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ORIGINAL PAPER

Remarkable impact of PAHs and TPHs on the richness and diversity of bacterial species in surface soils exposed to long-term hydrocarbon pollution

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Abstract Nowadays, because of substantial use of petroleum-derived fuels the number and extension of hydrocarbon polluted terrestrial ecosystems is in growth worldwide. In remediation of aforementioned sites bioremediation still tends to be an innovative, environmentally attractive technology. Although huge amount of information is available concerning the hydrocarbon degradation potential of cultivable hydrocarbonoclastic bacteria little is known about the in situ long-term effects of petroleum derived compounds on the structure of soil microbiota. Therefore, in this study our aim was to determine the long-term impact of total petroleum hydrocarbons (TPHs), volatile petroleum hydrocarbons (VPHs), total alkyl benzenes (TABs) as well as of polycyclic aromatic hydrocarbons (PAHs) on the structure of bacterial communities of four different contaminated soil samples. Our results indicated that a very high amount of TPH affected positively the diversity of hydrocarbonoclastic bacteria. This finding was supported by the occurrence of representatives of the α-, β-, γ-Proteobacteria, Actinobacteria, Flavobacteriia and Bacilli classes. High concentration of VPHs and TABs contributed to the predominance of actinobacterial isolates. In PAH impacted samples the concentration of PAHs negatively correlated with the diversity of bacterial species. Heavily PAH polluted soil samples were mainly inhabited by the representatives of the β-, γ-Proteobacteria (overwhelming dominance of *Pseudomonas* sp.) and Actinobacteria.

Keywords PAHs · TPHs · Bacterial diversity · Canonical correspondence analysis · PCA · T-RFLP

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Introduction

Gasoline and diesel fuels are the most widely used, essential fossil fuel products. Accidental spillages during transportation, leakages from storage units or pipelines led to the widespread pollution of terrestrial and aquatic ecosystems with toxic aliphatic hydrocarbons (e.g. TPHs), alkyl-benzenes (e.g. BTEX-benzene, toluene, ethyl-benzene, xylenes), VPHs and PAHs (Philp and Atlas 2005; Pavelescu et al. 2008). Among the aforementioned chemicals the removal of genotoxic BTEX and PAH compounds from the environment is of the greatest importance (Haritash and Kaushik 2009; Gonul and Kucuksezgin 2012). Bioremediation is the only sustainable way to achieve their elimination. In laboratory conditions the biodegradation of TPHs, as well as of BTEX by naturally occurring microorganisms is widely demonstrated (Mazzeo et al. 2010;

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Singh and Celin 2010). In contrast, it has been found that the number of bacterial taxonomic groups which perform the degradation of PAHs in soils is limited. Sphingomonads proved to be the most common PAH-degrading isolates (Johnsen 2005; Takeuchi et al. 2001). Besides, PAH biodegradation ability of members of the genera *Corynebacterium*, *Aeromonas*, *Rhodococcus* and *Bacillus* has also been reported (Mrozik et al. 2003).

Notwithstanding that the biodegradation of hydrocarbons in laboratory conditions is well studied scarce information is available regarding their long-term impact on the diversity and structure of the bacterial population in situ. In order to achieve an efficient in situ bioremediation is essential to know how different xenobiotic compounds affect the endogenous bacterial population in their natural habitats. Recent studies have demonstrated that the presence of PAHs exerts negative effect on the diversity of soil microbial population. Zhou et al. (2009) found that both the exposure time and the PAH concentration (even at a concentration as low as 20 mg l^{-1}) reduced the microbial diversity in mangrove sediments. Although the presence of PAHs reduces the bacterial diversity their selective pressure may promote the emergence of distinctive species with increased PAH degradation ability (Ben Said et al. 2010; Thavamani et al. 2012).

Fingerprinting techniques such as terminal restriction fragment length polymorphism (T-RFLP) (Liu et al. 1997) and denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993) enable us to detect differences in the structure and diversity of microbial communities of different environments. These whole-community fingerprinting approaches have been used recently successfully to study complex bacterial communities, to estimate the diversity and relative abundance of individual bacterial species within detectable bacterial communities (Hewson and Fuhrman 2004; Fedi et al. 2005; Sipilä et al. 2008; Iannelli et al. 2012; Vajna et al. 2012). In addition, using traditional selective cultivation dependent microbiological methods we are able to isolate site specific hydrocarbonoclastic bacterial strains which could form the basis of a new bacterial inoculant for bioremediation purposes.

The present work aims (i) to determine how long-term presence of different hydrocarbon pollutants (TPHs, alkyl-benzenes, VPHs and PAHs) influence the in situ diversity of bacterial communities of four geographically distant, non-linked soils; (ii) to assess relationships between the concentration- and type of hydrocarbon pollutants and the structure of microbial communities by using multivariate statistical analyses and, (iii) to obtain a site specific hydrocarbon-degrading bacterial strain collection which may be applicable in subsequent bioremediation attempts.

Materials and methods

Soil sampling and chemical analyses

Soil samples (300 g, thoroughly mixed representative samples from three distinct points of the sampling area) showing varying degree of contamination with TPHs, TABs, VPHs and PAHs were obtained from four different sites in central Romania. Contaminated samples were taken in the vicinity of former diesel-oil storage units from Sândominic (46°35′0″N/25°47′0″E) and Bălan (46°38′59″N/25°48′36″E); in vicinity of an abandoned oil well from Ghelinţa (45°56′35″N/26°14′28″E); and from the perimeters of a woodworking plant, polluted with gasoline and lubricating oils from Remetea (46°47′36″N/25°27′1″E). Pollution history of samples from Bălan, Sândominic and Ghelinţa is around 20 years. At sampling site from Remetea the hydrocarbon pollution is recurring due to the uncontrolled hydrocarbon spills during machinery maintenance. Samples serving as controls were taken from adjacent non-contaminated areas from the same soil types (Table 1). The soil type of studied areas from Bălan and Ghelinţa is lithosoil with low humus content and poor vegetation cover (<5 %). Sandy-loam textured, moderately acidic soils from Sândominic and Remetea are rich in humus. Due to the increased humus content these soils are more fertile and suitable for agricultural uses proven by the vegetation cover >50 %. Low phosphorous and potassium content values were recorded in Bălan and Remetea originated samples. In contrast, in samples from Sândominic and Ghelinţa these values were around the optimal levels. Soil samples with the lowest nitrogen content originated from Ghelinţa and Remetea (Table 1).

The top 10 cm of soil was sampled using a sterile spatula into sterile glass bottles (500 ml). Vessels were closed with cotton plugs for microbiological- and molecular microbiological analyses and with rubber septa for chemical analysis. Immediately after sampling, samples were stored at 4 °C using MobiCool Hybrid Thermoelektrik/Kompressor cool box (Conrad, De) and transported within 3 h to the laboratories. For classical microbiological and chemical analyses samples were further maintained at 4 °C (processing within 24 h), and at −20 °C for molecular biological investigations.

