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Microbial Community Structure and Diversity in Long-term Hydrocarbon and Heavy Metal Contaminated Soils

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ABSTRACT: The influence of long-term mixed organic and inorganic contamination on soil microbial activity, community structure and genetic diversity was investigated in soil samples from a coke oven plant located in Upper Silesia, Poland. The tested soils were heavily contaminated with polycyclic aromatic hydrocarbons (PAHs) and heavy metals. The microbial communities were characterized using the phospholipid fatty acid (PLFA) and the denaturing gradient gel electrophoresis (DGGE) methods. Analysis of the PLFA profiles showed that the biomass of Gram-negative bacteria and fungi was affected by heavy metals but not by PAHs. Similar results were obtained for total microbial activity measured as the rate of fluorescein acetate hydrolysis. Statistical analysis of the obtained results revealed that heavy metals rather than PAHs were primarily responsible for the reduction in microbial activity and the differences in the microbial community structure as showed by PLFA. The DGGE analysis showed that the most contaminated soil had a very low biodiversity and richness but a very high evenness index. The correlation analysis revealed that the biodiversity and richness indices were negatively correlated with PAHs but not with heavy metals. However, there was a positive relationship between the evenness index and tested metals as well as the PAH content. The partial 16S rRNA sequence analysis showed that some of the clones were closely related to the genera *Pseudomonas*, *Sphingomonas* and *Arthrobacter*, which are well-known hydrocarbon degraders. Obtained results indicated that a high level of contamination suppress the some bacterial community member giving finally a reduction in the genetic diversity.

Key words: DGGE, Heavy metals, Long-term mixed contamination, PAHs, PLFA

INTRODUCTION

Contamination on post-industrial sites is closely related to the type of activities carried out in the area. Some sites in Upper Silesia, Poland are contaminated with polycyclic aromatic hydrocarbons (PAHs) and heavy metals due to the coal deposits and the number of coal processing plants (Maliszewska-Kordybach *et al*., 2008, 2009; Rachwał *et al*., 2015). The co-occurrence of PAHs and heavy metals was commonly found in many industrialised and urbanised areas all over the world (Máthé *et al*., 2012; Thavamani *et al*., 2012; Rachwał *et al*., 2015). PAHs are hydrophobic organic pollutants, which are a major concern because some of them are considered as carcinogens, mutagens, and teratogens (IARC, 1983; Shaw and Connell, 1994). Unlike hydrocarbons, heavy metals cannot be degraded so they can stay in soil for a long time and exert negative pressure on soil microbial communities, especially on their enzyme activities (Kozdrój and Van Elsas, 2001; Macdonald *et al*., 2010, 2011). PAHs and heavy metals may accumulate in tissues of organisms and persist in the food webs and eventually become a risk to human health. Additionally, heavy metals may inhibit the biodegradation of hydrocarbons (Turpeinen, 2002; Sandrin and Maier, 2003; Almeida *et al*., 2013).

There is plenty of data about the influence of heavy metal or hydrocarbon pollution on soil microorganisms (Joynt *et al*., 2006; Muckian *et al*., 2009; Zhang *et al*., 2010; Sheik *et al*., 2012). However, there is lack of complete information on the impact of mixed and long-term contamination on the activity,

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structure and diversity of the entire soil microbial community. Some studies showed that the combined effect of PAHs and metals may be stronger or weaker than the expected impact of each contaminant individually *(Thavamani et al., 2012; Samia et al*., 2013). The effect of heavy metals and PAHs on soil microorganisms depends on the period of time in which soil biota are exposed to this contamination. The reaction of soil microorganisms is related to the process of ageing which resulted from the binding of PAHs to soil particles and decreases the bioavailability of these compounds to microbial cells (Ma *et al*., 2012; Tang *et al*., 2012). Long-term contamination can induce gradual changes in the microbial composition due to the selection of strains resistant/tolerant to the pollutants present in soil. Another reason for changes in microbial diversity is the horizontal transfer of genes responsible for metal resistance and/or organic pollutants degradation. The presence of hydrocarbon degradation genes on mobile genetic elements has been reported as a significant spreading mechanism for metabolic abilities among microorganisms in contaminated sites (Ma *et al*., 2006; Yagi *et al*., 2009). It has been observed that the microbial population in long-term contaminated environments can have a much higher biomass and activity than that measured in uncontaminated soils and that the initial reduction in biomass and activity is often temporary (Feris *et al*., 2004a; Joynt *et al*., 2006).

The aim of the present study was to determine the effects of long-term mixed organic and inorganic contamination on soil microbial communities in soil samples varying in the amount of heavy metals and PAHs. For this purpose, the total microbial activity expressed as fluorescein acetate (FDA) hydrolysis and the number of total, phenanthrene-tolerant and phenanthrene-degrading bacteria were measured. Moreover, to determine the microbial community structure, culture-independent techniques such as the phospholipid fatty acid (PLFA) and the denaturing gradient gel electrophoresis (DGGE) were used.

