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ORIGINAL ARTICLE

Assessment of soil potential to natural attenuation and autochthonous bioaugmentation using microarray and functional predictions from metagenome profiling

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Abstract

Purpose The use of autochthonous microorganisms for the bioaugmentation of areas contaminated with hydrocarbons has a high potential to overcome the limitations associated with the difficulty of allochthonous microorganisms to adapt. The prediction of bioremediation effects of autochthonous bioaugmentation can be improved by employing the rapid methods of the direct detection of genes crucial to the hydrocarbon biodegradation. This study aimed to evaluate the potential of microflora originating from soils with different levels of anthropogenization for application in autochthonous bioaugmentation by using microarray and functional predictions from metagenome profiling.

Methods Analyses based on the modern techniques of molecular biology—DNA microarrays and next-generation sequencing—coupled with the functional predictions of metagenome profiling.

Results Studies indicated that the metapopulations of all analyzed stations possess the ability to biodegrade petroleum hydrocarbons. It was established that the long-term supply of hydrocarbons in the areas characterized by strong anthropogenization resulted in increasing the biological decomposition of aromatic and polycyclic aromatic compounds. In contrast, areas with a low level of anthropogenization were characterized by a higher potential to decompose aliphatic hydrocarbons. Although alpha-biodiversity decreased when the consortia was isolated and cultivated under laboratory conditions with hydrocarbons as the sole carbon source, microbial communities with genetic biodegradation potential increased, which was confirmed by the analysis involving the loss of selected hydrocarbon fractions in aqueous systems.

Conclusions The presented studies indicated the vast potential for the application of isolated autochthonous microflora on soils permanently contaminated with hydrocarbons. The prediction of bioremediation effects may be improved by employing the rapid method of the direct detection of genes crucial to the biological decomposition of hydrocarbons, with DNA microarrays developed in the framework of this study.

Keywords Autochthonous bioaugmentation · Microarray · Hydrocarbon biodegradation · Biodiversity

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Introduction

Increased demand for solid fossil fuels, their intense exploration and distribution result in the unintended release of petroleum hydrocarbons to the natural environment (Adams et al. 2015; Singh et al. 2017; Zivelytea et al. 2017). It is believed that hydrocarbons are among the most widespread contaminants in highly industrialized and developing countries (Macaulay and Rees 2014). Local contamination of soil may result from accidental leaks during the extraction, refining, transport, and storage of fossil fuels, as well as during the improper storage of petroleum products in underground tanks, the destruction of industrial pipelines and the illegal uptake of fuels (Das and Chandran 2011). Biological methods based on the natural ability of microorganisms to decompose organic compounds enzymatically comprise a promising remediation technology for contaminated areas. These methods are an alternative for chemical and physical methods and are characterized by their low operational costs and limited risk of transformation of xenobiotics to more toxic intermediates (Karigar and Rao 2011; Kumar et al. 2011; Macaulay and Rees 2014). Bioaugmentation is a strategy for the biological decomposition of petroleum hydrocarbons, and this strategy has been known since the 1970s and is based on the introduction of microbial populations characterized by a high biodegradation potential. This method is particularly promising in cases in which microorganisms do not possess the proper metabolic predispositions to biologically decompose petroleum hydrocarbons or their ratio in the population is relatively low (Adams et al. 2015). The efficiency of introducing allochthonous microorganisms with high biodegradation potential is broadly discussed in the literature. It is believed that the limited ability of the inoculated consortium to adapt to local conditions and the rivalry for the environmental niche with autochthonous microflora are the factors that are responsible for the long-term reduction in biodegradation efficiency (Ueno et al. 2007; Macaulay and Rees 2014). Several authors indicate that the effect of the stimulation of biodegradation efficiency does not occur or is only short-term and that it is insignificant in the removal of hydrocarbons (Bento et al. 2005; Szczepaniak et al. 2016). Furthermore, it is believed that the efficiency of biodegradation processes is correlated, not with biodiversity, but rather with the number of individual microorganisms, which are capable of decomposing petroleum hydrocarbons (Wu et al. 2016). A novel method to overcome the limitations associated with the adaptation of allochthonous microorganisms may be the autochthonous bioaugmentation (ABA) technique, which is based on the re-introduction of local microflora. To date, there are only a few research studies involving this field (Ueno et al. 2007; Nikolopoulou et al. 2013). Since the efficiency of all bioremediation techniques, including ABA, is correlated with the enzymatic potential of autochthonous microflora, there is a need to develop molecular metagenomics tools that will allow for their assessment (Sierra-Garcia et al. 2014).

This study aimed to evaluate the potential of microflora originating from soils with different levels of anthropogenization and supply of petroleum hydrocarbons for the biodegradation of selected fractions of hydrocarbons based on the modern techniques of molecular biology—namely DNA microarrays and next-generation sequencing coupled with the functional prediction of metagenome profiling. Additionally, the ability of consortia to proliferate under laboratory conditions and the biodegradation efficiency of hydrocarbons under model conditions was analyzed. This analysis allowed the identification of the diverse enzymatic capabilities of soil systems and the determination of the possibility of using the ABA technology in cases of widespread contaminations.

Methods

Soil sampling

Three main types of study areas were defined: post-industry areas with a high level of anthropogenization (A), moderate level of anthropogenization (B), and low level of anthropogenization under the statutory form of natural protection (C). In each area, a sampling site was established (supplementary materials, Table S1) with an area of 5 m × 5 m. A composite sample was gathered from each site (composed of 10 partial samples of topsoil layer) using a Shelby tube sampler (2-cm diameter, 10-cm depth). Additionally, a model system for soil contamination with a low level of anthropogenization was prepared. Approx. 50 g was taken from the composite soil sample, and then, 5 g of diesel oil (DO) was added and left at room temperature for 2 months. The soils with the addition of DO were labeled type D (supplementary materials, Table S1). The selected hydrocarbon concentrations of the soil samples were presented as a supplementary material, Table S2.