Chemical analyses of soil samples were performed by Hungarian Standard (MSZ) analytical techniques in an accredited laboratory (Wessling Hungary Ltd.). For determination of PAHs MSZ 21470-84 (2002) analytical standard and Hewlett Packard-6890 gas chromatograph coupled to mass spectrometry (HP-6890-GC/MS) was used. VPHs were detected in accordance with MSZ 21470-92 (1997) analytical standard using HP-6890-GC apparatus coupled with photoionization/flame ionization

Table 1 Designation and general characteristics of the investigated soil samples

Study site	Sample designation	Sample description	Soil characteristics within studied area (0–20 cm)								
			G.[a] (%)	Sa. (%)	Si. (%)	C. (%)	pH	H_T (%)	N_i	P_m (ppm)	K_m (ppm)
Bălan	BNCS	Bălan non-contaminated soil	80.2	10	7.2	2.5	7.1	1.3	1.2	3.2	21
	BCS	Bălan contaminated soil									
Ghelinţa	GNCS	Ghelinţa non-contaminated soil	70.9	17.6	6.4	4.9	5	0.5	0.4	13.6	96
	GCS	Ghelinţa contaminated soil									
Remetea	RNCS	Remetea Non-contaminated soil	28.3	25.9	20.8	24.8	5.7	6.2	0.3	1.4	8.5
	RCS	Remetea contaminated soil									
Sândominic	SNCS	Sândominic non-contaminated soil	27.6	29.8	20.4	22.2	5.4	3.4	1.5	10.6	100
	SCS	Sândominic contaminated soil									

[a] *Abbreviations G.* gravel, *Sa.* sand, *Si.* silt, *C.* clay, H_T total humus, N_i nitrogen index, P_m phosphorous mobile, K_m potassium mobile

(PID/FID) detector. The concentration of TPHs (C_5–C_{40}) and total alkyl-benzenes (including BTEX compounds) was determined by using MSZ 21470-92 (1998) analytical standard and HP-6890-GC PID/FID apparatus. The pedological characterization (soil texture, pH, humus-, K-, Ni-, and P content) of soil samples was carried out by Pedological and Agrochemical Research Institute, Harghita County (Romania), as described in our previous study (Máthé et al. 2012).

Isolation of hydrocarbon-degrading microorganisms

Isolation of hydrocarbonoclastic bacteria was performed after selection and enrichment steps in BBH (Bacto Bushnell-Haas) mineral mediums (George-Okafor et al. 2009) supplemented with diesel oil as sole carbon source, as described previously (Máthé et al. 2012). The selective enrichment media contained 99 ml BBH mineral broth medium amended with diesel oil (1 %, v/v) and were inoculated with 1 g of contaminated soil. As a second selection, after an incubation period (14 days, 23 °C), the enriched cultures were serially diluted and inoculated onto solid BBH agar plates. The lid of Petri-dishes contained 250 µl of sterile diesel-oil as sole carbon and energy source. Diesel oil was used because of its remarkable PAH and BTEX content (Irwin 1997). Colonies with different morphologies were selected as diesel-oil degrading strains and were maintained on standard Nutrient Agar (Sigma Aldrich, Germany).

Molecular biological methods

DNA extraction

Isolation of environmental DNA from soil samples (0.75 g wet weight) was carried out using MoBio UltraClean™ Soil DNA Isolation Kit (MoBio Laboratories Inc., USA) according to the instructions of the manufacturer. For DNA isolation of hydrocarbon-degrading pure cultures V-gene Bacterial Genomic DNA Kit (V-gene, China) was used following the manufacturer's protocol for Gram (+) bacteria.

PCR amplifications

All PCR amplifications were carried out in a 50 µl reaction mixture containing 1X DreamTaq™ buffer with $MgCl_2$ (2 mM), 0.2 mM of each of the four dNTP, 0.3 µM of each primer, 1 U DreamTaq™ DNA Polymerase (Fermentas, Lithuania), 1 µl DNA template and molecular-grade water up to 50 µl. The temperature profile used for all primer sets was an initial denaturation for 3 min at 95 °C, followed by 32 cycles of denaturation for 0.5 min at 94 °C, annealing for 0.5 min at 52 °C and elongation for 1 min at 72 °C. The last step was a final extension for 10 min at 72 °C. All amplifications were performed using ABI GeneAmp 2700 thermo cycler system (Applied Biosystems, USA).

Bacterial community analysis by using T-RFLP

From environmental DNA the 16S rRNA encoding genes were PCR amplified using fluorescent-labeled 27F forward (5′-AGA GTT TGA TCC TGG CTC AG-3′) primer with HEX label and 519R (5′-ATT ACC GCG GCT GCT GG-3′) reverse primer (Lane 1991). The obtained PCR amplicons were digested with 1U of *Alu*I restriction endonuclease overnight at 37 °C. The generated fluorescent-labeled terminal restriction fragments (T-RFs) were purified by ethanol precipitation method, separated and detected by capillary gel electrophoresis using an ABI 310 Genetic Analyzer (Applied Biosystems, USA). The applied internal lane size standard was GeneScan™ 500 TAMRA™ (Applied Biosystems, USA). T-RFLP electropherograms were analyzed with the GeneMapper® Software

v3.7 (Applied Biosystems, USA). Only runs with total peak areas between 200000 and 400000 fluorescent units were accepted. T-RFs representing less than 1 % of the total fluorescence were considered as background noise (Hewson and Fuhrman 2004). For generate consensus profiles, runs of two replicates were aligned with the T-Align program (Smith et al. 2005) using 0.5 bp confidence interval.

Identification of isolated strains

Hydrocarbon-degrading pure cultures were grouped with Amplified Ribosomal DNA Restriction Analysis (ARDRA; Massol-Deya et al. 1995) using *Msp*I and *Hin*6I restriction endonucleases. Representative isolates were identified with 16S rRNA gene-based sequence analysis. The nucleotide sequence determination of obtained PCR products was performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and sequences were analyzed with ABI 310 Genetic Analyzer (Applied Biosystems, USA). The resulted sequences were edited and assembled using MEGA4 (Tamura et al. 2007). BLAST homology searches (Altschul et al. 1997) were made in the GeneBank database. EzTaxon server 2.1 carried out the determination of the closest type strain (Chun et al. 2007).

The sequence data obtained in this study were deposited in EMBL database under the accession numbers HE801226-HE801275. For the sake of completeness, 16S rRNA gene sequences obtained from previously isolated hydrocarbonoclastic bacteria (Máthé et al. 2012; deposited under accession numbers FR877562-FR877577) were also included in phylogenetic tree reconstruction.

Phylogenetic tree construction of 16S rRNA gene sequences was carried out with MEGA4 software with Kimura 2-parameter model and 1000 bootstrap resamplings to estimate the reproducibility of the tree.