MATERIALS & METHODS

The soil samples used in this study were obtained from a 120-year-old coke plant located in Zabrze, Upper Silesia, Poland. Coke production at this site began in the late 19th century. Based on our preliminary studies and the measurements of PAH and heavy metal contamination for this study, we chose soil samples (SI-SIV), which differed significantly in the level of contamination. The soil samples taken in the upper horizon (1-20 cm depth) had a strong hydrocarbon odour and contained tarry material. Fifteen different sub-samples (for each soil) taken from the areas of 25 m^2 were used for the experiment. In the laboratory, five sub-samples were randomly mixed and homogenised giving one replication. Finally, there were three replications of each soils. Soils were air-dried and sieved (mesh size 2 × 2 mm) to remove any large particles. The most physico-chemical properties of soils were determined according to methods described previously by Cycoń *et al*. (2010). The particle size of the soil was determined using the aerometric method (ISO 11277), while the pH values of the aqueous soil extracts (1:5, *w*/*v*) were measured in triplicate with a glass electrode using a Jenway pH-meter at 20°C (ISO 10390). The organic matter content (C_{org}) and the total nitrogen content (N_{tot}) were determined using the dichromate oxidation in the presence of concentrated sulphuric acid (Schinner *et al*., 1995) and the Kjeldahl method (ISO 11261), respectively. The 16 EPA priority PAHs in contaminated soils were identified and determined according to the EPA Standard Methods (USEPA, 1992, 1995). PAHs were identified using liquid chromatography coupled with a fluorescence detector (HPLC-FLD Agilent 1200). Metal contents were detected using atomic absorption spectrometry (SpectrAA 300 Varian) with a graphite furnace. The level of heavy metals, the sum of the 16 PAHs and the physicochemical properties are presented in Table 1.

Fluorescein diacetate (FDA) hydrolysis was used as a measure of the overall enzyme activity in the soil and assessed as described by Adam and Duncan (2001). For each of the soils tested triplicate 2-g subsamples were incubated at 30°C for 20 mian. on a rotary shaker with 15 ml of a 60 mM potassium phosphate buffer (pH 7.6) and 0.2 ml of FDA (1 mg/ml). The hydrolysis was stopped by adding 15 ml of a chloroform/methanol (2:1 *v*/*v*) mixture. The suspension was subsequently centrifuged at 3000 rpm for 5 min. The concentration of free fluorescein in the filtered solution was measured at 490 nm using a Thermo Spectronic Helios Epsilon spectrophotometer. An enzyme activity unit was defined as the µg of the substrate hydrolysed by 1 g of dried soil.

The total number of culturable microorganisms was determined by a viable count on serial agar plates (Cycoń and Piotrowska-Seget, 2007). The total heterotrophic and phenanthrene tolerant strains were enumerated on 0.1 TSA (tryptic soy agar) and 0.1 TSA media with phenanthrene added to a concentration of 50 mg/l, respectively. Moreover, the number of bacteria capable of using phenanthrene as their sole carbon source (phenanthrene degraders) was enumerated on

Table 1. General characteristics of soils used in the experiment

Parameter	Soils			
	SI	SII	SIII	SIV
Sand (%)	95	98	96	80
Silt (%)	5	2	4	2
Clay (%)	0	0	0	18
pH (in water)	7.2 ± 0.1	7.5 ± 0.1	7.3 ± 0.1	8.3 ± 0.2
Moisture (%)	20.1 ± 1.0	14.1 ± 0.7	13.4 ± 0.7	21.7 ± 1.1
Organic matter (%)	2.19 ± 0.12	1.95 ± 0.53	2.25 ± 0.3	8.79 ± 0.81
N_{tot} (%)	5.3 ± 0.1	0.1 ± 0.00	0.17 ± 0.00	0.09 ± 0.00
P_{tot} (%)	0.01 ± 0.00	1.24 ± 0.03	1.16 ± 0.03	0.01 ± 0.00
Conductivity (μS/cm)	367.3 ± 18.4	95.0 ± 4.8	248.1 ± 12.4	214.7 ± 10.7
Cd (mg/kg soil)	2.6 ± 0.4	3.1 ± 0.5	2.8 ± 0.5	3.1 ± 0.5
Pb (mg/kg soil)	57.8 ± 2.9	3378 ± 168.9	3478 ± 173.9	1843 ± 92.2
Zn (mg/kg soil)	247 ± 12.4	556 ± 27.8	504 ± 25.2	704 ± 35.2
Cu (mg/kg soil)	11.8 ± 0.5	143.8 ± 5.8	169.8 ± 6.8	99.7 ± 4.0
Ni (mg/kg soil)	10.5 ± 0.4	519.7 ± 20.8	666.4 ± 26.7	184.2 ± 7.4
∑16 PAHs (mg/kg soil)	171.4 ± 12.6	481.6 ± 47	604.6 ± 61.1	2137.1 ± 241.3

The values are the means of three replicates with the standard deviation, which was within 5% of the mean.

