be considered that possibly the oxygen availability is still satisfactory for aerobic biodegradation up to a depth of 30 cm.

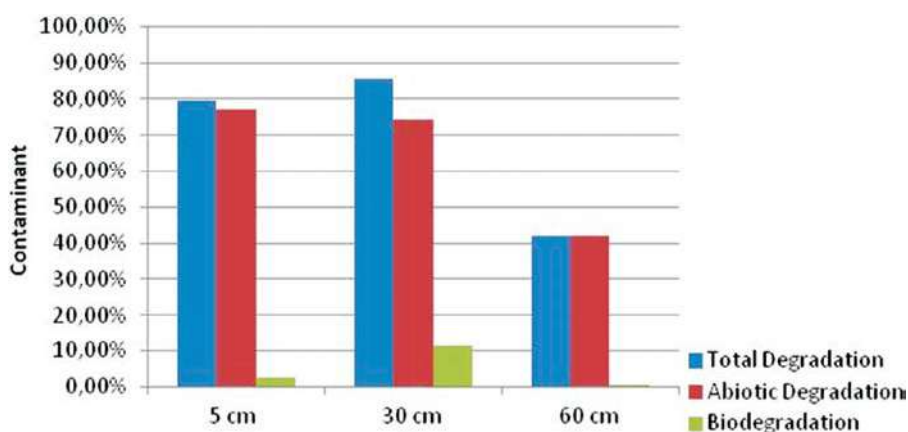


FIGURE 11.2 Biodegradation profile of the TPH fraction determined by the comparison between the decrease in contaminant levels observed in EXP B5 and CS B5 after 60 days of IB, at three different depths.

The rate described here can be considered low in relation to previous studies. Li et al. (2007) and Boopathy (2004) found that the natural biodegradation of pure diesel was around 20% and 4%, in studies with similar characteristics to those presented here, both for aerobic and anaerobic conditions. Additionally, considering the incubation time, it is quite plausible that biodegradation occurred only for less recalcitrant compounds in B5. In fact, in bioremediation assays by biopiles, Chemlal et al. (2013) described that the catabolism sequence of diesel starts with the simpler alkanes and ends with the aromatics. The TPH evaluated in this study were introduced in blends with biodiesel, i.e., with more easily biodegradable molecules. According to Prince et al. (2008), studying the B20 biodegradation, FAME present in the blend are readily degraded, that is, their catabolism precedes that of the simpler alkanes. It is supposed that FAME structures enable attack by various enzymes, which result in the oxidation of the molecules into acetic acid and other fatty acids. The latter, through a series of reactions, can be completely mineralized to CO_2 and H_2O (Jakeria et al. 2014).

11.4.2 (Bio)DEGRADATION PROFILE OF FAME

The FAME biodegradation percentages after 60 days of NA can be observed in Figure 11.3. It shows FAME (bio)degradation values for soil contaminated by B5 (see Figure 11.3A) and B100 (see Figure 11.3B), at the three depths.

