Acta Scientiarum. Biological Sciences
ISSN: 1679-9283
eduem@uem.br
Universidade Estadual de Maringá
Brasil

Serrano Silva, Ísis; Ragagnin de Menezes, Cristiano; Fonseca de Faria, Andréia; Gurgel da Costa, Josemar; Pepper, Susan; Britz, Margaret; Durrant, Lucia Regina
Application of molecular fingerprinting for analysis of a PAH-contaminated soil microbiota growing in the presence of complex PAHs
Acta Scientiarum. Biological Sciences, vol. 32, núm. 1, 2010, pp. 63-69
Universidade Estadual de Maringá
.png, Brasil

DOI: 10.4025/actascibiolsci.v32i1.7575

Application of molecular fingerprinting for analysis of a PAH-contaminated soil microbiota growing in the presence of complex PAHs

Ísis Serrano Silva[1*], Cristiano Ragagnin de Menezes[1], Andréia Fonseca de Faria[1], Josemar Gurgel da Costa[2], Susan Pepper[3], Margaret Britz[3] and Lucia Regina Durrant[1]

*[1]Departamento de Ciência de Alimentos, Universidade Estadual de Campinas, Cx. Postal 6121, 13083-862, Campinas, São Paulo, Brazil. [2]Departamento de Microbiologia, Universidade Federal do Rio Grande do Norte, Natal, Rio Grande do Norte, Brazil. [3]School of Food and Agriculture Systems, Faculty of Land and Food Resources, The University of Melbourne, Werribee, Australia. *Author for correspondence. E-mail: iserranosilva@gmail.com*

ABSTRACT. Polycyclic aromatic hydrocarbons (PAHs) constitute a group of priority pollutants which are present at high concentrations in the soils of many industrial contaminated sites. Pollution by these compounds may stimulate growth of organisms able to live in these environments causing changes in the structure of the microbial community due to some cooperative process of metabolization of toxic compounds. A long-term PAH-contaminated soil was stored for several years and used to analyze the native microbiota regarding their ability to grow on pyrene, benzo[a]pyrene, as well as in mixtures of LMW- and HMW-PAHs. Molecular profiles of the microbial community was assessed by PCR-DGGE of 16S rRNA gene, and the number of bands observed in DGGE analyses was interpreted as dominant microbial members into the bacterial community. Results of PAH-contaminated soil microorganisms showed different profiles in the degradative dynamics when some nutrients were added. Predominant species may play a significative role while growing and surviving on PAHs, and some other metabolically active species have emerged to interact themselves in a cooperative catabolism of PAHs.

Key words: polycyclic aromatic hydrocarbons (PAHs), biodegradation, PCR-DGGE, microbial community.

RESUMO. Aplicação de técnica molecular para análise da microbiota de solo contaminado em misturas complexas de HPAs. Os hidrocarbonetos poliaromáticos (HPAs) são considerados poluentes prioritários presentes em expressiva concentração no solo contaminado com derivados de petróleo. A poluição por esses compostos estimula o crescimento de microrganismos capazes de sobreviverem nestes ambientes contaminados, causando alterações na estrutura da comunidade microbiana do solo pelo processo de cooperação metabólica entre as populações. Um solo contaminado por um longo período de tempo foi coletado de uma área industrial (Port Melbourne, Austrália) e utilizado para análise da capacidade da comunidade microbiana em crescer em HPAs isolados e/ou em misturas como únicas fontes de carbono e energia. Os perfis moleculares foram obtidos por PCR-DGGE do fragmento da subunidade 16S do DNA ribossomal, sendo o número de bandas presentes nos géis de eletroforese interpretado como populações dominantes dentro da comunidade do solo. Resultados demonstraram diferentes perfis quando vitaminas e micronutrientes fizeram parte do meio líquido de crescimento da microbiota, além da presença dos HPAs. As espécies predominantes podem ter papel significativo na degradação desses compostos no solo a ser remediado, enquanto sobrevivem e interagem com outras populações por meio de mecanismos de catabolismo cooperativo.

Palavras-chave: hidrocarbonetos poliaromáticos (HPAs), biodegradação, PCR-DGGE, comunidade microbiana.