M9 medium (1 g NH_4Cl, 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mM thiamine/HCl, 20 g agar in 1 l water) with 100 mg/l phenanthrene. The plates were incubated at 28°C for 7 days to enumerate the number of total bacteria and 14 days for phenanthrene-tolerant and phenanthrene -degrading bacteria. The number of culturable bacteria of each group were expressed in log 10 colony-forming units (cfu)/g soil.

The composition and biomass of the total soil microbial community was assessed using the phospholipid fatty acid method according to Pennanen *et al*. (1996). Briefly, the lipids from 2 g of soil were extracted using a solution containing a chloroform:methanol:citric buffer (1:2:0.8 v/v/v) and separated into neutral, glycolipid and phospholipid fractions in silicic acid columns (Supelco Silica Tube, 3 mL, 500 mg). In order to obtain the fatty acid methyl esters (FAMEs), the phospholipids were subjected to a mild alkaline methanolysis. FAMEs were subsequently analysed using an Hewlett-Packard 6890 gas chromatograph system with an Ultra 2-HP capillary column (cross-linked 5 % phenyl-methyl silicone, 25 m, 0.22 mm i.d., 0.33 µm film thickness) with hydrogen as the carrier gas. FAMEs were detected using a flame ionisation detector (FID) and identified using the MIDI Microbial Identification System software (Sherlock TSBA6 library; MIDI Inc., Newark, DE, USA). Nonadecanoic acid (19:0) was used as the internal standard for the fatty acid quantitative analysis. Total PLFA biomass was calculated by summing all of the isolated fatty acids (Frostegård and Bååth, 1996). The fatty acid nomenclature was used according to Zelles (1999). The following PLFA were assigned to the microbial groups: i15:0, a15:0, i16:0, i17:0, a17:0 for Gram-positive bacteria, 16:1ω9, 16:1ω7, 18:1ω7, cy17:0, cy19:0 for Gram-negative bacteria, 18:2ω6,9 for fungi (Frostegård *et al*., 1993; Ehlers *et al*., 2010; Bird *et al*., 2011). In addition, to estimate the total bacterial PLFA biomass 15:0 and 17:0 fatty acids along with the above-mentioned bacterial biomarkers were used. Moreover, the relative abundances of PLFAs were used to calculate the ratio of Gram-negative (GN) bacteria to Gram-positive (GP) bacteria and bacteria to fungi.

The genetic diversity of bacterial community was assessed using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified DNA fragments as described previously (Cycoń *et al*., 2013). Total DNA was extracted from the soil samples using a GeneMATRIX

Soil DNA Purification Kit (Eur_x, Poland) as described in the protocol of the manufacturer and subjected to electrophoresis in 1% (w/v) agarose gel. A fragment of the V3 region of the bacterial 16S RNA gene was amplified using the primers F338 and R518. The forward primers contained a 40-bp GC-clamp attached to the 5' end (Muyzer *et al*., 1993). The PCR reaction mixture contained 1 × GoTaq Flexi Buffer (Promega), 2 mM $MgCl_2$, 0.2 mM dNTP Mix (Promega), 0.5 µM of each primer (Sigma-Aldrich), 0.2 µg of DNA and 1.5 U/µL GoTaq DNA Polymerase (Promega). PCR was carried out using a PTC-118 Thermal Cycler (BIO-RAD, CA, USA) as follows: (i) an initial denaturation step of 95°C for 10 min, (ii) 30 cycles of denaturation, annealing and extension (95°C for 1 min followed by 53°C for 1 min with an extension step at 72°C for 2 min) and (iii) a final extension at 72°C for 12 min. After amplification the products were puriûed using a QIAquick PCR Puriûcation Kit (Qiagen, USA) according to the protocol of the manufacturer and then analysed in 8% (w/v) polyacrylamide gel (37.5:1 acrylamide:bis-acrylamide), composed of a linear denaturing gradient ranging from 40% to 70%. The electrophoresis was run at 60°C in a 1 × TAE buffer for 14 h at a constant voltage of 80V using a DCode Mutation Detection System (Bio-Rad, USA). After this, the gels were stained with ethidium bromide (0.5 mg/mL) and visualised on a UV transilluminator. The DGGE profiles were analysed using a Quantity One® software (Bio-Rad) and the similarity values were calculated using the Dice coefficient. The phylogenic dendrogram based on the presence/absence of a band and band weighting (band density) were constructed using the unweighted pair-group method and the arithmetic averages (UPGMA). The Shannon-Wiener index (*H*), richness (*S*), and evenness (E_H) values were calculated as described previously (Cycoń *et al*., 2013). The distinct DGGE bands among all samples were excised and re-amplified using the same primers and amplification conditions. The products after amplification were puriûed and sequenced using a Big Dye® Terminator Cycle Sequencing Kit (Applied Biosystem) and an AbiPrism®3100 Genetic Analyzer in the research Centre "Genomed" (Warsaw, Poland). The obtained sequences were compared to known 16S rRNA gene sequences using the BLAST server at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) in order to search for close relatives.