In silico restriction analysis

According to Caretta and Brito (2011) *in silico* analysis is an effective technique in T-RF identification of T-RFLP fingerprints, although errors of deletion or insertion during the capillary electrophoresis step must be taken into account. For genus level identification of characteristic T-RFs *in silico* restriction analysis of isolates derived 16S rRNA gene sequences was carried out using MEGA4 software. First, it was checked if all sequences are in the same direction. Subsequently the terminal recognition site (AG↓CT) of *Alu*I restriction endonuclease for each sequence and thus the size of its *in silico* T-RF was determined. The obtained *in silico* restriction fragment size of a given genus was matched with the size of the community T-RFs.

Petroleum-hydrocarbon degradation potential of isolated strains

Isolated, ARDRA group-representative strains were tested for their ability to degrade and utilize different petroleum hydrocarbons (benzene, toluene, xylenes, n-dodecane, naphthalene–Analytical Reagents-Merck, Germany) as sole carbon and energy source as described previously (Máthé et al. 2012).

Statistical analyses

Microbial community structure similarities based on T-RFLP profiles were determined with principal component analysis (PCA). Shannon–Weaver diversity index (*H'*) for each T-RFLP electropherogram was also calculated. In order to assess the correlation between the abundance of each T-RF and environmental variables (different hydrocarbon pollutants) canonical correspondence analysis (CCA) was carried out using MVSP 3.21 (Multivariate Statistical Package, Kovach Computing Services) software (Ben Said et al. 2010). Statistical comparison of bacterial communities of soil samples based on T-RFLP results was performed with two-sample T test using *PAST* software (Hammer et al. 2001).

Results

Contaminant parameters of samples

The amounts of different hydrocarbon contaminants (related to dry weight soil) for each sample are summarized in Table 2. The highest TPH concentration (147,000 mg kg^{-1} soil) was measured in sample GCS. Sample SCS contained the highest amounts of high molecular weight (HMW, 154.91 mg kg^{-1}) and low molecular weight (LMW, 229.74 mg kg^{-1}) PAHs. The highest concentrations of BTEX (2.91 mg kg^{-1}), of total alkyl-benzenes (21.2 mg kg^{-1}) as well as of VPHs (1100 mg kg^{-1}) were recorded in sample RCS. Sample BCS contained moderate amounts of TPH, VPH, alkyl-benzenes and PAHs. In non-contaminated samples the concentrations of TPHs were around 50 mg kg^{-1}, the amounts of alkyl-benzenes (TAB, BTEX) and PAHs were under 0.5 mg kg^{-1} dry weight soil (Table 2).

Versatility of cultivable aerobic hydrocarbon degrading bacteria

According to distinct colony morphologies the isolation procedures resulted in total 91 types of colonies from the four different sites. 14 types of colonies were obtained

Table 2 Concentration of hydrocarbon pollutants in contaminated and non-contaminated soil samples

Pollutant concentration (mg kg^{-1})	Investigated soil samples							
	BNCS	BCS	SNCS	SCS	RNCS	RCS	GNCS	GCS
VPH	<25	157	<25	298	<25	1100	34	221
TPH	52	16400	<50	25100	50	18200	34	147000
Benzene	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	0.05
Toluene	<0.05	<0.05	<0.05	<0.05	<0.05	0.11	<0.05	<0.05
Ethyl-benzene	<0.05	<0.05	<0.05	<0.05	<0.05	0.45	<0.05	0.08
Xylenes	<0.25	<0.25	<0.25	<0.25	<0.25	2.3	<0.25	1.8
TAB	<0.5	4.6	<0.5	5.1	<0.05	21.2	0.05	3.5
NA	<0.09	5.6	<0.09	11.8	<0.09	3.3	<0.05	0.8
1MNA	<0.02	28.0	0.03	103	0.01	10.6	0.02	5.3
2MNA	<0.02	22.6	0.02	79.9	<0.02	13.2	0.02	3.9
ANA	<0.2	1.6	<0.2	9.9	<0.02	1.2	<0.02	0.7
FL	<0.02	4.0	<0.02	5.9	<0.02	1.9	<0.02	3.4
PH	<0.02	7.8	<0.02	16.1	<0.02	4.3	<0.02	12.4
ANTH	<0.02	0.9	<0.02	2.6	<0.02	0.7	<0.02	1.5
FLAN	<0.02	0.5	<0.02	6.7	<0.02	0.5	<0.02	1.1
PY	<0.02	1.3	0.05	16.5	<0.02	1.5	<0.02	2.9
B[a]ANTH	<0.02	0.2	<0.02	42.7	<0.02	0.2	<0.02	0.4
CH	<0.02	0.8	0.04	73.9	<0.02	0.2	<0.02	1.7
B[b]FLAN	<0.02	0.1	<0.02	4.37	<0.02	0.3	<0.02	1.7
B[k]FLAN	<0.02	0.02	<0.02	0.8	<0.02	0.1	0.02	0.2
B[e]P	<0.02	0.1	<0.02	5.5	<0.02	0.2	<0.02	4.0
B[a]P	<0.01	0.02	<0.01	2.2	<0.01	0.2	<0.01	0.3
I[123-cd]PY	<0.02	0.02	<0.02	0.5	<0.02	0.3	<0.02	0.3
D[a.h]AN	<0.02	0.02	<0.02	0.8	<0.02	0.04	<0.02	0.4
B[ghi]PERY	<0.02	0.02	<0.02	1.0	<0.02	0.2	<0.02	0.5
TPAH	<0.5	73.8	<0.5	384	<0.5	38.9	<0.5	41.5

TPHs total petroleum hydrocarbons, *TAB* total alkyl-benzenes, *VPH* volatile petroleum hydrocarbons, *NA* naphthalenes, *1MNA* 1-methylnaphthalene, *2MNA* 2-methylnaphthalene, *ANA* acenaphthene, *FL* fluorene, *PH* phenanthrene, *ANTH* anthracene, *FLAN* fluoranthene, *PY* pyrene, *B[a]ANTH* benz[a]anthracene, *CH* chrysene, *B[b]FLAN* benzo[b]fluoranthene, *B[k]FLAN* benzo[k]fluoranthene, *B[e]P* benzo[e]pyrene, *B[a]P* benzo[a]pyrene, *I[123-cd]PY* Indeno[1.2.3-cd]pyrene, *D[a.h]AN* dibenzo[a.h]anthracene, *B[ghi]PERY* benzo[g.h.i]perylene, *TPAH* total polycyclic aromatic hydrocarbons

from sample SCS, 27 from BCS, 22 from RCS and 28 from sample GCS, respectively. The 16S rRNA gene sequence based phylogenetic analysis revealed the distribution of isolates within six taxonomic groups (Bacilli, α-, β-, γ-Proteobacteria, Flavobacteriia and Actinobacteria).