Introduction

PAH-contaminated soil is a complex and dynamic biological system where native microbial communities are capable of degrading some low-molecular-weight polycyclic aromatic hydrocarbons (LMW-PAHs), such as naphthalene, phenanthrene, fluorene; however, the ability for high-molecular-weight (HMW-) PAHs degradation is still limited (JUHASZ et al., 2000). Bacteria act on the degradation of the PAHs through the production of dioxygenases, which incorporate atoms of oxygen at benzenic ring, resulting in the formation of cis-

dihydrodiol, followed by some dehydroxylated intermediates that enter in the TCA-cycle (KANALY; HARAYAMA, 2000). The native soil microbiota has different phases of adaptation to PAHs and by-products of PAHs degradation may influence the biodegradability rates of others PAHs, (BOUCHEZ et al., 1995). The presence of LMW-PAHs may increase the biodegradability of HMW-PAHs and metabolic activity of the microorganisms by providing alternative carbon and energy sources for growth (JUHASZ et al., 2000). The advantages of using native microorganisms for bioremediation include their better and faster adaptation and the avoidance of other impacts on the environment (CANET et al., 2001). Researches on *in situ* bioremediation have been pursued with selected bacterial strains isolated from the contaminated sites (MISHRA et al., 2001), where bioaugmentation is optimized with potentially degrader organisms, or biostimulated with ratios of oxygen and C:N:P, which provide greater efficiency of microbiota to degrade PAHs. Studies regarding ecology and diversity of the microbial community were limited in the past because only a minimal proportion of the microbial population is cultivable. Fingerprinting profiles of rDNA fragments amplified by PCR have been used to study microbial communities (TORSVIC et al., 1998), in freshwater lakes, estuaries, and soils (OVREÅS; TORSVIK, 1998). Over this last decade, the profile of bacterial populations in different matrices became more accurate with the application of molecular methods to directly detect DNA and RNA in microbial ecosystems, and this development in molecular biology has found extensive applications in the field of microbial ecology.

Denaturing Gradient Gel Electrophoresis (DGGE) is a useful tool to analyze the initial step of microbial communities composition when some factors are selecting specific populations (e.g. environmental disturbance by pollutants). This technique compares the fingerprinting profiles of samples to identify temporal and spatial differences in community structure or to monitor changes into the community due to the soil impact by contaminants. Based on the presence or absence of bands in each sample, similarity coefficient can be determined for DGGE profile when using general and/or specific primers. The use of group-specific primers allows the study of temporal and spatial changes of microbial populations, evaluating the long-term effect of bacterial inoculants on the stability and dynamic of microbiota in ecosystems (VAN ELSAS et al., 1998). In this present study our aim was to verify changes on the microbial community dynamics of a long-term contamination of soil from a manufacturing gas plant (MGP) site when growing in PAHs, identifying some predominant species involved in the biodegradation process.

Material and methods

Chemicals

PAHs (>98% of purity) were purchased from Sigma. Reagents for the denaturing gradient gel electrophoresys preparation were acquired from BioRad Laboratories (USA). DNA extraction and purification kits were obtained from MoBio Laboratories Inc. (USA) and Promega (Australia), respectively. Sequencing kit was purchase from Beckman Coulter Australia Pty. Ltd.

Soil samples and PAH experiments

The soil collected previously from an abandoned MGP-site located near Port Melbourne, Victoria, Australia, and containing high concentration of PAHs, were stored at 4°C for several years (JUHASZ et al., 2000). Aliquots of this contaminated-soil 'Bin#4' has been taken to be used in some growth experiments in the presence of PAHs.

PAH degradation experiments were performed with the original contaminated soil inoculated into 50 mL serum bottle flasks tightly closed with polypropylene lid and aluminum cap. Different treatments have been applied to 2 g-soil community in 20 mL of salt minimal medium containing or not vitamins and micronutrients (N) added in flasks containing: (A) LMW-PAHs phenanthrene and fluorene, 100 mg L^{-1} each; (B) pyrene, 100 mg L^{-1}; (C) benzo[a]pyrene, 50 mg L^{-1}; (D) HMW-PAHs benzo[a]anthracene and dibenzo[a,h]anthracene, 50 mg L^{-1} each; (E) coronene, 20 mg L^{-1}; and finally (F) a mixture of PAHs using the previous concentrations.

Treatments A-F were then carried out with salt medium and all the PAH combinations described above, where as treatments AN-FN were performed with the salt medium amended with micronutrients and vitamins plus the same PAH combinations. Flasks were incubated at 30°C/150 rev. $min.^{-1}$ in the dark for 30 days. Sampling was done each 7 days for genomic DNA extraction.