Isolation of consortia and preparation of the inoculum

Approx. 10 g of soil was taken from each sample, added to 90 mL of 0.9% solution of NaCl and shaken for 30 min (150 rpm). Next, the systems were left for 10 min to allow for the sedimentation of mineral particles, and then, 1 mL of the water phase was transferred to flasks containing tryptic soy broth (common nutritional medium to supports the growth of a wide range of bacteria) (Wanger et al. 2017) with the addition of 2% of glucose. The obtained consortia (ConA-ConD) were grown for 72 h under aerobic conditions on a shaker (130 rpm), then centrifuged (10 min, 4000 rpm), and rinsed three times with 0.9% solution of NaCl, centrifuging each

time according to the described procedure. The final inoculum was suspended in 0.9% solution of NaCl by normalizing the concentration based on optical density OD (600) to the value of 0.6 (Helios Delta Vis, ThermoFisher Scientific Inc., USA).

Biodegradation of hydrocarbons in model systems

Biodegradation was conducted in Duran-type flasks with baffles, in model aqueous systems with the addition of diesel oil as the sole carbon source. The experimental system was composed of 50 mL of mineral medium with microelements (Na_2HPO_4 6.21 g/L, KH_2PO_4 2.8 g/L, NaCl 0.5 g/L, NH_4Cl 1.0 g/L, $MgSO_4 \times 7H_2O$ 0.01 g/L, $FeSO_4 \times 7H_2O$ 0.001 g/L, $MnSO_4 \times 4H_2O$ 0.0005 g/L, $ZnCl_2$ 0.00064 g/L, $CaCl_2 \times 6H_2O$ 0.0001 g/L, $BaCl_2$ 0.00006 g/L, $CoSO_4 \times 7H_2O$ 0.000036 g/L, $CuSO_4 \times 5H_2O$ 0.000036 g/L, H_3BO_3 0.00065 g/L, H_2MoO_4 0.005 g/L, EDTA 0.001 g/L, HCl 37% 0.0146 mL/L), 1 g of diesel oil, and 250 μL of the microbial inoculum. Biodegradation was conducted for 7 days at 25 °C under aerobic conditions on a shaker (130 rpm). Each sample was prepared in six repetitions. Three were used for the further analysis of hydrocarbon biodegradation, and three were used for the analysis of the viability of consortia. Additionally, three internal controls without the addition of microorganisms were prepared to exclude the abiotic loss of hydrocarbons.

Analysis of the biodegradation of selected fractions of hydrocarbons

After 7 days of the biodegradation process, the samples were subjected to extraction. Approx. 2.5 mL of acetone was added to each system and shaken for 10 min (150 rpm). Next, 200 μL of internal standards was introduced: n-nonane (Sigma-Aldrich Inc.), n-octacosane (Restek Inc.), and acenaphthene-d10 (Restek Inc.) with concentrations of 6 mg/mL, 6 mg/mL, and 1.5 mg/mL, respectively, in an acetone/hexane mixture (2:3 *v*/*v*). Then, 25 mL of hexane (Poch Inc.) was added, and the samples were shaken again for 60 min (150 rpm). The samples were stabilized for 30 min until the phases were separated, and 2 mL of acetone (Poch Inc.) was added dropwise to remove any emulsions. The prepared extracts were diluted 25-fold in hexane and subjected to GC-MS analysis.

The GC-MS analysis was carried out using a Shimadzu 17A gas chromatograph coupled with a QP5000 mass spectrometer equipped with a Rxi-5MS COLUMN (Restek Inc.). The following separation conditions were employed: carrier gas (helium) flow 1.1 mL/min, injection port temperature 250 °C, splitless injection mode, injection volume 1.0 μL, and column temperature program 40 °C held for 1 min, ramped at rate of 15 °C/min to 300 °C, and held for 7 min. Detection conditions were as follows: interface temperature 230 °C, scanning interval 0.15 s, and scanning range 33–250 m/z.

Assessment of viability of consortia

Samples were taken from the model system after 24, 48, 72, and 168 h of biodegradation. To determine the viability of consortia, a commercially available ADP/ATP Ratio Assay Kit (Sigma-Aldrich Inc.) was used. Approx. 10 μL of each sample was transferred to a 96-well plate, and then, the ATP reagent (prepared in accordance with the manufacturers' protocol) was added. The samples were incubated for 60 s at room temperature, and then, the luminescence (RLUa) was measured using a SpectraMax M2e multi-mode plate reader (Molecular Devices Inc.). The samples were incubated for another 10 min, and the luminescence (RLUb) was measured again. Next, 5 μL of the ADP Reagent (prepared in accordance with the manufacturers' protocol) was added. After 60 s, the luminescence (RLUc) was measured for the third time. The ADP/ATP ratio was calculated based on the following formula:

$$\frac{ADP}{ATP} ratio = \frac{RLUc - RLUb}{RLUa}$$

Isolation of DNA and sequencing

Isolation of DNA

The isolation of the genetic material from the soil samples and the liquid cultures was conducted using the following kits: Genomic Mini AX Soil Spin (A&A biotechnology Inc.) and Genomic Mini AX Bacteria Spin (A&A Biotechnology Inc.), respectively, by following the manufacturers' recommendations. The validation of isolation efficiency was carried out with a fluorometric method using a Qubit 3.0 apparatus and Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific Inc.).