The obtained data were evaluated using a one-way analysis of variance (ANOVA). The statistical significance of any differences in the received data was performed by a post hoc analysis of the means using the least significant differences (LSD) test. The Pearson's correlation coefficient was also calculated to determine the correlations between the tested parameters. All statistical analyses were performed using the Statistica 10.0 PL software package.

RESULTS & DISCUSSION

The total concentrations of PAHs in tested soils varied from 171 to 2137 mg/kg dry soil (Table 1). In soil samples SII to SIV, the total concentration of the PAHs, Pb and Ni exceeded the Polish regulation guidelines for industrial areas (Dz.U., 2002). Pb, Cd and Zn had previously been reported as co-contaminants in PAH-polluted soils (Mielke *et al*., 2004; Thavamani *et al*., 2012). However, in our study, the Pearson's correlation analysis showed that there were significant ($P < 0.05$) positive relationships only between PAHs and Cd ($r = 0.64$) and Zn ($r = 0.82$). Moreover, we observed strong positive correlations between Pb and Cu content ($r = 0.99$) as well as Pb and Ni ($r = 0.96$).

The total microbial activity expressed as FDA hydrolysis activity was very high in the most and the least contaminated soil (Fig. 1). Moreover, the statistical analysis revealed that there were significant ($P < 0.05$) correlations between FDA hydrolysis activity and Pb ($r = –0.89$), Cu ($r = –0.83$) and Ni ($r = –0.87$) but no correlations with PAHs ($r = 0.24$). Based on these results, it can be assumed that heavy metals rather than PAHs were primarily responsible for the reduction in bacterial activity in tested soils. However, high FDA hydrolysis activity in the contaminated soils and no correlation with PAHs and heavy metals were observed by Gianfreda *et al*. (2005).

The total number of culturable bacteria in all soils ranged from 8.3 to 8.9 log 10 cfu/g dry soil (Fig. 2) and were similar to the results for PAH-contaminated soils reported by other authors (Margesin and Schinner, 2001; Lors and Mossmann, 2005; Lors *et al*., 2010). Reduced numbers of culturable bacteria are often observed in soils contaminated with PAHs *(Zhang et al*., 2010). In this study, pollutants did not affect the total number of culturable bacteria (Fig. 2). Since phenanthrene constituted a high percentage of the total hydrocarbon amount (data not shown), the number of phenanthrene-tolerant strains and phenanthrene degraders were established. The increasing level of PAH pollution was correlated with the number of phenanthrene-tolerant strains (Fig. 2). In soils with an increasing PAH content, an increment in the number of phenanthrene-tolerant strains was observed. Surprisingly, the number of phenanthrene degraders was the highest in the most contaminated soil and

there were no significant differences between soils with lower concentrations of PAHs. Our observations support the earlier published statements that a characteristic feature of contaminated soils is the development of microflora resistant to the presence of hydrocarbons and/or capable of degrading them (Johnsen *et al*., 2006; Johnsen and Karlson, 2007). As shown by the log 10 cfu counts, the numbers of phenanthrene-tolerant bacteria and phenanthrene degraders were significantly ($P < 0.05$) positively correlated with PAHs ($r = 0.89$ and $r = 0.90$). Similar results were obtained by Johnsen *et al*. (2006), who studied the potential of soil microbial communities to degrade PAHs in soil that varied in PAH content. The authors did not observe any differences in the total number of heterotrophic bacteria, although in the soil with the highest PAH concentration, the number of microorganisms capable of degrading phenanthrene was about 100 times higher than in the uncontaminated soil (Johnsen *et al*., 2006). This phenomenon may have resulted from the selection of degraders and the fact that these bacteria may use phenanthrene as an additional carbon source. Another reason may be the horizontal transfer of catabolic genes that encode the degradation of target contaminants (Ikuma *et al*., 2012; Bengtsson *et al*., 2013). In the present study, the number of microorganisms is certainly underestimated because they reflect only the culturable fraction of total bacteria. The high selectivity of the media used in the cultured method enable the growth of only about 0.1–