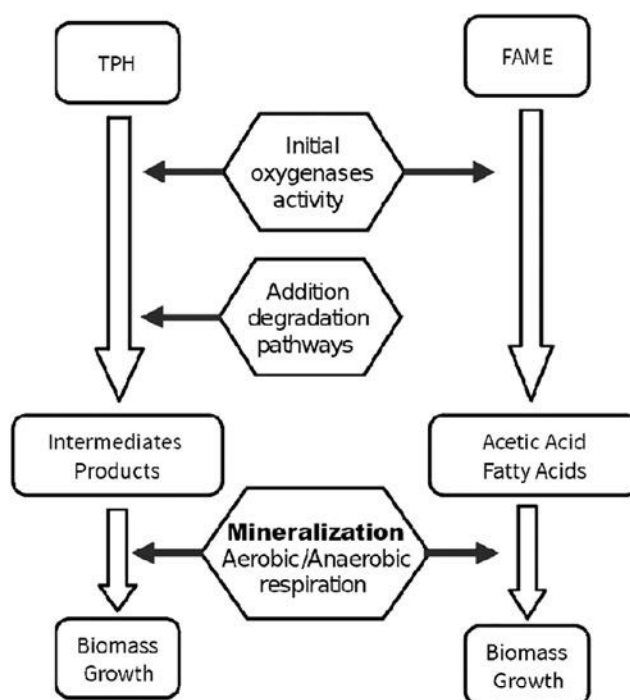


FIGURE 11.3 Relationship between suggested biodegradation pathways for diesel/biodiesel blends in impacted soil.

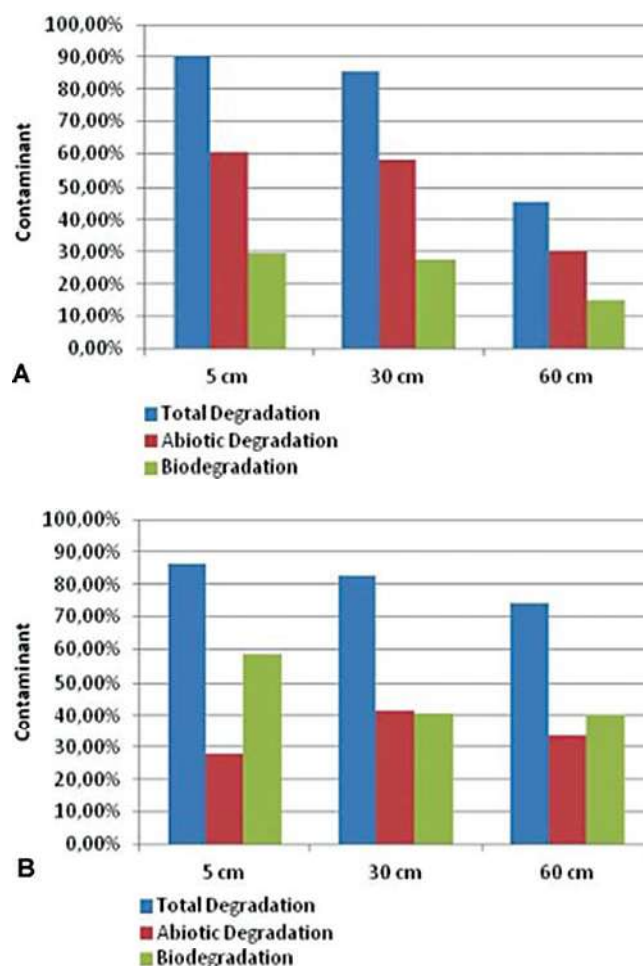


FIGURE 11.4 FAME fraction (bio)degradation profile was determined by comparing the decreases in contaminants observed in EXP B5 and CS B5 (A) and in EXP B100 and CS B100 (B) after 60 days of IB, at three different depths.

In this way, [Figure 11.3A](#) suggests the influence of column depth on the efficiency of FAME bioremediation (in B5 blend), especially when comparing rates at 5/30 cm (30%) to rates at 60 cm (15%). However, [Figure 11.3B](#) shows that FAME biodegradation was more effective in pure biodiesel (B100) than in B5 blend. Soil contamination by B100 presented biodegradation percentages of approximately 60% (5 cm) and 40% (30 and 60 cm).

FAME biodegradation occurred up to 60 cm in depth in both microcosms. However, higher bioremediation rates were observed in the soil surface horizon and, therefore, in the region where probably there is greater oxygen availability.

The (bio)degradation profiles indicate reduction in the bioremediation efficiency in soil contaminated by FAME and TPH concomitantly, when compared to B100 and

with literature data for diesel biodegradation. This decrease could be a consequence of similarities between the metabolic biodegradation pathways of hydrocarbon and FAME in a blend, mainly for the initial stages of aerobic metabolism (Jakeria et al. 2014), as shown in Figure 11.4. Based on this model, both co-stimulation—due to abundance of substrates and common intermediates as well as competition could be expected between the metabolic pathways.