PCR amplification and sequencing

Universal prokaryotic primers 515F-806R were used to amplify the V4 region of 16S rRNA (Caporaso et al. 2012). The PCR reaction was carried out in a volume of 25 μL (5 μL microbial template genomic DNA, 5 μL of each primer, 2.5 μL of PCR-grade water (ThermoFisher Scientific Inc.), and 12.5 μL of PCR Master Mix with the Taq polymerase (ThermoFisher Scientific Inc.). The following PCR reaction conditions were employed: initial denaturation 95 °C for 3 min; 35 cycles 1 min at 95 °C, 30 s at 52 °C, and 1 min at 72 °C; and final extension at 72 °C for 10 min. The amplicons

were purified using Clean-Up columns (A&A Biotechnology Inc.) and then used for the construction of libraries. Sequencing was conducted using a MiSeq (Illumina Inc., CA) apparatus with a MiSeq Reagent Kit v2 (2 × 250 bp) (Illumina Inc.). Details regarding the preparation of libraries were described in a previous study (Szczepaniak et al. 2016).

Analysis of data after sequencing

Raw data in the FASTQ format were imported to the CLC Genomics Workbench 8.5 software with the CLC Microbial Genomics Module 1.2 (Qiagen Inc.). The reads were demultiplexed, and paired ends were merged (mismatch cost = 2, min score = 8, Gap cost = 3, max unaligned end mismatches = 5). Next, the primer sequences were trimmed (quality limit = 0.05, ambiguous limit = *N*), and the identification and elimination of the chimeric reads were conducted. The output data were clustered independently based on two reference databases: SILVA v119 (Quast et al. 2013) and GreenGenes 13.5 (DeSantis et al. 2006) at a 97% probability level of open taxonomic units (OTUs). Based on the merged abundance table (clustered against SILVA v119), the alpha-biodiversity (number of OTUs) and beta-biodiversity (Bray-Curtis PCoA) factors were determined.

To predict the gene content from each OTUs table constructed against GreenGenes 13.5, the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) tool was used (Langille et al. 2013). The output data were a collection involving the abundance of key functional orthologues (KO) in each sample. Based on the Kyoto Encyclopedia of Genes and Genomes (KEGG), the KO in hydrocarbon biodegradation was selected, which allowed the prediction of the functional pathways (Kanehisa and Goto 2000). Finally, the data were grouped based on the EC classification. To compare the samples, the results were normalized by dividing the total predicted gene abundance for the number of OTUs in each sample.

Microarrays

Array preparation

Four round areas with a diameter of 9 mm (sub-matrices) were separated on SUPEROXY glass using 0.25-mm foil. Oligonucleotides with a sequence complementary to the amplified sequences were applied to fixed places on the four areas by the contact method using SpotBot 3 Microarrayer (Arrayit Inc.). Probe sequences were established using Primer3 Plus software (supplementary materials, Table S3) (Untergasser et al. 2007). On the 5′ end, each probe would possess a C12-AMINO modification and 18dT sequence preceding the correct oligonucleotide sequence. In each submatrix, a control for matrix preparation is applied with the following sequence: 5′-C12-AMINO-(dT)18-ACG TAC GTA CGT ACG TAC GTA CGT-Cy5-3′.

All four sub-matrices are printed on the glass using the same system. Upon application of the probes, the binding process was conducted for 12 h at room temperature and humidity < 30%, and then, the systems are rinsed two times with a 0.1% solution of sodium dodecyl sulfate and two more times with water.

DNA extraction and amplification

DNA was isolated from the soil and batch cultures as described in the previous section.

PCR amplification was conducted in two multiplex reactions. Two different fragments were amplified for each gene, and one was amplified for each multiplex reaction; they were detected using complementary probes (supplementary materials, Table S3). Multiplex 1 and 2 PCR amplifications were conducted in a 50 μL volume using specific starters (supplementary materials, Table S3) at the following conditions: 1× PCR buffer, $MgCl_2$ 2.5 mM, dNTPs 250 μM (A&A Biotechnology Inc.), BSA FractionV 0.16 mg/mL (Sigma-Aldrich Inc.), Glycerol (10% *w*/*v*), 5 U TaqPol (A&A Biotechnology Inc.), starters based on the Table S3 (supplementary materials), and 10 μL of DNA. The following temperature profile was used for the reaction: initial denaturation at 95 °C for 3 min, and then, 35 cycles denaturation at 94 °C for 1 min, annealing 60 °C for 1 min, elongation 72 °C for 1 min, and final elongation 60 °C for 15 min. Two studied samples are subjected to amplification—namely negative control for DNA isolation and positive control for PCR amplification.

The amplification products of Multiplex 1 and 2 were pooled and purified from the starters and nucleotides using the precipitation method on the clean-up columns (A&A Biotechnology Inc.). The purified products were rinsed from the column using a volume of 50 μL. Each test study includes the analysis of the positive control, the negative control, and the studied sample (two replications).

Matrix analysis

A 60-μL aliquot of the reaction mixture was applied to the separated submatrices for the positive control, the negative control, and the studied samples. The reaction mixture contained the following: 1× SBE Buffer, 0.75 μM of 7-propargylamino-7-deaza-2′,3′-dideoxyadenosine-5′thiphosphate-6-FAM, 5-propargylamino-2′,3′-dideoxycytidine-5′thiphosphate-6-FAM, 7-propargylamino-7-deaza-2′3′dideoxyguanosine-5′triphosphate6-FAM, 5-propargylamino-2′,3′-dideoxyuridine-5′triphosphate-6-FAM, 5-propargylamino-2′,3′-dideoxyuridine-5′triphosphate-6-FAM (Jena Bioscience Inc.), 5 U DynaSeq DNA Polymerase

(Finnzyme Inc.), and 20 μL of the purified PCR product. The matrix was covered with foil for coating PCR plates. Amplification was conducted in a thermocycler equipped with a microscopic glass amplification unit (Dual Flat Block GeneAmp PCR System 9700 Life Technologies Inc., Mastercycler Nexus Flat Eppendorf Inc.) at the following conditions: 30 cycles denaturation at 94 °C for 1 min, annealing/elongation 50 °C for 1 min, and over temperature 50 °C.

