INTRINSIC BIOREMEDIABILITY OF PETROLEUM HYDROCARBON CONTAMINATED SITES IN ROMANIA: DIVERISTY OF BACTERIAL COMMUNITY, CATECHOL DIOXYGENASE AND ALKANE-MONOOXYGENASE GENES

Tibor BENEDEK[1], István MÁTHÉ[2], András TÁNCSICS[3], Károly MÁRIALIGETI[4], Beáta ALBERT[5], Szabolcs LÁNYI[6]

Bacteriile endogene hidrocarbonoclastice joacă un rol important în procesul autoepurării solurilor contaminate cu diferite hidrocarburi. Prin intermediul sistemelor enzimatice microorganismele catalizează mineralizarea contaminanţilor în forme netoxice, cum ar fi CO_2 şi H_2O. Pentru evaluarea bioremedierii intrinseci este esenţială cunoaşterea structurii comunităţilor microbiene, precum şi numărul şi activitatea organismelor descompunătoare. Prin detectarea genelor funcţionale (C23O, C12O şi alkB) folosind primeri oligonucleotidici specifici, avem posibilitatea de a determina populaţia bacteriană care este activă în biodegradarea contaminanţilor. Metode convenţionale de microbiologie şi de biologie moleculară au fost folosite pentru realizarea lucrării.

Indigenous hydrocarbon-degrading bacteria play an important role in natural attenuation process of hydrocarbon contaminated soils. Through enzyme systems they catalyze the mineralization of contaminants into non toxic forms, into CO_2 and H_2O. In order to assess the intrinsic bioremediability is essential to know the microbial community structure, as well as the number and activity of degrading organisms. By detection of functional genes (C23O, C12O and alkB) using species-specific oligonucleotide primers we had the opportunity to elucidate the bacterial population that was actually active in biodegradation of contaminants. To achieve these goals conventional microbiological and molecular-biological methods were carried out.

Keywords: *alkB*, *alkM*, *C12O*, *C23O*, MNA, T-RFLP

[1] PhD student, Faculty of Applied Chemistry and Materials Science, University POLITEHNICA of Bucharest, Romania, benedektibor@sapientia.siculorum.ro
[2] Lecturer, Faculty of Sciences „Sapientia" University, Cluj-Napoca, Romania
[3] Lecturer, Szent István University, Gödöllő, Hungary
[4] PhD Eng., Faculty of Science, Eötvös Lóránd University, Budapest, Hungary
[5] Lecturer, Faculty of Sciences Miercurea Ciuc, Sapientia Hungarian University of Transylvania, Cluj-Napoca, Romania
[6] Prof., Faculty of Sciences Miercurea Ciuc, Sapientia Hungarian University of Transylvania, Cluj-Napoca, Romania

1. Introduction

In the environment numerous chemical compounds, arose from both natural and anthropogenic sources exert toxic effect to living beings, to biological systems. Huge development of oil- and gas industry, careless handling of petroleum derivatives contributed to the widespread pollution of soils and aquifers with hydrocarbon contaminants [1]. As a consequence of increased petroleum production and utilization of its derivatives the extension of hydrocarbon-polluted soils and aquifers through Romania is in growth [2, 3, 4].

Bioremediation is an attractive technology to decontaminate polluted environments. The use of microorganisms permits the breakdown of the offending contaminants (e.g. PAHs - polycyclic aromatic hydrocarbons; BTEX - benzene, toluene, ethylbenzene, xylenes) commonly found in different petroleum derivatives into harmless compounds via enzymatic activities [5]. One of the simplest strategies of bioremediation is the monitored natural attenuation (MNA) [6]. During MNA phylogenetic investigation of microbial communities by approaches targeting 16S rDNA, identification of catabolic genes involved in hydrocarbon degradation, determination of hydrocarbon-degrading bacterial counts and respiratory activity measurements are carried out. Together with identification of catabolic genes we have the opportunity to elucidate the structure of the microbial community that is actually functional in the contaminated environment [7]. Polymerase chain reaction (PCR) based methods are effective in detection of functional genes in the polluted environments [8].