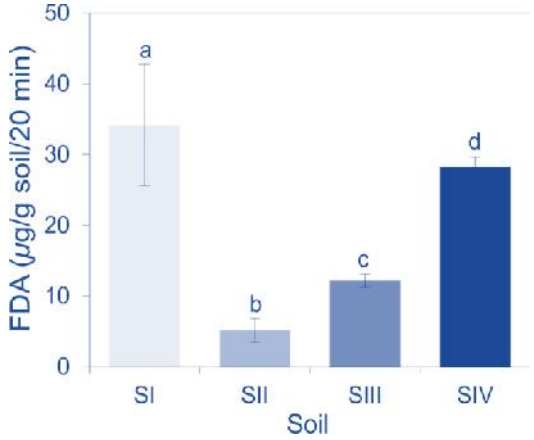

Fig. 1. Enzyme activity based on the fluorescein diacetate (FDA) hydrolysis assay in soils of different PAHs concentrations (SI: 171 mg/kg soil; SII: 476 mg/kg soil; SIII: 595 mg/kg soil, and SIV: 2137 mg/kg soil). The data presented are the means and standard deviations of three replicates. Different letters indicate significant differences ($P < 0.05$, LSD test) between soils

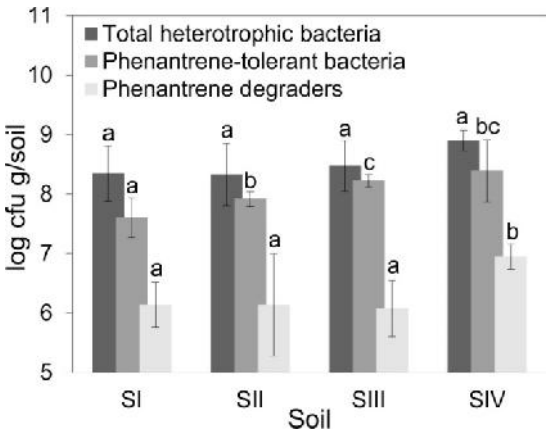

Fig. 2. The number of bacteria (log cfu/g) in soils of different PAHs concentrations (SI: 171 mg/kg soil; SII: 476 mg/kg soil; SIII: 595 mg/kg soil, and SIV: 2137 mg/kg soil). The data presented are the means and standard deviations of three replicates. Different letters within each bacterial group indicate significant differences ($P < 0.05$, LSD test) between soils

Table 2. The PLFA biomarkers and relative abundances of PLFAs (nmol/g soil) measured in soils

Parameter	Soil			
	SI	SII	SIII	SIV
TotPLFAs	52.98 ± 12.37^{a}	47.55 ± 4.44^{a}	45.93 ± 0.66^{a}	55.93 ± 9.15^{a}
GNBB	15.55 ± 4.45^{a}	17.04 ± 2.22^{a}	18.52 ± 0.40^{a}	18.84 ± 2.05^{a}
GPBB	8.36 ± 1.59^{a}	5.43 ± 0.44^{b}	5.50 ± 0.15^{b}	7.96 ± 1.01^{a}
FB	0.58 ± 0.12^{ab}	0.47 ± 0.03^{b}	0.67 ± 0.06^{a}	0.58 ± 0.51^{ab}
GNBB:GPBB	1.84 ± 0.18^{a}	3.13 ± 0.20^{b}	3.37 ± 0.03^{b}	2.37 ± 0.05^{c}
BB:FB	41.25 ± 2.06^{a}	47.97 ± 3.80^{b}	36.59 ± 3.54^{a}	30.42 ± 2.17^{c}

TotPLFAs: total biomass; GNBB: Gram-negative bacterial biomass; GPBB: Gram-positive bacterial biomass; FB: fungal biomass; SI: 171 mg/kg soil; SII: 476 mg/kg soil; SIII: 595 mg/kg soil, and SIV: 2137 mg/kg soil. The data presented are the means of three replicates with the standard deviation, which was within 5% of the mean. Different letters (within each parameter) indicate significant differences ($P < 0.05$, LSD test) between soils.

1% of the total microorganisms that are present in the soil (Amann *et al*., 1995; Hill *et al*., 2000; Johnsen and Karlson, 2007).

The total biomass (totPLFAs) extracted from tested soils did not vary significantly between tested soils (Table 2). Similarly, Pratt *et al*. (2012) showed no correlations between the level of pollutants and biomass (expressed as the content of phospholipid phosphate), activity or the diversity of PLFA patterns observed in PAH-contaminated sediments. In the presented study the correlation analysis confirmed that the total content of PLFAs was not related to the degree of contamination (Table 2). These observations are not in agreement with the findings presented by Slater *et al*. (2008), who found a decrease in the total PLFA content with an increasing level of PAH contamination in surface sediments in the vicinity of a harbor. In our study, the analysis of the PLFA profiles revealed that significant differences were observed only in the case of Gram-positive bacteria biomass (GPBB) and fungal biomass (FB) (Table 2).