After the termination of the reaction, the matrix was rinsed using a High Throughput Wash Station (Arrayit Inc.) twice for 2 min in deionized water and dried by centrifugation for 1 min at 500*g* in a Microarray High-Speed Centrifuge (Arrayit Inc.).

The matrices were analyzed using a GenePix 4300A scanner (Molecular Devices Inc.) in an ozone-free cell at excitation and emission wavelengths of 492 and 517 nm, respectively, for 6-FAM and 625 and 670 nm, respectively, for Cy-5 (spotting control).

Statistical analysis

To evaluate the significance of the differences among the analyzed systems, a nonparametric Kruskal–Wallis test was employed. Mann-Whitney test was used to test differences between groups. All data represent the mean and the standard deviation ($n = 3$).

Results

Biodegradation of hydrocarbons

The results regarding the efficiency of the biological decomposition of selected fractions of hydrocarbons by the consortia isolated from soils A–D are presented in Fig. 1. The analysis of samples without the addition of the microbial inoculum excluded the abiotic loss of hydrocarbons. The obtained results indicated that the consortia differed significantly regarding their biodegradation potential. In all studied systems, the most easily biodegradable fraction (> 94%) and the fraction characterized by the lowest quantitative diversity after 7 days of biodegradation (SD = 1.58%) were toluene. The highest diversity was observed in the alkane group (SD = 9.60%). The highest biodegradation potential for this group was exhibited by ConC, while ConB displayed the lowest potential. All consortia were characterized by a relatively high potential to biodegrade aromatic hydrocarbons, including polycyclic aromatic compounds. Interestingly, consortium ConD exhibited a significant, lower level of alkane biodegradation compared to that of ConC. The analysis also indicated a lower concentration of aromatic and polycyclic aromatic compounds in systems inoculated with ConD compared to that of ConC. Overall, taking the biodegradation of total petroleum hydrocarbons (TPH) into account, the highest efficiency of the biological decomposition of diesel oil was observed in the case of ConC.

Assessment of viability of consortia

To estimate the viability and proliferation of consortia, changes in the ADP/ATP ratio (supplementary materials, Fig. S1) were determined. All consortia after 24 h of biodegradation entered a phase of exponential growth. The lowest ADP/ATP ratio was noted in the case of ConB.

Metapopulation analysis

Taxonomic identification using the SILVA v119 database based on the V4 region of 16S rRNA allowed for a summary detection of 5 phyla, 10 classes, 23 orders, 61 families, 152 genera, and 389 species of microorganisms. The structure of the metapopulations of the soils and consortia cultivated under laboratory conditions is presented in Fig. 2. *Proteobacteria* was the dominant phylum in all samples. The presence of *Cyanobacteria* was observed only in SoilB (0.47%). Taking the comparison of the taxonomic composition of soils and the corresponding consortia into account, the highest differences were observed in the case of *Proteobacteria* (11.98% SoilA-ConA, 8.72% SoilB-ConB, 17.01% CoilC-ConC, and 17.63% SoilD-ConD) and *Bacterioidetes* (15.39% SoilA-ConA, 9.28% SoilB-ConB, 5.23% CoilC-ConC, and 18.04% SoilD-ConD) types. In all cases, except for SoilD, an increase in the percentage ratio of *Proteobacteria* in the consortia compared to that of the soil metapopulations may be observed, and these changes were mainly associated with the increased ratio of the *Gammaproteobacteria* class. The analysis of alpha-diversity expressed as the number of OTUs indicated significant differences among all types of collected soil samples (SoilA-SoilC) (Fig. S2). The addition of diesel oil to SoilC did not significantly influence the number of OTUs. All consortia, with the exception of ConC, were characterized by a lower alpha-diversity compared to that of the corresponding soils. Principal coordinate analysis (PCoA) with Bray-Curtis dissimilarity (Fig. 3) indicated significant differences between the populations of the studied sites and the cultivated consortia. The most notable changes between the composition of the soil metapopulation and the corresponding consortium were observed in the cases of ConB and SoilB, whereas the least significant changes were noted for ConC and ConB. ConD and SoilD exhibit intermediate characteristics between SoilA and SoilC, as well as ConA and ConC.

Predicted functional gene abundance

The PICRUSt tool was used to predict the functional potential of bacterial metapopulations based on the 16S RNA profile.