The results presented here agree with some data previously described in literature, which demonstrate that FAME are primarily metabolized by microorganisms in blends with diesel, aerobically and/or anaerobically. However, these studies have described an increase in general fuel biodegradation with no influence on the TPH metabolism (Prince 2008; Owsianiak et al. 2009; Cyplick et al. 2011; Wu et al. 2015).

11.5 CULTURABLE HETEROTROPHIC BACTERIA (CHB)—BIOMASS DENSITY AND MOLECULAR COMMUNITY STRUCTURE BY DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

Figure 11.5 shows the culturable heterotrophic bacteria (CHB) levels. It is possible to observe that the cell concentrations were equal to or higher than the values detected in pristine soil, in EXPB5 (see Figure 11.5 A/C) and EXPB100 (see Figure 11.5 B/D), even in aerobic or anaerobic cultivation. The biomass evaluation in EXPB5 (see Figure 11.5 A/C) suggests that the fuel catabolism had intensified after 30 days, probably after selection and acclimatization of native biodegrader populations. The

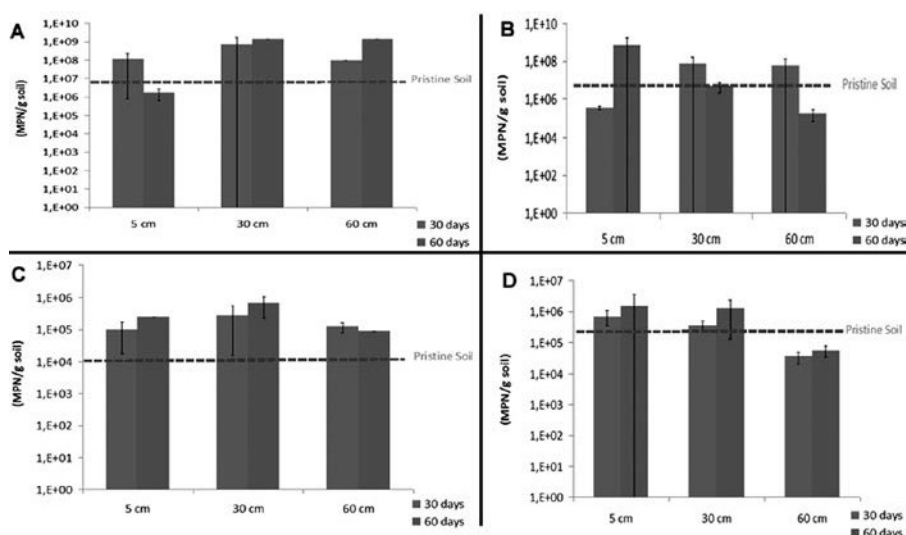


FIGURE 11.5 Culturable aerobic/anaerobic heterotrophic bacteria biomass from samples collected at EXPB5 (11.5 A/C) and EXPB100 (11.5 B/D) microcosms, as a function of depths and times. [Dashed line: cell concentration in the pristine soil.]