Extraction and purification of genomic DNA

Cells were removed from culture media by centrifugation at 1,500 g 4°C^{-1} for 5 minutes, and pellet containing also soil particles was suspended in a proper solution according to the UltraClean™

microbial isolation kit protocol provided by the manufacturer (MoBio Laboratories Inc, USA), using chemical/mechanical cells breakdown to release the genetic materials. Organic and inorganic substances as cells debris and proteins were then precipitated, followed by filtration of DNA in a silica membrane.

Genomic DNA was extracted, and a 1% agarose solution containing 0.5 µL ethidium bromide (5 mg mL^{-1}) was prepared in 50 mL 1 x TBE buffer pH 8 (from a 10 x TBE stock solution containing per liter: 108 g trizma-base, 55 g boric acid, 7.4 g EDTA). The samples (5 µL) added to 6 x loading buffer (1 µL, Genework) were loaded into the gel and run for one hour at 100 V with 1 x TBE as running buffer. Lambda DNA/*Eco* RI + *Hind* III marker (Fermentas) was run in parallel with DNA samples to estimate the amount and quality of DNA extracts, comparing the intensity of bands with the bands in the marker. The gel was photographed on a UV translumination using Quantity One computer program (BioRad).

The purification of DNA extracts was performed due to the presence of humic acid and some other substances that may interfere in the PCR amplification, using a Wizard® DNA Clean-up system protocol (Promega). Purified DNA templates were stored at -20°C to be used in PCR-DGGE experiments.

Polymerase chain reactions

The amplification reactions were prepared in a total volume of 25 µL, containing 2.5 µL of a 10 x PCR buffer (Roche; 100 mM Tris, 500 mM KCl, 15 mM $MgCl_2$), 0.5 µL of dNTP 40 mM (0.2 mM dATP, dCTP, dGTP and dTTP), 1 U Taq DNA polymerase enzyme (Roche), 10 µM of forward and reserve primers (Sigma-Genosys), and 10 ng of DNA template. A primer set from the V3 region into ribosome: GC clamp - PRBA338f (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-ACTCCTACGGGAGGC AGCAG - 3') and PRUN518r (5'-ATTACCGCGGCTGCTGG–3') was used to generate a 236bp product. The forward primer complements a conserved region among members of the Bacteria domain, and the reverse primer is based on a universally conserved region of this domain. The PCR cycle program was set with an initial denaturing step at 95°C for 4 minutes, followed by 30 cycles of 92°C for 1 minute (denaturation), 55°C for 1 minute (annealing), and then 72°C for 1 minute (extension). At the end of cycling, 10 minutes of a final extension was performed. Volumes of 1 µL of amplified fragments were run in a 1.5% agarose gel in 1 x TBE buffer. GeneRuler™ DNA Ladder Mix (Geneworks) was used as marker to analyze concentration and quality of the PCR products. To better estimate the concentration of amplified product to be loaded into DGGE wells, some dilutions from samples were made when necessary, and to check the reproducibility of the PCR products, replicate of amplifications and DGGE gels were run.

Denaturing gradient gel electrophoresis

DNA from the microbial community was analyzed to assess the total diversity and generate a profile of the community. Changes in profiles could indicate some changes into the community. The DGGE technique was performed in a DCode™ Universal Mutation Detection System (BioRad). PCR products (100-200 ng) added in 2 x loading buffer into a microtube were loaded onto 8% (w v^{-1}) polyacrylamide (bisacrylamide stock 37.5:1 – BioRad) gels in 0.5 x TAE buffer (20 mM Tris, 10 mM sodium acetate, 0.5 mM Na_2-EDTA, pH 8.0). The gels were made with denaturing gradient from 30 to 60%, where 100% of denaturant solution contains 7 M urea and 40% formamide. DGGE was run at 60°C for 3 hours and 200 V. After electrophoresis, the gel was soaked into glacial acetic acid 10% for 15 minutes, washed twice with ultra pure water (Milli Q system), followed by 15 minutes in methanol 50%, washed twice more, and then the final staining process with SYBR Green I nucleic acid gel dye (1:10,000 in 0.5 x TAE buffer, pH 8.0). The stained gels were photographed under UV translumination using the Quantity One computer program (BioRad).