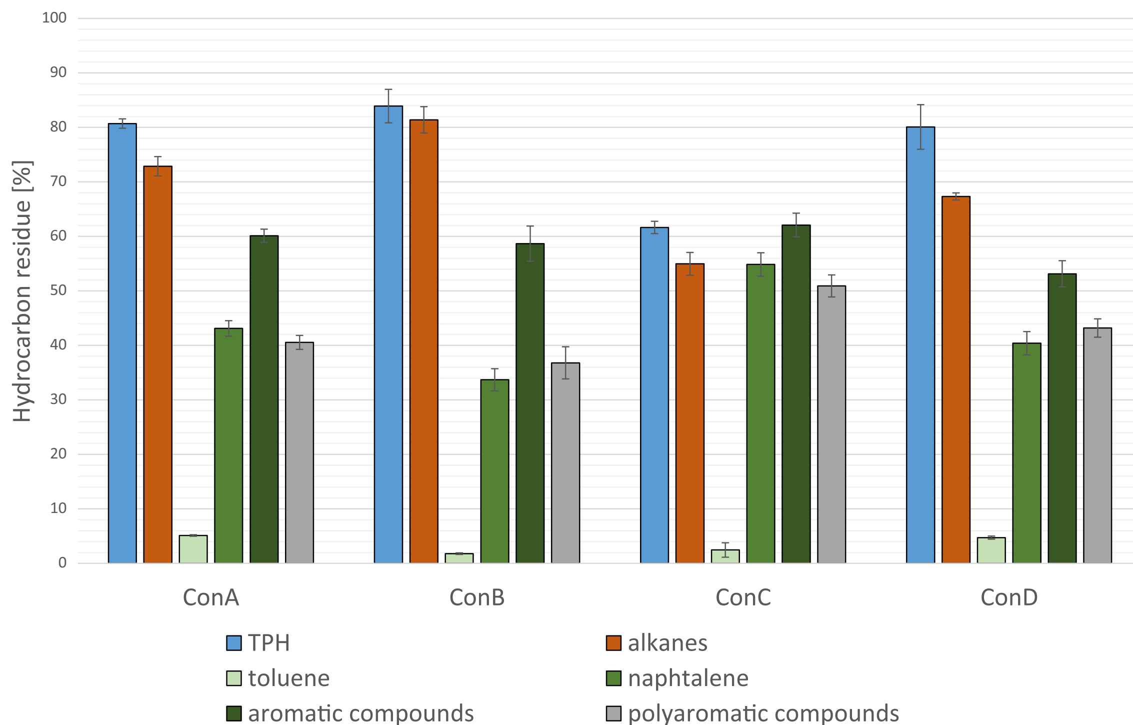


Fig. 1 Biodegradation efficiency of selected fractions of hydrocarbons by environmental consortia in model systems after 7 days of incubation

Reference genome coverage for all samples was calculated using the weighted Nearest Sequenced Taxon Index score (NSTI). The NSTI for all samples was in the range of 0.02–0.06, which indicates a good availability of reference genomes closely related to microorganisms in the sample (Langille et al. 2013). The total predicted gene abundance divided for the number of OTUs in each sample is presented in Figs. 4 and 5. A relatively high abundance of enzymes participating in alkane biodegradation was established in all samples: aldehyde dehydrogenase (EC 1.2.1.3) and alcohol dehydrogenase (EC 1.1.1.1). In the group of enzymes participating in the biodegradation of naphthalene, toluene, and polycyclic aromatic hydrocarbons (PAH), the highest indications were observed in the case of SoilB.

Microarray analysis

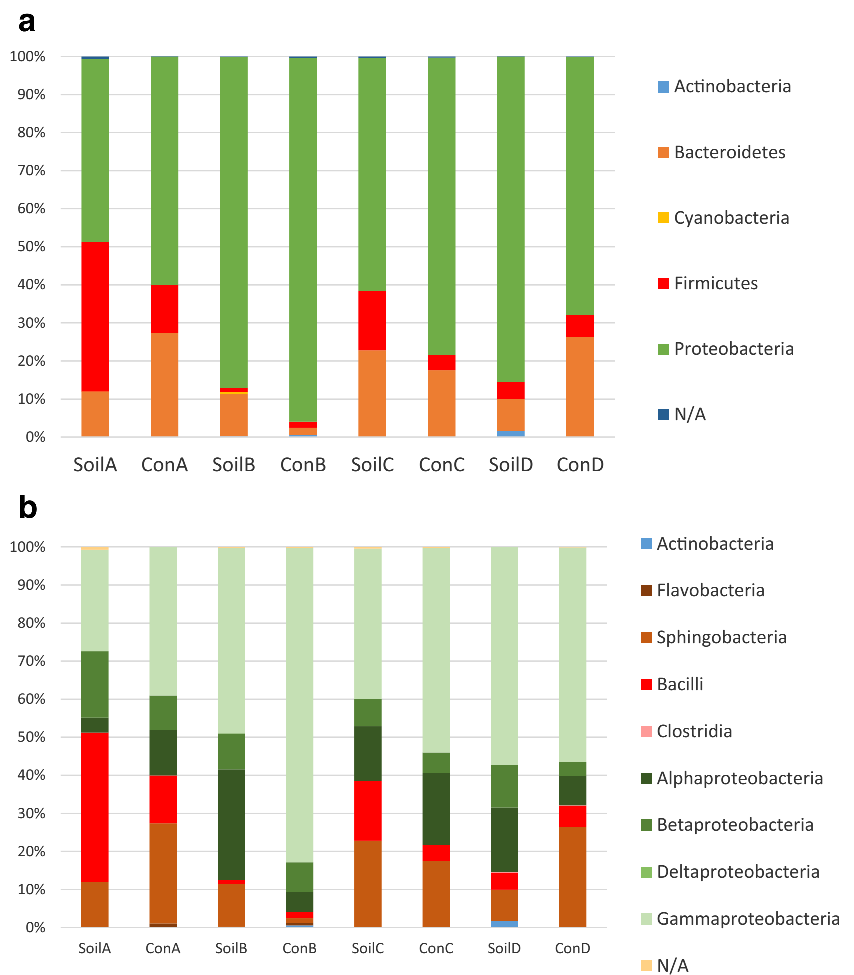
The results of the semi-quantitative analysis of the presence of copies of the selected genes are presented in Fig. 6. Genes *alkB1* and *alkB2* were characterized by the highest frequency of occurrence in SoilD, ConC, and ConD, whereas systems A and B exhibited the highest abundances of genes participating in the degradation pathways of aromatic and polycyclic aromatic hydrocarbons. In all cases, an increase in the share of analyzed gene copies in cultivated consortia compared to that in the respective soils was observed. SoilB and ConB exhibited the highest numbers of genes with a high abundance in the soil sample group and consortia group, respectively, whereas the lowest amounts were observed in the cases of SoilC and ConC.

