BACTERIAL COMMUNITY DYNAMICS AND ECOTOXICOLOGICAL ASSESSMENT DURING BIOREMEDIATION OF SOILS CONTAMINATED BY BIODIESEL AND DIESEL/BIODIESEL BLENDS

G. I. MATOS*, C. S. JUNIOR, T. C. OLIVA**, D. F. SUBTIL***, L. Y. MATSUSHITA*, A. L. CHAVES****, M. T. LUTTERBACH****, E. F. SÉRVULO***, S. N. AGATHOS*****, B. STENUIT*****

*Centro Federal de Educação Tecnológica Celso Suckow da Fonseca, CEFET/RJ, Brazil
**Instituto Federal de Educação Ciência e Tecnologia do Rio de Janeiro, IFRJ, Brazil
***Universidade Federal do Rio de Janeiro, UFRJ, Brazil
****Instituto Nacional de Tecnologia, INT/MCT&I, Brazil
*****Earth & Life Institute, UCL, Belgium

ABSTRACT

The gradual introduction of biodiesel in the Brazilian energy landscape has primarily occurred through its blending with conventional petroleum diesel (e.g., B20 (20% biodiesel) and B5 (5% biodiesel) formulations). Because B20 and lower-level blends generally do not require engine modifications, their use as transportation fuel is increasing in the Brazilian distribution networks. However, the environmental fate of low-level biodiesel blends and pure biodiesel (B100) is poorly understood and the ecotoxicological-safety endpoints of biodiesel-contaminated environments are unknown. Using laboratory microcosms consisting of closed reactor columns filled with clay loam soil contaminated with pure biodiesel (EXPB100) and a low-level blend (EXPB5) (10% w/v), this study presents soil ecotoxicity assessement and dynamics of culturable heterotrophic bacteria. Most-probable-number (MPN) procedures for enumeration of bacteria, dehydrogenase assays and soil ecotoxicological tests using *Eisenia fetida* have been performed at different column depths over the course of incubation. After 60 days of incubation, the ecotoxicity of EXPB100-derived samples showed a decrease from 63% of mortality to 0% while EXPB5-derived samples exhibited a reduction from 100% to 53% and 90% on the top and at the bottom of the reactor column, respectively. The dehydrogenase activity of samples from EXPB100 and EXPB5 increased significantly compared to pristine soil after 60 days of incubation. Growth of aerobic bacterial biomass was only observed on the top of the reactor column while the anaerobic bacteria exhibited significant growth at different column depths in EXPB100 and EXPB5. These preliminary results suggest the involvement of soil indigenous microbiota in the biodegradation of biodiesel and blends. However, GC-FID analyses for quantification of fatty acid methyl esters (FAMEs) and aliphatic hydrocarbons and targeted sequencing of 16S rRNA tags using illumina platforms will provide important insights into the profiles and underlying mechanisms of (bio)diesel biodegradation in soil environments.

INTRODUCTION

Air pollution reduction policies have motivated the development of environmentally-friendly technologies to generate and use renewable energy sources, equally efficient and with lower environmental impact (e.g., bioethanol and biodiesel produced from biomass (Rathman et al., 2005)). The biodiesel has been introduced into the Brazilian energy matrix within the framework of the Brazil's Biodiesel Production and Use Programme (PNPB,December 6, 2004) and the Law No. 11.097 - Introduction of Biodiesel in the Brazilian Energy Mix, 2005) , which regulates the production and commercialization of biodiesel blends. This blend is known as Diesel-B. The percentage of biodiesel commonly used in blends ranged from 5% (v/v) (B5)

(Faria, 2007) to 7% (v/v) (i.e., lower-level biodiesel blends). Due to the ever-increasing demand of biodiesel and blends in the Brazilian transportation system, environmental sites intentionally or accidentally contaminated by biodiesel blends are frequently detected throughout the national territory.