The structure of soil microbial community was assessed by calculating the relative abundance of PLFA specific for Gram-negative (GN), Gram-positive (GP) bacteria and fungi. The significantly ($P < 0.05$) higher value of the GNBB: GPBB ratio in soils SII and SIII in comparison with the least (SI) and the most (SIV) contaminated soils was detected (Table 2). As the correlation analysis revealed, there were significantly ($P < 0.05$) negative relationships between GPBB and concentrations of Pb ($r = –0.80$), Cu ($r = –0.77$) and Ni ($r = –0.83$). In contrast, FB was significantly ($P < 0.05$) positively correlated with the content of these three metals (Pb, $r = 0.58$; Cu, $r = 0.59$; Ni, r = 0.76). Additionally, there was no correlation between PAHs and bacterial or fungal biomass. Other results were obtained by Bengtsson *et al*. (2013), who analysed the within-site spatial heterogeneity of microbial community diversity at a creosote contaminated site. The authors found that PLFA 18:2ω6,9, which is an indicator of fungal biomass, was negatively correlated with PAH concentration. Interestingly, in our studies the lowest ratio of bacterial to fungal biomass was observed in the most contaminated soil – SIV. All of these factors may indicate that heavy metals rather than PAHs were the main driving force responsible for the observed differences in microbial community structure. Moreover, GN bacteria were more tolerant to heavy metals, especially Pb, Cu and Ni, than GP bacteria. These results also showed that fungi are less sensitive to long-term and high levels of metal and PAH contamination than bacteria. The high level of resistance of fungi to heavy metals and aromatic pollutants was already reported by many authors (Gianfreda and Rao, 2004; Wang *et al*., 2010; Qasemian *et al*., 2012).

The DGGE profiles (Fig. 3a) showed that numerous bands were present in all samples, suggesting that these bacteria were able to survive and propagate even in the highly contaminated soils. However, there were also changes in the presence of the bands and their relative intensities. Several of the bands observed in the DGGE profile from soils SI, SII and SIII were not present in the DGGE profile from soil SIV, showing that some bacteria could not survive in the most contaminated soil (Fig. 3a). These results indicate that contaminants can modify the structure and diversity

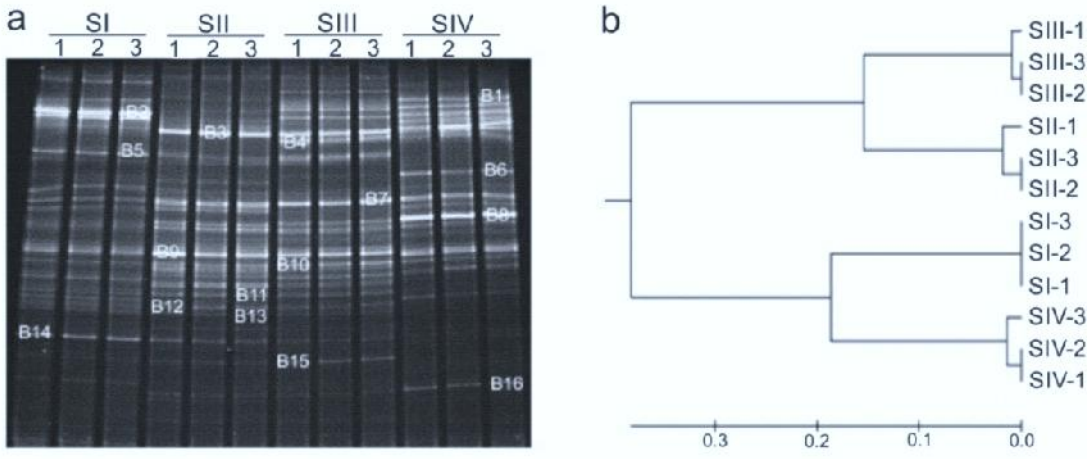

Fig. 3. DGGE profile (a) and phylogenic dendrogram (b) for PCR-amplified fragments of the 16S rRNA gene for soils of different PAHs concentrations (SI: 171 mg/kg soil; SII: 476 mg/kg soil; SIII: 595 mg/kg soil, and SIV: 2137 mg/kg soil; B1-B16: bands excised from the gel for re-amplification and sequencing)

of some bacterial communities while leaving other unchanged (Waldron *et al*., 2009). The cluster analysis based on DGGE patterns revealed differences in the bacterial communities among soil samples (Fig. 3b). Samples SII and SIII were clustered together, and these samples had similar level of PAHs and heavy metals especially Pb and Ni. Remarkably, the least (SI) and the most (SIV) PAH-contaminated soils showed more similarities in the DGGE profiles and were grouped together, which may indicate that PAH and metal content were not the only reason for observed differences in the genetic diversity of microbial communities.