The microbial community of the highly TPH impacted sample GCS proved to be the most variable. Besides proteobacterial, actinobacterial and bacilli isolates, two representatives of the class Flavobacteriia (*Chryseobacterium hungaricum*, *Chryseobacterium haifense*) were also obtained. The γ-Proteobacteria class was represented by species belonging to the family *Enterobacteriaceae* (*Klebsiella* sp., *Citrobacter* sp., *Enterobacter* sp.) and to the genera *Acinetobacter*, *Stenotrophomonas* and *Serratia*. In addition, the β-Proteobacteria class was represented by the members of the genera *Comamonas*, *Burkholderia*, *Cupriavidus* and *Pandoraea*. The lowest diversity of isolated strains was observed in case of the heavily PAH impacted sample SCS. Only the representatives of the α- and γ-Proteobacteria classes were found. While the α-Proteobacteria was represented by the genus *Ochrobactrum*, the majority of γ-Proteobacteria affiliated strains were members of the genus *Pseudomonas*. Beside pseudomonads the γ class of Proteobacteria was also represented by *Yersinia* sp. isolates. In sample RCS the isolates affiliated mainly to the γ-Proteobacteria (*Pseudomonas* spp. and *Thermomonas* sp.) and Actinobacteria (*Rhodococcus* spp., *Arthrobacter* sp., *Cellulomonas* sp. and *Micrococcus* sp.) classes. In the moderately polluted sample BCS the majority of hydrocarbonoclastic isolates affiliated with γ-Proteobacteria represented by the genus *Pseudomonas*. Besides, the representatives of the Actinobacteria (*Rhodococcus* sp., *Microbacterium* sp. and *Dietzia* sp.), α-Proteobacteria (*Caulobacter* sp.) and Bacilli

(*Bacillus* sp.) classes were also isolated. Isolates affiliated with *Bacilli* class were obtained from all samples excepting sample SCS (Fig. 1).

Fingerprinting of microbial communities assessed by T-RFLP

16S rRNA gene based T-RFLP fingerprinting of all samples provided T-RFLP profiles containing 12–28 T-RFs with relative abundances higher than 1 %. *H'* index calculated on basis of T-RFLP results was between 1.839 and 3.15. While the lowest number of T-RFs (12) and *H'* diversity index (1.839) value were recorded in heavily PAH impacted sample SCS, the largest number of T-RFs (28) and the second highest number of *H'* (3.081) were observed in its non-contaminated counterpart (SNCS). Statistical analysis revealed that the T-RFLP profiles based difference in bacterial communities of samples SNCS and SCS was significant ($P < 0.05$). Also a significant difference ($P = 0.0045$) was observed in case of Bălan originated samples too. While in sample BNCS the number of T-RFs was 23 with an *H'* diversity index of 2.774, in its contaminated counterpart, with the second highest PAH concentration, these values decreased to 17 and 2.232. No statistically significant difference ($P > 0.1$) was observed among bacterial communities of soil samples from Ghelinţa (GCS—T-RFs 18, *H'* 2.686; GNCS—T-RFs 19, *H'* 2.52). Interestingly, in contaminated sample RCS the number of T-RFs (27) and the value of *H'* diversity index (3.15) remarkably exceeded the values recorded in non-contaminated sample RNCS (T-RFs 23, *H'* 2.815; Table 3).

PCA based on T-RFLP profiles revealed remarkable difference between bacterial community structures of contaminated samples and non-contaminated ones. PCA plot pointed out that regardless of the geographical origin the non-contaminated samples in microbiological point of view were similar, formed a coherent group by the first two principal components. On the other hand, PCA showed that the contaminated samples were more distinct microbiologically. Interestingly sample RCS with the highest BTEX, TAB and VPH concentrations clustered together with non-contaminated samples (Fig. 2).

Canonical correspondence analysis of T-RFLP fingerprints showed the influence of different hydrocarbon pollutants, as well as of different soil properties on the structure and diversity of bacterial communities. The first two axes of the CCA described 73.14 % of the variation. Microbial communities of samples SCS and BCS were mainly influenced by PAHs; the bacterial population in sample GCS proved to be influenced by TPHs; in sample RCS the main selective driving force might be the presence of alkyl-benzenes and VPHs. CCA plot also pointed out the relationship between hydrocarbon pollutants and the members of bacterial communities. The most abundant bacteria represented within the 72 bp (39.9 %) and 153 bp (20.36 %) sized T-RFs were positively affected by elevated PAH concentration in sample SCS. The moderately PAH polluted sample BCS was dominated mainly by bacteria represented within the 147 bp (23.99 %) and 474 bp (28.42 %) sized T-RFs. Bacteria affiliated with T-RFs of 70 bp (7.34 %), 200 bp (7.79 %) and 207 bp (7.47 %) proved to be positively affected by the presence of alkyl-benzenes and VPHs. Bacteria affiliated with T-RFs of 56 bp (12.81 %), 58 bp (12.16 %), 183 bp (9.88 %) and 243 bp (10.13 %) were positively affected by elevated TPH concentration in sample GCS (Fig. 3; Table 3). As it is also evident from CCA the humus, nitrogen, phosphorous and potassium contents of soils also influence the actual structure of bacterial communities as well. The high diversity of bacterial population in sample RCS beside hydrocarbon pollutants (total alkyl-benzenes, VPHs) might be influenced by increased humus content.

In silico restriction analysis (Table 5) revealed that the ~72 bp sized T-RF with the highest relative abundance in sample SCS might be associated with the presence of Actinobacteria and γ-Proteobacteria classes; the ~147 bp sized T-RF with the presence of *Acidovorax* spp.; the ~153 bp T-RF might affiliate with *Comamonas* spp.; T-RF of ~207 bp might affiliate with presence of *Ochrobactrum* spp. Unfortunately, the *in silico* identification of the 474 bp sized T-RF found only in sample BCS was not achieved (Table 3).

Substrate specificity of the tested hydrocarbonoclastic bacterial strains

Hydrocarbon degradation potential of isolates originated from Bălan and Sândominic is discussed in detail in our previous work (Máthé et al. 2012).

Hydrocarbon degradation ability tests revealed that 58 % of the investigated strains were able to utilize benzene, 39 % toluene, 39 % xylene, 61 % naphthalene and 76 % n-dodecane as sole carbon source. Moreover we found that among the tested bacterial strains *A. gandavensis* RBB2, *C. humilata* RGB3, *R. erythropolis* RGG2, *C. basilensis* 1SG1, *Ch. hungaricum* GKN1, *M. luteus* GTN1, *R. qingshengii* K4 strains showed the highest degradation activity. They were able to utilize all of the tested hydrocarbons as sole carbon and energy source. On the other hand, strains *P. veronii* RGG4, *P. otitidis* GBN1, *K. oxytoca* GeT4 and *P. taiwanensis* GKN5A were not been able to degrade any of the tested hydrocarbons. In summary, hydrocarbon degradation ability tests revealed that the representatives of Proteobacteria and Actinobacteria classes were the most active in the biodegradation of tested hydrocarbons (Tables 4, 5).