Sequencing of predominant bands and single isolated colonies

Genomic DNA from predominant bands and isolated colonies were extracted and amplified using a set of primers 27-forward (5'-AGAGTTTGATCMTGGCTCAG-3') and 518-reverse (5'-ATTACCGCGGCTGCTGG–3'). Preparation of templates for sequencing was performed using CEQ Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter) according to the manufacturer protocol. Samples were sequenced in the Laboratory of Genetics and Molecular Microbiology in Australian Commonwealth Scientific and Research Organization (CSIRO). The sequence identification was performed using BLASTN from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/).

Results and discussion

A soil contaminated with PAHs indicated a reduction of bacterial diversity in previous

experiments when comparing with some agricultural soils (NAKATSU et al., 2000). Analyzing PCR-amplified 16S rDNA fragments of a bacterial biofilm community profile, the number of bands corresponded to the number of predominant members in the microbial community in DGGE. In a long-term contaminated environment, some species are liable to be dominated by those microorganisms able to survive in toxic contaminants, showing then less diversity but catabolically versatile bacterial community than those in non-impacted environment (LINDSTROM et al., 1999). When the original PAH-impacted soil was inoculated in liquid medium and/or nutrients (at day-zero), genomic DNA were also extracted, purified, amplified and loaded in the gels (Figures 1 to 4, lines 1, 6 and 11), showing two prominent bands as a initial soil profile. The presence of two dominant bacteria was verified prior to the incubation experiments in PAHs. Comparison of DGGE profiles of the microbiota 16S rDNA PCR amplification products has indicated absence of a high diversity of species in the PAH-contaminated soil; however, intensity of bands suggests that these species were abundant enough to be detected. A microbial population monitored in a field experiments to evaluate bioremediation of crude oil and all gram-negative bacteria showed evidence of environmental stress, even having strong bands at the initial day (VENOSA et al., 1999). Microorganisms have a wide range of responses to environmental stress, and changes of the population level include selection for more resistant species with a concomitant change in overall diversity (FORD, 2000).

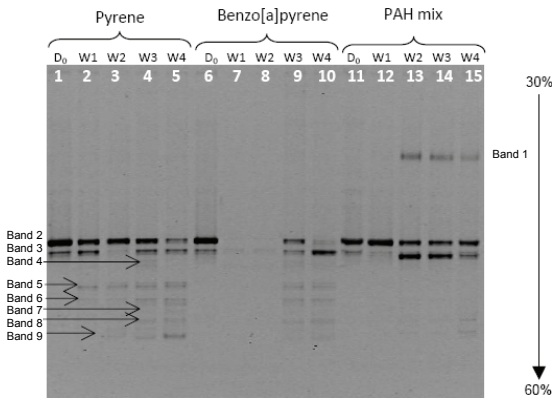

Figure 1. DGGE profiles of 16S rDNA amplicons of *Bacteria* domain obtained from PAH-contaminated soil incubated in minimal medium plus pyrene (lines 1-5); benzo[a]pyrene (lines 6-10); and mix of PAHs (lines 11-15) for 4 weeks (W1-W4). 'Day-zero' (D_0) is shown in lines 1, 6 and 11.

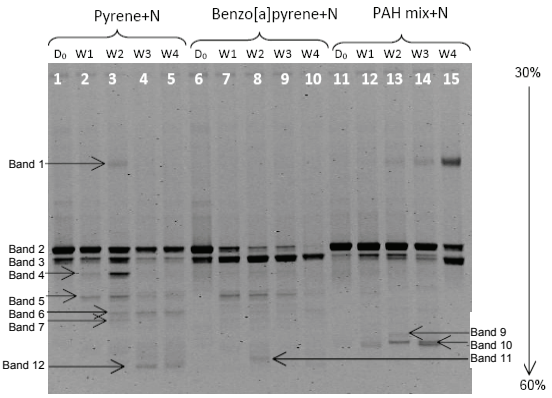

Figure 2. DGGE profiles of 16S rDNA amplification products from PAH-contaminated soil bacterial communities growing in minimal medium plus nutrients (vitamins and microelements) in pyrene (lines 1-5); benzo[a]pyrene (lines 6-10); and mix of PAHs (lines 11-15) for 4 weeks (W1-W4). 'Day-zero' (D_0) is shown in lines 1, 6 and 11.

