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Modifications of bacterial populations in anthracene contaminated soil

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ABSTRACT

Spiking soil with anthracene, a polycyclic aromatic hydrocarbon, inhibited soil microbial activity, so it remained to be seen if the bacterial population composition was affected. An initial PCR-DGGE analysis indicated that the bacterial population in the unamended soil was not affected by depth (0–2 cm, 2–8 cm and 8–15 cm) or incubation time (0, 14 or 28 days) while it changed in the contaminated soil over the time and after 28 days in the soil profile. A phylogenetic analysis was done of the uncontaminated soil at day 0, and in the 0–2 cm layer of the anthracene-spiked soil after 0, 14 and 28 days and in the 2–8 cm layer after 28 days. Sequences belonged to six different phyla, i.e. Acidobacteria, Actinobacteria, Chloroflexi, Gemmatimonadetes, Nitrospirae and Proteobacteria. However, 10% of the sequences remained as unclassified bacteria. Bacteria belonging to the Proteobacteria (63.2%) were always the most dominant followed by the Acidobacteria (22.8%) and the Gemmatimonadetes (3.6%). The percentage of Actinobacteria (4.1%), Chloroflexi (1.3%), and Nitrospirae (0.1%) was low and sequences belonging to these phyla were not always detected. Contaminating the soil had a momentaneous effect on the bacterial population as the percentage of Alphaproteobacteria, i.e. Sphingomonadales, and Gammaproteobacteria, i.e. the Xanthomonadales, reduced strongly while the percentage of Actinobacteria and Acidobacteria more than doubled. Already after 14 days, however, the percentage of Sphingomonadales, Actinobacteria and Acidobacteria was similar as in the unamended soil at day 0. After 28 days, the percentage of Burkholderiales and Xanthomonadales more than doubled in the 0–2 cm and 2–8 cm layer compared to the unamended soil at day 0. It was found that spiking soil with anthracene, incubation time and soil layer had an effect on the bacterial population, but the effect of the contaminant was transient and changes in the bacterial population with depth were only detectable in the contaminated soil after 28 days.

1. Introduction

Mineralization of polycyclic aromatic hydrocarbons (PAHs) is often low in soil. Kästner et al. (1995) found that only 0.6% of ^{14}C-labeled anthracene had been mineralized in soil amended with compost during the first 12 days and 23.8% after 104 days. However, no anthracene-derived CO_2 was emitted from the soil without compost. Brodkorb and Legge (1992) reported that approximately 19.5% of ^{14}C-labeled phenanthrene was mineralized after 21 days and 37.7% when *Phanerochaete chrysosporium* was added to soil. Although PAHs are degraded in soil, this did not always result in an increase in emission of CO_2 (Scelza et al., 2007). On the contrary, the emission of CO_2 is often reduced when a soil is spiked with PAHs. Eriksson et al. (2007) observed that the emission of CO_2 decreased in a soil contaminated with naphthalene, phenanthrene and pyrene. Vázquez Núñez et al. (2009) also reported a decrease in emissions of CO_2 when a saline–alkaline soil was contaminated with anthracene. This change in CO_2 emitted suggested that the added PAHs affected soil microbial activity. It can be speculated that when changes in microbial activity occurred, the microbial community composition might have been affected (Balser et al., 2002).

Soil conditions, such organic matter content, pH, salt content, O_2 diffusion, water content, temperature and structure, are known to change within the soil profile (Ussiri and Lal, 2009). Organic matter, which serves as *C*-substrate for most microorganisms, is often concentrated in the top layer of a forest soil, arable land or pasture (Vandenbygaart and Angers, 2006). As such, microorganisms are more diverse in the top soil layer than in the deeper soil layers (Fierer et al., 2003). However, the top soil layers are more prone to rapid and extreme changes in temperature and water content, which are known to affect survival of organisms in soil. For instance in an earlier study (Vázquez Núñez et al., 2009), we found that remediation of anthracene in soil was faster in the top 0–2 cm layer than in the lower soil layers and in a second experiment it was concluded that changes in soil conditions, i.e. light and a fluctuating water content, accelerated the removal of anthracene from soil.

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It remained unclear, however, how these changing soil conditions and the contaminant affected the microbial population structure.

Isolation of bacterial nucleic acids from natural environments has become a useful tool to detect and identify bacteria that cannot be cultured, to determine dynamics of selected bacteria, to reveal genotype diversity in different microbial ecosystems and to study how contaminants might affect bacterial populations (Ganzert et al., 2011; Nacke et al., 2011; Nemergut et al., 2011). Soil was spiked with anthracene or left unamended, while changes in the bacterial population structure were monitored. The water content in the columns of both the contaminated and uncontaminated soil was allowed to fluctuate freely as this resulted in the fastest removal of anthracene (Vázquez Núñez et al., 2009). Anthracene, which is a tricyclic aromatic hydrocarbon, was used as it might be carcinogenic and it is a known skin and eye irritant (IARC, 1983; Cai et al., 2007). The objective of this study was to investigate how spiking soil with anthracene affected the microbial population structure in different soil layers over time compared to an uncontaminated soil.