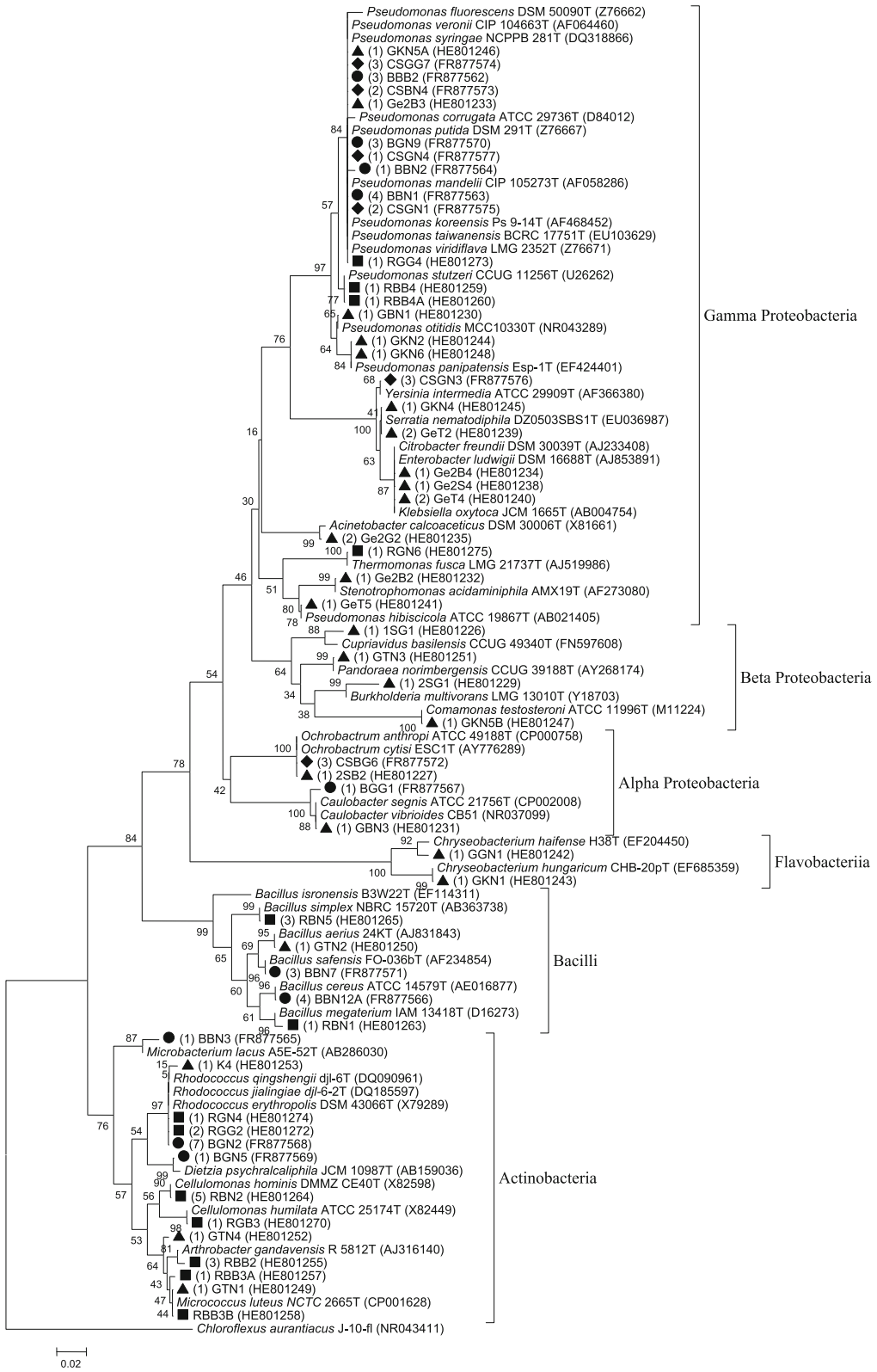

Fig. 1 16S rRNA gene based phylogenetic distribution of isolated hydrocarbonoclastic bacterial strains from contaminated samples. Isolates from sample BCS are marked with *filled circle*, from SCS with *filled diamond*, from RCS with *filled square* and from GCS with *filled triangle*. Number of isolates with the same ARDRA pattern is shown in parentheses. The tree was constructed by using neighbor joining method; *numbers* at the branch points represent percentage of 1,000 bootstraps. The names of the major divisions of prokaryotes are shown to the right of the corresponding cluster. *Cloroflexus aurantiacus* strain J-10-fl was used as an out-group

Table 3 The number of T-RFs and T-RFLP based diversity index values of samples, as well as the possible phylogenetic affiliation of representative T-RFs

Sample	No. of T-RFs (relative abundance >1 %)	*H'*	Type of dominant soil contaminants	T-RFs with the highest relative abundance	Possible phylogenetic affiliation of the T-RFs based on *in silico* digestion
BCS	17	2.232	TPH, TAB and PAH	146 bp (6.21 %)7	ND*
				147 bp (23.99 %)	*Acidovorax* sp.
				474 bp (28.42 %)	Not determined
BNCS	23	2.774	–	207 bp (20.46)	*Ochrobactrum* sp.
				230 bp (14.7 %)	ND*
GCS	18	2.686	High TPHs concentration	56 bp (12.81 %)	ND*
				58 bp (12.16 %)	ND*
				183 bp (9.88 %)	ND*
				243 bp (10.13 %)	ND*
GNCS	19	2.52	–	52 bp (20.72 %)	Not determined
				72 bp (14.82 %)	*Pseudomonas* sp.
					Cellulomonas sp.
					Microbacterium sp.
					Micrococcus sp.
				209 bp (13.22 %)	ND*
RCS	27	3.15	High TAB/VPH concentration	70 bp (7.34 %)	ND*
				200 bp (7.79 %)	ND*
				207 bp (7.47 %)	*Ochrobactrum* sp.
RNCS	23	2.815	–	59 bp (18.14 %)	ND*
				207 bp (10.42 %)	*Ochrobactrum* sp.
SCS	12	1.839	High PAH concentration	72 bp (39.9 %)	*Pseudomonas* sp.
					Cellulomonas sp.
					Microbacterium sp.
					Micrococcus sp.
				147 bp (14.85 %)	*Acidovorax* sp.
				153 bp (20.36 %)	*Comamonas* sp.
SNCS	28	3.08	–	207 bp (14.09 %)	*Ochrobactrum* sp.

ND* identification of the characteristic T-RF was not possible by applying the *in silico* digestion of 16S rDNA sequences of isolated hydrocarbonoclastic bacteria

Discussion

Bacterial diversity and microbial community shifts were investigated in case of four different non-linked contaminated sites. In order to assess the structure of bacterial communities and their relationships with environmental factors cultivation dependent and independent approaches, as well as comprehensive multivariate statistical analyses (PCA, CCA) were applied. Multivariate statistical analyses are of growing importance for characterization of bacterial populations in contaminated environments (Muckian et al. 2007; Ben Said et al. 2010). Sampling sites were chosen based on their distinct pollution history. Sample GCS was extremely polluted with TPHs; samples BCS and SCS were highly contaminated with PAHs; sample RCS was chosen because of its higher amounts of VPHs and alkyl-benzenes (TAB, BTEX) (Table 2). The amount of TPHs in sample GCS was approximately 6–9 times higher than in samples SCS, BCS or RCS. In sample RCS the concentration of VPHs was 4–7 times-, of TABs 4–5 times higher than in other contaminated samples. The concentration of total PAHs (384 mg kg^{-1}) in sample SCS was almost one order of magnitude higher than concentrations recorded in samples RCS, GCS. The amount of TPH in sample GCS more than seventy times-, the concentration of PAHs in sample SCS more than two times exceeded the permitted levels assessed by Ministry of Waters, Forests and Environmental Protection in Romania established for industrial areas (2000 mg kg^{-1} for TPHs and 150 mg kg^{-1} for PAHs; No. 759/03.11.1997) (Table 2).