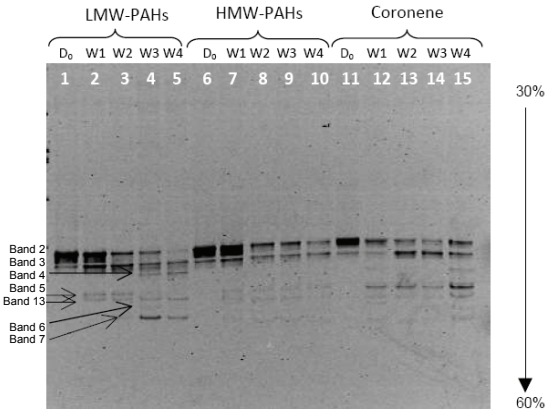

Figure 3. DGGE gel of 16S rDNA fragments of *Bacteria* domain obtained from PAH-contaminated soil incubated in minimal medium plus LMW-PAHs (lines 1-5); HMW-PAHs (lines 6-10); and coronene (lines 11-15) for 4 weeks (W1-W4). 'Day-zero' (D_0) is shown in lines 1, 6 and 11.

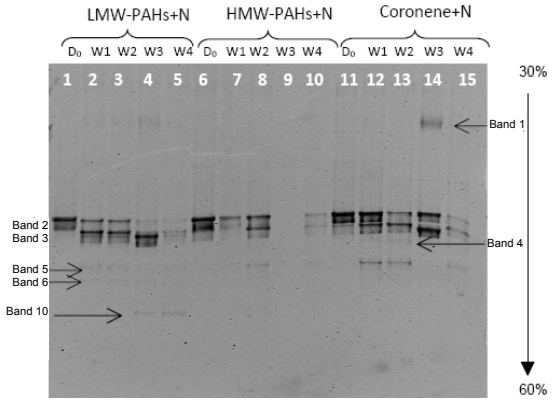

Figure 4. DGGE profiles of 16S rDNA fragments from PAH-contaminated soil bacterial communities incubated in minimal medium plus nutrients (vitamins and microelements) in LMW-PAHs (lines 1-5); HMW-PAHs (lines 6-10); and coronene (lines 11-15) for 4 weeks (W1-W4). 'Day zero' (D_0) is shown in lines 1, 6 and 11.

Analyzing soil microbiota in pyrene (Figures 1 and 2, lines 1 to 5), new bands were detected during the 4-week period, starting in the early week-1, suggesting adaptation of other species into soil able to grow using pyrene as carbon source. Band 2 showed less intensity during this period, probably by the action of new competitive microorganisms (bands 4 to 9), which were in less quantity before exposing soil with fresh PAH. Results from soil microbiota in benzo[a]pyrene (lines 6 to 10) indicated that the presence of this PAH caused a considerable impact into the community, once a very weak intensity of bands 2, 3 and 4 were visualized at weeks 1 and 2, showing a re-adaptation of these species from the week-3 on when previous predominant bands from day-zero are present again; however, specie correspondent to band 3 showed some predominance into the degradation process. When a mixture of PAHs was used as carbon source for the soil microbiota, bands from 'day zero' in this treatment were still predominant through the 4-week period. Nevertheless, the bands present in previous treatments were no longer showing defined bands (lines 11 to 15).

In the treatments performed with pyrene, benzo[a]pyrene and a mixture of PAHs plus trace elements and vitamins (Figure 2), some differences have also been shown on DGGE profiles. Microbiota incubated for a 2-week period in pyrene (line 3) showed the presence of band 2 and more predominance of band 4, suggesting facility to grown and survive into the medium when supplied with these nutrients, showing certain dependence to the production and activation of catabolic enzymes responsible for degradation of pyrene. Bands 5, 6 and new specie on band 12 remained in the pyrene profile up to 4 weeks (lines 1 to 5).

Results from soil microorganisms in benzo[a]pyrene plus nutrients (lines 6 to 10) indicated microbiota suffering less impact in the beginning of incubation than in medium with no nutrient addition (Figure 1, lines 6 to 10), however, less bands was visualized in the profile to week-4 in medium plus nutrients. During the 4-week period, the behavior of microbiota was near similar to day-zero. Band 2 was no longer predominant, as also observed to experiments with this PAH and medium without nutrients, where as band 3 remained predominant during all period. It may be possible that vitamins and trace elements help a faster growth of microbiota and its adaptation in the presence of benzo[a]pyrene.