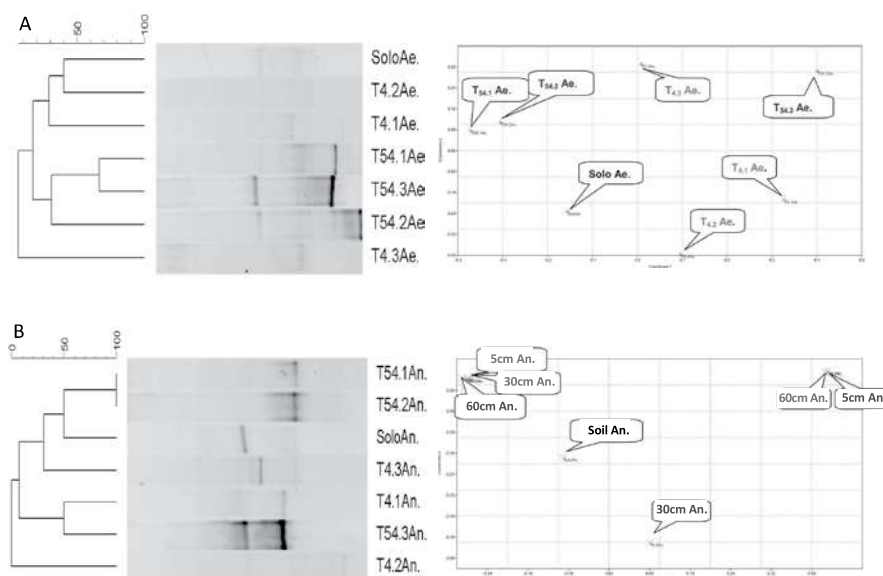


FIGURE 11.6 DNA fingerprinting by Denaturing Gradient Gel Electrophoresis (DGGE) followed by “Multidimensional Scaling” of culturable bacterial communities under aerobic (A) and anaerobic conditions (B), after 60 days. Blue – EXP B100 samples and Red – EXP B5 samples.

results of the cell growth agree with those verified in the biodegradation (TPH and FAME) analyses: low rates at 60 cm of reactor depth and the highest one in the intermediate zone (30 cm).

The CHB growth during B100 bioremediation is shown in [Figures 11.5 B/D](#), in aerobic and anaerobic cultures, respectively. The present data suggest populations are established quite early in this system and likely are influenced by aeration. It is possible to observe that after 30 days, aerobic growth decreases inversely to the anaerobic cultivation, starting from a depth of 30 cm. In addition, the highest values were observed in the superficial layer (Figure 11.5 B/D), which is consistent with the high FAME biodegradation at this site (around 60%). This dataset suggests that biodiesel biodegradation probably resulted in shorter periods of bioremediation.

11.6 CULTURABLE HETEROTROPHIC BACTERIA (CHB)— MOLECULAR COMMUNITY STRUCTURE BY DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

Molecular biology techniques have been used to better characterize communities involved in the bioremediation of biodiesel and blends (Meyer et al. 2014; Soriano et al. 2015). Figure 11.6 analyzes the molecular community structure of the putative

culturable biodegrader populations after 60 days of treatment. The molecular profiles by DGGE, dendrograms and Non-Metric Multidimensional Scaling (NMS) plots were obtained from pristine soil, EXPB5 and EXPB100, cultivated under aerobic (11.6 A) and anaerobic (11.6 B) conditions.

The data strongly contribute to the hypothesis of differential community structure, as consequence primarily of the fuel inserted in the microcosm. In relation to B5, that consists mainly of hydrocarbons, a distinct bacterial community is observed compared to that established for B100 contaminated soil. It is important to consider the toxic effect of TPH on the native bacteria, as well as the fact that biodiesel is subject to an easier catabolism, which allows the appearance of greater microbial diversity along the column. [Figure 11.6](#) also suggests that the presence of different contaminants is a selection factor for bacterial populations more relevant than depth, although it is possible to infer that this latter factor can contribute to the community structuring with greater influence for B100 than for B5.

11.6.1 CULTURABLE HETEROTROPHIC BACTERIA—DIVERSITY AT THE MICROCOSM SURFACE

As previously observed, the reactor surfaces (5 cm) presented the highest biodegradation rates for FAME. This fact in association with adequate biomass growth curves for CHB could suggest autochthonous bioremediation activity. In this way, the present work also sought to identify some of the prevalent bacterial genera that are putative biodegraders at this depth.