In pedological point of view samples originated from Bălan and Ghelinţa had similar texture with low humus contents (1.3 and 0.5 %, respectively). Samples from Remetea and Sândominic with also a similar soil structure had increased humus contents (6.2 and 3.4 %, respectively). The highest P concentrations were recorded in

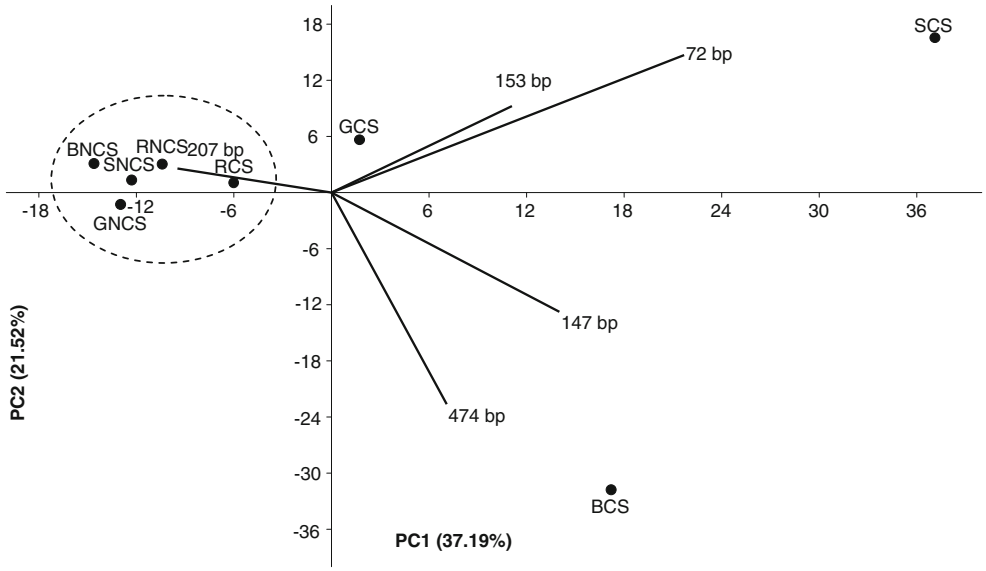

Fig. 2 T-RFLP/*Alu*I restriction based principal component analysis of bacterial communities from contaminated and non-contaminated soil samples. Only the first two principal components are shown (PC 1, PC 2) and the arrows represent the characteristic T-RFs with relative abundance higher than 20 %

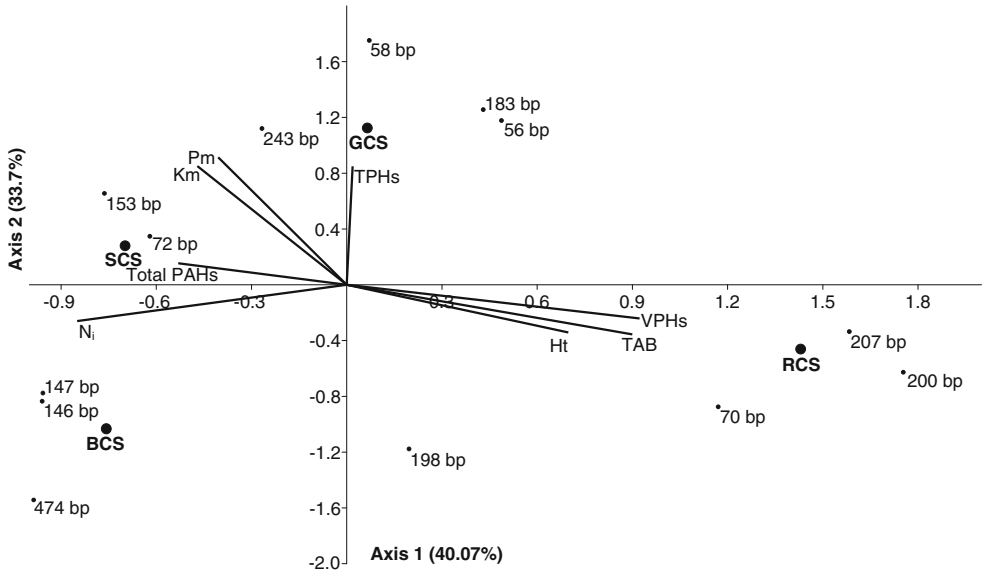

Fig. 3 CCA among the T-RFLP characterized bacterial communities of contaminated samples and different environmental parameters. *TPHs* total petroleum hydrocarbons, *VPHs* volatile petroleum hydrocarbons, *TAB* total alkyl-benzenes, *Total PAHs* total polycyclic aromatic hydrocarbons, H_t total humus, *Ni* nitrogen index, *Pm* phosphorous mobile, *Km* potassium mobile

samples from Ghelinţa and Sândominic (13.6 and 10.6 ppm, respectively). Bălan and Sândominic originated samples showed remarkably higher N index values (1.2 and 1.5, respectively) (Table 1).

Despite the fact that the investigated samples are geographically distant from each other, T-RFLP analysis revealed no statistically significant difference among the bacterial communities of similarly textured non-contaminated soil samples BNCS/GNCS ($P = 0.35$) and RNCS/SNCS ($P = 0.1$), respectively. On the other hand it has been found that a statistically significant difference exists between bacterial population of samples with similar soil structure but different pollution history (BCS/GCS $P = 0.0004$; RCS/SCS $P = 7.4355\text{E}{-14}$). Moreover, principal component analysis made it clear that among all samples only the PAH polluted samples SCS and BCS are outliers (Fig. 2).