To experiments in a mixture of PAHs added by nutrients, further new bands (9 to 11 in lines 11 to 15) could indicate enough growth of some other species when nutrients were present into the media. Band 1 was present after week-2, as observed in previous results with no addition of nutrients.

Samples corresponding to soil microbiota in pyrene, benzo[a]pyrene and a mix of all PAHs were loaded in the same gel to following profiles comparison of microbiota dynamic when using only pyrene - the most 4-ring degradable PAH by microorganisms served as a model to HMW-PAH studies, benzo[a]pyrene - the most toxic and mutagenic PAH with high resistance of biodegradation) and a mixture of several PAH – with probable metabolic cooperation and co-metabolic growth in the microbial community. When the soil microbiota was growing in pyrene, it seemed that in this period, a co-metabolic relationship has been expressed in gel due to the raise of some organisms using probably products of pyrene degradation generated by predominant bacteria in the first weeks. Regarding the microbiota performance into the mixture of PAHs (Figure 1), one additional specie was detected at week-2 (band 1), remaining on all period and suggesting ability to survive in several complex PAHs under cometabolism, probably growing by using intermediate metabolites as carbon source produced by other microorganisms when degrading PAHs. A microbial community isolated from contaminated soil could achieve more complete degradation of contaminants due to its ability to use byproducts of degradation as carbon source (RAMIREZ et al., 2001). Some species may not be affected by nutrients addition in pyrene, benzo[a]pyrene and a mixture of PAHs, however, some other bacteria showed more predominance intensively growing in the presence of nutrients; the appearance of new bands on the bottom of the gel have been detected as better performance of co-metabolism in a cooperative metabolization of PAHs.

Relating to the results from the HMW-PAHs medium, some LMW-PAHs can be added in the medium containing HMW-PAHs and induce their metabolism by activating metabolic common pathways, or stimulating co-metabolism by the use of some metabolic intermediate products from LMW-PAHs as carbon source to some other bacteria that will degrade HMW-PAHs, however, not using them to grow (BOONCHAN et al, 2000; MARCOUX et al., 2000; GAUTHIER et al., 2003). It is necessary to understand the whole microbial ecology, including microorganisms that are not responsible for the degradation, once they might influence the behavior of the degrading bacteria through microbial interaction (SEI et al., 2004).

The microbial community profiles in a mixture of LMW-, HMW-PAH and coronene individually (Figure 3) have indicated the same bands 2 and 3

verified in pyrene, benzo[a]pyrene and the mix of PAHs as predominant in the soil microbiota. In experiments with LMW-PAHs, an additional band 13 has appeared in the profile, remaining up to week-4 (lines 1 to 5). The dominant bands became weakly detected, specially band 2, indicating that such bacteria populations may receive some inhibition when phenanthrene and fluorine are used as carbon sources together, however, some other bacteria have developed some growth adaptation in the presence of these LMW-PAHs.

When the soil microbiota was growing in only HMW-PAHs, dominance of bands 2 and 3 has decreased, and any other band showed good resolution during the incubatio period. It is likely that the presence of only HMW-PAHs in the medium did not induce their degradation. In some other experiments it was verified that mainly HMW-PAHs were significantly degraded when nutrients were not added (VIÑAS et al., 2005). These authors also suggest that there are complex interactions between bacteria and nutrient conditions into the medium, influencing the biodegradation ability of microbiota.

Profiles of microbiota in coronene (lines 11 to 15) have shown the gradual presence of bands during the period of cultivation as shown in lines 12 to 15, when a total of 7 bands were present in this last period of incubation (week-4), indicating a better performance of microbiota when using coronene as sole carbon source, comparing with. It is possible that microorganisms could be used sub-products of coronene degradation by predominant bacteria at 'day zero', confirming metabolic cooperation in the degradation of coronene. Profile in coronene has near similarity than in pyrene in the same condition. In this case, the high number of aromatic rings of coronene did not show negative effect in the growth of soil microbiota, comparing with less complex PAHs.