Our aim was to investigate the intrinsic bioremediability of a crude oil contaminated soil sample, by detecting biodegradative functional genes encoding alkane-monooxygenase (*alkB, alkM*), catechol 1,2-dioxygenase (*C12O*) and catechol 2,3-dioxygenase (*C23O*) using PCR method. We also studied the diversity of microbial population of soil samples using conventional isolation methods and a cultivation independent molecular approach (T-RFLP - Terminal Restriction Fragment Length Polymorphism) [9]. Furthermore, the number and activity of indigenous hydrocarbon-degrading microorganisms was also determined using MPN (Most Probable Number) method and OxyTop flasks, respectively.

2. Experimental

2.1. Soil sampling

Crude oil contaminated soil sample (GBC) and a non-contaminated (GNC) one were collected in October 2010 from oil-field area of Ghelința, Covasna County, Romania. The hydrocarbon contaminated sample was taken from vicinity of an abandoned oil well from the upper 5 cm of the soil and placed into sterile

cotton-wool-plug closed glass bottles for microbiology analyses. Soil samples for molecular-biology analysis were stored at -20 °C while for microbiological investigations at 7 °C. Samples for chemical analysis were stored at 4 °C in bottles sealed with rubber septa.

Chemical analysis of soil samples was evaluated in accordance to European standard analytical techniques by the WESSLING Hungary Ltd. accredited laboratory.

2.2. Microbiological methods

2.2.1. Selection and isolation of oil-degrading soil microorganisms

Enrichment and selection of oil-degrading soil microorganisms from the contaminated soil sample was carried out. Therefore, 2 gram soil sample (GBC) was suspended in 98 ml BBH mineral salt (MS) solution (Bacto-Buschnell-Hass medium – 0.2 g $MgSO_4$, 0.02 g $CaCl_2$, 1 g KH_2PO_4, 1 g K_2HPO_4, 1 g NH_4NO_3, 0.05 g $FeCl_3$ x $6H_2O$, 1000 ml distilled water). Gasoline-BTX mixture (3:2; 10 g/L final concentration) as carbon source- and Tween 80 (1 g/L) as surfactant was added to MS media. After incubation (7 days, 23 °C) serial dilutions were made and plated on Nutrient Broth (NB) medium. Plates were incubated for 2 days at 27 °C.

2.2.2. MPN counts of total heterotrophic and hydrocarbon-degrading soil bacteria

MPN counts were performed in 96-well microtiter plates in two replicates. Serial dilutions of the soil suspensions were prepared in test tubes. The number of total heterotrophic soil bacteria was determined using NB liquid medium. For determination of oil-degrader counts 300 µl MS medium was used. After inoculation the MS medium was supplemented with resazurin indicator and 1 µl sterile gasoline as source of carbon. Plates were incubated for 4 days at 27 °C. Growth was evaluated visually based on turbidity of NB liquid medium, and on the basis of the colour change of the resazurin indicator.

2.2.3. Determination of microbial soil respiration

The microbial activity of contaminated soil was determined in 1 L bottles containing 50 g soil sample, a CO_2 trap system (50 ml 0.1 N KOH solution) and were closed with BOD measuring heads (OxyTop®-C controlled by the OxiTop® OC110 system, Germany). A non-contaminated microcosm was used as control. All microcosms were incubated at 27 °C for 30 days.

2.3. Molecular biological methods

2.3.1. Isolation of genomic and environmental DNA.

The DNA isolation of pure cultures was based on mechanical disruption of bacterial cells using a Retsch MM301 ball mill. Cells were suspended in 100 µl DEPC (diethylpyrocarbonate) treated water supplemented with sterile glass beads. Disruption of cell walls by shaking at 30 Hz for 2 minutes was followed by a centrifugation step at 10000 g for one minute. Supernatant of the samples were heated up to 99 °C and kept for 5 minutes at this temperature. A final centrifugation was carried out for 3 minutes at 10000 g.

Environmental DNA isolation from soil samples was performed by MoBio UltraClean™ Soil DNA Isolation Kit (MoBio Laboratories Inc., USA) according to the instructions of the manufacturer.

