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DISRUPTION AND RECOVERY OF BACTERIAL COMMUNITY STRUCTURE OF AN ATLANTIC FOREST SOIL AFTER EXPOSURE TO GASOHOL

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Abstract: This article reports the effects of gasohol on the genetic of a bacterial community of a tropical Atlantic Forest soil. Hydrocarbon and ethanol biodegradation was accompanied by CO_2 emission. Gasohol had an immediate impact on genetic structure of bacteria and on respiratory metabolism of soil microbial community. Cluster analysis of DGGE band pattern indicated a shift in the community structure between the fifth and fortieth days after contamination. At 60 days after contamination, the DGGE profile of the bacterial community in the contaminated soil was similar to that found in the non-contaminated control. Gasohol addition increased the respiratory rate of the soil, peaking at 3 days and returning to basal level at 15 days after contamination. We concluded that gasohol contamination causes a strong transient impact on soil microbial community structure that is completely reversed after a few days following contaminant removal. Secondary succession after contamination resulted in a bacterial community of identical genetic structure to that found before contamination. Our results point out to a high resilience of microbial community established in Atlantic Forest soil.

Keywords: hydrocarbon bioremediation; microbial ecology; microbial succession; resilience; Tropical forest soil.

INTRODUCTION

Gasohol is a volatile petroleum-derived liquid that is used primarily as a fuel in internal combustion engines. It is obtained by fractional distillation of petroleum, enhanced with a variety of additives, and is considered one of the most important automotive fuels (Demirbas 2009). A collateral effect of the high consumption of gasohol is the contamination of soils and water sources, including aquifers, due to leakages from underground storage tanks and accidental spillages (Corseuil & Marins 1997, Andrade *et al.* 2017).

The Atlantic Forest supports one of the highest plant and animal species richness and rates of endemism on the planet (Ribeiro *et al.* 2009), and is one of the 25 biodiversity hot spots of the world (Faoro *et al.* 2010). The same must be truth in respect to soil microbial diversity, although few studies have so far addressed the structure of soil microbial communities in this biome. Human activities can change the microbial diversity of soils and, therefore, it is important to evaluate the resilience of the microbial community of soils, mainly in areas with high importance for biodiversity conservation.

Soil contamination by petroleum hydrocarbons results in reduction of local microbial diversity, which leads to a decrease in microbial biomass, and genetic and functional diversity (Wardle & Giller 1996, Mummey *et al.* 2002, Khan *et al.* 2018), therefore, leading to loss of crucial ecological functions (Jung *et al.* 2016). The microorganisms play a key role in the biogeochemical cycles, and in this way they actively participate in the transfer of energy and nutrients in the soil (Baldrian 2017). The microbial activity affects directly both soil quality and the productivity of terrestrial ecosystems (Hill *et al.* 2000, Tótola & Chaer 2002, Birkhofer *et al.* 2008). Due to the importance of microorganisms for ecosystem functioning, it is essential to understand how these components of terrestrial ecosystems respond to natural variations and disturbances caused by human activities, including soil contamination by xenobiotics.

Fuel production, transport, and storage may lead to contamination of large areas, due to the massive volumes of these key energy sources consumed in modern human activities (Khan *et al.* 2018). Soil contamination by hydrocarbons leads to reduction of enzymatic activities (Alrumman *et al.* 2015) and changes of the structure of bacterial and fungal communities (Siles & Margesin 2018). Once a contamination occurs, remediation of the affected area must be immediately implemented to avoid environmental and economic effects of the contaminants. Remediation of environments contaminated with hydrocarbons can rely on physical, chemical, or biological methods. Physical and chemical treatments usually cause the dispersion of hydrocarbons and do not effective remove them from the environment; moreover, they have high costs. Compared with these methods, biological treatment using microorganisms is cost-effective, presents high efficiency and prevents secondary pollution (Khan *et al.* 2004, Bao *et al.* 2012). Bioremediation is an effective method for removal of contaminants, and the autochthonous microbial community can usually catabolize the contaminants (Martínez-Pascual *et al.* 2015).