Nine bacterial genera were isolated among all the substrates after 60 days. The prevalence of *Arthrobacter*, *Bacillus* and *Micrococcus* genera in the pristine soil could be noticed. After changes in the microenvironments caused by the fuels biodegradation, diversification of the microbial populations prevalent in microcosms could be observed. After 30 days, in the EXP B5 *Agromyces* sp., *Lysobacter* sp., *Sinomonas* sp. and *Arthrobacter* sp. (lasting up to 60 days) were observed, while in the EXP B100, *Bacillus* sp. and *Burkholderia* sp., appeared by the end of the incubation.

In relation to the populations described in EXPB5, it is worth highlighting those belonging to the genus *Arthrobacter*, which is widely cited in the literature for its participation in the biodegradation of hydrocarbons and derivatives (including diesel B) in different environments and laboratory assays (Prince 2005; Chen et al. 2015; Soriano et al. 2015). According to Paria (2008), the biodegradation efficiency of several recalcitrant compounds by *Arthrobacter* species is due to their capacity to produce different classes of biosurfactants. In this same way, the biodegradation activity of the genera isolated in the EXP B100 have been mostly described for hydrocarbons, as the metabolism of aliphatics has been attributed to *Bacillus* sp. (Van Beilen and Withold 2005; Chen et al. 2015), that of PAHs to *Burkholderia* sp. (Haritash and Kaushis 2009; Chen et al. 2015; Keskin et al. 2015), since data on the biodegradation of biodiesel are still scarce.

11.7 CULTURABLE MICROBIOME OF FUNGI

Fungi are also widely present in soils; therefore they are fundamental for the microbiota characterization during the TPH and FAME biodegradation process. Figure 11.7 shows the fungal biomass variation in the microcosms EXP B5 (see Figure 11.7 A) and EXP B100 (see Figure 11.7 B), respectively. The values related to the concentrations of fungi determined in the pristine soil and in those contaminated with fuels (diesel and biodiesel) are also indicated. Contaminant species from fuels have a common characteristic of robustness, given their ability to survive in an adverse environment, and possibly by the