All DNA samples were stored at -20 °C. Suitability of DNA preparations for PCR was checked by 16S rDNA amplification using 27-F and 1378-R primers.

2.3.2. Identification of strains

Strains were identified using the 16S rRNA gene-based sequence comparison. The 16S rDNA PCR products were purified with *EZ-10* Spin Column PCR Purification Kit (Bio Basic Inc., Canada) and used as template for sequencing reactions. The nucleotide sequence determination was performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and sequences were analyzed with ABI 3100 Genetic Analyzer (Applied Biosystems, USA). The obtained sequences were aligned against known sequences of the EzTaxon database [10]. Sequence similarity over 97% was accepted as species level identification.

2.3.3. Microbial diversity determination

A cultivation-independent method, T-RFLP was used for assessing the diversity of complex communities in soil samples. After isolation of DNA from the microbial community the 16S rRNA gene was amplified using fluorescently-labeled 27HEX forward and 519 reverse primers. Subsequently, the obtained PCR products were digested with 1U *Alu*I and *Msp*I restriction enzymes overnight at 37 °C thereby generating fluorescently-labeled terminal restriction fragments (T-RFs). For purification of obtained DNA fragments ethanol precipitation method was used [11]. Fragments were separated by capillary gel electrophoresis and T-RFs were detected using an ABI 310 Genetic Analyzer (Applied Biosystems, USA). The T-RFLP chromatograms were analyzed with the GeneMapper® Software v3.7 (Applied Biosystems, USA). Only runs with total peak areas between 200,000 and 400,000 fluorescent units were accepted. For consensus profiles runs were aligned with the T-Align program [12] using 0.5 bp confidence

interval omitting peaks below 0.2% relative abundance. The resulted matrix was visualized with PCA (Principal Component Analysis). Diversity indices were also calculated. Statistical analyses (PCA) and calculation of diversity indices were carried out using the PAST (Paleontological Statistics) software package [13].

2.3.4. Detection of functional genes (alkB, alkM, C23O, C12O)

Previously published primer sets were used to detect *C12O* and *C23O* genes from genomic and environmental DNA samples (Table 1).

Table 1

Primer sets for functional gene amplifications from environmental and genomic DNA

Targeted Gene	Primer pair	Sequence	Annealing T(°C)	Amplicon size (bp)	Ref.
C23O	XYLE1-F XYLE1-R	5'-CCGCCGACCTGATC(A/T)(C/G)CATG-3' 5'-TCAGGTCA(G/T)CACGGTCA(G/T)GA-3'	61.5	250	[15]
C23O	XYLE2-F XYLE2-R	5'-GTAATTCGCCCTGGCTA(C/T)GTICA-3' 5'-GGTGTTCACCGTCATGAAGCG(C/G/T)TC-3'	63.5	900	[15]
C23O	COM-F COM-R	5'-CGAGAACGTGCTGGGCATGAAG-3' 5'-AAGGCGATGTCGTGCGGC-3'	63	550	[16]
C23O	UNIV-F UNIV-R	5'-AAGAGGCATGGGGGCGCACCGGTTCGATCA-3' 5'-CCAGCAAACACCTCGTTGCGGTTGCC-3'	55	380	[17]
C12O	RHO-F RHO-R	5'-GCCGCCACCGACAAGTT-3' 5'-CACCATGAGGTGCAGGTG-3'	56	630	[18]
C12O	C12O-F C12O-R	5'-GCCAACGTCGACGTCTGGCA-3' 5'-CGCCTTCAAAGTTGATCTGCGTGGT-3'	57	282	[17]
C12O	AcC12O-F AcC12O-R	5'-ACACCACGAACAATTGAAGGACCG-3' 5'-TGCGAAGGGCGGTTACCATG-3'	57	450	This study
alkB	RalkB-F RalkB-R	5'-TACTACCGGTACTGCACCTAC-3' 5'-CCGTA(A/G)TG(C/T)TCGAGGTAGTT-3'	54	595	This study
alkM	AcalkM-F AcalkM-R	5'-CATCA(C/T)AAGCGTGC(A/C)GC-3' 5'-TTCCAGCTATGCTCTGGCAT-3'	54	340	This study
16SrRNA	27-F 1378-R 519-R	5' AGAGTTTGATCMTGGCTCAG 3' 5'CGGTGTGTACAAGGCCCGGGAACG 3' 5' ATTACCGCGGCTGCTGG 3'	52	1460/500	[19] [20] [19]