Discussion

ABA has been widely considered as a method with a high application potential due to the natural adaptation of autochthonous microorganisms to occupy their native soil environment. However, the efficiency of this method notably depends on the potential of microorganisms to biodegrade xenobiotics and proliferate under laboratory conditions (Dott et al. 1989; Vecchioli et al. 1990; Hosokawa et al. 2009). The terrestrial environment is a dynamic, multi-phase system, which results in the diversification of the local structure of autochthonous microbial metapopulations and, consequently, their metabolic profiles. The analysis of β-diversity based on the Bray-Curtis dissimilarity of soil samples indicated that the microbial communities of specific sites differ from each other. The highest distance was observed in the case of SoilC, representing urban areas, which may be associated with the co-existence of anthropogenic-based selection factors. The assessment of distances in the Bray-Curtis dissimilarity analysis for SoilA, SoilC, and SoilD indicated that the changes in the soil population were occurring because the supply of hydrocarbons is targeted and not accidental.

The *Sphingobacteriia* class is particularly sensitive, as its percentage ratio was lower in the metapopulation of SoilA,

Fig. 2 Relative changes in bacterial phyla (**a**) and classes (**b**) in soils A–D and consortia A–D



SoilB, and SoilD in comparison to SoilC. A decreasing tendency of biodiversity in environments characterized by high anthropogenization is also observed, especially in strongly contaminated areas (SoilA) in which the lowest number of OTUs was established. This outcome may result from the fact that only microorganisms with enzymatic profiles that are specialized for the biodegradation of xenobiotics and are characterized by their high resistance to the respective stress factors were capable of growth, due to the long-term effect of hydrocarbon contaminants. The reduction in biodiversity may also be caused by the formation of dead-end during the biodegradation of PAH, which has a toxic impact on part of the soil metapopulation (Ghosal et al. 2016).

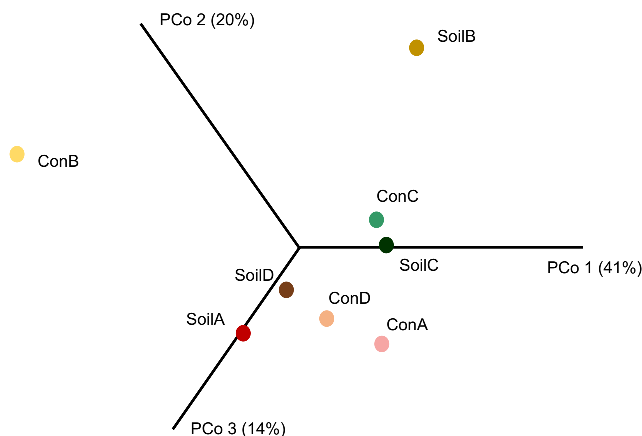


Fig. 3 PCoA with Bray-Curtis dissimilarity based on the 16S rRNA gene

The prediction of gene abundance based on 16S rRNA data indicated the important diversification of the soil enzymatic potential. The amounts of estimated gene copies encoding enzymes participating in the biodegradation of alkanes—EC 1.2.1.3 (aldehyde dehydrogenase) and EC 1.1.1.1 (alcohol dehydrogenase)—were particularly high in SoilD. This outcome may be associated with the fact that in soils permanently subjected to anthropression (SoilA and SoilB), the microorganisms adapted to the decomposition of hydrocarbons are characterized by lower bioavailability due to the dissipation of easily biodegradable hydrocarbons. In soil system type D, the highest amount of gene copies of alkane 1-monooxygenase (*alkB1* and *alkB2*) was also observed to be much higher than that in the case of SoilC; this outcome may indicate a rapid adaptation in the microbial community to the higher supply of hydrocarbons. Microbial metapopulations

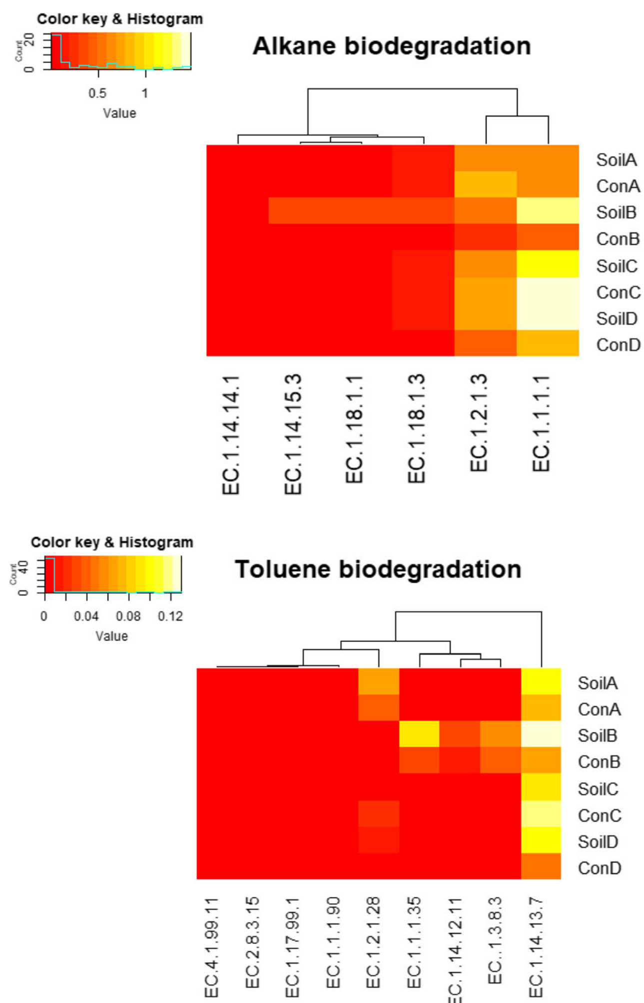


Fig. 4 Heatmap of enzymatic potential based on predicted gene abundance for alkane and toluene biodegradation