The use of nutrients in media containing soil microbiota in LMW-, HMW- PAHs and coronene (Figure 4) did not show any other new bands and predominant bands from day zero have a notable decrease in their intensities in the end of the 4-week period (lines 5, 10 and 15). The diversity of bands has also decreased and profiles showed inhibition of some organisms indicated by very little intensity of bands in the presence of nutrients. The presence of some essential growth factors such as trace elements and vitamins (e.g. used as co-factor of several enzymes involved in the degradation of pollutants) is associated with such inhibition of the microbiota for unknown reasons. A co-metabolic transformation seemed to be possible and explain some new bands appearing in the course period after predominance of some other species responsible for the initial PAH degradation. The genus *Pseudomonas* has been the subject of much research as regards its ability to degrade PAHs, been identifies as one of the predominant band from original Bin#4 in treatment with PAH. The presence of some other species indicated likely existence of different mechanisms for assimilating PAHs in liquid culture, once bacterial community growing under nutrient limitation they adapt their catabolic enzyme activities in relation to the type of compounds found in the feed media (LAPARA et al., 2006). A dominance of *P. putida* G7 decreased according to DGGE profiles, and other dominant microbial species have appeared in results obtained elsewhere (PISKONEN et al., 2005). This fact could be confirmed in this present work regarding to the two predominant bands in day-zero. Sequencing of the two predominant bands in the DGG, corresponding to the most predominant colonies on agar plate, identified as *Pseudomonas stutzeri* (98% of similarity) showing morphological and genetical differences (data not shown). The long exposure period of contaminants in the soil (e.g. the MGP-soil impacted with high levels of PAHs and stored for several years) may indicate great levels of catabolic activity (LEE et al., 2003; REID et al., 2002). Further researchers are necessary to better understand the microbial interaction within PAH-degrading microbiota, the regulatory mechanisms of PAH degradation, and also the co-metabolization through several catabolic activities to degrade such compounds.

Conclusion

The soil microbiota was capable of growing in PAHs, and when analyzing DGGE of PCR-amplified 16S rDNA fragments, predominant organisms related to PAH degradation has been detected. DGGE proved to be a very important first step tool for obtaining information related to the dynamic of all treatments, in the original soil and when growing in PAHs. The addition of nutrients could both increase the intensity of more dominant bands and also decrease some bands intensity and inhibit some other bands expression, depending on the presence of PAHs in different mixtures. Results have shown that contaminated soil microorganisms had different profiles in the degradative dynamic of PAHs when some essential factors of growth were added. Predominant species may play a significative role while growing and surviving on PAHs, and some other metabolically active species have emerged to interact themselves in a cooperative catabolism of PAHs.

References

BOONCHAN, S.; BRITZ, M. L.; STANLEY, G. A. Degradation and mineralization of high-molecular-weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures. **Applied and Environmental Microbiology**, v. 66, n. 3, p. 1007-1019, 2000.

BOUCHEZ, M.; BLANCHET, D.; VANDECASTEELE, J. P. Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain associations:inhibition phenomena and cometabolism. **Applied Microbiology and Biotechnology,** v. 43, n. 1, p. 156-164, 1995.

CANET, R.; BIRNSTINGL, J. G.; MALCOLM, D. G.; LOPEZ-REAL, J. M.; BECK, A. J. Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by native microflora and combinations of white-rot fungi in a coal-tar contaminated soil. **Bioresource Technology**, v. 76, n. 5, p. 113-117, 2001.

FORD, T. E. Response of marine microbial communities to anthropogenic stress. **Journal of Aquatic Ecosystem Stress and Recovery**, v. 7, n. 1, p. 75-89, 2000.

GAUTHIER, E.; DÉZIEL, E.; VILLEMUR, R.; JUTEAU, P.; LÉPINE, F.; BEAUDET, R. Initial characterization of new bacteria degrading high-molecular eright poycyclic aromatic hydrocarbons isolated from a 2-year enrichment in a two-liquid-phase culture system. **Journal of Applied Microbiology**, v. 94, n. 1, p. 301-311, 2003.

JUHASZ, A. L.; STANLEY, G. A.; BRITZ, M. L. Microbial degradation and detoxification of high molecular weight polycyclic aromatic hydrocarbons by *Stenotrophomonas maltophilia* strain VUN 10,003. **Letters in Applied Microbiology**, v. 30, n. 5, p. 396-401, 2000.

KANALY, R. A.; HARAYAMA, S. Bioregradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. **Journal of Bacteriology**, v. 182, n. 8, p. 2059-2067, 2000.