In order to detect *C12O* and *alkM* genes related to the genus *Acinetobacter* and *alkB* genes related to genera *Rhodococcus* new primer pairs were designed in this study. For development of *Rhodococcus* related RalkB- and *Acinetobacter* related AcalkM-primers following sequences were retrieved from GeneBank (accession numbers given in parentheses): *Rhodococcus opacus* B4 (NC_012522.1); *Rhodococcus jostii* RHA1 (CP000431.1), *Rhodococcus sp.* BCP1 (HM771646.1), *Rhodococcus erythropolis* PR4 (NC_012490.1); *Acinetobacter calcoaceticus* (AJ233398.1); *Acinetobacter sp.* ADP1 (NC_005966.1); *Acinetobacter baumannii* SDF (NC_010400.1); *Acinetobacter haemolyticus* (AY586401.3); *Acinetobacter sp.* MUB1 (EF080995.1). Obtained sequences were subjected to multiple alignments using the ClustalW algorithm and the *MEGA 5*

software [14]. Conserved regions were selected to design oligonucleotide primers for detection of genes.

2.3.5. PCR amplifications

All reactions took place in a 50 µl reaction mixture containing 1 U of Dream*Taq* polymerase (Fermentas, Lithuania), 1x Dream*Taq* buffer with $MgCl_2$ (2 mM), 0.2 mM of each deoxynucleoside triphosphate, 0.3 µM of each primer, and 1 µL of template DNA on a GeneAmp PCR System (Model 2400, Applied Biosystems, USA).

All PCR amplifications started with an initial denaturation step at 95 °C (3 min), followed by 32 cycles of 94 °C (0:30 min), annealing temperature given in Table 1 for 30 seconds, and 72 °C (1 min). The amplification was finished with an additional final extension for 10 minutes at 72 °C.

3. Results and Discussions

3.1. Soil description

The crude oil contaminated soil contained enormously high quantities of total petroleum hydrocarbons (TPH_{C5-C40} – 147,000 mg kg_{soil}^{-1}). Chromatography analysis indicated the presence of priority PAH pollutants, namely anthracene (1.50 mg kg_{soil}^{-1}), phenanthrene (12.4 mg kg_{soil}^{-1}), pyrene (2.98 mg kg_{soil}^{-1}), benzo[*ghi*]perylene (0.51 mg kg_{soil}^{-1}), benzo[*e*]pyrene (4.08 mg kg_{soil}^{-1}), benzo[*k*]fluoranthene (0.16 mg kg_{soil}^{-1}), benzo[*b*]fluoranthene (1.74 mg kg_{soil}^{-1}) and indeno[*1,2,3-c,d*]pyrene (0.26 mg kg_{soil}^{-1}). Concentration of total PAHs (41.5 mg kg_{soil}^{-1}) and BTEX compounds (< 5.48 mg kg_{soil}^{-1}) were under the intervention threshold limits specified by Ministry of Waters and Forest and Environmental Protection of Romania for industrial areas (Order Nr. 756, 3 November 1997).

3.2. Identification of hydrocarbon-degrading microorganisms

After enrichment and selection steps 11 strains were isolated from crude-oil contaminated sample according to their different morphologies. In order to classify this isolates, 16S rDNA sequencing was performed. Results (**Fig. 1**) revealed that most of the cultivable bacterial isolates belonged to *γ-Proteobacteria* (*Pseudomonas panipatensis, Pseudomonas taiwanensis, Acinetobacter calcoaceticus, Serratia nematodiphila, Stenotrophomonas acidaminiphila*). Representatives of the *β-Proteobacteria* (*Comamonas testosteronii, Pandoraea norimbergensis*) were also found beside the members of classes *Bacilli* (*Bacillus aerius*), *Actinobacteria* (*Micrococcus luteus*) and *Flavobacteria* (*Chryseobacterium hungaricum*).