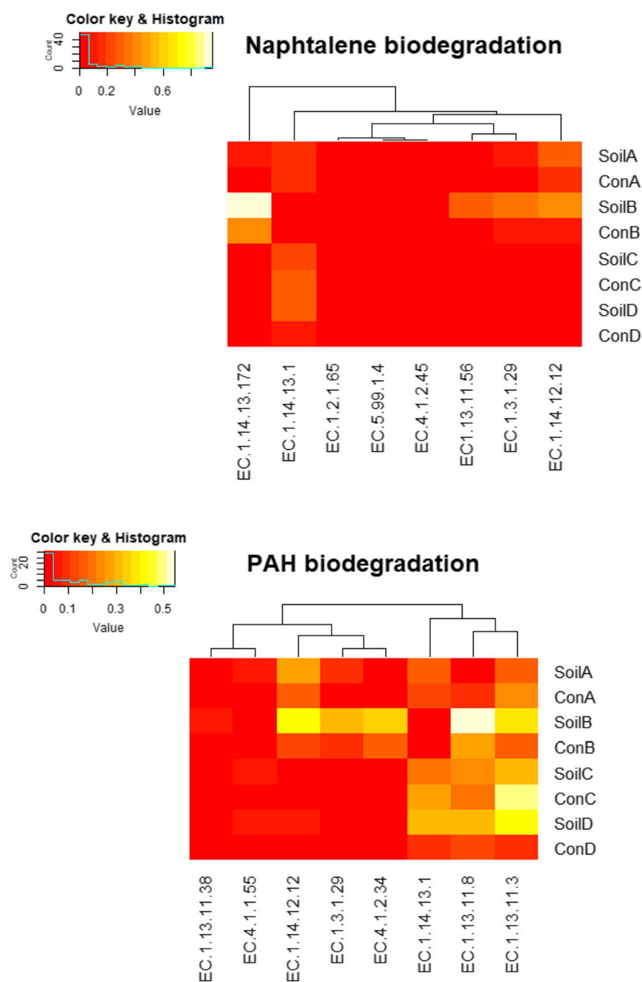


Fig. 5 Heatmap of enzymatic potential based on predicted gene abundance for naphthalene and PAH biodegradation

are characterized by the dynamics of qualitative and quantitative changes due to the changes in environmental factors and exposition to toxic compounds (Wyrwas et al. 2013). During bioremediation processes, the activation of microflora capable of degrading alkanes occurs first, whereas groups capable of degrading more complex structures tend to dominate in the long-term (Bento et al. 2005). This phenomenon is also reflected by the analysis of genes responsible for the biodegradation of aromatic and polycyclic aromatic hydrocarbons in soil samples. The highest estimated indications of enzymes initiating the pathway for the biological degradation of PAH, EC 1.14.12.12 (naphthalene 1,2-dioxygenase) and EC 1.3.1.29 (naphthalene dihydrodiol dehydrogenase), were present in SoilA and SoilB. The most important indications for the selected genes participating in the biodegradation of aromatic compounds *ndoB*, *nahG*, *cat 2*, *cat 3*, and *bphC* were also noted in such systems. In the case of toluene biodegradation, due to the relatively good representation of data associated with the KEGG, different biodegradation strategies may be distinguished. The prediction indicates the presence of the toluene-3-monooxygenase pathway in all analyzed soil systems, in which phenol 2-monooxygenase is used (EC 1.14.13.7). In SoilB, there are additionally high indications for the predictions of EC 1.14.12.11 (toluene 1,2-dioxygenase) and EC 1.3.8.3 (benzylsuccinyl-CoA dehydrogenase), indicating the presence of alternative pathways of

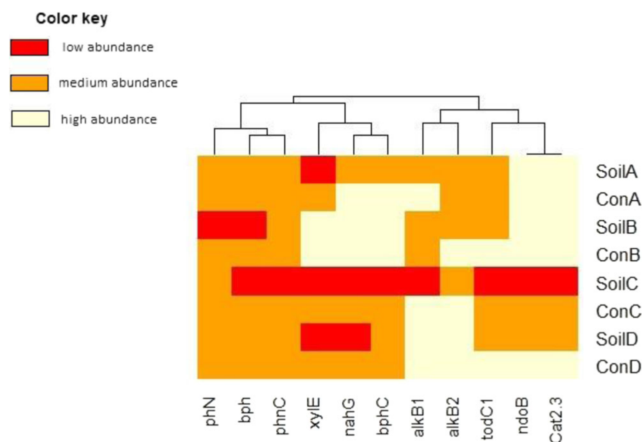


Fig. 6 Heatmap of gene abundance measured using microarrays

biological decomposition—namely dioxygenase mediated pathway and anaerobic toluene pathway with benzyl succinate intermediate, respectively. The presence of dioxygenase-mediated pathway confirms the high indication for the *xylE* gene in SoilB. (Parales et al. 2008). The results representing the predictions for several Enzyme Commission numbers (EC) are characterized by low indications, which may be associated with the limited availability of annotated reference genomes and the horizontal gene transfer in bacterial metapopulations mentioned in the literature (Jiménez et al. 2014). Furthermore, it should be noted that the relatively high results of representation for SoilB and ConB result from the lowest NSTI coefficient among all the analyzed samples (0.023 and 0.028, respectively).