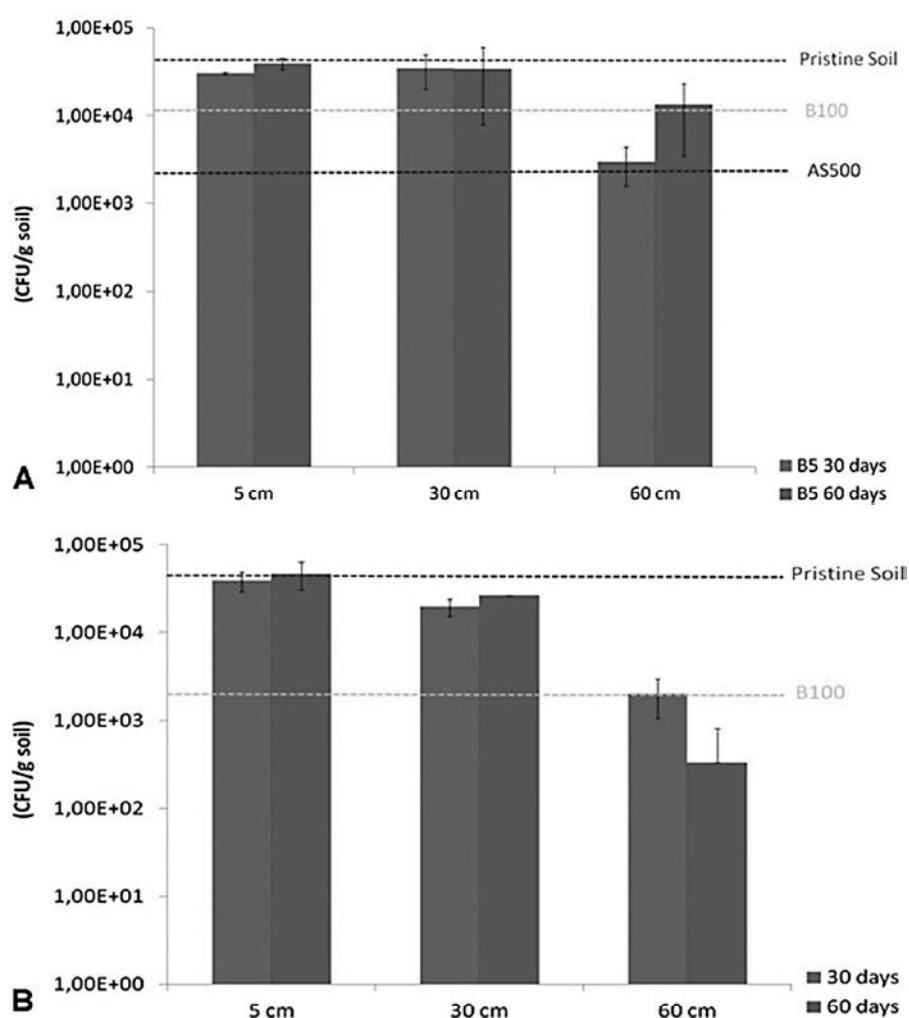


FIGURE 11.7 Quantification of culturable mycelial fungi, (A) EXP B5 and (B) EXP B100.

capacity to use these compounds as carbon sources. Therefore, besides the soil, the fungal microbiota of each microcosm may have had its origin in the fuels.

The fungi density in both microcosms remained, in general, around 10^4 CFU/g of soil during the experimental period, both in the superficial layer (5 cm) and in the intermediate layer (30 cm). Considering that this is the density of fungi in the pristine soil, it could be suggested that they participated in the fuels' degradation. However, in the deepest soil layer (60 cm), there was a decrease in the fungal population, most significantly in EXPB100 (Figure 11.7 B). This could be possibly due to the influence of oxygen availability to the fungal microbiota growth, which could limit its overall metabolic activity. Most mycelial fungi are obligate aerobes so they could indirectly indicate the soil oxygenation profile.

The prevalent fungi in the EXP B5 and EXP B100 microcosms at different depths (5 cm, 30 cm and 60 cm) and treatment times (30 and 60 days) are representative of the *Ascomycota* (mostly) and *Basidiomycota* phyla. The first phylum populations were present mainly in the pristine soil and diesel, remaining in higher proportions in the reactors. However, the main source of the *Basidiomycota* in the experimental microcosms was the B100 itself (data not shown).

Regarding identification, 19 genera of culturable fungi in pristine soil, fuels and microcosms were characterized. Through general data analyses of both microcosms, it was possible to verify that the main genus participants and, possibly, with highest catabolism capacity of contaminants, were *Fusarium* sp. and *Talaromyces* sp. These were the most prevalent, both in 30 and 60 days, in the different depths studied.

Particularly regarding the EXP B5 microcosm, it was observed that an initial effective selection of populations remains stable until the end of the assay. This means that the stable structure of the fungal community occurs early in the assay. From 30 days onwards, it is possible to determine the genera resistant to the toxic action of hydrocarbons and possibly responsible for TPH and FAME biodegradation present in the blend. In this context, the presence of *Fusarium* sp., *Penicillium* sp. and *Talaromyces* sp. populations in the superficial layers can be highlighted.

In the B100 bioremediation it was possible to observe an exclusive dynamics of fungal genera. It can be suggested that there was no stabilization of the local fungal community throughout the period of 60 days. The most prominent genera were *Aspergillus*, *Fusarium* and *Talaromyces*, originating from the soil; and *Phanerochaete* only initially detected in fuels (diesel and B100). It can be suggested that biodiesel, being less toxic and more easily metabolized than diesel, allowed the establishment of a greater fungal diversity culturable during the bioremediation.