Mainly the members of *Proteobacteria* (*α-,β-,γ- Proteobacteria*) class were isolated from polluted sample, whose hydrocarbon-degrading capacity is

well investigated in earlier studies [21, 22]. This fact is in correspondence with results of Kumar and Khanna (2010), who determined the bacterial community structure of a coal-tar-contaminated soil by establishing a clone library of 16S rRNA genes. They found that *Actinobacteria* and *Gammaproteobacteria* were the most predominant classes in contaminated sample [23]. Furthermore, Bakermans and Madsen (2002) also showed the presence of *Betaproteobacteria* in coal-tar-waste-contaminated aquifer [20].

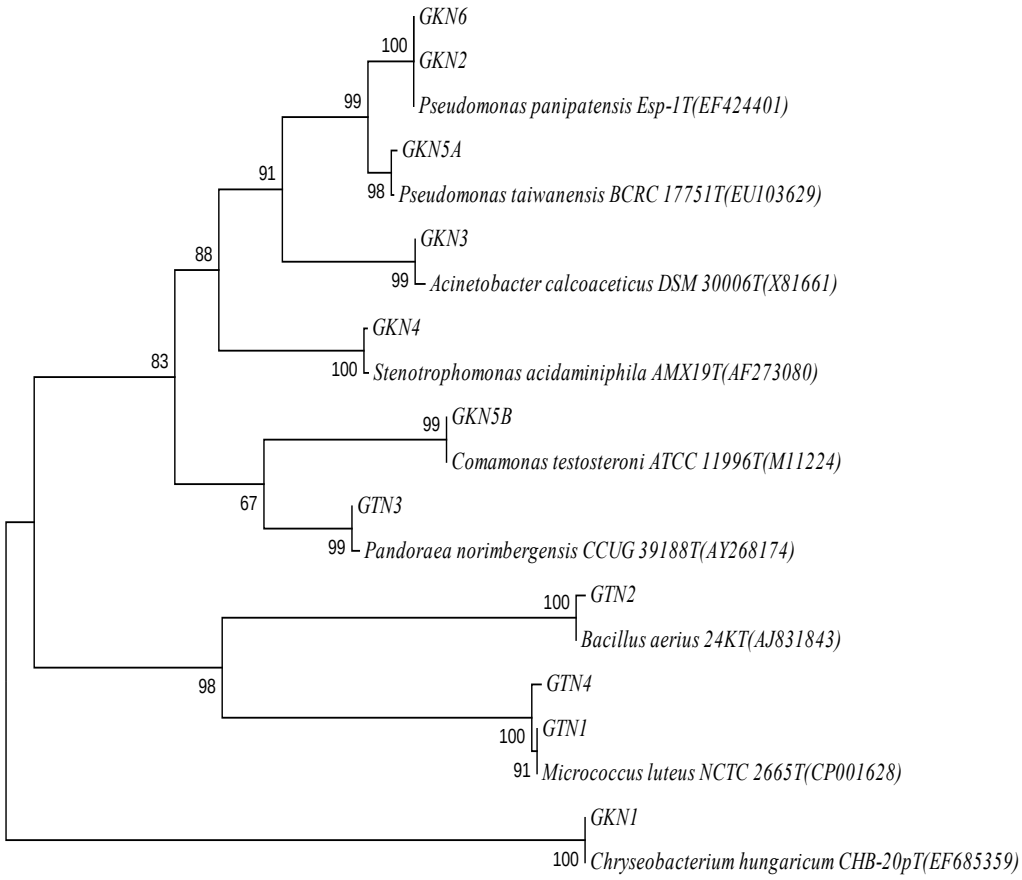

Fig. 1. Phylogenetic tree of microorganisms isolated from contaminated soil samples. The phylogenetic tree was generated by the neighbor-joining method using *MEGA 5* software. Number of bootstrap replications was 1000

3.3. Microbial counts and activity of soil microorganisms

Total heterotrophic bacterial counts in contaminated and non-contaminated samples were identical ($3 \cdot 10^5$ CFU g_{soil}^{-1}). The number of

hydrocarbon-degrading microorganisms in GBC sample ($3 \cdot 10^5$ CFU g_{soil}^{-1}) was higher with three orders of magnitude than in GNC sample ($2.5 \cdot 10^2$ CFU g_{soil}^{-1}).