T-RFLP analysis also pointed out a remarkable change in bacterial richness and diversity between samples of contaminated and non-contaminated sites. For all that

Table 4 Petroleum hydrocarbon degradation capacity of isolated bacterial strains

Isolated strains	Origin	Nearest cultured neighbor upon 16S rRNA genes (accession number)	Sequence homology (%)	Tested petroleum hydrocarbons				
				Benz.[a]	Tol.	Xyl.	Naph.	n-dod.
RBB2	RCS	*Arthrobacter gandavensis* (AJ316140)	99.4	++	++	+	++	+
RBB3B	RCS	*Micrococcus luteus* (CP001628)	99.7	–	–	+	++	++
RBB4A	RCS	*Pseudomonas stutzeri* (U26262)	100.0	+	+	–	–	–
RBN1	RCS	*Bacillus megaterium* (D16273)	98.8	–	–	–	+	–
RBN2	RCS	*Cellulomonas hominis* (X82598)	99.8	–	+	+	+	–
RBN5	RCS	*Bacillus simplex* (AB363738)	99.7	++	–	–	++	+
RGB3	RCS	*Cellulomonas humilata* (X82449)	99.3	+	++	+	++	++
RGG2	RCS	*Rhodococcus erythropolis* (X79289)	99.9	+	+	+	++	+++
RGG4	RCS	*Pseudomonas veronii* (AF064460)	99.6	–	–	–	–	–
RGN4	RCS	*Rhodococcus qingshengii* (DQ090961)	100.0	+	–	–	–	–
RGN6	RCS	*Thermomonas fusca* (AJ519986)	100.0	+	–	–	+	++
1SG1	GCS	*Cupriavidus basilensis* (FN597608)	97.9	+	+	+	+	+
2SB2	GCS	*Ochrobactrum anthropi* (CP000758)	99.3	+	–	–	+	++
2SG1	GCS	*Burkholderia multivorans* (Y18703)	97.7	+	–	–	–	++
GBN1	GCS	*Pseudomonas otitidis* (NR043289)	99.6	–	–	–	–	–
GBN3	GCS	*Caulobacter vibrioides* (NR037099)	99.8	–	+	+	+	+
Ge2B2	GCS	*Stenotrophomonas acidaminiphila* (AF273080)	99.4	++	–	–	++	++
Ge2B3	GCS	*Pseudomonas koreensis* (AF468452)	99.6	–	–	–	–	+
Ge2B4	GCS	*Enterobacter ludwigii* (AJ853891)	99.4	–	–	–	–	+
Ge2G2	GCS	*Acinetobacter calcoaceticus* (X81661)	99.8	+	–	–	+	+++
Ge2S4	GCS	*Citrobacter freundii* (AJ233408)	98.6	–	–	–	–	+
GeT2	GCS	*Serratia nematodiphila* (EU036987)	99.2	–	+	+	+	++
GeT4	GCS	*Klebsiella oxytoca* (AB004754)	100.0	–	–	–	–	–
GeT5	GCS	*Pseudomonas hibiscicola* (AB021405)	98.9	++	–	+	+	++
GGN1	GCS	*Chryseobacterium haifense* (EF204450)	98.02	–	–	–	++	++
GKN1	GCS	*Chryseobacterium hungaricum* (EF685359)	99.3	+	+	+	+	++
GKN5A	GCS	*Pseudomonas taiwanensis* (EU103629)	99.5	–	–	–	–	–
GKN5B	GCS	*Comamonas testosteroni* (M11224)	99.8	++	–	–	++	++
GKN6	GCS	*Pseudomonas panipatensis* (EF424401)	99.1	+	+	+	–	+
GTN1	GCS	*Micrococcus luteus* (CP001628)	99.8	+	++	+	+	+
GTN2	GCS	*Bacillus aerius* (AJ831843)	98.7	–	–	–	–	++
GTN3	GCS	*Pandoraea norimbergensis* (AY268174)	99.8	+	+	–	–	++
K4	GCS	*Rhodococcus qingshengii* (DQ090961)	100.0	++	++	++	++	+++

"–" blue colored samples, no degradation activity; "+" bluish pink samples, low degradation activity; "++" intense pink samples, medium degradation activity; "+++" colorless samples, the highest degradation activity

[a] *Abbreviations Benz.* benzene, *Tol.* toluene, *Xyl.* xylenes, *Naph.* naphthalene, *n-dod.* n-dodecane

samples SNCS (T-RFs 28, *H'* 3.03) and BNCS (T-RFs 23, *H'* 2.77) showed the highest bacterial diversity, the presence of PAHs reduced remarkably the number of T-RFs (SCS-12, BCS-17) and the value of *H'* diversity index in case of these sites (SCS-1.83, BCS-2.23). This finding highlights that a long-term pollution in an increased degree with PAHs reduces significantly the diversity of bacterial population even in presence of adequate nutrient supplies. In this context, Andreoni et al. (2004) also found that a long-term exposition to PAHs manifests in a poor bacterial diversity ($H' = 0.89$). Additionally needs to be noted that according to Muckian et al. (2007) the type of PAH ring structure has a greater influence on the bacterial community structure than total PAH concentration. The adverse effect of PAHs on the soil bacterial population was also unambiguously proven by the cultivation of hydrocarbonoclastic bacterial species from samples SCS and BCS. Sândominic originated isolates affiliated only with two classes of bacteria: α- and γ-Proteobacteria. Isolates of sample BCS affiliated only with Actinobacteria and γ-Proteobacteria classes. Multivariate statistical analysis (CCA) indicated a positive relationship of *Acidovorax* spp.

Table 5 Predicted T-RF lengths of *in silico Alu*I digested 16S rDNA sequences of isolated strains

Isolated strains	Origin	*In silico* restriction site of isolates (bp)	Nearest cultured neighbor	Accession number of 16S rDNA type sequences	Affiliation
RBB2	RCS	204	*Arthrobacter gandavensis*	AJ316140	Actinobacteria
RBN2	RCS	72	*Cellulomonas hominis*	X82598	
RGB3	RCS	72	*Cellulomonas humilata*	X82449	
BGN5	BCS	171	*Dietzia psychralcaliphila*	AB159036	
BBN3	BCS	72	*Microbacterium lacus*	AB286030	
RBB3B/GTN1	RCS/GCS	72	*Micrococcus luteus*	CP001628	
RGG2/BGN2	RCS/BCS	232	*Rhodococcus erythropolis*	X79289	
RGN4	RCS	232	*Rhodococcus qingshengii*	DQ090961	
GTN2	GCS	73	*Bacillus aerius*	AJ831843	Bacilli
BBN12A	BCS	74	*Bacillus cereus*	AE016877	
RBN6	RCS	251	*Bacillus isronensis*	EF114311	
RBN1	RCS	74	*Bacillus megaterium*	D16273	
BBN7	BCS	67	*Bacillus safensis*	AF234854	
RBN5	RCS	73	*Bacillus simplex*	AB363738	
GGN1	GCS	460	*Chryseobacterium haifense*	EF204450	Flavobacteriia
GKN1	GCS	460	*Chryseobacterium hungaricum*	EF685359	
BGG1	BCS	143	*Caulobacter segnis*	CP002008	α-Proteobacteria
GBN3	GCS	143	*Caulobacter vibrioides*	AJ009957	
CSBG6	SCS	207	*Ochrobactrum anthropi*	CP000758	
D6	BCS	143	*Phenylobacterium falsum*	NR042277	
D5	BCS	148	*Acidovorax defluvii*	Y18616	β-Proteobacteria
GKN5B	GCS	153	*Comamonas testosteroni*	M11224	
1SG1	GCS	155	*Cupriavidus basilensis*	AF312022	
GTN3	GCS	141	*Pandoraea norimbergensis*	AY268174	
D3	SCS	234	*Sulfuritalea hydrogenivorans*	AB552842	
Ge2G2	GCS	234	*Acinetobacter calcoaceticus*	X81661	γ-Proteobacteria
2SG1	GCS	278	*Burkholderia multivorans*	Y18703	
Ge2S4	GCS	75	*Citrobacter freundii*	AJ233408	
Ge2B4	GCS	74	*Enterobacter ludwigii*	AJ853891	
GeT4	GCS	74	*Klebsiella oxytoca*	AB004754	
BBN1	BCS	72	*Pseudomonas fluorescens*	Z76662	
GeT5	GCS	75	*Pseudomonas hibiscicola*	AB021405	
Ge2B3	GCS	72	*Pseudomonas koreensis*	AF468452	
CSGN1/BGN9	SCS/BCS	236	*Pseudomonas mandelii*	AF058286	
GBN1	GCS	72	*Pseudomonas otitidis*	NR043289	
GKN6, GKN2	GCS	72	*Pseudomonas panipatensis*	EF424401	
RBB4A, RBB4	RCS	72	*Pseudomonas stutzeri*	U26262	
GKN5A	GCS	72	*Pseudomonas taiwanensis*	EU103629	
BBN2	BCS	72	*Pseudomonas putida*	Z76667	
CSGN4	SCS	236	*Pseudomonas syringae*	DQ318866	
RGG4/CSGG7	RCS/SCS	72	*Pseudomonas veronii*	AF064460	
CSBN4/BBB2	SCS/BCS	72	*Pseudomonas corrugata*	D84012	
GeT2	GCS	75	*Serratia nematodiphila*	EU036987	
Ge2B2	GCS	76	*Stenotrophomonas acidaminiphila*	AF273080	
RGN6	RCS	75	*Thermomonas fusca*	AJ519986	
CSGN3	SCS	161	*Yersinia intermedia*	AF366380	