According to Harms, Schlosser and Wick (2011), most xenobiotic degrading fungi belong to the *Ascomycota* and *Basidiomycota* phyla. Among the genera known as degraders of aliphatic hydrocarbons, polyaromatic hydrocarbons, chlorophenols and pesticides, are *Acremonium*, *Fusarium*, *Penicillium* and *Phanaerochaete* (Chang 2008; Harms et al. 2011). The information about the biodiesel degrading fungal genera, presented here, is still scarce and more studies in this direction are extremely necessary.

Figure 11.8 shows the most prevalent fungal species observed in the microcosms. In total, 36 species were isolated and identified, the putative biodegrader species originating mainly from the soil. The greatest diversity was verified for the genera *Aspergillus*, *Talaromyces*, *Penicillium*, *Fusarium* and *Cladosporium*.

Fungal Species		Microcosms
<i>Fusarium solani</i>		EXPB100 / EXPB5
		
<i>Talaromyces pinophilus</i>		EXPB100 / EXPB5
		
<i>Penicillium citrinum</i>		EXPB5
		
<i>Cladosporium perangustum</i>		EXPB100
		
<i>Phanerochaete magnoliae</i>		EXPB100
		

FIGURE 11.8 Most frequently fungal species isolated after 60 days of assay.

Each microcosm revealed distinct profiles. The most frequently isolated species were *Fusarium solani* and *Talaromyces pinophilus*, suggesting they are the main biodegraders in the systems analyzed. These species were observed acting from the superficial to the deepest soil layers, where the fuels were detected in the highest concentration. *F. solani* is highlighted in the literature because it has biodegradative activity for different hydrocarbons, alone or in consortium (Veignie et al. 2004; Rafin et al. 2009; Wu et al. 2010; Thion et al. 2012). However, there is a lack of information about its ability to metabolize particularly pure diesel and biodiesel, as well as their blends.

Particularly in EXP B5, *Penicillium citrinum* shows a certain activity in the B5 biodegradation, having been isolated throughout the soil column. This species has already been reported as degrading hydrocarbons (Oudot et al. 1993; April et al. 2000). In relation to EXP B100, *Cladosporium perangustum* and *Phanerochaete magnoliae* were present also at the different reactor depths. *P. magnoliae* was also observed originally in B100, which suggests its importance for biodiesel biodegradation, since it appeared as a colonizer of such fuel. In fact, the literature describes that some species of this genus can express the ability to degrade xenobiotics (Prenafeta-Boldu et al. 2006; Pinedo-Rilla et al. 2009; Harms et al. 2011; Gadd 2016).

In order to complement the culturable fungal microbiome description, the presence of yeasts was verified. They were detected in all substrates used to probe the

microcosms composition (soil and fuels). In this context, it is possible to highlight the populations of the genera *Cryptococcus* and *Aureobasidium* in EXPB5. Both have already been characterized by their capacity for biodegradation of hydrocarbons either by direct metabolism or mediated by biosurfactants (Van Hamme et al. 2003; Van Beilen & Witholt 2005; Prince 2005; Corradi da Silva et al. 2006).

11.8 ECOTOXICITY ASSESSMENT

The bioremediation process reported in this chapter was a consequence of TPH and/or FAME reduction mediated by autochthonous microbial populations. However, this biodegradation of the contaminants is not necessarily related to the decrease of toxicity after treatment, mainly considering petroleum hydrocarbons (Dorn and Salanitro 2000).

In order to verify the aforementioned relationship, the degree of soil toxicity was evaluated immediately after the contamination and after 60 days of bioremediation. The model used for the ecotoxicity tests was the mortality of earthworms (*Eisenia fetida*). This organism has been found to be a very sensitive indicator to the presence of soil contamination.