Several microbial species of bacteria, archaea, and fungi can degrade hydrocarbons present in gasohol (Leahy & Colwell 1990, Habe & Omori 2003, El Fantroussi & Agathos 2005, Rodrigues *et al.* 2015). *In situ* microbial bioremediation processes can be classified as natural attenuation, bioaugmentation, or biostimulation (Yu *et al.* 2005). As reported by Alvarez *et al.* (2011), natural attenuation relying on the microbial soil community of an Atlantic Forest soil from Rio de Janeiro State was the most cost-effective approach for bioremediation of soil upon hydrocarbons contamination. However, no information about the impact of hydrocarbon contamination on the structure of microbial communities of Atlantic Forest soils was found in the literature.

This study was carried out to evaluate the response of a soil microbial community from the Atlantic Forest to gasohol contamination, in terms of genetic structure.

MATERIAL AND METHODS

Soil sampling and contamination

The soil used in this work was collected from the upper 5 - 15 cm layer of the Mata da Biologia, located in the campus of the Universidade Federal de Viçosa, which is part of the Atlantic Forest biome, Brazil (20°45'31.5" S 42°52'04.8" W). Twenty samples of soil were collected to result in a composite sample of 2 dm^3. All experiments were conducted with three replicates.

The soil was air-dried at room temperature, sieved at 2mm and thoroughly mixed before chemical and granulometric analyses. The soil received application of nutrients (200 mg/dm^3 P, 300 mg/dm^3 N-NH_4 and 100 mg/dm^3 K) to stimulate microbial biodegradation of hydrocarbons. The pH was adjusted between 6.5 and 7.0 with $CaCO_3$ and $MgCO_3$ at the proportion of 5:1 (w/w). Soil samples (20 g) were transferred to plastic pots and humidity was adjusted to 60% of the water holding capacity. The pots were maintained at 25°C throughout the incubation period. One week after fertilization, gasohol (containing 25% ethanol) was added to the contaminated treatment (20 mL/kg). Experimental control consisted of non-contaminated soil.

The experiment was conducted for two months. Samples (5 g) were taken at 0, 5, 10, 15, 25, 40, and 60 days after contamination and used to evaluate genetic diversity of bacteria.

Soil respiration

A separate experiment was set to study the metabolic response of the soil microbial community to gasohol contamination. The experiment consisted

of fertilized (refers to 1.1 for dosage) soil samples (10 g dry weight) in 100 mL Gibco glass flasks. Soil moisture was kept at 60% water holding capacity. The flasks were kept in a water bath (22°C) and connected to a respirometer (Sable Systems International, Las Vegas, NE). A CA-2A infrared CO_2 detector was used to measure CO_2 emission by the soil samples at 5 minutes intervals. Respiration was measured during 60 days, with three replicates for each treatment (non-contaminated control and gasohol-contaminated, 20 mL/kg).

DNA extraction and PCR-DGGE Fingerprint

Total DNA was extracted from 2 g soil samples at 0, 5, 15, 25, 40, and 60 days after the onset of the experiment (van Elsas *et al.* 1997) and then purified using the Wizard Plus SV Minipreps DNA Purification System (Promega). Integrity of DNA samples was checked by agarose gel electrophoresis. On day zero, DNA extraction occurred 2h after soil contamination.