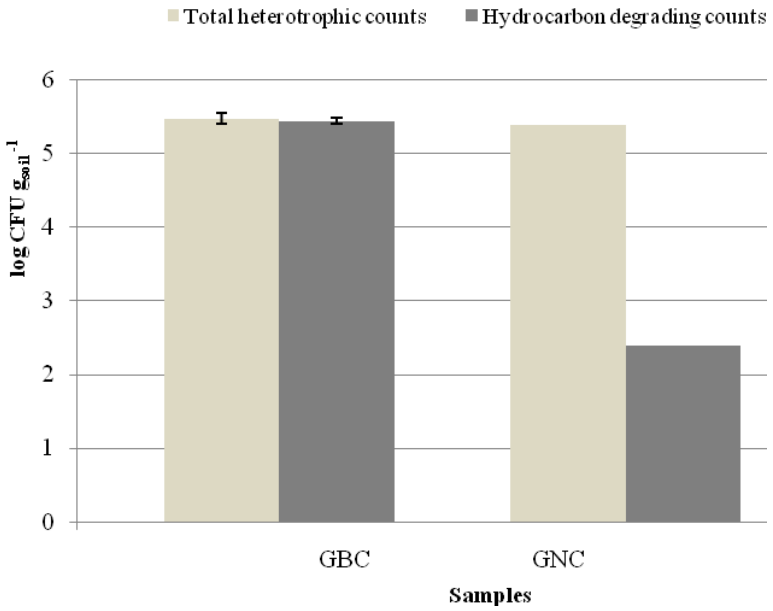

Fig. 2. Results of hydrocarbon-degrading and total heterotrophic bacterial count determination (CFU-Colony Formation Unit; GBC-Contaminated sample from Ghelința; GNC-Non-contaminated sample)

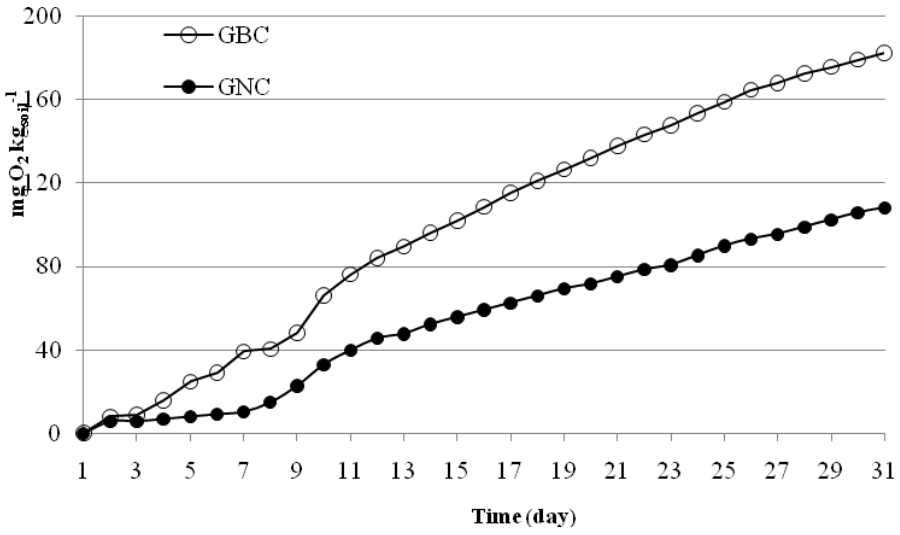

Fig. 3. O_2 consumption profiles of non-contaminated (GNC) and hydrocarbon contaminated (GBC) soils from Ghelința

Furthermore, in GBC sample the numbers of total cultivable- and hydrocarbon-degrading bacterial counts were equal (Fig. 2).