LAPARA, T. M.; KLATT, C. G.; CHEN, R. Adaptations in bacterial catabolic enzyme activity and community structure in membrane-coupled bioreactors fed simple synthetic wastewater. **Journal of Biotechnology**, v. 121, n. 1, p. 368-380, 2006.

LEE, P. H.; DOICK, K. J.; SEMPLE, K. T. The development of phenanthrene catabolism in soils amended with transformer oil. **FEMS Microbiology Letters**, v. 228, n. 2, p. 217-223, 2003.

LINDSTROM, J. E.; BARRY, R. P.; BRADDOCK, J. F. Long-term affect of microbial communities after a subartic oil spill. **Soil Biology and Biochemistry**, v. 31, n. 12, p. 1677-1689, 1999.

MARCOUX, J.; DÉZIEL, E.; VILLEMUR, R.; LÉPINE, F.; BISAILLON, J. G.; BEAUDET, R. Optimization of high-molecular-weight polycyclic aromatic hydrocarbons degradation in a two-liquid-phase bioreactor. **Journal of Applied Microbiology**, v. 88, n.1, p. 655-662, 2000.

MISHRA, S.; JYOT, J.; KUHAD, R. C.; LAL, B. Evaluation of inoculum addition to stimulate *in situ* bioremediation of oily-sludge-contaminated soil. **Applied and Environmental Microbiology**, v. 67, n. 4, p. 1675-1681, 2001.

NAKATSU, C. H.; TORSVIK, V.; OVREÅS, L. Soil community analysis using DGGE of 16S rDNA polymerase chain reaction products. **Soil Science Society of America Journal**, v. 64, n. 7, p. 1382-1388, 2000.

OVREÅS, L.; TORSVIK, V. Microbial diversity and community structure in two different agricultural soil communities. **Microbial Ecology**, v. 36, n. 1, p. 303-315, 1998.

PISKONEN, R.; NYYSSÖNEN, M.; RAJAMÄKI, T.; ITÄVAARA, M. Monitoring and accelerated naphthalene-biodegradation in bioaugmented soil slurry. **Biodegradation**, v. 16, n. 2, p. 127-134, 2005.

RAMIREZ, N.; CUTRIGHT, T.; JU, L. K. Pyrene biodegradation in aqueous solutions and soil slurries by Mycobacterium PYR-1 and enriched consortium. **Chemosphere**, v. 44, n. 5, p. 1079-1086, 2001.

REID, B. J.; FERMOR, T. R.; SEMPLE, K. T. Feasibility of using muchroom compost for the bioremediation of PAH-contaminated soil. **Environmental Pollution**, v. 118, n. 1, p. 65-73, 2002.

SEI, K.; INOUE, D.; WADA, K.; MORI, K.; IKE, M.; KOHNO, T.; FUJITA, M. Monitoring behavior of catabolic genes and change of microbial community structures in seawater microcosms during aromatic compound degradation. **Water Resources**, v. 38, n. 1, p. 4405-4414, 2004.

TORSVIK, V.; DAAE, F. L.; SANDAA, R. A.; OVREÅS, L. Novel techniques for analyzing microbial diversity in natural and perturbed environments. **Journal of Biotechnology**, v. 64, n. 1, p. 53-62, 1998.

VAN ELSAS, J. D.; DUARTE, G. F.; ROSADO, A. S.; SMALLA, R. K. Microbiological and molecular biological methods for monitoring microbial inoculants and their effects in the soil environment. **Journal of Microbiological Methods**, v. 32, n. 2, p. 133-154, 1998.

VENOSA, A. D.; STEPHEN, J. R.; MACNAUGHTON, S. J.; CHANG, Y.; WHITE, D. C. Microbial population changes during bioremediation of an experimental oil spill. In: BELL, C. R.; BRYLINSKY, M.; JOHNSON-GREEN, P. (Ed.). **Microbial biosystems**: new frontiers. Kentville: Atlantic Canada Society for Microbial Ecology, 1999. p.759-765.

VIÑAS, M.; SABATÉ, J.; ESPUNY, M. J.; SOLANAS, A. M. Bacterial community dynamics and polycyclic aromatic hydrocarbon degradation during bioremediation of heavily creosote-contaminated soil. **Applied and Environmental Microbiology**, v. 71, n. 1, p. 7008-7018, 2005.

Received on July 1, 2009.
Accepted on July 8, 2009.