Earthworm mortality rates for soil immediately after contamination and at the end of NA (60 days) were compiled in Figure 11.9 for microcosms B5 (see Figure 11.9A) and B100 (see Figure 11.9B). It can be noticed that the same test was done simultaneously with the uncontaminated pristine soil (negative control), that showed 0% mortality. Comparative analysis revealed the highest toxicity degree for TPH (see Figure 11.9A), although FAME contamination alone caused 63% mortality (see Figure 11.9B). The 14-day mortality readings show that there was a decrease in the toxicity of the contaminated soil. It must be pointed out that in EXP B100, after 60 days of bioremediation, toxicity reached values equal to those of the negative control.

11.9 FINAL REMARKS AND CONCLUSIONS

The ever-increasing participation of biodiesel in the worldwide energy landscape has made available more knowledge about the fate of this fuel, especially for diesel/biodiesel blends, in cases of environmental contamination occurrence. This chapter has demonstrated the dynamics of culturable microbial populations participating in the B5 and B100 biodegradation in artificially contaminated soil. The monitoring of

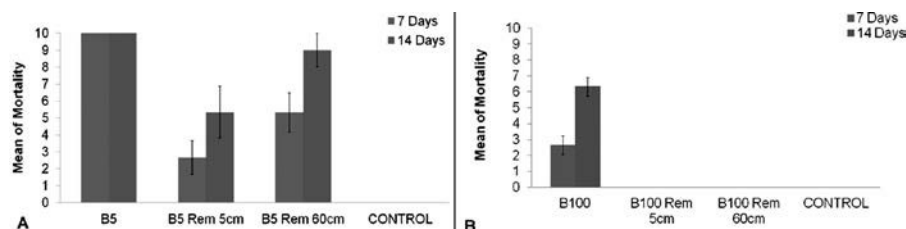


FIGURE 11.9 Ecotoxicological evaluation using *Eisenia fetida*.

the natural attenuation bioremediation was adopted as a treatment strategy in order to simulate the effect of blending petroleum hydrocarbons and fatty acid methyl esters on the autochthonous microbial community profile and in the biodegradation activity under natural conditions.

These results demonstrate the participation of yeasts and, mainly, bacteria and filamentous fungi in the NA bioremediation of contaminated soil up to 60 cm deep. The majority of the microbial species originated from the pristine soil, but the fuels also contained microorganisms that participate in the biodegradation. Effective bioremediation was observed for the contaminated soils, for pure biodiesel (B100) and low concentration blends (B5). However, the efficacy of microbial biodegradation for B100 was higher, promoting a reduction of almost 60% of the initial mass of FAME in the soil in 60 days. The presence of hydrocarbons (B5) negatively influenced FAME biodegradation, in the same way that the presence of FAME, even in low concentrations, seems to impair TPH degradation.

This observation is probably due to the differentiation of the microbial community observed in the soils as a consequence of contaminant action. Possibly these populations, even including TPH biodegraders, preferentially metabolized FAME as a carbon source, as seen in the (bio)degradation profile, which may have influenced the efficiency of the biodegradation capacity in the EXPB5 microcosm. This finding raises questions concerning previous studies that had observed an increase in biodegradation of blend contaminated systems directly related to the biodiesel addition under aerobic (Bücker et al. 2011; Lisiecki et al. 2014) and anaerobic conditions (Sørensen et al. 2011).

The data presented here are intended to assist in the monitoring and evaluation of the impacts on soils caused by contamination from biodiesel and diesel/biodiesel blends. In addition, more suitable procedures to remediate areas contaminated by such fuels can be better defined by predicting the pathways of their biotransformation in association with the microbiota involved. In this way, more improved techniques of removal of these soil contaminants, including bioaugmentation and/or biostimulation, could be applied more accurately and efficiently.

NOTES

1. Nutrient Broth: Meat peptone (10 g/L); Meat extract (3 g/L); K_2HPO_4 (1 g/L); NaCl (8.5 g/L).
2. Reducing solution: Sodium thioglycollate (0.124 g/L); Ascorbic acid (0.10 g/L); Resazurin (4.0 mL); NaCl (8.5 g/L).

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