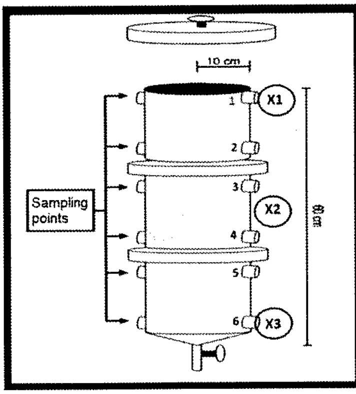
There is scant information available on potential environmental impacts of diesel/biodiesel blends when they are released into natural ecosystems. Compared to diesel, biodiesel is more likely prone to be degraded by microorganisms due to the lower complexity of its chemical structure (Fernández-Alvarez et al., 2007). However, further studies are required to obtain a comprehensive understanding of the environmental fate and ecotoxicological impacts of biodiesel and to develop bioremediation processes for mitigation of the noxious effects caused by biodiesel in natural ecosystems. The present work presents the dynamics of culturable heterothrophic bacteria (aerobic and anaerobic), as well as metabolic dehydrogenase activity and ecotoxicity level of samples collected from reactor columns filled with clay loam soil artificially contaminated by biodiesel (B100) and diesel/biodiesel mixture (B5).

MATERIAL AND METHODS

Laboratory microcosm studies and experimental design

The soil samples used for this study were collected by the Technological Research Institute (IPT) from a site not contaminated by biodiesel/diesel in São Paulo city, SP, Brazil. The soil samples were sieved with a 2 mm mesh, mixed to obtain homogenized samples, stored in plastic bags and transported to the laboratory for further processing at room temperature. The biodiesel and diesel samples were obtained from a distribution center to gas stations in the metropolitan area located in Rio de Janeiro city, RJ, Brazil.

Intrinsic biodegradation experiments were performed in four poly(vinyl chloride) (PVC) reactor columns incubated at 25-28°C for 60 days (Figure 1).



Assay	Contaminant	Description
EXP B5	B5	Soil (20% humidity) contaminated by B5 blend (10% (v/w) contamination)
EXP B100	B100	Soil (20% humidity) contaminated by B100 (10% (v/w) contamination)
CS - B5	B5	Sterile soil contaminated by B5 blend (three times autoclaving and 2% (v/w) formaldehyde)
CS - B100	B100	Sterile soil contaminated by B100

Figure 1 Microcosm incubations in PVC reactor columns. Incubation conditions: 21 kg clay loam soil, 20% humidity, 10% (v/m) contaminant, outer temperature: 25-28 °C, incubation time: 60 days.

Microbiological and Analytical Methods

Quantification of total culturable heterotrophic (TH) bacteria was carried out using MPN technique with nutrient broth under aerobic conditions and Thioglycollate medium for enumerating anaerobic bacteria in flasks purged with N_2. The Dehydrogenase activity (DHA) was measured over the course of incubation using triphenyltetrazolium chloride (TTC) method. At the end of the incubation time, ecotoxicological tests using earthworms (*Eisenia fetida*) were perfomed for 14 days.

RESULTS AND DISCUSSION

The figure 2 shows the results of ecotoxicological assays for EXPB100 and EXPB5. Samples collected on the top of the column reactors exhibited a higher decrease of ecotoxicity, probably due to the higher biodegradation rates of biodiesel/diesel under aerobic conditions.

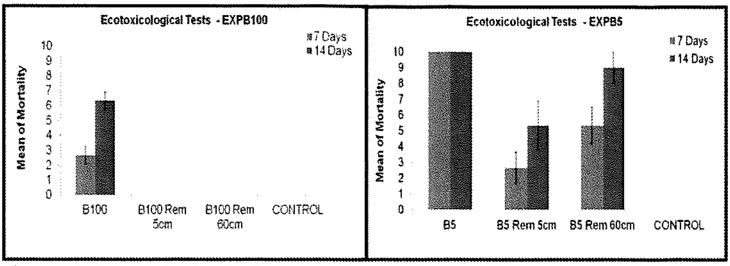


Figure 2 Ecotoxicological tests with earthworms Eisenia fetida carried out for 14 days at room temperature, according to the standard Brazilian technical procedure (ABNT, 2007). Control: pristine soil.