The most contaminated soil (SIV) had a very low biodiversity (Fig. 4a) and richness (Fig. 4b) but a very high evenness index (Fig. 4b), which may indicate that such a high level of contamination exerts strong pressure on microbial communities. Zhou *et al*. (2002) suggested that a high evenness in organic matter-rich soils may indicate a low competition. Máthé *et al*. (2012) compared the endogenous microbiota of soil contaminated solely with hydrocarbons and soil contaminated with hydrocarbons and metals using culture-based approaches and the molecular T-RFLP technique. The authors suggested that the T-RFs number and the diversity index in highly PAH-impacted samples were significantly lower due to the high level of PAHs. In addition, several other studies (Bachoon *et al*., 2001; Zhou *et al*., 2009) claimed that polycyclic hydrocarbons present even at low concentrations reduce the microbial diversity. This assumption is in contrast to our results because the least contaminated soil (SI) had a lower level of biodiversity as indicated by the *H* and *S* indices than the two more contaminated soils – SII and SIII (Fig. 4a,b).

The correlation analysis revealed that the *H* and *S* indices were significantly ($P < 0.05$) negatively correlated with PAHs ($r = –0.87$ and $r = –0.91$) but not with heavy metals. However, there was a significant ($P < 0.05$) positive relationship between the E_H index and each tested metal (Cd, $r = 0.75$; Pb, $r = 0.75$; Zn, $r = 0.92$; Cu, $r = 0.81$; Ni, r = 0.61) as well as the PAH content ($r = 0.67$). These results are in agreement with the remarks of Máthé *et al*. (2012) and Thavamani *et al*. (2012) that the co-occurrence of heavy metals and PAHs might promote the emergence of species able to survive and multiply in the presence of both types of contaminations at the same time. Moreover, Thavamani *et al*. (2012), who investigated mixed contaminated soils from a former gas manufacturing plant, revealed that there was a connection between heavy metals and PAHs that influenced soil enzyme activities and genetic diversity. Furthermore, Vivas *et al*. (2008), who studied soils historically contaminated by heavy metals and hydrocarbons, showed that this mixed pollution caused genetic and metabolic alterations in the microbial communities. Some authors suggested that the number of DGGE bands may vary by as much as a factor of 10 between more or less contaminated

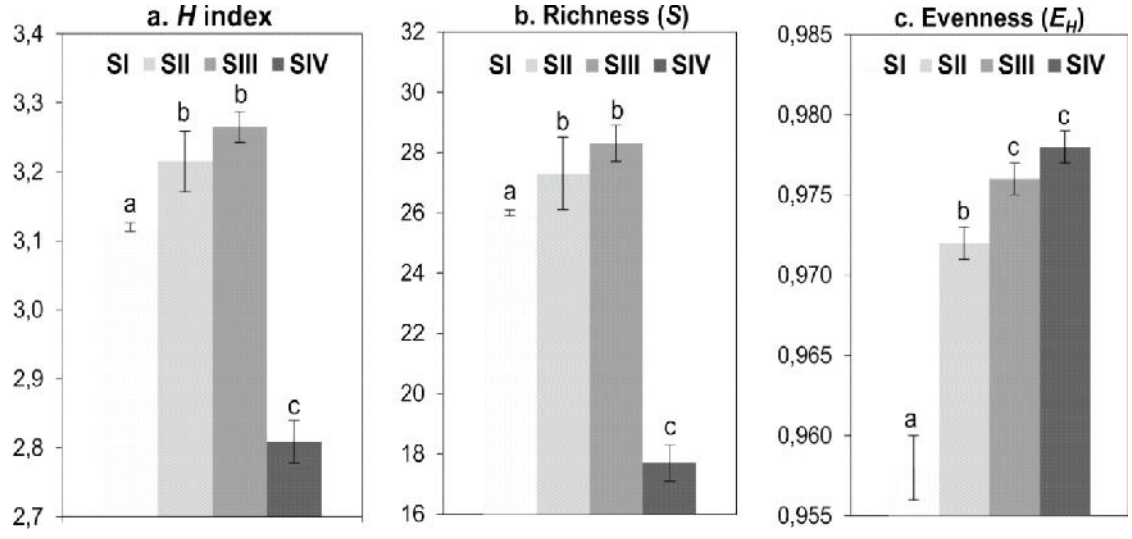

Fig. 4. Results of the DGGE analysis - *H:* Shannon-Wiener index (a), *R*: richness (b), and E_H: evenness (c) for soils of different PAHs concentrations (SI: 171 mg/kg soil; SII: 476 mg/kg soil; SIII: 595 mg/kg soil, and SIV: 2137 mg/kg soil). Different letters (within each parameter) indicate significant differences ($P < 0.05$, LSD test) between soils