2. Materials and methods

2.1. Sampling site, collection and characterization of soil

Details of the sampling site located near the ex-convent of Acolman in the State of Mexico (N.L. 19°38′, W.L. 98°55′) at an altitude of 2250 m above sea level and with a mean annual temperature of 14.9 °C and average annual precipitation of 624 mm (mainly from June through August) can be found in Betancur-Galvis et al. (2006). The soil was cultivated mainly with maize for >20 years, receiving a minimum amount of inorganic fertilizer without being irrigated. Soil was sampled at random by augering the 0–15 cm top-layer of three plots of approximately 0.5 ha. The soil from each plot was pooled separately, passed separately through a 5-mm sieve so that three soil samples were obtained and characterized. The size particle distribution of the clayey soil was 60 g sand kg^{-1}, 270 g silt kg^{-1}, and 670 g clay kg^{-1}. The soil with pH 6.3 and electrolytic conductivity (EC) 0.8 dS m^{-1} had a water holding capacity (WHC) 896 g kg^{-1}, organic *C* content 19.0 g kg^{-1}, and total nitrogen content 1.4 g kg^{-1}. The bulk density of soil was 1.1 g cm^{-3}. The soil was adjusted to 50% of WHC and conditioned in drums containing distilled water and 100 ml 1 M NaOH to trap the CO_2 evolved for a week.

2.2. Treatments, conditioning in the greenhouse and soil sampling

Two different treatments were applied to the soil. One treatment in which dichloromethane and anthracene were applied to soil and one treatment that was left unamended and served as control.

Nine sub-samples of 1 kg soil from each plot were amended with 15 ml dichloromethane containing 0.05 M anthracene, and thoroughly mixed. As such, the soil was contaminated with 550 mg anthracene kg^{-1}. The amount of anthracene applied to soil were such that its removal and the factors controlling it could be studied in an aerobic incubation within a reasonable time, i.e. between 28 and 112 days (Betancur-Galvis et al., 2006). In experiments with earthworms (*Eisenia fetida*), it was also found that they resisted that amount of anthracene (Contreras-Ramos et al., 2008). Additionally, nine sub-samples of 1 kg soil from each plot were not contaminated and considered the control.

The sub-samples were placed under vacuum in a desiccator for 30 min to remove all dichloromethane and the soil was added to polyvinyl chloride (PVC) tubes (length 25 cm and ⌀ 10.5 cm). A 15 cm soil layer was thus obtained. The 18 PVC tubes (9 amended with anthracene and 9 left unamended) were not covered so that the upper soil layer could dry out and light could reach the soil surface. Every other day in the evening, a sub-sample of 2 g soil were taken from each soil column weighted and dried overnight at 100 °C. The next morning, the dried soil was weighted, the water content calculated and the soil discarded. The soil columns were then adjusted to 50% WHC with distilled water as determined at the onset of the experiment.

The soil columns were placed in a greenhouse. The conditions in the greenhouse were not controlled so the natural day/night cycle was followed. After 0, 14 and 28 days, three PVC tubes were selected at random from the anthracene-amended and uncontaminated soil, the soil was removed from the tube taking care that three soil layers were obtained: the 0–2 cm, the 2–8 cm and the 8–15 cm soil layer. The concentration of anthracene was determined in each soil layer (Vázquez Núñez et al., 2009).

2.3. DNA extraction and purification, and PCR amplification of 16S rDNA

A 2 g sub-sample of soil was taken from each soil layer at the beginning (day 0), in the middle (day 14) and at the end (day 28) of the experiment and 1 g soil was extracted for DNA immediately. A PCR-DGGE analysis was done to identify possible changes in the microbial population structure in the soil profile. The uncontaminated soil showed no differences with depth and over time. A dendrogram of the DGGE patterns was made of the contaminated soil with the 0–2 cm layer of the uncontaminated soil as control.

DNA was extracted from soil with a modified method based on direct cell lysis technique of Guo et al. (1997). Details of the modified technique can be found in Valenzuela-Encinas et al. (2008).

The universal primers pair 46F (5′ GCC TAA CAC ATG CAA GTC 3′) and 1540R (5′ AAG GAG GTG ATC CAG CCG CA 3′) were used to amplify the V1–V9 highly variable regions of 16S rDNA, ca. 1500 bp (Edwards et al., 1989; Yu and Morrison, 2004). The nested PCR for DGGE was performed using the primer 46F plus GC clamp and the primer 534R (Muyzer et al., 1993). Details of the PCR amplifications can be found in Valenzuela-Encinas et al. (2009).

2.4. DGGE analysis

The DGGE was done using a DCode system (Biorad Laboratories). The 6% (w/v) polyacrylamide gel (acrylamide–bisacrylamide [37:1]) was done with a denaturing gradient of 45–65% where 100% denaturant contains 7 M urea and 40% formamide. Twenty five μl of nested PCR product was loaded on polyacrylamide gel and electrophoresis was done in 1× TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA (pH 8.0)) at 48 V and 60 °C for 19 h. Similarities between the DGGE patterns of the three layers of the uncontaminated soil at day 0 were determined by digitalizing the DGGE gels and compared to the three layers of the contaminated soil at day 0, 14 and 28. It was assumed that each band located in the same position in different lanes, had the same sequence and thus the same ribotype.

Every ribotype found was codified as 1, but when absent as 0. The display was analyzed in a GELDOC 2000 BIORAD image digitizer (Hercules, CA, USA). The information was processed in an excel file and is suitable to be analyzed in NTSYSpc 2.02j Applied Biostatistics Inc. (St. Paul, MN, USA) (Rohlf, 2005). The matrix generated previously was used as the basis for similarity analysis using the correlation coefficient with the UPGMA clustering method. The best tree was developed with the Cophenetic Correlation Coefficient (CCC) so that statistically significant dissimilarities could be determined. The representation of the trees was done with TREE VIEW 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/rod.html) program.