The comparison involving the enzymatic potential of independent samples is therefore semi-quantitative. The use of microarray analysis overcomes these limitations. The method allows for the direct detection of any selected, defined genes in a metapopulation. However, considering the analysis of the metabolic pathways of hydrocarbon biodegradation and the abundance of enzymes participating in these processes, a complex analysis seems very challenging to conduct. The analyses of genes responsible for the initial stages of biological decomposition may be a good solution, which, as confirmed by the presented studies, allow the elucidation of the differences in terms of the genetic potential of bacterial metapopulations and initial prediction of the biodegradation efficiency of selected hydrocarbon fractions.

The comparison of the metapopulation structure of bacterial cultures (ConA-ConD) with their respective soils (SoilA-SoilD) indicates significant changes. The increase in the *Gammaproteobacteria* class in ConA, ConB, and ConC demonstrates that this class possesses preferences to proliferate in a hydrocarbon-rich environment. Therefore, it displays a high application potential for ABA. The lack of significant changes in ConD results from the fact that the concentration of the hydrocarbon substrate in SoilD was at least as high as that in the liquid cultures. This outcome allows the conclusion that the increase in the *Gammaproteobacteria* ratio did not result directly from preferences to grow in a liquid medium. The increase in the *Gammaproteobacteria* ratio due to the increased supply of n-alkanes and cycloalkanes in an aqueous environment was also described in several reports in the literature (Dubinsky et al. 2013; Mason et al. 2014; Ferguson et al. 2017). Furthermore, some microorganisms belonging to this class, e.g., *Pseudomonas* sp., are capable of degrading several groups of hydrocarbons: alkanes, cycloalkanes, and aromatic compounds (Sydow et al. 2016).

Another interesting relation may be observed in the case of *Sphingobacteria.* This class seems to be particularly sensitive to anthropogenic stress factors. In systems A and D, in which the concentration of contaminants in the soil was highest and the deterioration of aerobic conditions could also occur, an increase in the *Sphingobacteria* ratio in cultivated consortia was observed. A different relation is observed in the cases of ConB and ConC. The cultivation under laboratory conditions results in the notable reduction in the number of OTUs. Some soil microorganisms are not capable of efficient growth in vitro (Pham and Kim 2012). The analysis of the ADP/ATP ratio indicated that all consortia in a holistic approach are characterized by the ability to proliferate under laboratory conditions using diesel oil as the sole carbon source. The highest indications were noted for ConC and ConD, which are reflected by the TPH biodegradation efficiency. A similar tendency is observed in the case of alkane biodegradation, which is associated with the fact that alkanes constitute more than 60% (*w*/*w*) of diesel oil (Liang et al. 2005). The biodegradation of toluene occurred at a high efficiency in all the analyzed systems. This outcome may result from the fact that microorganisms capable of decomposing this compound are widely distributed in nature. Furthermore, toluene is characterized by a relatively high solubility in water in comparison to other diesel oil hydrocarbons, making it an easily degradable substrate (Singh and Celin 2010). Predictions involving the use of the PICRUSt tool indicated that there is a lack of an anaerobic toluene pathway with benzyl succinate intermediate in ConB, as observed in SoilB, which may be associated with the improvement of aerobic conditions. Moreover, the decrease in the number of EC indications for toluene-3-monooxygenase pathway in ConA, ConB, and ConD in comparison to that for the soil samples most likely results from the reduction in alpha biodiversity. In the case of the biodegradation of polycyclic aromatic hydrocarbons, the highest efficiency was established in soils with a moderate and high level of anthropogenization. The long-term supply of hydrocarbon contaminants resulted in the succession of the metapopulation towards the biodegradation of poorly available hydrocarbon fractions. This phenomenon has found its confirmation in ConD, in which a higher biodegradation efficiency of aromatic and polycyclic aromatic hydrocarbons in comparison to ConC was observed, whereas the biodegradation potential of alkanes was decreased. The decrease in the estimated abundance for EC 1.14.12.12 (naphthalene 1,2-dioxygenase) in ConA and ConB, in comparison to that for the soil samples, may result from the fact that there was a supply of more easily degradable hydrocarbons in the case of laboratory cultivations with the addition of diesel oil and—similar to toluene—from the reduction in biodiversity. The dynamics of the metapopulation change in terms of how hydrocarbon contamination is a targeted process. However, in this field, further studies are required because of the complexity of the soil environment.

Conclusions

The efficiency of ABA strictly depends on the genetic potential of soil metapopulations. The process of cultivating autochthonous microorganisms in liquid systems with a selective

factor—diesel oil hydrocarbons as the sole carbon source—notably contributed to the increase in the genetic application potential of the obtained inoculates. The studies indicated that soils with a high level of anthropogenization, with long-term exposition to a high concentration of petroleum compounds and from uncontaminated areas, exhibit a hydrocarbon biodegradation potential. To increase the efficiency of soil treatment processes and determine the range of future implementations, appropriate tools that allow the evaluation of the presence of genes crucial for the proper metabolic pathways are necessary. Microarrays are a good alternative for bioinformatic predictions that require costly sequencing procedures. Due to the spontaneous, dynamic changes that occur in the metapopulations from the moment of contamination, the use of microarrays at a semi-quantitative level of evaluation seems to be sufficient. Notably, in the case of the presence of microorganisms with specific metabolic preferences, they can dominate the system as a result of succession processes and efficiently improve the bioremediation efficiency.

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Data availability The sequencing datasets generated and analyzed during the current study are available in the SRA repository, with the identifier SRP145038 (https://www.ncbi.nlm.nih.gov/sra/SRP145038).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights and informed consent This research does not involve human participants and/or animals; therefore, no informed consent is needed.

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