(147 bp sized T-RF), *Comamonas* spp. (153 bp sized T-RF) and *Pseudomonas* spp. (72 bp sized T-RF) with high PAH concentrations (Fig. 3) in both samples SCS and BCS. Members of these genera are also reported to grow on PAHs as sole carbon source and to tolerate high PAH concentrations (Skowasch et al. 2002; Erikson et al. 2003; Pannu et al. 2003; Singleton et al. 2009). T-RFs of 72 bp (39.59 %), 147 bp (14.87 %) and 153 bp (20.36 bp) represented 74.75 % of the total relative abundance in highly PAH impacted sample SCS. Taking into account the fact that more than 57 % of the SCS isolates belonged to the genus *Pseudomonas*, and the fact that the *Pseudomonas* affiliated 72 bp sized T-RF (Table 3) represented ~40 % of the relative abundance in sample SCS, we may assume that the pseudomonads are the most active within highly PAH contaminated environments. This finding is in accordance with reports of other studies which also found the dominance of *Pseudomonas* species in different PAH impacted environments (Ma et al. 2006; Bamforth and Singleton 2005; Johnsen et al. 2005; Liu et al. 2010). The present study provides further evidence for the overwhelming dominance of proteobacterial and actinobacterial isolates within highly PAH contaminated soils.

In contrast with high PAH contaminated samples, a notable bacterial richness and diversity increase was observed in highly VPH and TAB contaminated sample RCS (T-RFs-27, *H'*-3.15) compared to sample RNCS (T-RFs-23, *H'*-2.81). This finding may indicate that the actual concentration of VPHs (1100 mg kg^{-1}), TABs (21.2 mg kg^{-1}) as well as of PAHs (38.9 mg kg^{-1}) did not decrease the diversity of bacterial population; on the contrary they led to the emergence of other distinctive species. This observation is in contrast with findings of Zhou et al. (2009) who stated that a PAH concentration as low as 20 mg l^{-1} might affect negatively the bacterial diversity. Isolation procedure in sample RCS resulted in the overwhelming dominance of Actinobacteria affiliated species, represented by the members of the genera *Rhodococcus*, *Arthrobacter*, *Cellulomonas* and *Micrococcus*. Beside actinobacterial isolates, representatives of γ-Proteobacteria and Bacilli classes were also obtained.

Regarding the effect of TPHs on the bacterial diversity no remarkable difference was observed among Ghelinţa originated samples (T-RFs: GCS-18, GNCS-19; *H'*: GCS-2.68, GNCS-2.52). The increased bacterial diversity and the fact that low difference was observed among the number of T-RFs and *H'* diversity index values of contaminated GCS- and non-contaminated GNCS samples may indicate that the enormously high concentration of TPHs (147000 mg kg^{-1}) did not influence negatively the structure of the bacterial community. Furthermore, it might be speculated that in the lack of humus the need of carbon and energy of microorganisms probably was satisfied by the alternate carbon sources in form of TPHs. The positive effect of TPHs on the structure of bacterial population was also supported by the isolation of hydrocarbonoclastic bacteria. The most diverse bacterial strains were obtained in case of sample GCS. Apart from isolates affiliated with α-, β-, γ-Proteobacteria, the representatives of Actinobacteria, Flavobacteriia and Bacilli phyla were also found (Fig. 1). These observations are in contrast with findings of Rosano-Hernández et al. (2012). They claimed that the bacterial diversity changes inversely to the degree of TPH concentration. On the other hand, the positive effect of TPHs on the structure of bacterial community was also demonstrated in several other studies (Nie et al. 2009; dos Santos et al. 2011; Lee et al. 2011; Li et al. 2005).

This work contributes to a better understanding of the long-term impact of different types of hydrocarbons (PAHs, TPHs, TABs and VPHs) on the structure of bacterial population is soils. However, it must be noticed that the diversity of microbial communities in investigated soils may be influenced also by the availability of nutrients (humus, N, P, K etc.; Buckley and Schmidt 2002) as well as other soil characteristics.

Conclusions

High TPH concentration positively influenced the bacterial diversity, proven by the presence of representatives of α-, β-, γ-Proteobacteria, Actinobacteria, Flavobacteriia, Bacilli classes in sample GCS.

In sample RCS with the highest alkyl-benzenes (TAB and BTEX) and VPH concentrations the predominance of Actinobacteria was observed. It was established that the actual concentration of these hydrocarbons increased the bacterial biodiversity of contaminated sample.

PAH concentration of ~40 mg kg^{-1} might not decrease the bacterial diversity as demonstrated through the example of samples RCS and GCS.

It was observed that despite of the optimal nutrient supply (N, P, K and humus) a concentration of PAHs above 70 mg kg^{-1} exerted remarkable negative effect on the structure and diversity of bacterial communities as found in case of samples BCS and SCS.

Isolates A. *gandavensis* RBB2, *R. erythropolis* RGG2 and *C. humilata* RGB3 from sample RCS, *R. qingshengii* K4, *M. luteus* GTN1, *Ch. hungaricum* GKN1 from sample GCS due to their broad hydrocarbon degradation potential are suitable for biodegradation of hydrocarbon contaminants.

Considering all of the results it can be stated that high concentration of PAHs decrease significantly the microbial diversity even in presence of optimal nutrient supply, while other hydrocarbons (TPHs, BTEX and VPHs) can increase

microbial diversity even in soils with lower nitrogen supply.

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