The decrease of ecotoxicity after laboratory microcosm incubations was higher for soils contaminated by pure biodiesel (B100) compared to soils contaminated by B5. The earthworm mortality using EXPB100 samples showed a decrease from 63% to 0% while EXPB5 samples from 100% to 53% and 90% on the top and at the bottom of the reactor column, respectively. The initial mortality observed for EXPB100 is lower than that measured for EXPB5 because biodiesel is known to be less toxic than petroleum diesel in acute toxicity tests. The correlation of ecotoxicity decrease with (bio)diesel biodegradation is still lacking in this study but some authors have already observed biostimulation of diesel biodegradation in the presence of biodiesel (Pasqualino et al., 2006; Taylor & Jones, 2001; Cyplik et al., 2011). Biodiesel is a non-petroleum-based diesel fuel (a mixture of mono-alkyl esters), principally saturated, monoenoic and dienoic C16, C18 and/or C22 alkyl (usually methyl) esters, made by transesterification of plant oils. Biodiesel and biodiesel blends are rapidly biodegraded in soil under aerobic and anaerobic conditions and the fatty acid methyl esters present in biodiesel are degraded at about the same rate as the n-alkanes (typically nC12 to nC24) that are the main components of petroleum diesel (Prince, 2010). In addition, biodiesel can also stimulate the biodegradation of diesel components and can act as a solubilizing agent of crude oil (Pereira & Mudge, 2004)

The figure 3 presents the results of the deydrogenase assays. The metabolic dehydrogenase activity significantly increased in both microcosms compared to the pristine soil, after 60 days of incubation. It was also observed that the dehydrogenase activity was higher at the bottom of the reactor columns in EXPB5 and EXPB100.

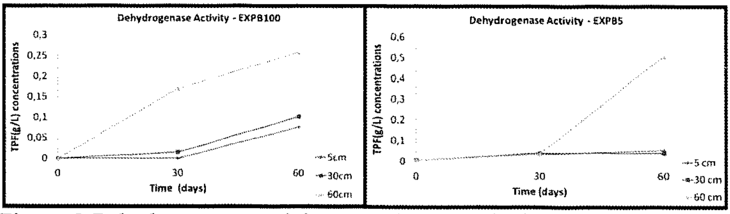


Figure 3 Dehydrogenase activity tests (TCC method) over the course of the incubation.

Soil dehydrogenase enzymes are the major representatives of the oxidoreductase enzymes class in the soil environment and DHA is considered as a suitable indicator of metabolic activity of soil microorganisms, such as the biodegradation of petroleum hydrocarbons. Although they can be active using different terminal electron acceptors, some studies have demonstrated that microorganisms under anaerobic conditions produce most dehydrogenases. In addition, oxygen limitation in soil has been associated with an increase of DHA (Wolinska & Stepniewska, 2012; Brzezińska 2001). This condition can be observed in the deepest zones of the column reactors due to fuels and water percolation.

The figure 4 presents the quantification of culturable heterothrophic bacteria. Compared to the pristine soil (control), the bacterial biomass is generally higher in both microcosms. However, differences between aerobic and anaerobic populations were observed for each reactor. Growth of aerobic bacterial biomass was only observed on the top of the reactor column in EXPB100 while the anaerobic (and anaerobic facultative)bacteria exhibited significant growth at different column depths in EXPB100 and EXPB5. These data suggest that biodiesel and biodiesel blends can be potentially biodegraded using aerobic and anaerobic degradation pathways.

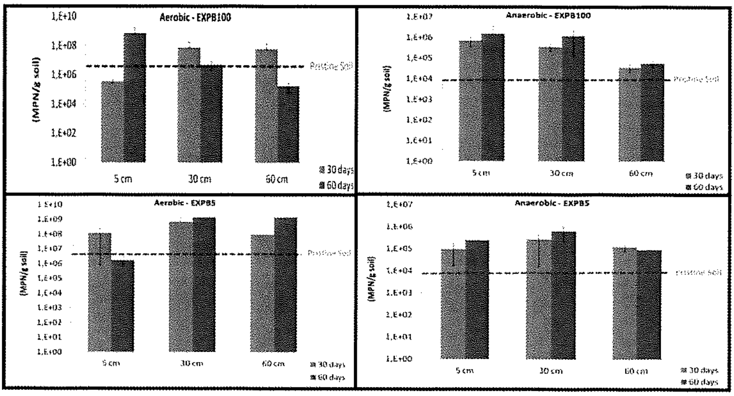


Figure 4 Total culturable heterothrophic bacteria quantification from samples collected at different column depths after 30 days and 60 days of incubation.

These preliminary results suggest that biodegradation of biodiesel and its blends catalyzed by native soil microorganisms could potentially occur in soil columns and natural attenuation of biodiesel-contaminated soils can be a promising bioremediation strategy. However, chemical analyses using GC-FID will be useful to understand the biodegradation profiles of B100 and B5,. In addition, the present work also seeks to decipher the bacterial community structure and dynamics in the investigated microcosm incubations using target sequencing of 16S rRNA tags using illumina platforms.

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