Bacterial 16S rRNA genes were amplified by PCR using the oligonucleotides described by Muyzer *et al.* (1993) in a Mastercycler Gradient thermocycler (Eppendorf). The reaction mixture (25 µL) consisted of 50 ng total DNA, *Taq* 1X buffer, 2.4 mM $MgCl_2$, 0.2 mM dNTP, 10 pmol of each initiator oligonucleotides, 10 µg BSA, 0.5 µL formamide and 1U *Taq* DNA polymerase (Promega). The amplification cycles were those described by Peixoto *et al.* (2002). An aliquot of the amplification reaction (5 µL) was used to check PCR products by agarose gel electrophoresis. The remainder PCR amplicons were applied onto an 8% (wt/vol) polyacrylamide gel containing a linear gradient of 45 - 70 % denaturant (where 100% denaturant solution contains 7 M urea and 40% (vol/vol) formamide). Electrophoresis was performed in 1X TAE buffer at 60°C, at a constant voltage of 100V for 16 h. After electrophoresis, the gel was stained for 10 min with SYBR Green I (Sigma). The PCR-DGGE banding profiles were analyzed with Gel Pro Analyzer R 3.1 software (Media Cybernetics Inc., Maryland-USA). Cluster analysis was performed with Pearson correlation coefficient for the total lane pattern after background subtraction with the BioNumerics software v. 5.10 (Applied Maths, St. Martens Latem, Belgium) and using the unweighted pair group method with arithmetic mean (UPGMA) method (Duineveld *et al.* 2001).

RESULTS

Soil respiration

The addition of gasohol caused a rapid increase of the respiration rate in soil (Figure 1). The respiration rate peaked at the third day, decreasing steadily until reaching the basal level of the non-contaminated control at day 15, indicating complete removal of the newly added substrates (gasohol components).

Effect of gasohol on soil DNA

The amount of total DNA recovered from the contaminated soil was significantly lower than from the non-contaminated soil (< 50 ng and > 2 mg per sample, respectively; Figure 2). This difference was attributed to a strong toxic effect of gasohol on the microbial soil community components. Forty-days after gasohol addition, the amount of DNA recovered from the contaminated soil was similar to that recovered from the non-contaminated control, indicating the recovery of the soil microbial biomass.

Microbial community fingerprints

The analysis of the bacterial community profile by DGGE revealed a similar genetic profile in all control samples during the 60-day incubation period (Figure 3A). The genetic profile of the contaminated and control soils shared many common DGGE bands (bands at same position), but with different intensity. A clear separation in two main clusters was detected upon a UPGMA analysis of DGGE profiles (Figure 3B). The contaminated soil at the

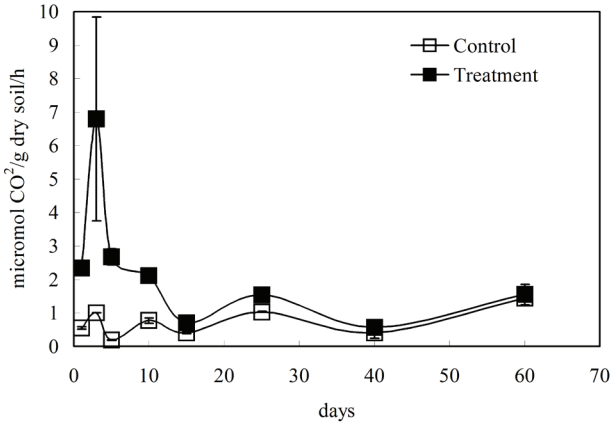

Figure 1. Basal respiration rate in either uncontaminated or gasohol- contaminated soil of a tropical forest. The soil samples were incubated at 22°C and kept at 60% water holding capacity.