Fig. 3 presents the cumulative O_2 consumptions of two microcosms. GNC sample displayed a lag-phase of 7 days during which consumption of oxygen increased slightly. An exponential increase in O_2 consumption was observable in GBC sample starting from the first day of experiment, probably due to the biodegradation of pollutants. By the end of experiment GBC sample showed the largest O_2 consumption (182 mg O_2) in contrast with GNC (108 mg O_2). The higher O_2 consumption in GBC sample is most possibly due to the hydrocarbon-degrading organisms which could use the hydrocarbon pollutants as a source of carbon and energy.

Microbial germ counting and respiratory activity measurement results suggest that within GBC sample the endogenous bacteria adapted to the polluted environment and are able to proliferate under these circumstances.

3.4. Microbial diversity determination (T-RFLP)

Based on the PCA and cluster analyses of T-RFLP runs it can be concluded that there was no representative difference between the microbiota of the investigated soils (data not shown). On basis of the Shannon-Weaver index GBC sample had the lowest diversity, compared to the GNC sample, probably due to the selective environment caused by the high pollution with hydrocarbons (Table 2).

Table 2

Diversity indices of investigated soils calculated on basis of T-RFLP chromatograms

Diversity indices	GBC	GNC
Shannon-Weaver	3.018	3.263
Simpson	0.9226	0.9194

T-RFLP results revealed that GNC and GBC samples share common species like *Pandoraea norimbergensis*, *Micrococcus luteus*, *Chryseobacterium hungaricum*. *Stenotrophomonas acidaminiphila* was found only in GBC sample.

3.5. Functional gene abundances

Functional gene-targeted analyses were performed on the GBC and GNC environmental DNA samples, as well as on genomic DNA of some isolated strains.

PCR amplification with primer sets showed in Table 1 yielded no products from environmental DNA samples. PCR amplification of *Pseudomonas* related *C23O* genes with *XYLE1* primer pair exhibited amplicons of expected size in *Pseudomonas panipatensis* GKN2 and GKN6 strains, as well as in *Pseudomonas taiwanensis* GKN5A strain. Positive PCR amplification was obtained of

Acinetobacter calcoaceticus GKN3 related *C12O* and *alkM* gene segments when amplified with *AcC12O* or *AcalkM* primer pairs designed in this study.

The expected band of 550 bp was gained after PCR amplification of *Comamonas testosteroni* GKN5B related *C23O* gene when using *COM* primer set. Amplification of *C23O* and/or *C12O* related genes in *Acinetobacter* and *Pseudomonas* isolates, when using primer sets published by Sei et al. (1999) exhibited PCR-negative results. Although, *RalkB* primer set gave negative results in environmental *alkB* gene detection, amplified the expected 595 bp DNA fragment in previously published [4] *Rhodococcus* isolates.

4. Conclusions

Although, samples showed the same count of total heterotrophic bacteria, the number of hydrocarbon-degrading microorganisms differed remarkably. In GBC contaminated sample the hydrocarbon-degrading counts were higher with three orders of magnitude than in non-contaminated one.

The increased microbial activity in GBC sample might be due to the degradable carbon sources in the form of pollutants which were used as energy source in metabolic pathways of endogenous microbiota.

Most of the isolated strains from GBC sample belonged to the classes of *β-, γ-Proteobacteria*, whose hydrocarbon-degrading capacities are well known. The representatives of these classes possessed *C23O* or *C12O* functional genes responsible in "*meta*" and "*orto*" cleavage of aromatic rings.

The results of catechol 1,2-dioxygenase and alkane-monooxygenase related gene detection confirmed the suitability of *AcC12O*, *AcalkM* primer sets designed in this study for PCR detection of biodegradative genes in *Acinetobacter* species. Applicability of *RalkB* primer set for detection of *Rhodococcus* related *alkB* genes was also supported by PCR-positive results in pure *Rhodococcus* cultures.

Although, we were able to isolate potential hydrocarbon-degrading organisms functional gene detection from environmental DNA samples was not successful. This fact might be explained with the low number of expected species (*Rhodococcus* spp., *Acinetobacter* spp., *Pseudomonas* spp., *Comamonas* spp., *Sphingomonas* spp.) and suggests the occurrence of other hydrocarbon-degrading organisms.

In order to enhance the intrinsic bioremediation of contaminated site biostimulation (N-, P addition, aeration) of endogenous microorganisms is recommended.

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