Table 3. The homology assay results based on the partial 16S rRNA sequence for bands excised from the gel after DGGE

Band[a]	Closest match[b]	Accession number[c]	Similarity[d] (%)	Presence in soil[e]			
				SI	SIII	SIII	SIV
B1	*Pseudomonas* sp.	AB772894, JN680232	95	+	-	+	+
B2	Pseudomonas sp. / *Pseudomonas aeruginosa*	HM113677 / Q884172	100	-	-	+	+
B3	Uncultured *Bacteroidetes* bacterium	DQ432374	100	+	+	+	+
B4	*Phenylobacterium* sp.	KJ008916	98	-	-	+	+
B5	Uncultured *Brevundimonas* sp.	JF923629	100	+	+	+	-
B6	*Sphingomonas hunanensis*	KF923436	100	-	-	+	+
B7	*Sphingomonas* sp.	AF177917	100	+	+	+	+
B8	Uncultured *Sphingomonas* sp.	GQ289421	94	+	+	+	+
B9	*Sphingomonas oligophenolica* / *Sphingomonas* sp.	HG794335 / F710032	99	+	+	+	+
B10	Uncultured bacterium	KF623272	95	+	+	+	+
B11	Uncultured *Hyphomicrobiaceae* bacterium	GQ351484	96	+	+	+	-
B12	Uncultured *Parvibaculum* sp.	DQ912804	97	+	+	+	-
B13	Uncultured bacterium	GQ351462	94	+	+	+	-
B14	*Arthrobacter* sp.	HM222679	96	+	+	+	-
B15	Uncultured *Methylovirgula* sp.	KC297188	95	+	+	+	-
B16	*Methylocystaceae* bacterium	KJ000026	96	-	+	+	+

[a]Band number as marked in the DGGE profile in Fig. 3; [b]Closest match to sequence obtained by comparison with BLAST search; [c]Numbers indicate the GenBank accession number; [d]Similarity was the ratio of identical sequence between the closest sequence from database entry and the band sequence, which was obtained after BLAST; [e]Based on the presence of band in the DGGE profile; SI: 171 mg/kg of soil; SII: 476 mg/kg of soil; SIII: 595 mg/kg of soil, and SIV: 2137 mg/kg of soil.

sites in the same area. One explanation may be the spatial resource heterogeneity created by the organic contaminants (Feris *et al*., 2004b; Bengtsson *et al*., 2013). Another very important issue is the bioavailability of metals, which is strongly dependent on pH (Vig *et al*., 2003). It is well established that low pH affects bioavailability by increasing the metal solubility and thereby influence microbial activity. In our study, the pH was neutral or even slightly alkaline, which indicates that heavy metals were most likely in an inaccessible form and hence may not affect the microbial communities (Ross, 1994; Antoniadis *et al*., 2008). Another reason may be the organic matter (OM) level because in OM-rich soils some contaminants may not reduce the microbial diversity. For example, the studies of Zhou *et al*. (2002) showed that very high levels of chromium contamination did not greatly reduce the microbial diversity in high-organic-matter soils.

The specific DGGE bands of interest (indicated in Fig. 3a) were isolated, re-amplificated and sequenced. The accession numbers and organism names of the closest matches to the sequences of excised DGGE bands are given in Table 3. Some of the clones were closely related to the genera *Pseudomonas*, *Sphingomonas* and *Arthrobacter*, which are well-known previously reported hydrocarbon degraders (Shi *et al*., 2001; Máthé *et al*., 2012; Festa *et al*., 2013). Besides these strains, many uncultured strains were identified, which mainly belonged to *α*-proteobacteria (Table 3). Members of *α*-proteobacteria were also dominant in other highly PAH-polluted soils and are suggested to be characteristic for chronic pollution (Vinas *et al*., 2005; Labbé *et al*., 2007; Cheema *et al*., 2015).

CONCLUSIONS

The long-term presence of PAHs and heavy metals had a significant impact on the soil microbial community structure and diversity. The obtained results clearly showed that microbial populations are still active and diverse despite some severe contaminant levels. We observed that in long-term and

mixed organic and inorganic contaminated soils, heavy metals are primarily responsible for inducing changes in the activity and structure of soil microbial communities. However, genetic diversity was influenced by both contaminants. One reason for this fact may be the naturally occurring degradation of hydrocarbons and their binding to soil particles. By contrast, metals are not degraded and remain in soil for a long time. Moreover, there are many factors other than pollution that may change the bacterial community in soil.

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