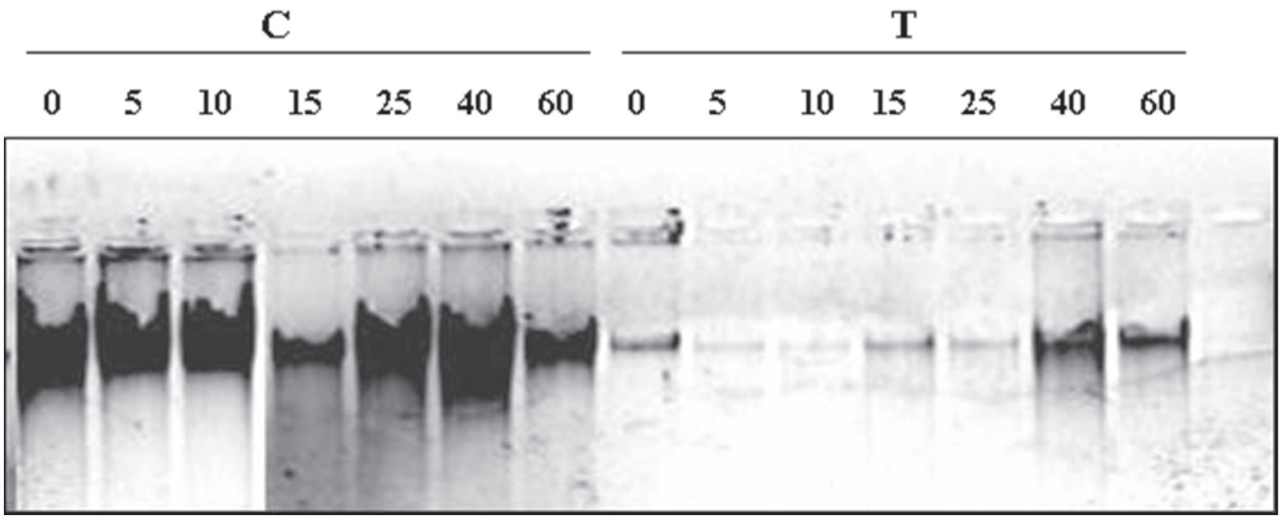

Figure 2. Agarose gel electrophoresis of total DNA extracted from the soil microbial community. Samples were taken from control soil (C_0 - C_{60}) and gasohol-contaminated soil (T_0 - T_{60}) and nucleic acids were isolated as described in material and methods.

onset of the experiment (T_0) grouped with the non-contaminated control soil (≈ 90% similarity). The same applies to the contaminated soil at day 60, indicating the recovery of the bacterial community structure after the biodegradation of the gasohol components (Figure 1).

DISCUSSION

The addition of gasohol caused significant and immediate increase in the respiration rate of the forest soil. The respiration rate of the contaminated soil returned to values similar to the non-

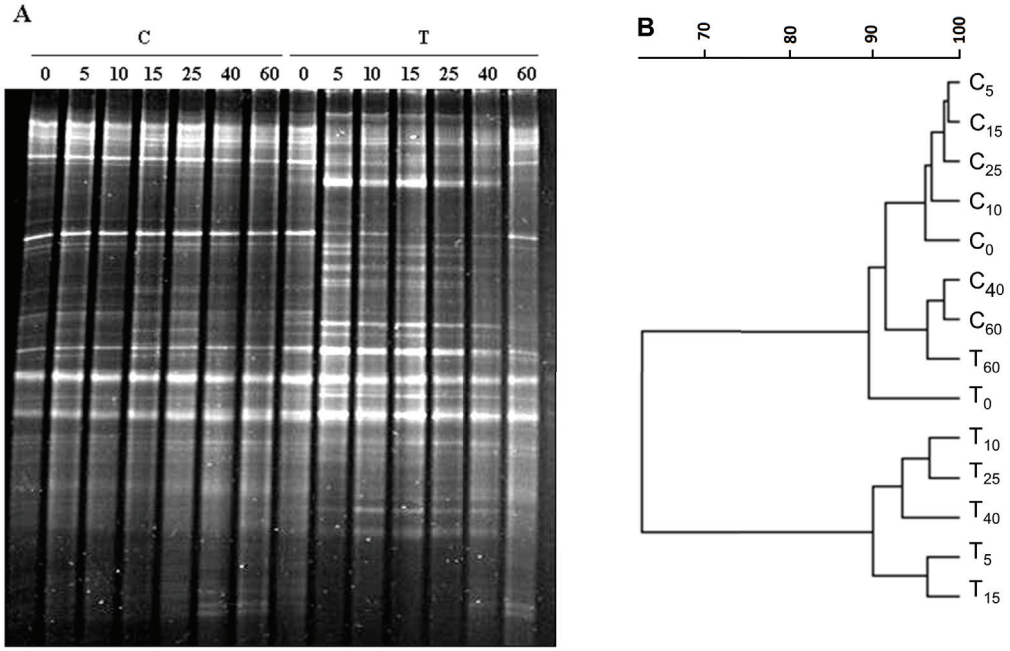

Figure 3. DGGE Analysis of PCR-amplified 16S ribosomal DNA from uncontaminated and contaminated soil DNA extracts. Gel gradient ranged from 45 to 75% denaturant (A). Soil samples were collected from 0 to 60 days after contamination from both control (C) and gasohol-contaminated (T) soil. Cluster analysis of DGGE profiles (B). Similarity dendrogram (UPGMA, Pearson correlation coefficient) of eubacterial banding patterns of control (C) or gasohol-contaminated (T) soils at differing sampling times, representing the percentage of similarity between the DGGE band profiles. The similarity dendrogram (scale 0–100) was calculated from PCR-DGGE profiles shown in Fig. 3A.

contaminated soil at 15 days after gasohol addition. This decline was attributed to the exhaustion of the newly available carbon sources resulting from the mineralization activity and partial loss of gasohol components by volatilization during air injection. The amount of total DNA recovered after 45 days from contamination is in agreement with the return of soil respiration of the contaminated treatment to basal level after 15 days, suggesting that the contaminants had been eliminated. The low yield of DNA from the contaminated soil on day zero, after only two hours of contamination (compared to the control), can be explained by the solvency potential of gasohol, and may not be an indicative of a strong and immediate effect on the microbial structure or biomass. This is reinforced by the similar DGGE profile between the contaminated and non-contaminated (control) soil.

The impact of gasohol contamination on the microbial community was shown by either significant reduction in the amount of DNA recovered from the contaminated soil and the shift in the genetic profile. The steep decrease in DNA recovery until day 10 coincided with the period of the highest shift in bacterial community profile (Figure 3A).

The biodegradation of contaminants by the microbial populations and loss of the volatile molecules during air injection paved the way to the reestablishment of the soil microbial populations. In the present case, the secondary succession that followed the contamination and contaminant removal lead to a microbial structure similar to that present in non-contaminated soil. This indicates a strong resilience of microbial the community present in soil of the Brazilian Atlantic Forest to soil contamination by gasohol.

The DGGE analysis showed the prevalence of some ribotypes in either non-contaminated control and in gasohol-contaminated soil, indicating the stability of some bacterial populations in the soil. We do not exclude the possibility that some or most of these populations were present as resting stages (*e.g.*, endospores). However, because tropical forest soils are generally rich in a diverse organic carbon pool, the dominant bacteria tend to be generalist, and therefore able to use different C sources (Laverman *et al.* 2005, Novak *et al.* 2017, Chen *et al.* 2019). Similar results were found by Evans *et al.* (2004) working with oil-contaminated soil.

The detection of new ribotypes upon contamination of soil with gasohol (T_5 - T_{40}) suggests the enrichment of some microbial populations that were present at low abundance in the non-contaminated soil, which were favored by the newly added carbon sources, indicating that these populations can use efficiently the components of gasohol as substrates. This in turn offers competitive advantage, resulting in the increase of the hydrocarbonoclastic populations. An additional explanation is that ecological niches previously occupied by naturally dominant populations that were inhibited (or even eliminated) by the toxic effects of the contaminants became available to these newly dominant populations (due to reduced competition). Independently of the intrinsic behavior of individual populations in response to soil contamination by gasohol, structure of the bacterial community was reestablished after only 60 days upon contamination, which is an evidence of the strong resilience of this component of tropical forest soil ecosystem.

Finally, the bacterial community of Atlantic Forest soil responds promptly to contamination by gasohol, in terms of both genetic profile and metabolic activity. Genetic profile and respiration rates returned to basal levels in just a few days, indicating a great resilience of the bacterial community in tropical Atlantic Forest soil. Future studies using other methods can reinforce the high resilience of the microbial community from Atlantic Forest soils contaminated with gasohol compounds and the key populations involved in contaminant removal.

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